Environmental influences on innate and adaptive immune responses against *Mycobacterium tuberculosis*

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Declaration

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Abstract

The evaluation of the immune responses in peripheral blood and at the site of disease of people with differential outcomes following *M.tb* exposure will lead to the discovery of host biomarkers that will increase our understanding of the protective and non-protective immune responses against the bacterium.

The main study consisted of a number of pilot studies and the objectives of the studies were:

(1) To determine the background and stimulated whole blood cytokine profiles of children and adults of the community;

(2) to establish biomarker profiles in whole blood of children with different *M.tb* infection phenotypes;

(3) to investigate the anti-mycobacterial whole blood immune responses in HIV infected and uninfected children;

(4) and to investigate the role of the innate immune system during human tuberculosis disease.

The study designs were as follow:

(1) Adults and children were enrolled in order to determine cytokine profiles in the community. Whole blood was stimulated with BCG and ESAT-6 or left unstimulated. Eighteen cytokines were measured in supernatants of each condition. Progression to active tuberculosis in the years after study participation was assessed by searching for patient entries in the tuberculosis register.

(2) Children with known tuberculosis exposure in their households and with *M.tb* infection as assessed through interferon-γ release assays, children with exposure but no infection and a control group with no exposure and no infection were investigated. Whole blood was stimulated in QuantiFeron tubes overnight and 21 cytokines were measured in antigen stimulated and unstimulated supernatants by multiplex cytokine arrays.

(3) HIV infected and uninfected children were enrolled in a hospital based study. Whole blood interferon-γ responses against specific mycobacterial antigens were investigated in a diluted 7 day whole blood assay and compared to QuantiFeron supernatants from the same participants.

(4) Tuberculosis diseased adults were enrolled before the onset of treatment and innate and adaptive cell populations were investigated upon start of treatment and at treatment end. In addition, pleural effusion fluid was collected from tuberculosis and cancer patients and innate cell populations further investigated.

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The studies were performed in Cape Town, South Africa and included Tygerberg Academic Hospital and the surrounding neighbourhoods of Ravensmead, Uitsig and Elsies River.

The main findings of the studies included:

(1) We showed age related cytokine differences in our study community. Tuberculosis progressors had significantly higher levels of IL-10 in the unstimulated sample several years before the onset of tuberculosis disease.

(2) Cytokines that distinguished best between children with tuberculosis infection and no infections were all cytokines that correlated with interferon-γ (interferon-γ was used to make the classification of *M.tb* infected and uninfected). Higher IL-1β and lower IL-17 levels in children with tuberculosis exposure without subsequent M.*tb* infection compared to children with no exposure were shown.

(3) HIV infected children showed better responses after 7 day whole blood antigen stimulation compared to the overnight stimulation in QuantiFeron tubes. TB10.4 stimulation in HIV infected TST positive children gave higher interferon-γ responses than ESAT-6 and CFP-10.

(4) The presence of myeloid derived suppressor cells is shown during tuberculosis disease circulating in peripheral blood. Upon treatment a decrease in the population is observed. No differences were seen in the myeloid derived suppressor cell frequencies between tuberculosis and cancer patients, however significantly lower frequencies were seen in healthy controls.

The immune response against *M.tb* is complex and interactions between the different cell types are essential to control and fight infection and disease. In this thesis we presented new biomarkers that play important roles during different stages of *M.tb* pathogenesis from exposure to infection and even during disease. These may shed light on mechanisms of protection against *M.tb*, relevant to development of tuberculosis diagnostics and vaccine strategies. Combinations of multiple biomarkers including cytokines and chemokines and cell subsets are required to characterize biosignatures relevant to the diagnosis of tuberculosis infection and disease.

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Opsomming

Deur die immuunreaksie te ondersoek, in heelbloed en in die setel van infeksie, in mense met verskillende uitkomste van *M.tb* blootstelling sal lei tot die ontdekking van biologiese merkers en sal bydra tot ons begrip van die beskermde en nie-beskermde immuunreaksies teen die bakterium.

Die hoofstudie het bestaan uit 'n aantal loodsstudies en die doel van die studies was:

(1) Om die sitokienprofiele in gestimuleerde heelbloed, asook agtergrond waardes, van kinders en volwassenes te bepaal, in die gemeenskap;

(2) om die profiele van biologiese merkers in heelbloed van kinders met verskillende *M.tb* infeksie fenotipes te bepaal;

(3) om die anti-mykobakteriële immuunreaksies in heelbloed by MIV geïnfekteerde en nie-geïnfekteerde kinders te bepaal;

(4) om ondersoek in te stel na die doel van die aangebore immuunsisteem tydens tuberkulose siekte.

Die studie ontwerpe was soos volg:

(1) Volwassenes en kinders het deelgeneem aan die ondersoek van sitokienprofiele in die gemeenskap. Heelbloed is gestimuleer met BCG en ESAT-6 of is ongestimuleerd gelaat. Agtien sitokiene is gemeet in die bo-stand verkry van elke kondisie. Mense wat aktiewe tuberkulose siekte in die jare na die studie ontwikkel het, is geïdentifiseer deur die pastiëntinligting in die tuberkulose-register.

(2) Kinders met gedokumenteerde huishoudelike tuberkulose blootstelling en met *M.tb* infeksie, soos bepaal deur vrygelate interferon-ã toetse, kinders met blootstelling maar geen infeksie en 'n kontrole groep met geen blootstelling en geen infeksie, is ondersoek. Heelbloed is gestimuleer in die QuantiFeron buise oornag en 21 sitokiene is gemeet in die antigeen gestimuleerde en ongestimuleerde bostande deur die multiplex sitokienpaneel.

(3) MIV geïnfekteerde en nie-geïnfekteerde kinders het deelgeneem aan 'n hospitaal baseerde studie.

Heelbloed interferon-ã reaksies teen spesifieke mykobakteriële antigene is bestudeer in 'n verdunde 7 dag heelbloed toets en vergelyk met die QuantiFeron bostande van dieselfde deelnemers.

(4) Siek tuberkulose volwassenes wat nie op behandeling is nie, het deelgeneem. Die aangebore en verworwe selpopulasies is bepaal aan die begin van behandeling asook voor die einde van

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behandeling. Verder is pluralevog van tuberkulose en kanker pastiënte bestudeer vir aangebore selpopulasies.

Die studies is uitgevoer in Kaapstad, Suid-Afrika en sluit in Tygerberg Akademiese Hospitaal en die gemeenskappe van Ravensmead, Uitsig en Elsiesrivier.

Die hoofbevindinge van die studies sluit in:

(1) Ons het gewys dat daar ouderdomsverwante sitokien verskille in die studie gemeenskap is. Mense wat tuberkulose siekte ontwikkel het, het beduidende hoër vlakke van IL-10 in die ongestimuleerde monsters getoon 'n paar jaar voor die begin van die siekte.

(2) Sitokiene wat die beste onderskeiding gewys het tussen infeksie en geen infeksie was sitokiene wat ook korrelasie getoon het met interferon-ã (interferon-ã is gebruik om die klassifikasie te maak van *M.tb* infeksie of geen infeksie). Hoër IL-1â en laer IL-17 vlakke in kinders met tuberkulose blootstelling en sonder *M.tb* infeksie, is gewys wanneer dit vergelyk is met kinders sonder blootsteling.

(3) MIV geïfekteerde kinders het beter reaksies getoon na 7 dag heelbloed antigeen stimulasie as met die oornag stimulasie in QuantiFeron buise. TB10.4 stimulasie in MIV geïnfekteerde TST positiewe kinders het hoër interferon-ã reaksies getoon as na stimulasie met ESAT-6 en CFP-10.

(4) Die teenwoordigheid van miloïed afgeleide onderdrukkende selle in heelbloed, is getoon tydens tuberkulose siekte. Na behandeling is 'n afname in die populasie gesien. Geen verskille is gesien in die aantal miloïed afgeleide onderdrukkende selle tussen tuberkulose en kanker pastiënte nie, alhoewel beduidende laer getalle is waargeneem in gesonde kontrole deelnemers.

Die immuunreaksie teen *M.tb* is kompleks en interaksies tussen die verskillende seltipes is belangrik om infeksie en siekte te kontroleer en te beveg. In die tesis het ons nuwe biologiese merkers geïdentifiseer wat belangrike funksies het, tydens die verskillende stadiums van *M.tb* patogenesiteit, van blootstelling tot infeksie asook tydens siekte. Dit kan gebruik word as biologiese merkers betrokke by die immuunreaksie teen *M.tb* en sal bydra tot die diagnose van tuberkulose infeksie en siekte.

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Abbreviations

ADA	Adenosine deaminase
ANOVA	Analysis of variance
ART	Antiretroviral therapy
BCG	Bacillus Calmette Guérin
CD	Cluster of differentiation
CFP-10	Culture filtrate protein-10
DCs	Dendritic cells
DOH	Department of Health
ELISA	Enzyme linked immunosorbent assays
ESAT-6	Early secretory antigenic target-6
FACS	Fluorescent activated cell sorter
GM-CSF	Granulocyte macrophage colony stimulating factor
HIV	Human immunodefiency virus
IGRA	Interferon gamma release assays
NO	Nitric oxide
iNKT cells	Invariant natural killer T cells
IFN-γ	Interferon gamma
INH	Isoniazid
IL	Interleukin
M. tb	*Mycobacterium tuberculosis*
mDCs	Myeloid dendrtitic cells
MDSCs	Myeloid derived suppressor cells
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NTM	Non-tuberculous mycobacteria
PBMCs	Peripheral blood mononuclear cells
PCA	Principal component analysis
PHA	Phytohemagglutinin
PPD	Purified protein derivative
pDCs	Plamacytoid dendritic cells
RNA	Ribonucleic acid
SEB	Staphylococcal enterotoxin B
TST	Tuberculin skin test

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Th cells	T helper cells
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TB	Tuberculosis
QFT	QuantiFeron Gold In-tube

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“And though every single human in the stands or in the commentary boxes was at a complete loss for words, the man who in his life had uttered fewer words than any of them knew exactly what to say.

That'll do, pig. That'll do.“

Babe (2005)

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Chapter 1

The immune responses against *Mycobacterium tuberculosis*

Introduction

This review will give a brief overview of the immune response to tuberculosis with particular focus on the cell types studied during the thesis work.

Tuberculosis

During the industrial revolution, pulmonary tuberculosis was one of the main causes of death. However, during the early 19th Century tuberculosis was romanticized amongst the middle class (Rosner and Markowitz, 1991), but for the working class the disease was much more threatening. Life expectancy was low and the infant mortality rate high. Within one year (1838-1839), more than 60 000 people in England and Wales were killed by tuberculosis.

During the 1890's many countries set out to educate people on the prevention of tuberculosis. The people were educated on healthy life style and living conditions. The United Kingdom followed suit and in 1902 the International Union against Tuberculosis was founded, based in Berlin. After the First World War the office reopened in Geneva in 1920 (Croft and Croft, 2005). The Union encouraged tuberculosis control throughout Europe. Most patients received medical care in sanatoriums but no real progress was made. It was only after governments took steps to improve housing and poverty that the number of tuberculosis cases was reduced. With the discovery of new antibiotics between 1945 and 1960 many more people were cured.

It was towards the latter part of the previous century that a re-emergence of tuberculosis was seen and again it is the poor who were the victims (Comas and Gagneux, 2009). Today more than ninety percent of tuberculosis cases occur in developing countries (Tuberculosis Alliance). Tuberculosis is prevalent again in densely populated areas often without basic services. The disease exacerbates poverty and this in turn increases the likelihood of others to contract tuberculosis.

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It has been long after the discovery of *Mycobacterium tuberculosis* and it remains one of the most common infectious causes of death around the world. In addition, with humans as the only natural hosts for *M. tuberculosis,* this bacterium also has the ability to establish latent infections and has spread to nearly a third of the world's population (WHO, 2008).

M. tuberculosis

M. tuberculosis (*M.tb*) belongs to the genus Mycobacterium that includes around 50 more species, often referred to as non-tuberculous mycobacteria or NTMs. Tuberculosis is defined as a disease caused by members of the *M.tb* complex. This complex includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canettii* (Alexander *et al*., 2010; Gutierrez *et al*., 2005).

For the diagnosis of tuberculosis disease it is relied on *M.tb* cultures in combination with chest X-rays of diseased individuals; however for the diagnosis of infection other methods are employed. The tuberculin skin test (TST) with purified protein derivative (PPD, soluble antigens derived from *M.tb*), illicit a classic delayed type hypersensitivity response and has been used for years to identify *M.tb* infection. The TST has been the subject of controversy, largely due to its lack of specificity in those who have been vaccinated with bacille Calmette-Guérin (BCG) or those exposed to possible cross-reactive NTM. False-positive results can lead to unnecessary treatment, (Vanhoof *et al.,* 2003). In addition, patients undergoing immunosuppressive therapy more often display negative TST results compared with general population (Ponce de León *et al*., 2005). Another major disadvantage is that the TST requires two visits to the clinic. Many countries use the BCG vaccine as part of their tuberculosis control programmes. According to the WHO (2008), BCG is the most commonly used vaccine worldwide and in South Africa all children are routinely vaccinated with BCG.

More recently, other promising antigens that have been identified found predominantly in bacterial cell lysates or culture filtrates (or both). These antigens include α-crystallin (Rv2031c, HspX, Geluk *et al*., 2007), early secreted antigenic target, ESAT-6 (Rv3875, Demissie *et al*., 2006), culture filtrate protein-10, CFP10 (Rv3874, Skjøt *et al*., 2000) and TB10.4 (Rv0288, Skjøt *et al*., 2002) some of which are being tested as potential tuberculosis vaccine candidates.

TB10.4 is expressed by both *M.tb* and BCG and is a promising vaccine candidate against *M.tb* infection. The vaccine is part of a fusion protein subunit vaccine HyVac4 based on TB10.4 and Ag85B that is currently in clinical trials (Dietrich *et al*., 2005). ESAT6 is a T cell stimulatory antigen

and is recognised by IFN-γ secreting T cells that are present in greater numbers in patients with active disease than in those who are uninfected (Ravn *et al*., 2005). ESAT6- and CFP10-induced IFN-γ responses have been shown to be useful in discriminating infected individuals from healthy controls (Brock *et al*., 2001). The importance of these recombinant antigens is in their immune recognition by T cells specific to infected individuals. Work by Demissie *et al*. (2006) have shown that immune responses to ESAT6 are more likely to be associated with active infection and that of α-crystallin might be a better marker of latency in patients with tuberculosis. Studies have shown that ESAT-6/CFP-10 is more specific than PPD in detecting latent tuberculosis infection (Lalvani *et al*., 2001; Doherty *et al*., 2002). Therefore, ESAT-6 and CFP10 have been used for the development of commercial assays to detect *M.tb* infection

The recently introduced commercial interferon gamma (IFN-γ) release assays (IGRA) QuantiFERON-TB Gold In-Tube (QFT; Cellestis, Victoria, Australia) and T-Spot.*TB* (Oxford Immunotec, Oxford, United Kingdom) utilise the antigens ESAT-6 and CFP-10, which are present in *M.tb* but absent from most NTM and BCG. A third antigen, TB7.7, has been included in the QFT test. TB7.7 (Rv2654) has been shown to be 100 % specific in low risk tuberculosis patients, however only 30 % sensitivity is recorded for patients with active tuberculosis (Rothel, 2006). The IGRAs appear to be as sensitive as the TST in detecting latent *M.tb* infection (Pai, 2006) and have good specificity that seems unaffected by BCG vaccinations. However, there is no 'gold standard' for latent *M.tb* infection.

Host immune responses

The initial stages of infection consist of inhaled bacteria being phagocytosed by alveolar macrophages that in turn lead to the recruitment of more immune cells and the onset of granuloma formation (Peyron *et al*., 2008). The granuloma consists of a mass of cells including macrophages that differentiates into special cell types including foamy macrophages (Peyron *et al*., 2008; Russell *et al*., 2010). As previously stated *M.tb* can enter a period of latency in the granuloma that can last for years, but only one in ten individuals in this infected population develops active disease (WHO, 2008). It is well known that during this time infected persons have a positive TST and IGRA aiding in the detection of latent infection. Reactivation of *M.tb,* the progression from infection to disease, is increased by immunosuppressive triggers. These triggers can include other concurrent infections like HIV infection (Flynn & Chan, 2001); immunosuppressive therapies like tumour necrosis factor (TNF) neutralization therapy for other

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diseases (Solovic *et al*., 2010); diseases like diabetes (Perez-Gusman *et al*, 2000); and even old age (Horsburgh *et al*, 2010).

Apart from causing disease, helminths also affects the incidence and progression of *M.tb* by exerting immune modulatory effects like the induction of a T helper2 immune response. Research has shown that chronic helminth infection may affect the ability of the host to control mycobacterial infections (Babu *et al*., 2009; Elias *et al*., 2006, 2008; Tristão-Sá *et al*., 2002; Diniz *et al*., 2001). Helminth induced immune modulation may therefore have important consequences for co-infections since helminths are prevalent in most areas where tuberculosis is a big health concern. However, the exact environmental conditions that influence the progression to tuberculosis disease in the host remain to be determined. However, a recent study showed that the foamy macrophages seem to be a key participant in both supporting persistent bacteria and contributing to the tissue pathology that leads to the release of bacilli (Russell *et al*., 2009; 2010) adding to our current knowledge.

Innate responses

The innate response contributes to host immune responses against *M.tb*. When *M.tb* enters the lung, factors at epithelial surfaces may already interfere with mycobacterial invasion. Consequent innate immune responses may decrease the inoculum size during the initial infection and may lead to a T helper 1 (Th1) immune responses downstream. During latent infection, innate mechanisms probably work as the effector arm of T cell mediated protective immunity to contain *M.tb* (Korbel *et al*., 2008). During reactivation and active tuberculosis disease the function of the innate immune responses is still not entirely clear. One explanation could be that the innate immune response may either be ineffective at the time of reactivation and disease due to inadequate signals from adaptive immune response (Korbel *et al*., 2008); or even promote immunopathology when not regulated. The innate immune response is very important in the early recognition of *M.tb* (Balboa *et al*., 2010) and subsequent triggering of a pro-inflammatory response to invading microorganism.

The innate cells include antigen presenting dendritic cells (DCs), cytotoxic natural killer (NK) cells, phagocytic macrophage, granulocytes and γδ T lymphocytes (Holtmeier & Kabelitz, 2005), some of which will be discussed later in this chapter. These cells work in conjunction with each other and communicate with the help of cytokines, chemokines and direct contact between cells. Cytokines and chemokines secreted by innate immune cells play a crucial part in the host defense against *M.tb* (Russell, 2007) by inducing further activation and cytokine production in a

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complex process of regulation and cross-regulation. This cytokine network plays a crucial role in the inflammatory response and the outcome of mycobacterial infections.

Some of the cytokines important in the innate response to *M.tb* infection are indicated in Figure 1 and shortly discussed below. TNF-α, a major pro-inflammatory cytokine, is produced by monocytes, macrophages (Mootoo *et al*., 2009), and DCs upon *M.tb* recognition. TNF-α plays a key role in granuloma formation (Toossi *et al*., 2000) and induces macrophage activation. TNF-α has a crucial role in protective immunity against *M.tb* infection. Firstly, it was confirmed in tuberculosis patients where TNF-α is present at the site of disease (Fenhalls *et al*., 2000) and secondly, it has been shown that TNF receptor deficient mice (Ehlers *et al*., 2000) developed *M.tb* infection.

IL-1β, another pro-inflammatory cytokine involved in the host response to *M.tb*. IL-1β is mainly produced by monocytes, macrophages, and DCs (TeKippe *et al*., 2010; Roach *et al*., 1993). In tuberculosis patients, IL-1β is highly expressed (Schauf *et al*., 1993) and present at the site of disease (TeKippe *et al*., 2010). Mice studies have confirm the important role of IL-1β in tuberculosis. IL-1R-deficient mice display an increased mycobacterial growth after infection with *M.tb* (Juffermans *et al*., 1998).

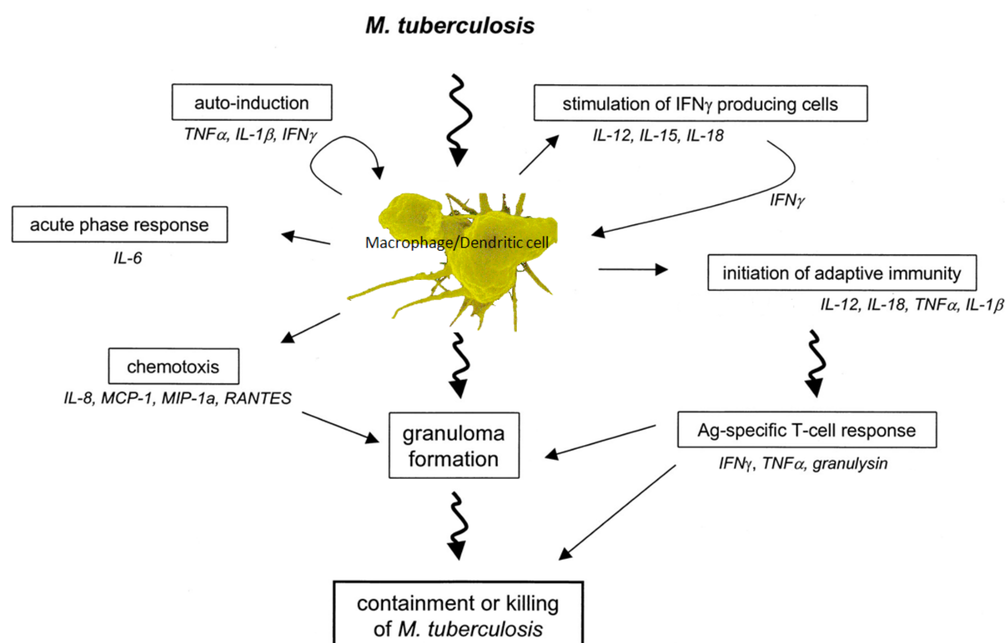

Figure 1: Inflammatory response upon immune recognition of *M.tb* by macrophages and dendritic cells. Cytokines that play a role is indicated in italics. Adapted from Van Crevel *et al*., 2002.

IL-12 acts as a regulatory cytokine which connects the innate and adaptive immune response to *M.tb* (as reviewed by Trinchieri, 2003) and is also an important cytokine in the host defense against *M.tb*. IL-12 is known to be produced after the phagocytosis of *M.tb* (Ladel *et al*., 1997). In tuberculosis, IL-12 has been detected in lung infiltrates (Taha *et al*., 1999), in pleurisy (Budak *et al*., 2008) and in granulomas (Bergaron *et al*., 1997). IL-12 has a crucial role in IFN-γ production (Vankayalapati *et al*., 2003).

The effects of pro-inflammatory cytokines can be inhibited by anti-infammatory cytokines. IL-6 is a cytokine that has both pro- and anti-inflammatory properties (Van Heyningen, 1997). It is produced early during mycobacterial infection and at the site of infection (Correia *et al.,* 2009). IL-6 can also be harmful during infection, as it has been shown to inhibit the production of TNF-α and IL-1β (Schindler *et al*., 1990).

Dendritic cells (DC)

Dendritic cells play a central role in the initiation of the immune response against *M.tb* (Balboa *et al*., 2010). Upon *M.tb* infection pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) recognize specific chemical signatures found in *M.tb*. Once DCs have a presentable antigen, they become activated into mature dendritic cells and begin to migrate to the lymph node (Banchereau & Steinman, 1998). The initial interaction between DCs and naive T cells and the surrounding cytokine environment influence the quality of immune response. DCs have been sub divided into myeloid DCs (mDCs) and plasmacytoid DC (pDCs). mDCs originate from a myeloid precursor, express CD11c (identified by cell surface expression of HLA-DR and CD11c and absence of CD123) amongst other markers and secrete IL-12 that lead to a T-helper 1(Th1) type immune response. mDCs are the biggest population and are found in blood, tissue, and lymph nodes (Mendelson *et al*., 2006). pDCs originate from a lymphoid precursor and express CD123 (identified by cell surface expression of HLA-DR and CD123 and absence of CD11c) and secrete interferon (IFN)-α and induce a Th2 type responses and play a role in the immune response to viral infections (Siegal *et al*., 1999).

However, subsequent studies suggest that during inflammation and viral infection, pDCs are also able to drive a Th1 response, whereas mDCs may be influenced by cytokines like IL-4 and IL-10 to stimulate a Th2 response (Kwadowaki *et al*., 2000; Cella *et al*., 2000). Therefore, the *in vivo* mechanisms by which dendritic cells control the Th1/Th2 balance are more complex than previously thought and may depend on various factors such as the cytokine microenvironment

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and the pathogen to which DCs are exposed (Moser *et al*., 2000). Interestingly, it is known that different microbial components induce mDCs to produce different levels of IL-12, which leads to diverse types of immune responses appropriate to eliminate pathogens (Kadowaki, 2007).

The study of the role of DCs during tuberculosis disease is held back by various factors as discussed by Mendelson *et al*. (2006) and included low cell frequencies during disease. Any decline in the ability of myeloid DCs to undergo maturation and to produce IL-12 may result in impaired stimulation of Th1 immunity (Mendelson *et al*., 2006) which is vital for the control of *M.tb* infection and disease. Taken together, multiple factors, including Th1 and/or Th2 signals from DC subsets, may dictate the quality of T cell responses (Ito *et al*., 2005) during active tuberculosis disease.

Myeloid derived suppressor cells (MDSCs)

MDSCs have recently been described as the myeloid cells with immune suppressive activity during cancer and other diseases. This population consists of immature myeloid derived cells that include monocytes, dendritic cells and granulocytes. Myeloid precursor cells originate in the bone marrow upon which they migrate as an immature population to the periphery were they mature into macrophages, dendritic cells or neutrophils. Under pathological processes like inflammation, infections and tumors these immature cells differentiate into MDSCs. The MDSCs produce immune suppressive factors like arginase I and inducible nitric oxidase synthase initiating the release of nitric oxide and reactive oxygen species that plays a role in programmed cell death and other immunosuppressive mechanisms (Ribechini *et al*., 2010).

Most of the current information on the role of MDSCs in immune responses has come from studies on cancer. However, MDSCs also regulate immune responses during bacterial and other infections, inflammation, traumatic stress and sepsis (as reviewed by Gabrilovich and Nagaraj, 2009). In addition, MDSCs were also observed in mice primed with *M.tb* in the form of complete Freund’s adjuvant (Zhu *et al*., 2007). Still no evidence exists to show the presence of this cell population during tuberculosis disease in humans.

In humans, MDSCs are most commonly defined as cells that express the myeloid marker CD33 but lack the expression of markers of mature myeloid and lymphoid cells and the MHC class II molecule (HLA-DR) (Corzo *et al*., 2009). Several other surface molecules have also been used to identify additional subsets of suppressive MDSCs, including CD80, CD115 (macrophage colony stimulating factor receptor) or CD124 (IL-4 receptor chain) (Schmid & Varner, 2010). Yang *et al*. (2010) reported that expression of CD80 is involved in the immunosuppressive activities of

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MDSCs. Ribechini *et al*. (2010) showed that in whole blood of cancer patients there are two subpopulations functionally able to suppress the immune response, CD14+ monocytes and CD15+ polymorphonuclear cells.

All current data suggest that MDSCs are a group of phenotypically heterogeneous myeloid cells that have common biological activity (as reviewed by Gabrilovich & Nagaraj, 2009; Van Ginderachter *et al*., 2010). MDSCs exhibit flexibility of maturation, differentiation and activation in response to the cytokine microenvironment and pathogen-derived triggers to which they are exposed as for other myeloid cell types. Hence, their expansion, phenotype and mechanism of suppression can vary depending on the range of host and pathogen derived factors they encounter (Van Ginderachter *et al*., 2010). During cancer, expansion and activation of this population would lead to immunosuppressive MDSCs (Almand *et al*., 2001; Gallina *et al*., 2006). In addition, it has been shown that during chronic helminth infections, the MDSCs can mediate the suppressive function (Goni *et al*., 2002; Brys *et al*., 2005). However, the presence of MDSCs at the site of disease, as identified with the current cell markers available to us, does not necessarily mean that these cells are immunosuppressive cells. Instead, these cells can be in an immature state and will still mature into DCs or macrophages upon the correct signal.

Natural killer cells (NK cells)

NK cells are effector cells of the innate immune system and are essential as first line defence against invading *M.tb*. Development of NK cells takes place in the bone marrow from where mature NK cells move to peripheral blood. NK cells present in secondary lymphoid organs are activated by T cell cytokines (Barcelos *et al*., 2008) therefore, it can be seen as a potential link between adaptive and innate immunity (Fehniger, 2003). Mature NK cells are characterized by granules, which include granzymes and perforin, and are capable of rapidly producing IFN-γ and other cytokines. Killer cell immunoglobulin like receptors (KIRs), are a family of cell surface proteins found on NK cells (Vilches & Parham, 2002). They regulate the killing function of these cells by interacting with MHC class I molecules, which are expressed on all cell types. This interaction allows them to detect cells that have a characteristic low level of MHC class I on their surface (Vilches & Parham, 2002).

NK cells are a distinct subpopulation of lymphocytes defined as CD3- CD16+ and/or CD56+ (Robertson *et al.,* 1990). In the peripheral blood, most NK cells are mature NK cells (CD3- CD16+ CD56+) (Barcelos *et al*., 2006; Barcelos *et al*., 2008) and little is known about the biological properties of the other NK cell subsets during tuberculosis infection. However, it is known that NK

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cells play a protective role during tuberculosis and reduced activity of NK cells has been reported in diseased tuberculosis patients (Nirmala *et al*., 2001).

Natural killer T cells (NKT cells)

NKT cells are narrowly defined as a T cell lineage expressing NK receptors that include NK1.1 (Bendelac *et al*., 2007) and a semi invariant CD1d restricted αβ T cell receptor. Phenotypic studies have demonstrated that NKT cells are distinct from T cells in that they express CD56, a NK cell marker as mentioned previously (Barcelos *et al*., 2008). NKT cells play an innate protective response against intracellular pathogens like *M.tb* and are under the control of the antigen presenting cells and soluble factors in the microenvironment (Barcelos *et al*., 2008). NKT cells has been shown to produce large quantities of Th1 (IFN-γ) and Th2 (IL-4) cytokines upon activation. Previous studies proposed to use NKT cell frequencies during tuberculosis disease as a prognostic marker for disease activity (Zahran *et al*., 2006) as NKT cells were shown to stop responding in mice during the course of BCG infection (Chiba *et al*., 2008).

Invariant natural killer T cells (iNKT cells)

iNKT cells are a subgroup of NKT cells that recognise glycolipid antigens presented by the antigen presenting molecule CD1d. Almost all of the CD1d restricted NKT cells display an invariant TCR repertoire, consisting of an invariant Vα24 chain paired with a semi invariant Vβ11 chain (Wilson & Delovitch, 2003; Yu & Porcelli, 2005). iNKT, like NKT cells, have the ability to regulate the innate and adaptive immune systems by rapid activation and the ability to release large amounts of Th1 and/or Th2 cytokines and perforin and granzymes (Kaer, 2007). Unfortunately, there is much less information on the role of iNKT cells during human immune responses. Some recent studies have shown that iNKT cells are activated during *M.tb* infection (Montoya *et al*., 2008).

Adaptive responses

The innate and adaptive immune responses are closely connected. Macrophages and dendritic cells, the primary cell types involved in the innate immune response to *M.tb* (Balboa *et al*., 2010), play an important role in the onset of the adaptive response. Two different lymphocytes play a role in this part of the immune system and they are B cells and T cells. B cells derive from the bone marrow and upon activation transform into antibody secreting plasma cells (James & Herzenberg, 2007) T cells also originate from the bone marrow but mature in the thymus (Romagnani, 1995).

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T lymphocytes

Activated dendritic cells are potent stimulators of naive T cells, because they express large amounts of the costimulatory B7 and CD40 molecules (Delves & Roitt, 2000). T cells can be divided into cytotoxic T cells (CD8+ T cells) and T helper cells (CD4+ T cells). The function of the CD8 T cells is to kill cells that may have an intracellular infection e.g. virus infection. CD8 T cells recognize peptides from foreign proteins expressed on MHC class I molecules. The Th cells can be further divided into several subsets based on their cytokine production (as represented in Figure 2), which can activate or suppress other immune cells or immune functions. Th1 cells that produce IFN-γ are very important for clearing intracellular infections (Ruedaad *et al*., 2010). Th2 cells that produce IL-4 and IL-13 are more involved in inducing antibody responses crucial for the clearing of parasites and extracellular pathogens. Th17 cells that produce IL-17 have been suggested to be important for fighting extracellular pathogens. There are also T regulatory cells that regulate the immune responses and maintain tolerance by producing TGF-β. One important feature of lymphocytes is the constant recirculation through blood, tissues and lymphoid organ. This enables them to encounter antigens carried from infected sites by macrophages and DCs (Balboa *et al*., 2010).

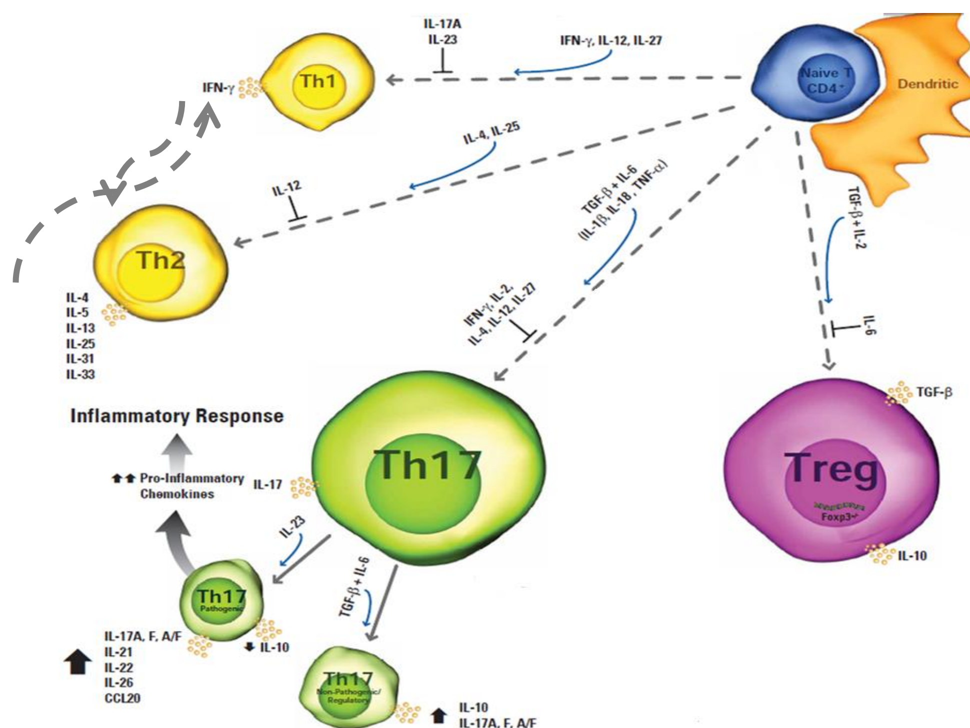

Figure 2: Downstream events upon T cell activation. A balance of the Th1, Th2, Th17 and Treg cell subsets and their cytokine expression is required in order to contain *M.tb*. Adapted from eBiosciences (http://www.ebioscience.com/ebioscience/litreq.asp).

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Th1 immune responses

NK and CD4+ T cells secrete Th1 pro-inflammatory cytokines like IFN-γ and TNF-α upon IL-12, IL-18, IL-23, IL-27 and IFN release by antigen presenting cells like DCs (as indicated in Figure 2). This leads to the activation and proliferation of Th1 type cells. Th1 type cells are the main producers of IFN-γ during *M.tb* infection (Ruedaad *et al*., 2010). IFN-γ plays the main role in determining susceptibility to tuberculosis disease (Jouanguy *et al*., 1996), disease severity (Demissie *et al*., 2006), and treatment outcome (Sahiratmadja *et al*., 2007). A good IFN-γ response is essential for protective immunity against tuberculosis (Britton & Palendira, 2003; and as reviewed by Fletcher, 2007). A study showed that mice which lack either IFN-γ or the IFN-γR, are highly susceptible to mycobacteria. That show the importance of IFN-γ for the control of tuberculosis (Flynn *et al*., 1993). In addition to IFN-γ, TNF-α is important in controlling primary tuberculosis infection. TNF-α is a cytokine that activates monocytes and it also helps to maintain the integrity of the tuberculous granulomas in which *M.tb* is contained (Stenger, 2005). In addition, as mentioned anti-TNF treatment will increase the susceptibility to tuberculosis disease highlighting the importance of this cytokine.

Th2 immune responses

The immune response of latently infected and tuberculosis diseased persons is predominately of Th1 type, however a balance exist between the Th1 and Th2 immune responses. IL-10 is an anti-inflammatory cytokine and is produced by alternatively activated macrophages (Shaw et al., 2000), DC, Th2 and subsets of T regulatory cells. IL-10 down regulates IL-12 production, decreases IFN-γ production and regulates antigen presentation (Sahiratmadja *et al*., 2007). In human tuberculosis, IL-10 production was higher in anergic patients, both before and after treatment, suggesting that *M.tb*-induced IL-10 production suppresses an effective immune response (Boussiotis *et al*., 2000). IL-10 also activates B cells and Th2 type cells while inhibiting Th1 type cytokine production. Activated Th2 type cells secrete IL-4, which also inhibits Th1 cell activity. Th2 promoting cytokines for example TGF-β, which is produced by *M.tb* stimulated macrophages (Toossi *et al*., 1995) as well as regulatory T cells, is thought to contribute to inhibition of bacterial growth. The Th2 promoting cytokines are required to balance the *M.tb* induced Th1 type response and therefore aid the containment of immunopathology. TGFβ and IL-10 seem to work in synergy: TGFβ selectively induces IL-10 production and both cytokines play a role in the suppression of IFN-γ production (Othieno *et al*., 1999).

Th17 immune responses

In mycobacterial infections IL-23 drives activation of Th17 cells and IL-17 production. IL-23 expression has been shown to increase during *M.tb* infection. IL-17 seems to play a role in granuloma formation (Curtis & Way, 2009). The absence of IL-17 during pulmonary *M.tb* infection only slightly alters the inflammatory response (Khader *et al*., 2005; Umemura *et al*., 2007). This suggests that IL-17 plays a minor role early in the response, and that the pathogenic role occurs as the balance between IFN-γ and IL-17 is altered in favour of IL-17.

Tuberculosis in South Africa

Pulmonary tuberculosis leads to 1.3 million deaths worldwide (WHO, 2008). South Africa has around 960/100000 population tuberculosis cases per year (Figure 3). South Africa is also the country with the highest incidence of new and relapse cases per year (303114 cases; WHO, 2008). With the increase of multi-drug resistant cases as well as the association of tuberculosis with HIV infection there is a clear problem in South Africa.

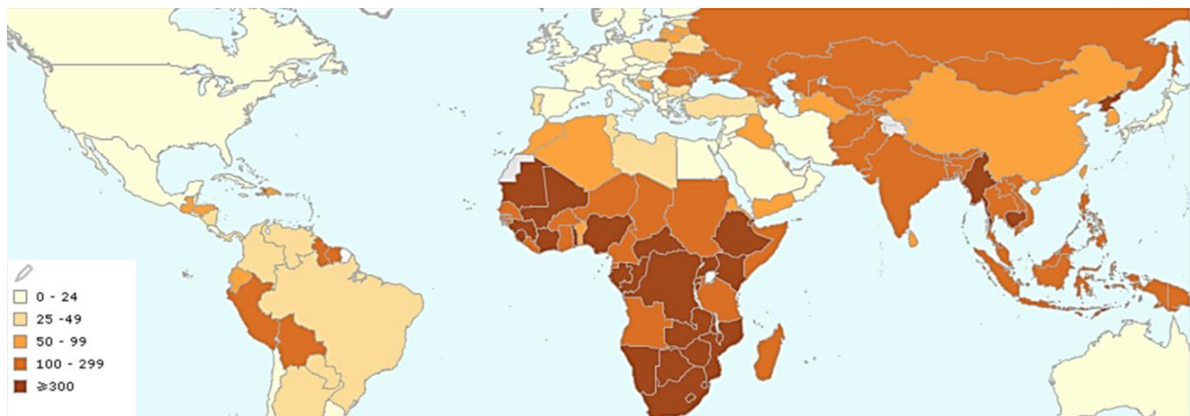

Figure 3: World incidence of tuberculosis (per 100000 population per year) as recorded in 2008.

Our study area includes the suburbs of Ravensmead and Uitsig which are in close proximity to Tygerberg Hospital with a HIV prevalence of approximately 2% (Kritzinger *et al*., 2009). Data from ongoing epidemiological surveillance in this area indicate that from 1996 to 2008 4930 tuberculosis disease episodes were recorded. The average annual increase in notification rates (1997-2008) was 27.7 per 100000 population per year for new tuberculosis cases. Pediatric tuberculosis cases represented 16 % of the total disease burden in this area (Marais *et al*., 2006).

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During the same period, 15.1 % of notified tuberculosis cases were also HIV infected. Annual risk of *M.tb* infection in Ravensmead and Uitsig was 4.1 % in 2006 (Kritzinger *et al*., 2009). This high annual risk of infection reflects the high rate of *M.tb* transmission in this community. Various factors including diagnostic delays (Meintjes *et al*., 2008), poverty and environmental factors contribute (Den Boon *et al*., 2007) to this occurrence.

Study hypothesis and objectives

Hypothesis

The evaluation of immune responses of people with differential outcomes of *M.tb* exposure will lead to the discovery of host biomarkers that will increase our understanding of the immune response against this organism and aid us in diagnosing and treatment of this disease. In the future, it will aid in vaccine development that is important for the control of *M.tb*.

Objectives

To begin with, in Chapter 2 we determined the background and *M.tb* stimulated peripheral whole blood cytokine profiles of children and adults in our study area, Ravensmead and Uitsig. The cytokine patterns of tuberculosis progressors were assessed. In Chapter 3, we aimed to establish biomarker profiles in peripheral whole blood of children with different *M.tb* infection and exposure phenotypes. In Chapter 4, we investigated the anti-mycobacterial peripheral whole blood immune responses in HIV infected and uninfected children. Lastly, in Chapter 5 we investigated the role of the innate immune system in the protective mechanism during human *M.tb* disease and aimed to compare cell frequencies in pleural effusions compared to peripheral blood.

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Chapter2

Cytokines patterns after acute exposure to *Mycobacterium tuberculosis*

Introduction

The aim of this chapter was to determine the cytokine profile of individuals in a high tuberculosis incidence setting. The cytokine patterns of tuberculosis progressors were also assessed. This was done by measuring the cytokine profiles in stimulated whole blood samples of community participants. This would aid the discovery of biomarkers for tuberculosis infection and disease specific to this community where latent infection is extremely high. Biomarkers will aid in tuberculosis diagnosis and early determination of treatment outcome and can be used in the future in conjunction with current available tests to add specificity and sensitivity in tuberculosis detection in this study area. Previous work has been done on specific disease phenotypes in the same community but never before has a study included large number of healthy community participants with no known tuberculosis disease. The information gained in this work will serve as reference *M.tuberculosis* specific cytokines levels in this community with high and on-going tuberculosis exposure.

Methods

Participants

The investigation was approved by the Ethics Review Committee of the Faculty of Health Sciences, University of Stellenbosch, South Africa (95/072). All participants in this study or their legal guardians provided written informed consent for participation in the study. The enrolment strategy was to target large households with healthy children and young adults. Priority was given to the largest households to allow later reconstruction of nuclear families. This family based enrolment strategy was performed for a subsequent genetic study focusing on heritability of anti-mycobacterial immunity. As part of the bigger study 346 healthy participants were recruited from the Ravensmead and Uitsig suburbs in Western Cape, South Africa. In this area in 2008, 375 tuberculosis cases were notified with an estimated notification rate of 1045/100 000 residents (DOH, 2008). BCG vaccination at birth is routine in the study area. All participants belong to the Cape Coloured ethnic group. There was no requirement for the participants to be household

contacts of tuberculosis cases. If TST reactivity was known at time of enrolment, households that contained both TST positive and TST negative subjects were prioritized. Participants who had confirmed tuberculosis disease 2 years prior to study were excluded. In addition, those who were HIV positive, pregnant, or undergoing chemotherapy were also excluded. The Mantoux skin test was carried out on all participants using *M. tuberculosis* PPD (2 T.U., Statens Serum Institut, Copenhagen, Denmark) and skin reactivity was read between 48-72 h after the test using a set of callipers calibrated to the nearest 0.5 mm. Skin test results were available for 311 of the participants. We completed a review of the tuberculosis treatment register in 2010 at both clinics were names, addresses and birth dates were matched to the data available in the register, to ascertain whether any participants were treated for tuberculosis during or after completion of the study.

Whole blood stimulation

Heparinised whole blood (10 mL) was taken from participants and transported to the laboratory within 2 hours at 37 ^{o}C. Blood was diluted 1 in 10 with RPMI (Gibco, Invitrogen, Carlsbad, California, USA) and stimulated in quadruplicate with live whole *M. bovis* bacillus Calmette-Guerin (BCG, Danish 1331 strain, Statens Serum Institut, 1.2 x 10^6 organisms/ml), early secreted antigen-6 (ESAT-6; Statens Serum Institut, 20 mg/ml). Staphylococcal enterotoxin B (SEB, Sigma Aldrich, St. Louis, Missouri, USA, 10 µg/ml) was used as positive control. An unstimulated negative control was included. Cultures were incubated at 37 ^{o}C with 5 % CO_2 for 7 days, before the supernatants were harvested.

Multiplex immunoassay

Multiplex immunoassay was only performed on the supernatants of whole blood after 7 day stimulation.

The Multiplex assays were done according to manufacturer's instructions (Lincoplex, cat no. HCYTO-60K, Millipore, Billerica, MA, USA). In short, the 96-well filter plate was blocked with assay buffer and an appropriate matrix diluent, in this case RPMI with glutamine (Gibco, Invitrogen, Carlsbad, California, USA), was added to all the wells. The 18 antibody covered beads were sonicated separately and then added together. This was followed by adding the samples and the mixed beads in duplicate to the appropriate wells. After 1 hour incubation on a shaker, the fluid was removed by a vacuum pump followed by two wash steps with wash buffer. The biotinylated detection antibody cocktail was added into each well and left to incubate on a shaker for 30 min where after the streptavidin-phycoerythrin was added to each well. After 30 min incubation on a shaker, a vacuum pump gently removed the contents. After another two wash steps sheath fluid

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was added to all the wells and put on a shaker for 5 min to allow the beads to resuspend. The beads were analysed immediately on a Bio-plex array reader (Bio-rad, Hercules, CA, USA). A standard curve was used in each assay ranging from 3.2 pg/ml to 10 000 pg/ml. Quality controls were provided with each kit to measure the reliability and precision of the selected cytokines. All 18 cytokines measured fell into the manufacturer's expected range. As additional quality control, a 7 day PHA stimulated supernatant from a healthy volunteer was used on all plates in this experiment. For all subsequent analysis the average of the duplicate measurements was used.

Statistical analysis

All statistical analyses were conducted using Statistica version 9 (StatSoft, Tulsa, OK, USA). The nonparametric Wilcoxon matched pairs test was used to determine differences between ESAT-6 and BCG stimulated and unstimulated. Spearman's nonparametric correlation coefficient was used to measure correlations. $P \leq 0.05$ was considered significant.

Principal component analysis and heat maps

Principal component analysis (PCA) was performed and heat maps generated using the Omics Explorer, Version 2.0 Beta (Qlucore AB, Lund, Sweden). The PCA operation does not make any assumptions regarding the data.

PCA is appropriate when you have obtained measures on a number of observed variables, in this case cytokines, and wish to develop a smaller number of artificial variables (called principal components) that will account for most of the variance in the observed variables. PCA is a variable reduction procedure used when you believe there is some redundancy in the measured cytokine concentrations. In this case, redundancy means that some of the cytokines are correlated with one another. Reduction of number of variables, by combining two or more variables into a single factor This transformation is defined in such a way that the first principal component has as high a variance as possible (that is, accounts for as much of the variability in the data as possible), and each succeeding component in turn has the highest variance possible under the constraint that it be orthogonal to the preceding components. The principal components may then be used as predictor in subsequent analyses. The q values are the name given to the adjusted p-values found using an optimised false discovery rate approach. The smaller the q the better. The false discovery rate approach is optimised by using characteristics of the p-value distribution to produce a list of q-values. Qlucore has the added advantage of generating heat maps from the PCA analysis. Heat map plots are coloured using normalized data.

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Each variable has been normalized to mean 0 and variance 1. The colour red corresponds to +2 and green to - 2.

Results

Demographics

The median age of participants was 13.67 years and the sex distribution was approximately equal, with 49.1 % males and 50.9 % females (Table 1). One hundred sixty seven (48.3 %) participants had a positive TST. Fifty-eight participants were between the ages of 0 and 7 years, 164 were between the ages of 8 and 15 years and 124 were aged between 16 to 40. Tuberculosis register reviews revealed that 9 participants had developed active tuberculosis within 3 years after recruitment into this study, at which time tuberculosis had been excluded.

Table 1: Demographic and clinical characteristics of study subjects

	All subjects (n=346)	Preschool (n=58)	School-going (n=164)	Adults (n=124)
Age groups (years)	0 - 40	0 - 7	8-15	16 - 40
Median age (years)	13.67	5.88	12.67	19
Female[1]	176 (50.9%)	25 (43.1%)	74 (45.1%)	77 (62.1%)
TST positive[1]	167 (48.3%)	11 (19.0%)	77 (47.0%)	79 (63.7%)
TST unknown[1]	35 (10.1%)	6 (10.3%)	13 (7.9%)	16 (12.9%)
TB progressors[1,2]	9 (2.6%)	1 (1.7%)	1 (0.6%)	7 (5.6%)

[1]Numbers of subjects, followed by the percentages of subjects relative to the total number of subjects in each age group in brackets, are shown; [2]Tuberculosis progressors developed active disease within 3 years after study recruitment. TST = tuberculin skin test; TB = tuberculosis.

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Cytokines concentrations in population

In the unstimulated samples IL-1Ra (29.9 pg/ml), sCD40L (11.5 pg/ml), IL-4 (11.0 pg/ml), IL-8 (43.3 pg/ml), MCP-1 (52.5 pg/ml), RANTES (1478.5 pg/ml) and IP-10 (16.0 pg/ml) were found above the lower limit of detection for the assay (Table 2).

The unstimulated concentration of RANTES was by far the highest measured. After ESAT-6 stimulation only sCD40L (7.4 pg/ml; $p = 0.437$) did not increase significantly from the unstimulated levels. IL-7 (0 pg/ml) was undetectable and IL-12p70 (0.6 pg/ml) was still below 1 mg/ml even after stimulation. The low concentration measurement made it difficult to include in statistical analysis and it was left out with IL-7. After stimulation with ESAT-6 the biggest increases in cytokine concentrations was seen for IL-1α (76.2 pg/ml; $p < 0.001$), IL-12p40 (13.3 pg/ml; $p < 0.001$), IL-8 (9581.0 pg/ml; $p < 0.001$) and MIP-1α (125.2 pg/ml; $p < 0.001$), ranging from a 132.5 fold increase to 1252 fold increase from unstimulated levels. After stimulation with BCG the biggest increases in cytokine concentrations was seen for IL-1α (115.9 pg/ml; $p < 0.001$), IFN-γ (428.2 pg/ml; $p < 0.001$), IL-8 (11100.7 pg/ml; $p < 0.001$), MIP-1α (258.0 pg/ml; $p < 0.001$) and GM-CSF (91.1 pg/ml; $p < 0.001$) ranging from a 256.4 fold increase to 2580 fold increase from unstimulated levels. The BCG stimulation resulted in highest median cytokine concentrations for all cytokines, apart from IL-1Ra and IL-12p40 that was higher after ESAT-6 stimulation.

Table 2: Median responses for 18 cytokines as measured by multiplex assay

	ESAT-6 stimulated			BCG stimulated			Unstimulated
Cytokine	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)
IL-1Ra	945.1	31.6	p<0.001	870.1	29.1	p<0.001	29.9
TNF-α	114.9	87.0	p<0.001	173.0	131.0	p<0.001	1.3
IL-1α	76.2	174.3	p<0.001	115.9	265.0	p<0.001	0.4
IFN-γ	23.9	73.4	p<0.001	428.2	1317.5	p<0.001	0.3
IL-12p40	13.3	132.5	p<0.001	10.2	102.3	p<0.001	0.1
SCD40L	7.4	0.6	p=0.437	22.9	2.0	p<0.001	11.5
IL-12p70	0.7	1.2	p=0.003	0.8	1.3	p<0.001	0.6
IL-17	1.0	9.9	p<0.001	5.9	59.2	p<0.001	0.1
IL-10	45.1	47.0	p<0.001	47.9	49.9	p<0.001	1.0
IL-4	12.0	1.1	p=0.024	12.9	1.2	p=0.001	11.0
IL-13	1.3	12.6	p<0.001	4.2	42.4	p<0.001	0.1
IL-8	9581.0	221.3	p<0.001	11100.7	256.4	p<0.001	43.3
MCP-1	3485.3	66.4	p<0.001	3573.8	68.1	p<0.001	52.5
RANTES	1502.3	1.0	p=0.020	1924.1	1.3	p<0.001	1478.5
IP-10	134.5	8.4	p<0.001	891.1	55.7	p<0.001	16.0
MIP-1α	125.2	1252.0	p<0.001	258.0	2580.0	p<0.001	0.1
GM-CSF	8.2	82.3	p<0.001	91.1	910.8	p<0.001	0.1
IL-7	0.0	0.0	p=0.126	0.0	0.0	p=0.346	0.0

Supernatants from unstimulated and stimulated whole blood were tested for 18 cytokines. Median fold differences and *P* values for significance testing by the Wilcoxon test are also presented.

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Cytokines that correlate with age

A negative correlation was found with IL-4 (r = -0.23 (unstimulated); r = -0.21 (ESAT-6); r = -0.2 (BCG)) and increasing age, regardless of stimulation (Table 3). Negative correlations were also found for the unstimulated concentrations of sCD40L (r = -0.17), IL-1α (r = -0.15), IL-17 (r = -0.13) and IL-12p40 (r = -0.11). After BCG stimulation, a negative correlation with age was only seen for IL-10 (r = -0.12). All negative correlations indicate that there is a trend for these cytokines measured to decrease with age. After stimulation, a positive correlation was seen with increasing age for MIP-1α (r = 0.13), sCD40L (r = 0.18), IL-17(r = 0.20), IL-1Ra (r = 0.21), GM-CSF(r = 0.24), IP-10 (r = 0.27) and IFN-γ (r = 0.29). The only unstimulated cytokine to show a positive correlation with age was IL-8 (r = 0.11).

Median cytokine concentrations within the age groups (as described in Table 1) were also assessed (Table 4). RANTES (1573.4 pg/ml) and IL-10 (57.5 pg/ml) as measured after ESAT-6 stimulation were found to be higher in children 0 – 7 years although not significant (Table 4). After BCG stimulation RANTES (2130.4 pg/ml), IL-13 (5.6 pg/ml) and IL-12p40 (12.9 pg/ml) showed highest concentrations in the age group 8 – 16 years, again not significant. Unstimulated concentrations of RANTES (1703.1 pg/ml) showed a similar pattern with the highest concentrations in the age group 8 – 16 years.

Considering that, young children were included in the study (Table 1) the possibility exists that recent BCG vaccination can lead to false positive TST results. In addition, since correlations with cytokine concentrations were found, although relatively low, it can also have an influence on other analyses. In order to assess if age should be corrected for in subsequent analyses, a one-way ANOVA test of variance was performed. It was found that a positive TST was associated with children older that 16 years and a negative TST with children under 12 years of age (Figure 4) indicating that no age adjustments is necessary. Further analysis were performed in children under 16 years looking at specific cytokine concentrations and it was found that adjusting for age had no effect on the outcome of results (comparative plots shown in appendix 1).

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Table 3: Spearman correlations of cytokines in relation to age

Cytokines	Spearman r value	Spearman p value	# cases	Observed trend
IL-4 (Unstim)	-0.23	p<0.010	345	decrease with age
IL-4 (ESAT6)	-0.21	p<0.010	346	decrease with age
IL-4 (BCG)	-0.20	p<0.010	346	decrease with age
SCD40L (Unstim)	-0.17	p<0.010	345	decrease with age
IL-1α (Unstim)	-0.15	p<0.010	345	decrease with age
IL-17 (Unstim)	-0.13	p=0.020	345	decrease with age
IL-10 (BCG)	-0.12	p=0.028	346	decrease with age
IL-12p40 (Unstim)	-0.11	p=0.036	345	decrease with age
IL-8 (Unstim)	0.11	p=0.033	345	increase with age
MIP-1α(ESAT6)	0.13	p=0.014	346	increase with age
IL-17 (BCG)	0.13	p=0.014	346	increase with age
SCD40L (ESAT6)	0.18	p<0.010	346	increase with age
IL-17 (ESAT6)	0.20	p<0.010	346	increase with age
GM-CSF (BCG)	0.21	p<0.010	346	increase with age
IL-1Ra (ESAT6)	0.21	p<0.010	346	increase with age
IL-1Ra (BCG)	0.22	p<0.010	346	increase with age
GM-CSF (ESAT6)	0.24	p<0.010	346	increase with age
IP-10 (ESAT6)	0.27	p<0.010	346	increase with age
IFN-g (ESAT6)	0.29	p<0.010	346	increase with age
IP-10 (BCG)	0.29	p<0.010	346	increase with age
IFN-γ (BCG)	0.30	p<0.010	346	increase with age

Supernatants from unstimulated and stimulated whole blood were tested for 18 cytokines. Stimulation condition is shown in brackets. *P* values for significance testing are also presented. The condition under which cytokine was measured is indicated in brackets after cytokine name.

Table 4: Median responses for 18 cytokines stratified according to age

	ESAT-6 stimulated			BCG stimulated			Unstimulated		
Cytokine	Median (pg/ml)			Median (pg/ml)			Median (pg/ml)		
Age bracket	**0-7**	**8-16**	**17-40**	**0-7**	**8-16**	**17-40**	**0-7**	**8-16**	**17-40**
RANTES	1573.4	1469.7	1471.8	1517.7	2130.4	1741.1	1312.7	1703.1	1279.8
IL-1Ra	899.3	855.5	1260.9	828.3	804.1	1054.1	30.1	28.0	35.9
IL-4	17.2	12.1	10.5	15.4	13.0	10.5	15.2	11.4	9.2
IL-7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-8	8905.6	9424.7	9726.9	10870.8	11147.7	11125.4	41.3	36.0	56.6
IL-10	57.5	43.5	41.4	55.3	47.9	44.7	1.5	0.9	0.9
IL-12p70	0.7	0.7	0.8	0.8	0.7	0.8	0.6	0.6	0.6
IL-13	0.6	1.2	1.9	4.1	5.6	3.6	0.0	0.0	0.0
IL-17	0.5	0.8	2.4	4.0	5.8	6.5	0.0	0.0	0.0
IL-1α	64.0	77.2	93.7	97.8	117.4	124.8	2.0	0.6	0.0
IFN-γ	2.6	19.7	147.5	211.3	366.8	770.8	0.4	0.3	0.3
GM-CSF	3.4	6.0	32.3	53.1	89.5	120.7	0.0	0.0	0.0
TNF-α	121.1	109.1	124.7	159.2	170.2	191.2	1.5	1.4	1.2
MCP-1	3204.5	3546.1	3407.1	3523.4	3600.2	3550.3	87.1	47.6	52.2
SCD40L	4.7	5.9	15.9	19.4	21.3	25.9	12.8	12.0	10.1
IL-12p40	13.5	14.7	7.4	1.1	12.9	10.0	0.0	0.0	0.0
MIP-1α	103.4	92.2	161.9	187.7	258.3	287.0	0.0	0.0	0.0
IP-10	43.0	125.9	412.7	436.8	619.2	1474.4	19.0	13.6	16.2

Supernatants from unstimulated and stimulated whole blood were tested for 18 cytokines

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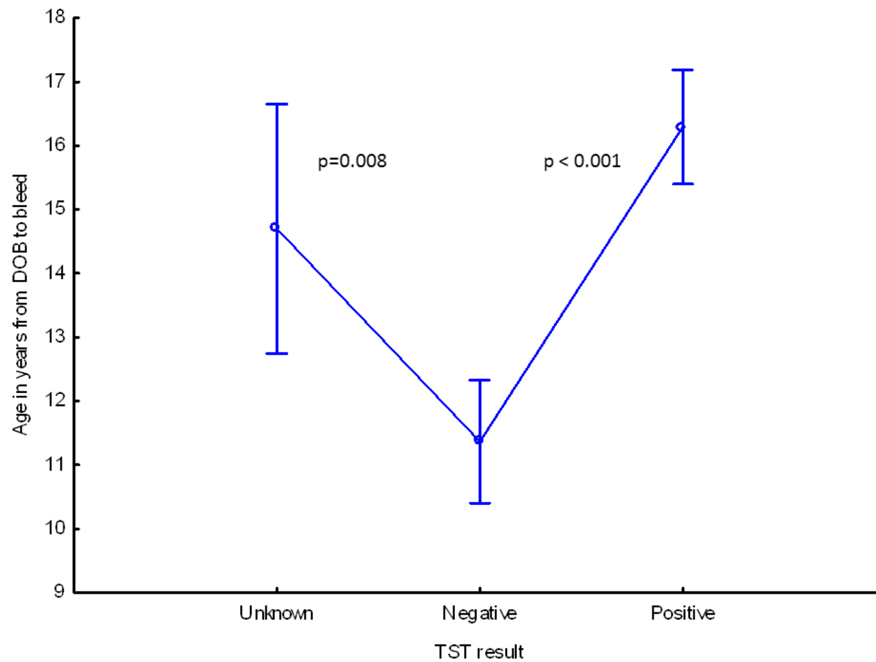

Figure 4: ANOVA analysis of age and TST results of the whole study group. P values for significance testing are also presented.

Cytokines that correlate with IFN-γ

IFN-γ is used in commercial assays as a proxy for tuberculosis infection. To assess which other cytokines measured correlate with this Th1 cytokine Spearman correlation was performed. Even though most cytokines did correlate with IFN-γ, the highest correlations was seen with IL-13 ($r = 0.79$, $p < 0.01$; $r = 0.72$, $p < 0.01$), IL-17 ($r = 0.80$, $p < 0.01$; $r = 0.68$, $p < 0.01$), GM-CSF ($r = 0.92$, $p < 0.01$; $r = 0.89$, $p < 0.01$), sCD40L ($r = 0.73$, $p < 0.01$; $r = 0.76$, $p < 0.01$), TNF-α ($r = 0.5$, , $p < 0.01$; $r = 0.62$, $p < 0.01$) and IP-10 ($r = 0.91$, $p < 0.01$; $r = 0.75$, $p < 0.01$) after stimulation with ESAT-6 (Figure 5) and BCG (Figure 6),respectively.

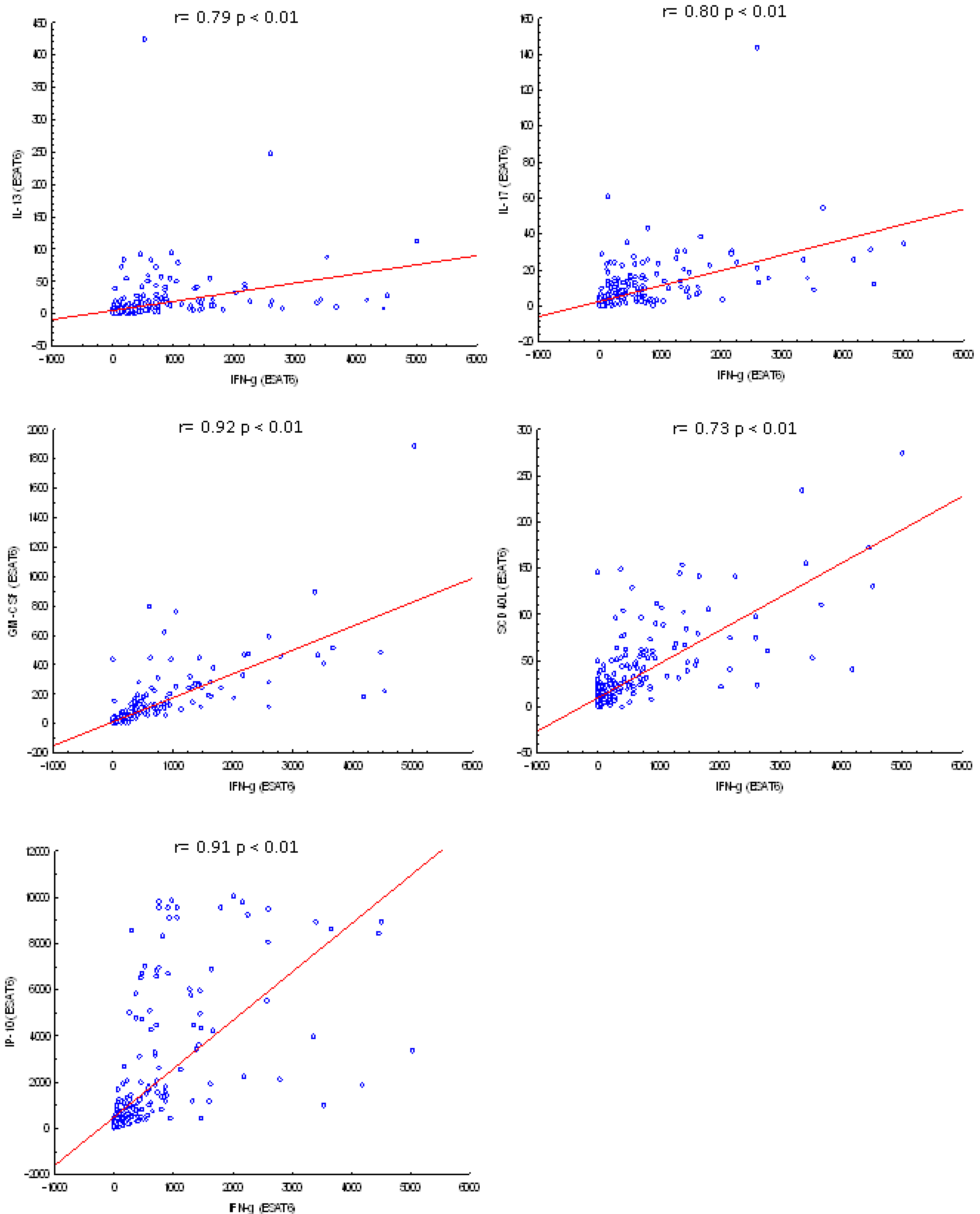

Figure 5: Cytokines that correlate with IFN-γ after ESAT-6 stimulation. Graphs are representative of the cytokines that showed strong correlation (r > 0.6) with IFN-γ. P values for significance testing are also presented.

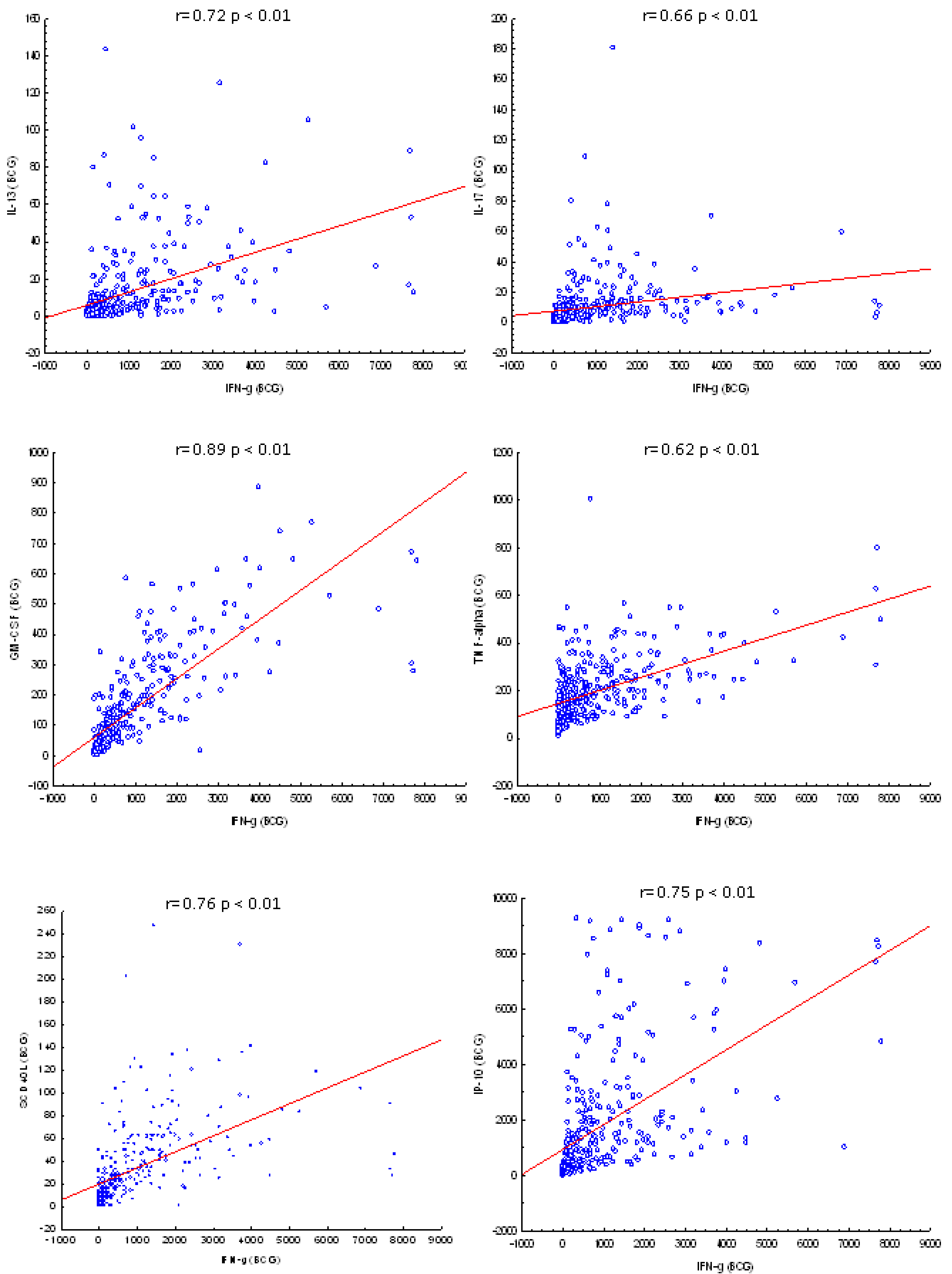

Figure 6: Cytokines that correlate with IFN-γ after BCG stimulation. Graphs are representative of the cytokines that showed strong correlation (r > 0.6) with IFN-γ. P values for significance testing are also presented.

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Cytokine differences between latently infected and uninfected participants

The TST result was used to group participants into clinical phenotypes of latently infected (a positive TST) and uninfected participants (a negative TST). Unstimulated values were subtracted from the stimulated values and used for PCA (Figure 7). PCA based analysis applying most stringent conditions within the data set of the whole group, differentiates TST positive individuals from TST negatives (as in panel B) in a highly significant fashion ($p = 6 \times 10^{-37}$, $q = 5.27 \times 10^{-36}$). After ESAT-6 stimulation similar results was found as seen in panel D again highly significant ($p = 3 \times 10^{-36}$, $q = 2.63 \times 10^{-36}$), but after BCG stimulation no clear difference was seen between the TST positive and negative group after applying a cut off ($p =9.5 \times 10^{-15}$, $q = 1.93 \times 10^{-14}$). Heat maps (Figure 8) after PCA showed that within the whole group, sCD40L, IFN-γ and IL-13 responses (all after ESAT-6 stimulation) were the best markers to distinguish between latently infected and uninfected healthy participants. After analysing the ESAT-6 stimulated samples similar results were found (Panel C). However, upon BCG stimulation, sCD40L, IFN-γ and GM-CSF showed to best ability to distinguish between latently infected and healthy participants.

Cytokine differences between tuberculosis progressors and healthy participants

In the unstimulated samples significantly higher concentrations of IL-10 was seen in participants who progressed to tuberculosis disease within 3 years after study enrolment ($n = 9$; $p = 0.033$). This was in comparison to healthy participants ($n = 338$) (including those who were previously treated for tuberculosis prior to enrolment into the study ($n = 18$; $p = 0.046$) (Figure 3). The demographics for tuberculosis progressors are included in Table 1.

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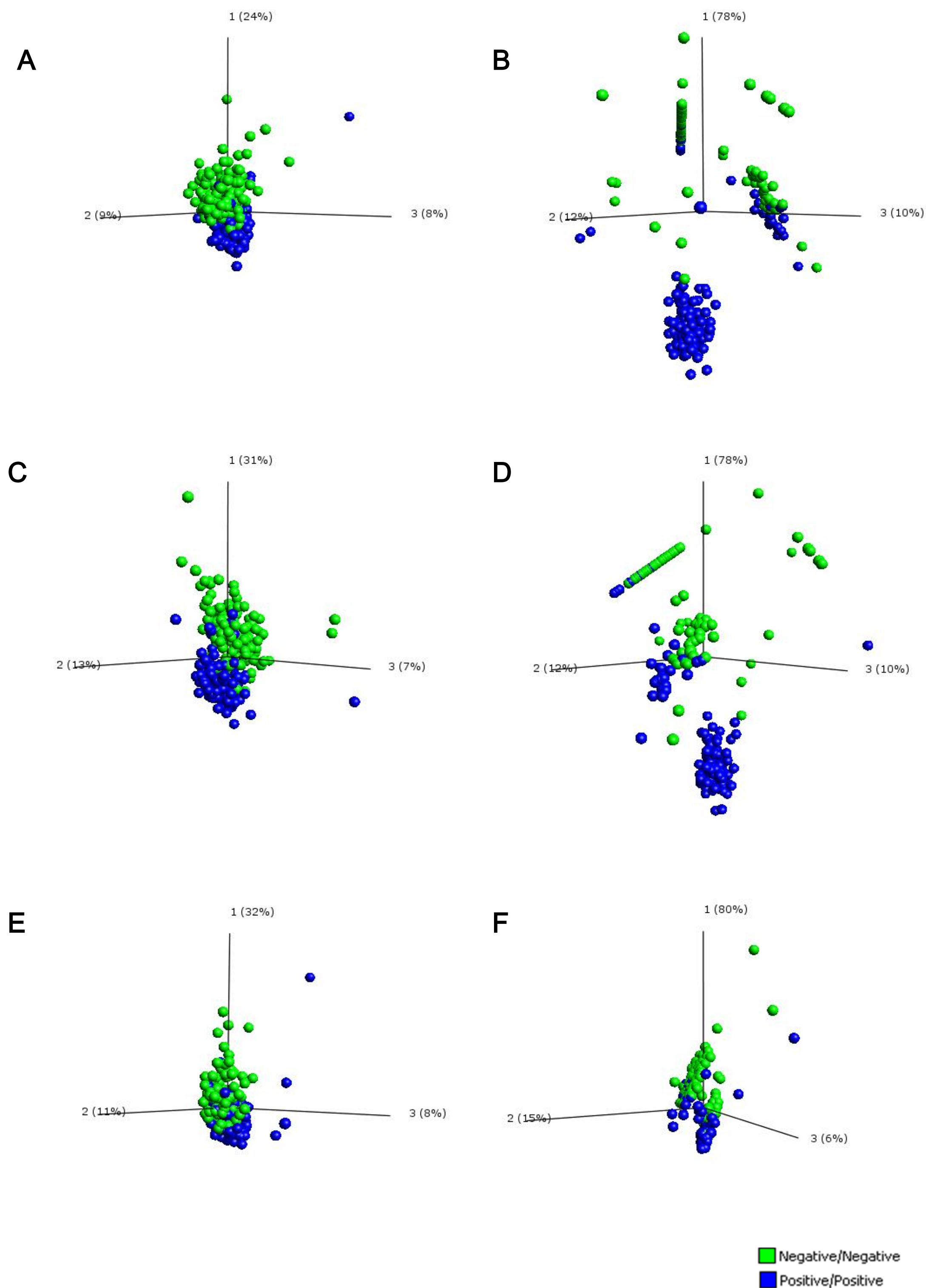

Figure 7: PCA plots of all the study participants that are TST positive (in green) and TST negative (in blue). The whole group as seen before analysis (A) and (B) after PCA ($p = 6 \times 10^{-37}$, $q = 5.27 \times 10^{-36}$). After ESAT-6 stimulation, the group as seen before analysis (C) and (D) after PCA ($p = 3 \times 10^{-36}$, $q = 2.63 \times 10^{-36}$). After BCG stimulation, the group as seen before analysis (E) and (F) after PCA ($p = 9.5 \times 10^{-15}$, $q = 1.93 \times 10^{-14}$).

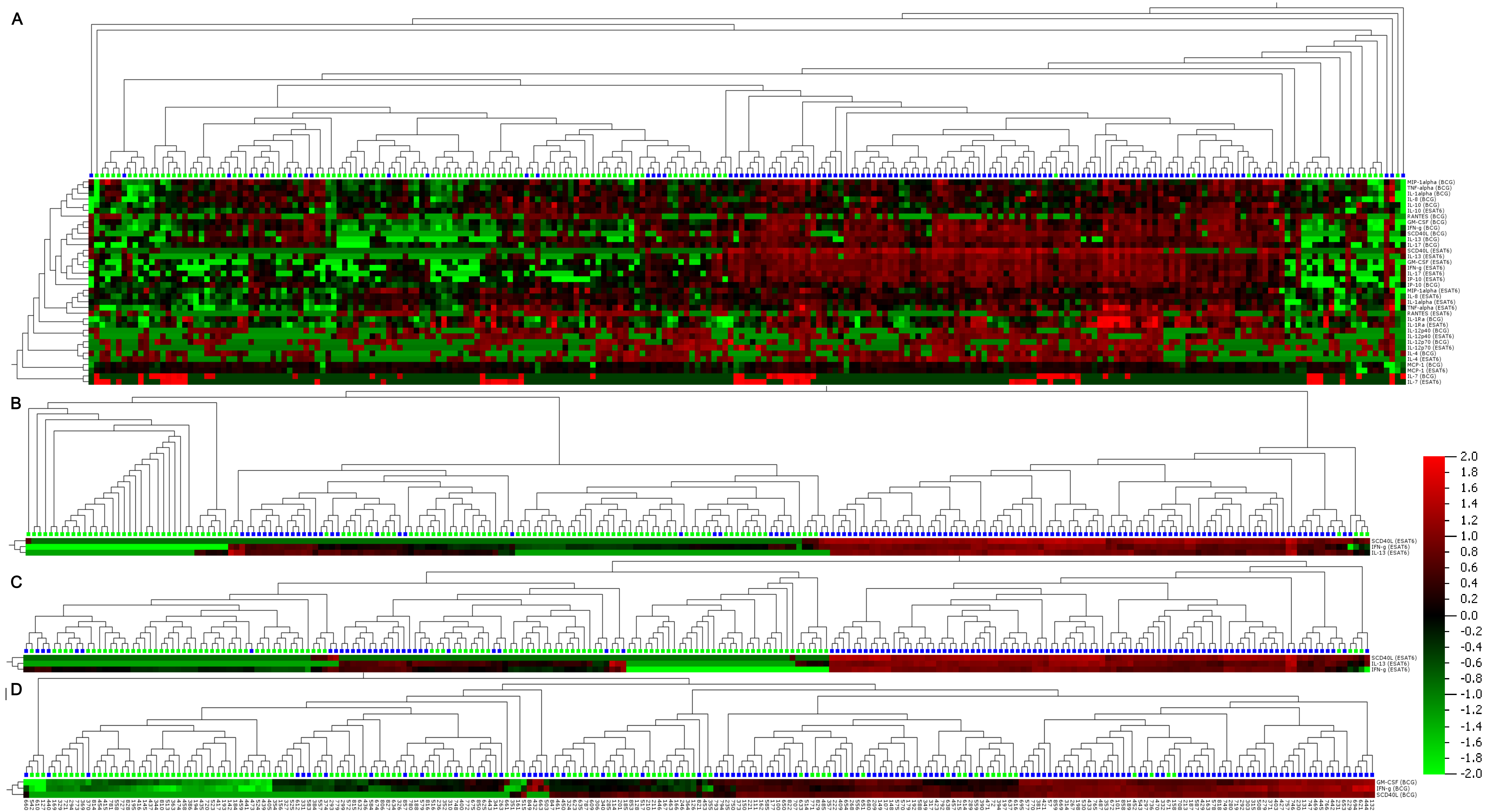

Figure 8: Heat map showing cytokine responses after PCA in the whole study group ordered by hierarchical clustering (Spearman correlation with average linkage) creating a condition tree, upper horizontal edge of heat map; study grouping are the coloured blocks at the top of each profile. Heat map rows are the cytokines and columns are the participants. Heat map plots are coloured using normalized data. Each variable has been normalized to mean 0 and variance 1. The colour red corresponds to +2 and green to - 2. First panel is representative of the whole study group before PCA analysis (A) applyng the same conditions as mentioned for PCA. After analysis of the whole group, ESAT-6 and BCG stimulated samples, 3 cytokines showed differences between the TST positive (in blue) and TST negative (in green) groups (B). After analysis of the ESAT-6 stimulated group (C) and the BCG stimulated group (D) more cytokines showed differences between the TST negative and TST positive groups.

Negative/Negative
Positive/Positive

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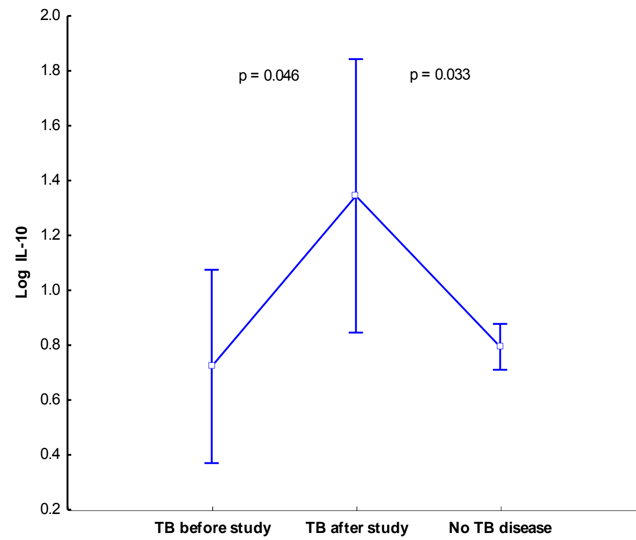

Figure 9: Unstimulated IL-10 concentration measured in participants with a history of tuberculosis before study enrolment (n = 18), those who developed tuberculosis after the study (n = 9) and healthy participants (n = 319; infected and uninfected). P values for significance testing are also presented.

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Discussion

Given the high tuberculosis notification (Verver *et al*., 2002) and transmission rate in this community (Kritzinger *et al*., 2009) it is expected that we will see high latent infection rates amongst people of the study community. Hence, we present cytokine levels that can be seen as reference values for this community specific to a high tuberculosis incidence setting. Reference values in such a large cohort can be useful in future studies especially for children as they may vary physiologically with age and differ from those of adults (Berdat *et al*., 2003). The measurement of the levels of cytokines of immune activation can provide reliable information regarding the disease diagnosis, disease stage, prognosis and the evaluation of therapy (Aziz *et al*., 1998). Reference values therefore can be used as a guide to assess immune status during disease and/or infection.

The Multiplex assay enabled the investigation of 18 cytokines in the same sample. Cytokine concentrations were determined in unstimulated, ESAT-6 and BCG stimulated plasma samples. In the unstimulated samples, 11 cytokines tested showed low or undetectable concentration. One reason could be that these cytokines are not being produced in this 7 day culture or that these cytokines are being produced but at levels below the sensitivity of the Multiplex assay. Alternatively, IL-Ra, sCD40L, IL-4, IL-8, MCP-1, RANTES and IP-10 are spontaneously produced in the unstimulated samples during the 7 day culture suggesting that *in vivo* activated cells now continue to produce cytokines *in vitro*. In addition, the pro-inflammatory cytokines, IL-1Ra and IL-8 that are present could stimulate the further production of the chemo attractant cytokines, MCP-1 and RANTES. The possibility exist that the cytokines are produced to regulate the cellular process under unfavourable culture conditions. Since BCG is prepared from a strain of the attenuated live *Mycobacterium bovis,* a more robust, and non-specific, immune response is seen against this vaccine. Whereas ESAT-6, that is absent from this vaccine strain, is a small protein and specific to *M.tb* therefore a more tuberculosis specific immune response is seen in the cytokines measured (as discussed in Chapter 2). Apart from IL-1Ra and IL-12p40 all cytokines were produced at higher median concentrations after BCG stimulation than after ESAT-6 stimulation.

Cytokines that showed correlation with age had relatively low r-values indicating that even though a correlation is significant, age is not the only role player in this correlation. The possibility exist that other factors, which were not taken into account in the present study, play a role in the correlations seen here. Other factors can include for example genetic variations within the group of children. Subsequent analysis showed that in this group, which consisted mostly of children, no further adjustment for age is needed. A study in a similar community near Cape Town found in young children that there were no independent associations between ethnicity, gender, age, over-crowding

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and TST results (Pan *et al*., 2010), leaving us confident that no further adjustments for these factors are needed.

Cytokines that showed a negative correlation with age, IL-4, sCD40L, IL-1α, IL-17, IL-10 and IL-12p40, were all mainly produced by the adaptive immune cells and it is known that the immune system does undergo age related changes and that these mainly occur in the adaptive part of the immune system (Weiskopf *et al*., 2009). As young children predominantly have a Th2 type immune response one would also expect Th2 cytokines, like IL-4, to decrease with age. Upon stimulation with either ESAT-6 or BCG, cytokines that showed a positive correlation with age are mainly Th1 and pro-inflammatory cytokines. This is expected since BCG and ESAT-6 is known to elicit mainly a Th1 directed immune responses (Ellner *et al*., 2000). Since a positive TST is associated with older age, we can conclude that the immune responses of the older children are due to exposure and/or infection to tuberculosis and should be a Th1 type immune response. The cytokines showing the highest association with age is IFN-γ, mainly produced by Th1 cells, and IP-10 that is induced by IFN-γ and TNF-α. This is of great interest as risk of tuberculosis infection is age related but that exposure in young children may have been recent, whereas adults may have had recurrent exposure. Taken together we found that a balance of pro- and anti-inflammatory as well as Th1 and Th2 cytokines is present at all ages.

Regardless of stimulation conditions, most cytokines tested correlated highly with IFN-γ, the cytokines with the highest correlations being IL-13, IL-17, GM-CSF, sCD40L, TNF-α and IP-10. The role of Th2 cytokines in the response to microbial antigens has been debated (Rook *et al*., 2001). It is possible that IL-13, which is closely related to IL-4 (as another Th2 cytokine), is produced in response to the high levels of IFN-γ. The cytokines that were produced after stimulation, especially those that did not show a strong correlation with IFN-γ, were of main interest. These cytokines can possibly be unique to the anti-tuberculosis immune response and can be useful biomarkers for infection and disease.

Several cytokines concentrations were significantly higher in the tuberculosis infected group (as determined by a positive TST) than the uninfected group (as determined by a negative TST). This included two cytokines that did not show a good correlation with IFN-γ and are of main interest. In the unstimulated samples, MCP-1 and IL-1Ra were found to be higher in the tuberculosis infected group. Higher MCP-1 concentrations in unstimulated samples might be due to a non-mycobacterial specific response (as also suggested by Lalor *et al*., 2010). Since we did not find MCP-1 to show significant differences after stimulation in the TST positive group that might just be the case that it is non-specific. IL-1Ra, MIP-1α and IL-1α were also higher in the tuberculosis infected group after

stimulation. As production of IL-1Ra is seen in unstimulated samples as well as stimulated samples, it could mean that this production is not specific to mycobacterial antigens and similar results were found by another study (Ruhwald *et al*., 2009). Little is known about the role of IL-1α in tuberculosis, however earlier work has suggested that it plays a role in tuberculosis susceptibility (Bellamy *et al*., 1998). In addition, Lalor *et al*. (2010) found that after BCG vaccination IL-1α was induced in infants in the United Kingdom. MIP-1α and other chemokines as found in our investigation have also been shown to be important against protection against tuberculosis (Mendez-Samperio *et al*., 2008) and therefore an increase in chemokines can be beneficial to the anti-tuberculosis immune response. IL-1α and MIP-1α should be investigated further for it diagnostic ability. After ESAT-6 stimulation, significantly higher IL-8 and IL-10 concentrations were seen in the tuberculosis infected group in our study. PCA found that after ESAT-6 stimulation, sCD40L, IFN-γ and IL-13 concentration were higher in the TST positive population compared to the TST negative population. As discussed it was found that these cytokines all showed high correlation with IFN-γ and is part of the tuberculosis specific immune response. Similar results were found after BCG stimulation with sCD40L, IFN-γ and GM-CSF concentrations being higher in the TST positives. The immune determinants of vaccination-induced protection against tuberculosis are not fully understood (Mansoor *et al.,*2009). However, the Th1 cytokine response, characterised by IFN-γ, TNF-α and IL-2 production, is widely thought to be essential (Mansoor *et al.,*2009) and now we see that more cytokine that correlate with IFN-γ play an important role.

One notable finding of this study is the presence of IL-10 that was produced at significantly higher concentrations in tuberculosis progressors than in healthy participants with and without infection, who did not progress to disease. The concentrations in the healthy uninfected participants corresponded well with those previously treated for tuberculosis disease prior to study recruitment. IL-10 is an anti-inflammatory cytokine and primarily produced by monocytes, is known to have important effects during immune regulation and inflammation. It is known to down regulate the expression of Th1 cytokines and also suppress the antigen presenting capabilities of APCs. A significant increase in IL-10 levels has been reported previously in tuberculosis patients (Hirsch *et al*., 1999) compared to TST positive people. In addition, Djoba Siawaya *et al*. (2009) also showed that the levels of IL-10 were significantly higher in slow responders early during treatment suggesting that an early increased anti-inflammatory response during treatment may lead to the delay of sputum culture conversion in patients. Two genetic studies also found that IL-10 plays a role during tuberculosis. They concluded that IL-10 had some specific effect on tuberculosis, by determining the disease form and severity, and that IL-10 is not necessarily associated with susceptibility to disease (Pacheco *et al*., 2008; Ansari *et al*., 2009).

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In a study that investigated serum IL-10 concentrations before and after tuberculosis treatment it was found that IL-10 concentrations are significantly elevated before treatment and decrease again after completion of treatment (Hari Sai Priya *et al*., 2009). However, Hirsch *et al*. (1999) showed that in tuberculosis patients on chemotherapy for active disease, IL-10 levels returned to baseline levels as soon as 3 months. It is clear from other work that active tuberculosis is associated with elevated IL-10 levels, but here we show that even up to 3 years prior to the development of tuberculosis disease and initiation of treatment, IL-10 levels were significantly up regulated compared to healthy controls. In the present study a very small number of participants progressed to tuberculosis disease and comparison to a large group of healthy controls may not be wholly appropriate. It is also important to consider that more differences between cytokines could potentially show if the sample size was bigger. There are other limitations to this study. No clinical data were available for these participants apart from TST results. In addition, no data were collected capturing tuberculosis exposure of these participants. Data collected retrospectively were done by using the local tuberculosis register whereas follow up data of these participants would have been ideal.

In summary, we propose that the cytokine concentrations presented here are representative of a community with high levels of tuberculosis exposure and infection. We also present age related references cytokine values. Our results suggest that the outcome of *M.tb* infection is dependent on a balance of pro- and anti-inflammatory factors. Receiver operating characteristic (ROC) analysis can be used to define diagnostic cut offs for positivity of interesting cytokines and this can also be used to determine the percentage of responders. Future studies should include the flow cytometric analysis of cell types that can be responsible for the IL-10 production under these conditions. In addition, transcriptional analysis of the IL-10 gene can also be done and since IL-10 regulates the immune profile, future research should be directed towards determining the exact role of IL-10 during tuberculosis infection as this cytokine is definitely an important mediator in progression of tuberculosis disease.

Acknowledgements

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Chapter 3

Cytokine profiles in children with documented *Mycobacterium tuberculosis* exposure and infection

Introduction

The aim of this work presented in this chapter was to determine the differences in cytokine profiles of children in a high tuberculosis incidence setting with known tuberculosis exposure and infection and those who have no known exposure and no infection. In Chapter 2, we identified cytokines that were able to distinguish between TST positive and TST negative children and adults. Therefore, we decided to investigate the immune response against tuberculosis further. Here we included an exposure gradient of tuberculosis and identified children with documented exposure. The QFT was included as a measure of infection in addition to the TST. The investigation was done by measuring the cytokine profiles in stimulated plasma samples of participants over time. The information gained from this study will reveal the dynamics of the immune response against tuberculosis infection and exposure in children. Identification of biomarkers will aid in tuberculosis diagnosis and early determination of treatment outcome and can be used in the future in vaccine design.

Methods

Participants

Participants in this nested immunological sub study were included from a larger on-going prospective cohort study of HIV uninfected children with different degrees of *M.tb* exposure. Participants were recruited and followed up between February 2008 and May 2009. Enrolment was postponed if a TST was placed or if the child was immunized in the preceding 6 weeks. All participants were investigated for tuberculosis disease through a symptom-based questionnaire, mycobacterial culture or sputum (gastric aspirate in children < 5 years of age) and chest radiography. Since no gold standard exist for measuring *M.tb* infection a formula is used to calculate a 'contact score' for each household member (Shams *et al.*, 2005; Hesseling *et al.*, 2009). This score quantifies the extent of *M.tb* exposure and therefore was used as proxy for infection. Children exposed to newly diagnosed adult tuberculosis index cases in the household and children selected from "control" adjacent neighbouring households without a known tuberculosis index case, were included in this study. All participants were followed serially at 3 months and again at 6 months after

recruitment. During this time no significant changes in tuberculosis exposure was seen. In this high tuberculosis incidence setting of Ravensmead and Uitsig the notification rate of tuberculosis in children was 407/100000 in 2004 (Marais *et al*., 2006). In Khayelitsha alone, it is estimated that the tuberculosis incidence rate was 1500/100000 in 2009, amongst the highest in the world (DOH, 2009). BCG vaccination at birth is routine in the study area. The Mantoux skin test was carried out on all participants using *M. tuberculosis* PPD (2 T.U., Statens Serum Institut, Copenhagen, Denmark) and induration (mm) was read between 48-72 h after the test using the ball-point pen and ruler method (Jordan *et al*., 1987; Bouros *et al*., 1989). QFT was completed at baseline, month 3 and month 6; TST at baseline and month 3. A positive TST was defined as an induration of ≥ 10 mm as per National Tuberculosis Control Management Guidelines (DOH, 2009).

The investigation was approved by the Ethics Review Committee of the Faculty of Health Sciences, University of Stellenbosch, South Africa (N05/07/129). All participants in this study or their legal guardians provided written informed consent for participation in the study.

Eligibility

Study groups were chosen based on two criteria, 1) *M.tb* contact score (Hesseling *et al*., 2009; Table 5), and 2) *M.tb* infection status as measured by the TST and QFT tests. When the *M.tb* contact score was ≥ 4 (determined by variables described in Table 5) the participant was classified as 'Tuberculosis Exposed', < 4 the participant were 'Tuberculosis Unexposed'. When the TST and QFT were both positive the participant was classified as 'Infected' and when both tests were negative 'Uninfected'. Participants were included in this study when their infection and exposure status remained unchanged for the next 6 months. In each the exposed infected and exposed uninfected groups 17 participants were included and in the unexposed uninfected control group only 11 participants were included.

QuantiFeron Gold In tube (QFT)

The QFT test was performed on all study subjects and interpreted for latent tuberculosis infection according to the manufacturer's instructions (Cellestis Limited, Carnegie, Victoria, Australia). In short, 1mL of whole blood was stimulated in each a nil, mitogen (PHA coated) or antigen tube (coated with a peptide cocktail of ESAT-6, CFP-10 and TB7.7). Tubes were incubated overnight at 37 °C with 5 % CO_2 and plasma was assayed for IFN-γ production by ELISA. For the ELISA plate was coated with conjugate and samples, controls and standards were added to the appropriate wells. As additional quality control, a 7 day PHA stimulated supernatant from a healthy volunteer was used on all plates in this experiment. Plates were incubated at room temperature for 2 hours and then washed. Enzyme substrate solution was added to all wells and the plate mix on a micro plate shaker

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for 5 min. This was followed by incubation at room temperature in the dark for 30 min. Enzyme stopping solution was added to each well and mixed again. Plate was read at 450 nm, with a 650 nm reference filter. A standard curve was used in each assay ranging from 0.25 IU/mL to 4 IU/mL. QFT analysis software (Cellestis) was used to analyse raw data and to calculate results.

Multiplex immunoassay

The Multiplex assays were done according to manufacturer's instructions (Lincoplex, cat no. HCYTO-60K, Millipore, Billerica, MA, USA). In short, the 96-well filter plate was blocked with assay buffer. The 21 antibody covered beads were sonicated separately and then added together. This was followed by adding the samples and the mixed beads in duplicate to the appropriate wells. After 1 hour incubation on a shaker, the fluid was removed by a vacuum pump followed by two wash steps with wash buffer. The biotinylated detection antibody cocktail was added into each well and left to incubate on a shaker for 30 min where after the streptavidin-phycoerythrin containing the detection antibody cocktail was added to each well. After 30 min incubation on a shaker, a vacuum pump gently removed the contents. After another two wash steps sheath fluid was added to all the wells and put on a shaker for 5 min to allow the beads to resuspend. The beads were analysed immediately on a Bio-plex array reader (Bio-rad, Hercules, CA, USA). A standard curve was used in each assay ranging from 3.2 pg/ml to 10 000 pg/ml. Quality controls were provided with each kit to measure the reliability and precision of the selected cytokines. All 21 cytokines measured fell into the manufacturer's expected range. As additional quality control, a 7 day PHA stimulated supernatant from a healthy volunteer was used on all plates in this experiment. For all subsequent analysis, the average of the duplicate measurements was used.

Statistical analysis

All statistical analyses were conducted using Statistica version 9 (StatSoft, Tulsa, OK, USA) and Graphpad Prism (version 5 for Windows, GraphPad Software, San Diego California, USA). All results were transformed using the logarithm base-10 to reduce variance in distributions. The nonparametric Wilcoxon matched pairs test was used to determine differences between stimulated and unstimulated. For all further analysis unstimulated (nil) values have been subtracted from the antigen stimulated values. Groups (exposed infected compared to exposed uninfected and exposed uninfected compared to unexposed uninfected) were analysed by nonparametric ANOVA with the Dunn's post-test. The degree of association between cytokines was calculated using Spearman's nonparametric correlation coefficient. $P \leq 0.05$ was considered significant.

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Principal component analysis and heat maps

Principal component analysis (PCA) was performed and heat maps generated using the Omics Explorer, Version 2.0 Beta (Qlucore AB, Lund, Sweden). The PCA operation does not make any assumptions regarding the data.

PCA is appropriate when you have obtained measures on a number of observed variables, in this case cytokines, and wish to develop a smaller number of artificial variables (called principal components) that will account for most of the variance in the observed variables. PCA is a variable reduction procedure used when you believe that, there is some redundancy in the measured cytokine concentrations. In this case, redundancy means that some of the cytokines are correlated with one another. This transformation is defined in such a way that the first principal component has as high a variance as possible (that is, accounts for as much of the variability in the data as possible), and each succeeding component in turn has the highest variance possible under the constraint that it be orthogonal to the preceding components. The principal components may then be used as predictor in subsequent analyses. Qlucore has the added advantage of generating heat maps from the PCA analysis. Heat map plots are coloured using normalized data. Each variable has been normalized to mean 0 and variance 1. The colour red corresponds to +2 and green to ‐2.

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Table 5: Formula for calculating *M.tb* contact score

Variable	Weight assigned
Relationship to TB index	
No known TB contact	0
Non-household TB contact	1
Relative/other contact in household with TB	2
Secondary caregiver (care provider during day) in household with TB	3
Primary caregiver in household with TB	4
Infectivity of TB index case	
No known TB contact	0
Sputum acid-fast negative	2
Sputum acid-fast positive	4
Type of exposure to TB index case	
No known TB exposure	0
Lives and sleeps in different house	1
Lives and sleeps in same house	2
Sleeps in same room	3
Duration (total hours) average contact per day with TB index case	
No known TB contact	0
0-3 hours	1
4-7 hours	2
8-11 hours	3
≥ 12 hours	4
Total contact score (maximum = 15)	

Table reproduced from Hesseling *et al*. (2009) with permission. TB = tuberculosis.

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Results

Demographics

Forty five participants were included in the study. The median age ranged from 3.4 to 7.5 years with 58.8 % male and 41.2 % female (Table 6). Exposed infected group consisted of QFT and TST positive and positive contact score individuals. Exposed uninfected group consisted of QFT and TST negative and contact score positive individuals. The unexposed uninfected group consisted of individuals that are QFT and TST negative as well as contact score negative. TST results indicated that TST indurations increased within 3 month in the positive group (Figure 10).

Table 6: Demographic and clinical characteristics of study subjects

	Exposed Infected (n=17)	Exposed Uninfected (n=17)	Unexposed Uninfected (n=11)
Median age (years)[1]	6.8 (1.4-14.5)	3.4 (1-10.1)	7.5 (0.6-11.4)
Median TST induration at baseline (mm)[1]	17 (11-23)	0 (0)	0 (0)
Median TST induration at 3 months (mm)[1]	20 (14-28.7)	0 (0)	0 (0)
Median contact score[2]	7 (4-15)	8 (4-13)	0 (0-3)
Female[3]	7 (41.2%)	9 (52.9%)	11 (100.0%)

[1]Median is shown with range in brackets; [2]High contact score defined as ≥4 and a low score defined by <4; [3]Numbers of subjects, followed by the percentages of subjects relative to the total number of subjects in each age group in brackets, are shown. TST = tuberculin skin test.

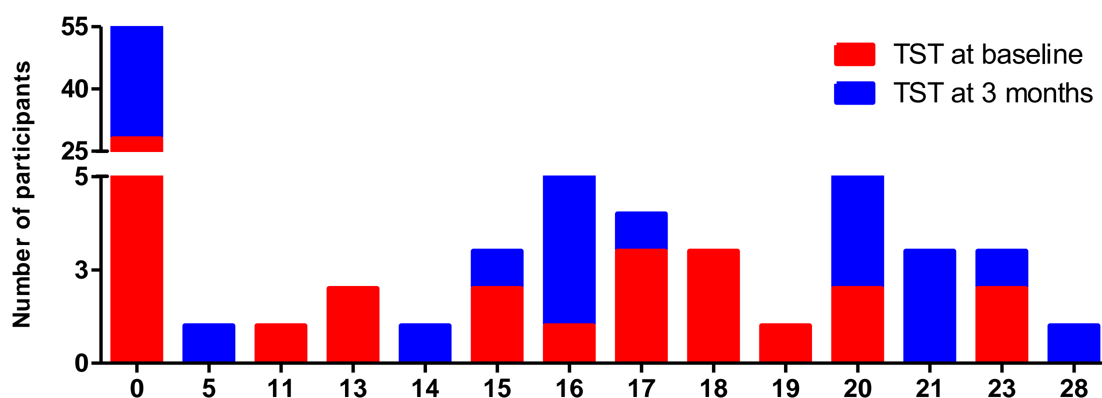

Figure 10: Distribution of TST readings (in mm) between measurement at baseline and at 3 months.

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Cytokine concentrations in population

Considering that, only young children were included in the study (Table 6) we do not expect to see any age related variance in the data and this was confirmed by doing a one-way ANOVA test of variance. Further analysis were performed in children under 16 years looking at specific cytokine concentrations and it was found that adjusting for age had no effect on the outcome of results (comparative plots shown in appendix 1). Higher unstimulated values were found compared to the work presented in Chapter 2. A number of factors could contribute to the higher values: firstly, it could be due to changes in the optics of the luminex machine over the years; secondly a number of operators were involved in running these tests; and thirdly it could be contributed to the differences study populations. Careful analysis of the reader data did show that high readings were found for all subjects and cannot be contributed to individual samples.

In the unstimulated samples, all cytokines were found above the lower limit of detection for the assay (Table 7 and Table 8). The unstimulated concentration of IL-8 was the highest measured at 10020.4 pg/ml (exposed infected), 17234.5 pg/ml (exposed uninfected), 9027.0 pg/ml (unexposed uninfected) at baseline (Table 7). Regardless of the group IL-1α (exposed infected, $p = 0.0004$; exposed uninfected, $p = 0.0016$; unexposed uninfected, $p = 0.0076$) and IP-10 (exposed infected, $p = 0.0003$; exposed uninfected, $p = 0.0056$; unexposed uninfected, $p = 0.0058$) was significantly increased after stimulation with the mycobacterial antigens. MCP-1 (exposed infected, $p = 0.0003$; exposed uninfected, $p = 0.0148$; unexposed uninfected, $p = 0.0058$) was decreased after stimulation in all three groups. In the unexposed uninfected group IL-8 ($p = 0.0409$), IL-10 ($p = 0.0208$), G-CSF ($p = 0.0128$) and TNF-α ($p = 0.0329$) were all significantly decreased upon stimulation.

In the exposed uninfected group IL-1β ($p = 0.0065$) was significantly increased upon stimulation. In the exposed infected group IL-8 ($p = 0.0395$), IL-10 ($p = 0.0231$) and G-CSF ($p = 0.0148$) were decreased upon stimulation. IL-2 ($p = 0.0003$), IL-5 ($p = 0.0277$), IL-13 ($p = 0.0049$) and IFN-γ ($p = 0.0031$) were only increased in the exposed infected group.

Table 7: Median responses in 21 cytokines as measured at recruitment in all participants with and without tuberculosis exposure and infection

	Exposed Infected				**Exposed Uninfected**				**Unexposed Uninfected**			
	M.tb antigen stimulated			Unstimulated	*M.tb* antigen stimulated			Unstimulated	*M.tb* antigen stimulated			Unstimulated
Cytokine	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)
IL-1β	148.5	1.4	p=0.4074	106.7	178.8	2.3	p=0.0065	77.7	71.0	0.8	p=0.3281	87.5
IL-2	538.8	49.4	p=0.0003	10.9	11.9	1.0	p=0.5695	12.3	14.8	1.2	p=0.1823	12.6
IL-4	150.9	1.1	p=0.4631	132.6	202.2	1.1	p=0.6874	176.5	620.3	1.3	p=0.9292	476.7
IL-5	6.3	1.4	p=0.0277	4.6	2.9	1.1	p=0.7960	2.6	8.2	1.6	p=0.9292	5.2
IL-6	1434.2	0.8	p=0.2274	1695.2	1057.9	1.4	p=0.3812	763.3	1309.1	0.5	p=0.0619	2395.6
IL-7	67.0	1.0	p=0.0684	70.0	79.7	1.1	p=0.8684	75.8	75.6	0.8	p=0.0619	90.6
IL-8	4965.0	0.5	p=0.0395	10020.4	17608.8	1.0	p=0.7226	17234.5	4379.5	0.5	p=0.0409	9027.0
IL-10	48.0	0.5	p=0.0231	96.3	24.3	0.7	p=0.8068	33.7	50.5	0.4	p=0.0208	122.6
IL-12p70	18.5	0.9	p=0.5540	20.8	22.4	1.0	p=0.3560	23.2	27.5	0.9	p=0.7221	30.4
IL-13	55.8	2.6	p=0.0049	21.9	16.2	1.3	p=0.8684	12.7	14.4	1.3	p=0.7213	10.7
IL-15	23.0	1.2	p=0.1239	18.5	28.1	1.0	p=0.4074	28.8	23.3	1.1	p=0.4769	21.5
IL-17	4.4	1.2	p=0.4631	3.8	0.0	1.0	p=0.6002	0.0	4.8	1.5	p=0.8785	3.1
IL-1α	2263.9	3.3	p=0.0004	676.9	670.8	1.4	p=0.0016	487.8	1698.2	1.7	p=0.0076	1002.3
IFN-γ	339.4	9.6	p=0.0031	35.3	54.5	1.1	p=0.5862	48.4	45.8	1.1	p=0.5940	43.1
G-CSF	316.2	0.7	p=0.0148	456.0	721.5	1.1	p=0.1626	681.6	273.2	0.5	p=0.0128	497.4
GM-CSF	162.6	1.0	p=0.7226	162.9	102.3	1.1	p=0.7226	94.8	168.0	0.9	p=0.0912	185.0
TNF-α	129.1	1.4	p=0.7946	94.9	113.8	0.9	p=0.4074	130.5	54.4	0.4	p=0.0329	133.4
Eotaxin	212.6	1.2	p=0.3088	179.1	187.2	1.0	p=0.0929	196.6	221.2	0.9	p=0.1823	242.1
MCP-1	1371.6	0.3	p=0.0003	5434.7	3274.9	0.8	p=0.0148	4363.8	1208.7	0.3	p=0.0058	3897.6
MIP-1α	2166.9	1.1	p=0.4631	1913.7	2470.8	1.1	p=0.7946	2173.4	1457.9	0.6	p=0.0505	2244.2
IP-10	29989.8	19.8	p=0.0003	1513.4	1517.1	1.4	p=0.0056	1052.4	3869.7	4.7	p=0.0058	824.0

Supernatants from unstimulated and stimulated whole blood were tested for 21 cytokines. Median fold differences and *P* values for significance testing by the Wilcoxon test are also presented. Values in red are representative of significant decrease from unstimulated median. *M.tb = Mycobacterium tuberculosis*.

Table 8: Median responses in 21 cytokines as measured at month 6 in all participants with tuberculosis exposure and with or without infection

	Exposed Infected				Exposed Uninfected			
	M.tb antigen stimulated			Unstimulated	*M.tb* antigen stimulated			Unstimulated
Cytokine	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)
IL-1β	156.5	1.2	p=0.0395	128.2	78.1	1.0	p=0.8313	78.5
IL-2	520.3	71.1	p=0.0003	7.3	8.2	1.1	p=0.6417	7.4
IL-4	112.8	1.3	p=0.0386	88.5	107.1	0.8	p=0.5862	129.1
IL-5	7.9	3.7	p=0.0065	2.1	0.7	0.9	p=0.7580	0.8
IL-6	1881.3	0.8	p=0.5228	2220.5	1899.5	0.8	p=0.0929	2510.7
IL-7	70.6	0.9	p=0.6529	78.3	67.6	1.0	p=0.3560	67.3
IL-8	8899.6	1.0	p=0.4348	8561.3	8463.8	0.9	p=0.2274	9392.8
IL-10	44.6	0.7	p=0.3318	68.4	75.9	0.7	p=0.1128	113.0
IL-12p70	13.2	1.2	p=0.4348	11.0	14.2	0.9	p=0.4380	15.8
IL-13	124.8	7.6	p=0.0036	16.5	7.6	1.0	p=0.1914	7.9
IL-15	14.2	1.6	p=0.0129	9.1	8.2	1.1	p=0.9433	7.5
IL-17	3.5	1.1	p=0.1477	3.3	1.7	0.7	p=0.9375	2.3
IL-1α	2686.1	2.3	p=0.0003	1191.1	1255.5	1.7	p=0.0005	760.6
IFN-γ	344.4	15.6	p=0.0003	22.1	14.8	1.0	p=0.4925	15.6
G-CSF	331.1	1.0	p=0.6192	317.4	344.4	0.8	p=0.0099	452.4
GM-CSF	250.7	1.0	p=0.0495	252.5	228.3	1.0	p=0.5228	230.9
TNF-α	182.8	1.9	p=0.0217	95.9	73.5	0.6	p=0.0049	125.7
Eotaxin	169.8	1.0	p=0.0086	175.8	182.6	0.9	p=0.6529	195.0
MCP-1	6779.1	0.8	p=0.4631	7980.9	3255.9	0.4	p=0.0031	8032.3
MIP-1α	3558.0	1.3	p=0.0313	2815.2	3171.5	1.0	p=0.4631	3032.6
IP-10	26445.9	7.5	p=0.0003	3542.7	1996.3	2.2	p=0.0004	891.5

Supernatants from unstimulated and stimulated whole blood were tested for 21 cytokines. Median fold differences and *P* values for significance testing by the Wilcoxon test are also presented. Values in red are representative of significant decrease from unstimulated median. *M.tb* = *Mycobacterium tuberculosis*.

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At month 6 (Table 8) IL-1α (exposed infected, $p = 0.0003$; exposed uninfected, $p = 0.0005$) and IP-10 (exposed infected, $p = 0.0003$; exposed uninfected, $p = 0.0004$) was increased significantly upon stimulation. In the exposed infected group IL-1β ($p = 0.0395$), IL-2 ($p = 0.0003$), IL-4 ($p = 0.0386$), IL-5 ($p = 0.0065$), IL-13 ($p = 0.0036$), IL-15 ($p = 0.0129$), IFN-γ ($p = 0.0003$), TNF-α ($p = 0.0217$) and MIP-1 ($p = 0.0313$) was increased upon stimulation. Whereas GM-CSF ($p = 0.0495$) and eotaxin ($p = 0.0086$) were decreased upon stimulation. In the exposed uninfected group G-CSF ($p = 0.0099$), TNF-α ($p = 0.0049$) and MCP-1 ($p = 0.0031$) were all decreased upon stimulation.

Cytokines that correlate with IFN-γ

IFN-γ is used in commercial assays as a proxy for tuberculosis infection. To assess which other cytokines measured correlate with this Th1 cytokine Spearman correlation was performed. The highest correlations was seen with IL-2 ($r = 0.90$, $p < 0.001$), IP-10 ($r = 0.76$, $p < 0.001$) and IL-13 ($r = 0.71$, $p < 0.001$) after stimulation (Table 9). IL-2 ($r = 0.73$, $p < 0.001$) and IL-15 ($r = 0.72$, $p < 0.001$) showed strong correlation with IFN-γ in the unstimulated group (Table 9).

Cytokine differences in the whole group

PCA applying multiple group comparison, for comparing more than two groups simultaneously, differentiates exposed infected from both exposed uninfected and unexposed uninfected in a significant way ($p = 9.6 \times 10^{-9}$, $q = 2.2592 \times 10^{-8}$; Figure 14). Heat map of the whole study group indicates that there are clear differences between the 3 study groups (Figure 13 A) but only after the multiple group comparison IFN-γ and IL-2 (as measured at month 6) and IL-2 (as measured at baseline) showed significant differences (Figure 13 B).

Cytokine differences between exposed infected and exposed uninfected participants

IL-2 (baseline, $p \leq 0.001$; month 6, $p \leq 0.001$), IL-13 (baseline, $p \leq 0.01$; month 6, $p \leq 0.001$), IL-1α (baseline, $p \leq 0.001$; month 6, $p \leq 0.01$) and IP-10 (baseline, $p \leq 0.001$; month 6, $p \leq 0.001$) was increased in the exposed infected when compared to the exposed uninfected group. At month 6 IL-5 ($p \leq 0.001$) and TNF-α ($p \leq 0.01$) was increased in the exposed infected when compared to the exposed uninfected group (Figure 11, B-H). Unstimulated IL-17 showed significant differences ($p < 0.05$) between the exposed infected and exposed uninfected groups at baseline (Figure 12). PCA applying two group comparison differentiates exposed infected from exposed uninfected significantly at baseline by IP-10 and IL-2 ($p = 1.6 \times 10^{-4}$, $q = 9.2385 \times 10^{-4}$, Figure 13 C) and at month 6 by IL-13 and IL-2 ($p = 9.6 \times 10^{-8}$, $q = 3.0172 \times 10^{-9}$, Figure 15 C).

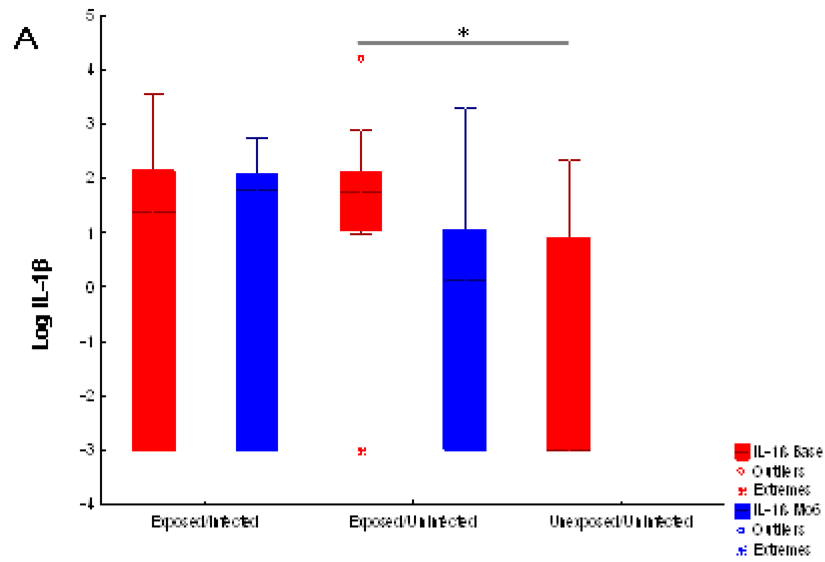
Cytokine differences between exposed uninfected and unexposed uninfected participants

IL-1β ($p \leq 0.05$) was increased in the exposed uninfected group when compared to the unexposed uninfected group at baseline. At month 6 the IL-1β concentration in the exposed uninfected was comparable to the other study groups again (Figure 11 A). Unstimulated IL-17 showed significant differences ($p < 0.05$) between the exposed uninfected and unexposed uninfected groups at baseline (Figure 12). PCA applying two group comparison differentiates exposed uninfected from unexposed uninfected significantly at baseline by IL-1β and IL-17 ($p = 0.0078$, $q = 0.4457$, Figure 13 D) and at month 6 by IL-13 and IL-2 again ($p = 0.0016$, $q = 0.0125$, Figure 15 D).

Table 9: Cytokines that correlate with IFN-γ after *M.tb* specific antigen stimulation and in the unstimulated samples

***M.tb* antigen stimulated**			**Unstimulated**		
Cytokines	Spearman r value	Spearman p-value	**Cytokines**	Spearman r value	Spearman p-value
IL-2	r = 0.90	p < 0.01	**IL-2**	r = 0.73	p < 0.01
IP-10	r = 0.76	p < 0.01	**IL-15**	r = 0.72	p < 0.01
IL-13	r = 0.71	p < 0.01	**IL-13**	r = 0.50	p < 0.01
IL-1a	r = 0.51	p < 0.01	**IL-12p70**	r = 0.48	p < 0.01
TNF-a	r = 0.26	p = 0.09	**IL-5**	r = 0.47	p < 0.01
IL-5	r = 0.25	p = 0.10	**G-CSF**	r = 0.46	p < 0.01
IL-1B	r = 0.22	p = 0.15	**IL-4**	r = 0.45	p < 0.01
IL-17	r = 0.20	p = 0.18	**IL-1B**	r = 0.40	p = 0.01
IL-15	r = 0.19	p = 0.20	**IL-17**	r = 0.38	p = 0.01
GM-CSF	r = 0.14	p = 0.37	**IL-8**	r = 0.38	p = 0.01
MIP-1a	r = 0.12	p = 0.41	**IL-7**	r = 0.37	p = 0.01
IL-8	r = 0.09	p = 0.56	**IL-10**	r = 0.36	p = 0.02
IL-6	r = 0.08	p = 0.59	**IP-10**	r = 0.34	p = 0.02
IL-12p70	r = 0.07	p = 0.65	**IL-1a**	r = 0.34	p = 0.02
MCP-1	r = 0.05	p = 0.73	**MIP-1a**	r = 0.28	p = 0.06
G-CSF	r = 0.03	p = 0.83	**TNF-a**	r = 0.21	p = 0.18
IL-7	r = 0.02	p = 0.91	**MCP-1**	r = 0.18	p = 0.24
IL-4	r = -0.06	p = 0.71	**IL-6**	r = 0.10	p = 0.53
IL-10	r = -0.07	p = 0.66	**GM-CSF**	r = 0.09	p = 0.58
Eotaxin	r = -0.13	p = 0.41	**Eotaxin**	r = -0.06	p = 0.68

M.tb = Mycobacterium tuberculosis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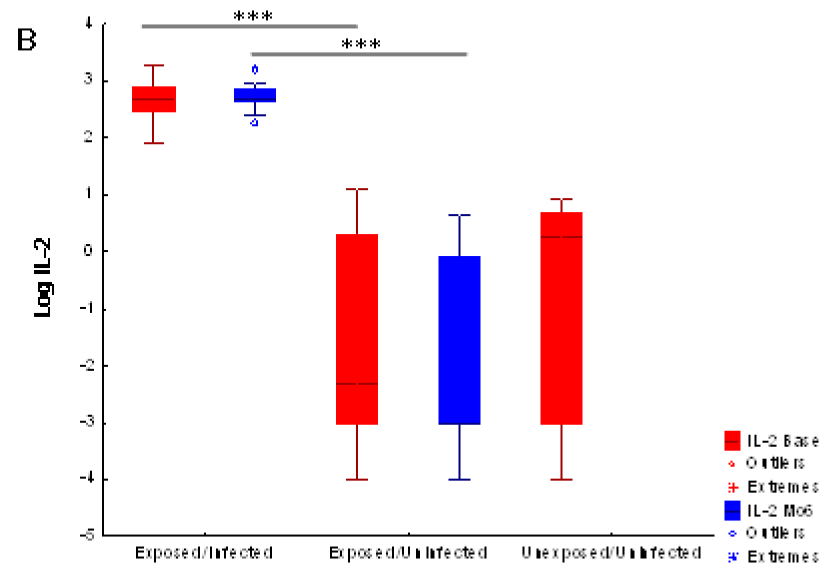
Log IL-1β
*
Exposed/Infected
Exposed/Uninfected
Unexposed/Uninfected


B
Log IL-2
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Exposed/Infected
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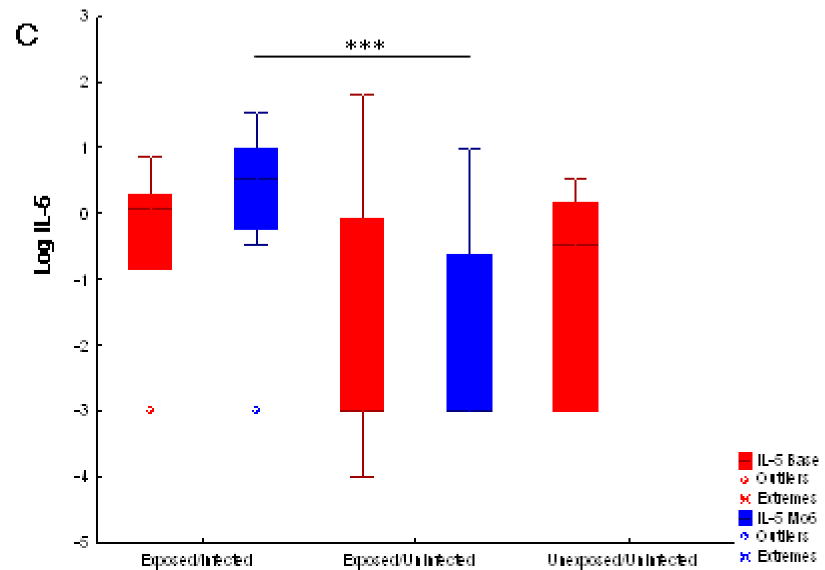
Unexposed/Uninfected
IL-2 Base
Outliers
Extremes
IL-2 Mo6
Outliers
Extremes


C
Log IL-6
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Exposed/Infected
Exposed/Uninfected
Unexposed/Uninfected
IL-6 Base
Outliers
Extremes
IL-6 Mo6
Outliers
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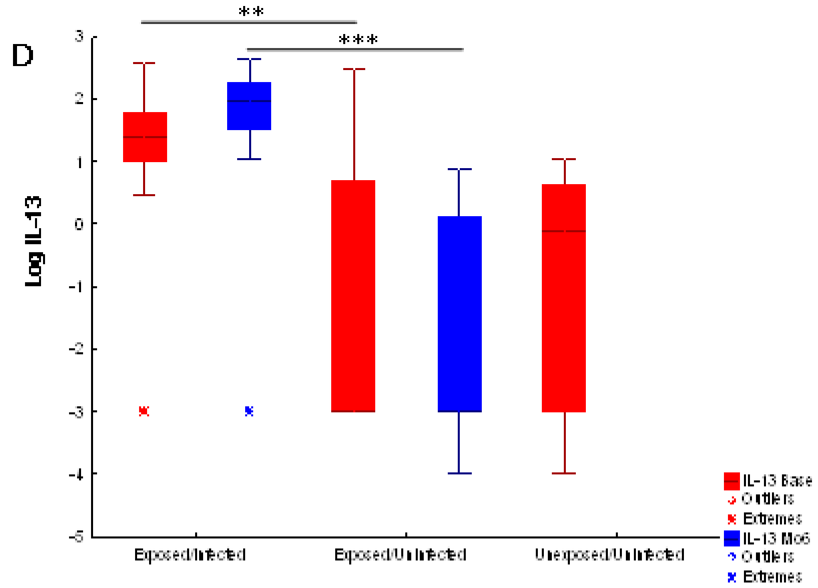

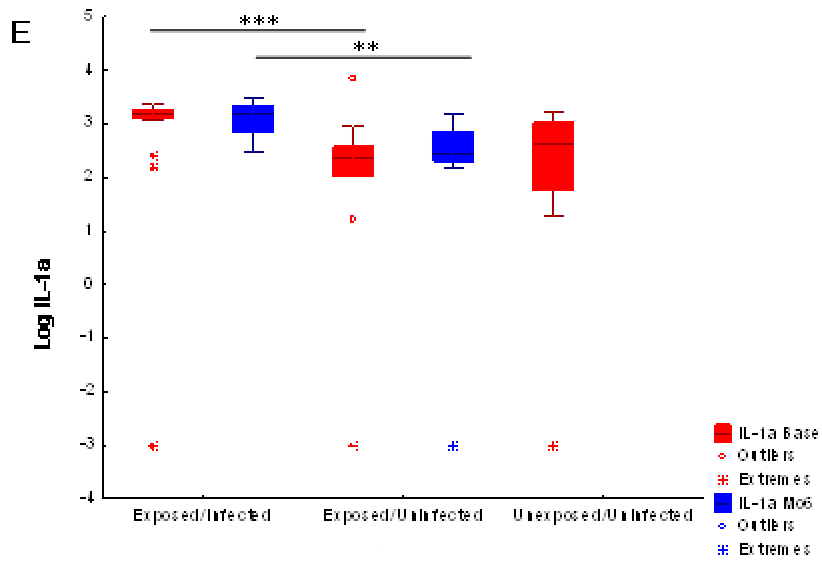

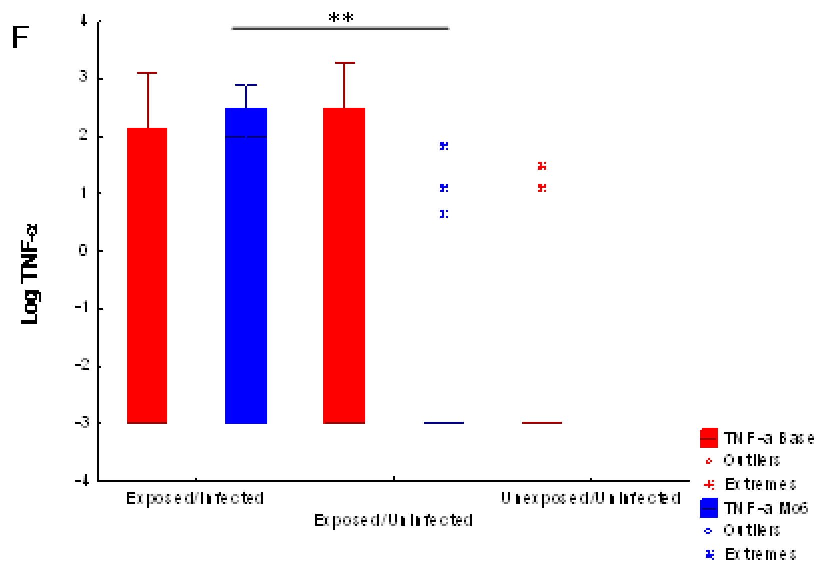

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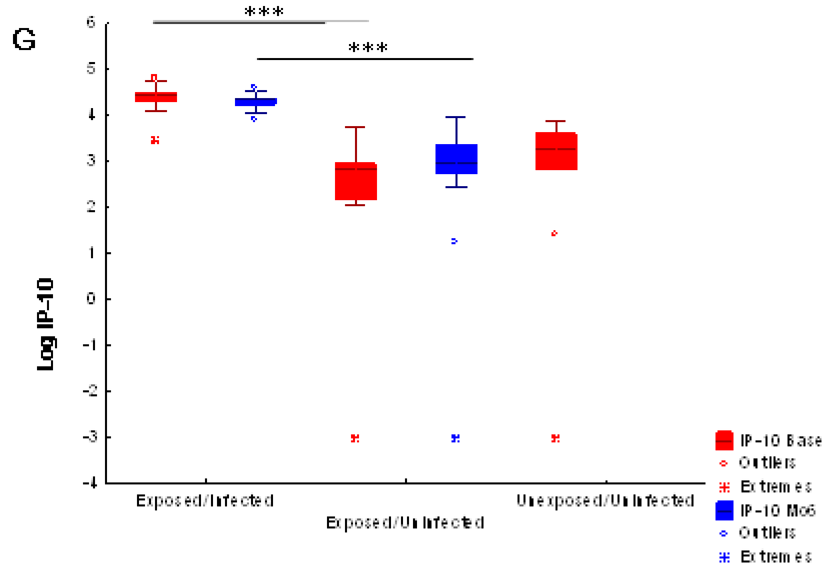

Figure 11: Cytokines that showed significant differences between the study groups. Unstimulated values were subtracted from stimulated values. Cytokines that showed significant differences between the exposed infected and exposed uninfected groups (B – H) as well as the exposed uninfected and unexposed uninfected groups (A). Red bars are baseline readings and blue bars month 6 readings. * denote significance of $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

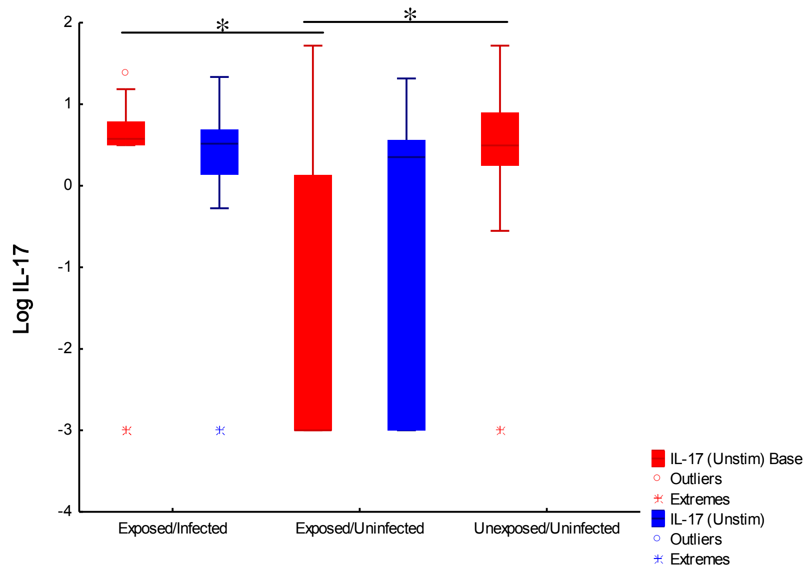

Figure 12: Unstimulated cytokines that showed a significant difference between the study groups. IL-17 showed significant differences between the exposed infected and exposed uninfected groups as well as the exposed uninfected and unexposed uninfected groups. Red bars are baseline readings and blue bars month 6 readings. * denote significance of $P \leq 0.05$.

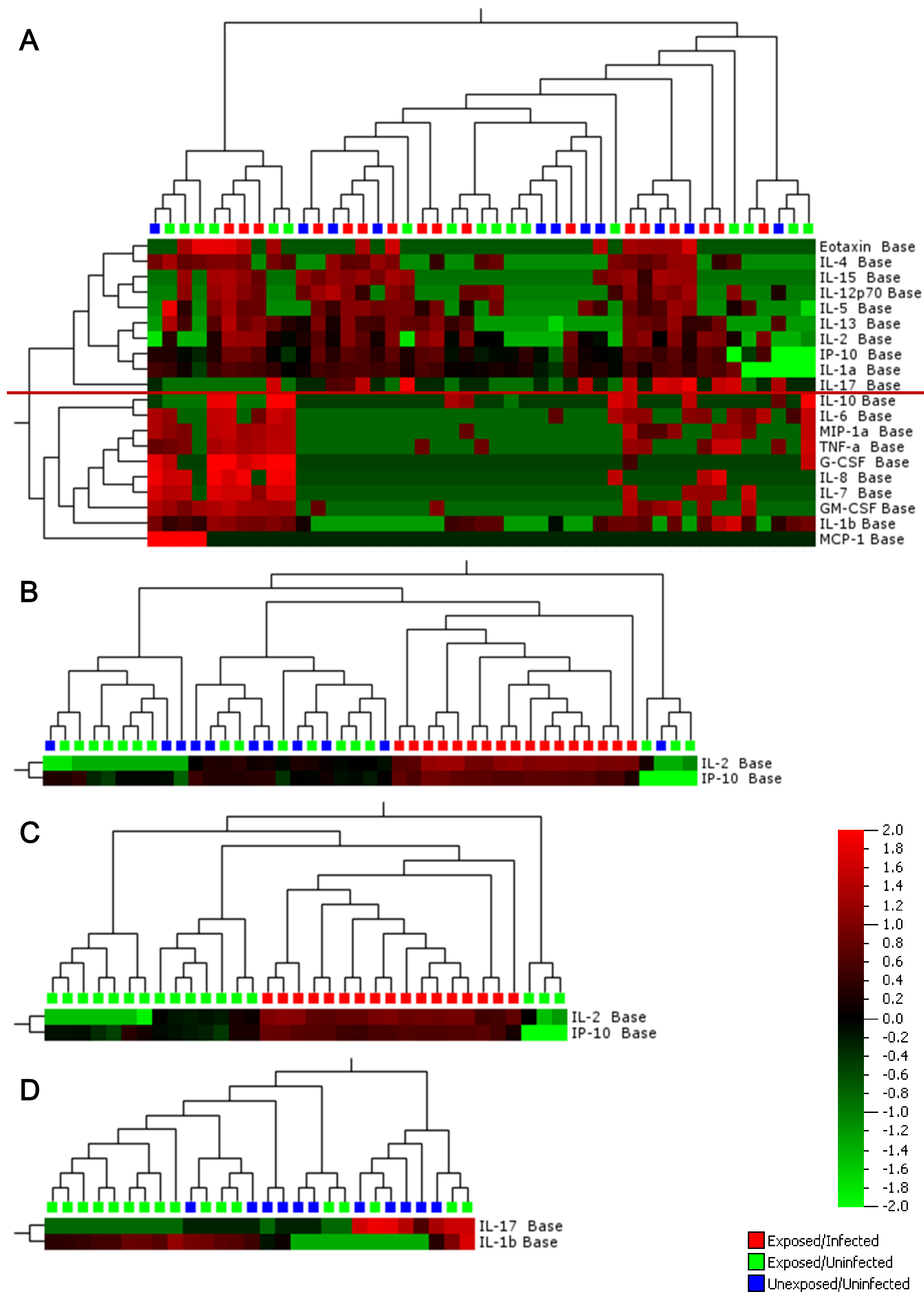

Figure 13: Heat maps at baseline ordered by hierarchical clustering (Spearman correlation with average linkage) creating a condition tree, upper horizontal edge of heat map; study grouping are the coloured blocks at the top of each profile. Heat map rows are the cytokines and columns are the participants. The whole study group (antigen – nil values) as seen before analysis (A) and after PCA (B). Red line on (A) indicates a split in the branch in the hierarchical clustering. Exposed Infected group compared to the exposed uninfected group after PCA (C). Exposed uninfected compared to unexposed uninfected after PCA (D).

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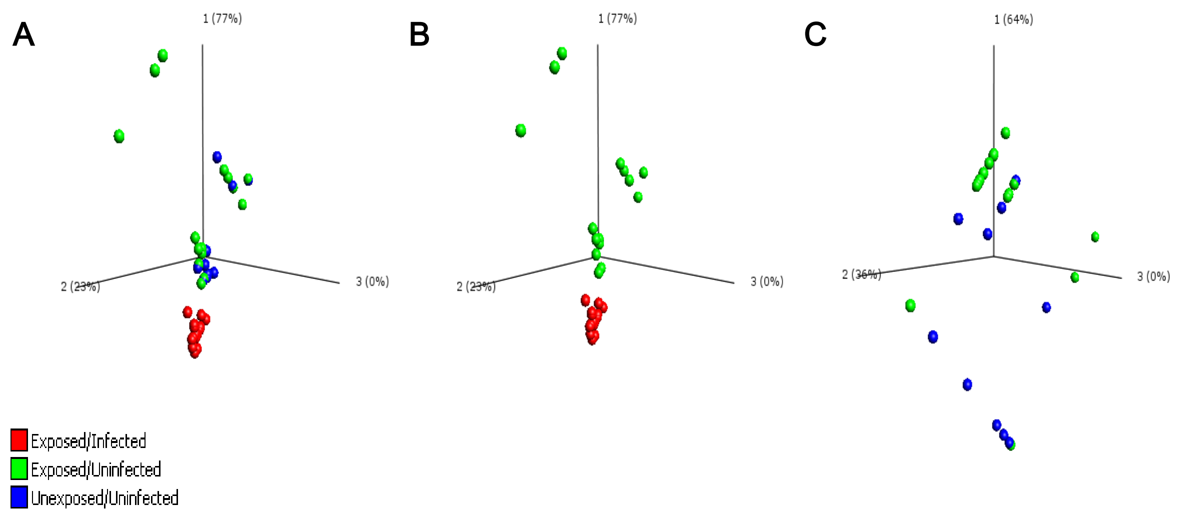

Figure 14: Principal component analysis (PCA) plots at baseline. The whole group as seen (A) after analysis. Exposed Infected group compared to exposed uninfected group after PCA (B). Exposed uninfected compared to unexposed uninfected after PCA (C).

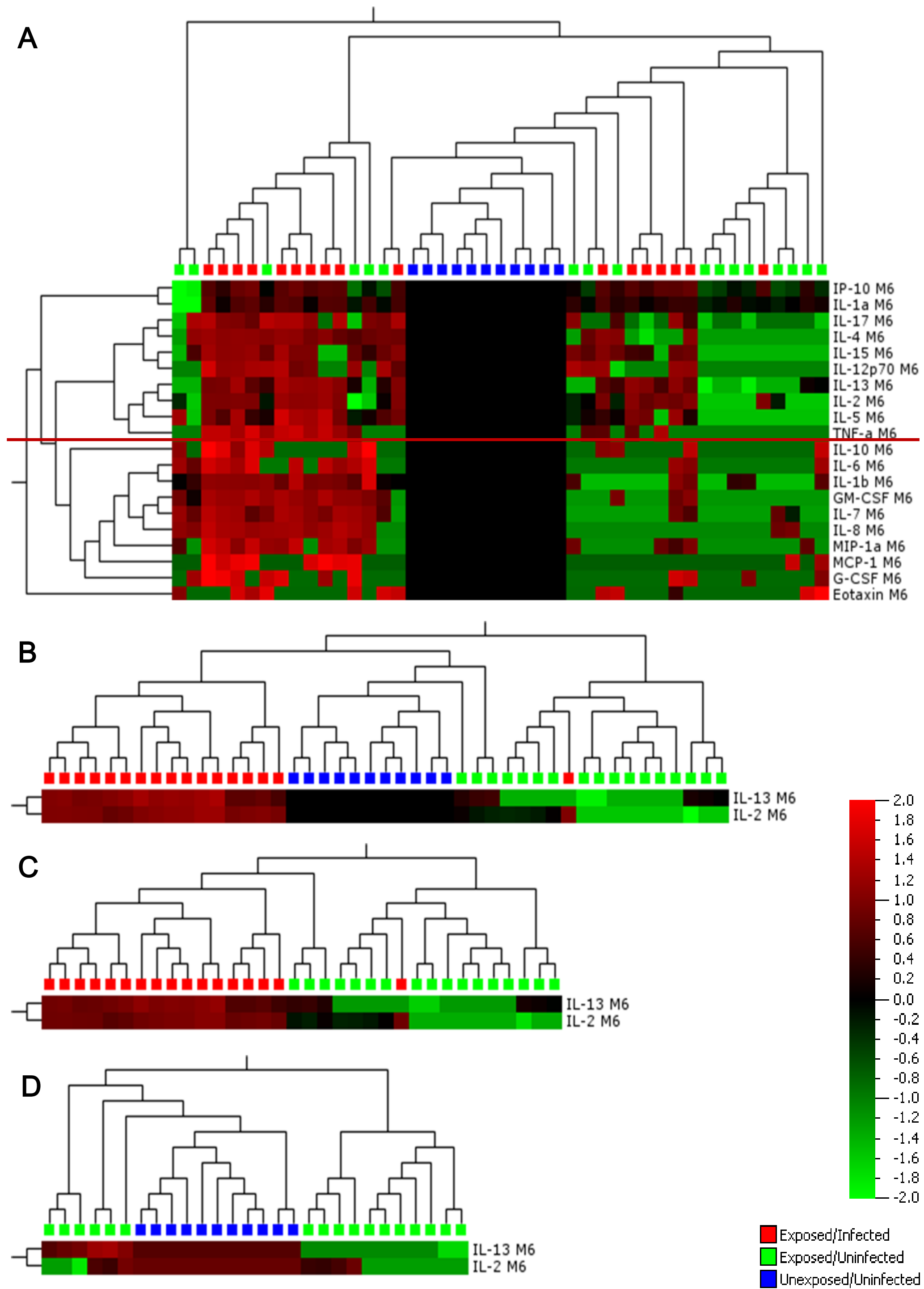

Figure 15: Heat maps at 6 months ordered by hierarchical clustering (Spearman correlation with average linkage) creating a condition tree, upper horizontal edge of heat map; study grouping are the coloured blocks at the top of each profile. Heat map rows are the cytokines and columns are the participants. The whole group (antigen – nil values) as seen before analysis (A) and after analysis (B). Red line on (A) indicates a split in the branch in the hierarchical clustering. Exposed Infected group compared to exposed uninfected group after PCA (C). Exposed uninfected compared to unexposed uninfected after PCA (D).

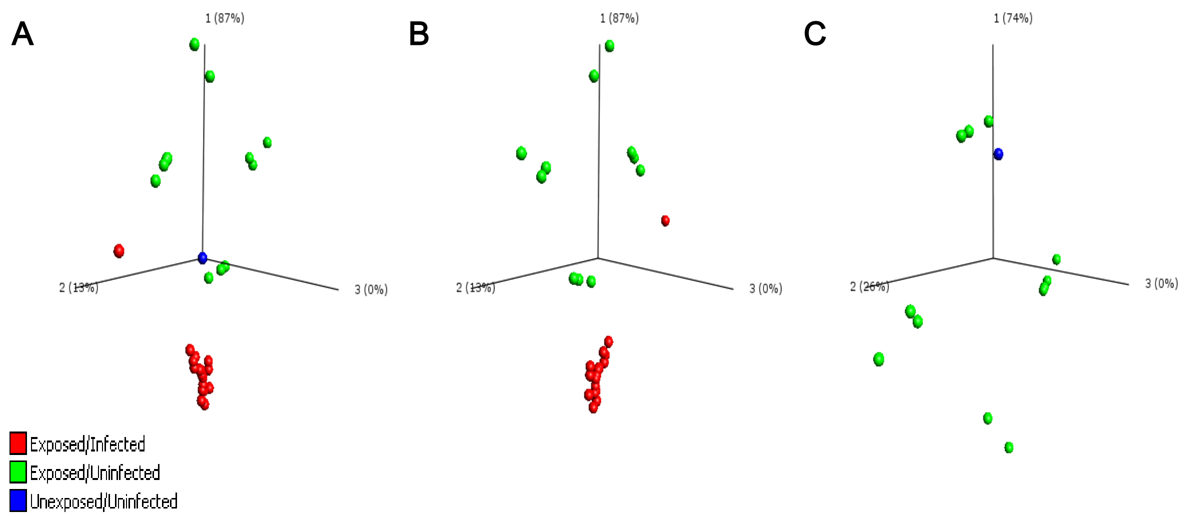

Figure 16: Principal component analysis at 6 months. The whole group as seen after PCA (A). Exposed Infected group compared to exposed uninfected group after PCA (B). Exposed uninfected compared to unexposed uninfected after PCA (C).

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Discussion

This study was designed with the main aim to investigate the differences in the cytokine profiles of children living in households with tuberculosis exposure and those children living in neighbouring households with no known tuberculosis exposure. In the tuberculosis exposed group there was distinguished between children with known tuberculosis infection and those who were not infected at time of recruitment. Infection was measured by a positive result in the commercial QFT assay and the TST.

As discussed in Chapter 1, the QFT assay is used for the detection of latent tuberculosis by measuring IFN-γ production after whole blood stimulation with ESAT-6, CFP-10 and TB7.7. Several studies have demonstrated the specificity and sensitivity of this assay, and excellent results obtained in those with latent tuberculosis (Arend *et al*., 2001; Mazurek *et al*., 2005; Pai *et al*., 2006). IGRAs have demonstrated excellent specificity and shown a closer correlation with the exposure gradient than the TST (Ewer *et al*., 2003; Menzies *et al*., 2007; Arend *et al*., 2007). Therefore, we can use the QFT as a measure of infection in our study. Cytokine concentrations were determined in unstimulated and the QFT stimulated plasma samples. In the unstimulated samples, all cytokines tested were above the lower limit of detection. After 7 day stimulation some cytokines were not detected by the Multiplex assay as found in Chapter 2. In this investigation after overnight stimulation, all measured cytokines were detected by the same assay.

Investigating the effects of whole blood stimulation, we saw that IL-1α (a pro-inflammatory cytokine) and IP-10 (an IFN-γ induced chemokine) was significantly increased and MCP-1, another chemokine, decreased upon stimulation in all study groups. The same trends were seen for IL-1α, IP-10 and MCP-1 at month 6. At baseline in the tuberculosis exposed infected group, IL-2 and IFN-γ, both Th1 cytokines, and the Th2 cytokine, IL-13 was significantly increased. However, in the same group IL-8 (chemokine), IL-10 (anti-inflammatory cytokine) and G-CSF (hematopoietic cytokine) were decreased upon stimulation. In the exposed but uninfected group, IL-1β (a pro-inflammatory cytokine) was significantly increased upon stimulation. From the results, we see that stimulation resulted in an increase of cytokines that are released by adaptive immune cells. When one thinks about the immune response against *M.tb* you would normally expect to see Th1 cytokines. However, we see both Th1 and Th2 cytokines are up regulated. This could be to maintain a balance in the immune response against *M.tb* antigens. In the inflammatory cytokines and chemokines we also see a balancing effect during stimulation. It has been shown that IL-10 and IL-13 down regulate *M.tb* induced IL-8, IP-10, RANTES and MCP-1 secretion in a cell line (Wright & Friedland, 2003)

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contributing to the complexity of the immune response and indicating how the response is regulated.

Hierarchical clustering, based on correlations, in the heat maps of the whole group showed all cytokines group into two distinct groups the first containing cytokines (containing IP-10, IL-1, IL-2, IL-4, IL-17, TNF-α amongst others) that are mainly involved in the inflammatory response, cellular movement and immune cell trafficking. In the second group (containing IL-1β, IL-10, IL-6, GM-CSF, G-CSF, MCP-1) in cellular movement, hematological system development and function and immune cell trafficking. This indicates that the first group of cytokines, containing those that correlate strongly with IFN-γ (IL-2, IL-13, IL-1a and IP-10) are all involved in the inflammatory response against *M.tb* and are up regulated as indicated by the red blocks in Figures 13 and 15.

Cytokines that showed differences between the tuberculosis infected and uninfected groups were IL-5 (at month 6) and IL-13, both Th2 cytokines, IL-2, IL-1α and TNF-α, which are all Th1 cytokines, IP-10, a chemokine, and IL-17 (only in unstimulated), a Th17 cytokine. These results were confirmed by PCA, applying most stringent conditions, that showed that it is possible to distinguish between exposed infected and the exposed uninfected by IL-2 and IP-10 at baselines and IL-2 and IL-13 at 6 months. Coincidentally, these cytokines showed to correlate positively with IFN-γ as expected since IFN-γ is widely used as a proxy for tuberculosis infection. IP-10 is induced by IFN-γ and therefore we did expect to see differences in IP-10 concentrations (as it showed high correlation with IFN-γ in this chapter as well as Chapter 2) between our study groups. This finding is supported by other studies for instance work done by Whittaker *et al*. (2008) and Lighter *et al*. (2009) who found that IP-10 could be used as a biomarker in children to determine tuberculosis infection. IL-1α was one of the cytokines identified by Chegou *et al*. (2009) to distinguish latent tuberculosis infection from active disease. Chegou's study, however, did not include children and therefore IL-1α should be further investigated for its diagnostic value and should not be disregarding in tuberculosis disease, as our study did not include a diseased group.

The fact that we do not see IFN-γ in this analysis does not mean that IFN-γ is not important to distinguish between infected and uninfected children. PCA apply very stringent condition and in this case, IL-2 showed bigger difference between the two groups investigated. Distinct IFN-γ and IL-2 profiles of antigen specific CD4+ T cells have recently been associated with different clinical disease states in tuberculosis (Millington *et al*., 2007). Even more recently, a modified QFT that simultaneously measures IL-2 and IFN-γ levels allowed differentiation between latent infection and active disease as reported by Glück (2010). From these results, we can conclude that IL-2 would be

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beneficial to measure in order to distinguish between tuberculosis infection and no infections as well as infection and disease.

IL-1β (higher in exposed group compared to the unexposed) and IL-17 (IL-17 unstimulated value is higher in the unexposed group compared to the exposed) are the cytokines that showed differences between tuberculosis exposed uninfected and the unexposed uninfected groups. IL-1β is a pro-inflammatory cytokine and is now believed to be an important molecule that participates in the inflammatory response required for the immunological control of infectious diseases like *M.tb*. IL-1β is the main cytokine for the activation of the innate immune system. IL-1β increases have been described in those infected or fighting reactivation of *M.tb* (Garlanda *et al*., 2007; Kindler *et al*., 1989). A genetic study did find that a functional polymorphism in the IL-1β gene influences the susceptibility to tuberculosis in a Colombian population (Gomez *et al*., 2006). This cytokine has also been shown to induce IL-10. IL-10, an anti-inflammatory cytokine, is known to have important effects during immune regulation and inflammation. It is known to down regulate the expression of Th1 cytokines and suppress the antigen presenting capabilities of antigen presenting cells. The fact that we found IL-1β to be higher in the exposed group indicates that early immune activation took place and that the innate immune systems of these children are sensitized to *M.tb*. The negative QFT and TST are proving that the adaptive immune system is not involved in this immune reaction. This highlights the importance of measuring innate cytokines for the detection of tuberculosis infection.

IL-17, the other cytokine that showed differences between tuberculosis exposed uninfected and the unexposed uninfected groups, is a pro-inflammatory cytokine, and has been linked to a number of chronic inflammatory conditions (Smith *et al*., 2010). IL-17 is mainly produced by Th17 cells but has also been shown that innate γδ T cells also have the ability to produce IL-17 in response to IL-1β (Sutton *et al*., 2009). The Th17 cells development and expansion is driven by the cytokines IL-1, IL-6, TGF-β, IL-21 and IL-23 (Sutton *et al*., 2009). It was thought that Th17 cells may play an indirect role to *M.tb* infection (Kader *et al*., 2007) but was proven wrong when a distinct subset of memory Th17 cells with specificity for *M.tb* was described, that suggested that they have an important contribution to the human anti-mycobacterial immune response (Scriba *et al*., 2008). IL-17 has been shown to be higher in TST negative adult participants in a recently study done by Babu *et al*. (2010). In addition, Van de Veerdonk *et al*. (2010) showed the presence of memory Th17 cells in TST negative people, who were not BCG vaccinated, that release IL-17 upon *M.tb* stimulation. Upon exposure, we see a drop in IL-17 concentrations and therefore lower levels is observed than in the unexposed (that are known to be TST negative). The reason for the decline in this cytokine in newly exposed children should be further investigated as well as the pathways involved.

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At month 6, after PCA the cytokines that distinguish best between the tuberculosis exposed and unexposed is IL-2 and IL-13, with higher concentrations measured in the unexposed group. This same cytokines showed differences between the tuberculosis infected and uninfected. Only because IFN-γ is below the detection levels of the QFT in the unexposed individuals we cannot say with absolute certainty that no tuberculosis infection took place. Previous results did indicate that the innate immune system is sensitized to *M.tb* in these individuals. It is possibly that since the adaptive immune system is not involved that the QFT is not enough to detect early infection. Other possible reason is that IL-2 could be produced spontaneously and that it is not specific to the stimulation. More work needs to performed to confirm this result. Future work should include flow cytometry to confirm the origin of the cytokines. The question should also be asked if this is only a phenomenon in peripheral blood and what exactly happens at site of disease.

In summary, we showed that IL-1β plays an important role in early tuberculosis exposure (initial exposure that sensitize the innate immune system) and higher IL-1β concentration upon *M.tb* antigen stimulation is indicative of recent exposure as IL-1β is one of the first cytokines to be secreted upon immune activation. In combination with IL-17, IL-1β can be used in future studies where receiver operating characteristic (ROC) analysis can be used to define diagnostic cut offs for positivity for these cytokines. These cytokines identified can also be tested in conjunction with currently used ones in order to improve on the specificity and sensitivity of tuberculosis detection.

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Chapter 4

Interferon-γ responses in HIV infected and uninfected children to *Mycobacterium tuberculosis*

Introduction

In the preceding two chapters, we identified multiple cytokine in children and adults that were able to distinguish between tuberculosis infection and exposure. As we are interested in environmental influences on the immune response we decided to investigate the immune responses in HIV infected and uninfected children. In the other chapters, we stimulated with well known tuberculosis antigens. Therefore, we decide to investigate other tuberculosis antigens in HIV. The reason behind this is that some work in our lab did show that other antigens showed some promise in determining tuberculosis infection in HIV infected adults. The aim of this chapter was to determine the anti-tuberculosis responses in HIV infected and healthy children who live in a high tuberculosis incidence setting. This was done by measuring the IFN-γ responses against known *M.tb* antigens in stimulated whole blood of participants. The information gained in this work will highlight the differences in the anti-tuberculosis immune response between HIV infected and healthy children. Knowledge gained from this work will help us to improve on current tuberculosis diagnostic tests and aid in vaccine development in the future.

Methods

Participants

Sixty children between the ages of 3 months and 15 years were recruited from Tygerberg Hospital in the Western Cape, South Africa. Children with known HIV infection were enrolled consecutively from the pediatric HIV OPD clinic and HIV unexposed, uninfected children from pediatric OPD clinics were enrolled concurrently between June 2008 and January 2009. Enrolment was postponed if a TST was placed or if the child was immunized in the preceding 6 weeks. All participants were investigated for tuberculosis disease through a symptom-based questionnaire, mycobacterial culture or sputum (gastric aspirate in children < 5 years of age) and chest radiography.

Children were excluded when they weighed less than 5 kg, had documented anaemia, or had been on tuberculosis treatment for active disease the last 12 months. Children were not excluded when a

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TST had been done in the last 3 months or if they were on isoniazid (INH) or anti-retroviral (ARV) therapy. In this high tuberculosis incidence setting the notification rate of tuberculosis in children was 620/100000 in 2008 (DOH, 2008). The estimated HIV prevalence among South African children aged 2- 14 years was 2.5 % in 2008 (DOH, 2008) and the incidence of tuberculosis in HIV infected children in the absence of INH preventive therapy was 9.2 % (Zar *et al.*, 2007). A questionnaire was completed at recruitment documenting deworming history, household exposure to smoke, if children are on INH therapy and additionally in HIV infected children ART and CD4 counts were documented. The tuberculin skin test was carried out on all participants using *M. tuberculosis* PPD (2 T.U., Statens Serum Institut, Copenhagen, Denmark) and skin reactivity was read between 48-72 h after the test using the ball-point pen and ruler method (Jordan *et al*., 1987; Bouros *et al*., 1989). Skin test results were available for all of the participants. A positive TST in HIV uninfected children was defined as an induration of ≥ 10 mm and in HIV infected children as ≥ 5 mm as per National Tuberculosis Control Management Guidelines (DOH, 2009).

The investigation was approved by the Ethics Review Committee of the Faculty of Health Sciences, Stellenbosch University, South Africa (N07/10/254). All participants in this study or their legal guardians provided written informed consent for participation in the study.

Whole blood stimulation

Heparinised whole blood (maximum 6 mL) was taken from participants and transported to the laboratory within 2 hours. Blood was diluted 1 in 5 with RPMI (Gibco, Invitrogen, Carlsbad, California, USA) containing 2 mM L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA) and stimulated in triplicate with antigen and mitogen; an unstimulated negative control was included in a volume of 100 µL/well, giving a final volume of 200 µL/well. Cultures were incubated at 37 ^{o}C with 5 % CO_2 for 7 days, before the supernatants were harvested and stored at – 80 °C.

Antigens

Common tuberculosis antigens were selected based on previous work in our laboratory. NTM PPD were also included to assess the cross reactivity with *M.tb* PPD. Whole blood was stimulated with ESAT-6 (2 µg/mL), CFP-10 (2 µg/mL), α-crystallin (2 µg/mL), TB10.4 (2 µg/mL), MPT64 (2 µg/mL), BCG (10 x 10^5 cfu/mL), *M.tb* PPD (2 µg/mL), *M. terrae* PPD (10 µg/mL), *M. scrofalaceum* PPD (10 µg/mL) and *M. avium* PPD (10 µg/mL) (all Straten Serum Institut, Copenhagen, Denmark) and P24 (10 µg/mL) (Fitzgerald Industries International, Acton, MA USA). SEB (0.1 µg/mL) (Sigma-Aldrich) was used as mitogen.

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ELISA

Stimulated supernatants were thawed to measure IFN-γ concentration (in pg/ml) by ELISA. Plates were coated with purified mouse anti-human IFN-γ ELISA capture antibody (1 mg/mL; Becton, Dickinson and Company (BD), San Jose, California, USA) and incubated for 5h at 4 °C. Plates were washed and blocked with phosphate buffered saline (PBS; Lonza BioScience, Walkersville, Maryland, USA) containing 10 % heat inactivated fetal calf serum (FCS; Sigma-Aldrich) for 2 hours at room temperature. This was followed by another wash and then samples, controls and standards (recombinant human IFN-γ ELISA standard, 25 µg, (BD) were added in duplicate to the appropriate wells. As additional quality control, a 7 day PHA stimulated supernatant from a healthy volunteer was used on all plates in this experiment. Plates were incubated overnight at 4 °C. Plates were washed and biotinylated anti-human IFN-γ ELISA detection antibody (0.5 mg/mL; BD) were added. All plates were incubated for 45 min at room temperature followed by a wash step and then avidin peroxidase (2.5 µg/mL; Sigma-Aldrich) was added to all wells. Plates were incubated at room temperature for 30 min followed by a washing step and OPD Fast (Sigma-Aldrich) was added to all wells and plates were left to develop in the dark at room temperature. Reaction was stopped with the addition of 2 M H_2SO_4 and the plate was read at 492 nm, subtracting the diluent blank from all wells. A standard curve was used in each assay ranging from 31.25 pg/mL to 4000 pg/mL. The lower point was chosen to be double the limit of detection of the reader.

QuantiFeron Gold In tube (QFT)

The QFT test was performed on all study subjects and interpreted for tuberculosis infection according to the manufacturer's instructions (Cellestis Limited, Carnegie, Victoria, Australia). In short, 1mL of whole blood was stimulated in each a nil, mitogen (PHA coated) or antigen tube (coated with a peptide cocktail of ESAT-6, CFP-10 and TB7.7). Tubes were incubated overnight at 37 °C with 5 % CO_2 and plasma was assayed for IFN-γ production by ELISA. For the ELISA, the plate was coated with conjugate and samples, controls and standards were added to the appropriate wells. As additional quality control, a 7 day PHA stimulated supernatant from a healthy volunteer was used on all plates in this experiment. Plates were incubated at room temperature for 2 hours and then washed. Enzyme substrate solution was added to all wells and the plate mix on a microplate shaker for 5 min. This was followed by incubation at room temperature in the dark for 30 min. Enzyme stopping solution was added to each well and mixed again. The plate was read at 450 nm, with a 650 nm reference filter. A standard curve was used in each assay ranging from 0.25 IU/mL to 4 IU/mL. QFT analysis software (Cellestis) was used to analyse raw data and to calculate results.

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Data analysis

Statistical analyses were performed using Statistica version 9 (StatSoft, Tulsa, OK, USA) and Graphpad Prism (version 5 for Windows, GraphPad Software, San Diego California, USA). The whole blood IFN-γ assay and QFT IFN-γ results were transformed using the logarithm base-10 to reduce variance in distributions. IFN-γ responses to antigens were analysed by nonparametric ANOVA with the Bonferroni post-test. To determine the differences between IFN-γ responses between the whole blood assay and the QFT, nonparametric Wilcoxon matched pairs test was used with confidence interval of 95%. Degree of association was calculated using Spearman's nonparametric correlation coefficient. Multivariate analysis of risk factors for tuberculosis used logistic regression. $P \leq 0.05$ was considered significant.

Results

A total of 60 children were enrolled between April 2008 and December 2008 (Table 10) with equal sex distribution within the group. Of these, 32 were HIV infected and 28 HIV uninfected. Median age of the study group was 60 months, 58 months for the HIV uninfected and 59 months for the HIV infected children. In the study group 46.7 % of participants were TST positive and 66.7 % of participants were QuantiFeron In-Tube (QFT) positive. Approximately half of the participants were dewormed within the year preceding enrolment into this study. Seventy six point seven percent of all children were exposed to smoke in the household. Only seven participants were on INH preventative treatment. In the HIV infected group 56.3 % are currently on ART and the median CD4 count of these participants were 1204 cells/mm^3. A positive TST was more common among children > 5 years (58.1 %). A negative TST result was most common in the HIV infected group (Figure 17) with 20 out of 32 children being negative.

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Table 10: Demographic and clinical characteristics of participants stratified according to HIV status

	All (n=60)	HIV uninfected (n=28)	HIV infected (n=32)
Female[1]	30 (50.0%)	15 (53.6%)	15 (46.9%)
Median age in months[2]	60 (4-165)	58 (6-136)	59 (4-165)
TST positive[1]	28 (46.7%)	16 (57.1%)	12 (37.5%)
QFT positive[1]	20 (66.7%)	13 (46.4%)	7 (21.8%)
Dewormed[1]	29 (48.3%)	14 (50.0%)	15 (46.9%)
Exposed to smoke[1]	46 (76.7%)	26 (62.9%)	20 (62.5%)
On INH[1]	7 (11.7%)	5 (17.9%)	2 (6.3%)
On ART[1]	na	na	18 (56.3%)
Median CD4 count[2]	na	na	1204 (32-3617)

[1]Numbers of participants, followed by the percentages of participants relative to the total number of participants in each group in brackets, are shown; [2]Mean value with range in brackets. na: not applicable.

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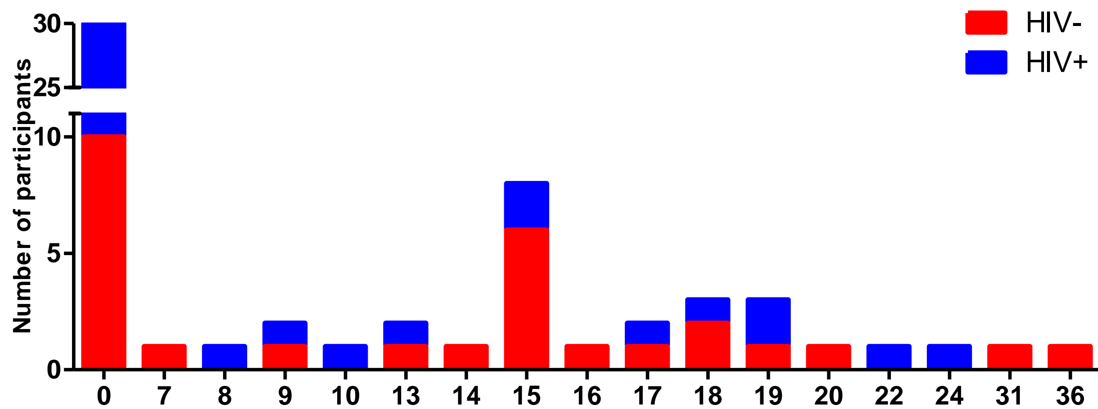

Figure 17: Distribution of TST readings (in mm) between the HIV uninfected (n=28) and HIV infected (n=32) participants.

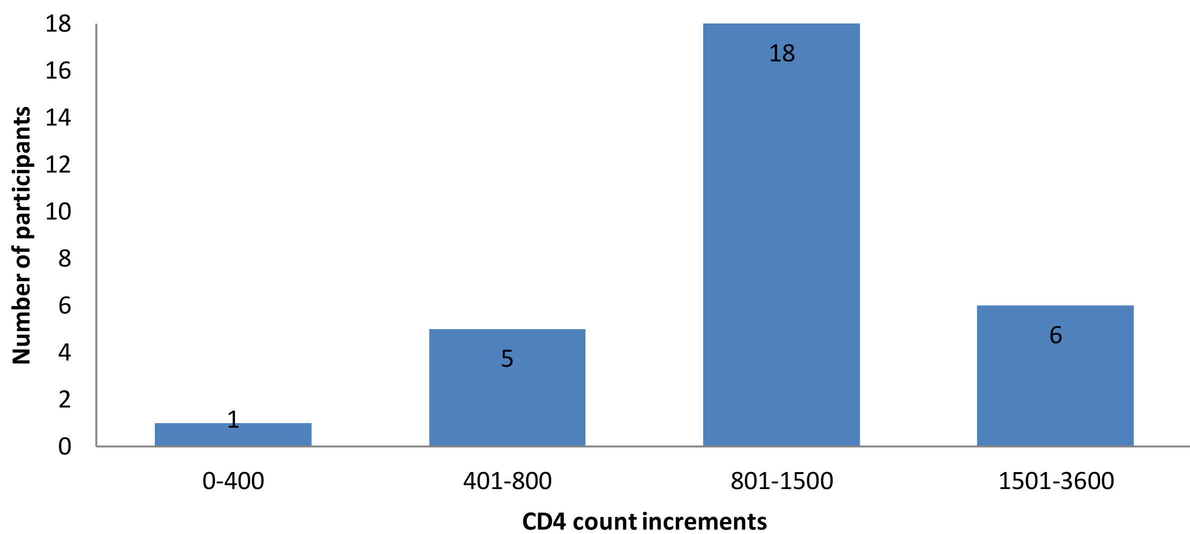

Figure 18: Distribution of CD4 counts in the HIV infected participants

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Considering that, only very young children were included in the study (Table 10) we do not expect to see any age related variance in the data and this was confirmed by doing a one-way ANOVA test of variance. Further analysis were performed in children under 16 years looking at specific cytokine concentrations and it was found that adjusting for age had no effect on the outcome of analysis (comparative plots shown in appendix 1).

When only the HIV positive patients with known CD4 counts (2 were not determined) were considered only one child had a CD4 count of below 400 cells/mm^3 indicating that the rest of the participants are not immune compromised (Figure 18). Higher lymphocyte counts are expected in young children and CD4 counts for HIV infected children fell into the range of 'healthy' CD4 counts. In practice, CD4 counts are not determined for HIV uninfected children and therefore, we are not able to correct for CD4 counts in this cohort.

Analysis between the HIV infected and HIV uninfected participants showed that IFN-γ responses following TB10.4 stimulation was significantly higher in the HIV uninfected (Figure 19). To analyse the relationship of TST to IFN-γ responses the magnitude of IFN-γ responses between TST positive and negative within the HIV infected and uninfected groups (Figure 20) were compared. In both HIV infected and uninfected the IFN-γ responses of the TST positive participants were consistently higher than those of the TST negatives. The differences were statistically significant within the HIV uninfected group after stimulation with ESAT-6 ($p < 0.01$), CFP-10 ($p < 0.01$), TB10.4 ($p < 0.001$), BCG ($p < 0.05$), *M.tb* PPD ($p < 0.001$), *M. terrae* PPD ($p < 0.05$) and *M. avium* ($p < 0.05$). Within the HIV infected groups differences were statistically significant after stimulation with TB10.4 ($p < 0.05$), BCG ($p < 0.05$) and *M.tb* PPD ($p < 0.001$).

Further analyses were done to investigate the magnitude of the IFN-γ response across age groups within the HIV infected and uninfected groups (Figure 21). Within the HIV uninfected group, IFN-γ responses against most antigens increased with age but not after stimulation with α-crystallin and MTP64, where a decline in responses in children > 5 years was observed. The only statistically significant difference was seen at > 5 years between the HIV infected and uninfected after *M.tb* PPD ($p < 0.05$) stimulation.

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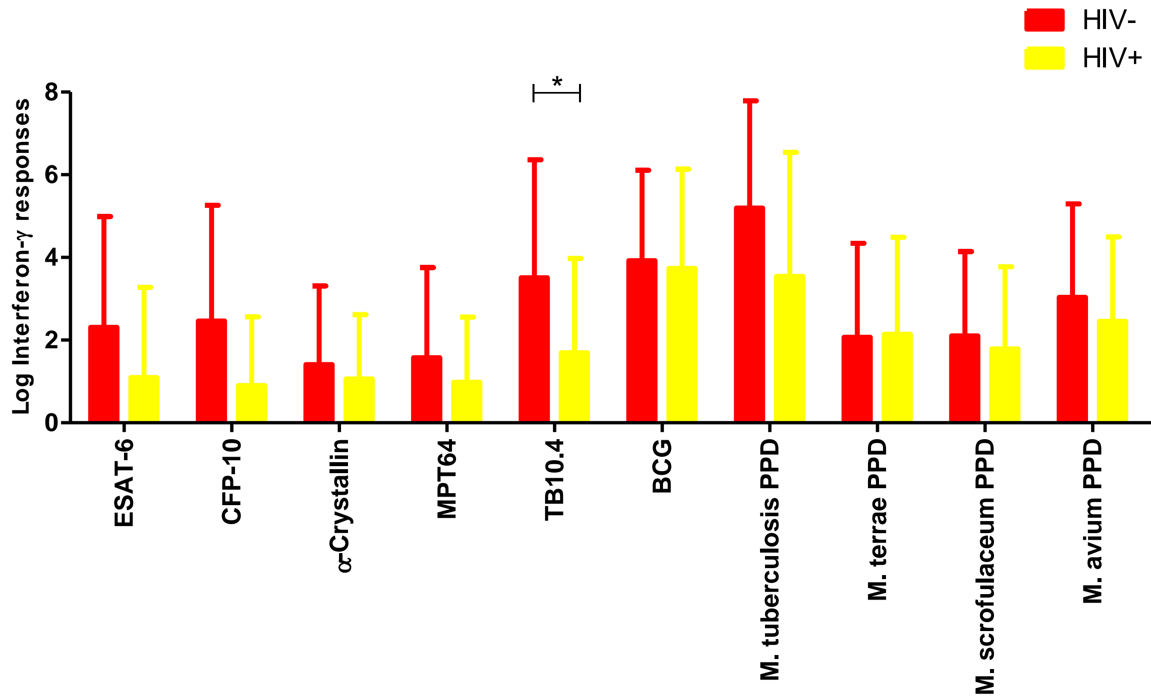

Figure 19: Comparison of whole blood IFN-γ responses in HIV uninfected (n=15) and HIV infected (n=15) participants as measured by ELISA. * denote significance of *P* ≤ 0. 05.

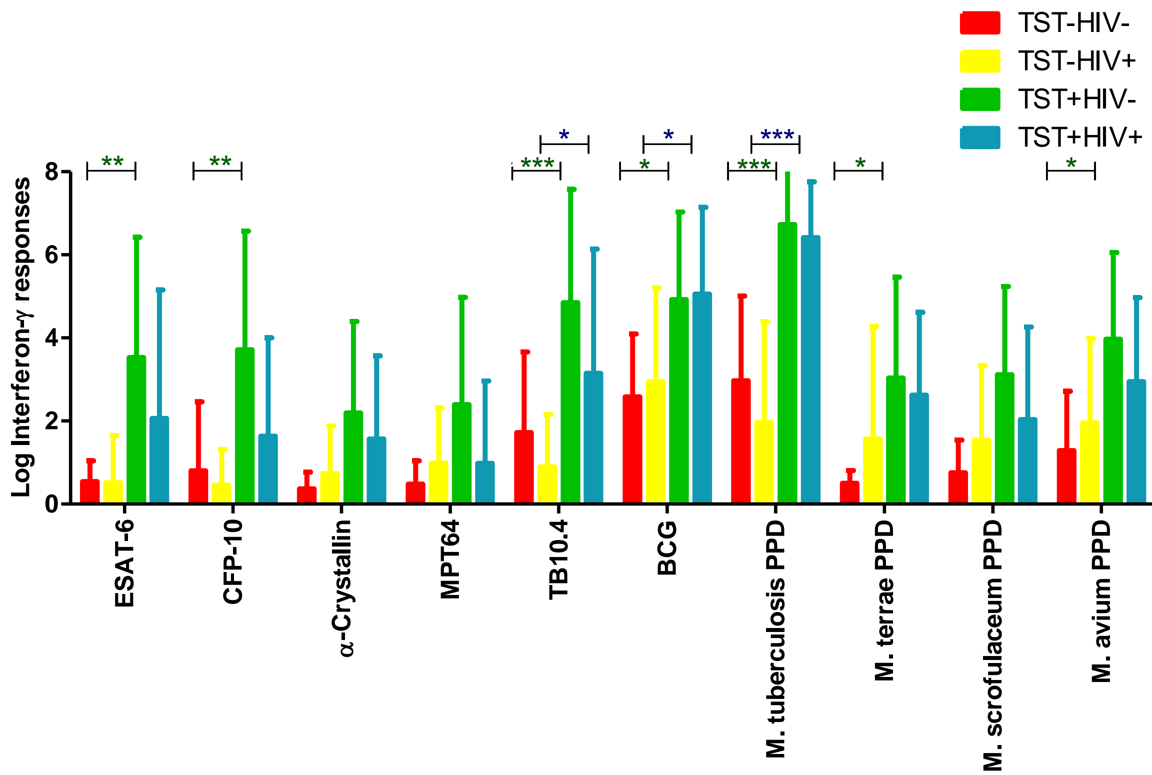

Figure 20: Comparison of whole blood IFN-γ responses in TST negative HIV uninfected (n=12), TST negative HIV infected (n=20), TST positive HIV uninfected (n=16) and TST positive HIV infected (n=12) participants as measured by ELISA. * denote significance of P ≤ 0.001, ** denote significance of *P* ≤ 0.01, * significance of *P* ≤ 0. 05.**

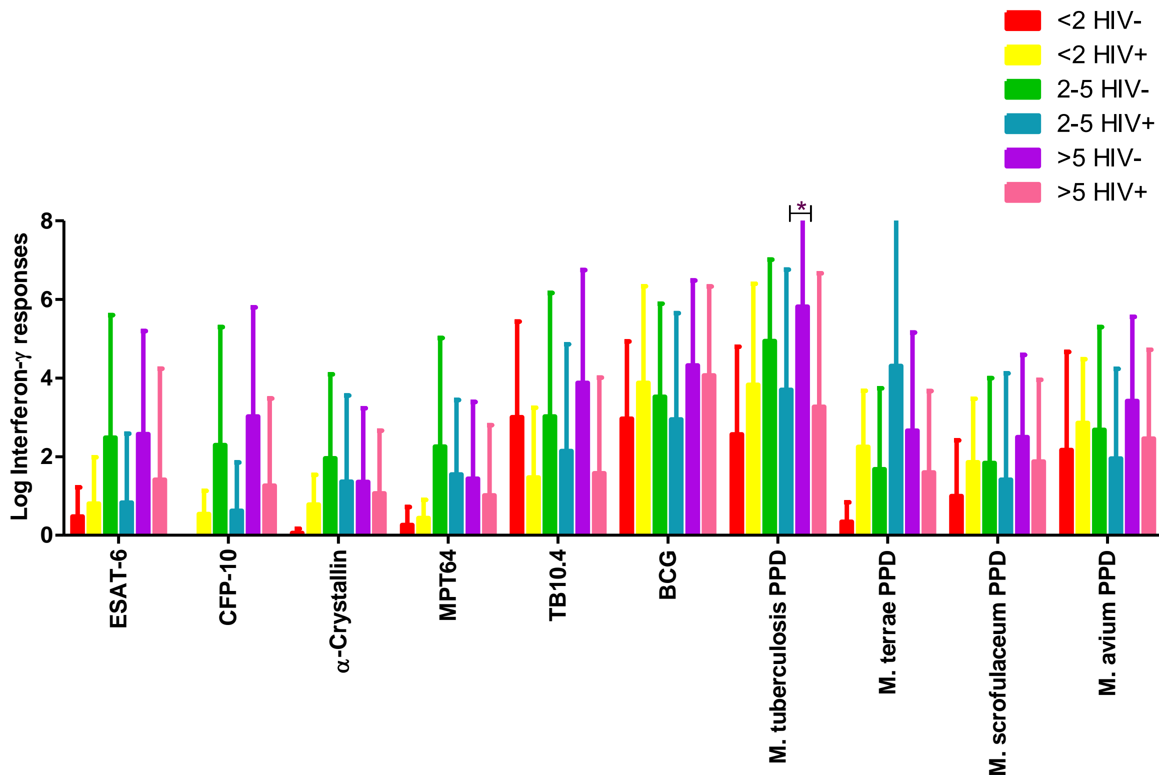

Figure 21: Comparison of whole blood IFN-γ responses in participants < 2 years of age and HIV uninfected (n=3) and HIV infected (n=9), between 2 and 5 years of age and HIV uninfected (n=9) and HIV infected (n=8), and > 5 years of age and HIV uninfected (n=16) and and HIV infected (n=15) as measured by ELISA. * denote significance of *P* ≤ 0. 05.

To compare the influence of deworming on the IFN-γ responses the magnitude of IFN-γ responses between different time points after routine deworming (Figure 22) was compared. The IFN-γ responses were consistently higher after 1 – 3 months following deworming, in the HIV uninfected compared to the HIV infected group. IFN-γ responses were statistically higher after stimulation with ESAT-6 ($p < 0.05$), CFP-10 ($p < 0.05$), TB10.4 ($p < 0.01$) and *M.tb* PPD ($p < 0.05$) in the recently dewormed HIV uninfected compared to HIV infected children. In addition, the relationship between TST and IFN-γ responses against the HIV antigen P24 (data not shown) in HIV infected children was investigated to assess the effect of latent infection (as measured with a positive TST) on the P24 induced immune response. No statistically significant differences were seen.

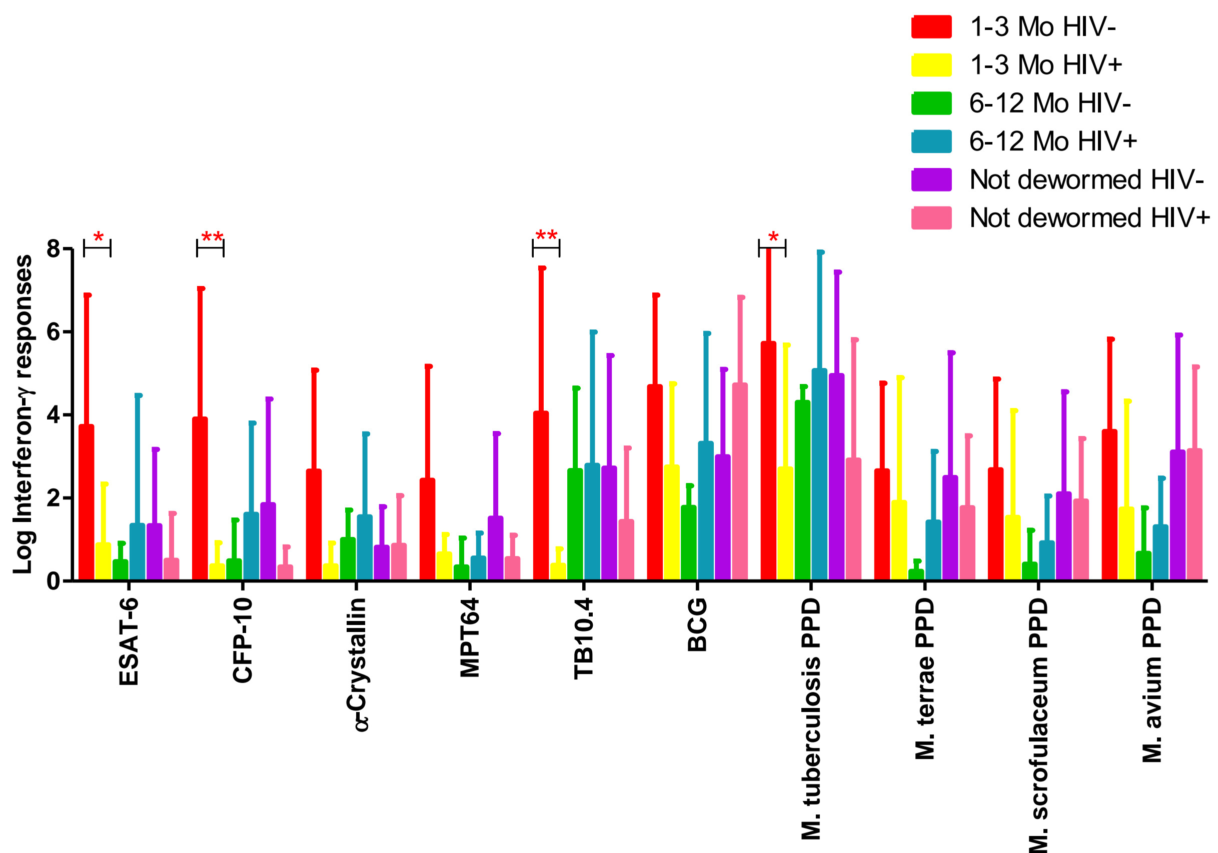

Figure 22: Comparison of whole blood IFN-γ responses in participants that were dewormed 1-3 months ago and are HIV uninfected (n=11) and HIV infected (n=8), those dewormed 6-12 months ago and are HIV uninfected (n=4) and HIV infected (n=7), and those not dewormed that are HIV uninfected (n=9) and HIV infected (n=11) as measured by ELISA. ** denote significance of $P \leq 0.01$, * significance of $P \leq 0.05$.

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The whole blood assay IFN-γ responses as measured after ESAT-6 ($r = 0.7301$, $p < 0.0001$) and CFP-10 ($r = 0.6981$, $p < 0.0001$) stimulation showed good correlation with the IFN-γ responses as measured by the commercial QFT (Figure 23). In the HIV infected group IFN-γ responses as measured after ESAT-6 ($r = 0.7067$, $p= 0.0003$) and CFP-10 ($r = 0.5416$, $p = 0.0166$) stimulation showed good correlation with the IFN-γ responses measured with the commercial QFT (Figure 24). From the correlation plots, it is clear that the high correlation is due to mostly low IFN-γ measurements (i.e. measured in those with no infection). Some discordant results were also found. The QFT showed a significantly lower IFN-γ response compared to the whole blood IFN-γ response after ESAT-6 ($p = 0.0001$) and CFP-10 ($p = 0.0005$) stimulation (Figure 25). In the HIV infected group QFT measured IFN-γ responses was again significantly lower than whole blood measured responses after ESAT-6 ($p = 0.0114$) and CFP-10 ($p = 0.0143$) stimulation (Figure 26). Analysis of IFN-γ responses between participants on ART treatment and those who were not on ART did not show any significant differences. The same was true for participants on INH treatment and those not on INH treatment (data not shown).

Univariate analysis of the group (Table 11) was performed to investigate the influences of HIV status, deworming, exposure to smoke, the QFT result and INH prophylaxis on specific *M.tb* antigen responses. Variables were included for the reasons as listed below. HIV status was included since it is known that HIV can have a negative effect on the immune response. Our results have shown the effect on deworming on the anti-tuberculosis immune response and therefore we chose to include this variable. Passive smoking was shown to be associated with *M.tb* infection in children living in a household with a patient with tuberculosis (Den Boon *et al*., 2007). Therefore, we wanted to investigate this further. The QFT was included in order to assess if the QFT result corresponds well with IFN-γ after *M.tb* antigen stimulation. INH is known to improve the anti-tuberculosis immune response (as discussed in Chapter 1) and is therefore included.

Univariate analysis showed that QFT positivity was significantly associated with IFN-γ responses as measured by the whole blood assay after stimulation with *M.tb* specific antigens (ESAT-6, $p < 0.0001$; CFP-10, $p < 0.0001$; α-crystallin, $p = 0.0208$; MPT64, $p = 0.0006$ and TB10.4, $p < 0.0001$). HIV infection was significantly associated with IFN-γ CFP-10 ($p = 0.0097$) and TB10.4 ($p =0.0089$) responses and exposure to smoking showed significant association with IFN-γ α-crystallin ($p = 0.0245$). In HIV infected children QFT positivity ($p = 0.0023$ and $p = 0.0004$ respectively) and ART ($p = 0.0136$ and $p = 0.0306$ respectively) showed a positive association with IFN-γ ESAT-6 and CFP-10 (Table 12). BMI and age showed no association with IFN-γ responses (data not shown) for either the whole study group or the HIV infected children.

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Multiple regression analysis (Table 13) was performed to assess the independent contributions of the independent variables, HIV, exposure to smoke, QFT, age, BMI and INH to the prediction of the dependent variable, antigen induced IFN-γ responses. The regression slope coefficient (β) indicates either a positive correlation exists (as indicated by a positive value) or if a negative correlation exist (as indicated by a negative value) between the independent and the dependent variable. Independent variables do not need to show a significant contribution to the model in order to be included.

Multiple regression analysis was performed by employing best subsets regression. Best subsets regression is a method used to help determine which independent variables should be included in a multiple regression model. This method involves examining all of the models created from all possible combination of predictor variables. Best subsets regression uses R^2 to check for the best model. First, all models that have only one predictor variable included is checked, and the two models with the highest R^2 are selected. Then all models that have only two predictor variables included are checked and the two models with the highest R^2 are selected, again. This process continues until all combinations of predictor variables have been taken into account.

Multiple regression done for the whole group (Table 13) showed limited association between the risk factors associated with *M.tb* infection and IFN-γ responses after whole blood stimulation. ESAT-6, CFP-10 and TB10.4 stimulated IFN-γ responses showed association with HIV status, exposure to smoke and QFT positivity. However, in addition to the above mentioned factors, after α-crystallin stimulation whole blood IFN-γ responses show some association with deworming ($p = 0.0327$).

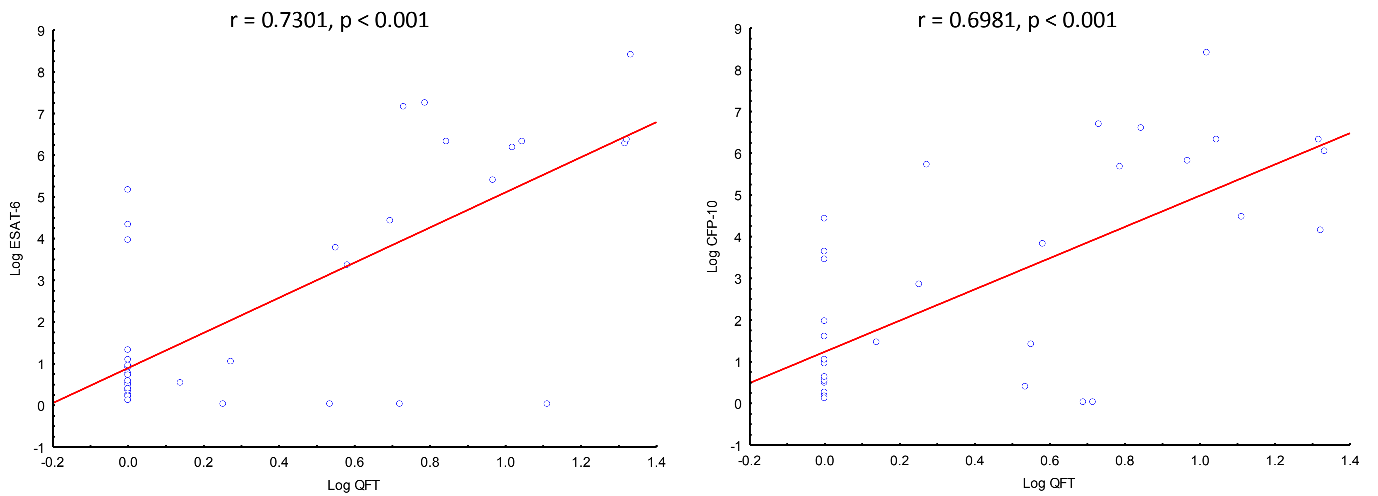

Figure 23: Correlation between whole blood IFN-γ ESAT-6 responses (left) / CFP-10 responses (right) as measured by ELISA and IFN-γ responses as measured by QFT in the study group.

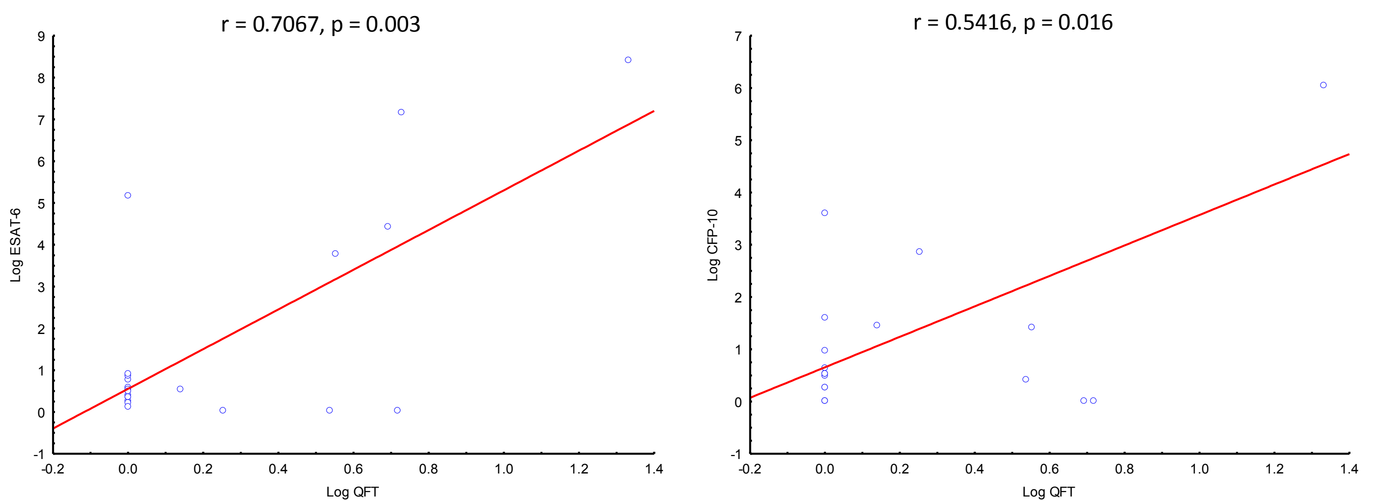

Figure 24: Correlation between whole blood IFN-γ ESAT-6 responses (left) / CFP-10 responses (right) as measured by ELISA and IFN-γ responses as measured by QFT in HIV infected children.

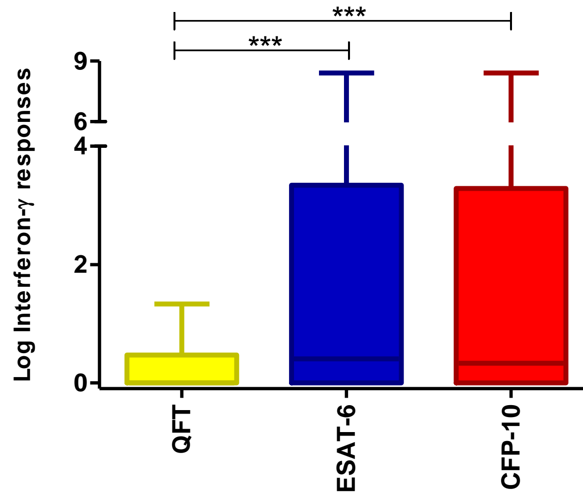

Figure 25: Comparison of whole blood IFN-γ responses in the QFT and whole blood ELISA after ESAT-6 and CFP-10 stimulation in the whole study group. * denote significance of P ≤ 0.001.**

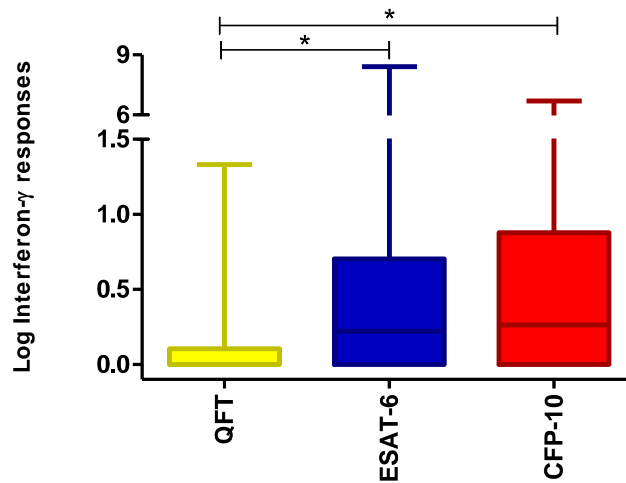

Figure 26: Comparison of whole blood IFN-γ responses in the QFT and whole blood ELISA after ESAT-6 and CFP-10 stimulation in only the HIV infected children. * denote significance of P ≤ 0.05.

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Table 11: Univariate analysis of factors associated with *M.tb* antigen specific IFN-γ production in children regardless of HIV status

	ESAT-6			CFP-10			α-Crystallin			MPT64			TB10.4		
	Mean (log pg/ml)	SE	p-value	Mean (log pg/ml)	SE	p-value	Mean (log pg/ml)	SE	p-value	Mean (log pg/ml)	SE	p-value	Mean (log pg/ml)	SE	p-value
HIV															
Negative	2.3	0.5	p=0.0599	2.5	0.4	p=0.0097	1.4	0.3	p=0.4368	1.6	0.4	p=0.2289	3.5	0.5	p=0.0089
Positive	1.1	0.4		0.9	0.4		1.1	0.3		1.0	0.3		1.7	0.5	
Deworm															
No	0.9	0.5	p=0.1219	1.0	0.5	p=0.1448	0.8	0.4	p=0.1299	1.0	0.4	p=0.5551	2.0	0.6	p=0.4457
Yes	2.0	0.4		2.0	0.4		1.6	0.3		1.3	0.3		2.6	0.5	
Exposure to smoking															
No	1.3	0.6	p=0.6899	0.9	0.6	p=0.2171	0.3	0.4	p=0.0245	0.8	0.5	p=0.3870	1.5	0.7	p=0.1328
Yes	1.6	0.4		1.8	0.3		1.5	0.2		1.3	0.3		2.8	0.4	
QFT															
Negative	0.4	0.3	p<0.0001	0.3	0.2	p<0.0001	0.9	0.3	p=0.0208	0.7	0.3	p=0.0006	1.4	0.4	p<0.0001
Positive	3.9	0.4		4.3	0.3		2.0	0.4		2.4	0.4		4.7	0.5	
INH prophylaxis															
No INH	1.6	0.3	p=0.6114	1.6	0.3	p=0.9946	1.1	0.2	p=0.2293	1.3	0.3	p=0.6589	2.3	0.4	p=0.0359
INH	2.1	0.9		1.6	0.9		2.0	0.6		1.0	0.7		4.6	1.0	

Mean concentration, standard error (SE) and *P* values for significance testing by ANOVA are presented. *M.tb* = *Mycobacterium tuberculosis*; HIV = human immunodeficiency virus; QFT = QuantiFeron Gold In tube; INH = isoniazid.

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Table 12: Univariate analysis of factors associated with *M.tb* antigen specific IFN-γ production in HIV infected children

	ESAT-6			CFP-10			α-Crystallin			MPT64			TB10.4		
	Mean (log pg/ml)	SE	p-value	Mean (log pg/ml)	SE	p-value	Mean (log pg/ml)	SE	p-value	Mean (log pg/ml)	SE	p-value	Mean (log pg/ml)	SE	p-value
Deworm															
No	0.5	0.6	p=0.4431	0.3	0.4	p=0.2424	0.9	0.4	p=0.8690	0.5	0.2	p=0.7629	1.4	0.6	p=0.9814
Yes	1.1	0.5		0.9	0.3		1.0	0.4		0.6	0.1		1.4	0.6	
Exposure to smoking															
No	1.0	0.6	p=0.7721	0.7	0.4	p=0.9780	0.3	0.4	p=0.0572	0.8	0.3	p=0.8347	1.4	0.6	p=0.8713
Yes	0.8	0.4		0.7	0.3		1.3	0.3		0.8	0.3		1.5	0.5	
QFT															
Negative	0.6	0.4	p=0.0023	0.4	0.3	p=0.0004	1.0	0.3	p=0.5720	0.8	0.3	p=0.3026	1.4	0.4	p=0.0949
Positive	3.4	0.8		2.9	0.6		1.4	0.6		1.6	0.6		3.3	1.0	
ART															
No ART	2.1	0.5	p=0.0136	1.6	0.4	p=0.0306	1.4	0.4	p=0.3317	1.5	0.4	p=0.0826	2.2	0.6	p=0.2316
ART	0.3	0.5		0.3	0.4		0.8	0.4		0.6	0.4		1.2	0.5	

Mean concentration, standard error (SE) and *P* values for significance testing by ANOVA are presented. *M.tb* = *Mycobacterium tuberculosis*; QFT = QuantiFeron Gold In tube; ART = antiretroviral therapy.

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Table 13: Multiple regression analysis of factors associated with *M.tb* antigen specific IFN-γ production in children

	ESAT-6			CFP-10			α-Crystallin			MPT64			TB10.4		
	β[1]	SE	p-value	β	SE	p-value	β	SE	p-value	β	SE	p-value	β	SE	p-value
Intercept	1.5	0.6	p=0.0219	1.1	0.5	p=0.0406	8.1	2.2	p=0.0010	0.8	0.4	p=0.0518	1.8	0.5	p=0.0013
HIV	-0.8	0.5	p=0.1201	-0.9	0.4	p=0.0301	-0.7	0.5	p=0.1718	-0.2	0.5	p=0.7380	-0.9	0.6	p=0.1475
Exposure to smoking	-0.9	0.6	p=0.1415	-0.3	0.5	p=0.4974	0.9	0.5	p=0.0857	1.7	0.5	p=0.0012	3.0	0.6	p<0.0001
QFT	3.3	0.5	p<0.0001	3.6	0.4	p<0.0001	-0.5	0.1	p=0.0005	Excluded			1.8	0.9	p=0.0672
Age in months	Excluded[3]			Excluded			0.9	0.6	p=0.1836	Excluded			Excluded		
Dewormed	Excluded			Excluded			1.6	0.7	p=0.0327	Excluded			Excluded		
BMI	Excluded			Excluded			Excluded			Excluded			Excluded		
INH	Excluded			Excluded			Excluded			Excluded			Excluded		

[1] Regression slope coefficient; [2]Multivariate regression models excluded the factors. Standard error (SE) and *P* values for significance testing by ANOVA are presented. *M.tb* = *Mycobacterium tuberculosis*; HIV = human immunodeficiency virus; QFT = QuantiFeron Gold In tube; BMI = body mass index; INH = isoniazid.

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Discussion

Tuberculosis disease is one of the most common infections seen in children in areas where HIV is prevalent (Swaminathan, 2004) and therefore a tuberculosis vaccine is needed. It is known that cell mediated immune responses, specifically those mediated by CD4+ T cells, are important for the control of both HIV and tuberculosis. In children with HIV infection, the CD4+ T cells will decrease over time and therefore these children are less capable of controlling the replication of *M.tb*. It is at this point when HIV infected children progress rapidly from primary infection to tuberculosis disease. Without the recruitment and activation of macrophages by the CD4+ T cell secreted cytokines in HIV infected children there is poor granuloma formation, little or absent caseous necrosis, poor containment of mycobacteria (Pilheu, 2000). Increased systemic immune activation and altered local cytokine micro environment at sites of *M.tb* infection have been implicated in enhanced HIV activity in patients with HIV and tuberculosis. While the *in vitro* IFN-γ response of mononuclear cells to mycobacterial antigens is impaired (Swaminathan, 2004). Similar changes have been observed with the other Th1 cytokines like IL-12 and IL-18 (Subramanyam *et al*., 2004).

This study investigated the IFN-γ concentrations of antigen stimulated whole blood when incubated with *M.tb* specific antigens in HIV infected and uninfected children in a high tuberculosis incidence setting. The information gained in the work highlight the differences in the anti-tuberculosis immune response between HIV infected and HIV uninfected children. This will aid to development of vaccines for this vulnerable population.

In both the HIV infected and the uninfected *M.tb* antigen induced IFN-γ responses in the TST positive groups were higher as expected, since TST positivity is used as a proxy for *M.tb* infection. In the HIV infected TST positive group no statistical differences were seen after ESAT-6 and CFP-10 stimulations when compared to the HIV infected TST negative group. This indicates that within the HIV positive group, one cannot distinguish between infection and no infection after ESAT-6 and CFP-10 stimulation. However, TB10.4 was found to induce higher IFN-γ responses in the HIV infected TST positive children compared to the HIV infected TST negative children, allowing for discrimination between infection and no infection.

Good correlation was found between the IFN-γ measured in the 7 day whole blood assay compared to the overnight measured IFN-γ after QFT. However, when specific responses were investigated it is clear that higher responses were obtained in the HIV infected after 7 day stimulation indicating that HIV infected children are more likely to respond to microbial specific antigens after 7 day stimulation. It should be taken into account that very small numbers were included in our study and larger studies is needed to confirm this result. Previous studies demonstrated higher sensitivity of a

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6 day whole blood assay for T cell responses in latent infected individuals, suggesting that prolonged *in vitro* stimulation induced central memory T cells (Leyten *et al*., 2007; Cehovin *et al*., 2007). In a recent study it was show that *M.tb* antigens, ESAT6/CFP-10 and TB10.4, induced stronger T cell responses in the 16 h assay (comparable to the QFT overnight assay performed here) but comparable proportions of IFN-γ producing T cells were detected in the 7 day assay (with restimulation during this time) (Schuck *et al*., 2009). The 7 day assay (with restimulation) detect specific T cell responses in latent infected individuals that are missed by the 16 h assay (Shuck *et al*., 2009). To summarize, the data suggest that the 7 day assay allows for a more accurate measurement of the IFN-γ response, which allows for better identification of latent tuberculosis in HIV infected children in our setting.

Univariate analysis further indicated that HIV infected anti-tuberculosis responses were significantly associated with CFP-10 and TB10.4 induced IFN-γ responses. In addition, the HIV infected children QFT positivity and ART showed a positive association with IFN-γ responses after ESAT-6 and CFP-10 stimulation. Since the ESAT-6 and CFP-10 (and TB7.7, but not investigated in this study) antigens are used in commercial IGRA (QFT) the possibility exist that it may not be sufficient to detect latent infection in the HIV infected children. A study by Mandalakas *et al*. (2008) found only moderate agreement between the TST and QFT in HIV infected children. It is well known that the TST is not accurate in the immune compromised. Another study found that ELISPOT assay is more sensitive than TST. However, the sensitivity of the ELISPOT assays is not sufficiently high to be used to rule out tuberculosis disease (Davies *et al*., 2009). From this work, it is clear that current tests are not sufficient to detect tuberculosis infection or disease in HIV infected children.

Kampmann *et al*. (2006) showed that IFN-γ measured anti-mycobacterial responses are lower in children under 5 years compared to children between the ages of 5 and 15 years. In this study, we found a similar trend in ages 2 to 5 years with anti-mycobacterial responses increasing as children get older. In general, T cell immune responses of younger children do demonstrate a decreased magnitude than observed in older children and it is known that younger children under 2 years are more likely to represent Th2 type immunity (Lewinsohn *et al*., 2004). One notable finding of this study is the increased anti-tuberculosis responses in children after recent deworming (in last 1 – 3 months). Since helminths is prevalent in most areas with high incidence of tuberculosis (Elias *et al*., 2006), controlling helminths, which is known to support a Th2 type immunity, can aid in controlling tuberculosis disease.

Univariate analysis of the whole group showed that QFT positivity was significantly associated with IFN-γ responses as measured by the whole blood assay after stimulation with *M.tb* specific antigens.

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Exposure to smoking showed significant association with IFN-γ after α-crystallin stimulation. It is now known that cigarette smoke affects both the innate and adoptive immune arms (Arnson *et al*., 2010). Passive smoking was also found to be associated with *M.tb* infection in children living in a household with a tuberculosis patient. (Den Boon, 2007). In previous studies, cigarette smoke was shown to augment the production of numerous pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 GM-CSF and to decrease the levels of anti-inflammatory cytokines such as IL-10. Cigarette smoke has also been shown to activate macrophage and dendritic cell activity (Arnson *et al*., 2010). More studies are needed to confirm this observation, but the possible association is a cause of great concern, considering the high prevalence of cigarette smoke and tuberculosis in this area. Multiple regression analysis done for the whole study group showed limited association between the risk factors associated with *M.tb* infection and IFN-γ responses after whole blood stimulation. HIV status, exposure to smoke and QFT positivity were shown to be predictors of the ESAT-6, CFP-10 and TB10.4 stimulated IFN-γ responses.

The biggest limitation to our investigation is the study numbers and it is possible that larger numbers would have shown more significant differences in all the groups tested. Future studies should include multiple cytokine analysis within these same groups by luminex. Flow cytometry would be useful to assess the differences between overnight and 7 day whole blood stimulations concerning cytokine production within these same groups.

In summary, our results suggest that detection of tuberculosis infection in HIV infected children could be more accurately measured using a 7 day whole blood assay. In addition, ESAT-6 and CFP-10, and TB10.4 can be used. Future studies should investigate the use of TB10.4 in detecting tuberculosis infection in HIV infected children. Our results showed that deworming HIV uninfected children more regularly have been shown to increase their anti-tuberculosis immune responses. Knowledge gained from this work will help us to improve on current tuberculosis diagnostic tests, especially in the HIV infected children.

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Chapter 5

Characterization of quantitative and functional innate immune parameters in *Mycobacterium tuberculosis* diseased adults

Introduction

We are interested the innate and adaptive immune system and the changes it undergoes during tuberculosis infection and disease. Here we aimed to identify the main innate populations present during tuberculosis disease. Limited blood volumes are available from children and it is not easy to get samples from the site of disease. For these reasons, we enrolled an adult cohort to answer our question. *Ex vivo* phenotyping of innate cell types in addition to cells of the adaptive immune system can provide novel information on the cells present at different stages of tuberculosis disease. Comparison of the different disease states will help to determine what is an important anti tuberculosis immune response and will therefore aid in future vaccine development. Phenotyping was performed on the following cell types: dendritic cells, myeloid derived suppressor cells, natural killer cells, invariant natural killer cells, natural killer T cells and T cells, in pulmonary tuberculosis patients before and after anti-tuberculosis treatment. Dendritic and myeloid derived suppressor cells were investigated in pleural effusions of pulmonary tuberculosis and cancer patients and compared to cell numbers in peripheral compartments.

Methods

Study participants

Recruitment took place between July 2009 and June 2010 as a sub study nested within two ongoing larger studies. The first was a study on patients with pleural effusions conducted at Tygerberg Hospital, which included tuberculosis and cancer patients. These patients were included in this study to investigate the difference in innate cell types at site of disease and peripheral compartments. All patients with an exudative pleural effusion according to Light's criteria (Light *et al*., 1972) were included, irrespective of ADA and lymphocyte counts. The main aim of the pleural effusion study is to investigate improved bacterial culture techniques on varying volumes of fluid collected with Dr Florian von Groote-Bidlingmaier as the principal investigator. As part of the study pleural fluid needle aspiration and a closed pleural needle biopsy were performed by experienced pulmonologists under ultrasound guidance. Study personnel took a detailed medical history.

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The participants of the pleural effusions study were stratified into two groups as follows:

'Tuberculosis' group: Comprised of 11 patients with pleural tuberculosis. When examination of pleural fluid revealed the presence of acid-fast bacilli by microscopy or *M.tb* by culture or when pleural biopsy specimens yielded a positive culture or granulomatous inflammation with caseous necrosis on histology the patient was diagnosed as having tuberculosis pleurisy. All *M.tb* strains identified were drug sensitive. The ages of the tuberculosis patients ranged between 19 and 70 years of age, 5 were male and 5 were HIV positive, the remaining six were either negative or the status was unknown as data was not collected.

'Cancer' group: Comprised of 10 cancer patients with malignant pleural effusions. Diagnosis was based on histological analysis of pleural biopsies or cytological analysis of pleural fluid. Diagnoses included non small cell lung cancer, small cell lung cancer, malignant mesothelioma, metastatic malignancies (breast cancer, endometrial carcinoma, renal cell carcinoma, gastric carcinoma, oesophageal carcinoma) and chronic lymphatic leukemia. The cancer patients were between 47 and 69 years of age and 5 were male. Since HIV testing was not performed routinely on older cancer patients at the study hospital, the HIV status of these patients was unknown.

The second study was community based and included tuberculosis patients and household contacts of active tuberculosis cases. Participants were recruited through 4 community clinics in the urban suburbs of Ravensmead, Uitsig, Adriaanse and Elsies River in the Western Cape Province, South Africa. This case/contact study was carried out as a sub-study of a collaborative longitudinal project to discover immune biomarkers of protection against tuberculosis in the context of high HIV prevalence. The biomarkers project is part of Grand Challenges in Global Health. An initiative launched in 2003 by the Bill & Melinda Gates Foundation and is thus known as the GC6-74 project or 'Biomarkers of protective immunity against TB in the context of HIV/AIDS in Africa' with Prof Stefan Kaufmann of the Max-Planck Institute for Infection Biology as project principal investigator and Prof Gerhard Walzl as principal investigator at Stellenbosch University. The sub-study required tuberculosis patients to be seen additionally at the end of treatment that was not in line with the design of the GC6 study and therefore customized case report and treatment outcome forms were created for 6 month follow up of tuberculosis patients (Appendix 2).

The participants of the community-based study were stratified into the following three groups:

'Before Treatment' group: Comprised of 20 patients newly diagnosed with active pulmonary tuberculosis by chest x-ray and confirmatory bacteriology before the onset of treatment. Ages ranged from 18 to 55 years; 7 were male. All were HIV negative.

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'End of Treatment' group: Comprised 12 tuberculosis patients from the same group as described above, in the last week of treatment (i.e. following 6 months anti-tuberculosis therapy). Drop out occurred to death, missed visits and relocation. Eleven participants in this group were considered cured if they had a two negative sputum culture during the last months of treatment. For one participant cure was defined according to a chest x-ray alone and the absence of symptoms without a sputum result. All were HIV negative.

'Household Contact' group: Comprised of 20 household contacts of active tuberculosis cases. Household contact was defined as living in the same house as an adult, sputum smear positive tuberculosis case that was diagnosed within the past three months. Ages ranged from 10 to 49 years and 10 were male. The Mantoux skin test was completed on the household contacts using *M. tuberculosis* PPD (2 T.U., Statens Serum Institut, Copenhagen, Denmark) and skin test reactivity was read between 48-72 hours after the test using a set of callipers calibrated to the nearest 0.5 mm. All household contacts were skin test positive (≥ 10 mm induration). All case/contact study participants agreed to answer a clinical questionnaire upon enrolment. All were HIV negative.

In addition to the above-cited groups, a control group were recruited:

'Control' group: Comprised of 17 healthy volunteer laboratory personnel with ages ranging from 23 to 35 years, without previous history of tuberculosis disease. 5 % of this study group were male. TST status of this group was not determined. All were HIV negative.

All patients were treated according to standard treatment protocols that are in use at their health care facility. No isoniazid chemoprophylaxis was offered to people with latent tuberculosis infection as this is not practiced in HIV negative and healthy adults in this country. Tuberculosis cases were treated under the direct observed therapy short course (DOTS) program and received standard doses of antibiotics. Treatment consisted of a 2 month intensive phase of rifampicin, isoniazid, pyrazinamide and ethambutol, followed by a 4 month continuation phase of rifampicin, isoniazid. The treatment of cancer patients depended on their type of malignancy.

This investigation was approved by the Ethics Review Committee of the Faculty of Health Sciences, University of Stellenbosch, South Africa (project numbers N10/02/055 and N05/11/187). All participants, or their legal guardians, provided written informed consent.

Sample collection

Heparinised whole blood (between 10 and 50 mL) was taken from all participants and transported to the laboratory within 2 hours. Whole blood was used directly for all subsequent analysis. For hospital participants pleural effusions (between 50 and 100 mL) were collected and transported on

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ice to the laboratory within 2 hours of collection. Effusions were spun down at 400 *g* for 10 minutes and the resulting cell pellets were washed twice before subsequent analysis.

Immunophenotypic cell staining

Aliquots of 100 µL whole blood or 100 µL of prepared pleural effusion cells were incubated with predetermined concentrations of monoclonal antibodies as described in Table 14. After mixing by vortex the tubes were incubated for 20 minutes at room temperature in the dark. One mL of FACS Lysing solution (BD Biosciences, San Jose, CA, USA) was added for hemolysis. Mixed by vortex and the preparation was incubated for 10 minutes at room temperature in dark. Preparation was mixed again and centrifuged for 1400 *g* for 5 minutes. Supernatant was discarded and resulting cell pellet was washed twice with 1 mL of wash buffer (phosphate buffered saline (PBS, Lonza, Walkersville, MD, USA) supplemented with 1 % fetal calf serum (FCS, Sigma Aldrich, St. Louis, Missouri, USA). In between the washing steps, the preparation was mixed by vortex and centrifuged at 1400 *g* for 5 minutes and supernatant discarded. Following the last washing step the resulting cell pellet was fixed by the addition of 200 µL 4 % paraformaldehyde (methanol free, Sigma Aldrich) and stored at 4 °C until analysis.

Immunophenotypic analysis

Flow cytometric acquisition was performed on the FACS Aria II or a FACS Canto II (BD Biosciences) due to logistical reasons. Instrument calibration was checked daily according to the manufacturer's instructions (BD Biosciences). Compensation settings were adjusted with the use of antibody-capture beads (CompBeads, BD Biosciences). Further compensation adjustments were made based on fluorescence minus one (FMO) controls (Baumgarth & Roederer, 2000). In multicolor experiments, it is not possible to set gates based on an entirely unstained or fully isotype stained control. In FMO controls one antibody is left out of the panel at a time in order to define the correct position for gates. Using a general acquisition gate on the forward scatter and side scatter dot plot at least 100000 events were acquired for each cell antibody panel and analysis were performed on FlowJo analysis software (version 7.5 and 7.6, Tree Star, Ashland, OR).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5 for Windows, GraphPad Software, San Diego California, USA). Non-parametric tests were employed. The differences between tuberculosis patients before and after treatment was analysed by the Friedman's test for repeated measures, with the Dunn's post test correction. The differences between tuberculosis patients, before and after treatment, their household contacts and controls was analysed by the Kruskal-Wallis test, with Dunn's post test for identifying differences between pairs when the *P* for multiple

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comparisons was ≤ 0.05. Kruskal-Wallis test with Dunn's post test correction was used to determine differences between tuberculosis and cancer patients when comparing pleural and whole blood cell frequencies.

Table 14: Antibody combinations used in this study to identify and characterise frequencies of DCs, MDSC and T-cells. A) Combinations used on the FACS Aria and B) combinations used on the FACS Canto II in tuberculosis study. C) Combinations used on the FACS Canto II in the pleural effusions study

A	DC	MDSC	T cells
FITC	LIN1	LIN1	CD4
PE	CD83	CD33	6B11
PE.Cy5		CD80	CD8
PE.Cy7	CD123		CD3
APC	CD40	CD11b	CD56
APC-AlexaFluor750	HLADR	HLADR	HLADR
Pacific Blue	CD86		CD16
Qdot605	CD11c		CD45RA

B	DC	MDSC	T cells
FITC	LIN1	LIN1	CD3
PE	CD83	CD33	6B11
PE-Cy5	CD11c	CD80	CD8
PE-Cy7	CD123		CD45RA
APC	CD40	CD11b	CD56
APC-eFluor 780	HLADR	HLADR	HLADR
Pacific Blue	CD86		CD16
V500			CD4

C	DC	MDSC
FITC	LIN1	LIN1
PE	CD123	CD33
PE-Cy5		CD80
PE-Cy7	CD11c	
APC		CD11b
APC-eFluor 780	HLADR	HLADR
Pacific Blue		CD14
V500		

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Results

Gating strategy

Different gating strategies were employed in order to identify the cell populations of interest as described in Table 15. All analyses were started by gating on populations of interest on the SSC/FSC plots (Figure 27). For the identification of all lymphocytic populations, the first gate was on the lymphocyte populations (Figure 27 A). For the dendritic cells, the gate was set on the lymphocyte and monocyte populations (Figure 27 B) and for identification of the myeloid derived suppressor cells the gate was set on the lymphocyte, monocyte and granulocyte populations (Figure 27 C).

From the lymphocyte gate on the SSC/FSC plot CD3 negative and positive cells were defined (Figure 28 A). This was then used to identify the following lymphocyte populations: T cells, natural killer cells (Figure 28 B), natural killer T cells (Figure 28 C) and invariant natural killer cells (Figure 28 D) as described in Table 15.

Non-lymphocytic cell populations were first identified by the lack of LIN1 (lineage marker 1 cocktail (BD Biosciences) contains CD3, CD14, CD16, CD19, CD20, and CD56; used as exclusion marker) expression. Dendritic cells subpopulations, plasmacytoid and myeloid dendritic cells (Figure 29 B), and myeloid derived suppressor cells (Figure 29 A) were identified as described in Table 15.

Table 15: Antibodies used in the identification of different cell populations present during tuberculosis disease and cancer

Cells types identified	Monoclonal antibody combinations
T cells	CD3+
CD4 T cells	CD3+ CD4+
CD8 T cells	CD3+ CD8+
Natural killer cells	CD3- CD16+ CD56+
Natural killer T cells	CD3+ CD56+
Invariant Natural killer T cells	CD3+ 6B11+
Plasmacytoid dendritic cells	LIN1- HLA-DR+ CD123+
Myeloid dendritic cells	LIN1- HLA-DR+ CD11c+
Myeloid derived suppressor cells	LIN1-/lo HLADR-/lo CD33+ CD11b+

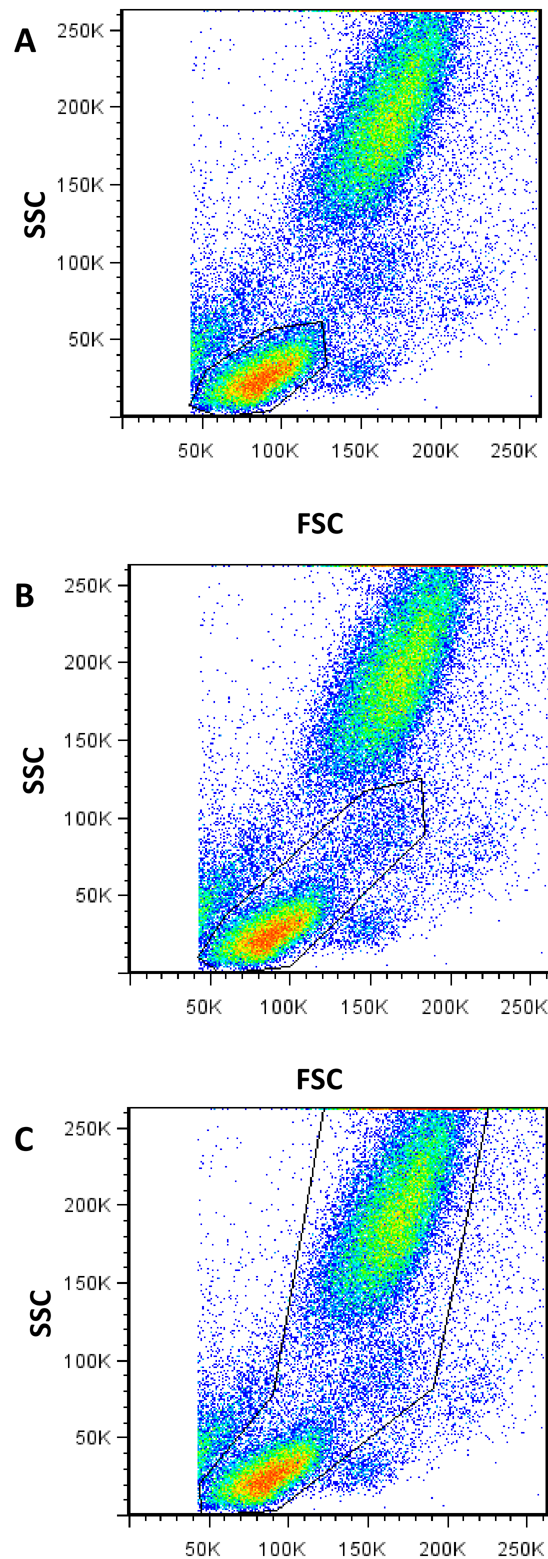

Figure 27: Gating strategy on the SSC and FCS dot plots for analysis. (A) For analysis of the lymphocyte-derived populations a gate was constructed. (B) For dendritic cell analysis a gate was constructed around the lymphocytes and monocytes and (C) for myeloid derived suppressor cell analysis around lymphocytes, monocytes and granulocytes.

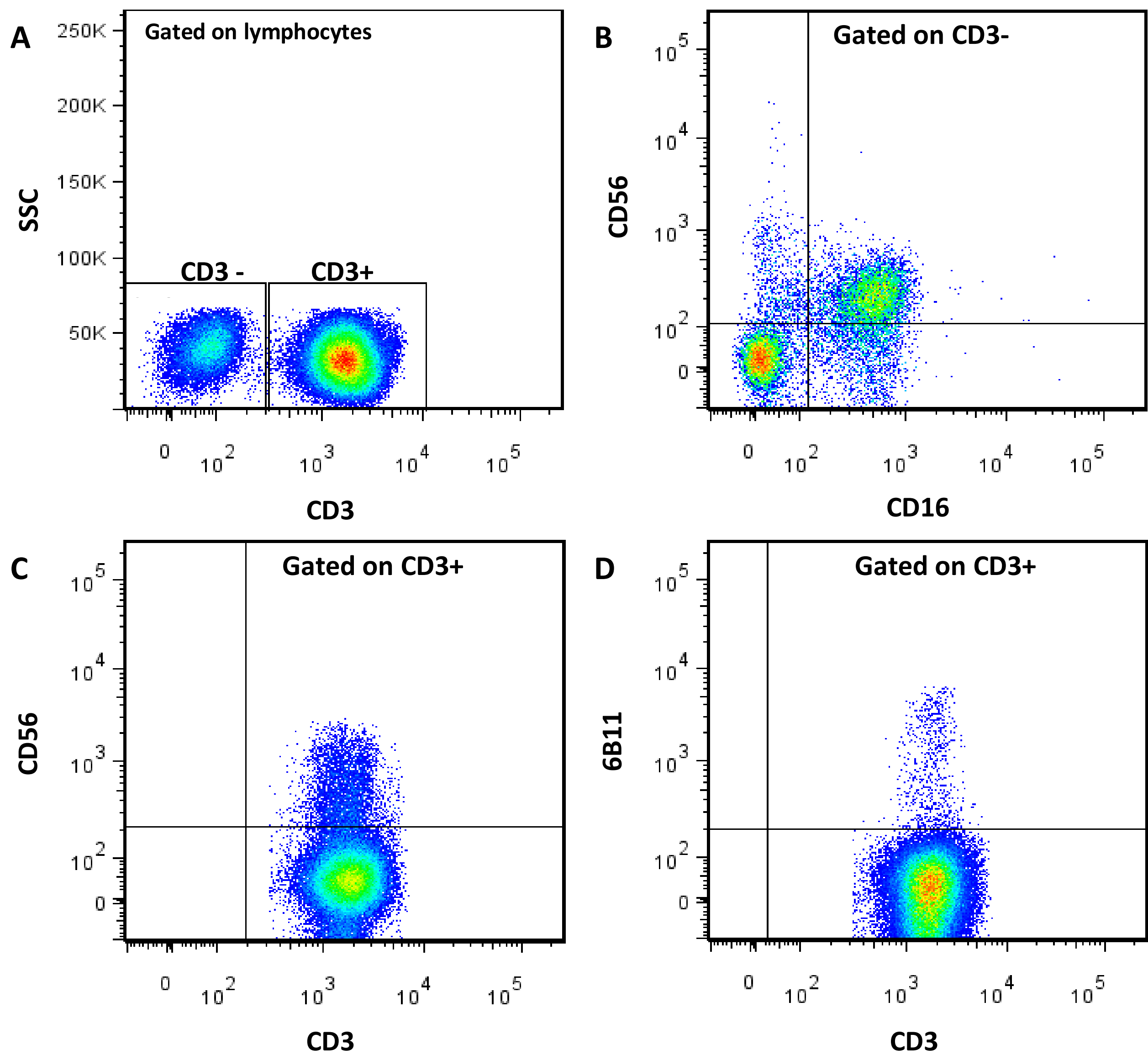

Figure 28: Gating strategy for lymphocytic cell populations. From the lymphocyte gate on the SSC/FSC plot (A) CD3 negative and positive cells were defined. Natural killer cells were identified by gating on the CD3 negative cells that express CD56 and CD16 (B). Natural killer T cells were identified by gating on the CD3 positive cells that express CD56 (C). Invariant natural killer T cells were identified by gating on CD3 positive cells that express the 6B11 clone of the invariant T cell receptor α chain (D).

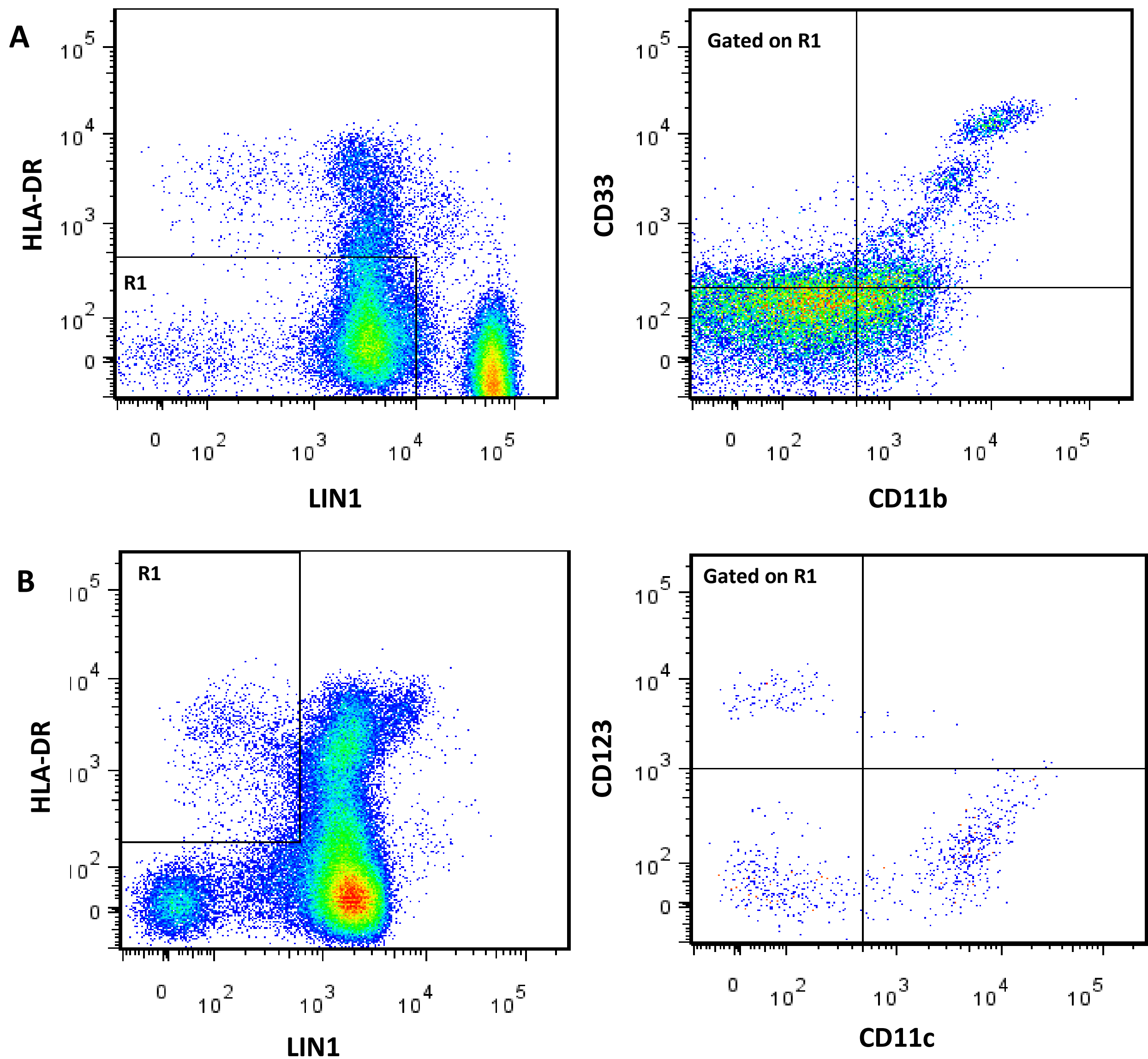

Figure 29: Gating strategy for non-lymphocytic cell populations. From the lymphocyte, monocyte and granulocyte gate on the SSC/FSC plot (A) LIN1 and HLA-DR negative and low staining cells were defined. These immature myeloid derived cells were investigated for the positive staining of both CD33 and CD11b to identify the myeloid derived suppressor cells. Dendritic cells were identified by gating on the lymphocytes and monocytes on the SSC/FSC plot that was used to define (B) LIN1 negative and HLA-DR positive staining cells. Subpopulations of can be identified as CD123 positive staining plasmacytoid dendritic cells and CD11c positive staining myeloid dendritic cells.

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Differences in T cells and T cell subsets following tuberculosis treatment

There was a predominance of CD4+ T cell frequencies over CD8+ T cell frequencies in the 3 groups as expected (Figure 30 A). Friedman analysis showed no significant differences between the frequencies of CD4+ and CD8+ cells in tuberculosis patients before and at end of treatment (data not shown). Natural killer T cell frequencies at the end of treatment were comparable to that before the start of treatment. However, natural killer T cells were significantly increased at the end of tuberculosis treatment compared to that of household infected contacts (Figure 30 B). Friedman analysis of the natural killer T cells showed no significant differences before and at end of treatment (data not shown). An increase in invariant natural killer cell numbers were seen in tuberculosis patients at the end of treatment compared to before treatment but not significantly so (Figure 31 A). Phenotypic changes were observed for invariant natural killer T and natural killer T cell as the expression of the molecule CD45RA+ increased (Figure 31 B). Compared to the before treatment group and household contacts CD45RA+ was significantly higher ($p \leq 0.001$) in the end of the treatment group. CD45RA is expressed on resting iNKT and NKT cells and upon activation; these cells will lose their CD45RA expression. This indicates that following treatment more resting cells are circulating than during infection. No differences were seen on invariant natural killer T cells for the molecules CD16, CD56 and HLA-DR; the same was observed for the molecules CD16 and HLA-DR on the natural killer T cells (Figure 31 B).

Levels of classic and pre-natural killer cells remained mostly unchanged between the 3 study groups (Figure 32 A). However, in tuberculosis patients at the end of treatment there was a significant decrease in mature NK cells when compared to before treatment (Friedman test; $p \leq 0.01$, Figure 32 B).

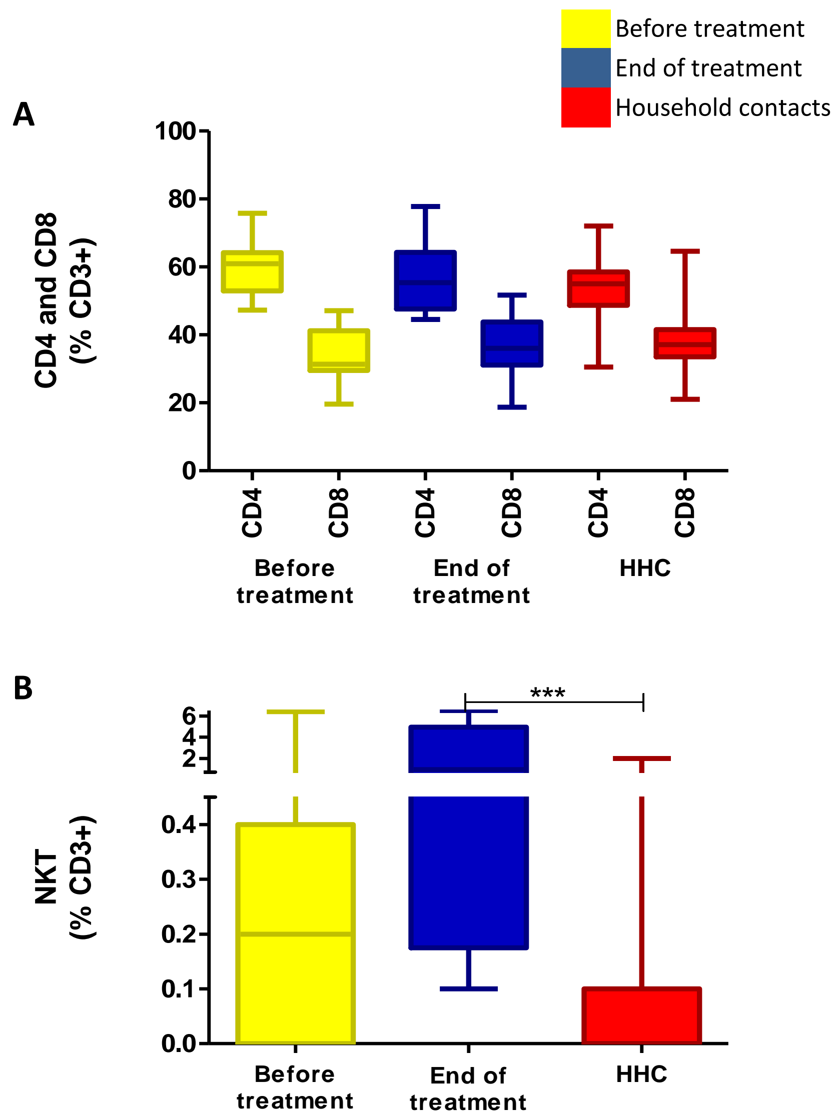

Figure 8: Analysis of CD4+ and CD8+ T cells (A) and natural killer T cells (B) frequencies before and at the end of treatment in whole blood by flow cytometry. The control group consisted of household contacts (HHC) of active tuberculosis cases. * denote significance of $P \leq 0.001$.**

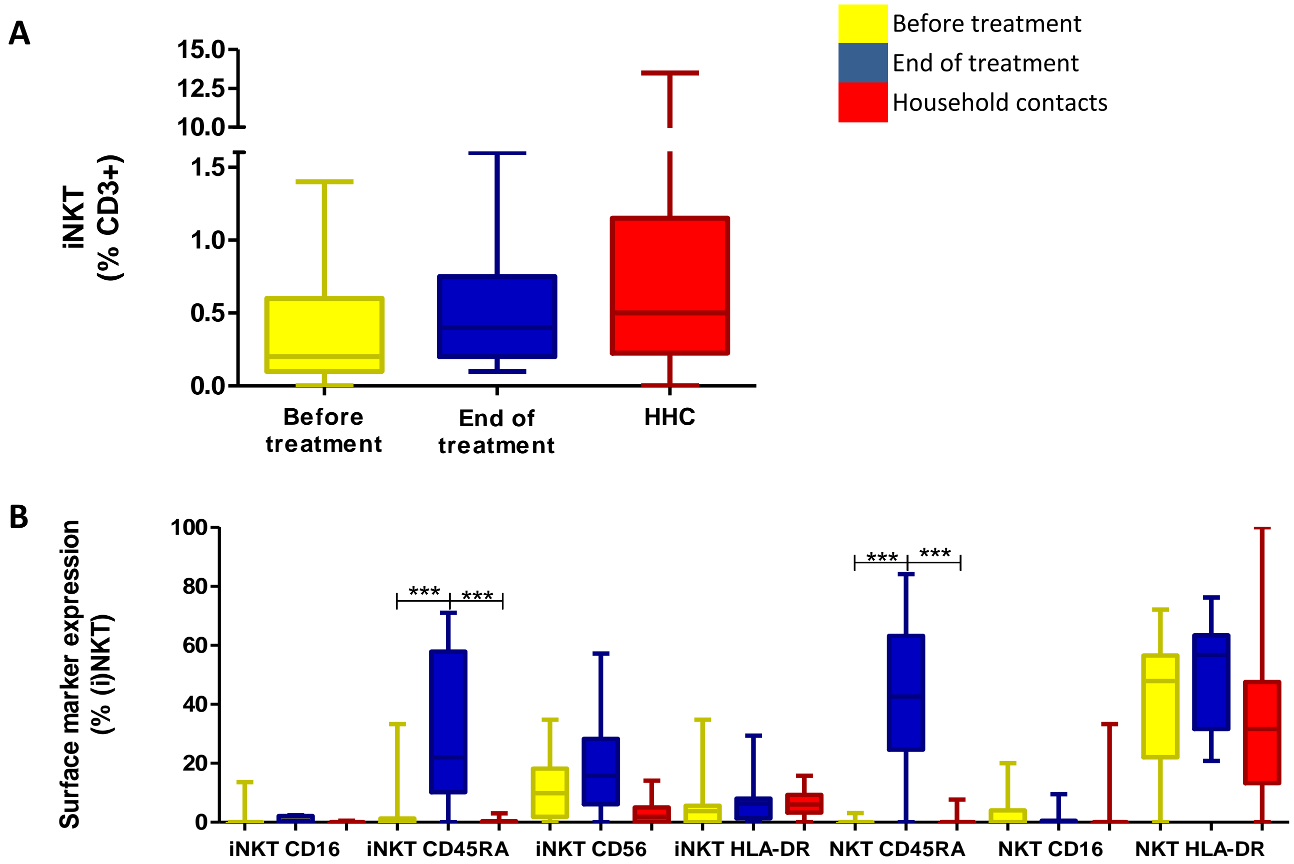

Figure 31: Analysis of invariant natural killer T cells (A) before and after treatment in whole blood by flow cytometry. Analysis of surface marker expression on invariant natural killer T cells and natural killer cells, before and at end of treatment. The control group consisted of household contacts (HHC) of active tuberculosis cases. * denote significance of $P \leq 0.001$.**

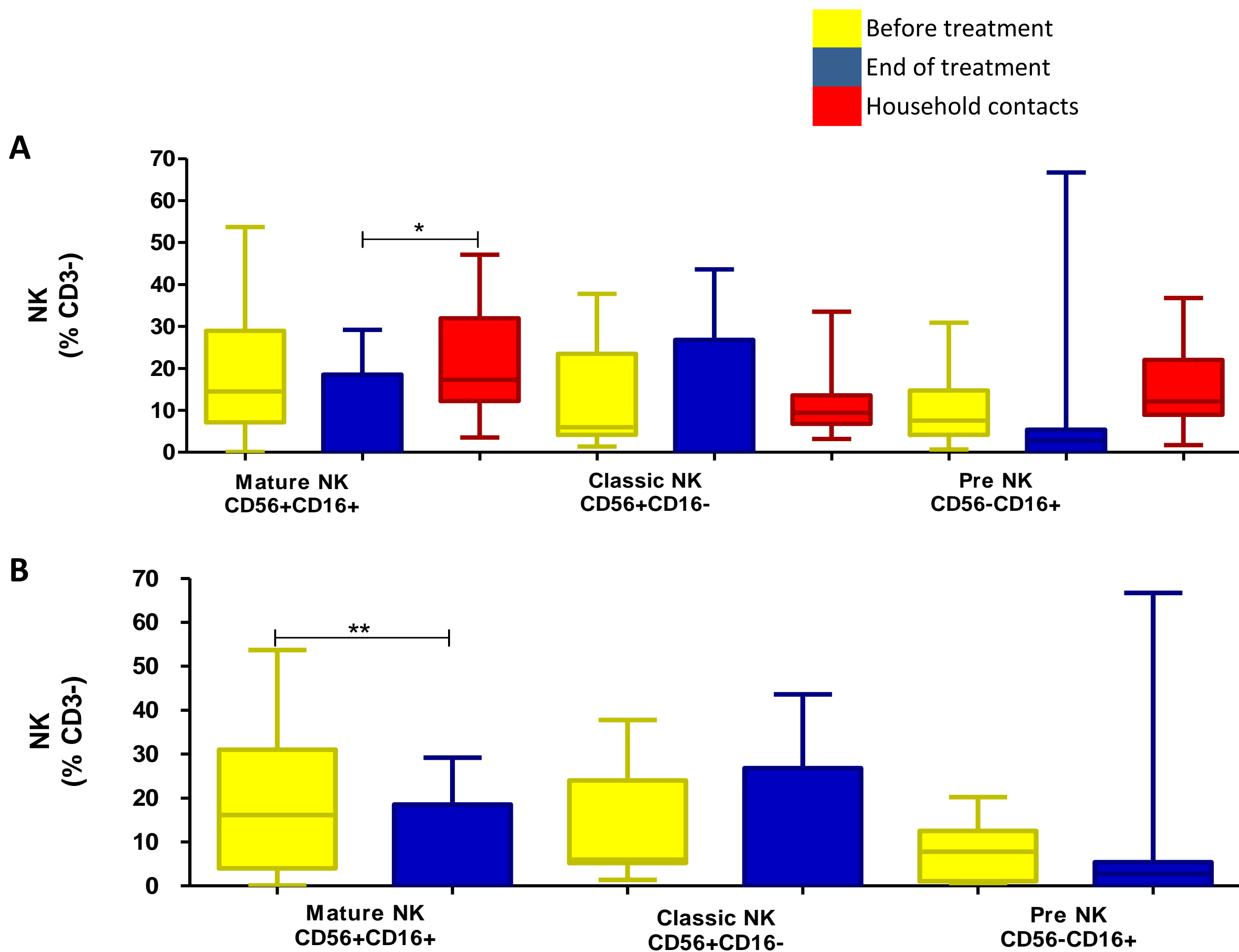

Figure 32: Analysis of natural killer cells and subsets before and at end of treatment in whole blood by flow cytometry. The control group consisted of household contacts (HHC) of active tuberculosis cases (A). Friedman analysis before and at end of treatment (B). ** significance of $P \leq 0.01$, * significance of $P \leq 0.05$.

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Differences in myeloid derived suppressor and dendritic cells numbers following tuberculosis treatment

Phenotypic analysis of the myeloid derived suppressor cells (LIN1-/lo HLA-DR-/lo CD33+CD11b+) showed that significantly reduced numbers were seen in tuberculosis patients at end of treatment compared to before treatment (Figure 33 A); confirmed by Friedman analysis ($p \leq 0.05$) (Figure 33 B). Tuberculosis patients before treatment and HHC had significantly more CD33+CD11b+ cells compared to healthy controls (Figure 33 A). Phenotypic changes where oberved in the myeloid derived suppressor cells as the expression of the molecule CD80+ (Figure 33 C). The CD80+ cells were significantly increased after treatment compared to tuberculosis patients before treatment ($p \leq 0.001$) and household contacts ($p \leq 0.001$) and the healthy controls ($p \leq 0.05$). Friedman analysis showed that the CD80+ myeloid derived suppressor cells were significantly increased at the end of treatment ($p \leq 0.01$) (Figure 33 D).

Phenotypic analysis of total dendritic cells showed no significant differences between the 3 groups (Figure 34 A). Nevertheless, in the subsets, plasmacytoid dendritic cells (LIN1-HLA-DR+CD123+) ($p \leq 0.01$ and $p \leq 0.001$) and myeloid dendritic cells (LIN1-HLA-DR+CD11c+) ($p \leq 0.01$ and $p \leq 0.01$), significant differences were seen in tuberculosis patients at the end compared to before treatment initiation and household contacts. Friedman analysis however showed no differences in dendritic cell or subset numbers (Figure 34 B).

Phenotypic changes were observed in the plasmacytoid subset with the molecule CD86 highly expressed ($p \leq 0.001$) in tuberculosis patients compared to household contacts. However, expression of CD86 is comparable with that of household contacts at end of treatment (Figure 34 C). Friedman analysis showed no significant differences for this molecule on plasmacytoid dendritic cells. The molecule CD40 showed significant differences between all 3 groups, for both the plasmacytoid and myeloid subsets. Friedman analysis showed significant differences in tuberculosis patients before and at the end of treatment with higher expression of CD40 at the end of treatment. This was true for the plasmacytoid ($p \leq 0.01$) and myeloid dendritc cells ($p \leq 0.05$) (Figure 34 D).

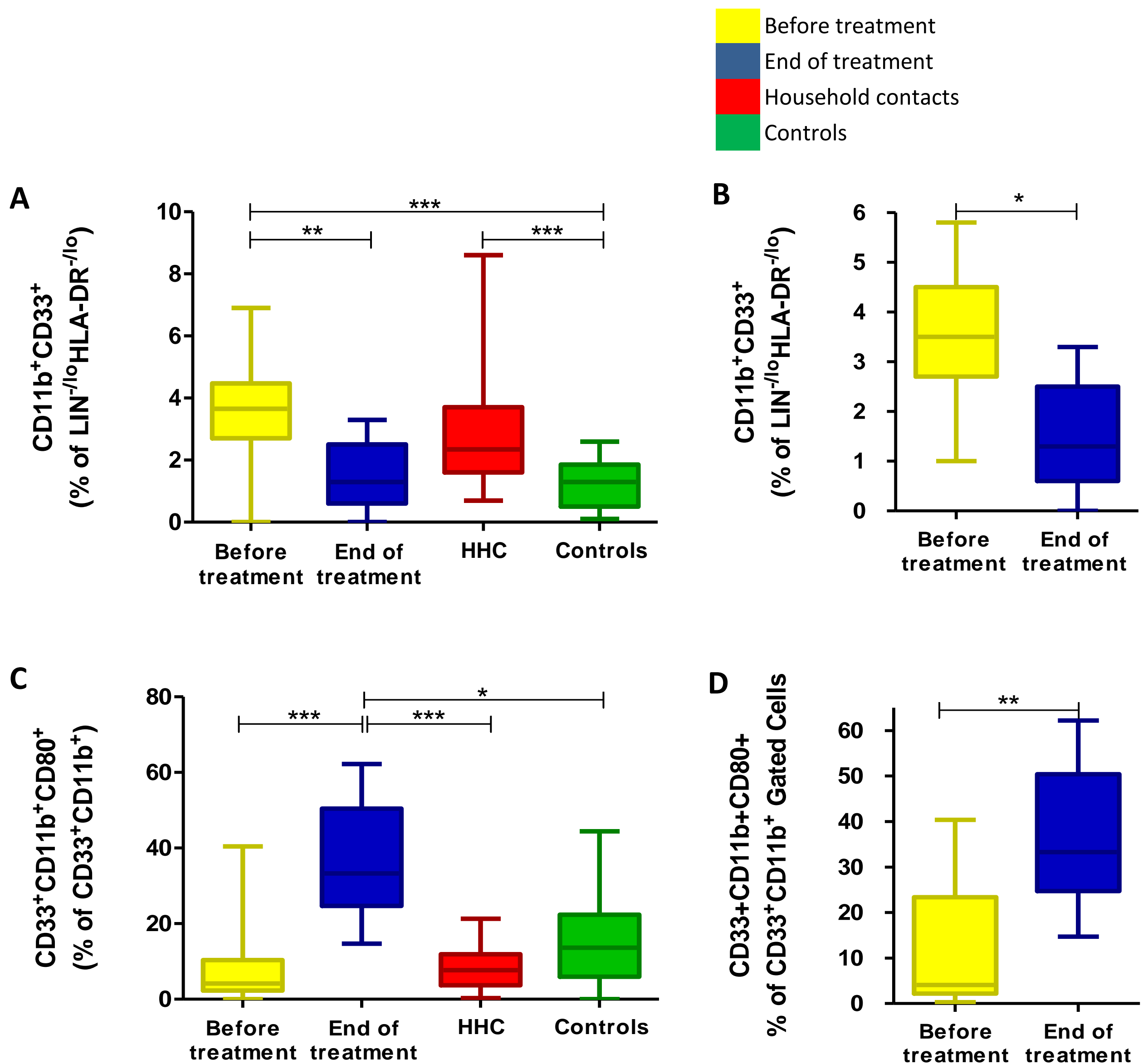

Figure 33: Analysis of myeloid derived suppressor cells (A) and CD80+ subsets (C) before and at end of treatment treatment in whole blood by flow cytometry. Household contacts (HHC) of active tuberculosis cases and healthy laboratory controls were included (A). Friedman analysis of before and at end of treatment (B) of myeloid derived suppressor cells and (D) of the CD80+ subset. * denote significance of $P \leq 0.001$, ** significance of $P \leq 0.01$, * significance of $P \leq 0.05$.**

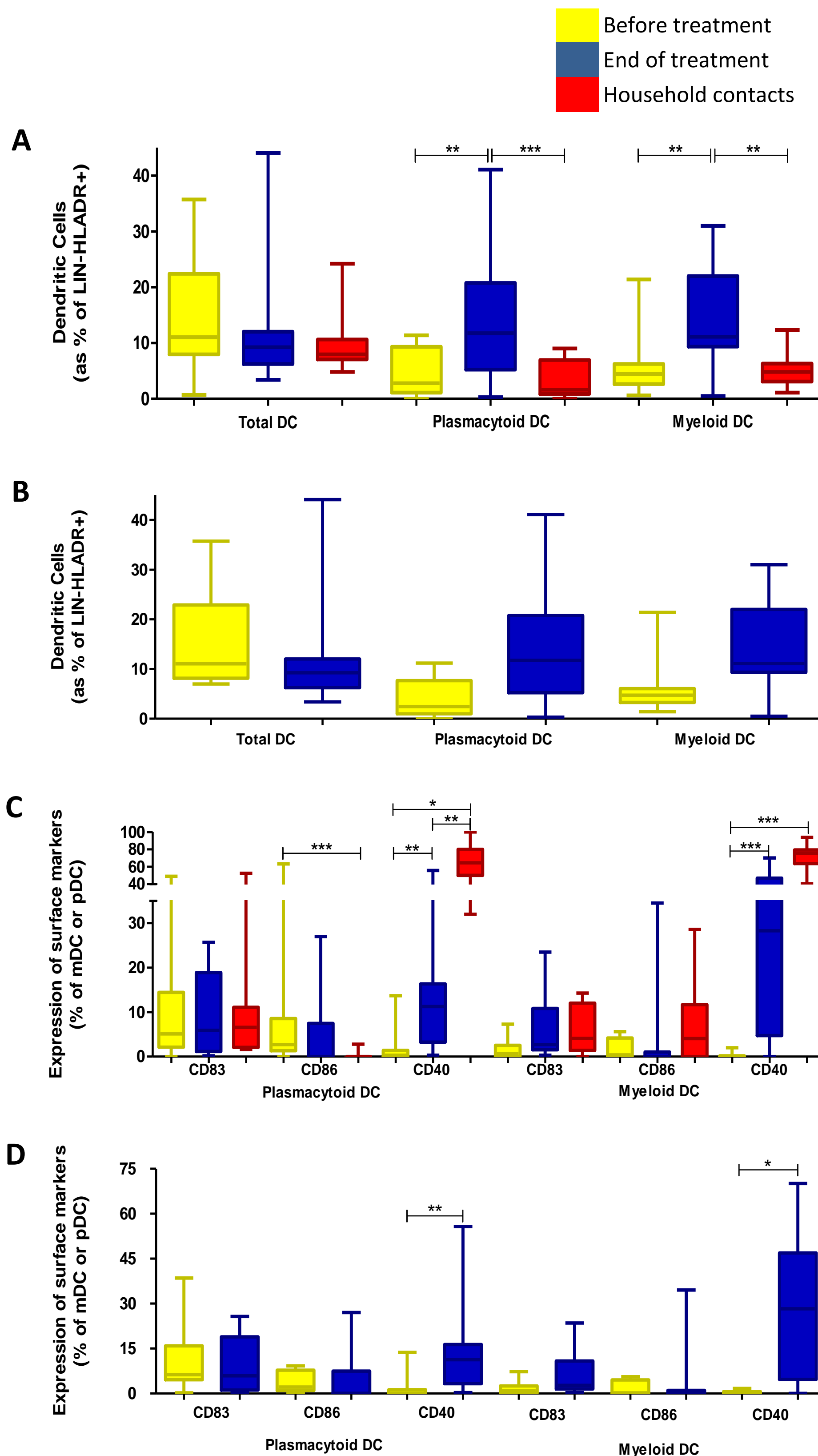

Figure 34: Analysis of dendritic cells (A) before and after treatment in whole blood by flow cytometry. The control group consisted of household contacts (HHC) of active tuberculosis cases Friedman analysis (B) before and at end of treatment of dendritic cells. Analysis of surface marker expression on dendritic cells subsets, before and at end of treatment. * denote significance of *P* ≤ 0.001, ** significance of *P* ≤ 0.01, * significance of *P* ≤ 0.05.**

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Differences in myeloid derived suppressor and dendritic cells between site of disease and peripheral blood

Myeloid derived suppressor cells have previously been shown to have a suppressive effect on the innate immune system in cancer patients. We found no differences between myeloid derived suppressor cell numbers in pleural effusions and whole blood of cancer and tuberculosis patients (Figure 35 A). However, higher numbers were observed to be circulating in whole blood compared to the site of disease (pleura) even though not significantly so (Figure 35 A). No phenotypic changes where oberved in the expression of the molecule CD80+ between all groups compared (Figure 35 B). Within the myeloid derived suppressor cell population a CD33hi population was identified and further identified to be CD14+. This population therefore can be defined as LIN1-/lo HLA-DR-/lo CD33hi CD11b+CD14+ and seems to be a subpopulation within the myeloid derived suppressor cells. In cancer patients significant differences were seen for the molecule CD14+ ($p \leq 0.05$) with higher numbers in pleural effusions than whole blood (Figure 35 C).

Dendritic cell numbers were comparable in pleural effusions of cancer and tuberculosis patients (Figure 36 A). In the whole blood of tuberculosis patients, significantly higher numbers ($p \leq 0.05$) were found to be circulating compared to whole blood of cancer patients. As for myeloid derived suppressor cells, higher numbers of circulating dendritic cells were seen in blood compared to the pleura even though not significantly so (Figure 27 A). The plasmacytoid and myeloid dendritic cell subsets showed no differences in percentages in all groups compared (Figure 36 B).

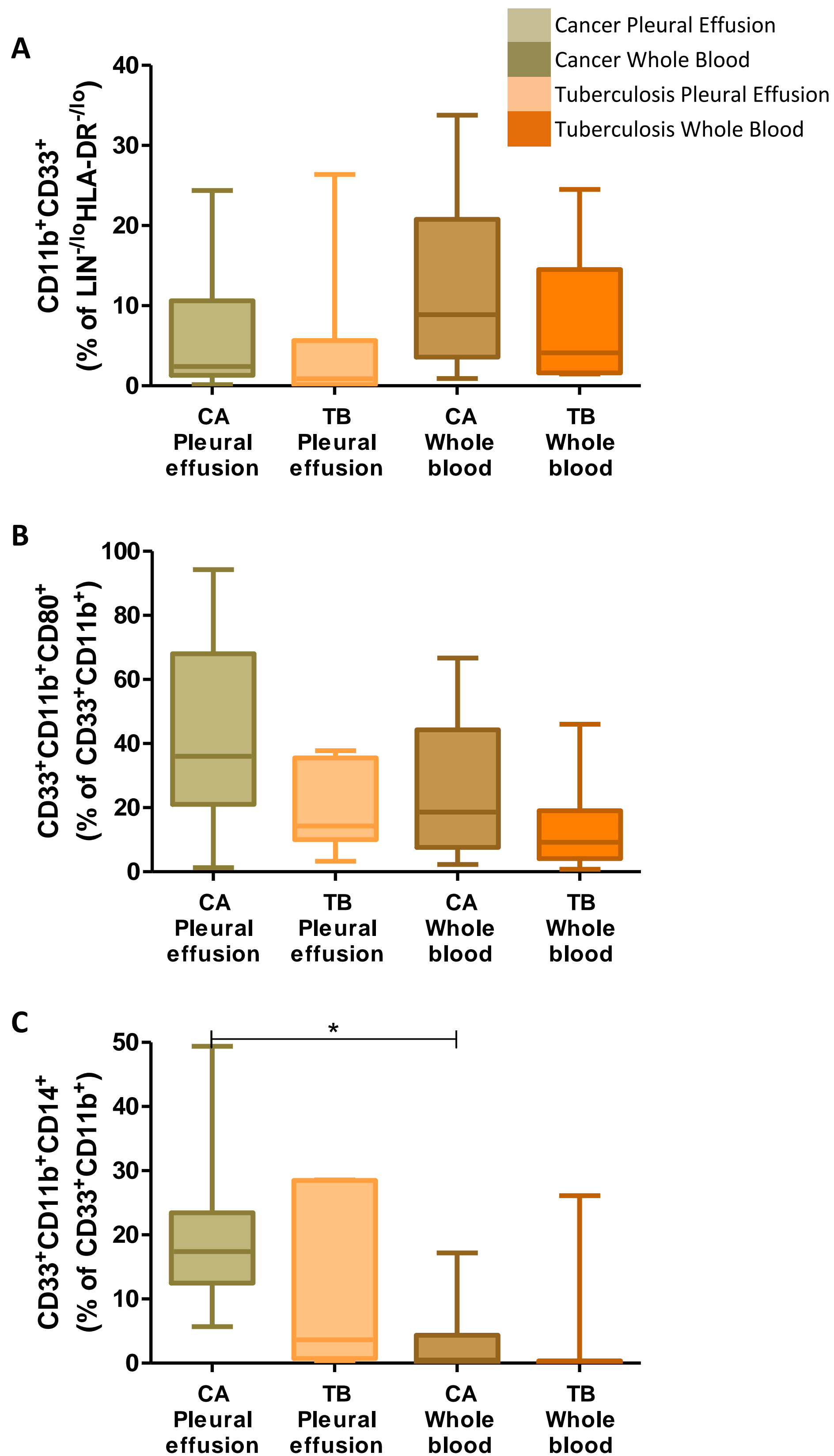

Figure 35: Analysis of myeloid derived suppressor cells (A), the CD80+ subsets (B), and the CD14+ subset(C) in pleural effusions and whole blood of cancer and tuberculosis patients by flow cytometry. * denote significance of $P \leq 0.001$, ** significance of $P \leq 0.01$, * significance of $P \leq 0.05$.**

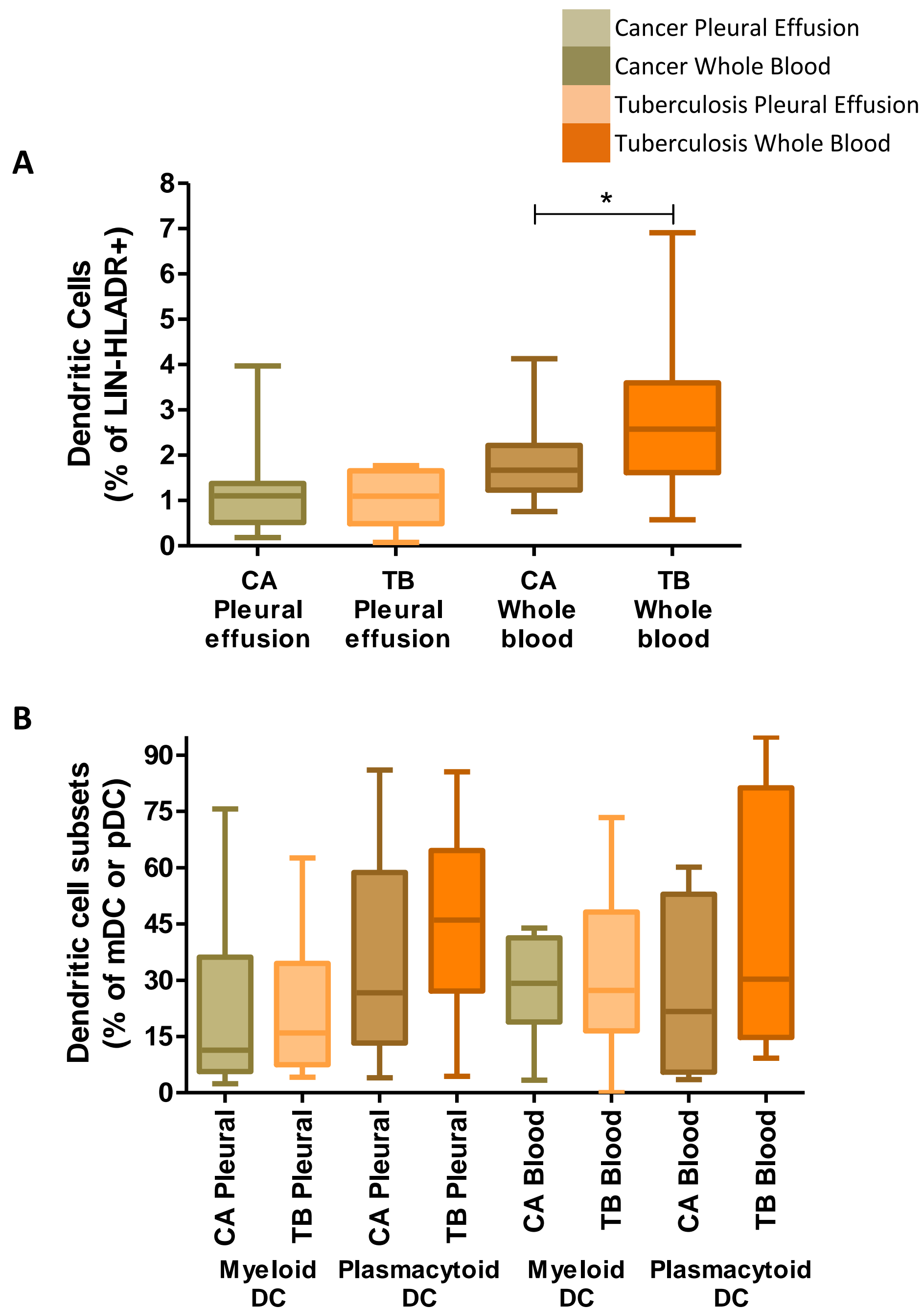

Figure 36: Analysis of dendritic cells (A) and the dendritic cell subsets (B) in pleural effusions and whole blood of cancer and tuberculosis patients by flow cytometry. * denote significance of $P \leq 0.001$, ** significance of $P \leq 0.01$, * significance of $P \leq 0.05$.**

Discussion

Few studies have looked at the differences in innate cell numbers in the peripheral blood and other compartments before and at completion of pulmonary tuberculosis treatment. It is important to assess parameters before and after treatment in order to understand how tuberculosis disease affects the immune system. Investigating these parameters will also allow for vaccine development in the future. Here we have evaluated the frequencies of these cells before and in the last week of tuberculosis treatment in order to allow for the investigation of immunological changes following successful completion of therapy. In addition, differences between circulating and site of disease innate cell frequencies were investigated.

In this study, the T cell frequencies observed are as expected. We observed a predominance of CD4+ over CD8+ T cells in whole blood in tuberculosis patients. CD4+ T cells percentages declined slightly at the end of treatment, not significantly so, but were still higher than CD8+ T cells. It has been shown that during anti-tuberculosis treatment a decrease in sputum bacillary load is observed and hence CD4+ T cell numbers might decrease due to weaker stimulation of the immune system (Belkaid & Rouse, 2005). Nevertheless, the importance of CD8+ T cells during *M.tb* infection should not be overlooked (Caccamo *et al*, 2009). Unfortunately, T cell frequencies were not assessed at site of disease and should be included in future studies.

In this study no difference were seen before and after tuberculosis treatment in NKT and iNKT cell populations. NKT and iNKT cells are involved in innate immunity and there is increasing evidence that suggests that cells are activated and play a protective role against pathogens during infection. A study by Sutherland *et al*. (2009) found that NKT frequencies of active tuberculosis cases compared to that of TST positive household contacts. In this study TST negative controls were also included and with treatment they found that NKT cell frequencies compared to that found in the TST negatives. More studies are investigating the role of these cells during bacterial infections. Chiba *et al*. (2008) found that NKT cell responses are self-terminating during the course of infection. Work by Sada-Ovalle *et al*. (2008) showed that the innate control of bacterial replication is dependent on iNKT cells. In addition, iNKT cells, even in limited numbers, are sufficient to restrict *M.tb* replication *in vivo* by rapidly producing large amounts of IFN-γ when activated. Since we saw, no differences in NKT and iNKT frequencies during treatment we can only concluded that they are present during active tuberculosis and still present at the end of treatment.

Most of the circulating NK cells were found to be mature NK cells during tuberculosis treatment and this is similar to what was found by Barcelos *et al*. (2006). We also saw significant decreased

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NK frequencies at the end of treatment. It is known that NK cells are critical in the innate immune system and sustain the early innate immune responses until adaptive immune responses can be mounted.

NK cells can be classified as classic NK, pre-NK, and mature NK cells. The role of NK cells during *M.tb* disease and infection is not clear, but it has been suggested that recruitment of pre-NK cells to the site of disease could be the linkage between the innate and adaptive immune systems (Sun & Lanier, 2009). Our results confirm the presence of NK cells during early tuberculosis disease and a decline in mature NK numbers at end of treatment.

Myeloid derived suppressor cells comprise of immature DCs, immature macrophages and granulocytes. Recent studies published have shown that alteration of cytokines during infections cause a progressive accumulation of myeloid cells in the spleen, lymph nodes and bone marrow. Mice primed with *M.tb* displayed an expansion of this population (Zhu *et al*., 2007). Here we showed that MDSC were significantly decreased after treatment in tuberculosis patients and that healthy controls have far lower numbers compared to tuberculosis patients and their household contacts. Further analysis showed that the MDSC frequencies were comparable to that of cancer patients both in whole blood as well at the site of disease. Within the myeloid derived suppressor cell population a CD33hi population was identified and defined as LIN1-/lo HLA-DR-/lo CD33hi CD11b+CD14+. Further identification and analysis is need. It would also be interesting to determine the function of the different MDSC populations.

Comparable to other studies, higher frequencies of MDSCs were found to be circulating than at the site of disease, even though not significantly so, implying that some of these immature cells mature at the site of disease, possibly due to the cytokine micro environment. Study groups are now focusing on how these cells suppress the immune system, especially in cancer. Work by Nagaraj *et al*. (2010) shed some light on the suppressive mechanism of these cells. They found when incubating peptide loaded MDSCs with antigen specific CD8+ T cells it would lead to the nitration of the molecules on the surface of CD8+ T cells. MDSC dependent generation of reactive oxygen species (ROS) and peroxynitrite causes nitration of tyrosines in the T cell receptor and CD8 molecules, preventing the ability of T cells to respond to specific peptides. This is localized to the site of physical interaction between MDSC and T cells. This only involves the T cell receptor specific for the peptide presented by MDSCs and will not induce signalling downstream of the T cell receptor.

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Recently a group investigated the early immune responses against BCG and found an accumulation of MDSCs after vaccination in mice (Martino *et al*., 2010). Upon BCG phagocytosis, the MDSCs produced nitric oxide (NO). However, despite NO production, they were unable to kill BCG or *M. smegmatis*, a commonly used non-pathogenic laboratory strain. Martino *et al*. (2010) concluded that the MDSCs are propathogenic cells, cells that dampen the early T cell response and might play an important role in allowing BCG to persist. From this work, we can conclude that this population does play a very important role during tuberculosis infection and disease. However, their exact function during tuberculosis disease still needs to be elucidated since we cannot conclude that the presence of these immature cells necessarily means that they have immunosuppressive activity. However, side-by-side comparison of pathogen induced with cancer induced MDSCs are complicated by the diversity of MDSCs phenotypes elicited and to some extent different tumors (Van Ginderachter *et al*., 2010).

Total dendritic cells were lower in tuberculosis patients at the end of the treatment. However, for the two subsets we showed that numbers increased at the end of treatment even though not significantly so. Furthermore, significant higher expression of the co-stimulator molecule CD40 was seen after treatment. It is known that the capacity of *M.tb* stimulated T cells to produce IFN-γ in modulated by signalling through CD40 on APC and CD40 ligand on T cells (Samten *et al*., 2000). CD40 ligand expression is reduced in peripheral blood T cells from tuberculosis. In addition, CD40 ligand expression increase after successful anti tuberculosis treatment and thus leads to the restoration of IFN-γ production (Samten *et al*., 2000).

In the two different experiments, the one assessing cells before and after treatment and the one comparing pleural fluid and whole blood; we saw very different frequencies in mDCs and pDCs. The only explanation for this could be the time delay before processing the blood. In the first experiment blood was transported to the lab from the field sites and this resulted in a time delay compared to the second experiment were whole blood was taken in hospital and processed within the hour.

Mendelson *et al*. (2006) found that patients with pleural tuberculosis have increased numbers of mDCs compared to healthy controls. Because we did not include healthy controls for this analysis, it is not possible to confirm the work by Mendelson *et al.* (2006). We found similar levels of mDCs and pDCs in diseased tuberculosis patients and their household contacts. When evaluating DC frequencies at the site of disease compared to circulating DCs, it was found that circulating DCs are higher compared to the site of disease even though not significantly so. This means that

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during infection and disease, DCs that have taken up antigens, are moving to the lymph nodes to present the antigens to T cells. Therefore, similar numbers is seen circulating in tuberculosis patients and their household contacts and a decline is seen at site of disease when compared to the periphery. Again, as for MDSC, the DC frequencies were comparable to that found in cancer patients but during cancer circulating cells were significantly lower compared to that seen during tuberculosis. A high incidence of lung cancer in this population often makes it difficult to differentiate between lung cancer and tuberculosis (Schuurmans *et al*., 2007). Here we also see that the cells present during these diseases are very comparable.

Strength of this work is that all cellular phenotyping were done directly *ex vivo* within a few hours of phlebotomy in order to see a realistic reflection of cell frequencies and to minimize handling and manipulation of cells. Sample size was limited and larger studies may show differences. During the follow up after treatment, only 12 patients were included due to non-compliance or for various other reasons. In future studies, this can be overcome by enrolling larger study groups. From this work, and the work of others, it is clear that the immune response against *M.tb* is complex and that interactions between the different cell types are essential to control/fight infection. More work should be done to look at innate cell types at different time points throughout treatment to better our understanding of the innate immune defence during tuberculosis treatment and disease. Functional studies should be performed to elucidate the role of MDSC during tuberculosis. Knowledge gained in the study of innate immune responses will aid in the diagnosis and treatment of tuberculosis and assist in vaccine development in the near future.

Acknowledgments

I would like to thank Dr Florian von Groote-Bidlingmaier and the GC6 study nurses for sample collection. I would also like to thank Dr Gillian Black for guidance in planning the study.

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Chapter 6

Discussion

Introduction

Defining correlates for protective immunity and disease susceptibility to *M.tb* in humans, and especially children, in a high tuberculosis incidence setting is not easy. IFN-γ secreted predominantly by adaptive immune cells is the most commonly used biomarker, which is a significant, but not sufficient, component of protective immunity against *M.tb*. As seen in the thesis the intricacy of the immune response against *M.tb* makes it apparent that other biomarkers are required. When investigating the immune response, the focus should be on the cell subsets present in a compartment and the overall cytokine microenvironment provoked by the presence of the cells. As soon as we have a clear picture of what occurs in different immune compartments, we can aim to identify biomarkers of infection and disease. The main obstacle is concurrent infections and as seen in this work helminths and HIV that are both predominant in this area lead to decreases in the protective immune response against *M.tb*. Here we discuss the most important findings from our evaluation of immune responses in of people with differential outcomes of *M.tb* exposure, as it is important to search for novel set of additional biomarkers.

Summary of findings

Cytokines patterns after acute exposure to *Mycobacterium tuberculosis*

We identified the cytokines produced in 346 children and adults (ages ranging from 0 to 40 years) in our study setting upon stimulation with ESAT-6 and BCG (Chapter 2). No previous studies were reported where background and *M.tb* specific cytokine values were determined in a large cohort. Hence, the values we see for this community can be used as reference values although genetic and environmental factors would have to be considered when extrapolating to other populations. We identified biomarkers for tuberculosis infection and disease specific to this community where latent infection is extremely high. Biomarkers will aid in tuberculosis diagnosis and early determination of treatment outcome and can be used in the future in conjunction with current available tests to add specificity and sensitivity in tuberculosis detection in this study area. Previous work has been done on specific disease phenotypes in the same community .Almost half of the participants were TST positive and upon ESAT-6 stimulation, we found that sCD40L, IFN-γ

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and IL-13 concentrations were higher in the TST positives compared to the TST negatives. However, after BCG stimulation sCD40L, IFN-γ and GM-CSF showed higher concentrations in the TST positive group compared to the TST negative group. Interestingly all these cytokines found to show differences also correlated very well with IFN-γ, leaving us to wonder if any other cytokines are really necessary to distinguish between these two infection groups. Other cytokine identified with potential diagnostic value is IL-1α and MIP-1α and it should be investigated further.One of the most important questions in tuberculosis pathogenesis is why some people progress to disease while others do not. In this investigation, we did included families from our study setting and retrospectively used information from the national tuberculosis register to assess outcome of *M.tb* infection in the 3 years following the study. The key finding from this investigation is the potentially important role of IL-10 as predictive marker for subsequent development of tuberculosis disease. We have identified study participants who progress to tuberculosis disease after the completion of the study and found a significantly higher IL-10 production in this group compared to those with previous tuberculosis and those who remained disease free. To our knowledge, this is the first study to show that even years before the progression to tuberculosis disease an increase in IL-10 levels is evident. Those found to progress to disease were all adults and hence we are still not closer to understanding why children might develop active disease. We conclude that the plasma IL-10 level may be suitable biomarkers for susceptibility to active tuberculosis in adults.

Cytokine profiles in children with documented *Mycobacterium tuberculosis* exposure and infection

In this investigation (Chapter 3) we compared the cytokine profiles in *M.tb* exposed and infected children with those who are exposed but uninfected as well as an unexposed and uninfected control group. Cytokine concentrations that were higher in the infected (QFT and TST positive group) compared to the uninfected (QFT and TST negative) were IL-2 and IP-10 at baseline and IL-2 and IL-13 at month 6. Here we showed again that these cytokines correlate highly with IFN-γ and that IFN-γ correlations were similar to that found in Chapter 2. Future work should include IL-2 in addition to IFN-γ in diagnostic assays in children. This would allow us to assess the importance of this cytokine in accurate identifying tuberculosis infection in children. The most important finding in this work is that IL-1β and IL-17 were able to differentiate between exposed and unexposed children. We showed significantly higher IL-1β levels at baseline in the exposed children compared to the unexposed however, 6 months later this difference was no longer apparent. This indicates that higher IL-1β and lower IL-17 production play a very important role

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during early exposure and that this cytokine signature is associated with protection from infection or at least generation of adaptive immunity against *M.tb*. This investigation identified these 2 cytokines that can be used in HIV uninfected children to determine recent tuberculosis exposure in conjunction with the exposure gradient all ready used. More studies should be performed in order to determine the effectiveness of these cytokines in HIV infected children.

Interferon-γ responses in HIV infected and uninfected children to *Mycobacterium tuberculosis*

In this chapter (Chapter 4), we investigated IFN-γ production in HIV infected and uninfected children in response to *M.tb* specific antigens. In addition, we compare the IFN-γ production in the 7 day whole blood assay compared to the overnight stimulation of the commercial QFT. HIV infected children have a higher risk of developing tuberculosis disease. However, very little has been done to investigate the effect of HIV infection on function of immune responses to specific mycobacterial antigens in children. Many studies now focus on the sensitivity and specificity of commercial IGRAs in different populations. Few studies have assessed these commercial assays in HIV infected children. Good correlation was found between the IFN-γ as measured by the 7 day whole blood assay and the IFN-γ measured by the overnight QFT. However, in the HIV infected participants lower IFN-γ concentrations were seen for most of the antigens tested compared to the HIV uninfected children. This shows that the *M.tb* specific immune response in the HIV infected children is weaker than that from healthy children. HIV infected children showed higher antigen specific responses after 7 day stimulation compared to the overnight stimulation in the QFT tubes. Clearly indicating that HIV infected children are more likely to respond to microbial specific antigens after 7 day stimulation. In addition, TB10.4 was found to induce higher IFN-γ responses in the HIV infected TST positive children. Since the ESAT-6, CFP-10 and TB7.7 antigens are used in commercial IGRA (QFT) the addition of TB10.4 might aid in the detection of tuberculosis infection in HIV infected individuals. In summary, our results suggest that detection of tuberculosis infection in the HIV infected children could be more sensitive when using a 7 day whole blood assay and in addition to the use of ESAT-6 and CFP-10, TB10.4 can be used. Future studies should investigate the use of TB10.4 in detecting tuberculosis infection in HIV infected children. With improved diagnosis of tuberculosis infection in HIV positive children, we would be able to target preventive therapy to tuberculosis and HIV infected children and reduce the risk of tuberculosis disease in these highly susceptible individuals.

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Characterization of quantitative and functional innate immune parameters in *Mycobacterium tuberculosis* diseased adults

Finally, innate and adaptive immune cell frequencies were evaluated before and at the end of tuberculosis treatment. In addition, differences between circulating and site of disease DCs and MDSCs frequencies were investigated. Few studies have looked at the differences in innate cell numbers in the peripheral blood and other compartments before and at completion of pulmonary tuberculosis treatment. However, compelling evidence exist indicating that the innate immune cells is important in the response to tuberculosis. Without a doubt, the presence of the MDSCs during active tuberculosis both in the peripheral blood and in pleural fluid is the important finding of this study. Our report is the first to show that MDSCs are present in humans during tuberculosis disease and that these cells decline in frequency upon tuberculosis treatment. MDSCs were first described during cancer and here we showed that the MDSC frequencies were comparable to that of cancer patients both in whole blood as well at the site of disease. Since we could only show the presence of these immature cells, it does not necessarily mean that they have immunosuppressive activity. Future studies should focus on the the molecular mechanisms involved in the induction of MDSCs, to characterise the mechanisms by which *M.tb* affects the expansion of MDSCs and the role of these cells in the pathogenesis of tuberculosis. It might be possible to use MDSCs in conjunction with other innate cell types as biomarkers to determine the extent of disease and to track the progress of tuberculosis treatment. More work should be done focusing on innate cell types throughout treatment at different time points to have better understand the innate immune defence during tuberculosis treatment.

Implications of main study findings

Recently in a review by Barry *et al*. (2009) a continuous response spectrum of the outcome of infection with *M.tb* was proposed instead of the traditional binary model of latent infection and active disease.

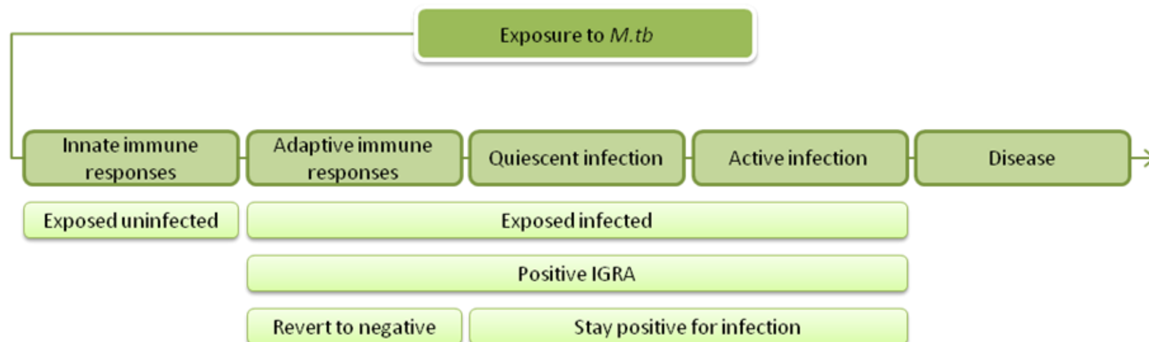

Figure 37: Spectrum of immune response to *M.tb* infection. Diagram adapted from Barry *et al*. (2009).

In this model (Figure 37), there are four different outcomes for latent infection. In the first, the innate immune system is able to successfully control the infection and the adaptive immune cells are never involved. Cytokines and chemokines secreted by innate immune cells, such as macrophages and DCs play a crucial part in the host defence against *M.tb* (Russell, 2007; Cooper & Khader, 2008). Differential induction of these molecules could contribute to variations in granuloma formation, latency and active disease as discussed earlier. The inflammatory response of macrophages to *M.tb* promotes local inflammation and innate control of bacterial infection by macrophages, neutrophils and natural killer cells (Van Crevel *et al*., 2002; Korbel *et al*., 2008). When the innate immune cells are not capable of controlling the infection adaptive T cells will be recruited to the site of infection in order to assist in fighting the infection. It will promote the migration of T cells from the draining lymph node to the site of *M.tb* infection where they exert adaptive control of *M.tb* through granuloma formation (Cooper, 2009). Upon involvement of the adaptive immune cells, immunological memory would be created. In the third part of the model, the adaptive immune cells would not be able to eradicate the infection completely and some bacteria would persist but would not replicate at this point. When the immune system then fails to control the bacteria in this non-replicating state active infection will occur. It is then at this

time when the person start to present with symptoms that progression to active disease takes place. Within this spectrum, we will look at the main findings of this investigation as set out in Figure 38.

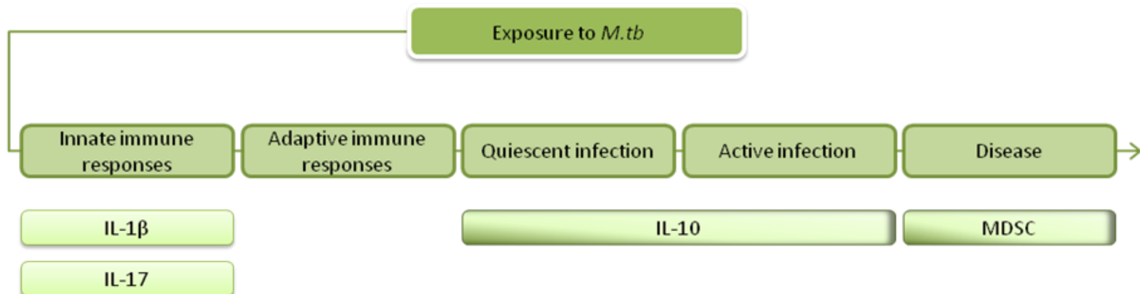

Figure 38: Summary of main findings of this investigation within the infection spectrum.

Effective anti-tuberculosis response starts with the host's innate immune response and IL-1β is a very early component of the innate immune response against infection. We showed that during *M.tb* exposure that IL-1β levels are increased in peripheral blood. Recent studies have shown that activation of the IL-1β inflammasome is an important first line of defence against *M.tb* (Mayer-Barber *et al*., 2010; Kleinnijenhuis *et al*., 2009). Previous reports did find IL-1β in the lungs of active tuberculosis patients that could be an insufficient delayed innate immune involvement. Therefore, we can conclude from our results that the levels of IL-1β in the exposed uninfected children indicates effective innate immune responses against *M.tb* and that the decline in IL-1β levels at 6 months is due to bacterial clearance. It remains to be seen what exactly is happening in the lungs, as site of disease, during initial exposure.

As described previously it has been shown that IL-1β induced inflammation increases IL-10 production. In our investigations, we observed that tuberculosis disease progressors have increased IL-10 levels up to 3 years before they were identified as active tuberculosis cases by the national tuberculosis treatment program. Taken into account the proposed model by Barry *et al*. (2009) we can conclude that from the stage of quiescent infection to the active disease stage can take any number of years. The high immune suppressive IL-10 levels measured in our study could have resulted from failed immune responses during the quiescent or early active stages of infection and may be caused by a range of genetic or environmental factors, including cytokine

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pathway defects, helminth infection or recurrent *M.tb* exposure. As a delicate balance between pro- and anti-inflammatory responses is required during persistent but controlled infection any disturbance in this balance by external influences, like recurrent *M.tb* infection could lead to establishment of active disease.

It is also known that MDSCs secrete IL-10 and therefore can be considered as an additional source of IL-10 during the onset of disease. Indeed as previously mentioned IL-1β, induced inflammation increases IL-10 production by MDSCs and induces MDSCs that ties in with what we have seen in our investigations. MDSCs may play a role in tuberculosis disease as a decline in numbers is seen after treatment. Since between 80 – 87 % (Constantoulakis *et al*., 2010) of active tuberculosis involves the lung the frequencies of MDSCs in the lungs becomes relevant. Although we did not examine MDSCs in the lung we did have an opportunity to investigate MDSCs frequencies at the site of disease by examining pleural effusions. However, more work must be done to compare cell frequencies in peripheral blood and in fluid from sites of disease, and in particular, the lung, as the immune response against *M.tb* can be compartment dependent. The question remains if MDSCs are involved in the pathogenesis of active tuberculosis infection or whether they are merely bystanders in the failed immune response against this organism.

Other environmental effects

It is known that an adaptive Th1 response is needed to control *M.tb* infection. Any environmental factor that decreases mycobacterial specific Th1 responses, including the promotion of Th2 or immune suppressive responses would negatively affect protection. The effect of co-infections on the anti-tuberculosis immune response should therefore be investigated. In this thesis, we did not have sufficient numbers of helminth-infected children to address the effect of helminth infection on anti-mycobacterial immune responses but we did show that the adaptive immune responses against *M.tb* are improved upon regular deworming. This may be due to a decrease in helminth induced Th2 or regulatory responses after deworming. Exposure to environmental mycobacterial and to high doses of *M.tb* is also known to induce a mixed Th1 and Th2 immune response and in addition, it was proposed that recurrent infections of *M.tb* might lead to a shift towards a Th2 type immune response. Recurrent exposure to NTMs or *M.tb* in our high transmission setting may therefore play a role in the high tuberculosis disease incidence in our study community.

As HIV mainly affect the adaptive immune system and more particularly the CD4+ T cells, it is easy to envisage how HIV infection can be detrimental to anti-mycobacterial immune responses. In our

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investigations, we showed that responses to mycobacterial antigens are diminished as measured by QFT in HIV infected children. Nonetheless, after 7 day stimulation increased IFN-γ responses are seen in the same HIV infected children. From our results, we propose that after the overnight stimulation (QFT) mostly effector memory is measured whereas after 7 days central memory to *M.tb* is measured. Studies have shown that central memory is poorly maintained during HIV infection, but in children provides us with a better and hopefully more accurate *M.tb* infection determination.

Future directions

The immune response to *M.tb* infection is extremely dynamic. The study of multiple cytokines by unbiased approaches like transcriptomics or by expanded targeted approaches like the multiplex cytokine arrays are particularly useful to increase our understanding of the different stages of latent infection and disease. In addition to these assays, *in vitro* assays, including trans-well cell culture experiments can help us to elucidate the functions of different cytokines during tuberculosis infection and disease.

Since children are more likely to be newly exposed to *M.tb* and as they represent a particularly vulnerable subpopulation the long-term follow up of children in household contact with adult tuberculosis cases is potentially of great value. For this purpose, the study of different protection phenotypes is helpful. This would provide an opportunity to investigate the contributors to the differential IL-1β level. Future work should focus on the role of the inflammasome during tuberculosis infection and disease.

Analysis of innate cell types such as myeloid derived suppressor cells coupled with cytokine levels would allow an understanding of how cells contribute to immune suppression during tuberculosis disease. Since it is sometime difficult to obtain enough blood or other bodily fluids from children most of this work needs to be done in adults. Functional assays need to be performed to assess the role of important innate cells in tuberculosis pathogenesis. Together, this will lead to a more comprehensive picture of the host innate immune response during infection. The information obtained through these future studies would be invaluable for future vaccination experiments.

All investigations need to be expanded to include approaches like transcriptomic (including deep sequencing, which also allows the evaluation of micro RNA and splice variants of immune

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molecules), metabolomics and proteomics to enable a comprehensive delineation of protective and non-protective anti-mycobacterial responses.

Conclusion

The immune response against *M.tb* is complex. Interactions between different cell types are essential to control *M.tb* exposure, infection and disease. In this thesis, we present new biomarkers that play important roles during the different stages of *M.tb* pathogenesis along the continuum of exposure, infection and disease. These may shed light on mechanisms of protection against *M.tb*, relevant to development of tuberculosis diagnostics and vaccine strategies. Combinations of multiple biomarkers including cytokines and chemokines and cell subsets are required to characterise biosignatures relevant to the diagnosis of tuberculosis infection and disease.

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Annexure 1

The following supportive data is included in this annexure.

1. Comparison of age vs TST results. One way ANOVA test of variance in the whole study group.
2. Comparison of age vs TST results. One way ANOVA test of variance in children under 16 years of age (as some studies performed only included younger children).
3. Comparative plots of IL-1α (log value) in study groups as described in Chapter 3. IL-1α was chosen as a representative for all other cytokines.
4. FMO plots for CD33 vs CD11b for the identification of MDSCs.

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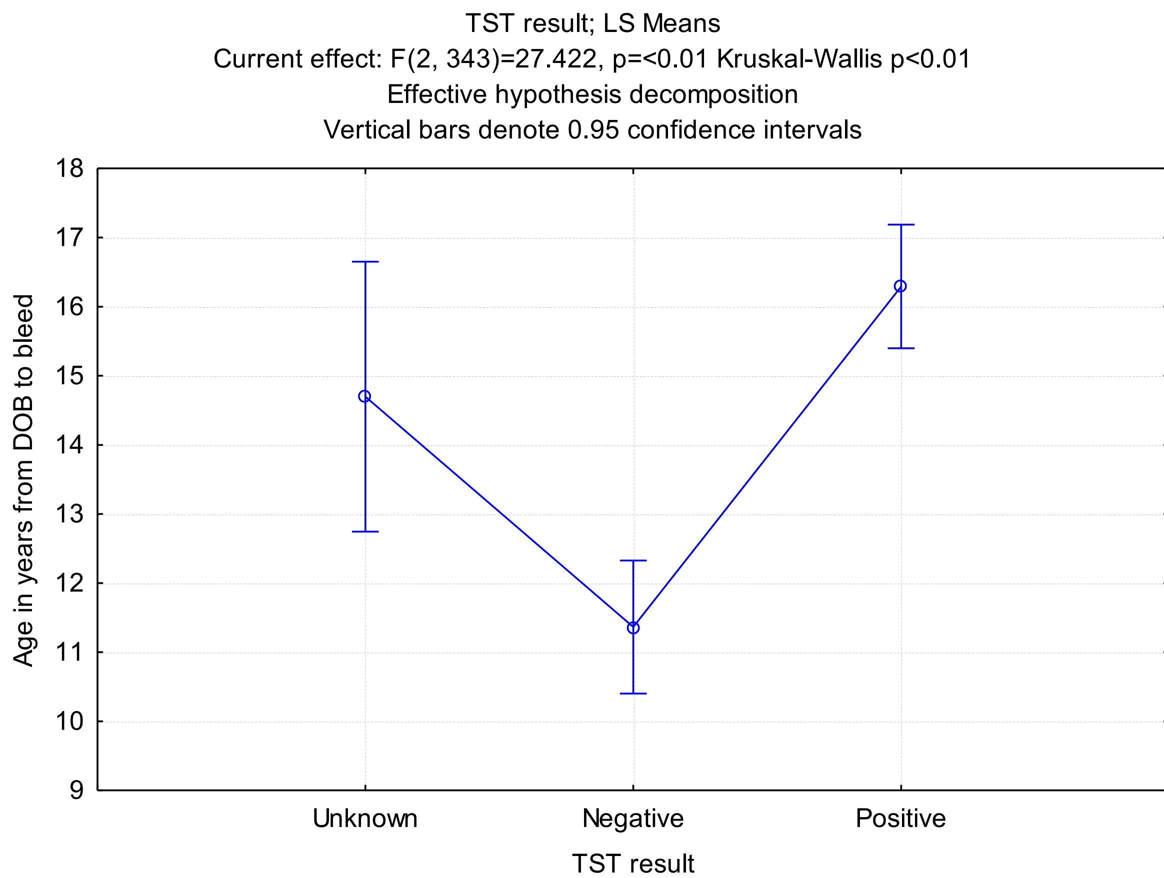
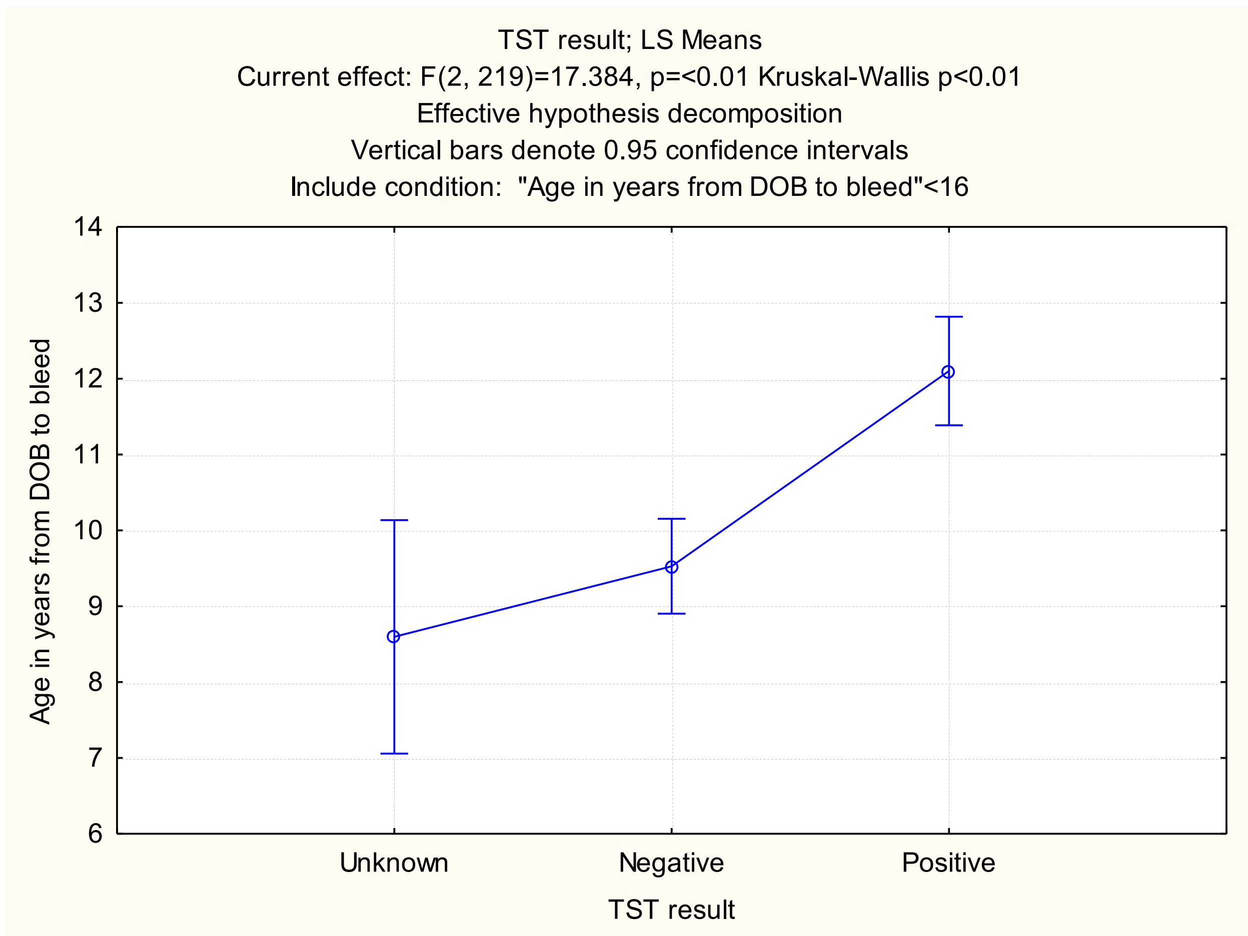
1. **Comparison of age vs TST results. One way ANOVA test of variance in the whole study group.**

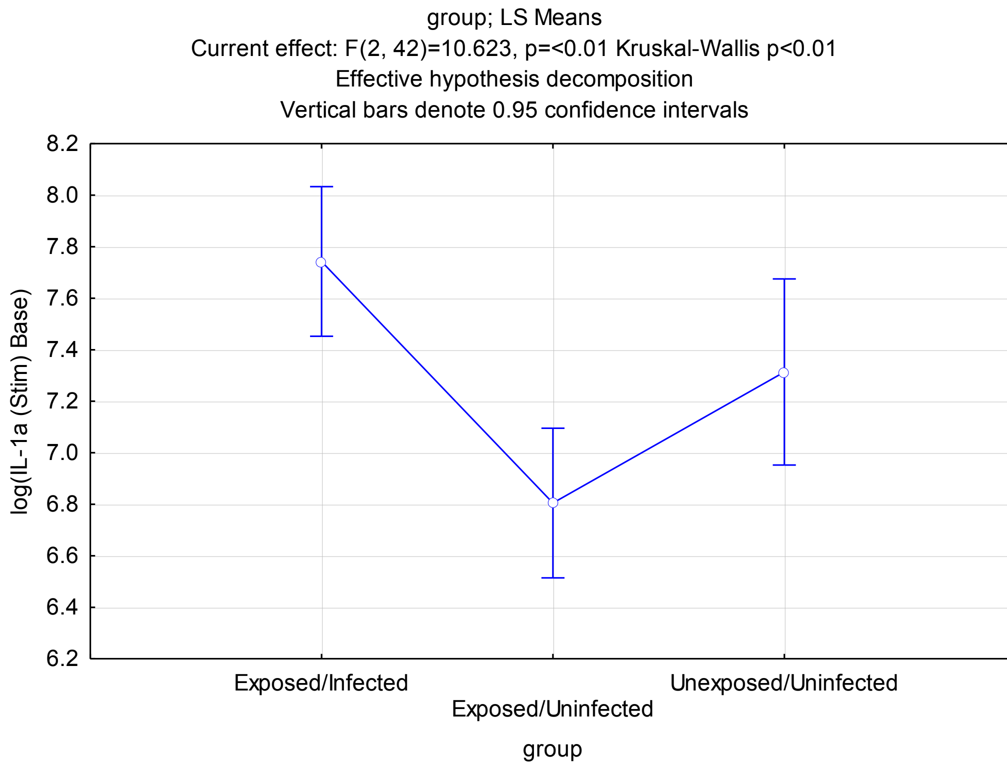
   This analysis was performed to confirm that TST positivity is not associated with recent BCG vaccination. Results indicate that a positive TST is associated with older age.

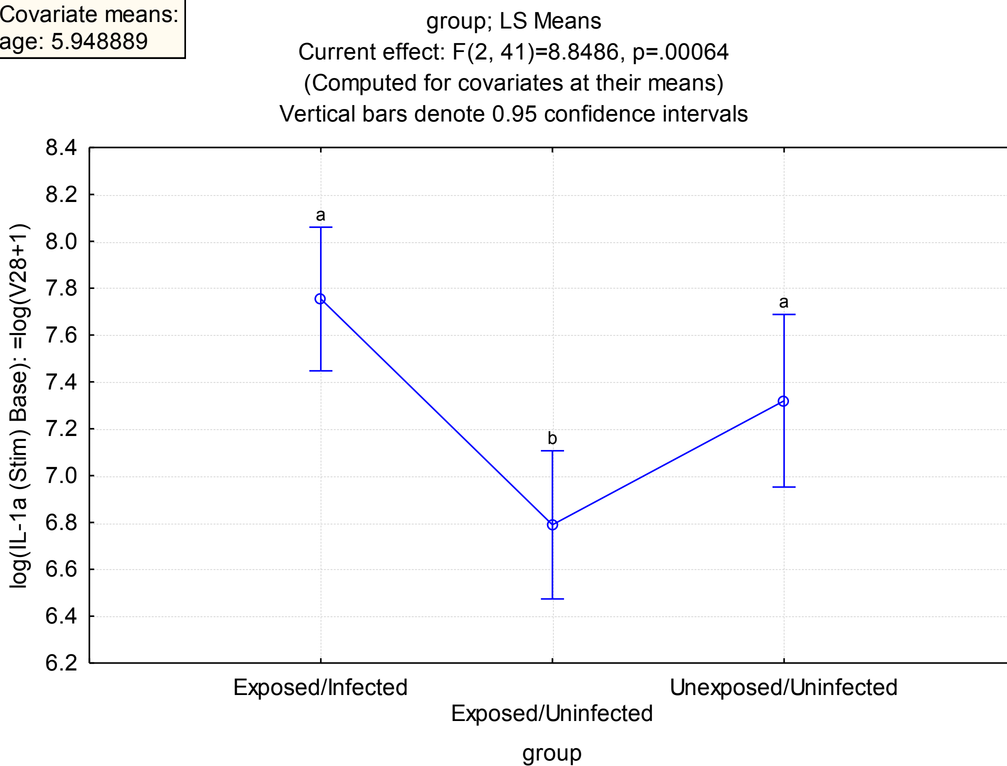


2. **Comparison of age vs TST results. One way ANOVA test of variance in children under 16 years of age (as some studies performed only included younger children).**



3. **Comparative plots of IL-1α (log value) in study groups as described in chapter 3. IL-1α was chosen as a representative for all other cytokines.**
   The first plot show results without adjusting for age. In the second plot age was included as a covariate and results did not change.





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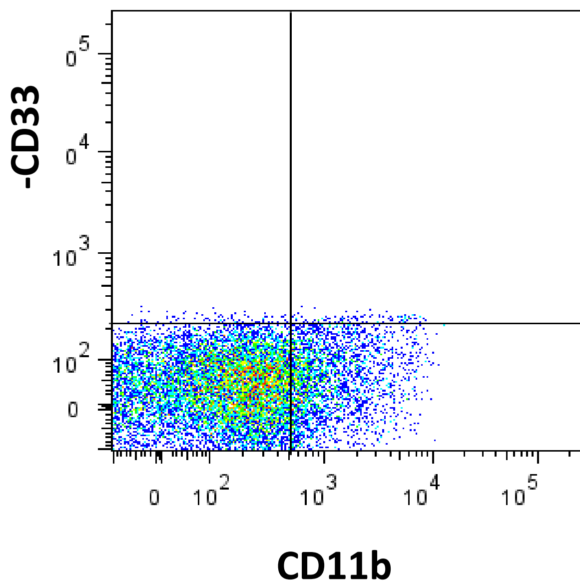
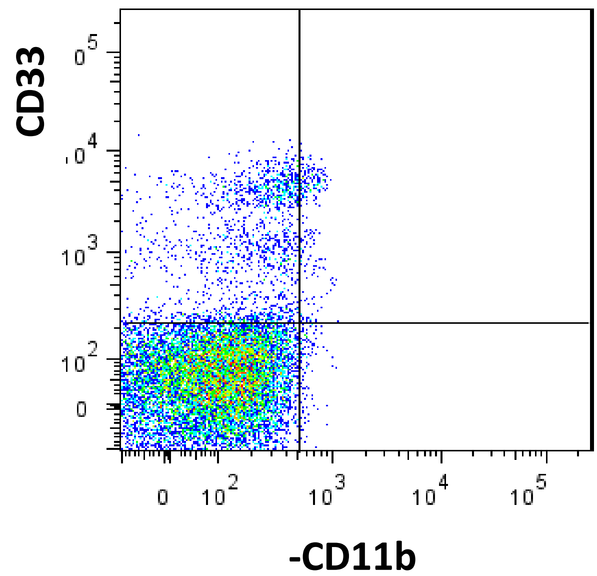
4. **FMO plots for CD33 vs Cd11b for the identification of MDSCs.**

   In the first plot the FMO control for CD33 is shown. Gate was set in order to determine the cut off between CD33 negative and positive (horizontal line). In the second plot the FMO control for CD11b is shown. Gate was set to determine the cut off between CD11b negative and positive (vertical line). The gating strategy was employed in the analysis of MDSCs.





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Annexure 2

The next pages are examples of the case report form, laboratory form and treatment outcome forms as used for the work as presented in Chapter 5. The mail merge function was used to insert participant numbers on the forms as recorded in the Excel database. All participant numbers are indicated by XXX on the forms in this example.

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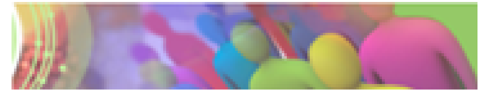
TB Treatment Response Study
Month 6 Case Report Form
January 2010—June 2010

TB TREATMENT
RESPONSE STUDY



Patient recruitment ID: Rxxx   Follow up ID: F6-xxx

Follow up date: DD/MM/YYYY

1. Personal Information

1.1 Date of Birth: DD/MM/YYYY

1.2 Gender: M / F

2. TB Treatment

2.1 When was TB treatment started? DD/MM/YYYY

2.2 Was TB treatment successfully completed? Y / N

2.3 If yes, when was TB treatment completed? DD/MM/YYYY

3. Culture Results

Month 2 culture:

3.1 Please indicate smear/culture results: Pos / Neg / Unknow

End of treatment culture:

3.2 Please indicate smear/culture results: Pos / Neg / Unknow

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TB Treatment Response Study
Month 6 Lab Form
January 2010—June 2010

TB TREATMENT RESPONSE STUDY

Patient recruitment ID: **R**xxxx
Follow up ID: **F6**-xxx

To be completed by study nurse:

Date blood taken	DD/MM/YYYY
Time blood taken at clinic	H
Time received in lab	H
Time PBMC isolation start	H

Blood tubes collected:

NaHep volume	
NaHep volume	
NaHep volume	
NaHep volume	
PaxGene volume	
EDTA volume	
Sputum	

ID on tubes and sputum pot should be **«F6-xxx»**

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TB Treatment Response Study
Month 6 Outcome
January 2010—June 2010

TB TREATMENT
RESPONSE STUDY

Patient recruitment ID: **Rxxxx**
Follow up ID: **F6-xxx**

Followed up time frame: **«1 week before» - «Follow up 2010»**

Is the patient followed up within the time frame? ☐ Y ☐ N

If no, please give reason and date of follow up:...

Please choose an outcome for patient:

1 Cured patient: a patient whose sputum smear is negative in the last month of treatment ☐

2 Treatment completed: treatment period completed but criteria for cure or failure are not met due to contaminated smear or as no smear was done ☐

3 Failed treatment: smear becomes positive again or remains positive at or after month 5 of treatment ☐

4 Not treated: less than 10 dosages TB treatment taken ☐

5 Defaulter: a patient who does not adhere to treatment regimen for at least 2 consecutive months ☐

6 Transferred out: transferred while on treatment to another reporting or recording unit, where treatment is continued but outcome is unknown ☐

7 Death while on TB treatment: any death, not necessarily from TB ☐

8 Missed visit: missed visit with study nurse ☐

In the case of a missed visit please obtain the outcome from the TB clinic records and note here: ....

Nurse initials: ....

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