Cellular immunity of naïve and BCG vaccinated neonates

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DEDICATION

I dedicate this thesis to my late mother, Esmé Owen. You were the epitome of selfless devotion to your family and made us all feel special and loved. You still inspire me.

and

To my father, Keith Owen, an impeccable gentleman and the bravest man I know. You always thought of others, even during times of great suffering, but sadly you passed away before hearing the outcome of my PhD. You taught me to never give up.

DECLARATION

____

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PUBLICATION

Results from this thesis have been published:-

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ABBREVIATIONS

---

^{3}HTdR	Tritiated thymidine
7AAD	7-Aminoactinomycin D
AA Mø	Alternatively activated macrophages
AFB	Acid-fast bacilli
AIDS	Acquired immunedeficiency syndrome
-APC	Allophycocyanin
APC	Antigen presenting cells
BCG	Bacille Calmette-Guérin
BCG-gfp	Bacille Calmette-Guérin expressing green fluorescent protein
BSA	Bovine serum albumin
CA Mø	Classically activated macrophages
CBMC	Cord blood mononuclear cells
CCR	Chemokine receptor
CD	Cluster of differentiation
CFP-10	Culture filtrate protein 10
CFU	Colony forming units
CM	Complete medium
CMI	Cell mediated immunity
Cos	Costimulants
CPD	Citrate phosphate dextrose
CpG DNA	Cytidine-phosphate-guanosine DNA
CPM	Counts per minute
CTL	Cytotoxic T lymphocytes
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule-grabbing non-integrin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot

ESAT-6	Early secreted antigenic target 6kDa protein
FACS	Fluorescence activated cell sorter
FB	FACS wash buffer
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
γδ	Gamma delta
g	Gravitational force
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
HEPES	N-2-Hydroxyethylpiperazine-N’-2-Ethanesulfonic Acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICC	Intracellular cytokine
ID	Intradermal
iDC	Immature dendritic cell
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGRA	Interferon-gamma release assay
IL	Interleukin
ION	Ionomycin
IQR	Interquartile range
iTreg	Induced regulatory T cells
kDa	Kilodalton
LAM	Lipoarabidomannan
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
mDC	Mature dendritic cell
MDR-TB	Multi-drug resistant TB
MFI	Mean fluorescent intensity
MHC	Major histocompatability complex
Mø	Macrophages
MOI	Multiplicity of infection

Mtb	*Mycobacterium tuberculosis*
NCR	Natural cytotoxicity receptors
NK	Natural killer
NKT	Natural killer T-cell
NRAMP1	Natural resistance-associated macrophage protein 1
nTreg	Natural regulatory T cells
OADC	Oleic acid albumin dextrose catalase
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Percutaneous
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PGE_2	Prostaglandin E2
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PPD	Purified protein derivative of *Mycobacterium tuberculosis*
PRR	Pattern recognition receptors
RD1	Region of difference one
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation
SEB	Staphylococcal enterotoxin B
SI	Stimulation index
SLE	Systemic lupus erythematosis
SNF	Supernatant fluid
TB	Tuberculosis
TBM	Tuberculosis meningitis
TCR	T cell receptor
Tfh	T follicular helper cells
TGF-β	Transforming growth factor beta
Th	T helper
Th1	Type 1 helper

Th2	Type 2 helper
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cells
TST	Tuberculin skin test
UNICEF	United Nations Children’s Fund
UV	Ultraviolet
WCB	Whole cord blood
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant TB
ZN	Ziehl-Neelson

ABSTRACT

Despite more than 95% vaccination coverage with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG), tuberculosis (TB) remains epidemic in South Africa. The dichotomy that successful pregnancy is usually associated with a type 2 (Th2) cytokine profile in the newborn child, whereas immunity to *Mycobacterium tuberculosis* (Mtb) is associated with the development of a type 1 helper (Th1) cytokine response, could impact on the subsequent adaptive immune response to BCG vaccination. Previous studies have suggested that immune responses in early life may be defective and related to the immaturity of antigen-presenting cells and/or T cells.

In the absence of prior exposure to mycobacterial antigen, neonates must rely on the innate immune response to mycobacteria for protection against TB. To better understand neonatal immune responses to mycobacteria, cord blood was collected and immune responses compared to those of healthy tuberculin skin test positive (TST^+) adults, or BCG vaccinated 13 week old infants. Macrophages play a vital role in innate immunity to mycobacteria with classically activated macrophages reputedly promoting Th1 immunity, and alternatively activated macrophages dampening Th1 immunity. Therefore both these macrophage types were differentiated from cord blood and adult blood and subsequently compared. Innate cells were found to be similar to adult cells in maturation capabilities, phagocytic properties (as determined by the ability of the cells to phagocytose green fluorescent protein labelled BCG), and cytokine production following this infection. In addition, the capacity of cord blood and adult macrophages to kill and/or control the growth of both avirulent and virulent mycobacteria was evaluated and found to be similar (Chapter 3).

To identify possible differences between adult and cord blood dendritic cell (DC) function, a comparison of both immature and mature DC from adults and cord blood was made. DCs are the only antigen presenting cell capable of stimulating naïve T cells. It was shown that DC from cord blood had similar maturation capabilities as DC from adult peripheral blood and the cells were similarly functional as demonstrated by

their ability to induce allogeneic T cell proliferation and interferon gamma (IFN-γ) production (Chapter 4).

The secreted cytokine and intracellular cytokine responses following *ex vivo* stimulation of cord blood cells with BCG were evaluated and the cytokine profile compared to responses in vaccinated neonates. Similar cytokine profiles were detected in cord blood and in peripheral blood of neonates vaccinated with BCG at birth. However, prior to vaccination, IFN-γ was produced by natural killer cells, but not by T cells. Following vaccination IFN-γ was clearly detected within $CD4^+$ and $CD8^+$ T cells (Chapter 5).

In summary, the cells of the innate response of naïve neonates stimulated *ex vivo* with BCG were found to be similarly functional when compared to these cells from vaccinated neonates or adults. The absence of T cell activation by mycobacterial antigens prior to BCG vaccination of newborns resulted in lower levels of IFN-γ production following *ex vivo* exposure of the blood cells to BCG. However, the IFN-γ produced by NK cells of pre-vaccinated neonates would be expected to facilitate potential activation of macrophages for anti-mycobacterial function similar to macrophage activation obtained post vaccination exposure to antigen specific T cells. Thus, BCG vaccination induced a quantitative increase in production of the protective Th1 cytokine IFN-γ but not a qualitative change. These results may explain why BCG vaccination of newborns at birth does not protect against TB per se but only against the more severe types of TB, miliary TB and TB meningitis.

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Hypothesis

The hypothesis underlying the studies reported here is that innate immune response induced **protection** against Mtb infection/progression to disease engendered by BCG vaccination is **quantitatively different** (i.e. the level of cytokines detected and the extent of innate immune cell activation is lower before vaccination than after vaccination), but **not qualitatively** different (i.e. a similar profile of soluble mediators and similar functional activation of dendritic cells and macrophages are found prior to and post BCG vaccination) from the pre-vaccination innate **protective response** of neonates against this pathogen. Consequently, although BCG vaccination fundamentally modifies the acquired cell mediated immune response of the neonate, it does not significantly improve protection against progression from infection to active TB disease. Thus the essential soluble factors/components and innate cell activity that induce protection against TB are already produced by the naïve innate immune response. Acquired immunity, including antigen-specific T cell activation, results in more and/or more sustainable production of the same soluble factors/components that drive cell activation. Thus in high prevalence settings, such as the Western Cape in South Africa, BCG vaccinated neonates are only protected against the more severe forms of disease (miliary and TB meningitis) and not against TB per se (Mahomed *et al.*, 2006; Moyo *et al.*, 2010). As reported by Moyo *et al* even optimal BCG vaccination does not reduce the 1-2% incidence of TB seen in these Western Cape communities during the first 2 years of life (Moyo *et al.*, 2010).

CHAPTER 1

LITERATURE REVIEW

1.1 EPIDEMIOLOGY OF TUBERCULOSIS

1.1.1 Global epidemiology of tuberculosis

TB is caused by the bacterium Mtb and is the leading cause of death from a curable infectious disease, killing approximately 5000 people every day. These alarming statistics caused the World Health Organisation (WHO) to declare TB a global emergency in 1993 (WHO, 2004; Dye, 2006; WHO, 2006 B, 2009). About 2 billion people, or one-third of the world's population are latently or asymptomatically infected with TB (WHO, 2004, 2006 B). However, ninety percent of these latently infected individuals will never develop active TB. The ten percent that do develop TB are often usually not immune suppressed. However, if they are immune compromised, they are much more likely to develop active disease such as from human immunodeficiency virus (HIV) infection, advancing age or some other medical condition (WHO, 2006 B). Even though progression to active TB disease does not occur in the majority of those that are latently infected, the WHO reported 9.27 million new cases and 1.78 million deaths from TB in 2007 (WHO, 2009).

Given the fact that immune suppression increases the likelihood of developing active disease it is hardly surprising that Africa has the highest estimated incidence rate of TB with 363 per 100 000 population affected per year and 31% of all new TB cases globally (Figure 1.1) (WHO, 2009). It has been reported that co-infection with HIV increases the risk of developing TB 30-fold (Collins and Kaufmann, 2001 A), with Africa accounting for 79% of HIV positive TB cases globally in 2007 (WHO, 2009). Although the incidence of TB is falling or stable in five out of six WHO regions in the world, the global annual incidence of TB has increased since 1980 and is growing at approximately 1% per year, largely due to the spread of HIV in Africa where most deaths among HIV infected people are as a result of TB (Collins and Kaufmann, 2001 A; Corbett *et al.*, 2003; Dye, 2006; Harries and Dye, 2006; WHO, 2006 B, 2009). Antibiotic treatment of sputum positive pulmonary disease is the cornerstone of TB

control programs. However, the duration of treatment for TB is extensive and failure to complete treatment often gives rise to the evolution of drug resistant strains of Mtb (Maartens and Wilkinson, 2007; WHO, 2007 A). It is hardly surprising therefore that drug resistant strains of Mtb have emerged and the TB crisis has become even more serious with the increase in incidence of drug-resistant and multi-drug resistant TB (MDR-TB). More recently extensively drug-resistant TB (XDR-TB) has been reported which is alarming (Bhatt and Salgame, 2007). It has been estimated that 4% of TB patients worldwide are infected with MDR-TB (Maartens and Wilkinson, 2007). In 2007 there were 0.5 million cases of MDR-TB, of which 0.3 million were among people not previously treated for TB, a fact that is worrying, because it indicates the spread of the resistant Mtb (WHO, 2009). MDR-TB is defined as being resistant to rifampicin and isoniazid (WHO, 2007 A). XDR-TB is resistant to rifampicin and isoniazid (i.e. MDR-TB), as well as any quinolone, in addition to at least one injectable second-line agent (capreomycin, amikacin or kanamycin). XDR-TB is therefore essentially untreatable and the majority of people infected with XDR-TB will die (Maartens and Wilkinson, 2007; WHO, 2007 A).

Estimated number of new TB cases, by country, 2007

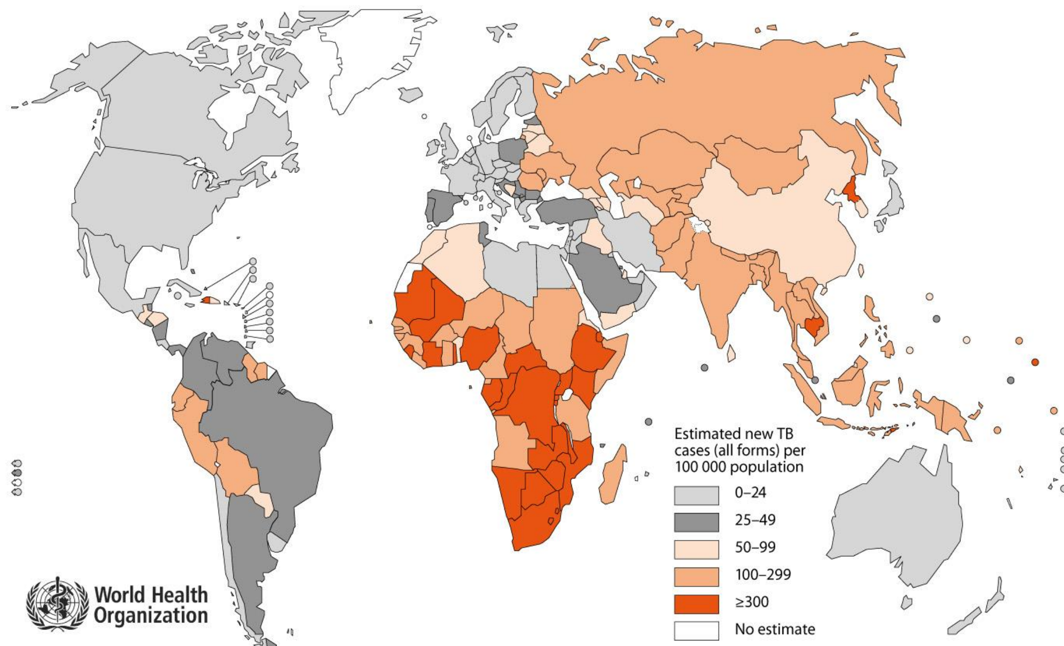


Figure 1.1 Estimated world incidence rates of tuberculosis distribution in 2007 (WHO, 2009).

1.1.2 Epidemiology of tuberculosis in South Africa

The incidence rate of TB in South Africa is extremely high. South Africa ranks 5th in the world after Nigeria for TB incidence of all forms with 948 per 100 000 population (WHO, 2009). In 2007 almost a third (31%) of all TB cases in the entire African region occurred in South Africa (WHO, 2009). Since 1995 the incidence of new TB cases has risen dramatically in South Africa and this increase is most likely due to the escalation in the incidence of HIV associated TB (WHO, 2006 A). Due to the enormous burden of TB worldwide, the Stop TB Partnership was established in 2000 with a goal of eliminating TB as a public health problem. This Partnership has set targets for 2005, 2015 and 2050. One of the targets is to halt and begin to reverse the incidence of TB by 2015, as well as to reduce by 50% the prevalence and mortality rates relative to 1990 levels. Although a few countries in Africa have recorded a decrease in prevalence, incidence and mortality of TB, South Africa is one of the countries in which this has not been achieved (WHO, 2007 A, 2009). In 2002, the Western Cape Province of South Africa had the highest notification rate of TB in the country and in 2003 and 2005 reported a TB incidence of 931 and 1037 per 100 000 population respectively (National Tuberculosis Control Program South, 2002; Schaaf *et al.*, 2007). Within the Cape Town metropole, which is situated in the Western Cape Province, and the area where this study was undertaken, the incidence of TB was 562 and 581 per 100 000 in 2000 and 2001 respectively (Soeters *et al.*, 2005). Children under 15 years of age, which is the WHO category for TB in children, form between 15-20% of this TB burden. Although these statistics for the incidence of TB in children are alarming, the true figures could be even higher, due to the fact that it is difficult to accurately diagnose childhood TB (Nelson and Wells, 2004; Marais *et al.*, 2006). Another disturbing fact is that the majority of resistant cases of TB have occurred in South Africa. In 2007, 85.4% of MDR-TB and 99.1% of XDR-TB cases were reported by South Africa (WHO, 2007 A).

1.2 *MYCOBACTERIUM TUBERCULOSIS,* THE PATHOGEN

The genus *Mycobacterium* is comprised mostly of soil dwelling environmental bacteria. Only a few members of the genus are successful pathogens, including *mycobacterium tuberculosis* the causative agent of TB. Mtb is a non-motile

pleomorphic rod with a complex, lipid-rich cell wall (Cosma *et al.*, 2003; WHO, 2004; Sundaramurthy and Pieters, 2007). The organism is a gram positive, obligatory aerobic intracellular pathogen of macrophages with a preference for growth in the lung, an environment rich in oxygen (Raja, 2004; WHO, 2004). The lipid-rich walls of the bacteria resist decolourisation with acid and Mtb is thus classified as acid fast. This attribute is utilised by the Ziehl-Neelsen (ZN) stain for acid-fast bacilli (AFB), which is used in the diagnosis of Mtb infection. In addition, the waxy cell walls of Mtb makes it relatively resistant to drying facilitating the spread of Mtb from one infected individual to the next within microdroplets of an aerosol that is formed when an infected person coughs or speaks (Frieden *et al.*, 2003). The organism is however sensitive to heat and ultraviolet (UV) light (WHO, 2004).

1.2.1 Pathophysiology and diagnosis of **Mycobacterium tuberculosis** *infection*

When Mtb containing aerosol droplets are inhaled by the host and deposited in the lung, alveolar macrophages and/or dendritic cells are the first host cells to encounter the bacilli and phagocytose them thereby establishing the lung as the primary site of Mtb infection (Frieden *et al.*, 2003; Raja, 2004). Mtb taken up by alveolar macrophages induce the immunological events (discussed in detail in section 1.7 on the host immune response to Mtb) which result in either the successful containment of infection or the progression of the infection to active disease (Frieden *et al.*, 2003; Hanekom *et al.*, 2007). The primary infection usually remains contained as a latent or dormant TB infection and active TB may never occur. Dormant Mtb survive in a non-replicating state but retain viability for extended periods of time. However, reactivation of this infection may arise following immune suppression. The greatest risk factor for reactivation of latent TB is HIV infection (Boon and Dick, 2002; Frieden *et al.*, 2003; Kashino *et al.*, 2006).

Progression to active disease is associated with the recruitment of additional mononuclear leukocytes to the site of infection and the formation of a cellular structure, known as a granuloma, which serves to restrict the spread and control the growth of the bacilli within infected macrophages. However, if the bacilli continue to grow within the macrophages of the granuloma, additional mononuclear leukocytes exit from the circulation, enlarging the cellular mass in the lung, ultimately leading to

the formation in the granulomas of central necrosis and liquefaction which can drain to give rise to a lung cavity. Bacilli growing at the cavity surface now have access to the airway and can be spread to others when the infected host coughs. If left untreated, each person with active TB will infect on average 10-15 people every year (Frieden *et al.*, 2003; WHO, 2004, 2006 B, 2007 A). In 2001 it was estimated that only 45% of TB cases were diagnosed and reported globally. Without treatment, approximately 50% of TB patients die of the disease (Collins and Kaufmann, 2001 B).

Macrophages form part of the innate immune response which is the initial host response to exposure to Mtb. Innate immunity is non-specific and protects against a wide range of bacterial infections. The migration of infected macrophages from the lung through the lymphatic system to the hilar lymph nodes initiates the activation of T cells and other components of the adaptive immune response. This response is a specific response to Mtb antigens (discussed in detail in section 1.7 on the host immune response to Mtb) (Frieden *et al.*, 2003; Hanekom *et al.*, 2007). Activated T lymphocytes produce soluble mediators that sustain macrophage activation inside the granulomas thus contributing to limiting both replication and further spread of Mtb (Schluger and Rom, 1998).

Common symptoms that aid in the diagnosis of active pulmonary TB include a persistent cough lasting longer than 2 weeks, fever, night sweats, weight loss, shortness of breath, haemoptysis (blood-stained sputum) and chest pain. The definitive diagnosis of active TB is dependent on the bacteriological culture of Mtb. The ZN stain for observation of AFB in sputum is an important aid in diagnosing active pulmonary TB infection. Although only 50-80% of Mtb culture positive individuals are positive for AFB in sputum this is important data to obtain, as smear positive TB is more infectious than smear negative TB. The chest radiograph, is also an important aid in the diagnosis of active pulmonary TB (Frieden *et al.*, 2003).

Following primary exposure, once an individual has developed adaptive immunity against Mtb they are usually positive for the TST (Frieden *et al.*, 2003). Thus, the TST, which uses purified protein derivative (PPD) of *Mycobacterium tuberculosis* as its test antigen, assists in the diagnosis of latent Mtb infection. Limitations to this

procedure include low sensitivity in immunocompromised patients and cross-reactivity with BCG vaccination and exposure to environmental mycobacteria (Huebner *et al.*, 1993; Zar, 2007). Peripheral blood tests are also available, which assist in identifying individuals that are latently infected with Mtb. One *in vitro* test, using whole blood, measures the amount of IFN-γ released by sensitised T cells when exposed to mycobacterial antigens. A commercial IFN-γ release assay (IGRA) such as Quantiferon TB, which uses Mtb specific antigens such as early secreted antigenic target 6 kilodalton (kDa) protein (ESAT-6) and culture filtrate protein 10 (CFP-10), is useful in diagnosing latent Mtb infection. Another IGRA, which is an enzyme-linked immunospot (ELISPOT) assay, T-SPOT.TB uses peripheral blood mononuclear cells to detect the number of T cells producing IFN-γ in response to these Mtb specific antigens. Results with the T-SPOT.TB assay are expressed as the number of spot-forming cells. The test is relatively sensitive and specific for the detection of latent TB infection. The correlation between TST and IGRA however has been low, with IGRA showing a greater sensitivity and specificity than TST (Bellete *et al.*, 2002; Frieden *et al.*, 2003; Mazurek and Villarino, 2003; Adetifa *et al.*, 2007; Zar, 2007).

1.2.2 **Mycobacterium tuberculosis** *infection in children*

It is difficult to accurately estimate the global burden of paediatric TB due to the difficulty of achieving a definitive diagnosis. The reasons for this include the increased presence of extra-pulmonary disease and a lack of universally accepted clinical standards in defining TB cases (Nelson and Wells, 2004). The accepted "gold standard" for diagnosis of TB in adults is the bacteriological culture of Mtb (Marais *et al.*, 2006; WHO, 2006 C, 2007 B). However, positive culture yields in children with suspected TB are reported as less than 30 to 40%. Furthermore, sputum smear microscopy for AFB, often the only diagnostic tool in endemic areas, is not only difficult to obtain, but rarely positive in children. Only 10 to 15% of children <10 years of age are sputum AFB smear positive (Marais *et al.*, 2006; Marais, 2007). Given these limitations, approximately 15-20% of TB cases in countries with a high incidence of TB and 11% worldwide have been reported to be children <15 years of age (Nelson and Wells, 2004; Marais *et al.*, 2006; WHO, 2006 C; Zar, 2007; WHO, 2007 B). As the WHO currently reports only smear-positive cases by age, and the

majority of cases in children are smear negative, the burden of TB in children is underestimated (Nelson and Wells, 2004; Marais *et al.*, 2006).

Diagnosis of childhood TB in endemic areas depends mainly on clinical features and the subjective interpretation of chest radiographs. In addition, the TST has limited diagnostic value in children in endemic areas, as this is often positive in healthy BCG vaccinated children (Marais *et al.*, 2006; Zar, 2007). TB symptoms in children include chronic unremitting cough, fever, a history of close contact with an adult with pulmonary TB, failure to thrive and fatigue (Houwert *et al.*, 1998; WHO, 2006 C; Marais, 2007; WHO, 2007 B). However, in countries where TB is endemic, factors such as non-specific clinical signs, coexisting malnutrition and variable interpretation of chest radiographs renders diagnostic confirmation even more difficult (Zar, 2007). This diagnostic dilemma has been compounded by the HIV epidemic in which chronic lung disease, immunologic anergy, coexisting malnutrition and non-specific clinical and radiological signs make definitive diagnosis of TB even more challenging. The consequences of undiagnosed or untreated paediatric TB are particularly serious as children are more likely to develop miliary (disseminated) and meningeal disease compared with adults (Marais *et al.*, 2006; Zar, 2007). Disseminated TB is defined as involvement of many organs simultaneously (Frieden *et al.*, 2003; WHO, 2004, 2006 B, 2007 A). TB meningitis is the most dangerous complication of miliary disease in children and childhood deaths from TB are usually caused by TB meningitis or miliary TB (Hussey *et al.*, 1991; Smith *et al.*, 1997; Frieden *et al.*, 2003; WHO, 2004; Marais *et al.*, 2006; WHO, 2006 A, 2007 A).

Children infected with TB differ from adults in both the immunologic and pathophysiologic response, which includes poor containment of the organism at the original site of deposition. The exact immune mechanisms underlying adult-type disease are unclear, but it has been noted that as children enter puberty, adult-type disease emerges and there is a striking rise in the incidence of TB at this age (Marais *et al.*, 2006; WHO, 2006 C, 2007 B). Staggering statistics indicate that following primary infection with Mtb, 43% of children younger than 1 year of age, 24% aged 1-5 years and 15% of adolescents progress to active TB compared to 5-10% of adults, who over a lifetime will develop active TB. Age therefore appears to be the most important variable that determines the risk of developing active TB in immune-

competent individuals. This finding suggests an inability of the developing immune system to mount a protective response against Mtb, the exact mechanisms of which are poorly understood (Nelson and Wells, 2004; Marais *et al.*, 2006). In very young infants who have never previously been exposed to TB, the innate immune cells would be the hosts' first line of defence. Given the alarming statistics of progression to active disease after a primary infection in young children, it is important to understand the innate response to Mtb and BCG in naïve individuals compared to that of adults. This study examined the innate response of naïve newborn neonates and compared it to that of TST^+ adults.

1.3 *MYCOBACTERIUM BOVIS* BACILLI CALMETTE-GUÉRIN, THE VACCINE

In 1904, at the Institut Pasteur in Lille, France, bacteriologist Albert Calmette and veterinarian Camille Guérin isolated the original *Mycobacterium bovis* from the udder of a tuberculous cow. Calmette and Guérin decreased the virulence of the bacteria by numerous culture cycles (passages) on bile-containing medium for a 13 year period from 1909-1921. All the current BCG vaccine strains are descendants of this original culture (Wittes, 2000; WHO, 2004). The vaccine became known as Bacillus Calmette-Guérin or BCG. Different laboratory strains have been cultured from the original strain of BCG and they show both phenotypic and genotypic differences (Wittes, 2000; Franco-Paredes *et al.*, 2006). All BCG vaccine strains have a deletion of region of difference one (RD1) which is present in both *Mycobacterium bovis* and *Mycobacterium tuberculosis*. This deletion is associated with the attenuation of the original *Mycobacterium bovis* strain (Ritz *et al.*, 2008).

1.3.1 BCG vaccination

Following preliminary experiments in cows, Calmette and Guérin administered their BCG vaccine, in 1921, orally to a baby whose mother had died of TB and whose grandmother was dying of the disease. The baby did not develop TB and suffered no side effects from the vaccine (Wittes, 2000). BCG vaccines have therefore been in existence for close to 90 years. The League of Nations recommended widespread BCG vaccination in 1928. The intradermal method of vaccination was initially

introduced in 1927 and the percutaneous method in 1931. The use of oral BCG vaccination was discontinued in 1973. BCG vaccination was included in the WHO Expanded Programme of Immunisation in 1974 (Fine *et al.*, 1999; Fine and Reichman, 2000; WHO, 2004; Hussey *et al.*, 2007). To date in countries where TB is endemic, BCG forms part of the national childhood immunisation program and >80% of neonates and infants are vaccinated with BCG (WHO, 2004). In South Africa, BCG vaccination became legally compulsory in 1973 (Jeena *et al.*, 2001). Currently about 120 million children are vaccinated worldwide with BCG each year (WHO, 2004; Trunz *et al.*, 2006; Ritz *et al.*, 2008). Although BCG is the most widely used vaccine, it is also the most controversial vaccine in current use (Fine, 1995). There are conflicting data on the protective efficacy of BCG; although BCG vaccination has a proven protective effect against TB meningitis (75-87%) and disseminated or miliary TB, protection against adult pulmonary TB is highly controversial. A meta-analysis of randomised trials estimated the overall protective effect of BCG against pulmonary TB at 50% with a range from 0% to 80%. Thus, BCG vaccination appears to provide only partial protection against pulmonary TB (Colditz *et al.*, 1994; Colditz *et al.*, 1995; Fine, 1995; Nelson and Wells, 2004; WHO, 2004; Walker *et al.*, 2006). TB usually spreads as a result of reactivation of latent TB in the form of adult pulmonary TB. While BCG may provide some protection against latent TB infection in children, the impact of BCG vaccination on TB transmission in adults seems fairly limited (Fine *et al.*, 1999; WHO, 2004; Ritz *et al.*, 2008).

The WHO and The United Nations Children's Fund (UNICEF) recommends that in countries with a high incidence of TB, a single dose of BCG vaccine be delivered by the intradermal (ID) route with a 25 or 26 gauge needle to all infants on the deltoid region of the right arm as soon as possible after birth. A 0.05ml dose is given to infants, whereas a 0.1ml dose is usually given to children and adults. The BCG vaccine is a live freeze-dried vaccine from the original attenuated strain of *Mycobacterium bovis* (Fine *et al.*, 1999; WHO, 2001, 2004, 2005). There is no consensus on which strain of BCG is optimal for vaccine usage, as a result of this, different strains of BCG are used for immunisation in different countries (Fine *et al.*, 1999; WHO, 2004).

1.3.2 Immune response of neonates to BCG vaccination

BCG vaccine administered either in infancy or adolescence induces memory to mycobacterial antigens that is still present and measurable for at least 14 years post vaccination in the majority of individuals (Weir *et al.*, 2008). Marchant and colleagues reported that in response to PPD, blood from unvaccinated infants demonstrated a predominantly Th2 response; in contrast, vaccination of neonates induced a Th1 memory response still evident at 1 year (Marchant *et al.*, 1999). Furthermore, Hussey and co-workers found that BCG vaccination at birth induced strong lymphoproliferation and Th1 cytokine response at 10 weeks of age following *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) with mycobacterial antigens (Hussey *et al.*, 2002). In addition, Murray *et al* reported a specific $CD8^+$ T cell proliferation, up-regulation of cytotoxic molecules and IFN-γ expression at 10 weeks of age following *ex vivo* stimulation of blood with BCG (Murray *et al.*, 2006). This same study, on 10 week old infants, recently reported cytokine profiles using multiparameter flow cytometry. Soares *et al* found antigen specific $CD4^+$ T cells expressing IFN-γ, interleukin (IL)-2 and tumour necrosis factor alpha (TNF-α) in various combinations following stimulation of peripheral blood with BCG. The number of $CD4^+$ T cells expressing IL-4 and IL-10 were low. In contrast, cytokine producing specific $CD8^+$ T cells were less frequent and produced mainly IFN-γ and/or IL-2 and less TNF-α, IL-4 and IL-10. An extremely important finding was that many BCG-specific $CD4^+$ and $CD8^+$ T cells did not produce IFN-γ, which has important implications for determination of host response to anti-TB vaccinations (Soares *et al.*, 2008). Although all these studies have shown an immune response following vaccination with BCG this does not mean that the response is protective, as no definitive correlates of protection to infection with TB have been demonstrated (Fletcher, 2007; Kagina *et al.*, 2010). Many studies have shown that the incidence of active TB infection does not change in response to BCG vaccination, only the severity of the disease is reduced (WHO, 2004; Mahomed *et al.*, 2006; Walker *et al.*, 2006; Moyo *et al.*, 2010).

1.3.3 Adverse effects of BCG vaccination

Although BCG vaccines are considered very safe with a low incidence of adverse effects, they cause a greater reaction than other vaccines in use today (Bannon, 1999;

Fine *et al.*, 1999). BCG is the only vaccine currently in use that induces a local ulcer (this was also the case with the smallpox vaccine) (Fine *et al.*, 1999). The local reaction to BCG administration usually subsides within 2-5 months, but may leave a superficial scar 2-10 mm in diameter which is used as an indicator of previous BCG vaccination (Fine *et al.*, 1999; WHO, 2004, 2005). In order to afford protection, BCG needs to activate cellular immunity hence the importance of lymphatic involvement (Fine *et al.*, 1999). Common side-effects include erythema, pain and swelling at the vaccination site. Local abscesses are reported in approximately 2% of vaccinated infants, which may be related to incorrect vaccine administration. Lymphadenitis and osteitis occurs in approximately 1% and 0.04% of infants respectively, mostly in Scandinavia and Eastern Europe. Serious adverse reactions are rare, with an incidence of <1 per million vaccinees. Disseminated BCG infection occurs in immunocompromised infants such as severe combined immunodeficiency and those with HIV infection (Franco-Paredes *et al.*, 2006).

1.3.4 Diverse responses to BCG vaccination

Vaccine trials evaluating the efficacy of BCG, have resulted in vastly diverse outcomes. As already mentioned the variable efficacy of BCG vaccination relates predominantly to adult pulmonary TB, as evidence consistently indicates that BCG vaccination protects children against TB meningitis and miliary TB (Fine *et al.*, 1999; Collins and Kaufmann, 2001 A). The lowest level of protection is reported in countries with the highest incidence of TB. Reasons for the large variation in results could be due to a number of factors including use of diverse strains of BCG. The original strain of BCG was never cloned and the organism has been maintained differently in various laboratories around the world. The resulting antigenic variation resulting from genetic diversity in these strains could explain discrepancies in efficacy of BCG vaccination (Fine and Rodrigues, 1990; Fine, 1995; Fine *et al.*, 1999; Ritz *et al.*, 2008; Delogu and Fadda, 2009). Some studies report differences in response to BCG vaccination in various geographic areas, such as in England compared to Malawi even though the same strain is used. The explanation for this could be related to variation in human exposure to environmental mycobacteria. Numerous species of mycobacteria are found in soil and water and are very common in tropical areas and in communities with poor hygienic standards, resulting in exposure of humans to

these bacteria. Two mechanisms for the difference in efficacy of BCG following exposure to non-tuberculosis mycobacteria have been postulated; 1) The "blocking hypothesis" which suggests that immunity induced by exposure to environmental mycobacteria restricts the growth of BCG and accelerates the clearance of BCG thereby decreasing protection against TB; or 2) The "masking hypothesis" which suggests, that BCG vaccination is unable to boost immunity further following the natural immunity attained by exposure to environmental mycobacteria. If BCG is administered soon after birth, no prior sensitisation with environmental mycobacteria would have occurred. However, in adults with prior exposure to these bacteria, BCG vaccination would not be expected to be as effective (Fine and Rodrigues, 1990; Fine *et al.*, 1999; Collins and Kaufmann, 2001 A; Martin, 2006; Weir *et al.*, 2006; Delogu and Fadda, 2009). In animal studies, infections with some species of mycobacteria have been shown to induce similar protection against Mtb as vaccination with BCG (Fine and Rodrigues, 1990). Another confounding factor could be human genetic variability which may influence the susceptibility of individuals to TB (Fine *et al.*, 1999; Collins and Kaufmann, 2001 A; Fletcher, 2007). Levels of nutrition and other factors know to influence general health status may negatively affect the efficacy of BCG vaccination. In addition, socioeconomic factors such as those caused by over-crowding and close contact could also be relevant, as this inevitably leads to high dose exposure to Mtb (Fine, 1995; Bannon, 1999). In addition, the virulence of clinical TB isolates may also account for variation in vaccine efficacy. Levels of UV light exposure could influence the outcome, as it is well known that BCG bacilli and dermal Langerhans cells, which are important in antigen presentation, are sensitive to UV exposure. Increased UV light exposure could explain why protection tends to be lower in tropical than temperate regions. Co-infection with helminths could also cause inconsistent results (Fine *et al.*, 1999; Collins and Kaufmann, 2001 A).

In 2000 the Department of Health in South Africa altered the route and strain of BCG vaccination from Tokyo 172 BCG delivered via the percutaneous (PC) route to Danish 1331 BCG administered by the ID route. Following this change, it was noted that the Danish 1331 BCG ID vaccine did not prevent more TB cases in children compared to the Tokyo 172 BCG PC vaccination; it did however reduce the proportion of children with disseminated disease (meningitis and/or miliary spread). Danish ID was 46% more effective than Tokyo PC BCG vaccination in preventing

disseminated TB. In addition, BCG by either PC or ID route reduced disseminated disease by 87% compared with those not vaccinated (Mahomed *et al.*, 2006). Furthermore, a randomised trial was conducted in South Africa comparing the efficacy of PC versus ID Tokyo 172 BCG vaccine in the prevention of TB in infants. No significant difference was found in the prevention of TB between the ID and PC vaccinated infants. Very few cases of disseminated TB were detected with no difference between the route of vaccination (Hawkridge *et al.*, 2008).

1.3.5 New candidate vaccines against TB

As infection with Mtb itself provides incomplete protection, which is clearly indicated by the fact that Mtb can remain dormant in the host for years, and that patients can be re-infected with a new Mtb strain after the first episode of TB was cured, it is hardly surprising that BCG fails to protect against pulmonary TB (Bandera *et al.*, 2001; Garcia de Viedma *et al.*, 2002; Shamputa *et al.*, 2007). The protection afforded in cattle against *Mycobacterium bovis* infection by BCG vaccination (its closest ancestral relative) is no better than its record against Mtb infection in humans (Vordermeier and Hewinson, 2006; Hope and Villarreal-Ramos, 2008). There is clearly a great need for the development of an improved TB vaccine. Several factors need to be borne in mind with this regard. Firstly, the vaccine needs to offer protection from active TB in individuals who may already be latently infected with Mtb. Secondly, the most important target for a new vaccine is adult pulmonary disease. This protection needs to be induced in populations where BCG vaccination is widely used and environmental mycobacteria as well as exposure to TB exists (Fine, 1995; Fine *et al.*, 1999). A major consideration must be safety of any candidate vaccines, especially in patients with acquired immunedeficiency syndrome (AIDS). The choice of adjuvant is also critically important as it should promote a Th1 response as these cells are important in adaptive immunity to Mtb (Collins and Kaufmann, 2001 A). Another factor to be considered would be the appropriate timing for administration of the vaccine. The ideal time for a vaccine against TB to be administered, in countries where TB is endemic, would be prior to exposure. A vaccination strategy which takes place shortly after birth would therefore be optimal in this environment. Another vaccine that could be used would be one that prevented the progression to active disease in people already latently infected with TB. This

strategy could act to improve the immunity to TB and be administered as a post-infection vaccination. Another option for a vaccination would be one that is given to patients during anti-tuberculosis treatment to enable this treatment period to be shortened or to reduce the risk of relapse. This therapeutic vaccine may be particularly relevant where MDR TB is found and where current TB measures are unable to control the incidence of TB (Hussey, 2007).

Because of the importance of DC in the development of specific immunity to TB, these cells have been investigated for their role in the development of vaccines against TB. The primary aim of TB vaccination is to target Th1 type cells with the aim of inducing a pool of memory cells that recognise and respond to Mtb. Since DC are important in priming Th1 cytokine polarisation, targeting a vaccine toward these cells vastly improves the immunogenicity of a number of vaccines (Collins and Kaufmann, 2001 A; Franco-Paredes *et al.*, 2006; Gupta *et al.*, 2007).

Several laboratories are also currently developing new subunit vaccines using different approaches. One is based on secretory antigens such as ESAT-6, TB10.4 and the antigen 85 complex, which is secreted from live mycobacteria early in the infection process and may trigger an earlier immune response (Fine *et al.*, 1999; Dockrell, 2008; Young *et al.*, 2008). Another tactic is based on recombinant DNA technology to modify BCG into a recombinant BCG vaccine against TB (Gupta *et al.*, 2007; Young *et al.*, 2008). An additional approach involves delivery of DNA encoding various specific mycobacterial antigens within plasmid carriers. This DNA is then taken up by host muscle cells, where it is translated into foreign proteins that induce specific antibody and T cell responses. DNA vaccines can invoke a good cellular immune response against TB from both $CD4^+$ and $CD8^+$ T cells (Fine *et al.*, 1999; Gupta *et al.*, 2007). A further strategy for a vaccine is one that could enhance a previous BCG vaccination, as effectiveness of neonatal vaccination declines after 10-15 years. This vaccine would aim to booster the previous BCG vaccination (Collins and Kaufmann, 2001 A). There is also much interest in the use of the BCG vaccine as a live vector to deliver a variety of recombinant antigens, thus acting as a "super vaccine" (Fine *et al.*, 1999).

In the last 15 years, much research has been conducted on TB vaccines and over 200 vaccines have been tested in animals. There are currently 4 vaccines which are being tested in human clinical trials; 1) MVA85A (Oxford University, UK) a subunit recombinant vaccine consisting of mycobacterial protein antigen 85 expressed in a non-replicating vector, a strain of the vaccinia virus; 2) Mtb72f (Glaxo Smith Klein Biologicals, Rixensart, Belgium) a combination of two immunogenic Mtb antigens Mtb39a and Mtb32a plus the adjuvants ASO2A or ASO1B; 3) Aeras-402 (Aeras Global Tuberculosis Vaccine Foundation, Rockville, MD, USA) a vaccine to be used in individuals who have already received BCG vaccination. This vaccine is a serotype 35 non-replicating adenovirus which contains DNA that expresses a fusion protein created from three Mtb antigens, 85A, 85B and TB10.4 (a member of ESAT-6 group of proteins). Another vaccine utilises *Mycobacterium vaccae,* which is an environmental saprophyte and thought to possess immunogenic properties to enhance the host immune response to Mtb. Vaccines from non-pathogenic mycobacterial strains are heat-inactivated and given to individuals in an attempt to institute short-lived infections in the host that should result in a protective immune response against Mtb. This *Mycobacterium vaccae* vaccine has been tested as a vaccine to be used in conjunction with TB treatment. Other promising candidates may be commencing human clinical trials shortly. All of these vaccines have been shown in animal trials to be either as good as BCG or better than BCG in reducing severity of TB disease or infection (Fine *et al.*, 1999; Gupta *et al.*, 2007; Hussey, 2007; Young *et al.*, 2008).

To determine the efficacy of new candidate vaccines in the prevention of TB, many study subjects need to be enrolled and monitored over an extended period of time, as active TB disease only manifests itself in a few individuals infected with Mtb and can do so many years after vaccination. The other factor that hampers vaccine development is the lack of definitive *in vitro* correlates of immune protection against TB, which would assist in determining the efficacy of vaccines. Currently the assessment of IFN-γ production by $CD4^+$ T cells following vaccination is used to determine protective memory immunity, but this does not appear to be an immune correlate of vaccine induced protection (Collins and Kaufmann, 2001 A; Goldsack and Kirman, 2007; Maartens and Wilkinson, 2007; Dockrell, 2008; Hanekom *et al.*, 2008).

In designing new vaccines with improved activity relative to BCG, the critically important factors that should be clearly understood are the hosts' initial response to BCG. This thesis will describe that response in naïve unvaccinated infants.

1.4 INNATE IMMUNITY

(The immune response to Mycobacterium tuberculosis is discussed in section 1.7).

The immune system consists of two interconnected components: the initial response referred to as the innate response and the second, acquired or adaptive immune response which is dependent on exposure to and response to specific antigens (Hanekom *et al.*, 2007). The innate response is the hosts' first line of defence against any invading organism and is not antigen specific. The innate immune system is comprised of several cell types, including macrophages, DC and natural killer (NK) cells (Marodi, 2006 B). Macrophages and DC express surface receptors that facilitate the recognition of pathogens (discussed in more detail under toll-like receptors in section 1.4.3). This pathogen recognition system is the key to the initiation of the innate immune response in the host (Bhatt and Salgame, 2007). Once pathogen recognition and engagement take place released soluble mediators facilitate the recruitment and accumulation of additional innate immune cells at the site of infection (Bhatt and Salgame, 2007). Macrophages have powerful antimicrobial properties which contribute to control of the infecting agents (Pieters, 2008). In addition, these innate cells produce soluble mediators or cytokines that initiate a cascade of events that ultimately results in the induction of a pathogen specific adaptive immune response by T cells (Trinchieri, 2003). In general, the innate response is capable of either averting an infection, or decreasing the pathogen load, but both the innate and the adaptive response is usually required for control of an established infection (Trinchieri, 2003).

1.4.1 Dendritic cells

DC are cells that specialise in uptake and processing of the antigen, and presentation to T cells; they are therefore central to the development of acquired immunity (Banchereau and Steinman, 1998). DC are found in peripheral tissues such as skin,

trachea and intestine where they recognise and bind pathogens or foreign antigen (Steinman, 2001; Guermonprez *et al.*, 2002). When DC encounter a foreign pathogen, they take it up into the cell, where it is degraded into peptides. These peptides are then presented to T cells in the context of major histocompatability complex (MHC) molecules expressed on the surface of the antigen presenting dendritic cells. DC thereby stimulate an antigen specific adaptive T cell response (Savina and Amigorena, 2007). Furthermore, DCs are the most potent antigen presenting cells (APC) in the immune system and the only cell capable of stimulating primary activation of naïve $CD4^+$ and $CD8^+$ T cells (Banchereau and Steinman, 1998; Dilioglou *et al.*, 2003 B; Sinha *et al.*, 2007; Randolph *et al.*, 2008). The DC has a large surface area comprised of long "dendritic" processes of membrane folds which assists in the capture and presentation of antigen to T cells (Banchereau and Steinman, 1998). For efficient priming and the generation of adaptive immunity in naïve T cells, immature DC need to mature and become activated (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Bhatt and Salgame, 2007). Immature DC have high phagocytic properties which is essential for the uptake of pathogens in the peripheral tissue. Following phagocytosis, the DC migrate to the draining lymph nodes. During this migration, and in response to cytokines and factors provided by the engulfed antigen, DCs undergo a process of maturation (Kaufmann, 2001; Bhatt and Salgame, 2007; Sinha *et al.*, 2007). This maturation process is associated with functional and phenotypic changes in the DC. Mature DC become fully competent APC by up-regulation of antigen presenting molecules and down-regulation of their phagocytic properties (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Mellman and Steinman, 2001). Mature DC therefore exhibit high levels of expression of the antigen presenting molecules MHC class I and II. In addition, as DCs mature, upregulation of costimulatory molecules CD40, CD80 (B7.1) and CD86 (B7.2) also occurs. This process is necessary for the activation of T cells (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Mellman and Steinman, 2001; Sinha *et al.*, 2007). CD40, one of the costimulatory molecules is a member of the tumour necrosis factor-receptor superfamily, the ligand of which is expressed on activated T cells (Lipscomb and Masten, 2002). CD83 another molecule expressed on DCs, is an immunoglobulin superfamily member that is upregulated during maturation of DCs and has been widely used as a marker for determining the extent of maturation of the cells (Lechmann *et al.*, 2002). DCs are therefore specialised in linking the innate and

adaptive immune responses by migrating to the lymph node where they activate naïve $CD4^+$ T cells and $CD8^+$ T cells into effector T cells capable of producing IFN-γ (Mellman and Steinman, 2001; Steinman, 2001; Bhatt and Salgame, 2007; Hanekom *et al.*, 2007; Savina and Amigorena, 2007).

1.4.2 Macrophages

Macrophages are a heterogeneous group of bone marrow-derived phagocytic cells of the innate immune system. These cells are found in large numbers in tissues exposed to external surroundings such as the lung where numerous pathogens, viruses, fungi and foreign particles are located (Bhatt and Salgame, 2007; Savina and Amigorena, 2007). Macrophages differentiate from circulating blood monocytes which have the ability to leave the blood and migrate into tissue (Randolph *et al.*, 2008). Macrophages are an essential effector arm of the innate response and particularly important against intracellular pathogens. Macrophages engulf pathogens via phagocytosis into phagosomes which then mature into phagolysosomes. Phagolysosomes have the ability to eliminate pathogens efficiently via acidification of phagosomes, activation of potent lysosomal proteases and subsequent proteolysis (Kaufmann, 2001; Nau *et al.*, 2002; Taylor *et al.*, 2005; Savina and Amigorena, 2007; Mosser and Edwards, 2008; Randolph *et al.*, 2008). Macrophage receptors, CD80 and CD86, present on the cell surface play a critical role in the activation of T cells. Both of these molecules bind to ligands which are present on T cells; CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (Orlikowsky *et al.*, 2003). For macrophages to carry out their effector functions, it is imperative that these cells are activated (Mosser, 2003; Mosser and Edwards, 2008; Pieters, 2008). Depending on the presence of cytokines, macrophages have the ability to differentiate into either classically or alternatively activated macrophages (Gordon, 2003; Mosser, 2003; Mosser and Edwards, 2008). Macrophages are classically activated as a result of exposure to two signals: The first signal is IFN-γ and the second signal is TNF-α which is usually produced by exposure of monocytes/macrophages to the bacterial products such as endotoxin lipopolysaccharide (LPS) found on the outer membrane of gram negative bacteria (Gordon, 2003; Mosser, 2003). IFN-γ is produced by NK, natural killer T-cells (NKT), gamma delta (γδ) T cells, mature DCs or antigen-specific T cells (Flynn and Chan, 2001; Raja, 2004; Pieters, 2008). In addition, for the

generation of classically activated macrophages an environment which includes IL-12 and IL-18 which are produced by APCs is required (Gordon, 2003). Although classically activated macrophages are not more phagocytic than resting mononuclear phagocytes, they have pro-inflammatory properties and an enhanced ability to kill and degrade intracellular pathogens (Dalton *et al.*, 1993; Mosser, 2003; Taylor *et al.*, 2005; Mosser and Edwards, 2008). Classically activated macrophages secrete cytokines such as TNF-α, IL-1β, IL-6, IL-12(p40) and IL-23, which stimulates Th1 immunity (Gordon, 2003; Mosser and Edwards, 2008). In contrast, non-classical or alternatively activated macrophages develop in the presence of IL-4 and IL-13, two cytokines usually produced during Th2-type responses (Gordon, 2003; Mosser, 2003; Mosser and Edwards, 2008). These alternatively activated macrophages play a role in allergic conditions such as asthma, tolerance of the histo-incompatible foetus, repair (including the central nervous system), wound healing, atherosclerosis and tumour immunity (Gordon, 2003; Mosser and Edwards, 2008). These alternatively activated macrophages produce little or no oxygen/nitrogen radicals and as a result are less efficient at killing intracellular pathogens. In addition these macrophages do not secrete IL-12(p40). The signature cytokine of these macrophages is the anti-inflammatory cytokine IL-10 and considerable amounts of this cytokine are produced by these alternatively activated macrophages (Mosser, 2003; Verreck *et al.*, 2004; Verreck *et al.*, 2006; Mosser and Edwards, 2008).

1.4.3 Toll-like receptors

Invading pathogens are recognised in the host via the pathogen associated molecular patterns (PAMP), on the bacteria. Some of the bacterial cell wall components, microbial nucleic acids and bacterial motility elements act as PAMPs and stimulate an immune response. Hosts cells identify these patterns via germline-encoded pattern recognition receptors (PRR) such as Toll-like receptors (TLR) which are present on host cells including macrophages and DCs. Mammalian TLR derive their name from the *Drosophila* Toll protein with which they share sequence similarity (Kaisho and Akira, 2001; Zarember and Godowski, 2002; Krutzik and Modlin, 2004; Xu *et al.*, 2004; Akira *et al.*, 2006; Pandey and Agrawal, 2006). In mammals, 13 members of the TLR family have been identified to date and the expression of 10 of these is known in humans (Pandey and Agrawal, 2006; Krishnan *et al.*, 2007). TLR can be

divided into subfamilies each of which recognises related PAMPs: TLR1, TLR2 and TLR6 recognise lipids; TLR5 recognise flagellin; TLR7, TLR8 and TLR9 recognise nucleic acids. Some TLR have the additional ability to recognise structurally unrelated ligands. TLR4 identifies a variety of ligands such as LPS and heat-shock proteins. LPS, has powerful immunostimulating properties and binds to CD14 on the surface of phagocytes. TLR 1, 2, 4, 5 and 6 are expressed on the cell surface, whereas TLR 3, 7, 8 and 9 are almost exclusively found on intracellular membranes; their ligands require internalisation to the endosome prior to signalling (Akira *et al.*, 2006; Berrington and Hawn, 2007). The macrophage response to TLR stimulation includes increased phagocytosis of bacteria, induction of anti-microbial molecules and secretion of pro-inflammatory cytokines such as IL-12. The DC response to TLR stimulation includes maturation of DCs and upregulation of the T cell costimulatory molecules CD40, CD80 and CD86 as well as the antigen presentation molecules MHC and secretion of pro-inflammatory cytokines. Although TLR are crucial for innate immunity, they are also necessary for the induction of an adaptive Th1 immune response (Kaisho and Akira, 2001; Blander and Medzhitov, 2004; Doyle *et al.*, 2004; Krutzik and Modlin, 2004; Verreck *et al.*, 2004; Krishnan *et al.*, 2007).

1.4.4 Natural killer cells

NK cells are a form of cytotoxic lymphoid cells and constitute an important component of the innate immune system. NK cells are critically important in early immune responses against both pathogens and tumours. These cells account for approximately 15% of all lymphocytes and are defined by their expression of CD56 and lack of expression of CD3 (Cooper *et al.*, 2001). In 1990 Ljunggren and Kärre proposed the “missing self” hypothesis, which described cells that were susceptible to NK cell attack (Ljunggren and Karre, 1990). “Missing self” is a term that is used to describe cells with low levels of MHC class I cell surface molecules, which can arise following viral infection or in tumour cells (Ljunggren and Karre, 1990). Although the exact mechanisms are not clearly understood, it is known that NK cells have both inhibitory and activating receptors that engage either MHC class I molecules, MHC class I-like molecules as well as molecules that are not related to MHC (Moretta *et al.*, 2000; Caligiuri, 2008; Biassoni, 2009). The receptors include the inhibitory killer cell immunoglobulin-like receptors that bind to MHC class I ligands and inhibitory

CD94-NKG2A receptors that bind non-classical MHC class I. Activating receptors that have been characterised on NK cells include NKG2D, the natural cytotoxicity receptors (NCR), the nectin and nectin-like receptors and NKp80 (Moretta *et al.*, 2000; Caligiuri, 2008; Biassoni, 2009). NK cells recognise target cells that have low MHC class I via a complex process involving both inhibitory and activating signals, the outcome of which may result in the killing of target cells (Moretta *et al.*, 2000; Caligiuri, 2008; Biassoni, 2009). In addition, NK cells are known to detect microbial infection via a non-TLR receptor system and have the ability to kill infected cells. Furthermore, the interaction between DCs and NK cells is essential in the initiation of an innate immune response via secreted cytokines (Akira *et al.*, 2006). In addition, NK cells and DCs are able to reciprocally activate one another (Hamerman *et al.*, 2005). NK cells are able to kill infected or transformed cells via perforin and granzymes or via Fas receptor. Perforin forms pores in the cell membrane of a target cell through which granzymes and associated molecules can enter the cell and induce apoptosis (Caligiuri, 2008). NK cells constitutively express numerous monokine receptors and produce IFN-γ rapidly in response to stimulation by cytokines such as IL-12 (Trinchieri, 2003). NK cells are a very important source of IFN-γ, especially in the absence of an acquired T cell immune response which serves to both enhance macrophage activation and to prime for the subsequent adaptive Th1 cytokine response (Trinchieri, 2003). In addition, IFN-γ has an antiproliferative effect on both viral and malignant-transformed cells (Trinchieri, 2003; Caligiuri, 2008).

1.5 T CELLS AND IMMUNITY

1.5.1 Polarisation and lineage of helper T cells

All $CD4^+$ (T helper lymphocytes) originate as naïve T helper (Th) 0 cells, a precursor cell which has the capacity of differentiating into distinctive $CD4^+$ effector Th cells (Weaver *et al.*, 2006). In 1986 Mosmann and colleagues categorised $CD4^+$ T cells into either Th1 or Th2 effector cells depending on their cytokine profiles (Mosmann *et al.*, 1986). This Th1-Th2 paradigm persisted for almost two decades however, recent advances have lead to a reclassification of T helper cells. Although controversy exists about the recognition of Th cell lineages, four discrete $CD4^+$ Th cell lineages are generally recognised namely Th1, Th2, Th17 and induced regulatory

T cells (iTreg) cells which are defined by the cytokines that they produce as well as by their function (Figure 1.2) (Zhu and Paul, 2008, 2010 A; Zhu *et al.*, 2010 C). iTreg are differentiated from naïve $CD4^+$ T cells in the periphery as opposed to natural regulatory T cells (nTreg) which emerge from the thymus as a distinct lineage (Zhu and Paul, 2008, 2010 B). The process by which commitment of Th cells develops is called polarisation and is mainly driven by cytokines (Kidd, 2003; Bashyam, 2007). Polarised helper T cells may develop from naïve precursor Th cells during activation in the lymph nodes which is initiated by T cell receptor (TCR) triggering, (signal 1) costimulation via costimulatory molecules (signal 2) and cytokines. Naïve Th cells are stimulated by DCs, to differentiate into effector $CD4^+$ Th cells (Smits *et al.*, 2001; Martino *et al.*, 2004; Park *et al.*, 2005; Bettelli *et al.*, 2007). Distinctive cytokines responsible for priming naïve $CD4^+$ T cells to differentiate into the respective Th cell lineage are as follows: IL-12 and IFN-γ prime for Th1; IL-4 and IL-2/ IL-7 for Th2; transforming growth factor beta (TGF-β) and IL-6/IL-21/IL-23 for Th17 and TGF-β and IL-2 for iTregs (Figure 1.2) (Martinez *et al.*, 2008; Miossec *et al.*, 2009; Zhu and Paul, 2010 A; Zhu *et al.*, 2010 C). Although controversial other potential Th cell lineages have been proposed, including Th3 cells, (TGF-β-producing $CD4^+$ T cells); Tr1 cells, (IL-10 producing $CD4^+$ T cells); Th9 cells, (IL-9 producing $CD4^+$ T cells); and T follicular helper cells (Tfh) which are found in follicular regions of lymph nodes and spleen (Zhu and Paul, 2010 A). Oral tolerance induces TGF-β-producing cells, which have been termed Th3 cells. Approximately 40% of these cells express Forkhead box P3 (Foxp3) which would imply that these Th3 cells contain iTregs (Zhu and Paul, 2010 A). Weiner however argues that Th3 cells form a unique T cell subset which primarily secretes TGF-β and has suppressive properties for both Th1 and Th2 cells (Weiner, 2001). Tr1 cells are $CD4^+$ T cells that express IL-10 and possess regulatory functions; however other Tregs also produce IL-10 although it does appear that Tr1 cells are distinct from $Foxp3^+$ IL-10 producing cells. IL-10 is also produced by Th1, Th2 and Th17 cells, therefore Tr1 cells may not be a separate lineage, but rather IL-10 producing Tregs as subsets of those cells (Zhu and Paul, 2010 A). In contrast, TCR stimulation of naïve $CD4^+$ T cells in the presence of TGF-β and IL-4 induces cells that produce IL-9 and it has been proposed that these cells be called Th9 cells. However, Th2 cells, Tregs and Th17 cells express IL-9 and this cytokine is therefore not a distinguishing factor.

These IL-9 producing cells may be subsets of Th2, iTreg and Th17 cells and not a separate lineage (Zhu and Paul, 2010 A). Tfh cells are found enriched in the edge of the B cell zones and follicular regions and germinal centres in the lymph nodes and spleen (Wan and Flavell, 2009). These cells were initially suggested as a separate lineage as they did not produce Th1, Th2 or Th17 cytokines (Zhu and Paul, 2010 A). However, Tfh cells have subsequently been shown to produce IFN-γ, IL-4 or IL-17A (Zhu and Paul, 2010 A). Wan *et al* argue that Tfh cells are distinct from Th17 cells and that they also have the ability to differentiate independent of TGF-β, which is essential for Th17 differentiation (Wan and Flavell, 2009). Tfh cells may in fact be a subset of Th1, Th2 or Th17 cells and further studies of these Tfh cells are expected which may resolve this matter (Zhu and Paul, 2010 A). However, Zhu and Paul maintain that the signature cytokines produced by these 'potential T cell lineages' are also produced by Th1/Th2/Th17/iTreg cells and are therefore not unique (Zhu *et al.*, 2010 C).

The cellular source of the respective cytokines required to stimulate naïve $CD4^+$ Th cells for specific differentiation are as follows; IFN-γ, important in Th1 cell differentiation is produced by NK cells, NKT cells, $CD8^+$ T cells and $CD4^+$ Th1 cells (Miossec *et al.*, 2009; Wan and Flavell, 2009). The main source of IL-12, which is essential in Th1 cell polarisation, is phagocytes and DCs (Fieschi and Casanova, 2003). IL-4, important in Th2 cell development, is produced by NKT cells and $CD4^+$ Th2 cells (Miossec *et al.*, 2009). TGF-β, important in Th17 and iTreg differentiation is produced by many cells including Treg cells and cells of the innate system, while IL-6, also a cytokine required for Th17 cell induction, is an acute phase protein and produced during inflammation by cells which include monocytes. IL-23 and IL-21, important in the development of Th17 cells, are mainly produced by innate and T cells respectively, but IL-21 is also produced by NK cells (Bettelli *et al.*, 2007; Miossec *et al.*, 2009; Wan and Flavell, 2009). IL-2, important in iTreg development, is produced mainly by activated T lymphocytes (Wan and Flavell, 2009).

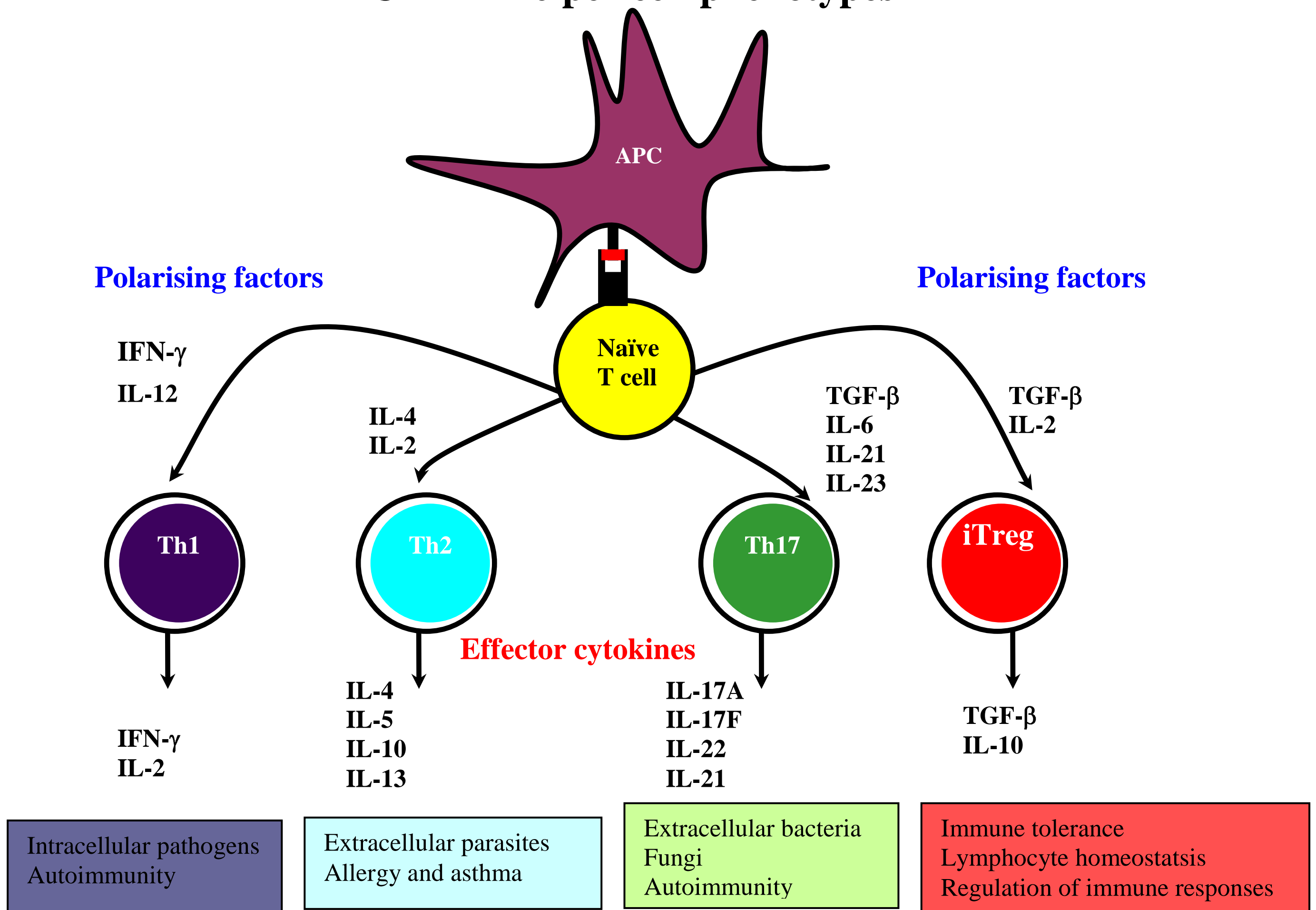


Figure 1.2 Schematic diagram of differentiation of naïve T cells into Th1, Th2, Th17 or iTreg cells.
Polarising cytokines as well as signature cytokines are indicated. Function of these CD4$^+$ Th cells is included (adapted from (Zhu and Paul, 2008).

1.5.2 Cytokine production by mature CD4$^+$ T cells

Mature Th1 cells secrete high levels of IFN-γ; whereas mature Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 as their signature cytokines (Figure 1.2) (Mosmann and Sad, 1996; Romagnani, 1997; Spellberg and Edwards, 2001; Kidd, 2003; Park *et al.*, 2005; Zhu and Paul, 2010 A). CD4$^+$ T cells, other than Th2 and Treg cells, can also produce IL-10, including Th1 and Th17 cells. IL-10 is important as a negative feedback mechanism in regulating the immune response to prevent host tissue damage (O'Garra and Vieira, 2007; Zhu and Paul, 2008). Th17 cells produce many cytokines including IL-17 (also called IL-17A), IL-17F, IL-22 and IL-21 (Figure 1.2) (Bettelli *et al.*, 2007; Martinez *et al.*, 2008; Miossec *et al.*, 2009; Zhu and Paul, 2010 A). iTregs express the master transcription factor, Foxp3, which is associated with the generation

of Tregs, which produce significant amounts of IL-10 and TGF-β (Figure 1.2) (Wan and Flavell, 2009). The cytokines produced by mature $CD4^+$ T cells, are key mediators of cell to cell communication and are responsible for a number of immune reactions including inflammation and other immune responses to infection (Makhseed *et al.*, 2000). Different $CD4^+$ Th cell lineages play distinctive roles in fighting pathogens, inducing inflammation and autoimmunity as well as maintaining self-tolerance and immune homeostatsis (Wan and Flavell, 2009). Cytokines produced by Th1 cells are pro-inflammatory and cellular immunity induced by these cells include activation of macrophages to combat intracellular pathogens; while Th2 cytokines are anti-inflammatory and are critical for IgE production, the recruitment of eosinophils and regulation of the humoral response and for elimination of extracellular parasites including helminths and nematodes (Figure 1.2) (Mosmann and Sad, 1996; Saito, 2000; Carp, 2004; Wilczynski, 2005; Bettelli *et al.*, 2007). Th17 cells play an important role against extracellular pathogens and fungi (Figure 1.2) (Bettelli *et al.*, 2007; Miossec *et al.*, 2009; Zhu and Paul, 2010 A). In addition, preferential IL-17 production during infection with various pathogens including Mtb has been reported, and alludes to the requirement for its presence such as in the clearance of pathogens (Bettelli *et al.*, 2007; Martinez *et al.*, 2008; Miossec *et al.*, 2009; Wan and Flavell, 2009). Furthermore, pathogen specific Th17 cells stimulated during mycobacterial infection were found to induce the expression of chemokines in mice which caused IFN-γ producing Th1 cells to relocate to the lung, as a control mechanism of mycobacterial infection at the site of pathology (Schmidt-Weber *et al.*, 2007; Martinez *et al.*, 2008). iTregs may be involved in self-tolerance and immune modulation (Figure 1.2) (Zhu and Paul, 2010 A). Excessive production of Th1 cytokines is linked to various autoimmune and inflammatory disorders, whereas enhanced Th2 cytokines are associated with atopic diseases, including allergies and asthma. Extreme production of Th17 cells plays an important role in severe autoimmunity in animal models (Bettelli *et al.*, 2007; Zhu and Paul, 2010 A). In addition, increased IL-17 expression has been detected in human autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and psoriasis (Bettelli *et al.*, 2007; Korn *et al.*, 2007; Martinez *et al.*, 2008; Wan and Flavell, 2009). Th1 and Th2 cytokines tend to be mutually inhibitory as IFN-γ secreted by Th1 cells suppresses the IL-4 secretion thereby inhibiting differentiation

of naïve Th0 cells into Th2 cells. Conversely, IL-4 and IL-10 inhibit secretion of IL-12 and IFN-γ, preventing the polarisation of Th0 cells into Th1 cells (Gajewski and Fitch, 1988; D'Andrea *et al.*, 1993; Mosmann and Sad, 1996; Carp, 2004; Bashyam, 2007). Both IL-4 and IFN-γ antagonise the development of Th17 cells, whereas IL-2 which acts as a growth factor for most T cells and T cell subsets appears to inhibit the expansion of Th17 cells (Bettelli *et al.*, 2007; Martinez *et al.*, 2008). Mature T cells that secrete both IFN-γ and IL-4 also known as Th0 cells are lymphocytes that failed to polarise during maturation and took on characteristics of both Th1 and Th2 cells (Street *et al.*, 1990; Mosmann and Sad, 1996).

1.5.3 The effects of Th1 cells on immunity

Th1 cells are the main regulators of type 1 immunity and IFN-γ is primarily responsible for the characteristic pro-inflammatory effect. It stimulates phagocytosis, the oxidative burst, and intracellular killing of microorganisms (Szulc and Piasecki, 1988; Marodi *et al.*, 2000). IFN-γ also upregulates the expression of class I and class II MHC molecules on a variety of cells, thereby stimulating antigen presentation to T cells (Volk *et al.*, 1986). It induces other cell types to secrete pro-inflammatory cytokines such as TNF-α and chemotactic cytokines or chemokines (Spellberg and Edwards, 2001). Cell mediated immunity (CMI) or adaptive immunity is made up of both T helper cells and cytotoxic T lymphocytes (CTL) or killer T lymphocytes, which are usually $CD8^+$ T cells. T helper cells are responsible for orchestrating and directing the immune response, whereas CTLs are killer cells that move to sites of infection and lyse infected cells. Both these types of effector cells play essential roles in eliminating or controlling chronic microbial infections (Esser *et al.*, 2003).

Protective immunity against intracellular pathogens requires a Th1 response characterised by the production of IFN-γ, whereas a Th2 response is effective against extracellular pathogens (Trinchieri, 2003). The induction of a Th1 immune response is dependant on the presence of IL-12, previously called the NK cell stimulatory factor. IL-12 binds to a heterodimeric receptor IL-12Rβ1 and IL-12Rβ2; expressed by both NK and T cells (Spellberg and Edwards, 2001; Trinchieri, 2003). IL-12 is a 75kDa glycoprotein heterodimer composed of two subunits linked by a disulphide bond. The smaller subunit (p35) has a molecular weight of 35 kDa while the larger

subunit (p40) has a molecular weight of 40 kDa. These two subunits together form the biologically active p70 heterodimer molecule. IL-12 stimulates both NK and $CD8^+$ T cells to produce IFN-γ, resulting in enhanced cellular immunity (Bastos *et al.*, 2004; Hunter, 2005; Kastelein *et al.*, 2007). For many intracellular pathogens, IL-12 is critical in the initiation of a CMI response whereas IFN-γ is the effector cytokine that maintains a Th1 immune response and has a direct cytotoxic effect on the invading pathogen (Frucht, 2002; Esser *et al.*, 2003; Fieschi and Casanova, 2003). IL-23 is a closely related heterodimeric cytokine, which is composed of IL-12p40 in association with p19 and is produced by activated macrophages and DCs. The p19 component is primarily synthesised by activated macrophages, DCs, T cells and endothelial cells, whereas only activated macrophages and DCs concurrently express IL-12p40. The importance of p19 in immune responses has been demonstrated through the increased synthesis in a TLR2 dependent manner during stimulation with bacterial products (Lankford and Frucht, 2003). Although IL-12 and IL-23 both stimulate the proliferation of T cells in humans, IL-12 also promotes IFN-γ production from both naïve and memory T cells (Frucht, 2002; Fieschi and Casanova, 2003; Lankford and Frucht, 2003).

1.5.4 The effects of Th2 cells on immunity

A Th2 response is characterised by a humoral or antibody-mediated immune response and Th2 cells stimulate high titres of antibody production (Lundgren *et al.*, 1989; Esser *et al.*, 2003). In particular, IL-4, IL-10 and IL-13 activate B cell proliferation, antibody production and class-switching from IgG to IgE, the latter being critically dependant on the presence of IL-4 or IL-13 (Snapper *et al.*, 1991; Mosmann and Sad, 1996; Foster, 2003).

IL-10 is possibly the most anti-inflammatory cytokine known and is produced by both innate and adaptive immune cells and can, in some cases, also restrict the Th2 responses (Rennick *et al.*, 1995; Rainsford and Reen, 2002; Bashyam, 2007). IL-10 inhibits secretion of pro-inflammatory cytokines, suppresses phagocytosis, the oxidative burst, intracellular killing and also inhibits antigen presentation to T cells, thereby causing T cell anergy (Spellberg and Edwards, 2001). IL-10 acts directly on macrophages and inhibits the production of pro-inflammatory cytokines such as TNF-

α, IL-6 and IL-12 and thereby Th1 differentiation (Conti *et al.*, 2003; Bashyam, 2007). IL-4 and IL-13 also inhibit phagocytosis and intracellular killing, suppress inflammatory cytokine production and may also induce T cell anergy (Spellberg and Edwards, 2001).

1.6 IMMUNE SYSTEM OF THE NEONATE

The WHO estimates that approximately 7.1 million infants aged between 1 and 12 months of age die annually of infections that are rarely fatal in older children and adults (Velilla *et al.*, 2006) and has been attributed to the immaturity of the neonatal immune system. The immaturity of the neonatal immune system was ascribed to T cells that are unable to become fully activated due to intrinsic deficiencies, which include low baseline expression of TCR/CD3 complex and adhesion molecules; defects in cytokine production and low $CD8^+$ T cell cytotoxic activity (Harris *et al.*, 1992; Rainsford and Reen, 2002; Velilla *et al.*, 2006). An immature immune system has not only been implicated in increasing the susceptibility of young infants to infectious diseases but also in preventing the induction of protective immune responses by vaccines (Ota *et al.*, 2002). Furthermore, evidence suggests that a newborn infant has a Th2-biased immune system which impacts on his/her ability to control Th1 dependent intracellular pathogenic infections (Marodi, 2002). Contrasting evidence with respect to the contribution of DCs in neonatal immune immaturity has been reported. It has been proposed that the reduced Th1 and CTL responses found in young infants is a result of the immaturity of DC (Salio *et al.*, 2003). It has also been suggested that neonatal DC are intrinsically biased against Th1 immune responses due to a defect in the synthesis of IL-12 (Goriely *et al.*, 2001; Langrish *et al.*, 2002; Amoudruz *et al.*, 2005). In addition, the antigen presenting capability in neonates (via production of costimulatory cytokines) is reportedly reduced in cord blood compared to adults (Cohen *et al.*, 2000 A). In contrast, other researchers report that DCs generated from cord blood progenitors are both mature and functional and when appropriately activated are able to prime efficient Th1 and CTL responses (Hunt *et al.*, 1994; Borras *et al.*, 2000; Goriely *et al.*, 2001; Liu *et al.*, 2001; Langrish *et al.*, 2002; Liu *et al.*, 2002; Salio *et al.*, 2003). Studies conducted on cytokines generated from cord blood cells have concluded that these cells have a reduced capacity in both cytokine production and response to cytokines and may not

therefore be capable of maintaining an inflammatory response. In addition, neonatal monocyte-derived macrophages are reported to be hypo-responsive to activation by IFN-γ (Marodi *et al.*, 2000; Cohen *et al.*, 2000 A; Marodi *et al.*, 2001). Taken together, these studies implicate DCs, macrophages and T cells for the increased susceptibility of neonates to infection.

1.7 HOST IMMUNE RESPONSE TO *MYCOBACTERIUM TUBERCULOSIS*

1.7.1 Innate immune response to Mycobacterium tuberculosis infection

After inhalation into the lung, Mtb bacilli travel to pulmonary alveoli where they encounter resident alveolar macrophages and immature DC. After recognition by phagocytic receptors bacteria are taken up by macrophages, immature DC and monocytes that have been recruited from the bloodstream (Kaisho and Akira, 2000; Medzhitov and Janeway, 2000; Takeda *et al.*, 2003; Goldsack and Kirman, 2007). Macrophages and DCs express TLR that recognise molecular patterns expressed on the pathogen as discussed previously. Mycobacterial components are recognised by TLR2 in association with TLR1/TLR6, or by TLR4 (Kaisho and Akira, 2000; Medzhitov and Janeway, 2000; Takeda *et al.*, 2003; Quesniaux *et al.*, 2004; Ferwerda *et al.*, 2005). Ligation of TLR by pathogen-specific ligands causes secretion of cytokines by the macrophage which recruits and stimulates NK cells and γδ T cells to produce IFN-γ, a cytokine which activates macrophages (Aderem and Ulevitch, 2000; Hanekom *et al.*, 2007; Russell, 2007). Activated macrophages that have phagocytosed mycobacteria subsequently transfer the bacteria to phagosomes which ultimately results in phagosome-lysosome fusion or maturation into lysosomes (Sundaramurthy and Pieters, 2007; Jordao *et al.*, 2008). The environment within the vacuole of activated macrophages is bactericidal due to the combined action of acidic proteases which causes a decrease in pH to approximately 5.2 and generation of reactive oxygen and nitrogen (Russell, 2001).

Following the uptake of Mtb immature DCs undergo maturation and result in functionally and phenotypically mature DCs that migrate to the draining lymph node where antigen is presented to naïve T cells to become activated effector T cells which subsequently migrate to the lung (Flynn and Chan, 2005; Hanekom *et al.*, 2007). This

process is essential for the induction of specific immunity (Flynn and Chan, 2005; Hanekom *et al.*, 2007). In addition to TLR, DCs also express the C-type lectin, DC-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN; CD209) which recognises a variety of microbes including Mtb. DC-SIGN is an important receptor for Mtb binding and uptake (Bodnar *et al.*, 2001; Tailleux *et al.*, 2003 A; Tailleux *et al.*, 2003 B; Sinha *et al.*, 2007).

Effector T cells that have become activated by mature DCs in the lymph node, migrate to the lung where they produce IFN-γ, mediating activation of macrophages and thereby increasing its ability to kill ingested mycobacteria (Hope *et al.*, 2004). T cells and macrophages in the lung form a cellular mass, the 'tubercle' or granuloma, which acts as a barrier to both contain and control infection to prevent dissemination of the bacilli (Hanekom *et al.*, 2007; Russell, 2007; Saunders and Britton, 2007; Pieters, 2008). A granuloma is a dynamic structure containing activated lymphocytes and continuously stimulated macrophages (Russell, 2007; Pieters, 2008). In latent TB infection, occurring in up to 95% of cases bacilli may remain contained in the granuloma (Hanekom *et al.*, 2007; Saunders and Britton, 2007). If the granuloma is unable to control the infection, the centre of the granuloma becomes necrotic as a result of widespread macrophage death (Cosma *et al.*, 2003; Hanekom *et al.*, 2007; Russell, 2007). Necrosis is followed by caseation in which the granuloma walls break down and resulting in transmission of the bacteria via the airways (Cosma *et al.*, 2003; Russell, 2007; Saunders and Britton, 2007). The cytokine TNF-α plays a central role in both granuloma formation and preservation and is thus important in controlling latent TB infection (Raja, 2004; Hanekom *et al.*, 2007; Saunders and Britton, 2007). Murine studies have indicated that Mtb infected mice deficient in TNF-α or TNF receptor are more susceptible to Mtb, have impaired granuloma formation and have much higher levels of bacteria compared to normal mice (Flynn *et al.*, 1995; Quesniaux *et al.*, 2004; Raja, 2004; Wong *et al.*, 2007). In addition, patients with Crohn's disease and rheumatoid arthritis treated with anti-TNF-α antibodies, had increased reactivation of TB including miliary and extrapulmonary disease. This would indicate impaired granuloma formation due to a reduction in TNF-α (Kaufmann, 2001; Wong *et al.*, 2007).

Macrophages play a conflicting role in the protection from TB, as it is the first cell to respond to Mtb, but also contributes to the dissemination of Mtb (Cosma *et al.*, 2003). Mtb utilises a variety of immune evasion strategies such as prevention of both migration and maturation of DCs to avoid elimination (Bhatt and Salgame, 2007). In addition, virulent Mtb invade and replicate in the macrophage within the phagosome by preventing maturation of the phagosome thereby restricting its acidification and limiting fusion with or into lysosomes. As such Mtb prevents degradation and processing and presentation of mycobacterial antigens to cells of the immune system (Kaufmann, 2001; Russell, 2001; Houben *et al.*, 2006; Russell, 2007; Sundaramurthy and Pieters, 2007; Hernandez-Pando *et al.*, 2009). Moreover, DC and macrophages that are infected with Mtb produce the anti-inflammatory cytokine IL-10 (Hope *et al.*, 2004; Raja, 2004; Bhatt and Salgame, 2007). While DC are still able to prime naïve cells towards an effective Th1 response, despite simultaneous secretion of IL-10; production by *in vitro* Mtb infected monocytes reduces production of IL-12 (Giacomini *et al.*, 2001; Hickman *et al.*, 2002; Hope *et al.*, 2004; Jang *et al.*, 2004; Bhatt and Salgame, 2007). In addition, IL-10 inhibits proliferation of IFN-γ producing T cells as well as decreasing the activity of $CD4^{+}$ and $CD8^{+}$ T cells via downregulation of costimulatory molecules on macrophages (Bhatt and Salgame, 2007). IL-10 may also inhibit the export of MHC class II molecules to the cell surface which would result in down-regulation of the T cell response (Hope *et al.*, 2004). Collectively therefore the induction of IL-10 by Mtb causes suppression of anti-Mtb immunity (Bhatt and Salgame, 2007). This is further demonstrated by the fact that depressed T cell IFN-γ responses in pulmonary TB is associated with IL-10 production from monocytes (Hirsch *et al.*, 1999). Nevertheless in murine studies using IL-10 knock-out mice, resistance to Mtb was not enhanced and transgenic over-expression of IL-10 resulted in reactivation of chronic disease (North, 1998; Turner *et al.*, 2002). However, as Th1 cytokines are often found in addition to IL-10, it is possible that the relative amounts of the two cytokines are important in establishing whether Th1 immunity is suppressed or not (Bhatt and Salgame, 2007), as T cells secreting both IFN-γ and IL-10 have been isolated from bronchoalveolar lavage fluid of TB patients (Gerosa *et al.*, 1999).

Other innate immune cells that participate in the control of Mtb infection are γδ T cells, NK cells and NKT cells. The importance of γδ T cells in the control of Mtb is suggested from bovine studies which indicate that γδ T cells, in the presence of IL-2, produced IFN-γ in response to a cell wall component of *Mycobacterium bovis* although the underlying mechanism remains unknown. Murine studies have shown that the γδ T cell response against Mtb precedes that of $CD4^+$ and $CD8^+$ T cells. Once activated, γδ T cells secrete IFN-γ and TNF-α, which enhances the bactericidal capacity of macrophages. In addition, patients with active pulmonary TB have reduced levels of γδ T cells which suggests an important role of these cells in preventing infection with Mtb (Wang *et al.*, 2004; Born *et al.*, 2006; dEsther and Ottenhoff, 2006; Bhatt and Salgame, 2007; Millman *et al.*, 2008). In murine studies, it was shown that NK cells were recruited to the lungs soon after Mtb infection, where they expanded and became a primary source of IFN-γ (Bhatt and Salgame, 2007). Both IL-2, produced by Th1 cells, and IL-12, produced by phagocytes and DC, enhances NK responses against Mtb. In early innate immunity, IL-12 drives production of IFN-γ from NK cells which augments the synthesis of IL-12 by DCs. NK cells may also directly lyse the pathogens or lyse infected monocytes (Raja, 2004; Bhatt and Salgame, 2007). NKp46 is expressed on the surface of either resting or activated NK cells and has been shown to participate in the killing of Mtb infected monocytes through the recognition of vimentin which is upregulated on the surface of infected monocytes. Upregulation of a NKG2D ligand on the Mtb infected monocyte occurs simultaneously, thereby becoming susceptible to NKG2D-mediated cell killing by NK cells (Biassoni, 2009). NK cells have also been shown to lyse infected human alveolar macrophages (Cooper, 2009 B). In addition, NK cells are also able to activate phagocytic cells at the site of Mtb infection via production of IFN-γ which further serves to boost the innate immune response to Mtb (Raja, 2004). Patients with active pulmonary TB were found to have reduced NK activity which implies that this activity is important in preventing the development of Mtb infection (Wang *et al.*, 2004). Furthermore, a significant reduction in NK activity has been found to be associated with MDR-TB, which highlights the role these cells have in controlling TB infection (Raja, 2004).

Ligation of TLR by pathogen-specific ligands causes secretion of cytokines such as IL-12 and TNF-α and chemokines which are crucial in acquiring specific adaptive immunity against Mtb (Aderem and Ulevitch, 2000). For effective control of Mtb infection, a co-ordinated response of cells of both innate and adaptive immune response is required (Flynn and Chan, 2001). IL-12 also plays a key role in driving the activation, differentiation and proliferation of antigen-specific T cells (Ottenhoff *et al.*, 2005; dEsther and Ottenhoff, 2006).

1.7.2 T cell immunity in TB

Th1 immunity is critical for protection against TB, which is demonstrated in patients with defective IFN-γ receptor and IL-12 receptor genes who are prone to serious and fatal mycobacterial infections including Mtb (dEsther and Ottenhoff, 2006; Hernandez-Pando *et al.*, 2009). The importance of $CD4^+$ T cells in immunity to TB is confirmed by increased disease in HIV infected individuals, in whom a reduction in $CD4^+$ T cells increases susceptibility to TB infection or reactivation of latent TB infection (Raja, 2004; Rosenzweig and Holland, 2005; Bhatt and Salgame, 2007). In addition, the IFN-γ and TNF-α produced by Th1 cells also has a direct lytic effect on intracellular organisms such as Mtb. The Th1 immune response is also associated with a strong cell-mediated CTL response. CTL use two major mechanisms, the perforin granule exocytosis pathway and the Fas ligand/Fas mechanism of apoptosis to kill cells infected with pathogens. Granulysin present within CTL, together with perforin, possess bactericidal properties and are able to kill intracellular as well as extracellular Mtb (Smith and Dockrell, 2000; Esser *et al.*, 2003; Hernandez-Pando *et al.*, 2009).

Recently other subsets of T cells producing proinflammatory cytokines IL-17 and IL-22 have been found in mice infected with TB (Khader and Cooper, 2008). Both IL-17 and IL-22 have been implicated in human pulmonary inflammation caused by Mtb infection (Scriba *et al.*, 2008). Th17 cells have also been found to play a role in TB infection in murine studies in the lung itself. These Th17 cells upregulated chemokines, thereby recruiting Th1 cells to the site of pathology (Cooper, 2009 B). In mice IL-23 is required for the generation of Th17 cells and γδ T cells also produce IL-17 in response to Mtb (Khader and Cooper, 2008). In healthy humans exposed to

Mtb, two new distinct mycobacteria-specific $CD4^+$ T cells, producing IL-17 and IL-22 have been reported in high frequencies in peripheral blood (Scriba *et al.*, 2008). Th17 cells were found to be distinct from Th1 cytokine producing cells and the IL-22 producing cells distinct from both Th17 and Th1 cells. In patients with active TB infection, IL-17 and IL-22 producing cells were reduced in the blood; which has already been established regarding IFN-γ producing cells in patients with pulmonary TB (Zhang *et al.*, 1995; Schwander *et al.*, 1998). In contrast however, Scriba and colleagues report increased IL-22 in the lungs of TB patients but not IL-17 and hypothesise that the reduced IL-17 could be as a result of inhibition by Th1 cells. Scriba and colleagues suggest that IL-17 and IL-22 may play an important role in the immune response against Mtb (Scriba *et al.*, 2008).

Multifunctional T cells producing IFN-γ, TNF-α and IL-2, as well as cells making TNF-α alone have been reported to be increased in the peripheral blood of TB patients compared to healthy contacts, whereas T cells producing IL-2 alone were reduced (Sutherland *et al.*, 2009). However, studies on infants infected with Mtb compared to uninfected controls showed no correlation with polyfunctional T cells producing IFN-γ, TNF-α and IL-2 in the peripheral blood and protection against TB (Kagina *et al.*, 2010). In addition, no role was found in specific $CD8^+$ T cell responses nor γδ T cell responses in protection against TB (Kagina *et al.*, 2010). The role of T cell subsets and cytokines in infection with Mtb and disease, are only beginning to be elucidated, however without definitive correlates of protection, the ability to clearly define this cellular response is limited (Cooper, 2009 B; Kagina *et al.*, 2010).

Increased levels of TGF-β have been found in TB patients in both blood and in the lung. TGF-β is a cytokine which downregulates cell mediated immunity and suppressors excessive inflammation thereby preventing tissue damage (Hernandez-Pando *et al.*, 2009). TGF-β or IL-10 another regulatory cytokine, which has previously been discussed, appear to have a role in preventing excessive inflammation in the lung (Hernandez-Pando *et al.*, 2009). The balance in proinflammatory and anti-inflammatory cytokines seems to be important in immunopathogenesis of TB (Hernandez-Pando *et al.*, 2009).

1.7.3 Regulatory T cells in immunity to TB

$CD4^+$ T cells that express both CD25 and Foxp3, with immunoregulatory properties have recently been described. These Treg cells make up 5-10% of circulating $CD4^+$ T cells in healthy adults, however only 1-2% of these cells expressing high levels of CD25 have a strong regulatory function (Guyot-Revol *et al.*, 2006; Hernandez-Pando *et al.*, 2009). An increase in Tregs both in the blood and in the lung has been reported in TB patients compared to those latently infected with Mtb, which would imply that these cells have a role in the establishment of an active infection (Hernandez-Pando *et al.*, 2009; Cooper, 2009 A). The Th1 immune response is important in controlling the replication of the Mtb and preventing dissemination of the pathogen, however it gives rise to significant immunopathology (Guyot-Revol *et al.*, 2006; Scott-Browne *et al.*, 2007). Tregs are important in the regulation of Th cells which suppress Th1 cells and IFN-γ production that favour the progression of the disease, but simultaneously limit immune-mediated host tissue damage (Guyot-Revol *et al.*, 2006; Scott-Browne *et al.*, 2007; Joosten and Ottenhoff, 2008; Hernandez-Pando *et al.*, 2009).

1.8 THE IMMUNE RESPONSE TO *MYCOBACTERIUM TUBERCULOSIS* INFECTION IN CHILDREN

The risk of developing an infection with Mtb in children depends on the proximity of exposure to an infected individual with cavitatory pulmonary disease (Newton *et al.*, 2008; Sablan, 2009). Children are at a much greater risk of progression to active disease than adults; which is greatest for children under 2 years of age (Lewinsohn *et al.*, 2004; Newton *et al.*, 2008; Mack *et al.*, 2009; Sablan, 2009). Several components of the immune system that appear to be important in controlling mycobacterial infection differ in adults and infants which are likely to contribute to the greater observed risk and greater disease severity seen in the paediatric population including miliary disease and TB meningitis (TBM) (Smith *et al.*, 1997; Hanekom *et al.*, 2007). Miliary disease occurs when breakdown of a local granuloma occurs into a blood vessel resulting in the spread of Mtb via the blood to distant organs. TBM may occur in a similar manner, as a result of Mtb in the blood stream, or it may be caused by dissemination from the lung or lymph nodes (Hanekom *et al.*, 2007). Innate pulmonary defences of the neonate and young infant are likely to be impaired (Smith

et al., 1997; Lewinsohn *et al.*, 2004). In addition, alveolar macrophage antimicrobial activity and recruitment of monocytes appears to be diminished (Smith *et al.*, 1997; Lewinsohn *et al.*, 2004; Newton *et al.*, 2008). These differences could result in a greater bacterial burden in the lungs of infants prior to the initiation of the antigen-specific immune response (Smith *et al.*, 1997; Newton *et al.*, 2008). The production by APC of cytokines such as IL-12, which may aid in the control of infection, may also be reduced (Smith *et al.*, 1997; Newton *et al.*, 2008). Neonatal monocyte derived DC have a defect in IL-12p35 expression and as IL-12 is essential in priming a Th1 response with production of IFN-γ, this implies reduced Th1 immunity in infants (Newton *et al.*, 2008). Furthermore, the ability of DC from infants to present antigen to naïve neonatal T cells appears reduced and does not reach adult competence until after 1 year of age (Smith *et al.*, 1997). Moreover, blood derived DC at birth are reported to be both functionally immature and less differentiated compared to adult DC (Lewinsohn *et al.*, 2004; Newton *et al.*, 2008). Less efficient antigen presentation by DC to naïve cells may delay the initiation of an antigen-specific T cell response and could result in diminished production of IFN-γ and TNF-α (Smith *et al.*, 1997). Although some of these differences between children and adults may not be profound, the cumulative effect would be a delayed and less effective host response against TB (Smith *et al.*, 1997).

1.9 RATIONALE FOR UNDERTAKING THIS STUDY

1.9.1 Rationale for undertaking studies of macrophage function

Let us consider the question of which component of the immune system, the innate or the adaptive response is the most crucial for protection against TB? In general, recent observations have suggested that it is the innate response that is the most important in determining susceptibility versus resistance to TB (Davila *et al.*, 2008). Evidence for a central role of the innate immune response to Mtb infection relies heavily on host genetic studies. Many of the factors shown to be involved in determining genetic resistance or susceptibility to mycobacterial infections are components of the innate system. For instance, TLR (Ogus *et al.*, 2004; Davila *et al.*, 2008; Leandro *et al.*, 2009), mannose receptors (Hoal-Van Helden *et al.*, 1999; Selvaraj *et al.*, 1999), vitamin D receptor (Bellamy *et al.*, 1999; Selvaraj *et al.*, 2000; Liu *et al.*, 2004;

Lykouras *et al.*, 2008; Leandro *et al.*, 2009; Gao *et al.*, 2010), natural resistance-associated macrophage protein 1 (NRAMP1) (Remus *et al.*, 2003; Liu *et al.*, 2004; Li *et al.*, 2006; Lykouras *et al.*, 2008; Takahashi *et al.*, 2008) and human leukocyte antigen (HLA) determinants have all been implicated (Selvaraj *et al.*, 1998; Remus *et al.*, 2003; Fernando and Britton, 2006; Berrington and Hawn, 2007). Since Mtb is an obligate intracellular pathogen of macrophages, it follows that what happens within the macrophage is of critical importance to the outcome of infection (Raupach and Kaufmann, 2001; Cosma *et al.*, 2003; Mosser, 2003; Chan and Flynn, 2004). The activation status of macrophages determines whether organisms such as Mtb, grow or are killed and also whether they persist and remain viable even in the absence of bacillary growth (Kaufmann, 2001; Russell, 2001; Mosser, 2003; Houben *et al.*, 2006; Pieters, 2008; Ray *et al.*, 2008). The contribution of other cells of the innate response, such as DCs, NK cells, NKT cells, γδ T cells, as well as the cells of the acquired immune response i.e. $CD4^+$ and $CD8^+$ T cells, appears to be the production of soluble mediators that confer activation of macrophages. These include IFN-γ and other macrophage activating or deactivating cytokines, chemokines, enzymes and cytotoxic molecules (Flynn and Chan, 2001; Raja, 2004; Berrington and Hawn, 2007; Pieters, 2008). In the absence of antigen-specific T cells, macrophages can be activated if IFN-γ and TNF-α are available from an innate immune cell source (Mosser, 2003). Since IFN-γ is produced by NK, NKT, γδ T cells and mature DC (all cells of the innate system), and TNF-α is produced by macrophages as well as other cells of the innate immune response, it is conceivable that, even in the absence of an adaptive T cell response, macrophages can be activated and exert a reasonable inhibitory effect on the growth of Mtb (Flynn and Chan, 2001; Raja, 2004; Wang *et al.*, 2004; Russell, 2007; Sinha *et al.*, 2007). However, these innate responses are usually short-lived and cannot be sustained over long periods of chronic infection (Flynn and Chan, 2001; Feng *et al.*, 2006; Hanekom *et al.*, 2007). What T cells add to this scenario is longevity and specificity of the response: when antigen-specific T cells are exposed to Mtb antigen they continue to release IFN-γ and other cytokines and chemokines for the duration of exposure to the antigen (Mosser and Edwards, 2008). Thus, as long as there is antigen in the system, i.e. as long as the organisms persist, T cells will continue to release mediators which lead to stimulation of the mononuclear phagocytes (Schluger and Rom, 1998; Mosser and Edwards, 2008). T cells also

respond efficiently to the re-introduction of antigen, for example, if following vaccination an individual is infected with Mtb, a very early and robust T cell cytokine response is observed. Thus, although T cells are not the main direct effector cell that determines Mtb growth or survival, they do provide the sustainable long term recognition of the pathogen which facilitates maintenance of a macrophage mediated protective response (Marchant *et al.*, 1999; Hussey *et al.*, 2002). This scenario is supported by the observation that following vaccination with BCG, infant leukocytes manifest increased expression of IFN-γ, IL-2 and TNF-α within $CD4^+$ T cells and IFN-γ and IL-2 in $CD8^+$ T cells (Kagina *et al.*, 2010) while T cells expressing IL-10 and IL-4 are infrequent (Soares *et al.*, 2008).

1.9.2 Rationale for studying dendritic cell function

The role of DCs, another central player in the innate immune response, in the induction of an adaptive antigen-specific T cell response to TB is well documented (Henderson *et al.*, 1997; Buettner *et al.*, 2005; Bhatt and Salgame, 2007). DCs specialise in the uptake of mycobacterial antigen and in processing and presentation of this antigen to T cells in the context of MHC molecules (Henderson *et al.*, 1997; Jiao *et al.*, 2002; Savina and Amigorena, 2007). DCs are the only APC capable of stimulating the primary activation of naïve $CD4^+$ and $CD8^+$ T cells (Banchereau and Steinman, 1998; Dilioglou *et al.*, 2003 B; Savina and Amigorena, 2007; Sinha *et al.*, 2007; Randolph *et al.*, 2008). Immature DC are highly phagocytic cells which enables them to take up the pathogen Mtb, but they are poor stimulators of the T cell response (Banchereau and Steinman, 1998; Buettner *et al.*, 2005; Munz *et al.*, 2005; Bhatt and Salgame, 2007). To enable DCs to become efficient as APC, they need to undergo a process of activation and maturation (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Bhatt and Salgame, 2007). Following the phagocytosis of Mtb in the lungs, and in response to cytokines and other soluble factors induced by the engulfed antigen, immature DC develop into functionally and phenotypically mature DC. This maturation process is associated with migration to the draining lymph nodes of the lung where the DCs encounter naïve T cells and activate them (Kaufmann, 2001; Gagliardi *et al.*, 2004; Bhatt and Salgame, 2007; Sinha *et al.*, 2007). DCs are therefore specialised cells that link the innate and adaptive immune responses (Steinman, 2001; Bhatt and Salgame, 2007; Savina and Amigorena, 2007).

1.9.3 The rationale for focussing on the cytokines IFN-γ and TNF-α

The central role of IFN-γ and TNF-α in macrophage activation to an increased bacteria-static or bactericidal state is well documented in a large number of published studies (Flynn and Chan, 2001; Mosser, 2003; Raja, 2004; Pieters, 2008; Ray *et al.*, 2008). Prior to activation, macrophages are relatively permissive for the intracellular growth of mycobacteria (Desai *et al.*, 1989; Bonecini-Almeida *et al.*, 1998). Permissive macrophages can support the survival of the organism in the host tissues for years, as seen in lepromatous leprosy: *Mycobacterium leprae* can replicate to very high numbers in permissive macrophages in the skin (Desai *et al.*, 1989). The transition from permissiveness to control of mycobacterial growth by macrophage is dependant on the activation of the cells by the cytokines IFN-γ and TNF-α (Flynn and Chan, 2001; Bogdan and Schleicher, 2006; Berrington and Hawn, 2007; Pieters, 2008; Ray *et al.*, 2008). As discussed above, sustainability of cytokine production and the quantity of IFN-γ produced are dependent on the activation of adaptive T cell immunity (Flynn and Chan, 2001). TNF-α activated NK cells also produce IFN-γ which can drive the maturation of DC and the subsequent production of IFN-γ by these cells (Flynn and Chan, 2001; Mellman and Steinman, 2001; Houben *et al.*, 2006; Berrington and Hawn, 2007; Sinha *et al.*, 2007; Ray *et al.*, 2008). Thus, these two cytokines, IFN-γ and TNF-α, orchestrate and affect the activation of macrophages to facilitate control of Mtb intracellular growth and survival (Flynn and Chan, 2001; Raja, 2004; Pieters, 2008).

1.9.4 Immune activation versus protective immunity

The immune response is an indication or measure of exposure to antigen, but not all immune activation is necessarily protective. The best example of this in TB patients is the fact that anti-Mtb specific antibodies are produced in response to infection, but in general have not been shown to be protective against progression to active disease (Glatman-Freedman and Casadevall, 1998). Another supporting observation is the fact that BCG vaccination does not induce a fundamental change in protection against TB in neonates in spite of the fact that it results in the activation of the acquired antigen-specific T cell responses against mycobacterial antigens (Marchant *et al.*, 1999; Hussey *et al.*, 2002; Soares *et al.*, 2008). Many studies have shown that the

incidence of active TB infection does not change in response to BCG vaccination, only the severity of the disease is reduced (WHO, 2004; Mahomed *et al.*, 2006; Walker *et al.*, 2006; Moyo *et al.*, 2010).

Clearly it is important to distinguish between an immune response to infection and protection against TB. The immune response that is generated by exposure to mycobacterial antigens provides some protection, but not all components of the immune response are correlates of protection. To date there is no definitive correlate of immune protection against TB that confirms a certain immune response as an indicator of protection against establishment of active Mtb infection or progression to active TB disease (Fletcher, 2007; Kagina *et al.*, 2010). Thus, many components of the immune response that can be measured may not be protective and the extent of activation of these components of the immune response may not predict clinical outcome.

The hypothesis underlying the studies in this thesis is that **protection** against TB engendered by BCG vaccination is **quantitatively different** (ie. different amount of activating mediators), but **not qualitatively** different (i.e a similar profile of activating mediators) from the pre-vaccination innate **protective response** of neonates against this pathogen. In this thesis, I will try to establish whether different components of the innate immune response against mycobacterial antigens are fully functional in cord blood before vaccination and/or before environmental exposure.

CHAPTER 2

METHODS AND OPTIMISATION OF ASSAYS

2.1 INTRODUCTION

In this thesis I hypothesise that **protection** against TB, engendered by BCG vaccination, is **quantitatively different**, but **not qualitatively** different from the pre-vaccination innate **protective response** in neonates. To accurately determine whether different components of the innate immune response against mycobacterial antigens were fully functional in cord blood before vaccination and/or before environmental exposure, it was necessary to optimise existing assays using naïve cord blood cells. Not only was it imperative that cord blood collection and cell separation was optimal but also that the *in vitro* culture of macrophages and DC, stimulation of cells, and assessment of secreted and intracellular cytokines could be measured. The primary aims were:-

1. To select the ideal anticoagulant for cord blood, which would be suitable for all the various assays that were performed.
2. To optimise the collection of cord blood, as it was critically important to ensure that sterility was maintained, that the cord blood was not contaminated with maternal blood, as well as ensuring the subsequent cell viability of cord blood derived cells that required subsequent *in vitro* culture.
3. To ensure the purity of cord blood mononuclear cells (CBMC) following separation. This required a different method to the standard one used for purification of PBMC, as the mononuclear fraction of cord blood is often contaminated with erythrocytes and nucleated erythroid precursors (Boyum, 1968; Ridings *et al.*, 1996).
4. To establish the culture of classically and alternatively activated macrophages from monocytes in order to determine macrophage function in the neonate.
5. To evaluate secreted cytokine production in the neonate (CBMC and purified $CD14^+$ monocytes).
6. Finally, to establish which cells were producing cytokines, prior to and following BCG vaccination, it was necessary to validate a flow cytometrically

based intracellular cytokine assay. Optimisation and validation of this intracellular cytokine assay included experiments to determine whether whole cord blood or CBMC would be superior. Titration of BCG was also undertaken to ensure optimal stimulation of cells. The addition of costimulants was also investigated as to whether they enhanced the intracellular IL-10 response as has been reported with intracellular IFN-γ (Hanekom *et al.*, 2004). Comparison between dual staining with both anti-CD3 and anti-CD8 or anti-CD8 monoclonal antibodies alone, for assessment of cord blood intracellular $CD8^+$ T cells.

2.2 ETHICAL CONSIDERATIONS

Human participation was according to the US Department of Health and Human Services and good clinical practice guidelines. This included protocol approval by the University of Cape Town Research Ethics Committee in South Africa. Written informed consent was obtained from all adult volunteers prior to the collection of peripheral blood and from the mothers prenatally for collection of cord blood, as well as prior to the collection of peripheral blood from 13 week old infants, where relevant. Patient anonymity was strictly maintained at all times.

2.3 MATERIALS AND METHODS

2.3.1 Study participants

Umbilical cord blood samples were collected from pregnant females undergoing elective caesarean section. Labour involves the release of pro-inflammatory cytokines and inflammatory mediators and it has been found that this can affect both infant's and mother's immune response and also influence the cytokine production of CBMC (Brown *et al.*, 2003; Protonotariou *et al.*, 2003; Amoudruz *et al.*, 2005). To prevent any effects of labour, cord blood was collected from mothers undergoing elective caesarean sections, which were performed prior to the onset of labour for mothers with breech presentations, large babies or with histories of prior caesarean sections. Although any effects that labour may have had on the cord blood cells was eliminated by the collection of cord blood following elective caesarean sections; the stress of surgery and/or any anaesthesia that the mother used could have had either

immunosuppressive or activating effects on the cord blood cells. This immunosuppressive or activating effect might have impacted on studies performed with innate and adaptive immune cells obtained from cord blood. However, in many studies involving neonates, cord blood is used as a means of collecting and studying neonatal cells, as it is unethical to collect large volumes of blood from a newborn infant (Trivedi *et al.*, 1997; Kovarik and Siegrist, 1998; Goriely *et al.*, 2001; White *et al.*, 2002; Rowe *et al.*, 2004; Amoudruz *et al.*, 2005).

Gestation period prior to the collection of cord blood, was always term (38 - 41 weeks), as preterm birth is defined as <37 weeks (Park *et al.*, 2005). Apgar scores, an assessment of newborn health, were recorded for each infant. Only babies with an immediate Apgar scores of ≥8 and the 5-minute Apgar scores of ≥9 were included in the study. Details on the mothers and babies for all samples collected for the studies in this thesis are included in Table 2.1.

All infants received Danish intradermal vaccination shortly after birth. For studies on cytokine responses of neonates after BCG vaccination, peripheral blood was collected at 13 weeks of age from six healthy infants in whom cord blood had been studied previously. Details of the six infants are indicated in Table 2.2. All the healthy TST^{+} adult volunteers are shown in Table 2.3.

Exclusion criteria of infants included foetal distress, maternal diabetes mellitus, maternal HIV, maternal infection and pre-eclampsia. The TB history of the mothers was not known, but none of the mothers had active TB at the time of caesarean section. As it has been reported that diabetes and pre-eclampsia can increase cytokine production both locally in the placenta, as well as in the maternal circulation, these patients were also excluded (Hauguel-de Mouzon and Guerre-Millo, 2006).

TABLE 2.1 Details on mother, baby and placenta from whom cord blood was collected. The chapter indicates in which section of the study the cord blood was utilised.

ID	Chapter	Age of mother	Gestational age	Baby sex	Baby weight (g)	Placenta weight (g)	Apgar score
1	Macrophage and cytokine	26	38	M	3380	635	8 and 9
2	Macrophage	32	39	M	3430	540	9 and 9
3	Macrophage	31	39	F	3680	800	9 and 9
4	Macrophage	26	38	F	3320	585	9 and 10
5	Macrophage	35	38	M	3340	560	9 and 9
6	Macrophage	38	38	M	3150	750	9 and 10
7	Macrophage	30	39	M	3210	520	9 and 9
8	Macrophage	32	39	M	3350	795	9 and 10
9	Macrophage and DC	30	38	M	3300	620	9 and 10
10	Macrophage	33	38	F	3380	700	9 and 9
11	Macrophage	22	38	F	3360	660	9 and 10
12	Macrophage	34	38	F	2710	600	9 and 10
13	Macrophage	34	38	M	3305	785	9 and 10
14	DC	30	38	F	3565	730	8 and 9
15	DC and cytokine	33	38	M	4155	960	9 and 10
16	DC and cytokine	31	38	M	2850	490	9 and 10
17	DC and cytokine	34	38	F	2925	670	8 and 9
18	DC	38	38	M	3300	620	9 and 10
19	DC	29	38	F	3480	670	9 and 10
20	DC	38	38	F	2960	630	9 and 10
21	DC	29	41	M	4150	765	9 and 10
22	DC	37	39	M	3520	720	9 and 10
23	DC	30	38	M	3300	620	9 and 10
24	DC	32	38	M	3930	975	9 and 10

ID	chapter	Age of mother	Gestational age	Baby sex	Baby weight (g)	Placenta weight (g)	Apgar score
25	cytokine	29	39	M	3195	620	9 and 10
26	cytokine	37	38	F	2770	650	9 and 10
27	cytokine	24	38	F	3220	610	9 and 10
28	cytokine	31	38	F	2840	420	9 and 9
29	cytokine	17	38	M	3395	620	9 and 10
30	cytokine	38	38	M	3040	760	9 and 10
31	cytokine	27	38	F	2990	605	9 and 10
32	cytokine	39	39	M	3405	665	8 and 9
				M	2960		8 and 9
33[a]	cytokine	32	38	M	2080	1100	9 and 9
34	cytokine	31	38	F	3160	1115	8 and 9
35	cytokine	31	38	F	2820	730	9 and 9
36	cytokine	18	38	F	2800	605	9 and 9
37	cytokine	36	38	M	2600	420	9 and 10
38	cytokine	32	39	F	2930	600	9 and 10
39	cytokine	31	38	F	4740	885	9 and 10
40	cytokine	19	38	M	2900	500	9 and 9
41	cytokine	29	38	F	3455	950	9 and 10
42	cytokine	26	38	M	3380	635	8 and 9
43	cytokine	26	38	M	3300	600	9 and 10
44	cytokine	27	38	F	2965	550	9 and 10

[a] denotes cord blood collection was from one of twins

TABLE 2.2 Details on infants in whom peripheral blood was collected at 13 weeks of age. Peripheral blood was collected and used to determine the BCG stimulated cytokine response following Danish BCG vaccination given at birth.

ID	Chapter	Age of mother	Gestational age	Baby sex	Baby weight (g)	Placenta weight (g)	Apgar score	Age in days
1	cytokine	29	39	F	3175	655	9 and 10	89
2	cytokine	29	39	M	3195	620	9 and 10	98
3	cytokine	31	38	F	2840	420	9 and 9	92
4	cytokine	31	38	F	2820	730	9 and 9	89
5	cytokine	34	38	F	2925	670	8 and 9	92
6	cytokine	32	39	F	2930	600	9 and 10	91

TABLE 2.3 Details of healthy adult volunteers from whom peripheral blood was collected.
The chapter indicates in which section of the study the peripheral blood was utilised.

ID	Chapter	Age	Sex
1	Macrophage and DC	40	M
2	Macrophage and DC	38	M
3	Macrophage	23	F
4	Macrophage and DC	35	M
5	Macrophage	47	M
6	Macrophage and DC	47	F
7	Macrophage and DC	45	F
8	Macrophage	23	F
9	Macrophage	26	F
10	Macrophage	25	F
11	DC	35	M
12	DC	47	M
13	DC	47	F
14	DC	35	F
15	DC	62	M

2.3.2 Method optimised for collection of cord blood

Cord blood was obtain from the umbilical vein and collected into a closed system by using a sealed blood donor bag to prevent both bacterial as well as maternal blood contamination. Prior to collection of cord blood, a standard blood donation bag (Sabax, Johannesburg, South Africa) was prepared by aseptically adding 2000 units of preservative free sodium heparin (Sigma, Steinham, Germany). Following delivery of the infant, the umbilical cord was clamped and the placenta was placed in a sterile bowl. The cord was then punctured with a 16-gauge needle connected to the heparinised blood donor bag and cord blood from the umbilical vein was allowed to

flow into the bag by gravity and the blood was gently mixed to prevent clotting. The cord blood sample was immediately transferred to the laboratory at room temperature (RT).

2.3.3 Method optimised for isolation of cord blood mononuclear cells from cord blood

CBMC were used for the determination of secreted cytokine responses of neonates to BCG prior to BCG vaccination. For isolation of CBMC, cord blood was diluted with an equal volume of phosphate buffered saline (PBS; without calcium or magnesium; Bio Whittaker, Walkersville, MD, USA) and carefully layered on Ficoll-Hypaque (Sigma-Aldrich, Steinham, Germany). The gradient was then centrifuged at 1200g for 30 minutes at RT. The interphase containing the mononuclear cells was removed and washed twice with PBS by centrifugation at 300g for 10 minutes. Cells were resuspended in PBS to the original volume of cord blood and the procedure repeated with another round of Ficoll-Hypaque density gradient centrifugation to ensure removal of erythroid precursors from CBMC. This was followed by three PBS washes. After the first and second gradient, CBMC were stained with anti-CD45 allophycocyanin (APC)-conjugated mouse anti-human monoclonal antibody (Caltag, Burlingame, CA, USA) to identify cells expressing the leukocyte common antigen. In addition, the CBMC were stained with anti-CD235a phycoerythrin (PE)-conjugated (Glycophorin A; BD Biosciences, San Jose, CA, USA) to identify nucleated erythroid precursors. These antibodies proved that the erythroid cells were removed from the CBMC as they express Glycophorin A but not CD45, whereas CBMC stain $CD45^{+}$ and are negative for Glycophorin A.

2.3.4 Surface staining of cells for flow cytometric evaluation

For surface staining of monocytes, macrophages and DC, approximately 1×10^5 cells were incubated for 15 minutes in the dark at RT with the relevant monoclonal antibody. Thereafter, cells were washed with fluorescence activated cell sorter (FACS) wash buffer (FB) comprising of 0.1% Sodium azide (Sigma-Aldrich, Steinham, Germany) and 1% AB serum (Western Province Blood Transfusion Service, South Africa) in PBS. Cells were subsequently resuspended in FB and 20 000 gated events were acquired on a FACsCalibur flow cytometer. Analysis was

performed using Cellquest software (BD Biosciences, San Jose, CA, USA). The addition of 7-aminoactinomycin D (7AAD; 1µg/ml; Sigma-Aldrich, Steinham, Germany) ensured that only viable cells were analysed. Analysis of cells stained with CD80, CD86, CD40, CD83, CD25 and HLA-DR included both the percentage of cells expressing a particular marker as well as geometric mean fluorescent intensity (MFI), as the upregulation of some markers results in an increase in MFI, while downregulation may result in a decrease in MFI. As CD80, CD40, CD86 and HLA-DR have continuous expression of these markers, it was more informative to compare the change in MFI over time. CD83 and CD25 have discrete expression, therefore MFI, as well as percentage of cells expressing a particular marker, was compared.

2.3.5 Peripheral blood separation and lymphocyte proliferation and IFN-γ evaluation

PBMC were obtained from healthy adult volunteers following separation of peripheral blood using Ficoll-Hypaque as previously described (Boyum, 1968). To compare the two different anticoagulants, peripheral blood from adult volunteers was collected into citrate phosphate dextrose (CPD) tubes (BD Biosciences, San Jose, CA, USA) or into syringes containing sodium heparin (Sigma, Steinham, Germany). Lymphocyte proliferation and IFN-γ evaluation was carried out. Purified PBMC were resuspended at 1 x 10^6 PBMC/ml in complete medium (CM) which consisted of 10% AB serum in RPMI-1640 (Bio Whittaker, Walkersville, MD, USA). 100µl of PBMC per well was plated into 96 U-well microtitre plate (Costar, Corning Incorporated, New York, USA) in triplicate for each stimulant. Unstimulated cells served as a negative control. Stimulants included phytohaemagglutinin (PHA; 5.75 x 10^{-3} mitogenic units/ml; Wellcome Research Laboratories, Beckenham, UK) and phorbol 12-myristate 13-acetate (PMA; 10ng/ml) + Ionomycin (ION; 1µg/ml; both from Sigma-Aldrich, Steinham, Germany) for 3 days and PPD (3µg/ml; Statens Serum Institut, Copenhagen, Denmark), Japanese BCG (BioVac Institute, South Africa) or Danish BCG (Statens Serum Institut, Copenhagen, Denmark) both at an average multiplicity of infection (MOI) of 10:1 BCG per monocyte for 6 days. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Supernatant fluid (SNF) was removed and stored at -80°C for later evaluation of secreted IFN-γ. 18hrs prior to the completion of the stimulation period, Tritiated thymidine (^{3}HTdR, Amersham

International, Buckinghamshire, UK) was added (2μCi/well) then the plates were harvested using an automated cell harvester (Titretek cell harvester, Flow Laboratories, Scotland). ^{3}HTdR incorporation was measured in a beta counter (Insta-Gel II, Packard, Meriden, USA) and results were expressed as a stimulation index (SI), where SI = mean counts per minute (CPM) in stimulated wells/mean CPM in unstimulated wells.

2.3.6 Purification of CD14$^+$ cells

Monocytes from CBMC and PBMC were purified using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for *in vitro* culture of macrophages and DC, and for determination of monocyte secreted cytokines. Cells were stored on ice to prevent any adherence of monocytes. The concentration of CBMC or PBMC was adjusted to 1 x 10^7cells/80μl Miltenyi buffer. The Miltenyi buffer consisted of 2mM ethylenediaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin (BSA; both from Sigma, Steinham, Germany) in PBS. 10μl of anti-CD14 microbeads were added per 80μl Miltenyi buffer and incubated at 4°C for 25 minutes. Cells were washed with Miltenyi buffer and pelleted at 4°C (300g). After the magnetic separation, CD14 negative cells were retained and CD14$^+$ cells were removed from the magnet using a syringe as per manufacturers' instruction. The purified CD14$^+$ and CD14$^-$ cells were washed twice in PBS to ensure the removal of any residual EDTA. Cells were stored on ice until required.

2.3.7 Method validated for stimulation of secreted cytokines

CBMC were dual stained with CD45-APC and CD14 PE antibodies (BD Biosciences, San Jose, CA, USA) to determine the percentage of monocytes present in CBMC. CBMC cultures were adjusted to a concentration of 3 x 10^5 monocytes per ml. CBMC were then plated at 100 μl per well in CM in 96 U-well microtitre plates. Purified CD14$^+$cells were set up in parallel at 3 x 10^5/ml and 100 μl per well and all tests were conducted in triplicate. By ensuring that the same numbers of monocytes were present as CBMC, or as purified CD14$^+$cells, it was possible to directly compare the monocyte secreted cytokines in cord blood (Chapter 5). CBMC and CD14$^+$ monocytes were stimulated with Danish BCG vaccine strain at a MOI of 5:1. Unstimulated cells served as a negative control and Staphylococcal enterotoxin B

(SEB; 0.1μg/ml) or LPS (1μg/ml; both from Sigma-Aldrich, Steinham, Germany) was used as a positive control. Microtitre plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After incubation (2 days for IL-10 and IL-12 and 6 days for IFN-γ, IL-5, IL-4 and IL-13 as determined following optimisation in section 2.4.3.2), SNF was removed and stored at -80°C for later evaluation of secreted cytokine evaluation.

2.3.8 Enzyme-linked immunosorbent assay

Cytokine evaluation was quantified by sandwich enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer, using cytokine-specific antibodies for IL-10, IL-5, IL-4, IL-13 and IFN-γ (Pharmingen, BD Biosciences, San Jose, CA, USA). Each plate included a standard curve of a recombinant human cytokine. IL-12 (total p40p35; Pierce Biotechnology, Rockford, IL, USA) and TNF-α (Assay Designs Inc., Ann Arbor, MI, USA) were purchased as single pre-coated plates.

The limit of detection for the cytokines was as follows:
TNF-α ≥8pg/ml;
IL-12 ≥5pg/ml;
IL-10 and IFN-γ ≥15pg/ml;
IL-5, IL-4 and IL-13 ≥10pg/ml.
For IFN-γ ELISA assays, the top of the standard curve was 5000pg/ml and any value that was above this level was repeated in dilution, so that accurate levels could be determined.

2.3.9 Modified method validated for intracellular cytokine evaluation in cord blood

An intracellular cytokine (ICC) assay was used to determine the cytokine response of neonates to BCG before and after BCG vaccination. This ICC assay evaluates the frequency of antigen-specific $CD4^+$ and $CD8^+$ T cells and was used to quantify the frequency of IFN-γ and IL-10 producing T cells, $CD14^+$ monocytes and IFN-γ producing $CD3^-CD56^+$ NK cells following a 12 hour stimulation. After this short period of incubation only memory T cells will produce IFN-γ and in this way only memory/effector T cells would be evaluated (Hanekom *et al.*, 2004). A schematic

diagram for the ICC methodology is indicated in Figure 2.1. On the day prior to collection of cord blood, 3 sets of 2-ml polypropylene tubes (Sarsted, Nümbrecht, Germany) were prepared for both whole cord blood (WCB), ICC and CBMC ICC (one set for evaluation of ICC $CD4^+$ and $CD8^+$ IFN-γ, the second set for the evaluation of ICC $CD3^-CD56^+$ NK cell IFN-γ and the third set for evaluation of ICC $CD14^+$ IFN-γ and IL-10 and ICC $CD4^+$ and $CD8^+$ IL-10) and the tubes stored at 4-8°C until required. Anti-CD49d and anti-CD28 costimulatory antibodies (final concentration of 1μg/ml of both antibodies; both BD Biosciences, San Jose, CA, USA) were added to the first two sets of tubes for evaluation of ICC $CD4^+$ and $CD8^+$ IFN-γ cytokines and $CD3^-CD56^+$ NK cell intracellular IFN-γ, but was excluded from the tubes for evaluation of $CD14^+$ ICC and IL-10 ICC (validation for exclusion of costimulants in IL-10 ICC evaluation is described in section 2.4.4.2). Two unstimulated tubes were included in each set of tubes; one stained as described below and the other stained with either IFN-γ or IL-10 isotypic control. PPD (20μg/ml) or Danish BCG clinical vaccine were used to stimulate the cells *ex vivo*. The lyophilised vaccine was reconstituted in RPMI and added to allow an average MOI of 3.6:1. The mean ± SD percentage of $CD14^+$ cells in unseparated cord blood was previously determined by dual staining with anti-CD45 APC-conjugated and anti-CD14 PE-conjugated antibodies (9.5% ± 5.5%). This figure of 9.5% was applied for calculating the concentration of BCG, so that MOI of 3.6:1 in whole cord blood ICC assay was used. SEB (10μg/ml) was utilised as a positive control.

Immediately following the collection of cord blood, whole unseparated cord blood (0.5-2ml) was added to the WCB ICC tubes and incubated in a 37°C waterbath for 7 hours. CO_2 is not necessary during this short incubation period. Brefeldin A (10μg/ml; Sigma-Aldrich, Steinham, Germany) was then added to capture newly formed intracellular cytokines in the Golgi apparatus. Tubes were then placed in a programmable waterbath, to change the temperature from 37°C to RT for a further 5 hours (i.e. a total of 12 hours at 37°C). CBMC were run in parallel and incubated in CM for a total of 18 hours (the last five hours in the presence of Brefeldin A) with a loose cap in a 37°C incubator with 5% CO_2. The WCB ICC tubes were harvested within 10 hours of the waterbath switching off. The CBMC tubes were harvested after 18 hours incubation. The method for both the WCB ICC and CBMC ICC was

identical following harvesting of the cells. Tubes were vortexed and cells harvested by addition of 2mM EDTA and incubated for 15 minutes to arrest activation and detach adherent cells from the tubes. The cells were then spun and five ml FACS lysing solution (BD Biosciences, San Jose, CA, USA), diluted 1:10 with distilled water, was added for 10 minutes to lyse erythrocytes and fix leukocytes. Cells were pelleted and resuspended in 10% dimethyl sulfoxide (DMSO; Merck, Midrand, South Africa) in foetal bovine serum (FBS; GIBCO, Invitrogen, Carlsbad, CA, USA) and cryopreserved at -80°C using step-wise freezing (Cryo 1°C freezing container, Mr Frosty, Nalgene, Thermo Fisher Scientific, NY, USA) until required.

When needed, cryopreserved tubes were rapidly thawed at 37°C, pelleted and washed with PBS and then incubated for 10 minutes with permeabilisation fluid, which consisted of 0.1% saponin, 0.1% BSA (both from Sigma-Aldrich, Steinham, Germany) in PBS. Cells were pelleted and 10ul neat AB serum was added to all tubes, to prevent non-specific staining and capping of bound antibody and the cells were incubated at RT for 10 minutes. Cells were then stained with the relevant fluorescence-conjugated antibodies; anti-CD3 fluorescein isothiocyanate (FITC), anti-CD4 FITC, anti-CD8 PE, anti-CD14 PE, CD56 PE, IL-10 APC (and its relevant isotypic control) or IFN-γ APC (and its relevant isotypic control; all from BD Biosciences, San Jose, CA, USA) in 50μl of permeabilisation fluid for 30 minutes at RT in the dark. Cells were washed once in permeabilisation fluid and resuspended in 1% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS. Flow cytometric acquisition was performed within 24 hours of fixation, during which time samples were stored at 4°C in the dark. For NK cells, a minimum of 15 000 gated events were acquired. During acquisition, events were acquired slowly (<200 gated events per second, to ensure that only single cells passed the laser at a time) as this has been found to ensure a low background unstimulated response which allows increased sensitivity of the measured stimulated response (Hanekom *et al.*, 2004).

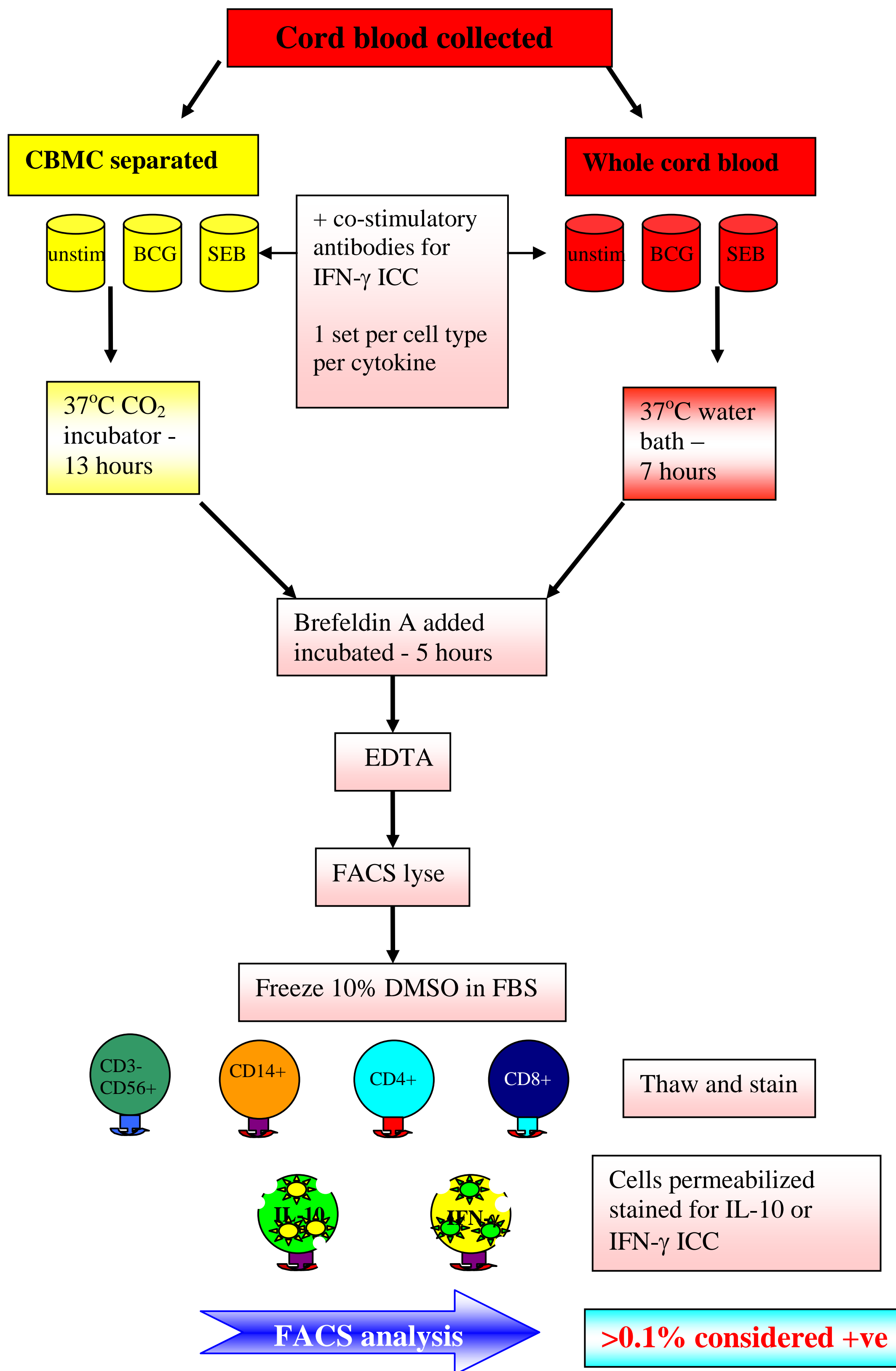


Figure 2.1 Schematic diagram showing details of method for determination of IFN-γ and IL-10 intracellular cytokines in cord blood.

2.3.10 Culture of monocyte-derived dendritic cells

CBMC or PBMC from adult volunteers were isolated and further purified for $CD14^+$ monocytes as previously described. Monocytes were resuspended in DC medium consisting of 5% AB in RPMI with 10mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES; GIBCO, Invitrogen, Carlsbad, CA, USA). Monocytes were plated at 2-3 x 10^6 per well in a 6 well tissue culture plate (Falcon; BD Biosciences, San Jose, CA, USA) in a total volume of 3ml DC medium, with IL-4 (1.6ng/ml) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.83ng/ml; both obtained from Pierce Endogen; Pierce Biotechnology, Rockford, IL, USA). Plates were incubated at 37°C with 5% CO_2 in a humidified atmosphere. Cells were viewed daily using an Olympus CK40 inverted light microscope (Olympus Corporation, Hamburg, Germany) to compare the morphology of the DC generated. On days 2 and 4, the cytokines were replenished by removal of 300µl of SNF from each well and the addition of 500µl DC medium per well, containing IL-4 and GM-CSF at the same concentrations as previously used. On day 6, immature DC (iDC) were harvested, washed and resuspended in DC medium for the culture of mature DC (mDC). Some iDC were retained for surface staining and flow cytometric evaluation of CD80, CD86, CD40, CD83, CD25 and HLA-DR as described in section 2.3.4. iDC were resuspended at 2 x 10^5 per ml in 10% AB serum in RPMI with IL-4 and GM-CSF at the same concentration as before, in 24 well tissue culture plates (Falcon; BD Biosciences, San Jose, CA, USA). The total volume per well was 2ml with 10% AB RPMI containing the following maturation cytokines: TNF-α (10µg/ml, Pierce Endogen; Pierce Biotechnology, Rockford, IL, USA), IL-1-β (10µg/ml; Pharmingen; BD Biosciences, San Jose, CA, USA) and Prostaglandin E2 (PGE_2; 1ug/ml; Sigma-Aldrich, Steinham, Germany) which have previously been utilised in adult studies to generate mDC (Hanekom *et al.*, 2003). Cells were cultured for a further 2 days in 37°C containing 5% CO_2 in a humidified atmosphere for maturation of DC. After 2 days incubation, EDTA in PBS was added to each well at a final concentration of 0.02% to facilitate the removal of adherent mDC and incubated for 10-15 minutes at 37°C until cells had loosened. mDC were washed 3 times with PBS and some mDC were retained for surface staining and flow cytometric evaluation of CD80, CD86, CD40, CD83, CD25 and HLA-DR. Mature and immature DC were photographed using the Zeiss AxioCam MRm camera attached to a Zeiss Axiovert 40CFL

microscope (both from Carl Zeiss MicroImaging GmbH, Göttingen, Germany) at x200 magnification.

2.3.11 Dendritic cell-induced allogeneic mixed lymphocyte reaction

To determine whether mature cord blood DC were functional compared to mature adult DC, the ability of mature DC (mDC) to present antigen and drive the proliferation of T cells *in vitro* was compared. In addition, the ability of mDC to stimulate the *in vitro* production of IFN-γ was also evaluated and compared (Chapter 4). mDC were used to stimulate allogeneic CD14 negative cells (used as a source of T cells with no monocytes, (Hanekom *et al.*, 2003) purified by magnetic beads as previously described in section 2.3.6. Autologous CD14 negative cells (used as a negative control) and allogeneic CD14 negative cells were resuspended at 1 x 10^6/ml viable cells in CM and 100µl plated per well of 96 U-well microtitre plate in triplicate. Wells containing CD14 negative cells alone were also included. mDC were added at ratios of mDC:CD14 negative cells of 1:50, 1:100, 1:300 and 1:1000 in triplicate. Wells containing mDC alone were also included. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 5 days. ^{3}HTdR was added (2µCi/well) for a further 18 hours to assess lymphoproliferation then harvested and ^{3}HTdR incorporation was measured in a beta counter. Results were expressed as delta CPM, or mean CPM in stimulated wells less mean CPM in wells containing CD14 negative cells alone.

2.3.12 Culture of polarised classically and alternatively activated macrophages from monocytes

The culture of polarised classically and alternatively activated macrophages from monocytes was necessary for all assays undertaken to determine macrophage function in neonates. Cord blood or peripheral blood was collected and separated for CBMC as previously described (2.3.3). CBMC were purified for CD14$^+$ cells using magnetic beads. CD14$^+$ monocytes were stained for surface staining following isolation and prior to culture of monocytes with anti-CD14, anti-CD80, anti-CD86 or anti-HLA-DR (all PE conjugated and from BD Biosciences, San Jose, CA, USA). Purified monocytes at 2-3 x 10^6 per well were cultured in 6 well tissue culture plates in 3ml culture medium, consisting of 10% FBS in RPMI-1640 for 6 days. Two sets of

monocyte cultures were set up for differentiation into the different macrophage types. For culture of classically activated macrophages (CA Mø), GM-CSF (5ng/ml) was included in the culture medium. For culture of alternatively activated macrophages (AA Mø), macrophage colony-stimulating factor (M-CSF; 50ng/ml; R&D Systems, Minneapolis, USA) was added. On day 3 or 4, medium was refreshed, by removing 300µl SNF from each well and adding 500µl 10% FBS RPMI and 5ng/ml GM-CSF for CA Mø or with 10% FBS RPMI containing 50ng/ml M-CSF for AA Mø (Verreck *et al.*, 2004; Ottenhoff, 2006). Following 6 days of culture, an Olympus CK40 inverted light microscope at a magnification of x200 was used to compare the morphology of the macrophages generated. Macrophages were photographed using the Zeiss AxioCam MRm camera attached to a Zeiss Axiovert 40CFL microscope at x200 magnification. Adherent cells were removed by vigorous pipetting and surface phenotyping was performed for each macrophage type using anti-CD14, anti-CD80, anti-CD86 and anti-HLA-DR to determine whether upregulation or down-regulation of these markers had occurred following differentiation into these two distinct macrophage types.

2.3.13 Culture of green fluorescent protein expressing-BCG

BCG expressing green fluorescent protein (BCG-gfp; a gift from Prof. B Ryffel, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, South Africa) was cultured to log phase in Middlebrook 7H9 broth (DIFCO, BD Microbiology Systems, Sparks, MD, USA). The media was supplemented with Hygromycin B (50µg/ml; Boehringer Mannheim, Germany) for selection of recombinant mycobacteria. The 7H9 broth was also enriched with 10% OADC (Oleic Acid Albumin Dextrose Catalase; BD Microbiology Systems, Sparks, MD, USA) in the presence of 0.02% glycerol (Fluka, Sigma-Aldrich, Steinham, Germany) and 0.05% Tween 80 (Merck, Darmstadt, Germany). The organisms were frozen in 10% glycerol at -80°C. To determine the colony forming units (CFU), 3 tubes of the frozen BCG were thawed and ultrasonically dispersed by sonication (Microson Ultrasonic Cell disruptor, Misonix Inc., Farmingdale, NY, USA) to disperse clumps of bacilli and obtain single cell suspensions. The BCG was then plated on Middlebrook 7H10 agar (DIFCO, BD Microbiology Systems, Sparks, MD, USA) using square agar plates with 6 grids in each direction (Falcon; BD Biosciences, San

Jose, CA, USA). Various dilutions of the broth culture were made from the thawed tubes and 6 replicates of each dilution were plated on the agar plates (10μl per square grid). This was performed in triplicate using dilutions from the 3 thawed tubes. The mean number of colonies were then determined on 6 replicates of each dilution from 3 thawed tubes by counting the number of colonies using an Olympus S751 microscope. CFU/ml could then be determined by multiplying the mean colony count (of 6 squares) by 100 (as 10μl was plated) and then multiplied by the dilution factor.

2.3.14 Culture of Danish BCG and laboratory strain of Mycobacterium tuberculosis (H37Rv)

Danish BCG and H37Rv were cultured to log phase in Middlebrook 7H9 broth enriched with 10% OADC in the presence of 0.2% glycerol and 0.05% Tween 80. The organisms were frozen in 10% glycerol at -80°C. CFU were then determined as described above for culture of BCG-gfp.

2.3.15 Infection of macrophages and dendritic cells with BCG-gfp and stimulation with LPS

To determine whether cord blood macrophages had similar phagocytic properties to those of adult macrophages, uptake of BCG-gfp was determined (Chapter 3). Macrophages were also stimulated with LPS and SNF collected from both BCG-gfp infected and LPS stimulated cells for cytokine evaluation (Chapter 3). In addition, phagocytic properties of cord blood iDC and mDC were compared to determine whether maturation of DC caused downregulation of the phagocytic capacity (Chapter 4). CA Mø or AA Mø that had been cultured for 6 days, were plated at 2 x 10^{5}/ml per well in a 24 well tissue culture plate (Falcon; BD Biosciences, San Jose, CA, USA). iDC that had been cultured for 6 days or mDC that had been cultured in the presence of maturation cytokines for a further two days were also plated at 2 x 10^{5}/ml per well in a 24 well plate. Sufficient wells were included for uninfected, infected with BCG-gfp and LPS stimulated for each macrophage type and either iDC or mDC to determine the uptake of BCG-gfp. CA Mø included GM-CSF in culture medium and AA Mø included M-CSF in 10% FBS in RPMI-1640. iDC and mDC with relevant cytokines were cultured in 10% AB serum in RPMI-1640. Cells were allowed to rest overnight. BCG-gfp was sonicated to obtain single cell suspensions. All relevant

wells were stimulated with LPS (10ng/ml) or infected with BCG-gfp (MOI of 1:1) for 24 hours. SNF was removed and stored at -80°C for cytokine evaluation. Following infection, macrophages were harvested (infected and uninfected cells) by vigorous pipetting. iDC were harvested by gentle pipetting and mDC were removed by the addition of EDTA in PBS to each well at a final concentration of 0.02% and incubated for 10-15 minutes at 37°C following which mDC were removed by pipetting. All cells were washed three times in PBS in an attempt to remove all BCG-gfp that had not been phagocytosed and were merely adherent to the surface of the cell. Surface staining was performed using anti-CD14, anti-CD80, anti-CD86 and anti-HLA-DR for each macrophage type that was cultured. Both iDC and mDC were stained with CD25, CD83, CD40, CD80, CD86 and HLA DR. Flow cytometric acquisition of cells infected with BCG-gfp was performed on a FACsCalibur and analysis was performed using Cellquest software to determine the percentage of cells infected with BCG-gfp. In addition, percentage of iDC and mDC staining positive for CD25 or CD83 and the MFI of macrophages stained with anti-CD14, anti-CD80, anti-CD86 and anti-HLA-DR was also determined.

2.3.16 Infection of macrophages with Danish BCG or H37Rv and determination of colony forming units

The control of intracellular growth of mycobacteria in cord blood macrophages was compared to the growth within adult macrophages, to determine whether neonatal macrophages were functioning optimally (Chapter 3). Macrophages that had been cultured for 6 days were plated at 1 x 10^{5}/ml per well in a 96-well flat bottom plate (Costar, Corning Incorporated, New York, USA). Sufficient wells were included for triplicate wells for day 1, day 4 and day 7 for both CA Mø and AA Mø. CA Mø contained GM-CSF and AA Mø included M-CSF supplemented with 10% FBS in RPMI-1640. Cells were allowed to rest overnight and then infected with sonicated Danish BCG or sonicated H37Rv (both at MOI of 1:1). Wells were infected for 24 hours and then washed three times with warm 10% FBS in RPMI-1640, to remove any extracellular BCG or H37Rv. Macrophages in day 1 wells were lysed with 0.3% saponin in PBS and the lysate plated onto Middlebrook 7H10 agar square agar plates with 6 grids in each direction. The lysate was plated out undiluted and then following various dilutions. Six replicates of each dilution were plated onto each grid of the

agar plate (10µl per grid). When CFU were determined, cultures were always set up in triplicate and plated onto a plate with 6 grids, so that the average of 6 replicates was determined in each of the triplicate cultures. The mean number of colonies was then determined from the 6 replicates by counting the number of colonies using an Olympus S751 microscope (Figure 2.2). CFU/ml could then be calculated by multiplying the mean colony count (of 6 squares) by 100 (as 10µl was plated) and then multiplied by the dilution factor, if relevant. This was repeated on day 4 and day 7, so that the antimicrobial activity of the macrophages could be determined over time. On day 4, the medium for the day 7 wells was refreshed by removing 100µl SNF from each well and adding 100µl 10% FBS in RPMI-1640 and GM-CSF for CA Mø or with 100µl 10% FBS in RPMI-1640 and M-CSF for AA Mø.

Figure 2.2 Photograph of typical Danish BCG colonies. Colonies were viewed through an Olympus S751 microscope at x15 magnification when counting CFU.

2.4 RESULTS

2.4.1 Evaluation of anticoagulants in collection of cord blood

CPD has been used as the anticoagulant for cord blood collection internationally and is also the only anticoagulant available in sealed blood donor bags in South Africa. However, sodium heparin is the recommended anticoagulant when whole blood ICC assays are performed (Biosciences, 2002). A comparison between the two different anticoagulants was therefore carried out. Peripheral blood was collected from an

adult volunteer, using either CPD or sodium heparin as anticoagulants. PBMC were isolated and used in evaluation of lymphocyte proliferation and secretion of IFN-γ, as indicated in section 2.3.5. Results show that there was no difference in the proliferative response when either CPD or sodium heparin was used as the anticoagulant (Figure 2.3 A). Similarly, IFN-γ secretion in response to all stimulants was not significantly different whether CPD or sodium heparin was used (Figure 2.3 B). Consequently sodium heparin was used as the anticoagulant of choice for all cord blood collection as it has been recommended for the evaluation of ICC (Biosciences, 2002).

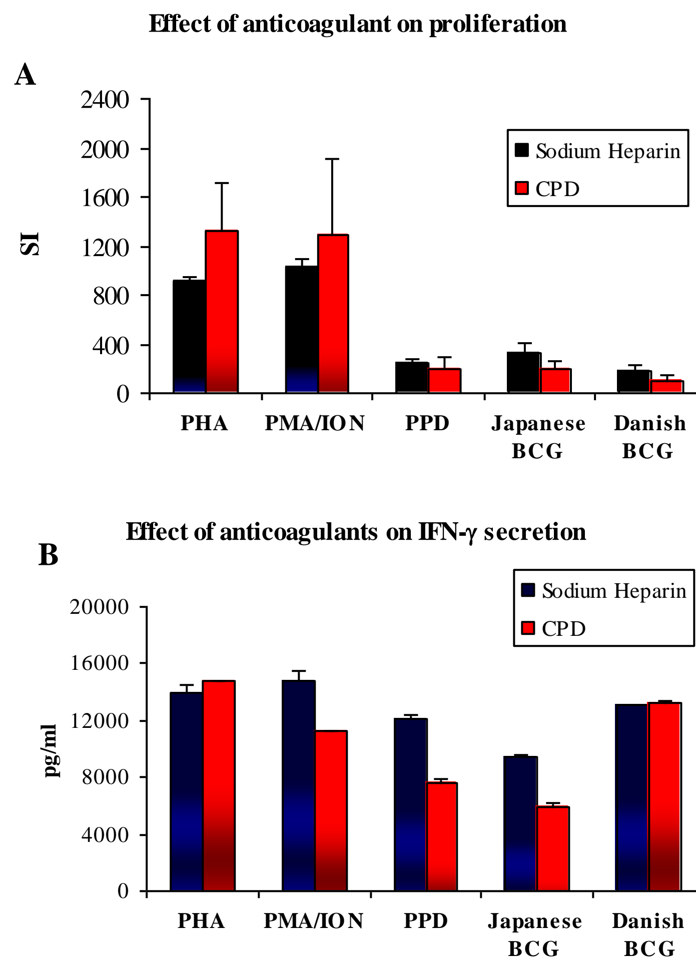


Figure 2.3 The effect of anticoagulants on PBMC proliferation and IFN-γ secretion. Adult peripheral blood containing either sodium heparin or CPD as an anticoagulant was used for PBMC isolation. PBMC were stimulated with PHA 5.75 x 10^{-3} mitogenic units/ml, PMA 10μg/ml and ION 1μg/ml, PPD 3μg/ml, Japanese or Danish BCG (both MOI 10:1) for lymphocyte proliferation (A) and IFN-γ quantification (B). Results for proliferation are expressed as a stimulation index (SI), where SI = mean counts per minute (CPM) in stimulated wells/mean CPM in unstimulated wells (A). Results for IFN-γ are mean + SD following subtraction of unstimulated response (B).

2.4.2 Optimisation of cord blood mononuclear cells isolation

Erythroid precursor cells can be found in high numbers in cord blood and are captured within the same density gradient as lymphocytes as they are nucleated and of similar size. Normoblasts appear to have a smaller sedimentation rate than adult erythrocytes, so centrifugation of the gradient was performed at a higher gravitational force (g) than that used for separation of PBMC. It has also been reported that high purity of CBMC was attained by using two rounds of Ficoll-Hypaque density separation therefore this was compared to a single density gradient, and centrifugation was conducted at a higher g force to that used for isolation of PBMC. In addition, centrifugation was carried out for different periods of time (Ridings *et al.*, 1996; Regidor *et al.*, 1999; Yang and Lin, 2001). Dual staining for both CD45 and Glycophorin A were compared after either one or two rounds of Ficoll-Hypaque density separation. Table 2.4 provides an indication of purity following one or two rounds of Ficoll-Hypaque gradient separation. In this study, normoblast contamination accounted for all contaminating cells, as the sum of $CD45^{+}$ and Glycophorin A^{+} cells was in every case approximately equal to 100% (Table 2.4). It was therefore decided to calculate the purity of CBMC by measuring the percentage of $CD45^{+}$ cells alone.

TABLE 2.4 CBMC dual stained with CD45 and Glycophorin A for determination of erythroblast contamination after Ficoll gradients.
CBMC were dual stained with anti-CD45 and Glycophorin A after either a single or two Ficoll gradients. Erythroid precursor cells stain CD45 negative and Glycophorin A^{+}, whereas CBMC stain $CD45^{+}$ and Glycophorin A negative.

	1st Ficoll		**2nd Ficoll**	
CB #	**$CD45^{+}$**	**Glycophorin A^{+}**	**$CD45^{+}$**	**Glycophorin A^{+}**
1	35%	65%	93%	7%
2	34%	66%	37%	53%
3	88%	8%	96%	3%
4	50%	46%	95%	4%
5	18%	81%	83%	15%

Ridings *et al* found that after a single round of Ficoll-Hypaque density separation, contamination with CD45 negative cells could exceed 50% (Ridings *et al.*, 1996). Our results were in agreement with those of Ridings *et al*, as Figure 2.4 indicates that similar rates of contamination ($CD45^{+}$; 44% ± 21%) were found after a single round

of Ficoll-Hypaque separation. After a second round of Ficoll-Hypaque density centrifugation, purity was >80% as indicated by $CD45^+$ staining (mean; 86% ± 19%) (Figure 2.4).

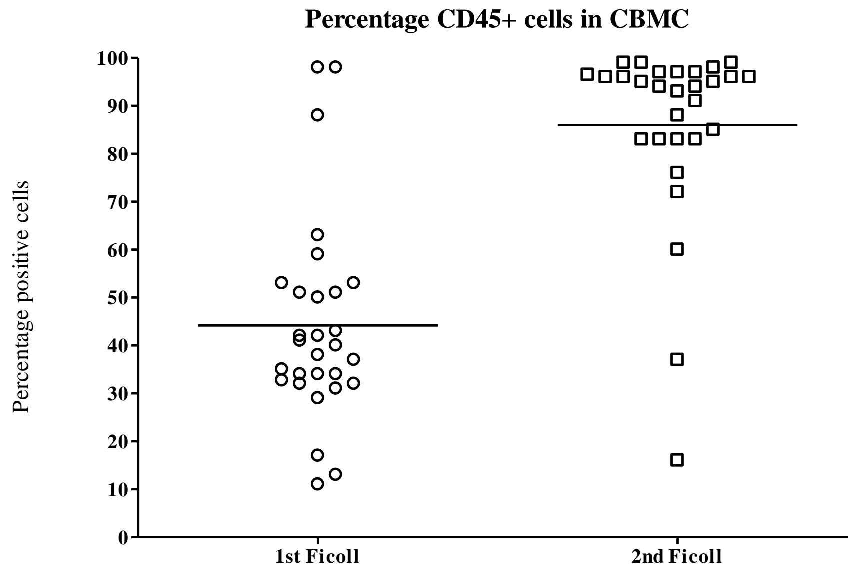


Figure 2.4 CBMC stained with CD45 for determination of erythroblast contamination after Ficoll gradients.
CBMC were stained with anti-CD45 APC after either a single or two Ficoll gradients. Erythroid precursor cells stain CD45 negative, while CBMC stain CD45 positive. Mean is indicated by a horizontal line. n=26 independent cord blood evaluations.

According to previous reports, two rounds of Ficoll-Hypaque density separation does not adversely affect the CBMC functions (Ridings *et al.*, 1996; Yang and Lin, 2001). As the purity of CBMC was much greater after two rounds of Ficoll-Hypaque density centrifugation and also by centrifugation at a higher *g* force than that used for PBMC isolation, this technique was used for CBMC purification as described in section 2.3.3.

2.4.3 Validation of secreted cytokines in cord blood

2.4.3.1 Optimisation of cell concentration for secreted cytokines

CBMC were isolated from cord blood and a portion was further purified for $CD14^+$ monocytes using magnetic bead separation. Initially CBMC were plated at 1 x 10^6/ml (100µl/well) in 96 well U-bottom plate and stimulated with Danish BCG (MOI 5:1). Monocytes were plated at 1 x 10^5/ml (100µl/well) in a 96 U-well microtitre plate and were similarly stimulated. Secreted cytokines produced by purified monocytes were

however very low. When staining for the percentage of monocytes in CBMC by dual staining with anti-CD45 APC and anti-CD14 PE to establish the percentage of monocytes in CBMC, it was found that the mean percentage was 27% ± 9%, as indicated in Figure 2.5.

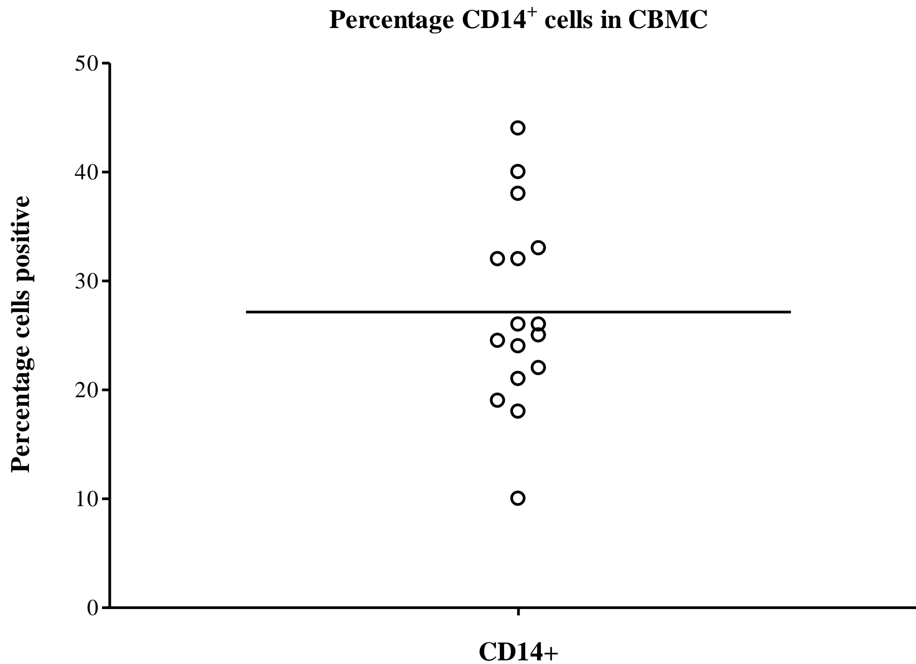


Figure 2.5 Percentage of $CD14^+$ monocytes in CBMC.
CBMC were dual stained with anti-CD45 and anti-CD14 to determine monocytes present in CBMC. Mean is indicated by a horizontal line. n=16 independent cord blood evaluations.

It was decided to plate out CBMC by standardising on the number of monocytes added to the 96-well tissue culture plates, so that cytokine production by monocytes could be directly compared. A comparison was made between secreted cytokines generated when using these different cell dilutions and by standardising on the concentration of monocytes added, enhanced secreted cytokines were detected (Table 2.5). As the mean percentage of monocytes in CBMC was found to be 27%, the concentration of CBMC was therefore adjusted so that 30% of the cells were $CD14^+$ following staining with anti-CD45 and anti-CD14 and flow cytometric evaluation. CBMC were then approximately 1 x 10^6/ml and 100µl were plated per well, which ensured that 3 x 10^5/ml were monocytes. Purified monocytes were also plated at 3 x 10^5/ml (100µl/well), so that these cytokine results from monocytes could be directly compared. There were insufficient cells for determination of $CD14^+$ IL-12 secreted cytokines.

It was preferable to standardise by the addition of 3 x 10^5/ml monocytes for both CBMC and $CD14^+$ cells stimulated for secreted cytokine evaluation, as there was an increase in the level of all cytokines measured. This method for determination of cell concentration was adjusted accordingly and used in all subsequent experiments in Chapter 5 for the evaluation of the cytokine response of neonates to BCG before BCG vaccination.

TABLE 2.5 Optimisation of CBMC and monocytes concentration for secreted cytokine evaluation.
CBMC and monocytes at different cell concentrations were stimulated for secreted cytokine evaluation in SNF. Mean results ± SD are indicated in pg/ml following subtraction of the unstimulated response. n = 3 to 9 for the different cytokines.

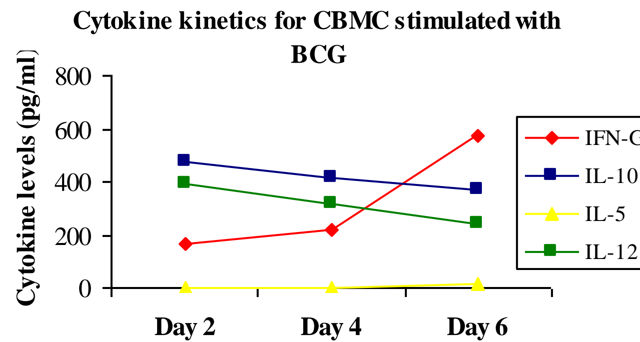
	CBMC secreted cytokines (pg/ml)	
	1 x 10^6 CBMC	**30% monocytes**
IFN-γ	962 ± 1047	5536 ± 5329
IL-10	608 ± 542	905 ± 846
IL-12	357 ± 171	851 ± 872
IL-5	19 ± 21	119 ± 85
	Monocyte secreted cytokines (pg/ml)	
	1 x 10^5 monocytes	**3 x 10^5 monocytes**
IFN-γ	0 ± 0	43 ± 39
IL-10	49 ± 59	255 ± 277

2.4.3.2 Optimisation of kinetics for secreted cytokines

To determine the optimal kinetics for generation of secreted cytokines of neonates to BCG before BCG vaccination, CBMC and $CD14^+$ monocytes at the optimal cell concentration as described above, were stimulated with BCG (MOI of 10:1), or SEB (0.1μg/ml) or LPS (1μg/ml) for 2, 4 and 6 days. The SNF was removed and the level of IFN-γ, IL-10, IL-12 and IL-5 determined by ELISA in pg/ml following subtraction of the unstimulated response. As indicated in Figure 2.6 A and B, the optimal incubation periods for IL-10 and IL-12 was 2 days for stimulation with BCG and LPS, while for IFN-γ optimal production was after 6 days for BCG stimulation. CBMC stimulated with SEB for IFN-γ evaluation produced 8246 ± 2554pg/ml on day 2, 19669 ± 10649pg/ml on day 4 and 29632 ± 18774pg/ml following 6 days of incubation. Although minimal amounts of IL-5 were detected, these peaked after 6

days. These optimal incubation times were subsequently utilised for secreted cytokine evaluation in cord blood (Chapter 5).

A



B

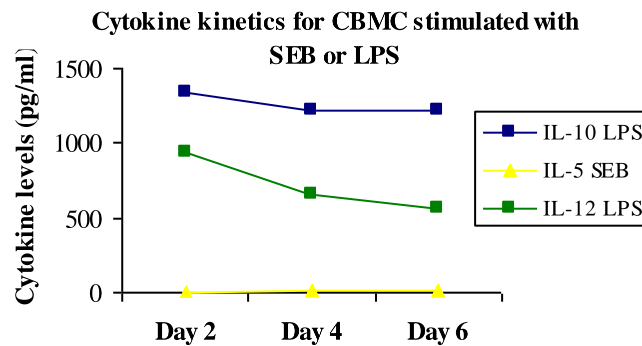


Figure 2.6 Optimisation of kinetics for secreted cytokine evaluation.
CBMC were stimulated with BCG (MOI 10:1) (A) or SEB 0.1µg/ml or LPS 1µg/ml (B) for 2, 4 and 6 days and secreted cytokines measured in the SNF. Mean results are indicated in pg/ml following subtraction of the unstimulated response. n = 3 to 7 for different cytokines.

2.4.3.3 Titration of Danish BCG for stimulation of secreted cytokines

To establish the optimal MOI of Danish BCG, CBMC and $CD14^+$ monocytes were incubated with Danish BCG at a MOI of 10:1, 5:1, 3.3:1 and 1:1. SNF was removed and cytokine levels determined in pg/ml following subtraction of the unstimulated response. The cytokine response for CBMC and for $CD14^+$ monocytes is indicated in Table 2.6.

TABLE 2.6 Titration of BCG for secreted cytokine evaluation.
CBMC and $CD14^+$ monocytes were incubated at MOI of 10:1, 5:1, 3.3:1 and 1:1 of Danish BCG. Mean results ± SD are indicated following subtraction of unstimulated results. n=3 to 9 for different BCG concentrations.

CBMC resuspended at 3 x 10^5 monocytes/ml				
	MOI 10:1	**MOI 5:1**	**MOI 3.3:1**	**MOI 1:1**
IFN-γ	2129 ± 3173	5536 ± 5329	1295 ± 1278	5073 ± 3098
IL-10	808 ± 651	905 ± 846	387 ± 39	372 ± 209
IL-5	35 ± 35	119 ± 85	36 ± 12	156 ± 139
IL-12	1049 ± 925	851 ± 872	779 ± 404	306 ± 161
$CD14^+$ monocytes resuspended at 3 x 10^5/ml				
	MOI 10:1	**MOI 5:1**	**MOI 3.3:1**	**MOI 1:1**
IFN-γ	15 ± 30	43 ± 39	45 ± 79	41 ± 38
IL-10	250 ± 282	255 ± 277	273 ± 305	194 ± 108
IL-5	0.3 ± 0.5	2 ± 2	1 ± 2	11 ± 3
IL-12	164 ± 175	242 ± 329	122 ± 20	115 ± 120

A single concentration of BCG needed to be used to stimulate the cord blood cells for cytokine evaluation, as there were insufficient cells to perform multiple BCG concentrations (Chapter 5). It was decided to use Danish BCG at an MOI of 5:1 for stimulation of secreted cytokines, as this gave maximum levels of IFN-γ and IL-10 and a strong IL-12 response when CBMC were used. In addition, when $CD14^+$ cells were used, an MOI of 5:1 resulted in the highest production of IL-12 and a good response for IL-10. The amount of IL-5 detected was consistently low.

2.4.4 Validation of intracellular cytokine evaluation in cord blood

ICC are regarded as rare events and sampling low numbers of a cell population is likely to increase false positive results, therefore results for ICC evaluation were only accepted when ≥20 000 gated events were obtained for $CD4^+$, $CD8^+$ and $CD14^+$ cells and ≥15 000 for $CD3^-CD56^+$ NK cells (Hanekom *et al.*, 2004). Flow cytometer settings and fluorescence compensation were standardised for each experiment using CaliBRITE beads and the FACSComp program (BD Immunocytometry Systems, San Jose, CA, USA). For every antigenic event, three dot plots were used, the gating strategy of which is shown in Figure 2.7. Although this figure indicates the analysis of $CD8^+$ IFN-γ^+ ICC, in a similar manner $CD4^+$ and $CD14^+$ ICC (IFN-γ and IL-10) and $CD3^-CD56^+$ NK cell IFN-γ^+ ICC was also determined. In the first dot plot in Figure 2.7, lymphocytes were selected in the gate R1 on the basis of their size and

scatter properties. In the second dot plot, $CD4^+$ or $CD8^+$ (FITC or PE) was plotted against side scatter and $CD4^+$ or $CD8^+$ cells were selected in the gate R2. In the third dot plot, either IL-10 or IFN-γ was plotted against forward scatter. The quadrant was set using the unstimulated and the unstimulated isotypic response, and this quadrant was then copied and pasted to each antigenic event to ensure exactly the same cut-off points were used for all assays. To address whether any autofluorescence had occurred, as cells that had been frozen were being analysed, the peridinin chlorophyll protein (PerCP) channel, that did not have any fluorochrome or staining antibodies present (FL3) was utilised. Only FL3 negative events were selected. Results were given as stimulated response minus unstimulated response. A result >0.1% was considered positive, as this is the threshold of detection for this assay. The threshold of detection was determined by 2SD of the unstimulated cytokine response (mean 0.041; SD 0.047; 2SD 0.094

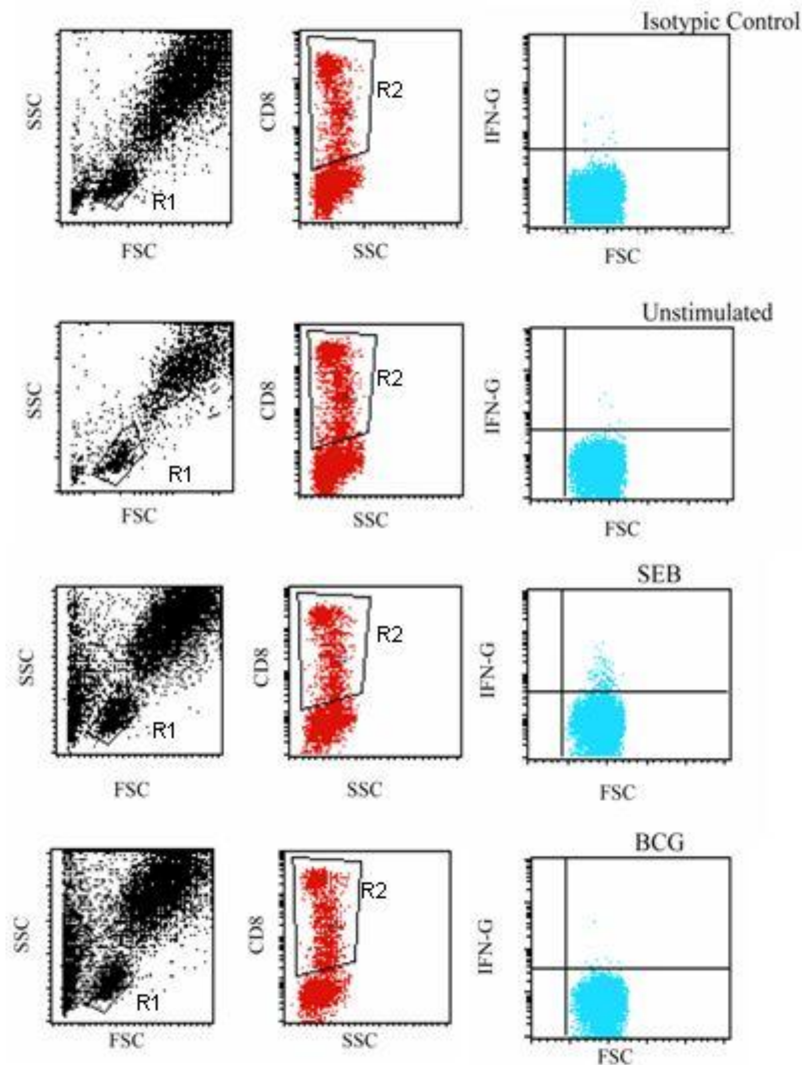


Figure 2.7 Flow cytometric gating strategy for measurement of intracellular cytokines.
In the first dot plot lymphocytes were selected by size and granularity in R1. In the second dot plot, $CD8^+$ cells were selected in R2. The last dot plot was IFN-γ plotted against forward scatter. In the top three horizontal dot plots, an unstimulated tube was stained with IFN-γ isotypic control. In the second three horizontal dot plots, an unstimulated sample was used to measure background IFN-γ. These two tubes were used to set the quadrants in the third dot plot. This quadrant was then copied and pasted in the dot plots for SEB and BCG stimulated samples to ensure exactly the same cut-off points were used for all assays.

2.4.4.1 Comparison of whole cord blood and cord blood mononuclear cell intracellular cytokine assay

A comparison was made between CBMC and whole cord blood to determine which was optimal for evaluation of ICC in cord blood. In all CBMC ICC results, it was found that when cells were stimulated with Danish BCG, the percentage of monocytes during flow cytometric acquisition was noticeably reduced; this reduction in monocytes correlated with the increase in MOI. It was assumed that the BCG had killed the monocytes. Data analysis was performed using FlowJo (Tree Star, Stanford

CT, USA) and this phenomenon is illustrated in Figure 2.8, which shows a representative dot plot of CBMC ICC in cord blood using donor number 25 (CB25). The dot plot in Figure 2.8 A is the unstimulated response and Figure 2.8 B is the PPD (20 μg/ml) response. In both these dot plots, the monocytes are visible and percentages are indicated as 31.11% and 24.2% respectively. Figure 2.8 C is the BCG at an MOI of 0.6 (21.10%). The dot plots D-F are the BCG response at MOI of 1.2 (11.66%), 1.8 (5.74%) and 3.6 (2.64%) respectively. As shown, when the MOI increased, the percentage of monocytes was considerably reduced.

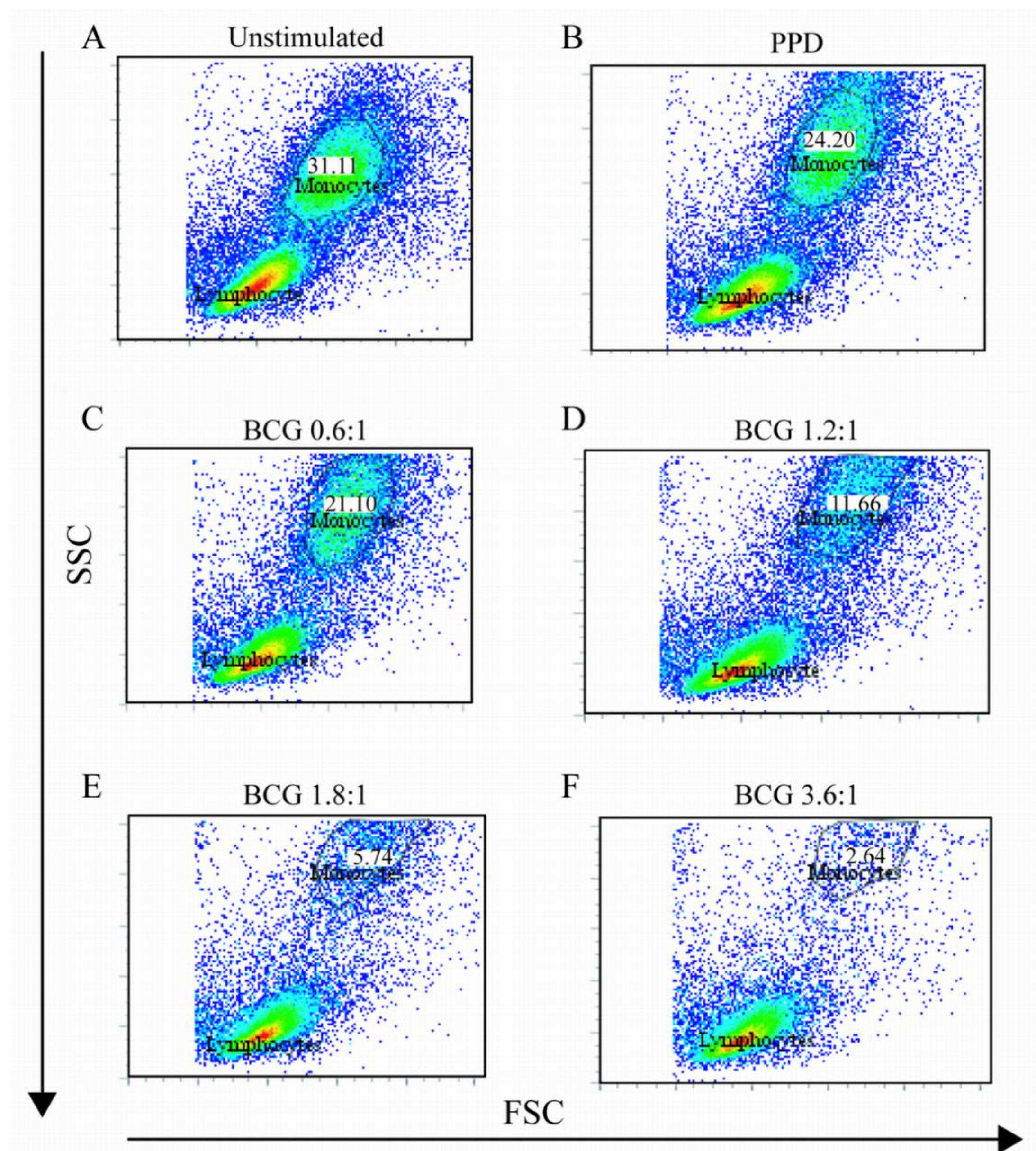


Figure 2.8 Flow cytometric dot plots of stimulated CBMC showing percentage of monocytes. Dot plots from donor number CB25 indicate monocytes in unstimulated (A) and PPD 20 μg/ml (B) and Danish BCG at MOI of 0.6:1 (C), 1.2:1 (D), 1.8:1 (E) and 3.6:1 (F).

This phenomenon of monocyte death did not occur in the WCB ICC of the same donor, as shown in Figure 2.9. Figure 2.9 A is the unstimulated response (27.4% monocytes) and Figure 2.9 B is the PPD (20 μg/ml) response (36.35%) and Figure 2.9 C is Danish BCG at an MOI of 1.8 (28%). In the dot plots, D-F, the BCG stimulated response is indicated at an MOI of 3.6 (31.78%), 5.4 (25.22%) and 10.8 (24.82%) respectively. Even at the highest MOI of 10.8 (Figure 2.9 F), the percentage of monocytes is similar to that of the other dot plots. A possible explanation for this phenomenon is that the organisms were phagocytosed by monocytes and the polymorphonuclear leukocytes that were present in the whole unseparated cord blood were able to take up any excess bacteria present that were not phagocytosed by the monocytes. Due to the fact that these polymorphonuclear leukocytes were absent in CBMC, this resulted in monocyte destruction. As this phenomenon did not occur in whole blood, it was decided to use whole cord blood for ICC evaluation in Chapter 5 rather than CBMC to avoid monocyte death.

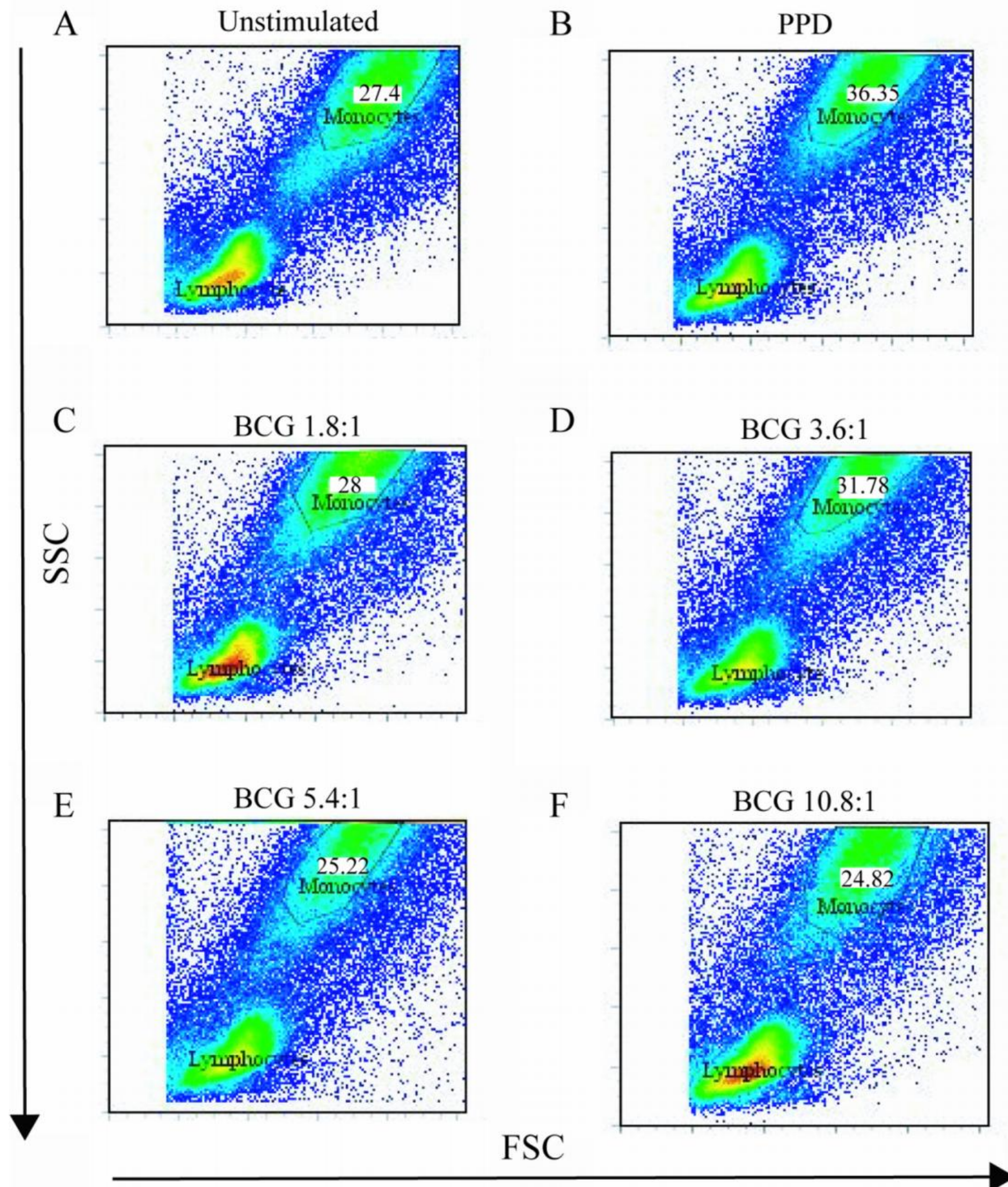


Figure 2.9 Flow cytometric dot plots of stimulated whole cord blood showing percentage of monocytes.
Dot plots from donor number CB25 indicate monocytes in unstimulated (A), PPD 20 μg/ml (B) and Danish BCG at MOI 1.8:1 (C), 3.6:1 (D), 5.4:1 (E) and 10.8:1 (F).

Figure 2.10 shows a comparison between CBMC and WCB ICC. CD4$^+$ IFN-γ$^+$ expression for donors CB25 and CB26 are compared in Figure 2.10 A. In Figure 2.10 B, CD4$^+$ IL-10$^+$ expression are shown for CBMC and WCB. In Figure 2.10 C and D, a comparison between CB26 CBMC and WCB can only be made as sample CB25 CBMC had <20 000 events for ICC CD8$^+$ evaluation. CD8$^+$ IFN-γ$^+$ ICC is indicated

in Figure 2.10 C and CD8$^+$ IL-10$^+$ ICC in Figure 2.10 D. CD4$^+$ IFN-γ^+ production by the SEB positive control was detected in both donors for both samples. SEB stimulated samples resulted in detectable CD4$^+$ IL-10$^+$ and CD8$^+$ IFN-γ^+ in donor 26 in both samples.

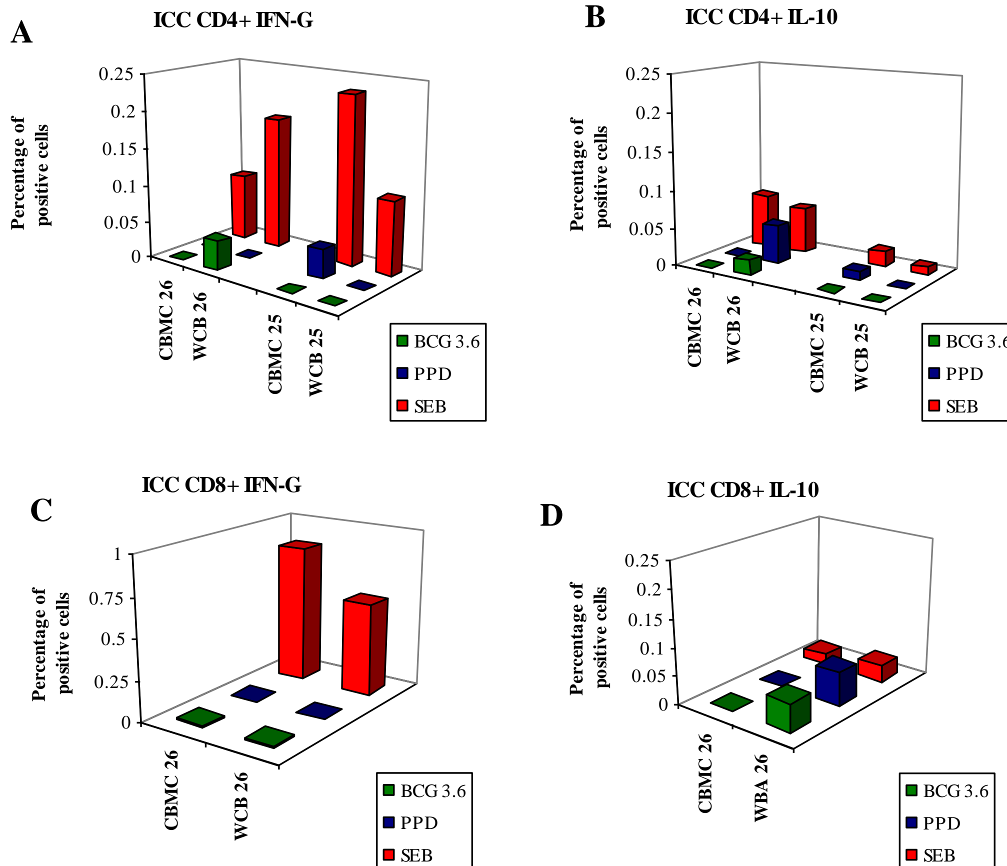


Figure 2.10 A comparison between CBMC intracellular cytokine (ICC) and whole cord blood ICC responses.
Results are indicated following stimulation with BCG (MOI 3.6:1), PPD 20 μg/ml or SEB 10μg/ml for donors CB25 and 26 for percentage CD4$^+$ IFN-γ^+ (A) and CD4$^+$ IL-10$^+$ (B). A comparison in a single donor for CD8$^+$ IFN-γ^+ (C) and CD8$^+$ IL-10$^+$ (D) is shown, due to the fact that donor 25 had insufficient gated events for CD8$^+$ ICC results to be acceptable. Note different scale for C.

As previously discussed, a minimum of 20 000 gated events was used as a cut-off point for any result to be acceptable for analysis. It was however difficult to obtain >20 000 gated events in all CBMC samples processed. Furthermore, due to death of monocytes following stimulation with BCG, analysis of CD14$^+$ ICC was not possible in CBMC ICC.

In ICC analyses, a response of >0.1% was considered positive; as this is the threshold of detection for the assay (2SD of background unstimulated samples). In this study, all BCG and PPD stimulated ICC results in cord blood were extremely low. When a comparison was made between CBMC and WCB ICC in the same cord blood sample, a similar trend was observed (note scales are different in Figure 2.10 C). It was decided to use WCB ICC in all cord blood ICC assays instead of CBMC ICC. The reasons for this are:

1. In all CBMC ICC assays, it was difficult to obtain >20 000 events;
2. Separation of mononuclear cells from cord blood could affect the ratio of different cytokine producing cells compared to ratios present in whole blood. It may therefore, be appropriate to utilise a technique involving whole blood, as it represents a more physiological environment and mimics the natural situation whilst also maintaining the cytokine milieu;
3. The unstimulated response in WCB ICC was usually lower than the CBMC ICC unstimulated response;
4. In this study, a comparison was to be made between the cord blood ICC result and the ICC result in the same infant at 13 weeks of age. In the 13 week old infants, there is a limit on the volume of peripheral blood that can be drawn, so a whole blood ICC assay was used at 13 weeks. For a direct comparison to be made, ICC in whole cord blood would therefore be preferable

2.4.4.2 Addition of costimulants for intracellular IL-10 evaluation

To further validate the ICC assay for determination of cytokine responses of neonates to BCG before and after vaccination with BCG (Chapter 5), it needed to be established whether addition of the costimulants, anti-CD49d and anti-CD28 would enhance IL-10 ICC response in cord blood. Addition of costimulants has been previously shown to augment peripheral blood IFN-γ ICC response (Hanekom *et al.*, 2004). The addition of costimulants did enhance ICC IFN-γ (Hanekom *et al.*, 2004) so the effect of costimulants on ICC IL-10 production was only tested. Figure 2.11 indicates IL-10 ICC results in $CD4^+$ (A) and $CD8^+$ (B) cells following stimulation with Danish BCG, PPD and SEB. Included are the percentages of $CD4^+$ or $CD8^+$ cells expressing IL-10 ICC with and without the addition of costimulants. Although

all results are below the threshold of detection for the assay of 0.1%, it can be seen in Figure 2.11 A and B that the addition of costimulants had no effect on the production of ICC IL-10 so they were excluded from the IL-10 experiments.

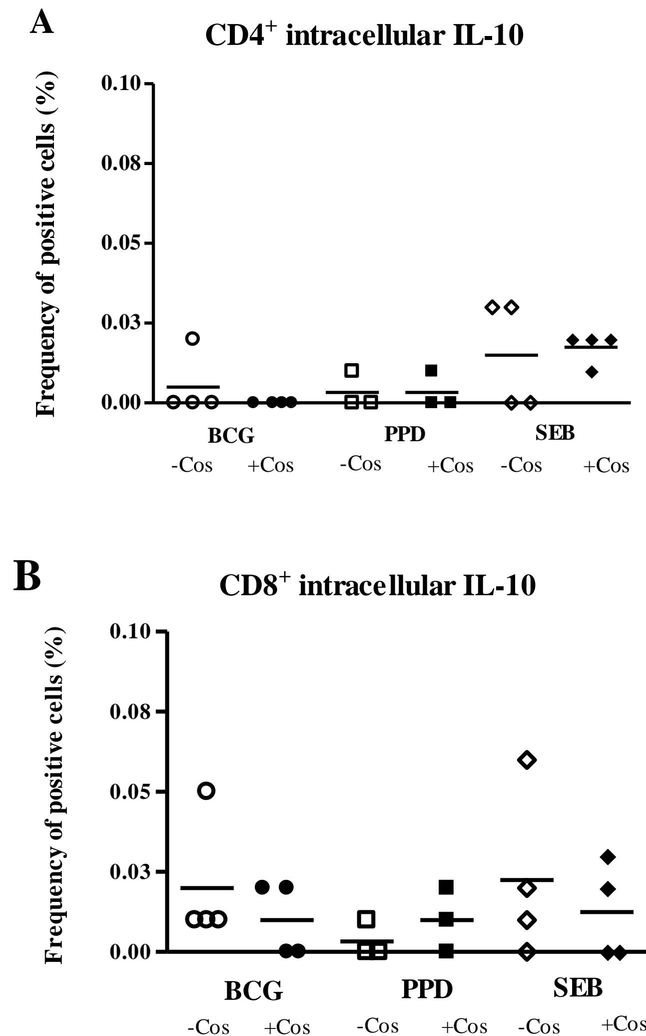


Figure 2.11 Costimulants do not enhance whole cord blood IL-10 ICC production. Whole cord blood was incubated with and without costimulants (Cos) anti-CD49d 1µg/ml and anti-CD28 1µg/ml and stimulated with Danish BCG (MOI 3.6:1), PPD 20µg/ml or SEB 10µg/ml. The percentage of $CD4^+$ (A) and $CD8^+$ (B) cells expressing IL-10 ICC was assessed. Unstimulated results have been subtracted from stimulated results. Mean is indicated by a short horizontal line. n=3-4 for different stimulants.

2.4.4.3 Effect of dual staining with anti-CD3 and anti-CD8 or anti-CD8 alone for detection of intracellular cytokines

For further validation of the ICC assay, a comparison was performed when staining for ICC, using dual staining with both anti-CD3 and anti-CD8 monoclonal antibodies or staining with anti-CD8 alone for the assessment of intracellular cytokines within

$CD8^+$ T cells in cord blood samples. As some NK cells are $CD8^+$ it is possible that staining with anti-CD8 alone could cause false positive results for ICC IFN-γ in cord blood. It must be emphasised that in ICC detection of stimulated peripheral blood samples obtained from 13 week old infants, dual staining with both anti-CD3 and anti-CD8 was always performed for assessment of $CD8^+$ ICC. Using anti-CD8 alone however, would be preferable in cord blood, because it would enable use of anti-CD4 and anti-CD8 in a single tube, thereby facilitating the assessment of ICC within $CD4^-$ $CD8^-$ double negative cells.

Table 2.7 indicates the mean percentage of positive cells ± SD obtained when anti-CD8 was used alone, or together with anti-CD3 for analysis of intracellular IFN-γ and IL-10 following stimulation with Danish BCG or SEB. All IFN-γ results were positive in the case of SEB (positive control), irrespective of whether dual staining or anti-CD8 alone was used and both mean and SD were very similar (Table 2.7). In contrast, all IFN-γ results were negative for the specific antigen BCG in every sample except for a single donor of 6 tested (CB28), which gave a low positive result (0.14%) when anti-CD8 was used alone, but negative when dual staining with anti-CD3 and anti-CD8 (0.07%) was used. All results for intracellular IL-10 were negative, but a similar trend was observed whether dual staining with both anti-CD3 and anti-CD8 or anti-CD8 alone was used (Table 2.7). Consequently, anti-CD8 was used alone to stain for ICC in cord blood (Chapter 5).

TABLE 2.7 WCB ICC results staining for $CD8^+$ alone or dual $CD3^+CD8^+$.
Whole cord blood (WCB) was stimulated with Danish BCG (MOI 3.6:1) or SEB 10μg/ml. Percentage of cells positive for intracellular cytokine (ICC) IFN-γ and ICC IL-10 was assessed within $CD8^+$ cells by staining with anti-CD8 alone or dual staining with anti-CD3 and anti-CD8. Unstimulated results have been subtracted from stimulated results. Mean ± SD is indicated. n=6 for ICC IFN-γ and n=4 for ICC IL-10.

	Intracellular IFN-γ	
	$CD3^+CD8^+$	**$CD8^+$**
BCG	0.02 ± 0.03	0.03 ± 0.05
SEB	1.3 ± 0.97	1.3 ± 0.86
	Intracellular IL-10	
	$CD3^+CD8^+$	**$CD8^+$**
BCG	0.003 ± 0.005	0.01 ± 0.005
SEB	0 ± 0	0.02 ± 0.03

2.4 .5 Yield of classically and alternatively activated macrophages

To enable the comparison of neonatal macrophage function to that of adults, classically and alternatively activated macrophages were cultured from monocytes (Chapter 3). A known number of viable monocytes, as determined by Trypan blue exclusion dye, were plated at the commencement of culture for macrophages. A cell count of viable macrophages was performed after 6 days of culture, and the number of each macrophage type noted. The yield of macrophages was determined by the number of macrophages recovered after 6 days culture divided by the number of $CD14^+$ monocytes plated *ex vivo* as a percentage. This was undertaken to determine whether cord blood monocytes had a similar capacity to differentiate into these distinct macrophage types as adult blood derived monocytes. The yields for monocyte-derived macrophages (Figure 2.12) from adult peripheral blood was slightly lower for CA Mø (CA Mø; 48.6 ± 23.3% and AA Mø; 37 ± 16.1%) to that of cord blood derived macrophages (CA Mø; 79 ± 25.9% and AA Mø; 49.4 ± 22.6%), although this difference was not significant. It could be concluded therefore that neonatal mononcytes had a similar capacity to differentiate into either classically or alternatively activated macrophages as adult monocytes.

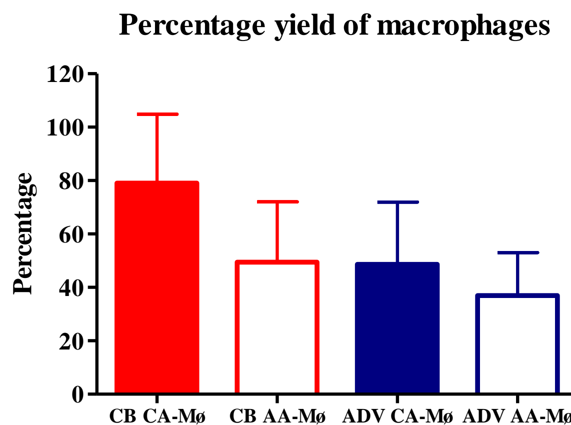


Figure 2.12 The yield of monocyte-derived macrophages from adult and cord blood.
The percentage yield was determined by the number of classically activated macrophages (CA Mø) or alternatively activated macrophages (AA Mø) obtained after 6 days culture, divided by the number of monocytes plated *ex vivo*. Mean (+ SD) is indicated. n=10 cord blood and n=9 adult blood independent evaluations.

CHAPTER 3

MACROPHAGE FUNCTION IN NEONATES

3.1 INTRODUCTION

Two types of phagocytic cells, mononuclear phagocytes including monocytes and macrophages and polymorphonuclear leukocytic phagocytes, form an important part of the innate immune system (Savina and Amigorena, 2007). Mononuclear phagocytes are a heterogeneous population of cells, comprising about 10% of mononuclear leukocytes in the peripheral blood. These cells have the capacity to differentiate into efficient phagocytes or macrophages, as they traffic from the bone marrow into the circulation and extravasate from the circulation into the tissues (Randolph *et al.*, 2008). Blood monocytes and tissue macrophages form one of the first lines of defence against microbial pathogens (Bhatt and Salgame, 2007; Savina and Amigorena, 2007). The macrophages are found not only as resident cells of normal peripheral tissue, but also accumulate in an activated state at the sites of pathogen entry. Macrophages engulf pathogens via phagocytosis into intracellular organelles known as phagosomes; those fuse with lysosomes and mature into phagolysosomes. Phagolysosomes have the capacity to kill pathogens efficiently via the low pH combined with potent lysosomal proteases and subsequent proteolysis (Kaufmann, 2001; Hope *et al.*, 2004; Savina and Amigorena, 2007; Pieters, 2008). Although the microbicidal activity of activated macrophages is generally efficient, some pathogens such as mycobacteria are able to survive and replicate within the macrophage phagosome. These pathogens can also enter into a non-replicating state and remain viable indefinitely (Cosma *et al.*, 2003; Bhatt and Salgame, 2007; Sundaramurthy and Pieters, 2007; Jordao *et al.*, 2008; Pieters, 2008). While macrophages are able to process antigens and present them to memory T cells via their surface MHC molecules, they are not as efficient as DC. Moreover, macrophages, unlike DC are not able to stimulate a primary T cell response (Banchereau and Steinman, 1998; Guermonprez *et al.*, 2002; Gogolak *et al.*, 2003; Savina and Amigorena, 2007; Sinha *et al.*, 2007).

Invading pathogens are recognised by host phagocytes via a range of cell surface receptors that bind to PAMP molecules found on the bacteria. Both macrophages and DC utilise cell surface PRR such as TLR to recognise and bind pathogens. The most well characterised PAMP is LPS which is found in the outer wall of gram negative bacteria. The receptor for LPS is TLR4. Mycobacterial components are recognised via TLR2 in association with TLR1 and/or TLR6, or via TLR4 (Kaisho and Akira, 2001; Zarember and Godowski, 2002; Xu *et al.*, 2004; Ryffel *et al.*, 2005; Akira *et al.*, 2006; Sundaramurthy and Pieters, 2007). PAMP molecules of pathogenic mycobacteria include Cytidine-phosphate-guanosine DNA (CpG DNA), 19-kDa lipoprotein, lipoarabidomannan (LAM) and mannosylated phosphotidylinositol (Ryffel *et al.*, 2005). CpG DNA signalling is mediated via TLR9 (Kaisho and Akira, 2001). Macrophage engagement of pathogens via TLR results in an increased phagocytosis of bacteria. Pathogen binding and internalisation causes the release of the cytokine TNF-α by the macrophage which recruits and stimulates NK, NKT and γδ T cells to produce IFN-γ and ultimately causes activation of the macrophages (Aderem and Ulevitch, 2000; Blander and Medzhitov, 2004; Doyle *et al.*, 2004; Krutzik and Modlin, 2004; Verreck *et al.*, 2004). TNF-α also synergises with IFN-γ in the activation of macrophages, resulting in enhanced bactericidal activity (Flynn and Chan, 2001; Raja, 2004; Russell, 2007; Sundaramurthy and Pieters, 2007; Pieters, 2008).

Activated macrophages are also a heterogeneous group of cells. The process of activation can result in either classically or alternatively activated macrophages. Classical activation of macrophages occurs following stimulation with IFN-γ, whereas alternative activation of macrophages results from the presence of IL-4 and IL-13, two cytokines usually produced in Th2-type responses (Gordon, 2003; Mosser, 2003; Mosser and Edwards, 2008). The role of CA Mø that have migrated to sites of inflammation and encountered pathogens is to control the growth and/or kill the pathogens. In contrast, the role of AA Mø, in the lung in particular, appears to be that of a regulatory macrophage. These AA Mø are thought to provide negative regulatory signals which would protect the host from overstimulation of an inflammatory

response (Mosser, 2003). CA Mø are therefore pro-inflammatory, whereas AA Mø are considered anti-inflammatory. One of the most important cytokines produced by CA Mø is IL-12, which contrasts with the signature cytokine of IL-10 for AA Mø (Gordon, 2003; Mosser, 2003; Verreck *et al.*, 2004; Verreck *et al.*, 2006). AA Mø have also been implicated in allergic conditions such as asthma, to tolerance of the histo-incompatible foetus, repair (including the central nervous system), wound healing, atherosclerosis and tumour immunity (Gordon, 2003; Mosser and Edwards, 2008).

Neonates are more susceptible to TB disease following infection than adults and once infected with TB, the severity of the disease is usually worse in infants (Smith *et al.*, 1997). The fact that human neonates are highly susceptible to infections, especially with intracellular pathogens, suggests that they may have an impaired immune system (Levy, 2005; Marodi, 2006 A; Kollmann *et al.*, 2009). Prior to exposure to the pathogens infants have a naïve adaptive immune system and therefore innate immunity is critically important for protection at this age (Marodi, 2006 B). A Th1 cytokine environment which includes IFN-γ and TNF-α is required for control of intracellular bacteria (Mosser, 2003). These soluble mediators play an important role in protective immunity including the activation of macrophages, a crucial step in the control of intracellular growth and survival of pathogens (Mosser, 2003). However, it is well documented that early in life neonates have a placenta-derived Th2 cytokine bias which serves as protection against placental and foetal damage (Roth *et al.*, 1996; Piccinni *et al.*, 2000; Szekeres-Bartho, 2002). This is because an excess of IFN-γ production at the maternal-foetal interface is a major cause of foetal loss (Wegmann *et al.*, 1993). Th2 polarisation results in a deficiency of the Th1 cytokines not only in utero but also after birth. This cytokine imbalance is considered central to the diminished neonatal immune response (Marodi, 2006 B). Studies have shown that stimulated neonatal cells had a deficiency in IFN-γ and IL-12 production (Wilson *et al.*, 1986; Lee *et al.*, 1996). In addition, neonatal macrophages stimulated with LPS have been reported to be defective in the production of pro-inflammatory cytokines including TNF-α (Joyner *et al.*, 2000; Chelvarajan *et al.*, 2004; Levy, 2005). Other investigators have found that neonatal macrophages were unable to efficiently

phagocytose pathogens (Marodi *et al.*, 1994; Johnston, 1998; Marodi *et al.*, 2000) and that neonatal macrophages were hyporesponsive to IFN-γ when compared to the response of adult peripheral blood derived macrophages (Marodi *et al.*, 1994; Marodi *et al.*, 2001; Marodi, 2006 B). Taken together, the low cytokine response to LPS, in particular that of TNF-α, the reduced macrophage activation by IFN-γ, together with the Th2 cytokine environment in neonates, could account for the increased susceptibility to infectious diseases seen in young infants.

In this chapter which is devoted to macrophage function, I attempt to test the hypothesis that macrophage activation would contribute to control of Mtb engendered by BCG vaccination is **quantitatively different** (i.e. difference in levels of macrophage activation marker expression prior to and post vaccination), but **not qualitatively** different (i.e. similar surface markers of macrophage function are expressed before and after vaccination with BCG) from the pre-vaccination macrophage **protective response** of neonates against this pathogen. The following properties of neonatal macrophages will be studied:

1. *In vitro* maturation of macrophages
2. Phagocytic properties of macrophages in tissue culture
3. Production of cytokines IL-10 and TNF-α by cultured macrophages
4. Phagocytic control of growth of the intracellular mycobacteria

As macrophages are a heterogeneous group of cells, both classically and alternatively activated macrophages will be cultured from monocytes and the function of both cell types determined. This heterogeneity of responses could be important in host immunity, as well as determining the outcome of intracellular bacterial infections. The function of macrophages listed above will be compared in cord blood and peripheral blood obtained from BCG vaccinated adults. While it would have been preferable to compare neonatal cord blood macrophages to those of peripheral blood macrophages obtained from BCG vaccinated infants, this is not ethically possible, as these studies require relatively high volumes of blood and there is a limit to the amount of blood that can be collected from these very young infants.

3.2 MATERIALS AND METHODS

3.2.1 Study Participants

Healthy, BCG vaccinated adult volunteers (n=10) including 4 male and 6 female, with a mean age (± SD) of 34.9 ± 9.9 years, were recruited for evaluation of macrophage function from peripheral blood. All infants in this study received Danish intradermal vaccination shortly after birth. Umbilical cord blood was collected from pregnant females undergoing elective caesarean section as described in Chapter 2. The gestation period of donors ranged between 38 - 41 weeks. Thirteen cord blood samples were collected from neonates, eight of which were male, for determination of macrophage function. The mean age of the mothers' was 31 ± 4.3 years and the mean gestation time in weeks was 38.3 ± 0.5. The weights of the neonates and of the placentas were 3301 ± 216g and 658 ± 99g respectively. The average placental weight is slightly higher than the described range of 400 – 600g (Perrin and Sander, 1984). Apgar scores are an assessment of newborn health, with 10 being the highest score. All infants had immediate Apgar scores ≥8 and 5-minute Apgar scores of 9 or 10. These values are regarded as healthy in the newborn.

3.2.2 Culture of macrophages

Macrophages were cultured from purified monocytes as described in Chapter 2 for culture of CA Mø (cultured with GM-CSF), and AA Mø (cultured with M-CSF). These macrophages were then used after 6 days in the presence of these cytokines for the studies described below.

3.2.3 Morphology of macrophages

An Olympus CK40 inverted light microscope at a magnification of x200 was used to compare the morphology of the macrophages generated. Cultured macrophages were photographed using the Zeiss AxioCam MRm camera attached to a Zeiss Axiovert 40CFL microscope at x200 magnification.

3.2.4 Phenotypic analysis of macrophages

PE-conjugated antibodies against CD14, CD80, CD86 and HLA-DR were used to determine basal surface expression of freshly isolated monocytes and compared to

macrophages after 6 days of culture, and then following infection with BCG. By measuring the surface expression of these molecules in cultured macrophages, I was able to compare the *in vitro* maturation of cord blood and adult blood-derived macrophages. HLA-DR, an important molecule in antigen presentation, was studied to determine whether neonatal APC function was deficient compared to that of adults. CD80 and CD86 are T cell costimulatory markers and are important in promoting a Th1 response. It was therefore important to determine the levels of expression of CD80 and CD86 on macrophages to determine the costimulatory properties in neonates compared to adults. The markers for HLA-DR, CD80 and CD86 expression are continuous, and therefore MFI as a means of comparison was used.

3.2.5 Measurement of BCG phagocytosis

To determine phagocytic properties of macrophages, CA Mø and AA Mø cells from adult and cord blood were infected with BCG-gfp, washed three times and the percentage of cells infected was determined by flow cytometric analysis, as described in Chapter 2. Uninfected cells served as a negative control.

3.2.6 TNF-α and IL-10 production by BCG infected or LPS stimulated macrophages

Production of cytokines from infected or stimulated macrophages was ascertained. The TNF-α and IL-10 macrophage cytokine response to BCG-gfp infection and to stimulation with LPS, as a positive control, was determined. CA Mø and AA Mø cells from adult and cord blood were infected with BCG (MOI of 1:1) or stimulated with LPS (10ng/ml) for 24 hours. The SNF was removed and stored at -80°C for determination of TNF-α and IL-10 levels by ELISA. The background response was subtracted from the stimulated response for IL-10 and results reported in pg/ml. The background unstimulated TNF-α results have not been subtracted from stimulated results as a single sample from CA Mø of adult origin had no unstimulated sample.

3.2.7 Enumeration of colony forming units from BCG and H37Rv infected macrophages

The ability of macrophages to control infection with mycobacteria was ascertained. Bactericidal activity of macrophages was assessed using the attenuated vaccine strain

Danish BCG, and compared to H37Rv, the standard virulent laboratory strain of Mtb. Macrophages were infected with Danish BCG or H37Rv at an MOI of 1:1. On day 1, day 4 and day 7, cells were lysed and the lysates plated onto Middlebrooks agar for determination of CFU/ml, as described in Chapter 2.

3.2.8 Statistical Analysis

Results are reported as mean ± SD. Normality assumption of data was tested using Shapiro-Wilks test. Owing to the fact that data were found to be non-normally distributed, non-parametric tests were used. To compare data from 2 groups, the Mann-Whitney test was used for unpaired data and the Wilcoxon test was used for paired data. To compare data from more than two groups, the Kruskal-Wallis ANOVA test was used for unpaired data and the Friedman ANOVA test was used for paired data. Data were analyzed by using Statistica (version 9, StatSoft, Inc., Tulsa, OK, USA).

3.2.9 Graphical Software

Graphs were generated using GraphPad PRISM (version 4.03, GraphPad Software Inc, San Diego, CA) and Microsoft Office Excel 2003 (Microsoft Corporation).

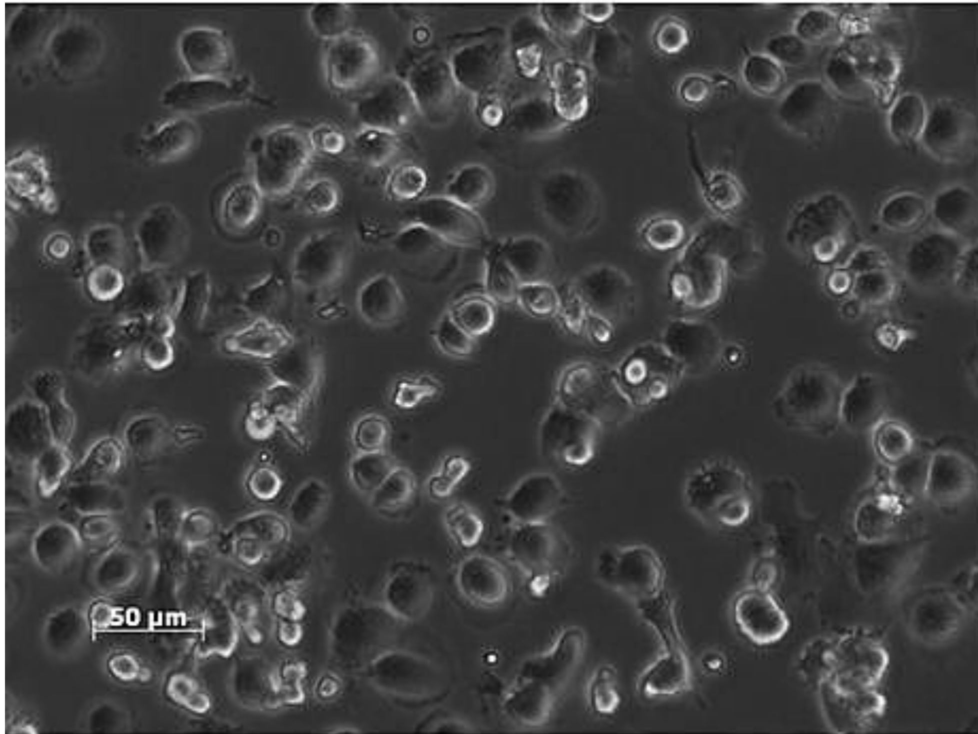
3.3 RESULTS

3.3.1 Morphology of macrophages

As mentioned in the methods section of Chapter 2, it is possible to culture and selectively differentiate PBMC and CBMC monocyte-derived polarised CA Mø or AA Mø *in vitro*. CA Mø, the effectors of Th-1 immunity, are obtained by culturing monocytes in the presence of GM-CSF. In contrast, AA Mø, which reputedly subvert type 1 immunity, are derived by the addition of M-CSF to cultured monocytes (Verreck *et al.*, 2004; Verreck *et al.*, 2006). To determine whether both cord blood and adult blood monocyte-derived polarised macrophages were similar, a microscopic comparison of macrophage morphology was undertaken. After 6 days of culture, in the presence of GM-CSF, CA Mø, from cord blood displayed classical adherent cells with “fried egg morphology” similar to that of CA Mø of adult blood monocyte

derived macrophages (Figure 3.1 A). In contrast, the majority of AA Mø (cultured in the presence of M-CSF) derived from both adult and cord blood displayed distinctly different morphology. These alternatively activated macrophages resembled adherent cells with stretched and spindle-like morphology (Figure 3.1 B).

A



B

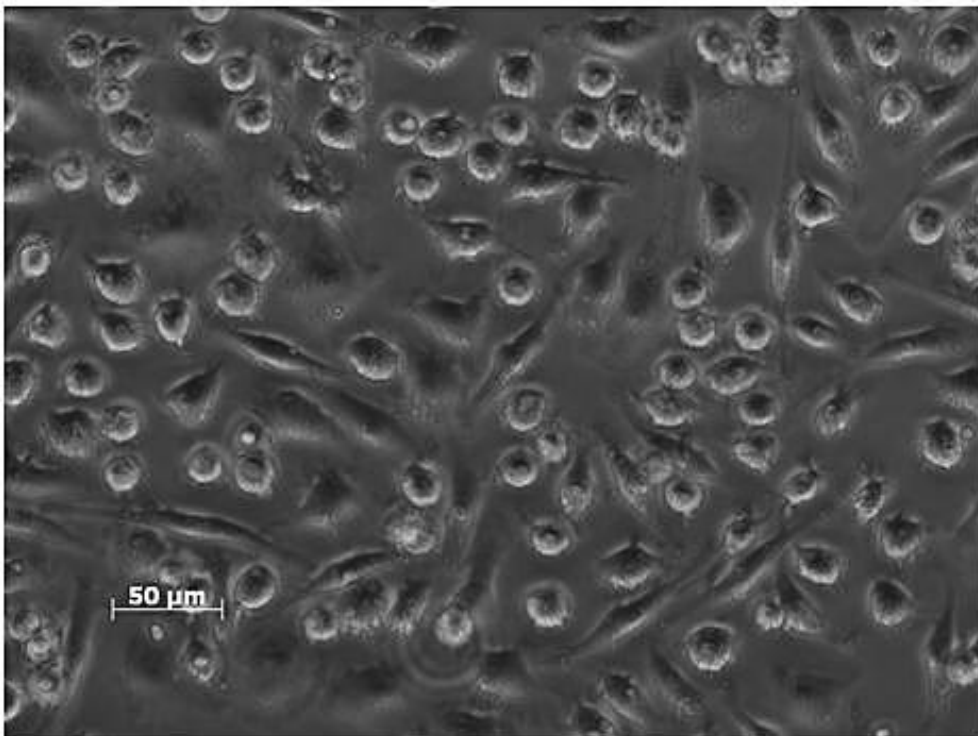


Figure 3.1 Typical morphology of cord blood derived classically activated macrophages (CA Mø) (A) and alternatively activated macrophages (AA Mø) (B) after 6 days in culture.
CA Mø display characteristic "fried egg morphology". The cells are essentially round and flattened (A). AA Mø display an elongated adherent spindle-like morphology (B). Magnification x200; phase contrast microscopy. The scale of 50µm is included in each photograph.

3.3.2 Monocyte and macrophage phenotypic evaluation

A comparison of basal surface expression of CD80, CD86, HLA-DR and CD14 molecules on cord blood monocytes to the level of expression of these markers on the macrophages matured *in vitro* (BCG infected and uninfected) was made. The phenotypic characterisation of cord blood monocyte derived macrophages was also compared to adult blood derived macrophages to determine whether the maturation of macrophages was similar. A histogram overlay indicating the change of MFI surface expression of CD14, CD80, CD86 and HLA-DR from monocytes to CA Mø in cord blood (Figure 3.2 A) and adult blood CA Mø (Figure 3.2 B) was generated. This overlay also indicates the difference in expression following infection with BCG. A similar histogram overlay is also shown for variation in surface expression from monocytes to AA Mø from cord blood (Figure 3.3 A) and adult blood (Figure 3.3 B).

Cord Blood derived
Classically activated macrophages

Adult Blood derived
Classically activated macrophages

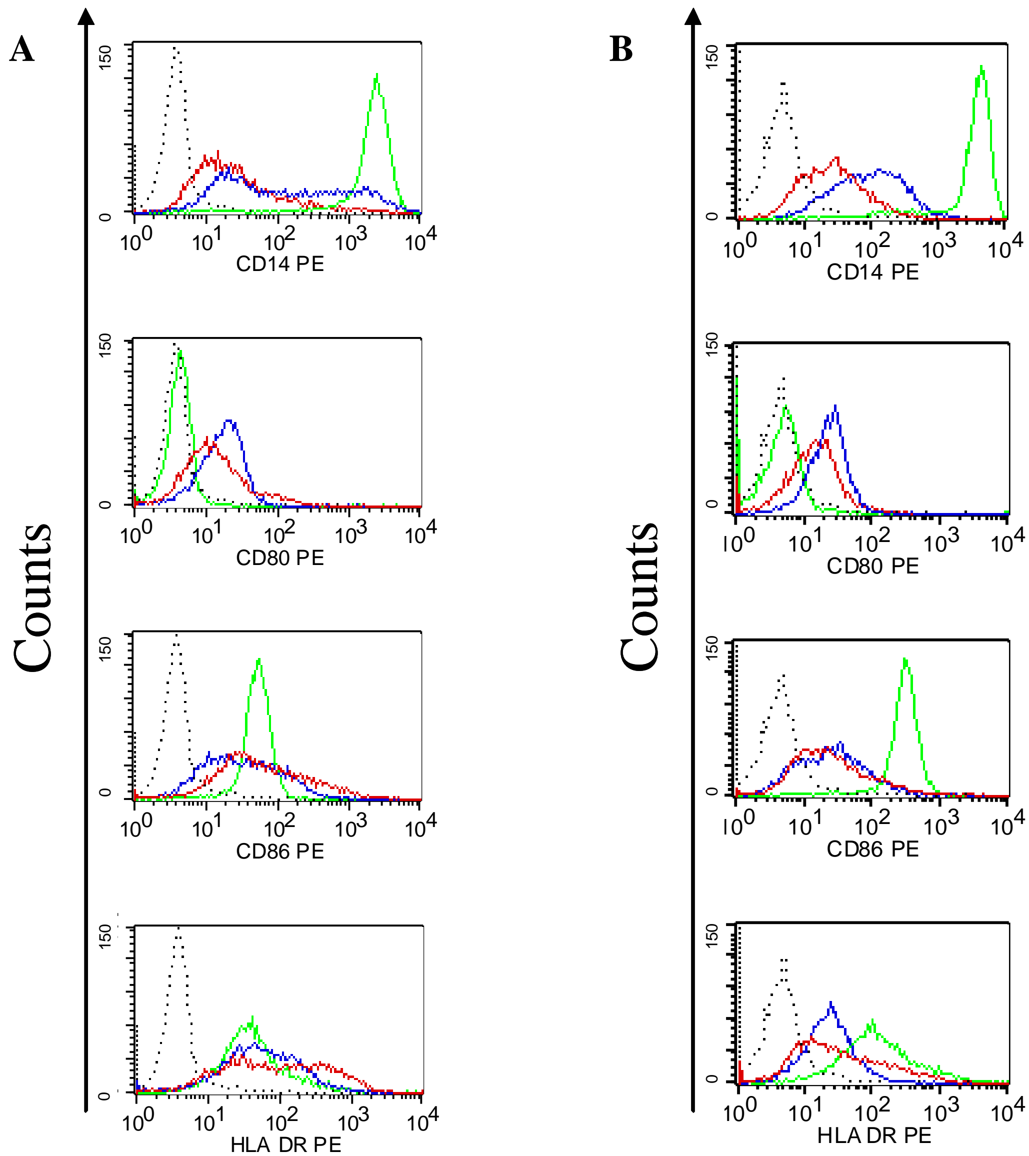


Figure 3.2 A histogram overlay of mean fluorescent intensity (MFI) staining of cell surface markers in cord blood and adult blood CA Mø.
The MFI of CD14, CD80, CD86 and HLA-DR of monocytes (green line), CA Mø (blue line), following 6 days culture and CA Mø after infection with BCG-gfp (red line) is indicated. The black dotted line is the negative control. The histogram overlays are representative of those obtained for cord blood (A) and adult blood cells (B) respectively.

Cord Blood derived
Alternatively activated macrophages

Adult Blood derived
Alternatively activated macrophages

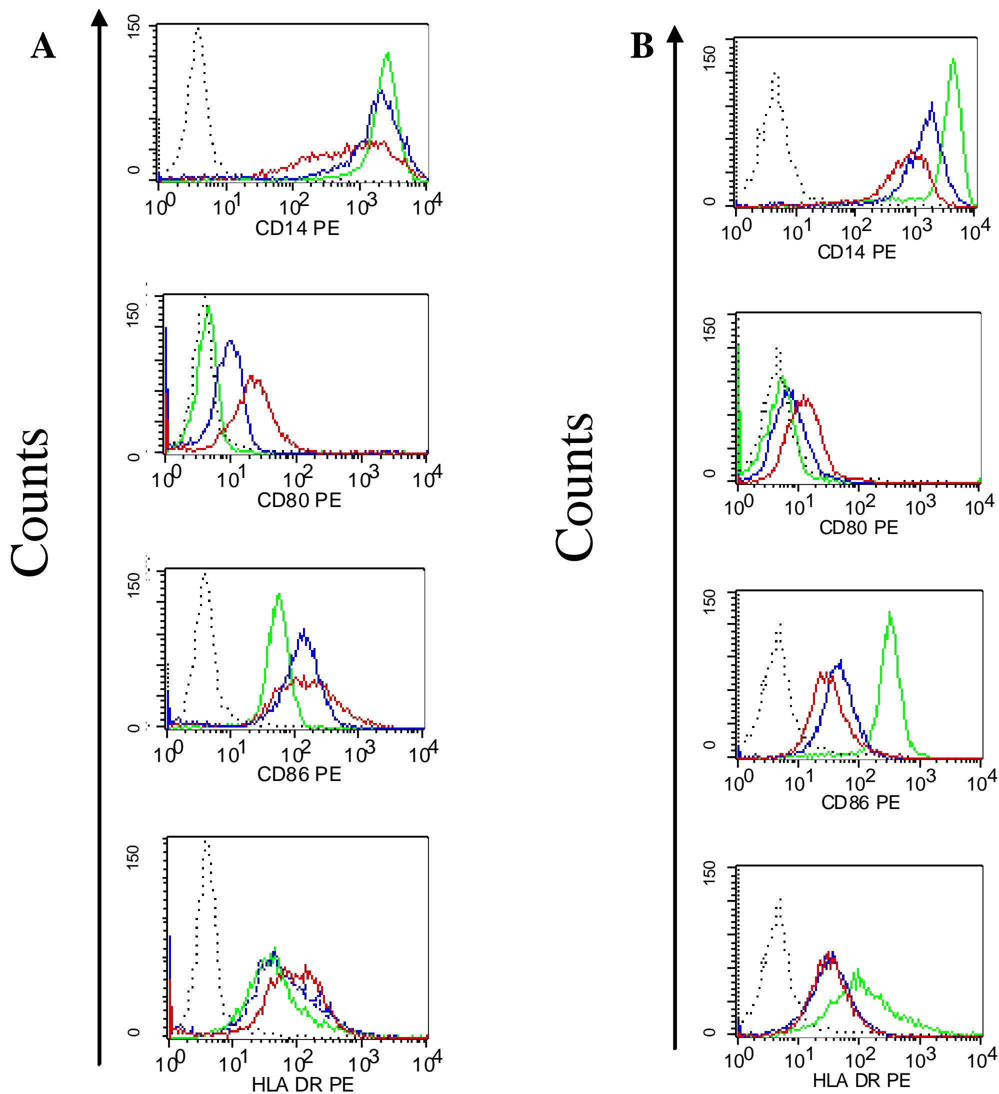


Figure 3.3 A histogram overlay of MFI staining of cell surface markers in cord blood and adult blood AA Mø.
The MFI of CD14, CD80, CD86 and HLA-DR of monocytes (green line), AA Mø (blue line), following 6 days culture and AA Mø after infection with BCG-gfp (red line) is indicated. The black dotted line is the negative control. The histogram overlays are representative of those obtained for cord blood (A) and adult blood cells (B) respectively.

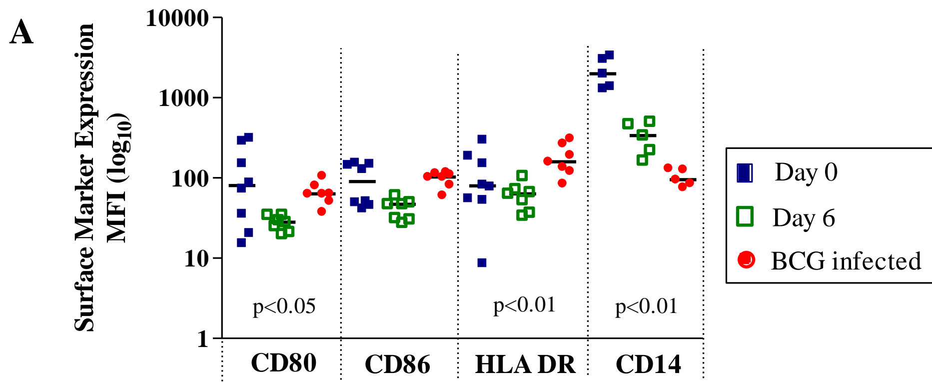
Basal expression of CD80 on monocytes was significantly higher in cord blood than in adult peripheral blood (Figure 3.4 A and B) ($p<0.05$; using Mann-Whitney test). In contrast, CD86 ($p<0.05$) and HLA-DR ($p<0.01$) expression in cord blood monocytes was significantly lower than that of adult monocytes (Figure 3.4 A and B). CD14

surface expression was similar for both adult and cord blood monocytes (Figure 3.4 A and B).

Surface phenotypic characterisation was carried out on macrophages that had differentiated from monocytes following 6 days in culture to assess activation. Surface expression for CD80, CD86 and HLA-DR in adult and cord blood CA and AA Mø was similar. The Kruskal-Wallis ANOVA test was used to compare the groups for surface phenotypic expression between cord blood and adult blood derived macrophages and no significant difference was obtained (Figure 3.4 and 3.5 A and B). However, the difference between CD14 expression on adult CA Mø and AA Mø did attain statistical significance ($p<0.05$). During differentiation from monocytes into macrophages, the surface expression of most markers on cord blood CA Mø changed significantly. The Friedman ANOVA test was used to compare the groups for changes in cord blood macrophage expression and while the change in surface expression of CA Mø CD86 ($p=0.066$) did not attain significance, CA Mø CD80 ($p<0.05$), CA Mø HLA DR ($p<0.01$) and CA Mø CD14 ($p<0.01$) and AA Mø ($p<0.05$) were significantly different. The surface expression of CD14 following differentiation into macrophages, was reduced in both cord blood and adult blood macrophages, but this reduction in expression of CD14 was greater for CA Mø (6.6- and 15.9-fold) than AA Mø (1.5- and 1.6-fold) from both cord and adult blood respectively (Figure 3.4 and 3.5 A and B).

Following infection of macrophages with BCG-gfp for 24 hours the expression for CD80, CD86 and HLA-DR was similarly retained in adult and cord CA Mø and AA Mø (Figure 3.4 and 3.5 A and B). Surface expression of CD14 in infected cord blood CA Mø was reduced, whereas in AA Mø it was only partially reduced (MFI; 103 ± 25; 714 ± 107) from cord blood (Figure 3.4 and 3.5 A and B). This is similar to what was found in BCG-gfp infected adult derived CA Mø and AA Mø (49 ± 14; 631 ± 409)

Phenotypic characterisation of cord blood CA Macrophages



Phenotypic characterisation of adult blood CA Macrophages

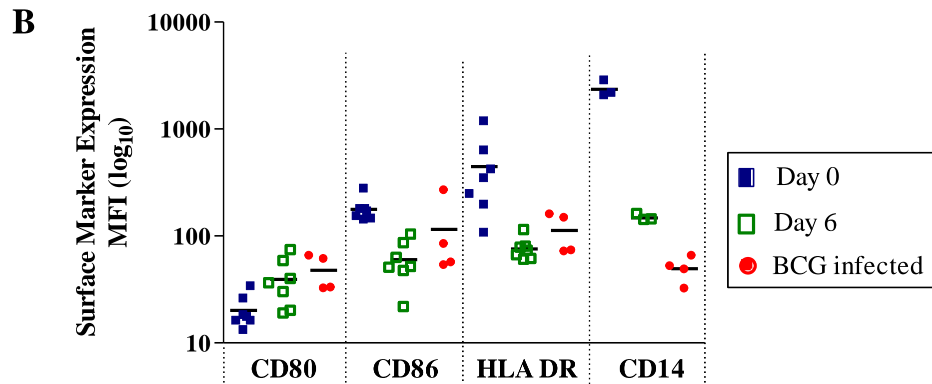


Figure 3.4 Surface phenotypic characterisation of monocytes (D0) and CA Mø after 6 days culture and following infection with BCG.
CA Mø were stained for surface expression of CD80, CD86, HLA-DR and CD14. MFI (log10) is indicated for cord blood (A) and adult (B). Significant changes in surface expression of cord blood CA Mø, using the Friedman ANOVA test are indicated. Mean MFI log10 for the individual data points shown is indicated by a short line.

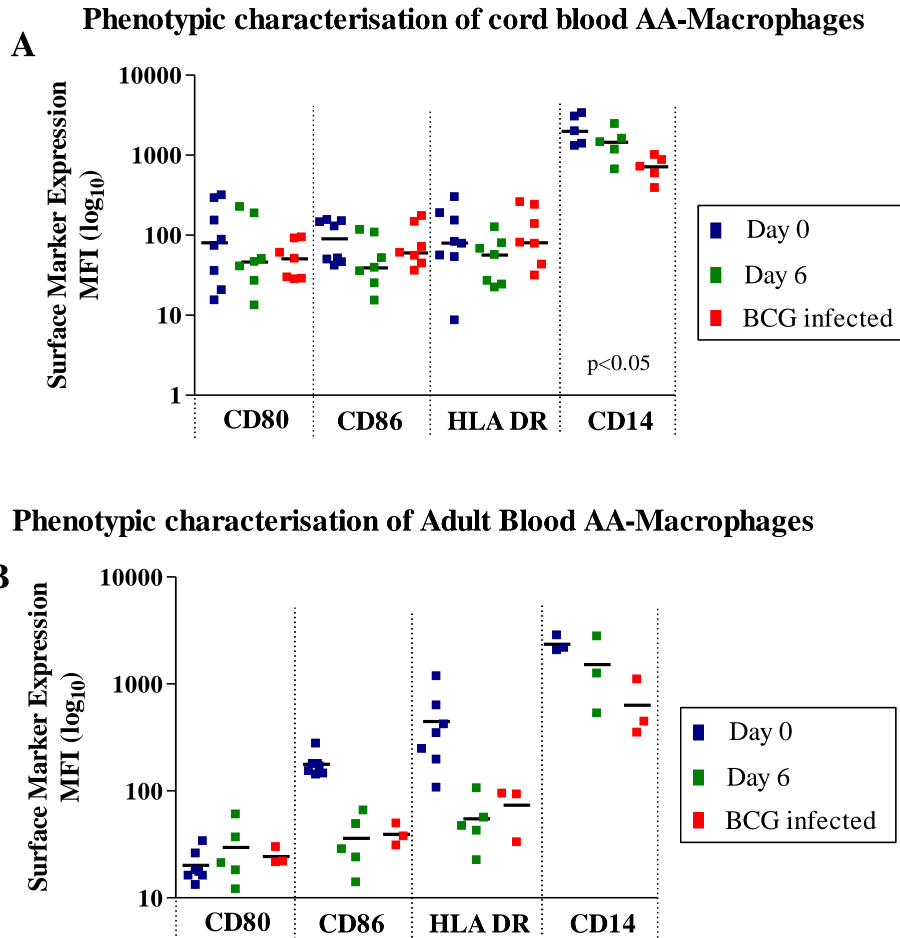


Figure 3.5 Surface phenotypic characterisation of monocytes (D0) and AA Mø after 6 days culture and following infection with BCG.
AA Mø were stained for surface expression of CD80, CD86, HLA-DR and CD14. MFI (log_{10}) is indicated for cord blood (A) and adult (B). Significant change in CD14 surface expression of cord blood AA Mø, using the Friedman ANOVA test are indicated. Mean MFI log_{10} for the individual data points shown is indicated by a short line.

3.3.3 Phagocytosis of BCG-gfp by macrophages

To determine whether cord blood derived macrophages were functioning optimally as phagocytic cells, the phagocytic properties of cord blood derived macrophages was compared to that of adult blood derived macrophages. The gating strategy utilised for the determination of the percentage of infected CA Mø is shown for uninfected (A) and infected (B) cells (Figure 3.6). CA Mø were selected by their size and granularity in gate R1, and the percentage of CA Mø staining positive for BCG-gfp is shown in the histogram. In histogram (A), uninfected cells give a background positive value of 0.2%, whereas in 24 hour BCG-gfp infected CA Mø indicate that 20% of the cells were positive for BCG-gfp (B). A similar gating strategy was used for determination of the percentage of AA Mø infected with BCG-gfp.

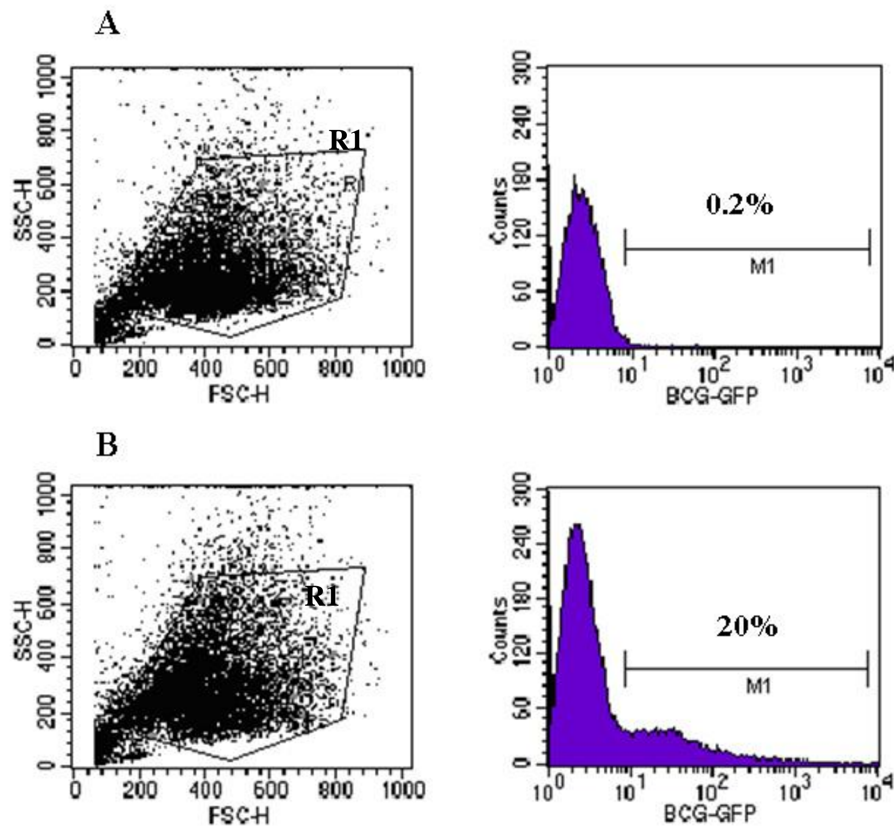


Figure 3.6 The gating strategy utilised for analysis of percentage of infected macrophages. In the dot plots, CA Mø are selected by size and granularity in R1 and the percentage of these cells infected with BCG-gfp is determined in the histogram. Uninfected CA Mø (A) or 24 hour BCG-gfp infected CA Mø (B) are indicated.

Adult and cord blood AA Mø infected with BCG-gfp at an MOI of 1:1 showed greater phagocytic properties compared to that of CA Mø, this difference attained significance in cord blood derived macrophages ($p<0.05$; Wilcoxon matched pairs test). Cord blood macrophages had a similar capacity to phagocytose BCG when compared to macrophages of adult origin.

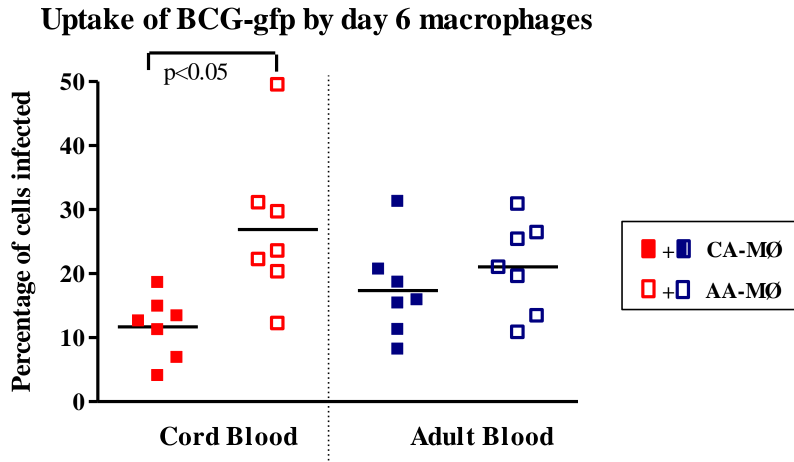


Figure 3.7 Phagocytosis of BCG-gfp by CA and AA Mø from adult and cord blood. Statistically significant difference using Wilcoxon matched pairs test, between uptake of BCG by CA and AA Mø from cord blood is indicated. Mean is indicated by a short line.

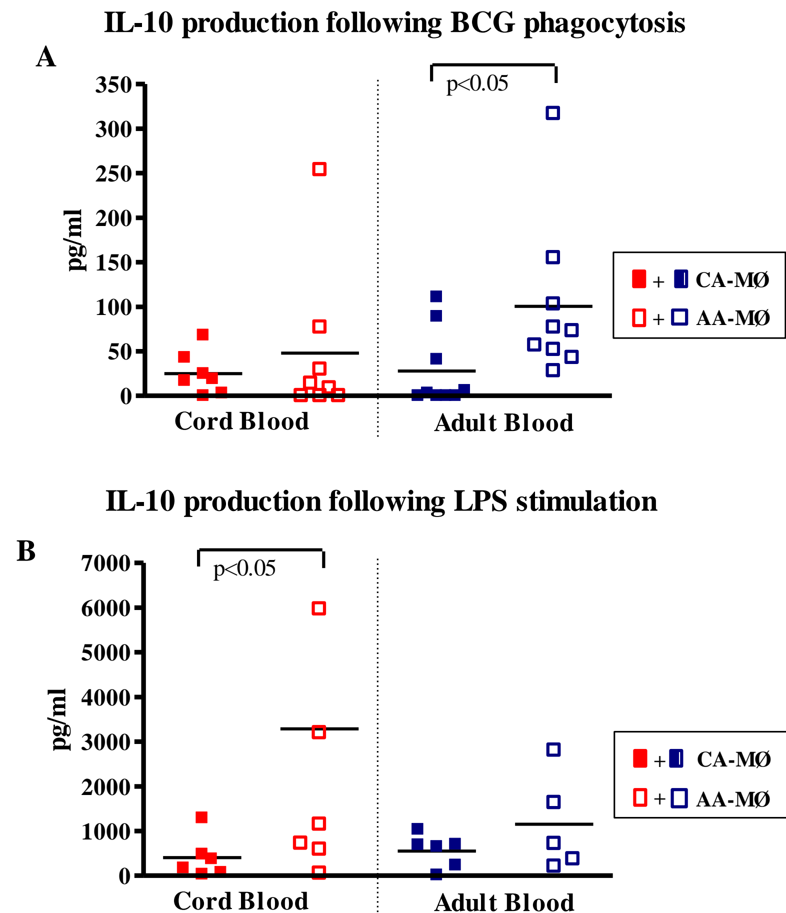


Figure 3.8 Amount of IL-10 produced by CA Mø and AA Mø infected with BCG-gfp (A) or stimulated with LPS (B).
Statistically significant differences are indicated using Wilcoxon matched pairs test. Mean results are displayed by a short line following subtraction of the background response.

3.3.4 TNF-α and IL-10 production by BCG-gfp infected or LPS stimulated macrophages

To further determine whether cord blood-derived macrophages were functioning optimally, the ability of cord blood macrophages to produce cytokines was compared to that of adult blood-derived macrophages. It was found that AA Mø stimulated with either BCG or LPS from both adults and cord blood produced more IL-10 than similarly stimulated CA Mø (Figure 3.8 A and B). Significantly more IL-10 was produced by adult AA Mø infected with BCG than adult CA Mø ($p<0.05$; Wilcoxon matched pairs test). In cord blood, significantly more IL10 was produced by AA Mø stimulated with LPS than CA Mø ($p<0.05$; Wilcoxon matched pairs test).

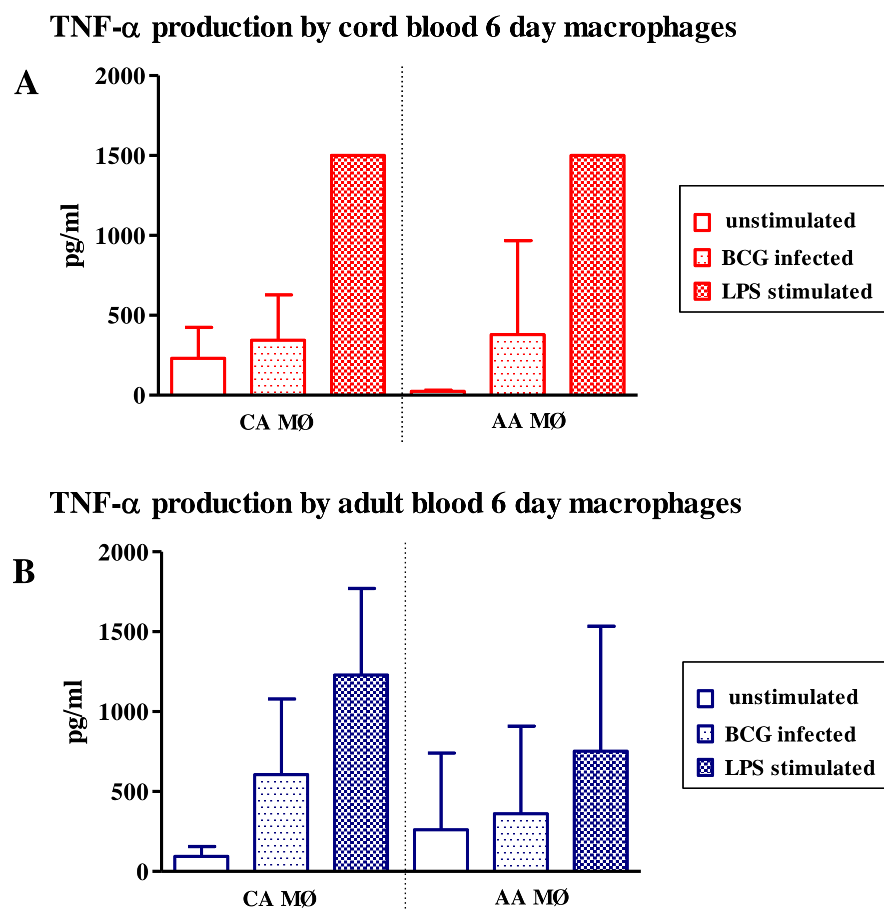


Figure 3.9 Amount of TNF-α produced by CA Mø and AA Mø unstimulated, infected with BCG-gfp or stimulated with LPS from cord (A) and adult (B) blood.
All LPS stimulated cord blood macrophages had >1500pg/ml of TNF-α, but are indicated in the graph as 1500pg/ml. Mean results and SD are indicated. The background results have not been subtracted. n=4 cord blood and adult blood derived macrophages.

In response to BCG infection, similar amounts of TNF-α were produced by cord blood macrophages compared to macrophages derived from adult blood. The background unstimulated TNF-α results have not been subtracted from stimulated results as a single sample from CA Mø of adult origin had insufficient cultured macrophages for an unstimulated sample. Although the range of results was large, CA Mø from adult blood produced slightly more TNF-α (606 ± 475pg/ml) than AA Mø from adult (361 ± 549pg/ml) or CA Mø (345 ± 283pg/ml) or AA Mø (379 ± 590pg/ml) from cord blood (Figure 3.9 A and B). In response to LPS stimulation, both CA Mø and AA Mø from cord blood produced >1500pg/ml of TNF-α. In contrast, CA Mø from adult blood (1230 ± 541pg/ml) produced slightly more TNF-α than AA Mø (752 ± 783pg/ml) in response to LPS stimulation, although not significantly more (Figure 3.9 A and B).

No IL-12 (total p40p35) was detectable in 6 day CA Mø or AA Mø from either adult or cord blood donors following stimulation with either LPS or infection with BCG.

3.3.5 Antimicrobial activity of macrophages

A critically important function of activated macrophages is the ability to control the growth of mycobacteria. The antimicrobial activity of cord blood derived macrophages against both Danish BCG and H37Rv was therefore compared to that of adult-derived macrophages. A comparison of CFU/ml in BCG and H37Rv infected CA Mø and AA Mø is indicated as means (Figure 3.10 A and B). These results from BCG and H37Rv infected macrophages were also compared using the CFU plated on day 1 as 100%, so that all results could be shown on a single graph (Figure 3.10 C). In addition, a summary of the results based on the data in Figure 3.10 A and B is indicated in Table 3.1 as a growth index. The growth index was defined as the number of CFU/ml on day 4 and day 7 divided by the number of CFU/ml on day 1. A growth index of >1 indicates multiplication within the macrophage, whereas a growth index of <1 indicates antimicrobial activity. Adult and cord blood AA Mø showed similar growth curves following BCG infection reaching a day 7 growth index of 1.76 and 1.70 respectively (Figure 3.10 A and C and Table 3.1). Adult CA Mø showed the best antimicrobial activity following BCG infection with a day 7 growth index of 1.54, contrasting with cord blood CA Mø which showed the worst antimicrobial

activity following BCG infection with a day 7 growth index of 2.31 (Figure 3.10 A and C and Table 3.1). These results indicate that both adult and cord blood derived macrophages limit the growth of BCG, but do not kill the organism i.e. the organism grows slowly. A different scenario is seen following infection of macrophages with virulent Mtb (H37Rv). A statistically significant increase in CFU of H37Rv were found in cord blood and adult blood derived CA Mø and AA Mø over time, using the Friedman ANOVA test ($p<0.05$ for both cell types of cord and adult blood origin) as indicated in Table 3.1. Cord blood derived AA Mø infected with H37Rv showed the best antimicrobial activity with a growth index of 2.46 and adult blood derived CA Mø the worst antimicrobial activity with a growth index of 8.82 (Figure 3.10 B and C and Table 3.1). A statistically significant reduction in CFU/ml of H37Rv was found in AA Mø compared to CA Mø in adult ($p<0.05$; Friedman ANOVA) and cord blood ($p<0.05$; Friedman ANOVA) derived cells, indicating that AA Mø were more bactericidal than CA Mø (Figure 3.10 B and C and Table 3.1). The results of H37Rv infected macrophages indicate that this pathogen survives and possibly grows, but is not killed. In summary, macrophages from both adult and cord blood, were more efficient at controlling intracellular BCG than intracellular H37Rv. H37Rv appears to grow better than BCG in CA Mø and possibly AA Mø (Figure 3.10 A, B and C and Table 3.1).

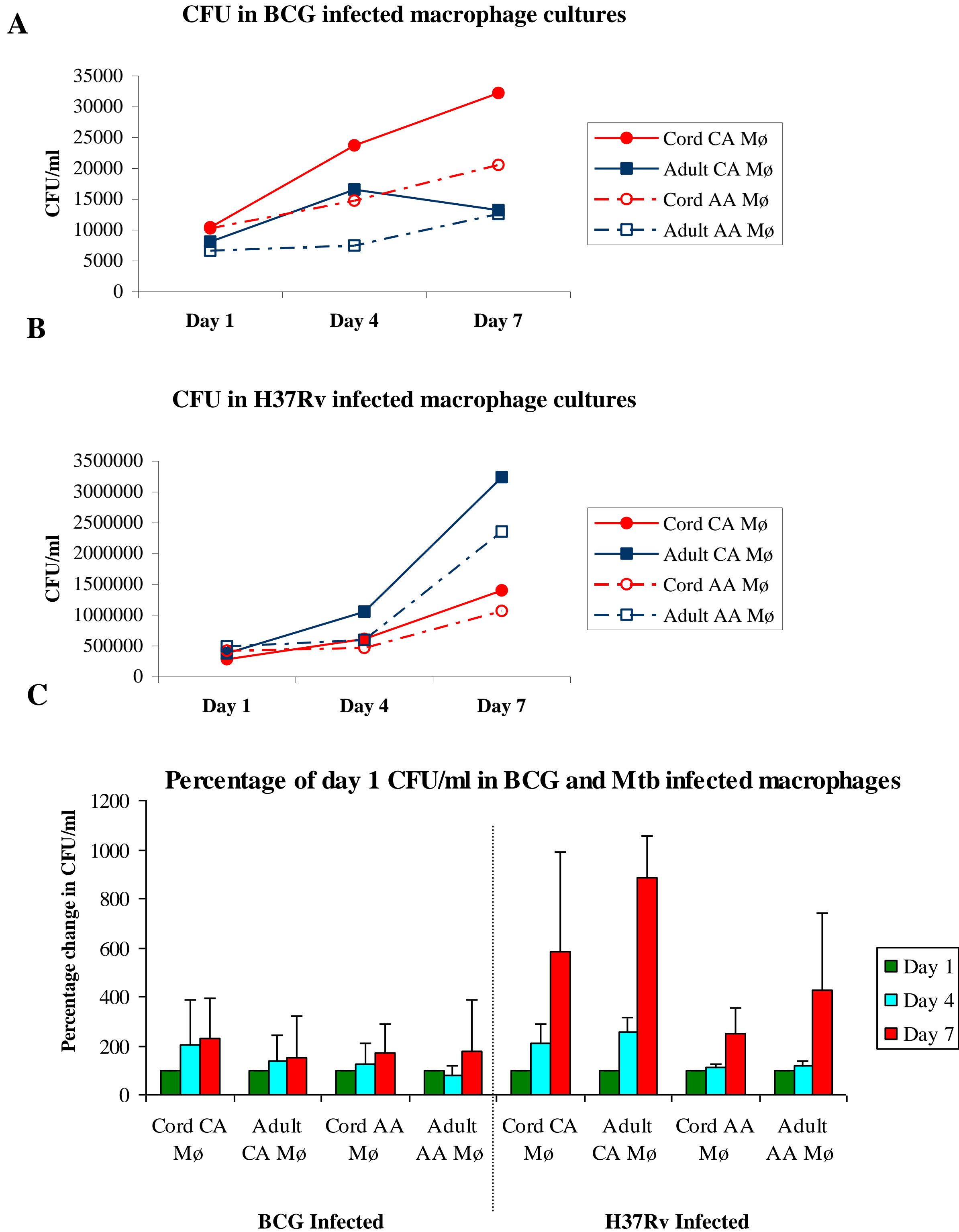


Figure 3.10 CFU/ml of BCG (A) or H37Rv (B) infected CA Mø and AA Mø over time and percentage change in CFU/ml over time (C).
The CFU/ml is indicated as means (A and B) and mean percentage change + SD (C). Please note different scales for BCG (A) and H37Rv (B) infected macrophages. n = 4-6 for cord blood and n = 3-5 for adults.

TABLE 3.1 Growth index of BCG and H37Rv in cultured macrophages.
Growth index (relative growth rate) is defined as the number of CFU/ml on day 4 and day 7 divided by the number of CFU/ml on day 1 for each separate individual and is indicated as means ± SD. Results are based on data in Figure 3.10 A and B.

Danish BCG infected MOI 1:1

	Day 1	Day 4	Day 7	p value
Cord Blood CA Mø	1	2.03 ± 1.81	2.31 ± 1.65	ns
Adult Blood CA Mø	1	1.37 ± 1.06	1.54 ± 1.69	ns
Cord Blood AA Mø	1	1.23 ± 0.85	1.70 ± 1.17	ns
Adult Blood AA Mø	1	0.77 ± 0.38	1.76 ± 2.08	ns

H37Rv infected MOI 1:1

	Day 1	Day 4	Day 7	p value
Cord Blood CA Mø	1	2.10 ± 0.78	5.80 ± 4.10	<0.05
Adult Blood CA Mø	1	2.56 ± 0.56	8.82 ± 1.73	<0.05
Cord Blood AA Mø	1	1.11 ± 0.13	2.46 ± 1.11	<0.05
Adult Blood AA Mø	1	1.17 ± 0.22	4.27 ± 3.16	<0.05

3.4 DISCUSSION

The hypothesis underlying the studies reported here is that "Macrophge mediated - protection against Mtb engendered by BCG vaccination is **quantitatively different** (i.e. difference in extent of macrophage activation marker expression prior to and post vaccination), but **not qualitatively** different (i.e. macrophages function is similar before and after vaccination with BCG) different from the pre-vaccination innate **protective response** of neonates against this pathogen". The importance of macrophages in innate immunity to TB is discussed in detail in section 1.9.1 of chapter 1 "Rationale for undertaking studies of macrophage function". As this thesis is studying the innate response of naïve infants using cord blood, the first step was a comparison of macrophage function in neonates to that of BCG vaccinated adult volunteers. While it would have been preferable to compare the function of macrophages from naïve infants to that of BCG vaccinated infants, this is not ethically possible as there is a limit of blood that can be collected from these infants.

An important observation in this study on macrophages was the confirmation that the differential maturation of classically activated macrophages compared to alternatively activated macrophages could be generated from both monocyte-derived cord and adult blood. This fact is clearly denoted in the morphology of these macrophages

which is dissimilar for CA Mø contrasted with AA Mø (Figure 3.1 A and B) and is also indicated in the graphs showing the phenotypic characterisation of CA Mø and AA Mø (Figure 3.4 and 3.5). Similar lower expression of CD14 was observed on the surface of CA Mø (MFI; 338 and 148) compared to AA Mø (MFI; 1465 and 1517) in cord and adult blood derived macrophages respectively. Thus, regardless of the source of cells used to generate the different population of polarised macrophages, similar differential maturation was seen. This also confirms previous studies: Verreck *et al* report that CD14 expression was consistently found to be greater on AA Mø compared to CA Mø (Verreck *et al.*, 2006).

Published reports indicate the importance of HLA-DR as an essential antigen presenting molecule. A reduction in the expression of this molecule might imply a diminished capacity for antigen presentation. In the present study significantly reduced levels of basal expression of HLA-DR ($p<0.01$) were found on cord blood monocytes compared to monocytes from adult blood (Figure 3.4). While some researchers report a significant reduction in HLA-DR expression on neonatal monocytes (Stiehm *et al.*, 1984; Roncarolo *et al.*, 1994; Birle *et al.*, 2003; Drohan *et al.*, 2004; Canaday *et al.*, 2006), others have reported a significantly higher expression of HLA-DR on cord blood monocytes (Marwitz *et al.*, 1988) and Keever *et al* report normal levels of HLA-DR expression on cord blood monocytes compared to adult monocytes (Keever *et al.*, 1995). In addition, previous studies indicate that CD80 was not expressed on neonatal monocytes (Dilioglou *et al.*, 2003 A; Han *et al.*, 2004) and a low baseline expression of the costimulatory molecules CD86 was found in neonatal monocytes (Velilla *et al.*, 2006). The current study was in agreement with Velilla *et al* regarding the reduction of CD86 expression on cord blood monocytes, as this was significantly lower ($p<0.05$) than the expression on adult monocytes (Figure 3.4 A and B). In contrast to the studies reporting a lack of CD80 expression on neonatal monocytes (Dilioglou *et al.*, 2003 A; Han *et al.*, 2004), I found that the surface molecule CD80 was expressed on cord blood monocytes in the resting state, and was significantly higher ($p<0.05$) than basal expression on adult blood monocytes (Figure 3.4 A and B). These conflicting reported results, particularly with neonatal monocyte expression of CD molecules may be explained in light of the publications demonstrating that monocytes are not a homogenous population of cells and are heterogeneous in the blood (Mosser and Edwards, 2008; Randolph *et al.*, 2008).

However, differences in the handling of the bloods *in vitro* could also affect some of the results.

While basal expression of these markers in monocytes is interesting, the phenotypic characterisation of macrophages derived from cord blood compared to those of adult origin is highly relevant to this study for determining whether cord blood macrophages are similar phenotypically to those derived from adults. Verreck and co-workers report that in adults AA Mø, but not CA Mø down-modulated their antigen presenting and costimulatory molecules HLA-DR and CD86 (Verreck *et al.*, 2004). In the current study a reduction in the expression of HLA-DR occurred in both CA Mø and AA Mø from adults, but was slightly more pronounced in AA Mø. Cord blood CA Mø had a significantly increased expression of CD80 and significantly reduced expression of CD86, HLA DR and CD14 during differentiation into macrophages (i.e. compared to the basal expression on cord blood monocytes). Cord blood AA Mø expression of CD14 also reduced significantly during the 6 days of culture. Ultimately however, on day 6 both cord blood-derived and adult blood-derived CA Mø as well as AA Mø had similar levels of phenotypic expression for CD80, CD86, HLA-DR and CD14 (Figure 3.4 and 3.5). The importance of HLA-DR expression in antigen presentation has been discussed, it also needs to be highlighted that CD86 and CD80 are essential co-stimulatory molecules and necessary for the activation of T cells (Orlikowsky *et al.*, 2003). Orlikowsky *et al* showed that CD86 but not CD80 was constitutively expressed on adult macrophages, but not in cord blood. In addition, Orlikowsky and co-workers reported upregulation of MFI expression of both CD80 and CD86 on cord blood macrophages after 48 hours in culture. This was significantly lower than the expression by adult macrophages after 48 hours in culture. These researchers argue that cord blood macrophages therefore have impaired co-stimulatory activity (Orlikowsky *et al.*, 2003). In the present study, the expression of the co-stimulatory molecules CD80 and CD86 and the antigen presenting molecule HLA-DR was similar in cord blood and adult blood macrophages after 6 days in culture. It is interesting to hypothesise then that the present study implies that neonatal macrophages do not have impaired co-stimulatory or antigen presenting properties

Following infection of macrophages with BCG, the expression of CD80, CD86, HLA-DR and CD14 was similarly retained in the polarised macrophages from both cord and adult blood (Figure 3.4 and 3.5). Following infection of macrophages with BCG-gfp and prior to measuring the uptake of bacteria by macrophages using flow cytometric evaluation, cells were washed three times in an attempt to remove any bacteria that were adherent to the cells and not engulfed by the cells. However, it is possible that some of the adherent bacteria may not have been washed off and caused a false positive result for uptake of bacteria. As all cell preparations were treated in the same manner, any false positive results from the presence of adherent bacteria would have caused all the results to be falsely elevated. Given this limitation, the phagocytosis of BCG-gfp by CA Mø from adult and cord was comparable, as was the phagocytosis of AA Mø from adult and cord blood. The uptake of BCG by AA Mø was slightly higher than that of CA Mø, particularly in those of cord blood origin where this difference attained statistical significance ($p<0.05$) (Figure 3.7). In contrast to this, Velilla and colleagues stated that cord blood macrophages show decreased phagocytic properties compared to adult derived macrophages (Velilla *et al.*, 2006). This difference in the present results compared to those of Velilla *et al* can be attributed to the fact that my comparison used CA Mø and AA Mø, whereas Velilla did not include polarising cytokines. Verreck *et al* report that binding and uptake of BCG-gfp was supported by both macrophage subsets in adults, but more efficiently by AA Mø than by CA Mø. The results obtained in this thesis are therefore in agreement with these published findings by Verreck and co-workers and those of Xu *et al* who report that AA Mø are potent phagocytes (Verreck *et al.*, 2004; Xu *et al.*, 2007). It is of interest to note that the AA Mø which showed the highest expression of CD14 (Figure 3.5) also demonstrated enhanced phagocytosis of BCG (Figure 3.7). CD14 is a receptor for LPS and TLR4 and TLR2 are co-receptors with CD14 for specific microbial ligands (Taylor *et al.*, 2005). As mycobacterial components are recognised via TLR2 in association with TLR1/TLR6, or via TLR4 this enhanced phagocytosis could be due to the increased expression of CD14 in these AA Mø (Kaisho and Akira, 2001; Zarember and Godowski, 2002; Xu *et al.*, 2004; Ryffel *et al.*, 2005; Akira *et al.*, 2006). The findings in this chapter, verify that cord blood macrophages are phagocytic and are in agreement with other published reports indicating that that AA Mø are more phagocytic than CA Mø. It can be confirmed

therefore that the phagocytic properties of cord blood-derived macrophages are similar to those of adult origin.

In cellular immune studies there is donor to donor variation in results as well as variation in the same donor; this was particularly true of experiments to determine whether macrophages had the ability to control infection with mycobacteria. Given this shortcoming, macrophages were found to limit the growth of Danish BCG, but did not kill the organism. In Danish BCG infected macrophages, CA Mø of adult origin were able to limit the infection better than AA Mø, whereas CA Mø of cord blood origin were least efficient at containing the infection (Figure 3.10 and Table 3.1). Using a luciferase-transfected BCG reporter strain, Verreck *et al* also reported a higher growth of this bacteria within AA Mø compared to CA Mø, which is similar to what was found in this study with adult macrophages (Verreck *et al.*, 2004). In contrast H37Rv was able to grow within the macrophages. This fact is highlighted by the significant increase in CFU/ml for both classically and alternatively activated macrophages from both adult and cord blood donors. However H37Rv infected AA Mø showed significantly enhanced limitation of growth of this pathogen in cord blood ($p<0.005$) and those of adult blood origin ($p<0.005$) compared to CA Mø (Figure 3.10 and Table 3.1). These findings are in agreement with other published reports that both pathogenic and non-pathogenic mycobacteria are phagocytosed by macrophages (Wong *et al.*, 2007; Jordao *et al.*, 2008). However, previous publications also report that non-pathogenic strains such as BCG are invariably killed by the macrophages, whereas the pathogenic strains such as Mtb resist killing by macrophages (Wong *et al.*, 2007; Jordao *et al.*, 2008). The findings in this current study were in agreement with these published reports regarding Mtb, however while the growth of BCG was limited, this organism was not killed. Nevertheless, compared to growth of the virulent H37Rv strain within the macrophage that of BCG was reduced. Marodi and co-workers report that neonatal monocyte-derived macrophages stimulated with recombinant IFN-γ or GM-CSF exhibit a reduced capacity to kill phagocytosed group B Streptococcus compared to adult macrophages (Marodi *et al.*, 2000). In this current study CA Mø from cord blood were the least efficient macrophage at killing BCG and CA Mø from adult blood the least efficient at killing H37Rv. As CA Mø are cultured in the presence of GM-CSF, this finding particularly in the light of those published by

Marodi and co-workers is of particular interest. In addition, AA Mø suppressed the growth of H37Rv more than that of CA Mø and were therefore the more efficient macrophage type at containing intracellular Mtb. This finding was particularly true for cord blood derived AA Mø. Similar to the enhanced phagocytosis of AA Mø and the higher expression of the molecule CD14, a similar theory may hold true for the bactericidal capacity of AA Mø compared to CA Mø. Results from the current study that found AA Mø suppressed the growth of H37Rv more than that of CA Mø, was contrary to a report by Mosser, who describes AA Mø as relatively poor at killing intracellular pathogens (Mosser, 2003). In addition Verreck and colleagues found that AA Mø showed a higher growth of luciferase-transfected *M bovis* BCG reporter strain compared to CA Mø (Verreck *et al.*, 2004). However, the heterogeneity of macrophages that had been cultured on different surfaces has been described by Kaplan and colleagues. In the study by Kaplan *et al*, macrophages displayed phenotypic differences that were induced in cells derived from *in vitro* differentiation of peripheral blood monocytes cultured on various culture surfaces (Kaplan and Gaudernack, 1982). The variation in culture conditions, such as serum supplements and culture surfaces may explain the differences obtained in the current study compared to that of other investigators.

Studies of cytokine response in neonates have yielded conflicting results. Some authors describe a decreased pro-inflammatory cytokine response to antigen, mitogen and LPS stimulation (Weatherstone and Rich, 1989; Bessler *et al.*, 2001; Brichard *et al.*, 2001; Velilla *et al.*, 2006); others a comparable response to LPS (Berner *et al.*, 2002) and yet others an increased cytokine response to LPS (Dembinski *et al.*, 2002). In the current study, low levels of IL-10 were produced by CA Mø from both neonates and adults following stimulation with BCG. AA Mø produced more IL-10 in response to LPS than CA Mø; which was significantly more enhanced in macrophages of cord blood origin ($p<0.05$); whereas IL-10 production from adult blood-derived AA Mø was significantly greater in response to BCG ($p<0.05$) (Figure 3.8). This is in accordance with published reports that IL-10 is the signature cytokine of AA Mø (Mosser, 2003; Verreck *et al.*, 2004; Verreck *et al.*, 2006). No IL-12 (total p40p35) was measurable in CA Mø or AA Mø from either adult or cord blood donors following stimulation with either LPS or BCG. The lack of IL-12 secretion is in agreement with Verreck *et al* who reported that CA Mø secreted high levels of IL-23

(p40/p19) heterodimer and IL-12p40 monomer, but no IL-12 (total p40p35), which required addition of exogenous IFN-γ; whereas AA Mø produced neither IL-23 nor IL-12 (Verreck *et al.*, 2004; Verreck *et al.*, 2006). In this thesis, adult blood-derived CA Mø produced more TNF-α irrespective of the stimulant, whereas cord blood-derived macrophages produced similar amounts of TNF-α when stimulated with BCG, but maximal amounts (i.e. >1500pg/ml) following LPS stimulation (Figure 3.9). If it had been possible to repeat the LPS stimulated cord blood macrophage TNF-α results in multiple diluted samples, it is possible that a distinction in production of this cytokine could have been found in CA Mø compared to AA Mø. Verreck *et al* report greatest TNF-α production from CA Mø, which is similar to what I found in adult blood-derived macrophages (Verreck *et al.*, 2006). Pro-inflammatory cytokines produced by macrophages such as IL-12 and TNF-α are important in immunity to TB. TNF-α has an important role in granuloma formation which aids in the containment of Mtb. However, anti-inflammatory cytokines such as IL-10, when produced in response to mycobacterial infection, possesses macrophage-deactivating properties. These properties include down-regulation of IL-12, which results in reduction of IFN-γ production by T cells. IL-10 is also reported to inhibit export of MHC class II molecules to the cell surface, which would hamper the APC function of cells infected with mycobacteria and result in the down-regulation of T cell responses (Flynn and Chan, 2001; Hickman *et al.*, 2002). In addition, *in vitro* IL-10 has been reported to suppress the production of IL-12 in monocytes infected with Mtb via the downregulation of costimulatory molecules on macrophages, reducing the activity of $CD4^+$ and $CD8^+$ T cells (Bhatt and Salgame, 2007). In this study, AA Mø were found to secrete increased amounts of the anti-inflammatory cytokine IL-10 which would suppress the inflammatory response to TB as discussed above. The suppressor activity of these AA Mø is reported by Mosser to protect organs from unwanted inflammatory or immune reactions, particularly in the lung (Mosser, 2003). However, in this current study, these AA Mø appeared to show opposing roles, as they also displayed increased phagocytosis and increased bactericidal properties against H37Rv. Although CA Mø are regarded as efficient stimulators of Th1-type cells (Verreck *et al.*, 2004) and I found in this study that adult cells secreted greater amounts of TNF-α, these cells were also less efficient at phagocytosis, as well as in the suppression of H37Rv intracellular growth. These findings highlight the

heterogeneity of macrophages and possibly indicate that both classically and alternatively activated macrophages are important in immunity and outcome of infection with the intracellular organism Mtb. In addition, results from this chapter indicate that with regard to the phagocytic properties, the phenotypic characterisation of macrophages, the presence of cytokines released by macrophages and the ability of macrophages to control the growth of virulent mycobacterium, cord blood derived macrophages are as proficient as those derived from adult blood.

CHAPTER 4

DENDRITIC CELL FUNCTION IN THE NEONATE

4.1 INTRODUCTION

The development of protective host immunity, with activation of an antigen specific response, is dependent on the presentation of foreign antigen to naïve T cells in the context of MHC molecules expressed on the surface of APC (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Savina and Amigorena, 2007). DCs are the most potent APC in the immune system and the only cell capable of inducing primary activation of naïve $CD4^+$ and $CD8^+$ T cells (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Dilioglou *et al.*, 2003 B; Sinha *et al.*, 2007; Randolph *et al.*, 2008). Resident blood or tissue DC are highly proficient at antigen uptake and poor stimulators of the T cell response (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Mellman and Steinman, 2001; Bhatt and Salgame, 2007; Savina and Amigorena, 2007). To present antigen efficiently, iDC need to differentiate into activated mDC via upregulation of antigen presenting properties and downregulation of their phagocytic capacity (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Mellman and Steinman, 2001; Munz *et al.*, 2005; Bhatt and Salgame, 2007). iDC, when charged with antigen in the periphery, must migrate to the site of T cell activation via efferent lymphatic vessels (Banchereau *et al.*, 2000). As these iDC mature, upregulation of the chemokine receptor (CCR)7 occurs enabling them to accumulate in the draining lymph nodes (Banchereau *et al.*, 2000; Lipscomb and Masten, 2002; Bhatt and Salgame, 2007). In the lymph node, DC mature both functionally and phenotypically, thereby acquiring the ability to effectively stimulate naïve T lymphocytes (Banchereau and Steinman, 1998; Banchereau *et al.*, 2000; Sinha *et al.*, 2007). mDC exhibit high levels of expression of the antigen presenting molecule MHC class I and II and also of the T cell costimulatory molecules CD40, CD80 and CD86 (Banchereau *et al.*, 2000; Lipscomb and Masten, 2002). MHC class II can increase 5 to 20-fold and CD86 can increase up to 100-fold (Mellman and Steinman, 2001). Antigen displayed on the surface of mDC is presented to naïve T

cells driving their maturation to become activated effector T cells capable of producing IFN-γ and other cytokines (Mellman and Steinman, 2001; Bhatt and Salgame, 2007; Hanekom *et al.*, 2007). In TB these antigen-specific T cells proliferate and migrate back to the lung where they produce cytokines required for the activation of macrophages in the infected granuloma. This process is essential for the induction of specific anti-mycobacterial pulmonary immunity (Kaufmann, 2001; Gagliardi *et al.*, 2004).

Cord blood DCs have been reported to be functionally immature. This observation has led to the assumption that neonatal APC lack the ability to deliver important Th1 polarising signals to T cells (Hunt *et al.*, 1994; Trivedi *et al.*, 1997; Upham *et al.*, 2002). Salio and co-workers reported that neonatal monocyte-derived DC have a lower capacity than adult DC to stimulate allogeneic $CD4^+$ T cells although these neonatal DC were capable of priming antigen-specific $CD8^+$ T cells to levels comparable to those of adult DC (Salio *et al.*, 2003). The observation that neonates have increased susceptibility to intracellular pathogens has also been taken as additional evidence of a possible defect in neonatal DC function (Macatonia *et al.*, 1995; Goriely *et al.*, 2001). Neonatal DC are reported to have a reduced ability to produce IL-12 compared to adult DC; they have been shown to have a deficiency in the production of bioactive IL-12(p70), attributable to defective expression of the IL-12(p35) gene (Goriely *et al.*, 2001). Since DC are essential for priming naïve T cells, as well as producing IL-12, a defect in these DC functions could contribute to the impaired immune responses found in the newborn (Macatonia *et al.*, 1995; Goriely *et al.*, 2001). This deficiency could also play a role in the poor IFN-γ production detected during most *in vitro* T cell responses in neonatal blood (Goriely *et al.*, 2001). The reduced capacity to produce IL-12 and to stimulate an allogeneic lymphocyte reaction, as well as poor IFN-γ production, has led to the assumption that mature neonatal DC may not reach the same level of maturity as adult DC (Ota *et al.*, 2002; Marchant and Goldman, 2005).

Another aspect that would suggest that neonatal DC are not as efficient as adult DC in priming an adaptive response, is the fact that umbilical cord blood transplantation is associated with a much lower risk of transplant rejection, as well as reduced severe

graft-versus-host disease (GVHD) compared to allogeneic bone marrow transplantation. GVHD is an immune reaction that results from the interaction between cell surface molecules on T cells and APC. Since DCs are the most potent APC they play a central role in the development of GVHD. The reduced GVHD mediated by cord blood transplantation compared to allogeneic bone marrow transplantation suggests that neonatal DC are not as efficient as APC compared to those of adult DC (Cohen *et al.*, 2000 B; Varis *et al.*, 2001; Nachbaur and Kircher, 2005).

However, the immune responses measured in infants following vaccination with BCG suggest that neonatal DCs are efficient APC. BCG is known to be a potent stimulator of DC via activation and maturation of the cells, resulting in a strong Th1 response measured in infants vaccinated at birth (Marchant *et al.*, 1999; Siegrist, 2001; Hussey *et al.*, 2002). Following vaccination, it has been suggested that BCG may remain viable for many years and that the continued presence of the bacilli during the maturation of the immune system in the infant may also play a role in the Th1 response via continuous stimulation and activation of DC (Siegrist, 2001). A published report by Hoft and colleagues demonstrated that viable BCG bacilli are definitely present a few months after vaccination. These researchers found viable replicating bacilli in the ulcerative drainage from intradermal BCG vaccine lesion but not in the circulation 2 months after vaccination (Hoft *et al.*, 1999). The continued presence of BCG and ongoing activation of DC could account for the fact that after vaccination the Th1 memory of neonates can still be detected up to one year later (Marchant *et al.*, 1999). In contrast, the Th1 memory response following infant BCG vaccination that is detectable up to 14 years later, in the majority of individuals from the UK, is probably due to later stimulation by environmental mycobacteria (Weir *et al.*, 2008).

The response of the vaccinated individual to mycobacterial antigens following BCG vaccination at birth, is reported to cause increases in both Th1 and Th2 cytokines, as well as antibody responses to vaccines which are unrelated to BCG. BCG therefore improves the immunogenecity of other disparate vaccines. Furthermore BCG vaccination during the first week of life is associated with reduced atopy in children which is mediated by Th2 cytokines (Ota *et al.*, 2002). These findings confirm the

suggestion that neonatal DC in the presence of BCG, have potent APC-activating properties and are capable of priming a Th1 response.

In this chapter, I attempt to test the hypothesis that similar DC function exists before and after BCG vaccination, but it is associated with more extensive DC stimulatory activity post vaccination versus prior to vaccination. The following properties of neonatal DC will be studied:

1. Ability of cord blood DC to differentiate and mature *in vitro*
2. Ability of cultured cord blood DC to present antigen and drive T cell proliferation *in vitro*
3. Ability of cultured cord blood DC to stimulate the *in vitro* production of the Th1 cytokine IFN-γ

These distinct phenotypic and functional characteristics of DC in the neonate will be studied using monocyte derived DC from cord blood. Monocyte derived DC have been shown to express a myeloid surface phenotype and induce Th1 differentiation, (Liu *et al.*, 2001). As the frequency of DC in human tissue including cord blood is extremely low (0.15% ± 0.03%), and similarly in peripheral blood (males, 0.28% ± 0.12%; females 0.32% ± 0.13%) (Ueda *et al.*, 2003) any experiment requiring functional DC cannot rely on direct isolation of mature cells from the blood or tissues. Rather as demonstrated by other investigators (Zhou and Tedder, 1996; Zheng *et al.*, 2000; Mellman and Steinman, 2001; Gogolak *et al.*, 2003; Hanekom *et al.*, 2003) experiments to evaluate the function of mature DC are usually carried out with *in vitro* matured cells. Healthy BCG vaccinated adults will be used as a control group with which to compare cord blood-derived DC. While I would have preferred to compare neonatal DC to those of BCG vaccinated infants, this is not possible, as ethically the amount of blood that can be collected from these young infants is not sufficient for the number of cells that must be used for the completion of the experimental work required for this comparison.

4.2 MATERIALS AND METHODS

4.2.1 Study Participants

Peripheral blood was collected from healthy BCG vaccinated adult volunteers over the age of 21 years. Peripheral blood from ten adult volunteers was collected for DC culture. The mean age (± SD) of the adult volunteers was 42.8 ± 9 years of which 6 were male and 4 female. All infants in this study were vaccinated shortly after birth with Danish BCG by the intradermal route. Umbilical cord blood was collected as previously described. Eleven cord blood samples were collected for culture of DC. Different assays were carried out dependant on both the volume of blood and the yield of DC obtained. The mean age of the mothers was 32.8 ± 3.5 years and the mean gestation period was 38.4 ± 1 weeks. Seven of the infants were male. The mean weights of the neonates and of the placentas were 3467 ± 464g and 714 ± 145g respectively. The mean placental weight is slightly more than the described range of 400 – 600g (Perrin and Sander, 1984). All infants had immediate Apgar scores ≥8 and 5-minute Apgar scores of 9 or 10, indicating that they were healthy.

4.2.2 Culture of dendritic cells

The method for culturing DC is described in detail in Chapter 2. Briefly, purified monocytes from both cord blood and adult volunteers were cultured for 6 days in 5% AB serum in RPMI-1640. IL-4 and GM-CSF, myeloid DC-promoting cytokines were included in the culture medium. GM-CSF (with other cytokines) is widely used for the generation of DC and the addition of IL-4 has been shown to inhibit macrophage differentiation and induce DC generation (Gogolak *et al.*, 2003). After 6 days in culture, monocytes differentiated into iDC. iDC were then cultured for an additional 2 days in the presence of a maturation cocktail to obtain mDC. Maturation cytokines consisted of IL-4, GM-CSF, TNF-α, IL-1-β and PGE_2.

4.2.3 Morphology of dendritic cells

An Olympus CK40 inverted light microscope at a magnification of x200 was used to compare the morphology of the iDC and mDC generated. DC were photographed using the Zeiss AxioCam MRm camera attached to a Zeiss Axiovert 40CFL microscope at x200 magnification.

4.2.4 Phenotypic analysis of dendritic cells

PE-conjugated antibodies against CD40, CD80, CD86, CD83, CD25 and HLA-DR were used to determine surface expression of iDC and mDC via flow cytometric evaluation. The percentage and MFI of iDC and mDC positive for CD83 and CD25 was determined in cord blood derived DC and compared to that obtained in adult DC. The MFI was determined for CD40, CD80, CD86 and HLA-DR. Non-viable cells were excluded by scatter characteristics and staining with 7AAD.

4.2.5 Measurement of BCG phagocytosis by dendritic cells

The phagocytic properties of iDC compared to mDC were determined by measuring the uptake of BCG-gfp as described in Chapter 2. DC were washed three times and the percentage of cells infected with BCG-gfp established using a flow cytometer. Uninfected cells were run in parallel and used as a negative control.

4.2.6 Phenotypic analysis of adult dendritic cells following BCG infection

DC infected with Mtb or BCG are reported to cause maturation and activation of the DC (Hickman *et al.*, 2002). This maturation and activation of DC can be detected by expression of the activation marker (CD25), maturation marker (CD83), antigen presentation marker (HLA-DR) and T cell costimulatory molecules (CD40, CD80 and CD86). To confirm whether infection with BCG caused maturation and activation, monocyte-derived iDC were cultured with maturation cytokines for 48 hours in the absence and presence of BCG (MOI 1:1). In addition, mDC were cultured in the presence or absence of BCG. Infected DC and uninfected DC were then stained for flow cytometric evaluation of maturation, activation, antigen presentation and T cell costimulatory markers.

4.2.7 Determination of functional capacity of dendritic cells using an allogeneic lymphocyte reaction

To determine whether mDC were functional, mDC were used to stimulate autologous T cells (as a negative control) and allogeneic T cells from an unrelated cord blood sample, as described in Chapter 2. $CD14^-$ cells were used as a source of T cells as performed by other investigators (Hanekom *et al.*, 2003) and these cells will be referred to as T cells. The ratio of mDC to T cells were 1:50, 1:100, 1:300 and

1:1000. SNF was collected and soluble IFN-γ levels determined by ELISA. Results were expressed as pg/ml after subtraction of cytokine levels in unstimulated cultures.

4.2.8 Statistical Analysis

Results are reported as mean ± SD. Normality assumption of data was tested using Shapiro-Wilks test. Owing to the fact that data were found to be non-normally distributed, non-parametric tests were used. To compare data from 2 groups, the Mann-Whitney test was used for unpaired data and the Wilcoxon test was used for paired data. To compare data from more than two groups, the Kruskal-Wallis ANOVA test was used for unpaired data and the Friedman ANOVA test was used for paired data. Data were analyzed by using Statistica (version 9, StatSoft, Inc., Tulsa, OK, USA).

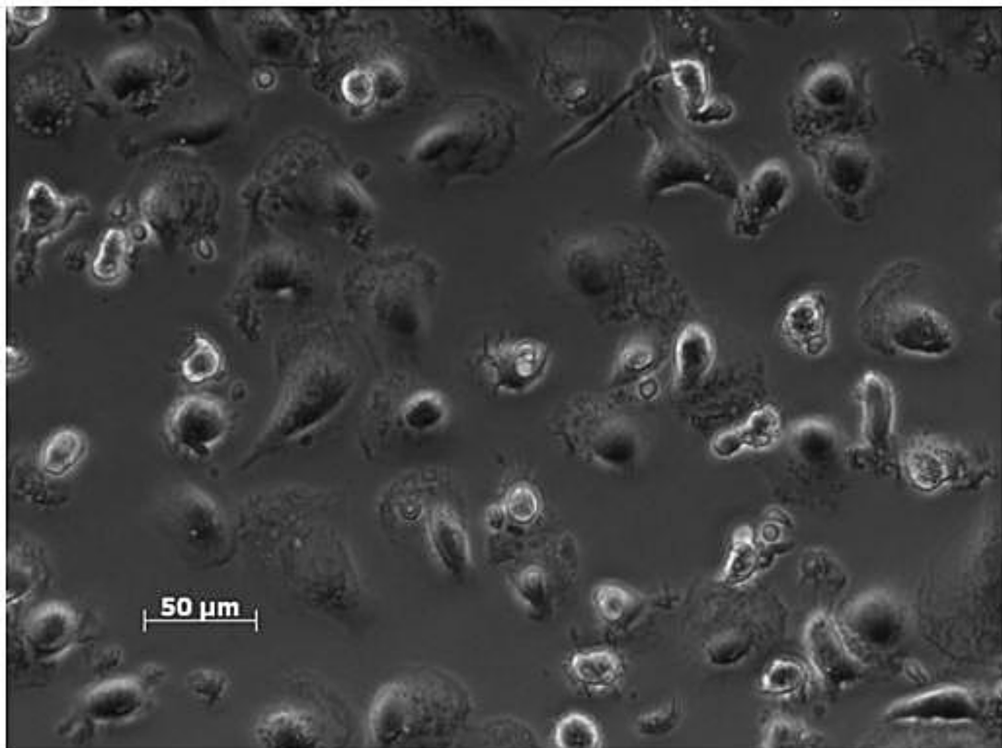
4.3 RESULTS

4.3.1 Morphology of DC

To determine whether monocyte-derived cultured DC had the ability to differentiate and mature, morphology of cord blood derived iDC and mDC was compared to iDC and mDC of adult blood origin. During differentiation from monocytes to iDC, cells become non-adherent and developed into larger cells irregular in shape with some visible cytoplasmic projections, which is typical DC morphology (Banchereau and Steinman, 1998). With the addition of maturation cytokines, mDC became slightly adherent to the plastic surface of the tissue culture plates (but the cells remain easily detached from the plates) and the projections were more prominent and highly ruffled. When these mDC are viewed by phase-contrast microscopy they extend large processes (>10μm), or veils in many directions from the body of the cell. These branched projections are known as dendrites, a term from which these cells derive their name (Banchereau and Steinman, 1998). Adult and cord blood derived immature and mature monocyte-derived DCs were shown to be similar morphologically when examined by phase-contrast microscopy. As can be seen in Figure 4.1 A, iDC from cord blood were irregular in shape with some visible cytoplasmic projections. In contrast, mDC displayed multiple fine dendrites

(processes) greater than 10μm in length (Figure 4.1 B) (Banchereau and Steinman, 1998). This morphology appeared similar to the morphology of adult iDC and mDC.

A



B

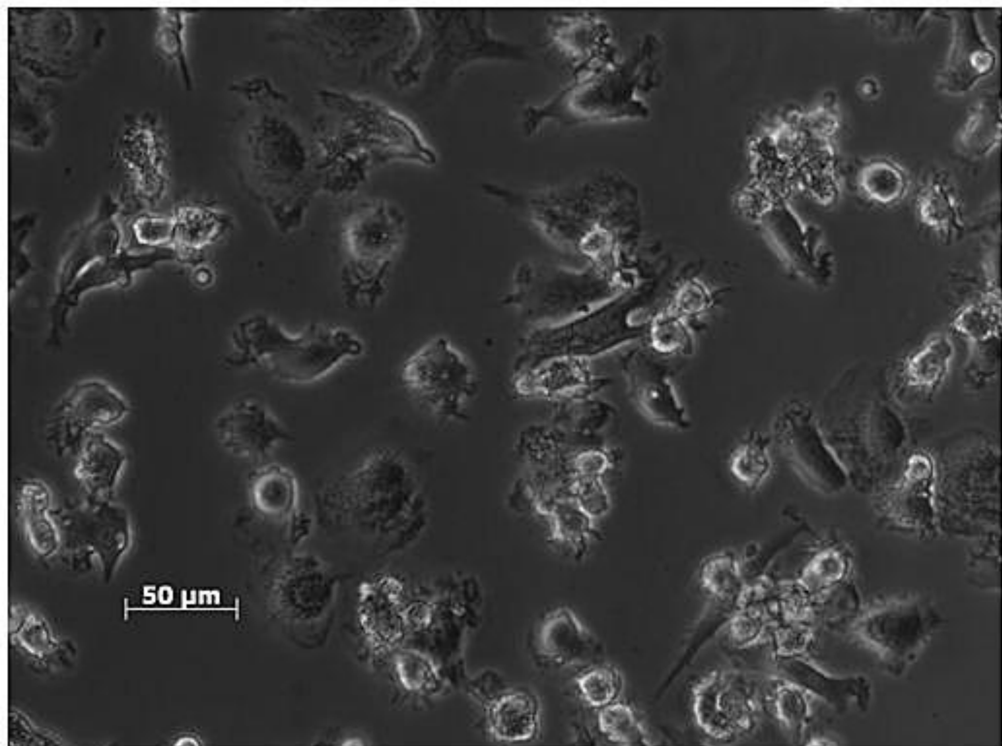


Figure 4.1 Typical morphology of cultured monocyte derived immature DC (iDC) (A) and mature DC (mDC) (B).
iDC from cord blood were irregular in shape with some visible cytoplasmic projections (A). In contrast mDC are highly ruffled and projections are more prominent. Magnification x200, phase contrast microscopy. The scale of 50µm is included in each photograph.

4.3.2 Flow cytometric analysis of DC

As DC mature functionally they express a number of surface markers that distinguish them from their precursor monocytes. These phenotypic changes are commonly used as indicators of DC functional maturation. To determine whether cord blood derived DC were able to differentiate and mature to a similar degree to that of adult DC, phenotypic characterisation of cord blood and adult blood iDC and mDC were therefore undertaken and compared. Figure 4.2 A shows an example of iDC and Figure 4.2 B is a representative example of mDC dot plots generated for analysis on the FACsCalibur using Cell Quest software. In the first dot plots, DCs are selected in R1 by their size and granularity. In the second dot plots, viable DC are selected in R2 i.e. they are 7AAD negative cells. In the third dot plots, MFI and percentage of cells expressing either CD83 or HLA-DR are indicated (y-axis). Using the Cell Quest software, the MFI and percentage of cells expressing CD83 can be determined. As mentioned previously in Chapter 2, CD83 and CD25 have discrete expression, whereas the other markers are continuous, in which case MFI as a means of comparison is more informative. Therefore, in future Figures, results for CD83 and CD25 will be given as both percentage cells positive for these markers as well as MFI. Only MFI will be shown for CD40, CD80, CD86 and HLA-DR

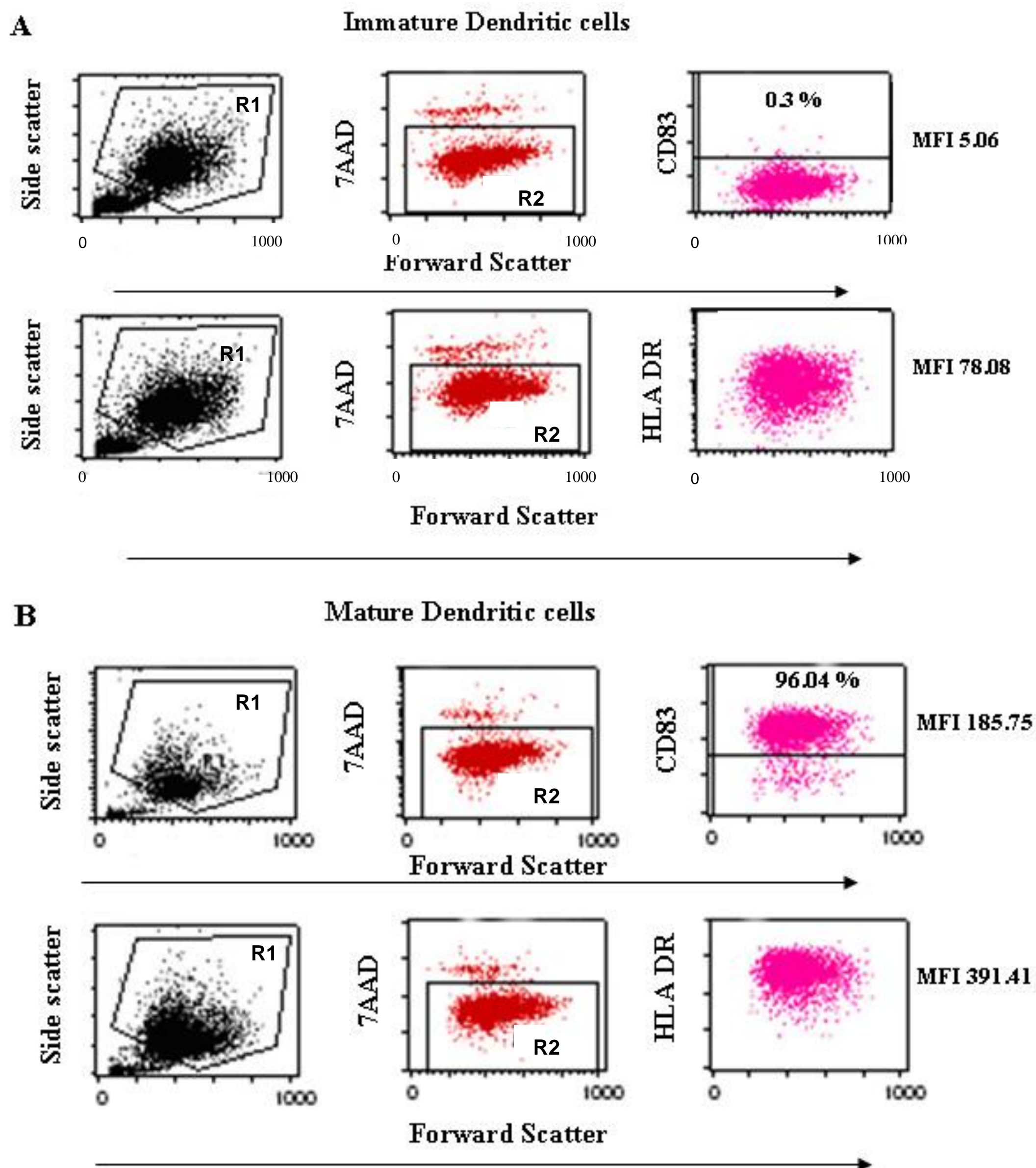


Figure 4.2 Gating strategy for cultured monocyte derived iDC (A) and mDC (B). In the first dot plots, DC are selected in R1 by size and granularity. In the second dot plots, viable DC are selected in R2 i.e. 7AAD negative cells. In the third dot plots, CD83 or HLA-DR MFI expression is indicated (y axis). Percentage expression is also included for CD83.

Using Cell Quest software, it was possible to generate a histogram overlay indicating the level of expression for a particular marker in iDC. A second line on the same graph indicates the expression of the same marker in mDC. Figure 4.3 is a representative example of cord blood DC (A) and adult blood DC (B) surface expression of iDC compared to mDC shown as an overlay. The green line represents the expression of iDC and the purple line the expression of mDC. Upregulation in

expression of CD83, CD80 and CD86, and an increase in the expression of CD25, CD40 and HLA-DR can be seen in both of these overlays.

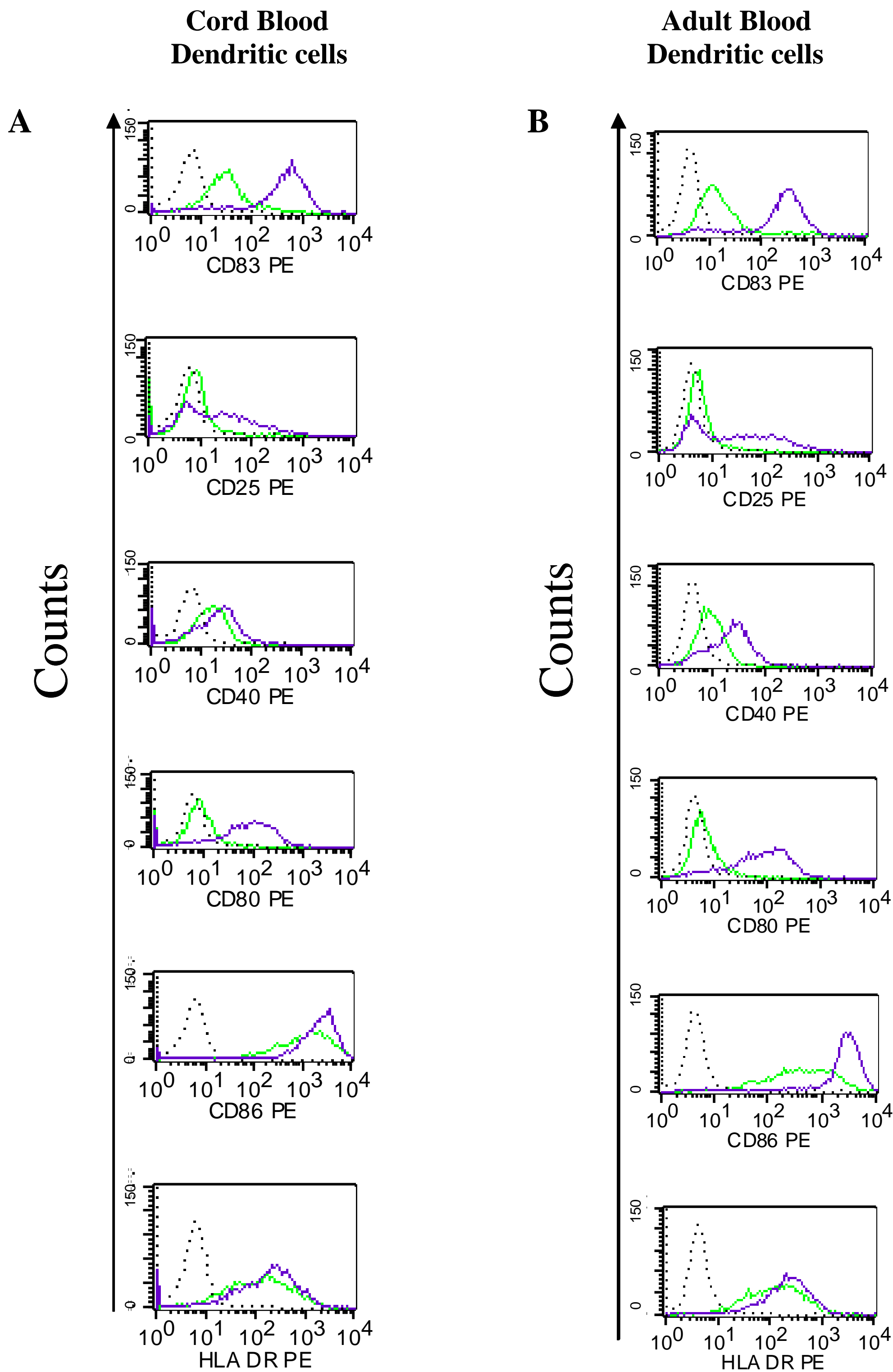


Figure 4.3 The histogram overlay of cord blood (A) and adult blood (B) monocyte-derived iDC and mDC.
The overlay indicates the change in expression for a particular marker in iDC and mDC. The green line indicates iDC and the purple line mDC. The black dotted line is the negative control. The histogram overlays are representative of those obtained for cord blood and adult blood cells.

4.3.3 Confirmation of in vitro *culture of mDC cells from cord blood*

To determine whether monocyte-derived cord blood DC had the same maturation capabilities of similarly derived adult DC, iDC (monocytes cultured for 6 days in presence of IL-4 and GM-CSF) and mDC (iDC cultured for an additional 2 days in presence of IL-4, GM-CSF, IL-1β, TNF-α and PGE_2) from adult and cord blood were stained with the DC maturation marker CD83 and the activation marker CD25 and these results compared. Cord blood derived mDC attained similar levels of expression of CD83 and CD25 to adult mDC (Figure 4.4 A and B). The percentage of cells expressing CD83 in cord blood iDC 10.1% (± 20.2%) and mDC 80.6% (± 13.8%) compared to adult blood iDC 12.9% (± 16.1%) and mDC 88.4% (± 9.4%) is shown in Figure 4.4 A and B respectively. Similar levels of expression of CD25, (the IL-2 receptor) were found in cord blood iDC 7.3% (± 7.1%) and mDC 57.3% (± 13.9%) and in adult blood iDC 1.0% (± 1.2%) and mDC 49.9% (± 16.9%) (Figure 4.4 A and B). In addition, statistically significant upregulation (using Wilcoxon matched pairs test) in the expression of CD83 (cord $p<0.005$; adult $p<0.05$) and CD25 (cord $p<0.05$; adult $p<0.05$) on iDC compared to mDC in both cord and adult derived cells occurred (Figure 4.4 A and B). These results confirm the culture of similarly matured DC from monocyte-derived cord compared to adult blood.

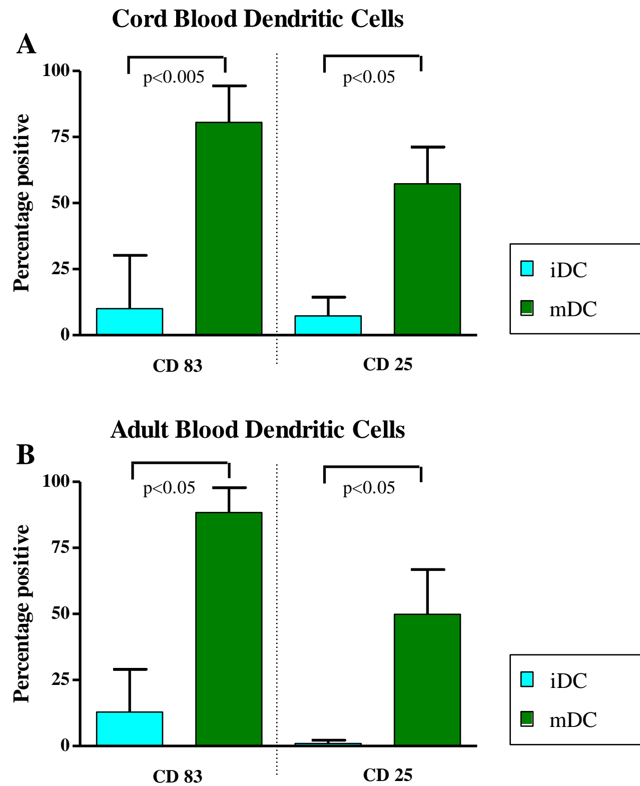
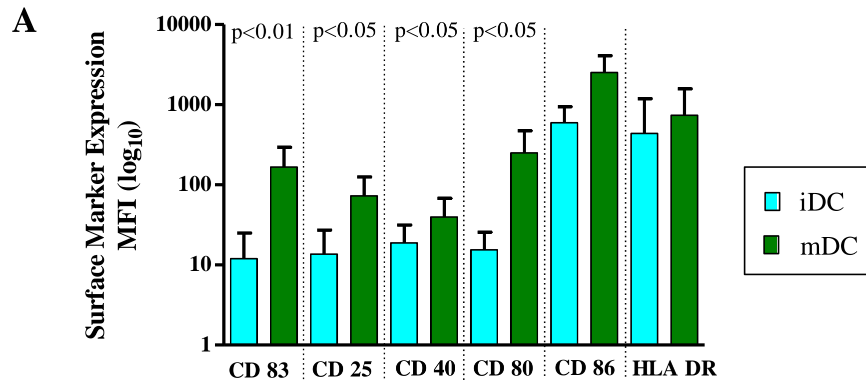


Figure 4.4 Maturation and activation of cultured iDC and mDC.
Percentage of cells expressing maturation (CD83) and activation marker (CD25) in cord blood (A) and adult blood (B). Mean percentage (+ SD) of cells staining positive for CD83 or CD25 is indicated. Statistically significant increase in expression is indicated using the Wilcoxon matched pairs test. n=6-10 cord blood (A) and n=7 adult blood (B) independent evaluations.

4.3.4 Phenotypic characterisation of immature and mature DC

To determine whether DCs from cord blood were similar to those obtained from adult blood, phenotypic characterisation of iDC and mDC was carried out. Significant upregulation (using Wilcoxon matched pairs test) in expression of CD83, CD25, CD40 and CD80 (p values for all markers <0.05 except cord CD83 p<0.01) occurred during maturation from immature to mature DC in both cord and adult blood derived cells (Figure 4.5 A and B). In addition, an increase in the expression of CD86 and HLA-DR occurred in mDC compared to iDC for cord blood DC and this increase reached significant levels for adult DC (p<0.05 for both markers) (Figure 4.5 A and B). Similar levels of expression of all these markers were measured in both cord blood (Figure 4.5 A) and adult blood (Figure 4.5 B) derived iDC and mDC, indicating similar differentiation and maturation abilities of cord DC compared to adult DC. To enable all results to be indicated in a single graph the MFI $\log_{10}$ results are shown.

Phenotypic characterisation of cord blood Dendritic Cells



Phenotypic characterisation of adult blood Dendritic Cells

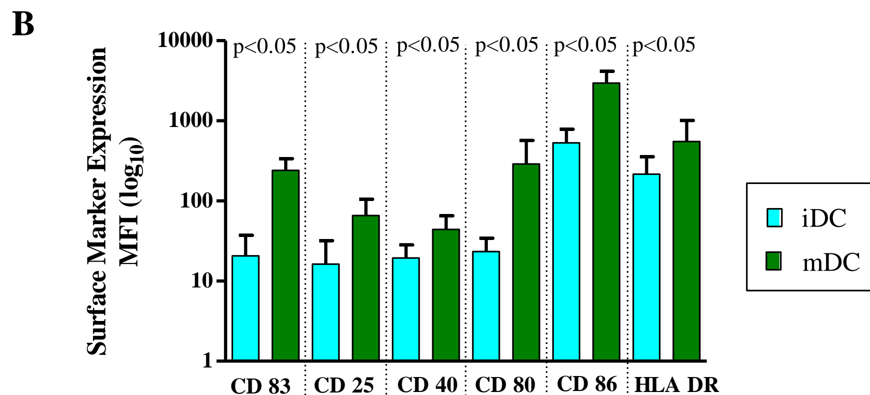


Figure 4.5 Phenotypic characterisation of iDC and mDC.
Monocyte-derived cord blood (A) and adult blood (B) iDC and mDC were stained for surface expression of CD83, CD25, CD40, CD80, CD86 and HLA-DR. Statistically significant increase in expression is indicated using the Wilcoxon matched pairs test. Results are expressed as mean MFI $\log_{10}$ (+ SD). n=6-10 cord blood (A) and n=7 adult blood (B) independent evaluations.

4.3.5 Phagocytosis of BCG-gfp by iDC and mDC

iDC are reported to be more phagocytic than mDC and as the cells mature, they become less phagocytic and the antigen presenting properties become upregulated (Savina and Amigorena, 2007). The phagocytic properties of iDC and mDC from cord blood were therefore compared to determine whether maturation could be confirmed for phagocytic properties. Prior to flow cytometric evaluation to determine the phagocytic properties, these cells were washed three times in an attempt to remove any bacteria that were adherent to the cells rather than engulfed by them. iDC from cord blood were found to be significantly more (p<0.05; Wilcoxon matched pairs test) phagocytic 25.6% (± 3.7%) as determined by increase in the uptake of BCG-gfp

compared to that of mDC 16.7% (± 2.9%) (Figure 4.6). iDC from adult blood engulfed 28.6% (± 3.2%) of BCG-gfp, whereas preliminary results from 2 volunteers indicated that there was a trend for mDC to be less phagocytic 19.5% (± 0.8%) as the DC matured (Figure 4.6).

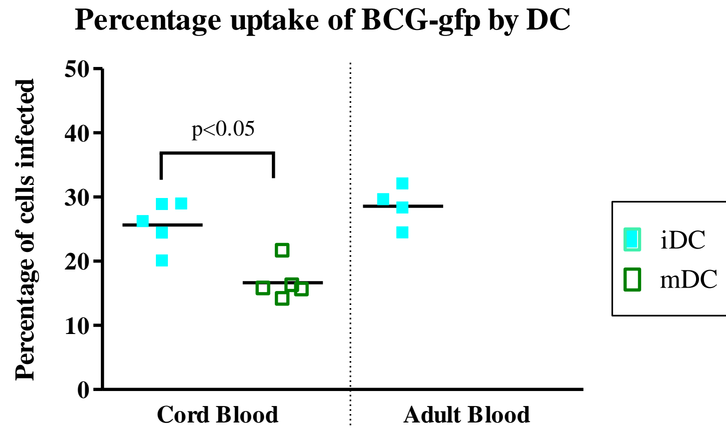


Figure 4.6 Phagocytic properties of iDC and mDC in cord blood.
Mean percentage of DC infected with BCG-gfp as determined by flow cytometric evaluation is indicated by a short line. Uninfected cells were used as a negative control.

4.3.6 Phenotypic evaluation of BCG infected DC compared to uninfected DC

A pilot study was undertaken to determine whether infection of monocyte-derived iDC and mDC with BCG-gfp caused upregulation in percentage expression of the maturation marker CD83 and activation marker CD25. Figure 4.7 indicates the mean percentage results obtained in cord blood and adult blood iDC and mDC. The results obtained are preliminary, but they seem to indicate a trend for further maturation and activation of imDC and mDC from both cord blood and adult blood derived DC following infection with BCG.

Effect of BCG infection on phenotypic maturation of DC

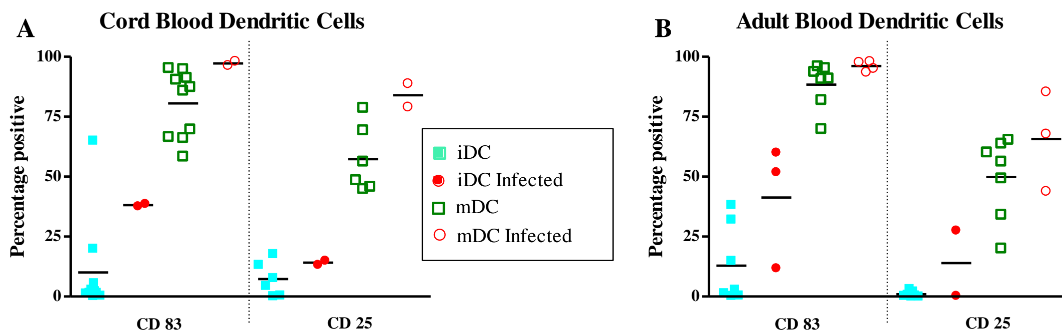


Figure 4.7 Pilot study indicating maturation and activation of DC following infection with BCG.
Percentage of cells expressing maturation (CD83) and activation marker (CD25) in cord blood (A) and adult blood (B). Mean percentage of cells staining positive for CD83 or CD25 is indicated by a short line.

4.3.7 Determination of neonatal mDC function

To determine whether these cord blood derived mDC were functional as APC, the ability of cord blood derived mDC to present antigen and to drive the proliferation of T cells *in vitro* was determined via an allogeneic lymphocyte reaction. The proliferative properties in CPM of these cord blood allogeneic lymphocyte reactions were compared to CPM from adult blood allogeneic lymphocyte reactions. A ratio of mDC to T cells of 1:50, 1:100, 1:300 and 1:1000 was used. As a negative control, autologous T cells and mDC from cord blood and autologous T cells and mDC from adult blood were cultured, and no proliferation was obtained. However, similar levels of proliferation were obtained when allogeneic T cells and mDC were cultured from both cord blood and adult blood derived cells (Figure 4.8). Greatest proliferation occurred at 1:50 for cord blood and 1:100 for adult blood which declined as the ratio of mDC to T cells decreased and was lowest at a ratio of 1:1000. These results indicate that cord blood mDC were functional and had the ability to present antigen and induce proliferation in an allogeneic lymphocyte reaction.

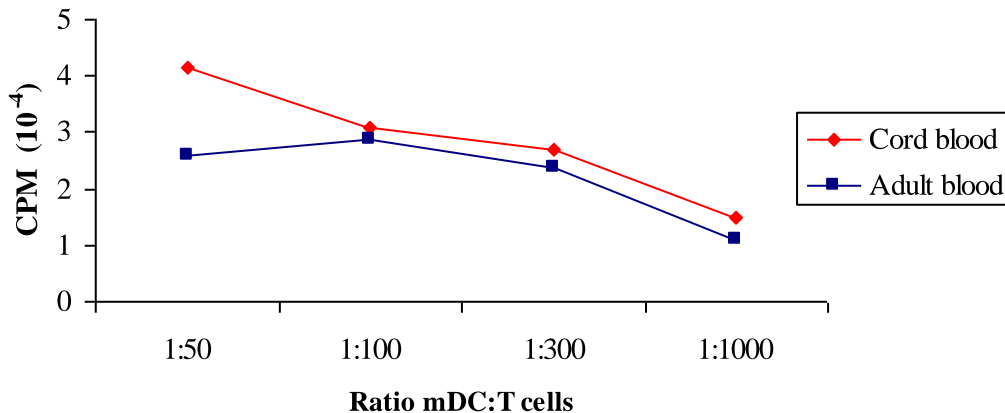


Figure 4.8 Proliferation of allogeneic T cells with mDC as APC.
T cells were cultured in the presence of allogeneic mDC from cord blood or adult blood. The mean of results in CPM is indicated for ratio of mDC:T cells of 1:50 to 1:1000. The unstimulated response has been subtracted from the stimulated response. In all experiments duplicate assays were set up using autologous T cells as a negative control, which showed no proliferation. n=4 cord blood and n=3 adult blood independent evaluations.

Another method to determine whether mDC from cord blood were functional was the evaluation of the ability of mDC to present antigen and stimulate the production of IFN-γ produced by T cells in an allogeneic lymphocyte reaction. In all experiments duplicate assays were set up using autologous T cells and mDC as APC in which only background levels of IFN-γ were measured. Adult T cells produced more IFN-γ than cord blood T cells although the range of results was greater in adult blood derived T cells. Maximum IFN-γ was measured at mDC:T cells of 1:50 (adults 1323pg/ml ± 1764pg/ml; cord blood 376 ± 308) and this amount declined as the ratio of mDC to T cells increased to 1:500 (adults 182pg/ml ± 190pg/ml; cord blood 87pg/ml ± 42pg/ml) (Figure 4.9). No IFN-γ was detectable in cord blood at a ratio of 1:1000. Cord blood derived mDC had the ability to stimulate the production of IFN-γ in an allogeneic lymphocyte reaction.

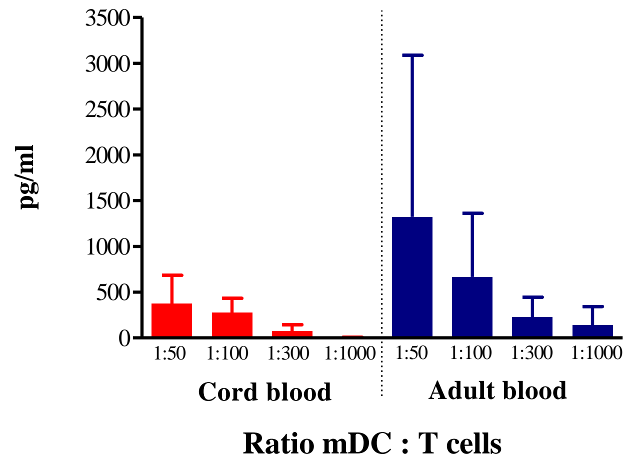


Figure 4.9 IFN-γ release from allogeneic T cells with mDC as APC.
T cells were cultured in the presence of allogeneic monocyte derived mDC from cord blood or adult blood in the ratio of mDC:T cells of 1:50; 1:100; 1:300 and 1:1000. The unstimulated response has been subtracted from the stimulated response. In all experiments duplicate assays were set up using autologous T^- cells which showed no IFN-γ release, as a negative control. n=3 independent evaluations for both cord blood and adult blood.

4.4 DISCUSSION

The hypothesis underlying the studies in this thesis is that **protection** against Mtb engendered by BCG vaccination is **quantitatively different** (i.e. more DC stimulation is seen post vaccination than prior to vaccination), but **not qualitatively** different (i.e. similar DC function exists before and after BCG vaccination) from the pre-vaccination innate **protective response** of neonates against this pathogen. The importance of DC as innate immune cells during Mtb infection has been highlighted in section 1.9.2 of Chapter 1 “Rationale for studying dendritic cell function”. This thesis is studying the innate response of naïve infants using cord blood, prior to vaccination or exposure to mycobacterial antigen. The second step was a comparison of DC function in neonates to that of BCG vaccinated adult volunteers.

The experiments in this chapter using neonatal DC from cord blood, confirm the following properties of neonatal DC: 1. Cord blood DC had the ability to differentiate and mature, which was demonstrated by the significant up regulation of maturation and activation markers to similar levels measured in adult DC. In addition, the phagocytic properties of DC, which diminished significantly during maturation

($p<0.05$), further indicated the normal maturation of these cells in cord blood. 2. Mature DC had the ability to present antigen and to drive the subsequent proliferation of T cells via an allogeneic lymphocyte reaction. 3. Cord blood-derived DC were able to stimulate the production of the Th1 cytokine IFN-γ in response to the allogeneic lymphocyte reaction, which confirms functional maturation of DC and their ability to effectively present antigen to naïve T cells. Furthermore, the morphology of cultured monocyte-derived DC from cord blood was comparable to DC cultured from adult blood, as determined by phase contrast light microscopy.

Langrish *et al*, reported reduced expression of CD25 and CD83 compared to adult DC (Langrish *et al.*, 2002), our findings that cord blood DC reached equivalent levels of maturation and activation as adult DC is therefore in contrast to this. In addition, both a reduction in HLA-DR expression (Hunt *et al.*, 1994; Petty and Hunt, 1998; Sorg *et al.*, 1999; Liu *et al.*, 2001; Langrish *et al.*, 2002; Drohan *et al.*, 2004) and similar levels of HLA DR expression in cord blood and adult blood DC have been reported (Zheng *et al.*, 2000). Canaday *et al* suggested that the decreased surface expression of HLA-DR on both monocyte-derived DC and neonatal DC could be a contributing factor to the decreased T cell response found in neonates, as the antigen presentation capabilities of neonatal DC would be suboptimal (Canaday *et al.*, 2006). The HLA-DR expression in cord blood mDC (740.1 ± 841.2) in this study had a slightly higher mean MFI expression than adult mDC but a greater range (552.9 ± 456.4). In this thesis neither a reduction in expression of HLA DR nor in antigen-presenting capabilities of cord blood derived DC were found. Furthermore, a reduction in cord blood DC CD40 expression compared to adult DC has been demonstrated (Goriely *et al.*, 2001; Drohan *et al.*, 2004). However in this study, CD40 expression in cord blood and adult blood mDC was comparable (39.7 ± 28.2 and 44.0 ± 21.0 respectively). With regards to CD80 expression of cord blood DC, a reduction in expression of this molecule has been reported compared to that of adults (Goriely *et al.*, 2001). Some researchers reported similar levels of expression of CD86 in cord and adult DC (Zheng *et al.*, 2000; Liu *et al.*, 2001), whilst others report lower expression in cord blood DC (Langrish *et al.*, 2002). In the current study, the expression of CD80 on the surface of cord blood mDC was equivalent to that of adult blood mDC of (249.4 ± 223.5 and 289.6 ± 281.3 respectively), as was CD86

expression (2517 ± 1569 and adult 2962 ± 1100). It was interesting to note that Dilioglou *et al* reported that the T cell co stimulatory molecule CD86 on cord blood monocyte-derived DC was found to be more effective in activating T cells than the CD80 molecule. These researchers found that blockage of the CD80 molecule on cord blood DC had negligible effects on T-cell responses, while CD86 blockage had detrimental effects on T-cell activation (blockage of CD80 on DC had 24% and CD86 had 71% reduction in naïve $CD4^+$ T cell activation respectively) (Dilioglou *et al.*, 2003 B). Although I measured similar levels of expression for CD80 and CD86, this was particularly interesting especially in the light of the report by Dilioglou *et al*; as the MFI of CD86 in the current study was approximately 10-fold more than the CD80 expression for both cord and adult mDC. The differences obtained in the expression of various markers in this study to that of published data could be attributed to the fact that different culture conditions, such as serum supplements and cocktail of lymphokines were used for the *in vitro* culture of DC from monocytes. This has the potential to induce different phenotypic and functional properties of the DC and in addition makes the comparison of results obtained from different studies particularly difficult (Dilioglou *et al.*, 2003 B). Another factor that might account for differences observed in DC, is the mode of delivery prior to collection of cord blood, as cord blood was collected following caesarean section and prior to commencement of labour in this study. However, in the other reported studies cord blood may have been collected following normal delivery and cytokines and hormones secreted during labour may account for these differences (Brown *et al.*, 2003; Protonotariou *et al.*, 2003), particularly cortisol, which is immunosuppressive and is induced during labour (Thornton *et al.*, 2003).

In this thesis I have observed that cord blood iDC were significantly more phagocytic than mDC ($p<0.05$) which is in agreement with published reports of adult DC (Steinman, 1991; Savina and Amigorena, 2007). In addition, published reports indicate that phagocytosis of pathogens by iDC causes maturation and activation of DC to become mature APC which then initiate adaptive immune recognition rather than the killing of the microbe (Bhatt and Salgame, 2007). This maturation and activation of DC therefore results in the differentiation of the DC to cells capable of stimulating an adaptive immune response, thereby modulating the immune response and link the innate and adaptive immune responses (Hope *et al.*, 2004; Savina and

Amigorena, 2007). Moreover, my pilot study indicated that up-regulation in expression of maturation and activation markers occurred on monocyte derived iDC and mDC following infection with BCG. This is in agreement with the findings by others, who reported that interaction of human DC with Mtb or BCG resulted in cell maturation and activation which was characterised by changes in cell surface phenotype (Hickman *et al.*, 2002; Liu *et al.*, 2003).

In this study, cord blood derived mDC were able to support an allogeneic lymphocyte reaction and proliferation of naïve T cells was obtained. In addition, these T cells had the ability to produce IFN-γ in response to allogeneic lymphocyte stimulation. While cord blood allogeneic lymphocyte proliferative responses reached similar levels to that of adult allogeneic lymphocyte responses, IFN-γ responses in cord blood allogeneic lymphocyte responses were lower than those of the adult allogeneic lymphocyte reaction, but the adult range was greater. These findings would imply that cord blood mDC were functional. Published reports provide conflicting results, with some studies reporting that cord blood monocyte derived DC showed a decreased ability to stimulate allogeneic responses compared to that of adults (Liu *et al.*, 2001; Velilla *et al.*, 2006). Matthews *et al* reported, that monocyte derived DC from cord blood caused greater proliferation in cord blood T cells than was found in adult T cells, yet equal amounts of Th1 cytokines were produced. Matthews and colleagues concluded that cord blood derived DC that were used to stimulate T cells, provided enough co-stimulation for these DC to induce naïve $CD8^+$ T cell derived Th1 cytokine production (Matthews *et al.*, 2000). In contrast Borras *et al* report that freshly isolated cord blood DC failed to induce a potent allo-stimulation of cord blood T cells, which these researchers concluded indicated that circulating DC were functionally incompetent (Borras *et al.*, 2001). Zheng *et al* measured a similar capacity of cord blood DC to induce allogeneic lymphocyte proliferation as adult DC (Zheng *et al.*, 2000). Salio and colleagues report that sufficiently activated cord blood monocyte derived DC could efficiently prime antigen-specific $CD8^+$ T cells capable of both cytolytic activity and IFN-γ secretion (Salio *et al.*, 2003). Langrish and colleagues found a reduced capacity of cord blood DC to induce IFN-γ production in $CD4^+$ T cells compared to adult DC (Langrish *et al.*, 2002). In contrast, Velila and colleagues suggest that functional defects in APC from neonates and young infants

contribute to the functional immaturity of the immune system of newborns. These authors propose that a defect of APC due to their incomplete activation and/or maturation in delivering costimulatory signals to T cells exists. They also propose that neonatal monocyte derived DC required a higher level of activation than adult mDC (Velilla *et al.*, 2006).

Once again, differences in these published reports could be due to the fact that different conditions were used for the *in vitro* culture of DC or mode of delivery of the infants studied. Circulating DC are exposed to cellular interactions and various cytokines which may alter the maturation or APC function of these cells (Schibler *et al.*, 2002). In the circulation, a difference in the number of DC or an imbalance in DC subsets could explain defects in APC function of neonatal DC. Cord blood cells are reported to have a reduced frequency of cytokine producing cells and a reduced ability to respond to cytokines compared to adult cells (Cohen *et al.*, 2000 B). An increase in IL-12 production of adult DC compared to cord blood DC has also been reported (Goriely *et al.*, 2001; Langrish *et al.*, 2002). In cord blood an increase in the ratio of plasmacytoid/lymphoid DC to myeloid DC compared to adult levels have been described (Borras *et al.*, 2001). Myeloid DC were the predominant DC type in adult blood, whereas in cord blood, lymphoid DC were increased, however the significance of this inverted ratio in cord blood is unclear (Borras *et al.*, 2001; Navarrete *et al.*, 2003). Myeloid DC produce large quantities of IL-12, a Th1 polarising factor (Navarrete *et al.*, 2003) and if myeloid DC are reduced in neonatal circulation compared to adult blood, this factor could explain differences in APC function of neonates. However, in contrast to the reports of an inverted ratio of DC, Schibler *et al* found that an imbalance of myeloid to lymphoid DC does not exist and therefore these researchers conclude that this finding does not cause the reduced adaptive immune response measured in neonates (Schibler *et al.*, 2002).

The ability of cord blood mDC to support an allogeneic lymphocyte reaction and to produce IFN-γ observed in the present studies, appears to indicate that the functional capacity of neonatal DC are intact. This finding has important implications for the development of vaccine strategies in early life. Marchant *et al* stated that identification of vaccines that target DC and result in T cell activation in human

neonates may result in a more efficient and better protective response against intracellular pathogens such as Mtb (Marchant and Goldman, 2005). In addition, Siegrist reported that the impairment of $CD4^+$ and $CD8^+$ T-cell function in early life results from suboptimal APC-T cell interactions and that these can be overcome by the use of specific adjuvants or delivery systems (Siegrist, 2001). Since DC can be used as adjuvants in vaccines for the regulation of antigen specific immunity, it appears from this current study that a vaccine to Mtb which targets DC may be beneficial (Steinman and Pope, 2002).

CHAPTER 5

CYTOKINE RESPONSES OF NEONATES TO BCG BEFORE AND AFTER BCG VACCINATION

5.1 INTRODUCTION

BCG was introduced as an antituberculosis vaccine over 90 years ago and has since become one of the most widely used of all vaccines (Fine *et al.*, 1999; WHO, 2004). Approximately 120 million children are vaccinated with BCG worldwide each year (WHO, 2004; Trunz *et al.*, 2006; Ritz *et al.*, 2008). BCG vaccination of children has proven protection against TB meningitis (75-87%) and miliary TB, but it is variably effective against pulmonary disease at all ages or against reactivation of latent pulmonary infection in adults (WHO, 2004; Walker *et al.*, 2006). Consequently, in spite of extensive BCG vaccination in many parts of the world, TB rates are high with about 9.27 million new cases and 1.78 million deaths from TB in 2007 (WHO, 2009). South Africa ranks 5th in the world after Nigeria for TB incidence of all forms with 948 per 100 000 population (WHO, 2009). Because TB is endemic in South Africa, infants are vaccinated with Danish BCG shortly after birth (Mahomed *et al.*, 2006). Despite >95% BCG vaccination coverage, the TB disease rate in babies remains very high in some areas of South Africa exceeding 2% per year in under 2 year olds (Hanekom, 2005). The nature of the cytokine-mediated immune response prior to and following BCG vaccination is the subject of this chapter.

Type 1 immunity is critical for protection against TB (Rosenzweig and Holland, 2005; Bhatt and Salgame, 2007). However, during pregnancy, the maternal cytokine milieu is reported to be dominated by Th2 type immunity, a response considered essential to prevent rejection of the foetus (Lin *et al.*, 1993; Wegmann *et al.*, 1993). Hormones such as progesterone and the cytokine IL-10 and other soluble mediators which are present at the maternal-foetal interface, suppress the Th1 response in the neonate while still *in utero*. Both of these factors are produced by trophoblasts which are known to be potent Th2 inducers (Krishnan *et al.*, 1996; Roth *et al.*, 1996;

Szekeres-Bartho *et al.*, 1996; Ng *et al.*, 2002; Kidd, 2003; Salem, 2004; Piccinni, 2006). Clinical evidence that supports this Th2 cytokine bias in pregnancy, includes the observation that a large percentage of women with rheumatoid arthritis (a Th1 cell-mediated autoimmune disorder) experience a temporary remission of their symptoms during pregnancy and a higher risk of post-partum relapse (Lin *et al.*, 1993; Wegmann *et al.*, 1993; Szekeres-Bartho, 2002; Aluvihare *et al.*, 2005). In contrast, patients with systemic lupus erythematosis (SLE; mediated by Th2 and excessive autoantibody production) usually experience an aggravation of their disease during gestation (Lin *et al.*, 1993; Wegmann *et al.*, 1993; Szekeres-Bartho, 2002; Kidd, 2003). After birth, the newborn infant is believed to have a Th2-biased immune system (Delespesse *et al.*, 1998; Amoudruz *et al.*, 2005). This immunologic bias during the first few months of life, appears to be associated with neonatal dependence on maternal antibodies that cross the placenta providing protection against extracellular organisms (Zinkernagel, 2001). In contrast, maternal lymphocytes required for a sustained Th1 protective immune response, do not cross the placenta and so newborn infants are not protected against intracellular organisms such as mycobacteria (Zinkernagel, 2001). Thus, to provide the infant with protection against infectious organisms, a rapid switch to production of Th1 cytokines is required (Mosmann and Sad, 1996; Flynn and Chan, 2001). This supposed shift to Th1 cytokine production is critically important and is the subject of this part of the current study (Wegmann *et al.*, 1993; Rosenzweig and Holland, 2005; Bhatt and Salgame, 2007).

Although murine neonatal immune responses have been extensively studied, little is known about the immune response of human neonates to mycobacteria prior to vaccination with BCG (Flynn and Chan, 2001). Since only very small volumes of blood can be obtained from newborns prior to BCG vaccination, most studies have utilised cord blood as a source for such studies. Ota and co-workers reported low levels of IFN-γ, IL-5 and IL-13 following stimulation of cord blood with PPD (Ota *et al.*, 2002). Another study that assessed responses of cord blood to mycobacteria found low levels of secreted IFN-γ, IL-10 and IL-5 following incubation with BCG (Hussey *et al.*, 2002). It has not been clear which cells produce these cytokines and how IFN-γ is induced in the naïve host prior to exposure to mycobacterial antigens

and the induction of an acquired T cell response. Marchant and colleagues also reported that in response to PPD, blood from unvaccinated infants demonstrated a predominantly Th2 response. In contrast, after BCG vaccination a Th1 memory response was noted in 2 month old neonates as well as at 1 year (Marchant *et al.*, 1999). Furthermore, Hussey and co-workers found that BCG vaccination at birth induced strong lymphoproliferation and Th1 cytokine response at 10 weeks of age following *in vitro* stimulation of PBMC with mycobacterial antigens (Hussey *et al.*, 2002).

In this chapter, I attempt to test the hypothesis that, cytokine-mediated **protection against Mtb** engendered by BCG vaccination is **quantitatively different** (i.e. higher amounts of soluble mediators are measured after vaccination), but **not qualitatively** different (i.e. a similar profile of cytokines are detected pre and post BCG vaccination) from the pre-vaccination cytokine-mediated **protective response** of neonates against the pathogen. The following properties of neonatal BCG-induced cytokine production will be studied:

1. Secretion of type 1 cytokines, IFN-γ and IL-12 and type 2 cytokines IL-5, IL-13, IL-4 as well as IL-10 a regulatory cytokine from BCG stimulated cultured cord blood mononuclear cells
2. Intracellular IFN-γ and IL-10 levels following *in vitro* BCG stimulation of cord blood cells **(pre vaccination)**.
3. Intracellular IFN-γ and IL-10 produced following *in vitro* BCG-induced stimulation of peripheral blood cells from infants' **post-BCG vaccination.**

The intracellular cytokine response in cord blood was compared to the response obtained in peripheral blood at 13 weeks post-BCG vaccination. Neonatal T cells obtained from cord blood are naïve and have not been exposed to mycobacterial antigen, therefore no IFN-γ is expected to be detected within these cells. In contrast, the production of IFN-γ by T cells following BCG-induced adaptive immunity is expected to be relatively high and long lived. Any IFN-γ produced by cord blood cells would be expected to be made by NK cells or other cells of the innate response. As the number of circulating NK cells is low (about 1 % of mononuclear leukocytes) a large volume of cord blood is required for sufficient gated events to be obtained in the evaluation of NK intracellular cytokines. However, it is not possible to determine

intracellular IFN-γ within NK cells post vaccination due to the small volumes of blood available. It would have been preferable to compare the cytokine response in the peripheral blood obtained from infants who had not received BCG vaccination to responses of infant bloods post BCG vaccination. However, not vaccinating infants with BCG is not ethical in South Africa where TB is endemic, and BCG vaccination has proven protection against miliary TB and TB meningitis.

5.2 MATERIALS AND METHODS

5.2.1 Study Participants

All infants in this study were vaccinated shortly after birth with Danish BCG by the intradermal route. A total of 23 infant cord bloods (i.e. prior to vaccination or exposure to mycobacterial antigen) were studied for the measurement of secreted and intracellular cytokines. Different assays were carried out on subsets of cord blood samples dependant on the volume of cord blood obtained. The mean age of the mothers was 29.5 (± 6) years and the mean gestation period was 38.1 (± 0.3) weeks. The TB history of the mothers was not known, but none of the mothers had active TB at the time of caesarean section. Twelve (52%) infants were female. The mean weights of the neonates and of the placentas were 3120g (± 515g) and 685g (± 194g) respectively. The mean placental weight was slightly more than the described range of 400 – 600g (Perrin and Sander, 1984). All infants had immediate Apgar scores ≥8 and 5-minute Apgar scores of 9 or 10, which would be considered healthy in the newborn infant. In addition, peripheral blood was collected at 13 weeks of age from six healthy infants in whom cord blood had been studied previously. The mean birth weight of these infants was 2981g (± 164g). The ratio of female to male infants was 5:1. The mean age at which peripheral blood was collected was 92 days (± 3 days).

5.2.2 **In vitro** *antigen stimulation for secreted cytokine evaluation in cord blood mononuclear cells*

To determine the quantity of secreted cytokines, CBMC and purified $CD14^+$ cells were incubated *in vitro* with Danish BCG. SEB was used as a positive control. The amount of secreted cytokines (IFN-γ, IL-12, IL-10, IL-5, IL-4 and IL-13) present in the culture SNF of CBMC and $CD14^+$ purified monocytes was expressed as pg/ml after subtraction of cytokine levels in unstimulated cultures.

5.2.3 Whole cord blood (pre-vaccination) and peripheral blood (post-vaccination with BCG) intracellular cytokine detection

ICC levels in whole cord blood and peripheral blood from 13 week old infants were determined as described in Chapter 2. WCB or peripheral blood (0.5-2ml) was exposed to lyophilised clinical Danish BCG vaccine. SEB was used as a positive control. Quantification of IFN-γ and IL-10 ICC was performed in $CD4^+$ and $CD8^+$ cells and $CD14^+$ monocytes. Intracellular IFN-γ only was evaluated in $CD3^-CD56^+$ NK cells (using 10-12ml WCB). ICC results are presented as percent of cells exposed to stimulant (BCG or SEB) that stained positive following subtraction of the unstimulated response. A result >0.1% was considered positive as this was the threshold of detection for this assay.

5.2.4 Statistical analysis

Results are reported as mean ± SD. Normality assumption of data was tested using Shapiro-Wilks test. Owing to the fact that data were found to be non-normally distributed, non-parametric tests were used. To compare data from 2 groups, the Mann-Whitney test was used for unpaired data and the Wilcoxon test was used for paired data. To compare data from more than two groups, the Kruskal-Wallis ANOVA test was used for unpaired data and the Friedman ANOVA test was used for paired data. Data were analyzed by using Statistica (version 9, StatSoft, Inc., Tulsa, OK, USA).

5.3 RESULTS

5.3.1 BCG-induced cord blood mononuclear and $CD14^+$ cell cytokine secretion

To determine whether Th1-type cytokines were produced by cord blood cells prior to BCG vaccination or whether there was a predominance of Th2-type cytokines, the profile and levels of cytokine production in cord blood were determined. CBMC were incubated *in vitro* with Danish BCG for optimum periods of time as previously described in chapter 2, which was 2 days (IL-10 and IL-12) or 6 days (IFN-γ, IL-13, IL-5 and IL-4) and cytokine release in culture SNF was measured. High levels of

IFN-γ, lower levels of IL-10 and IL-12 and minimal amounts of IL-13 and IL-5 were detected; IL-4 was not measurable (Figure 5.1 A). When very elevated levels of cytokine were detected, the samples were repeated in dilution so that accurate results could be obtained. When $CD14^+$ were purified from the CBMC and incubated with BCG, raised levels of both IL-10 and IL-12 and very low levels of IFN-γ were measured (Figure 5.1 B). IL-5, IL-13 and IL-4 were not produced by the $CD14^+$ monocytes. Thus, the high IFN-γ produced by BCG stimulated CBMC was derived primarily from populations other than monocytes, whereas IL-10 and IL-12 were produced mostly by these cells. When CBMC were stimulated with the polyclonal superantigen SEB, as a positive control, for general T cell reactivity in newborns, elevated levels of IFN-γ (27602 ± 20494) and lower levels of IL-10 (1953 ± 2825) were detected (Figure 5.1 C). Similar quantities of IL-12 (369 ± 292) and IL-13 (437 ± 342) were found and minimal IL-5 but no IL-4 were detected. In contrast, purified $CD14^+$ monocytes exposed to SEB produced very little IFN-γ and essentially no IL-10, IL-12, IL-5, IL-13 or IL-4 (Figure 5.1 D). Thus, while CBMC responded both to the microbial antigens BCG and to SEB, monocytes responded only to BCG. As monocytes do not respond to SEB, this result confirmed that the $CD14^+$ monocytes were not contaminated with T cells.

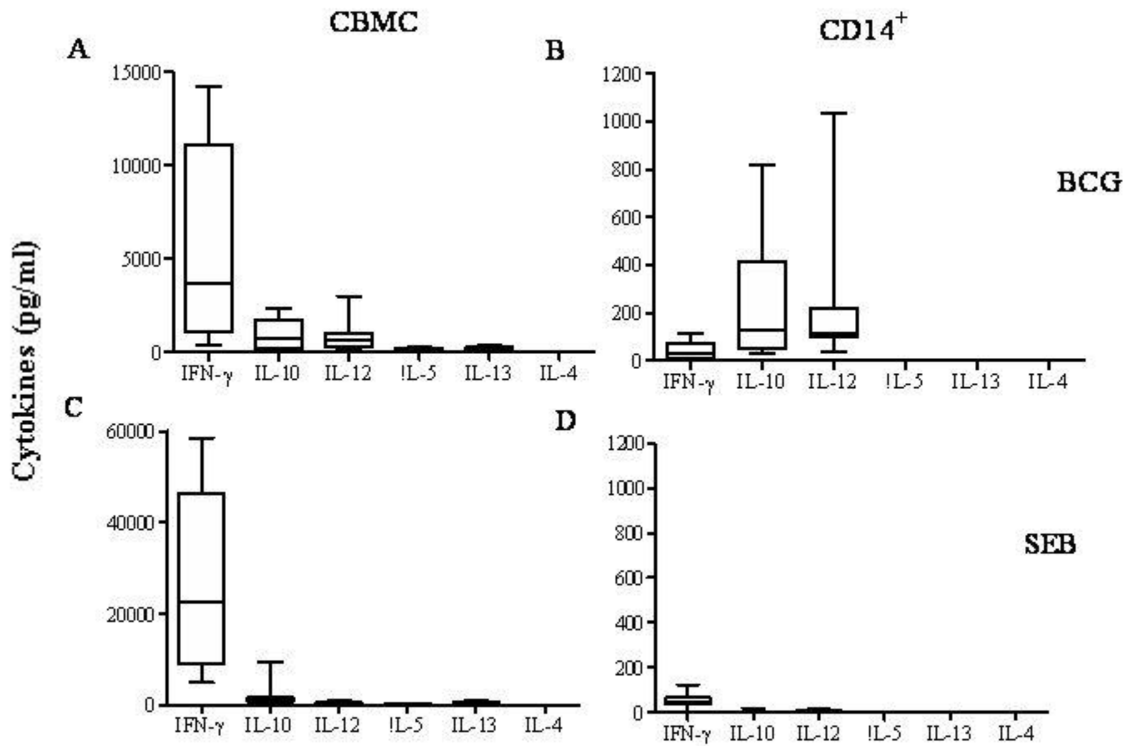


Figure 5.1 Secreted cytokines from cord blood mononuclear cells (CBMC) and CD14^{+} monocyte stimulated cells.
CBMC (A and C) and CD14^{+} cells (B and D) were stimulated with BCG or SEB and cytokine levels determined in SNF. IL-10 and IL-12 were measured after 2 days whereas IFN-γ, IL-5, IL-13 and IL-4 after 6 days stimulation. The unstimulated response has been subtracted from all results. Median and IQR are indicated by the line and box respectively. Minimum and maximum results are indicated by the whiskers. n=6-9 independent cord blood evaluations. Please note different scales for all graphs.

5.3.2 Whole cord blood intracellular cytokine production prior to BCG vaccination

As IFN-γ is an important cytokine and crucial in the control of Mtb, the fact that it was measured in BCG stimulated cord blood prior to vaccination with BCG was an important finding. It was essential to determine which cells were producing this cytokine. Because of the importance of IL-10 in the successful outcome of pregnancy, as reported by many authors, it was also crucial to identify which cells were producing this cytokine. To directly establish which cell populations produced IFN-γ and IL-10, cell-specific cytokine production was assessed by an ICC assay in WCB. ICC IFN-γ was determined within CD4^{+} and CD8^{+} T cells, CD4^{-}CD8^{-} cells, CD14^{+} cells and CD3^{-}CD56^{+} NK cells. ICC IL-10 was measured within CD4^{+} and CD8^{+} T cells, CD4^{-}CD8^{-} cells and CD14^{+} cells. Due to the fact that a large volume of blood was required for the determination of ICC IFN-γ within NK cells, this was only

performed when sufficient cord blood was obtained, which resulted in fewer results for this cell type. Figure 5.2 is not the gating strategy that was utilised (as shown in Chapter 2), but these dot plots generated on the FACsCalibur and analysed using Cell Quest software are used as a means of visual comparison of; ICC IFN-γ in $CD8^+$ T cells (A and B); ICC IFN-γ within $CD14^+$ cells (C and D); ICC IL-10 within $CD14^+$ cells (E and F). Figure 5.3 shows ICC IFN-γ within unstimulated $CD3^-CD56^+$ NK cells (A and B), and following SEB (C and D) or BCG stimulation (E and F) of WCB. The response of unstimulated cells was subtracted from the response of stimulated cells. The background frequency of IFN-γ was markedly higher in NK cells compared with T cells (Figure 5.3 A and B).

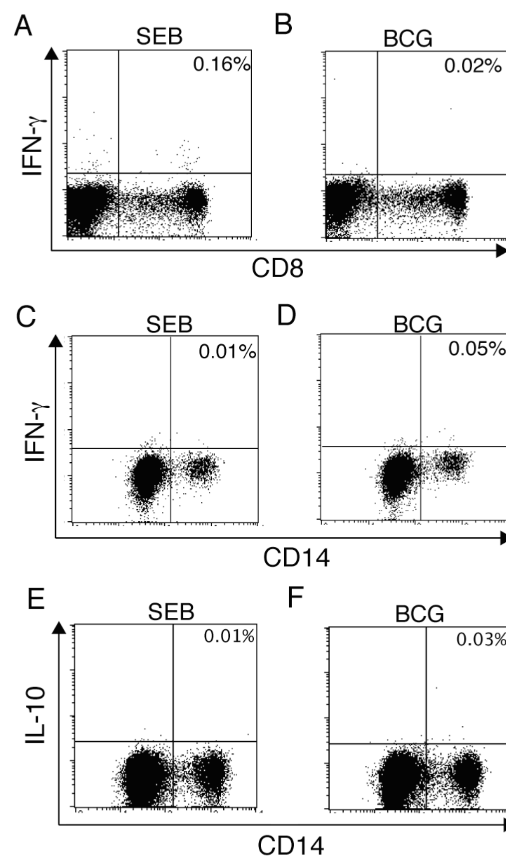


Figure 5.2 Flow cytometric dot plots from stimulated whole cord blood (WCB) for intra-cellular cytokine (ICC) evaluation.
IFN-γ ICC within $CD8^+$ cells (A and B) and $CD14^+$ cells (C and D) and IL-10 ICC within $CD14^+$cells (E and F) following SEB or BCG stimulation.

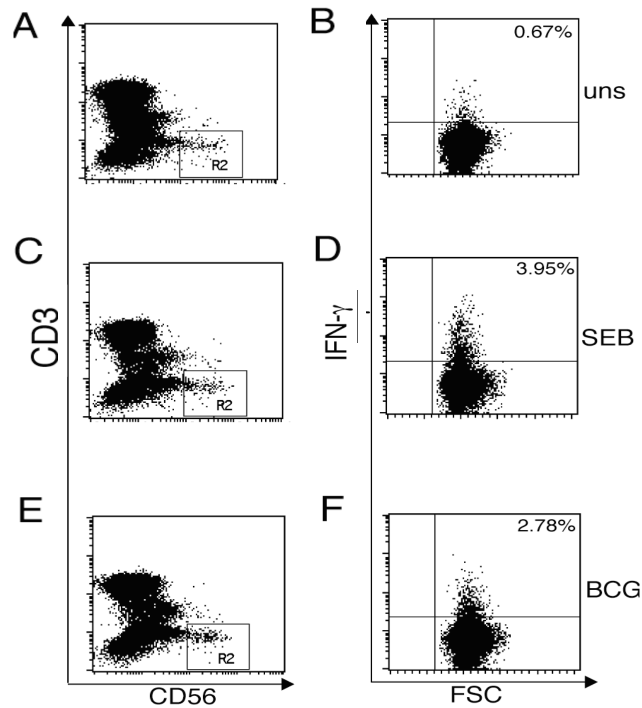


Figure 5.3 Flow cytometric dot plots from stimulated WCB for ICC evaluation of NK cells. NK cells defined as $CD3^-CD56^+$ were selected in R2 (A, C and E); only 1% of cells are indicated as 8–10 million cells were acquired for >15 000 gated events to be obtained. IFN-γ ICC within NK cells unstimulated (B), SEB (D) or BCG (F) stimulated; 100% of cells are shown.

The percentage of $CD4^+$ or $CD8^+$ cells, $CD4^-CD8^-$ cells, $CD14^+$ monocytes and $CD3^-CD56^+$ NK cells expressing intracellular IFN-γ after *ex vivo* stimulation of cord blood with Danish BCG is shown in Figure 5.4 A. Clearly, the IFN-γ produced by CBMC exposed to BCG was predominantly a product of NK cells, with a very minor contribution from $CD8^+$ cells, in two donors out of seventeen cord blood samples analysed. IL-10 was produced by $CD14^+$ monocytes and not by $CD4^+$ or $CD8^+$ cells (Figure 5.4 B). When SEB was used to stimulate cytokine production by whole cord blood, low levels of IFN-γ were produced by $CD4^+$ and $CD8^+$ cells as well as $CD4^-CD8^-$ cells compared to $CD3^-CD56^+$ NK cells (Figure 5.4 C). Similar to the observations made with BCG stimulated WCB, $CD8^+$ cells were more responsive in terms of IFN-γ production compared to the other T cell subsets. IL-10 was essentially not induced by stimulation with the polyclonal superantigen SEB (Figure 5.4 D).

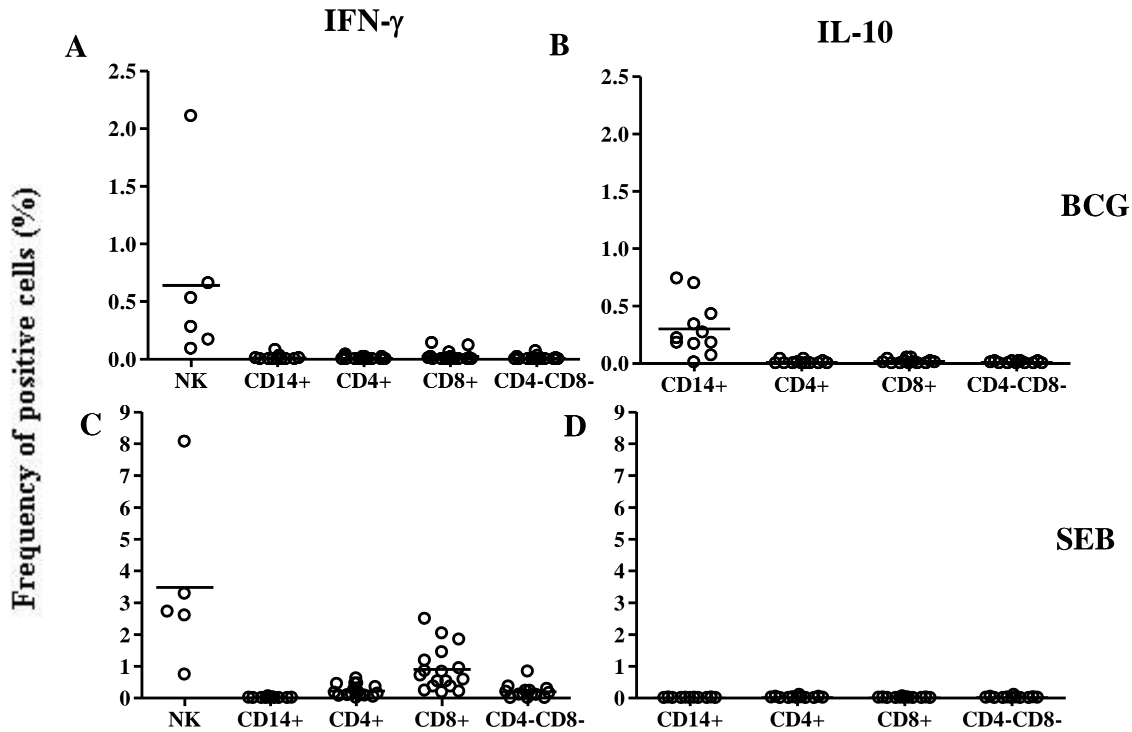


Figure 5.4 ICC response to whole cord blood stimulation with BCG or SEB.
IFN-γ ICC (A and C) and IL-10 ICC (B and D) following stimulation. Results are expressed as the percentage frequency of positive cells following subtraction of the unstimulated response. Mean is indicated by a short horizontal line. n=6-17 independent cord blood evaluations. Please note different scales.

5.3.3 Whole peripheral blood intracellular cytokine production post BCG vaccination

To determine whether the cytokine profile differed in infants following BCG vaccination, to that of cord blood; ICC production from infants at 13 weeks of age following BCG vaccination at birth was evaluated. Peripheral blood was stimulated *ex vivo* with BCG, and ICC producing cells identified, as described for cord blood. Since no more than 8mls of blood could be collected from these infants, it was not possible to determine the NK ICC response post vaccination, as these assays required a minimum of 2mls per treatment condition (i.e. 6mls for NK ICC evaluation alone) to ensure that at least 15 000 gated events were obtained. Figure 5.5 A and B shows representative examples of dot plots generated on the FACsCalibur and analysed using Cell Quest software from the peripheral blood of a 13 week infant for ICC IFN-γ in $CD8^+$ T cells following either BCG (A) or SEB (B) stimulation. To assure selection of only $CD4^+$ and $CD8^+$ T cells, a different gating strategy was used with 13 week old infants. $CD3^+CD4^+$ or $CD3^+CD8^+$ cells were selected and these cells

stained for the presence of ICC IFN-γ or ICC IL-10. Post BCG vaccination, T cells acquired the ability to produce IFN-γ in response to stimulation with BCG. An increase in the percent of $CD4^+$ and $CD8^+$ T cells producing intracellular IFN-γ between cord blood (Figure 5.4 A) and peripheral blood tested at 13 weeks after vaccination was found (Figure 5.6 A). Figure 5.6 B shows the ICC IFN-γ and IL-10 production by cells from 13 week infant peripheral blood following stimulation with SEB. IFN-γ levels in $CD4^+$ and more so in $CD8^+$ T cells increased and became strongly positive by 13 weeks of age. In addition, $CD4^+$ T cells obtained from some infants acquired the ability to produce IL-10 (Figure 5.6 B). During incubation with BCG, CD14 was downregulated therefore the ICC profile of these cells could not be determined accurately.

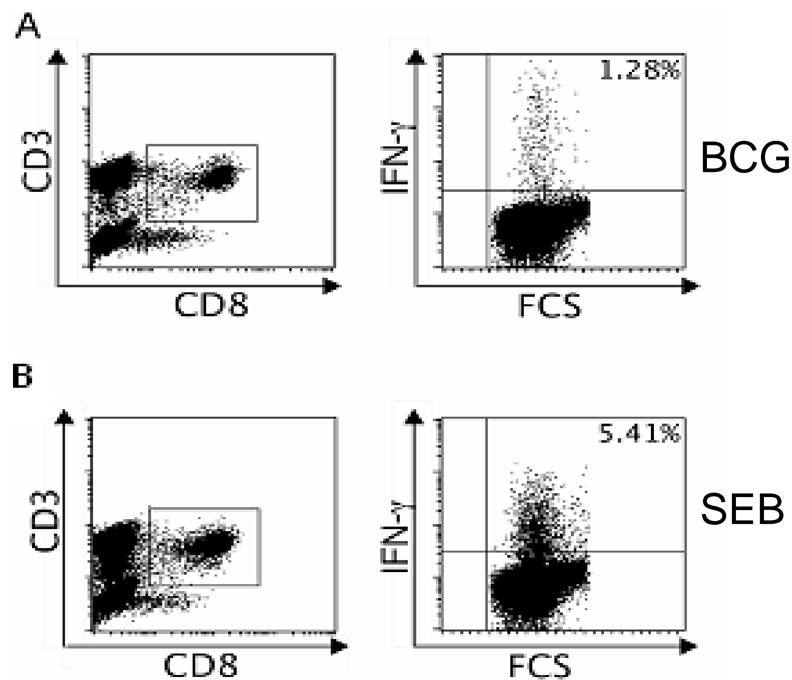


Figure 5.5 Flow cytometric dot plots for ICC evaluation from peripheral blood in 13 week old infants.
Dot plots of IFN-γ ICC within $CD3^+CD8^+$ T cells following BCG (A) or SEB stimulation (B).

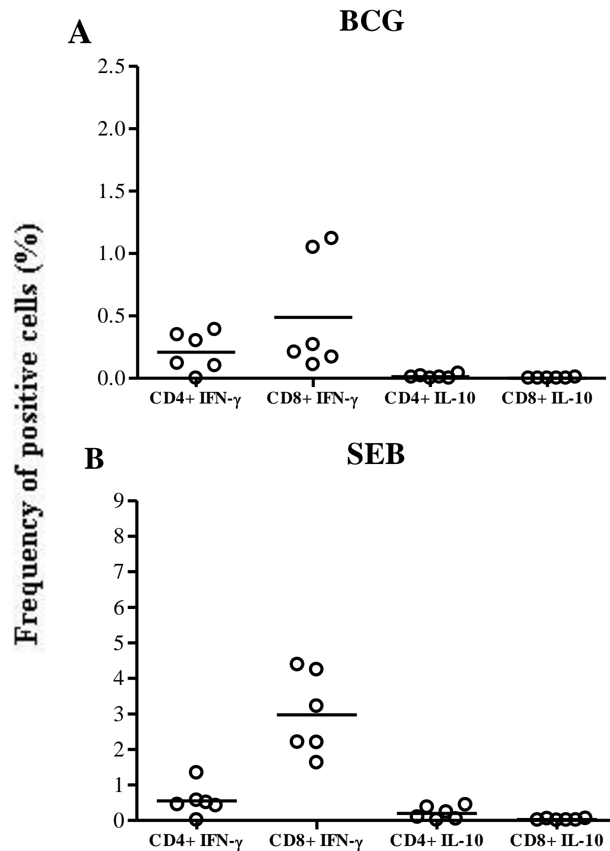


Figure 5.6 IFN-γ ICC and IL-10 ICC from peripheral blood in 13 week old infants following stimulation with BCG (A) or SEB (B).
Results are expressed as the percentage frequency of positive cells following subtraction of the unstimulated response. Mean is indicated by a short horizontal line. n=6 infants 13 weeks of age. Please note different scales.

Figure 5.7 shows the matched ICC response in the cord blood and in the peripheral blood at 13 weeks of age in the same infants. After *ex vivo* stimulation with BCG there was a significant increase in ICC IFN-γ in $CD8^+$ T cells ($p<0.05$; Wilcoxon test) when the matched infant bloods were compared with their response in cord blood (Figure 5.7 A). When the $CD4^+$ T cells were analysed, a small increase in ICC IFN-γ producing cells was noted in 4 of the 6 neonates (Figure 5.7 B). IL-10 expression was not induced in response to *ex vivo* BCG stimulation in either $CD4^+$ or $CD8^+$ T cells of any of the vaccinated neonates tested (Figure 5.7 E and F). When SEB induced T cell IFN-γ was examined, a significant increase in the percentage of $CD8^+$cells that were IFN-γ^+ (Figure 5.7 C) was seen ($p<0.05$; Wilcoxon test). Similar to the findings with BCG stimulation, $CD4^+$ T cells from vaccinated infants displayed a smaller increase upon SEB stimulation compared with $CD8^+$ T cells; consequently the results were not statistically significant (Figure 5.7 D). In contrast, *ex vivo* stimulation with SEB

resulted in a greater increase in the frequency of CD4^{+} IL-10^{+} T cells, which attained statistical significance ($p<0.05$; Wilcoxon test) in the blood of vaccinated neonates compared with their cord blood (Figure 5.7 H); which was not seen for CD8^{+} T cells (Figure 5.7 G).

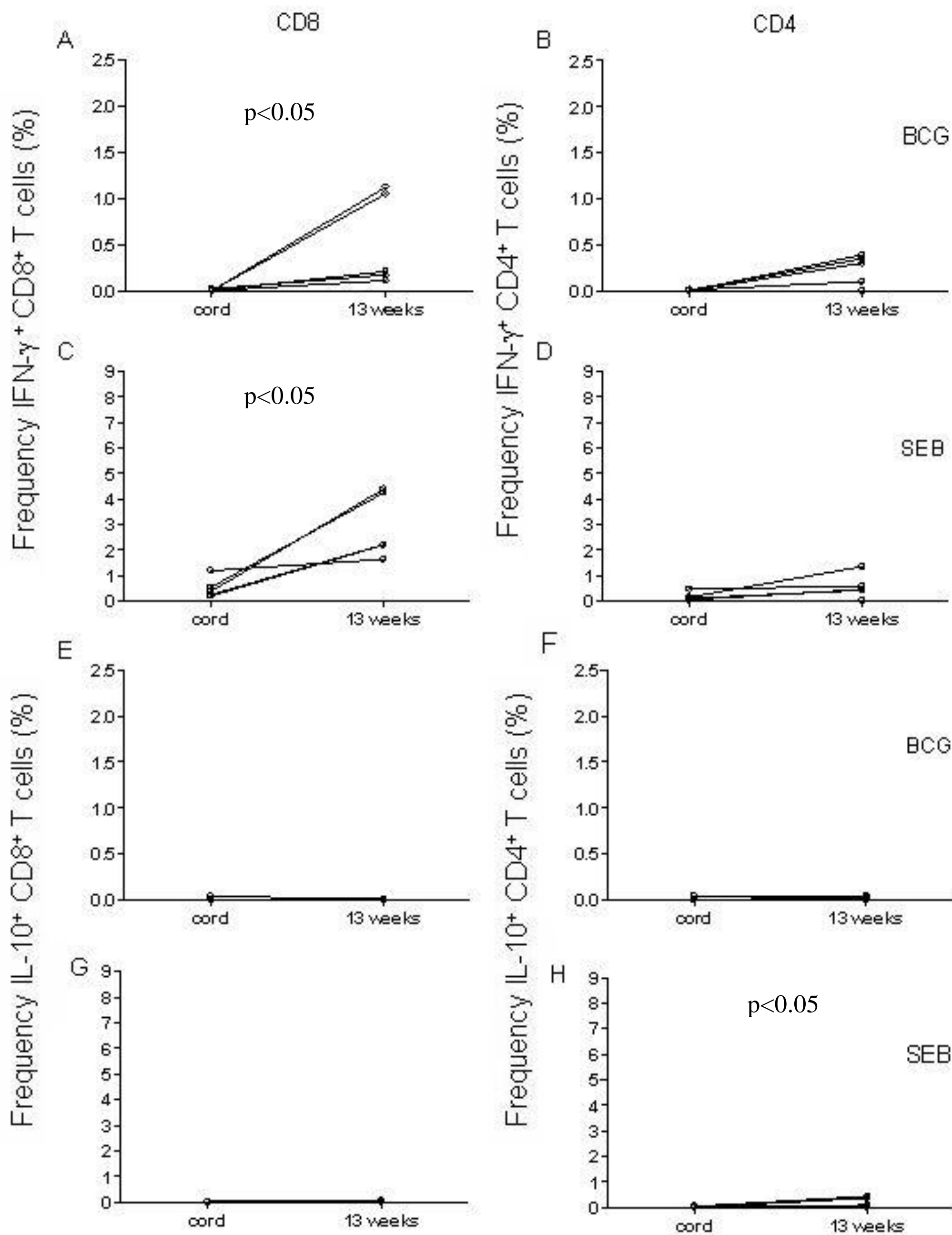


Figure 5.7 ICC response in stimulated WCB and peripheral blood at 13 weeks of age in the same infants.
IFN-γ ICC response in CD8^{+} T cells (A and C) and CD4^{+} T cells (B and D) following BCG or SEB stimulation. IL-10 ICC response following stimulation in CD8^{+} T cells (E and G) and CD4^{+} T cells (F and H). Results are expressed as the percentage frequency of positive cells following subtraction of the unstimulated response. Please note different scales in the graphs.

5.4 DISCUSSION

Experiments in this chapter show that the type 1 cytokines IFN-γ and IL-12 and the regulatory cytokine IL-10 were produced by CBMC after *in vitro* exposure to BCG. More IFN-γ was measured than IL-12 and IL-10, which were both detected at similar levels. Type 2 cytokines IL-5, IL-13 and IL-4 were secreted at very low levels or not at all. The source of both IL-5 and IL-13 cytokines is likely to be Th2 cells (Mosmann and Sad, 1996). IFN-γ appeared to be produced predominantly by NK cells. In contrast to the IFN-γ, IL-10 was produced by $CD14^+$ monocytes and not by T cells. These results indicate that immunologically naïve neonates have a type 1 cytokine response to mycobacteria prior to T cell activation and induction of acquired immunity.

The ability of T cells to produce IFN-γ in response to SEB stimulation mirrored that of BCG induced responses i.e. naïve $CD4^+$ T cells, not previously exposed to mycobacterial antigen, produced low levels of IFN-γ even in response to the superantigen SEB. The immune suppression associated with pregnancy, or the immature nature of the lymphocytes in cord blood could account for the very low $CD4^+$ and $CD8^+$ T cell IFN-γ response following exposure to SEB. $CD8^+$ cells in cord blood produced somewhat more IFN-γ when exposed to SEB. Similar to the observations with SEB in this study, White *et al* describe a lower cord blood $CD4^+$ T cell IFN-γ response, but a similar $CD8^+$ T cell IFN-γ response compared to naïve adult peripheral blood cells following stimulation with PMA + ION (White *et al.*, 2002). The fact that ICC IFN-γ was measured within SEB stimulated NK cells, which by definition lack TCR, could suggest that the NK cell IFN-γ response was an indirect response of the cells to cytokines produced as part of the super-antigen-induced T cell response.

NK cells are required for normal defences against several classes of extracellular pathogens, including bacteria, parasites, fungi and yeasts (Jouanguy *et al.*, 1999; Morrison *et al.*, 2003). NK cells have also been shown to play an important role in protective immunity against a number of intracellular bacterial pathogens including mycobacteria, listeria and salmonella (Emoto *et al.*, 1997; Lieberman and Hunter,

2002; Wick, 2004; Feng *et al.*, 2006). In addition, IFN-γ has been reported to be produced by human NK cells but not by T cells after stimulation of PBMC with *Staphylococcus aureus* and, the early production of IFN-γ by NK cells, rather than by T cells, was found to be essential in resistance to murine listeriosis (Dunn and North, 1991; Yoshihara *et al.*, 1993). In T cell-deficient mice, resistance to Mtb, was NK cell dependant and IL-12 was required for this innate response. These T-cell deficient mice were more resistant to Mtb than mice deficient in IFN-γ. Mice that were both T-cell and NK cell deficient were more susceptible to infection than only T-cell deficient mice and their lifespan was equivalent to that of IFN-γ deficient mice. The importance of NK cells for immunity against Mtb was further confirmed by experiments using naïve splenocytes from T-cell deficient mice. Following stimulation of these cells with the Mtb bacilli, IFN-γ was found only within NK cells. However, when splenocytes from both T cell and NK cell deficient mice were exposed to Mtb, no IFN-γ was detected (Feng *et al.*, 2006). Thus, NK cells are a very important source of IFN-γ production, especially in the absence of an acquired T cell immune response. Selective NK-cell derived IFN-γ could serve both to enhance macrophage activation and to prime for the subsequent adaptive Th1 cytokine response. The findings in this study of cord blood NK cells producing IFN-γ in response to BCG stimulation *in vitro,* seems to imply that innate immunity plays a role in neonatal responses to infections, including infection with pathogenic mycobacteria prior to an adaptive T cell response.

As newborn T cells are naïve and do not respond to mycobacterial antigens at birth, it is the role of NK cells to direct the host response towards a type-1 cytokine profile. BCG stimulation of IFN-γ production in human cells has previously been shown to require IL-12 and is dependent on CD40 costimulation (Mendez-Samperio *et al.*, 1999). In the present study, IL-12 was produced by BCG stimulated $CD14^+$ monocytes. Furthermore, IL-12 is an important cytokine that links innate and adaptive immunity and the IL-12 receptor, which is composed of β1 and β2 chains, is found on both T cells and NK cells (Bastos *et al.*, 2004; dEsther and Ottenhoff, 2006). IL-12 is therefore critical in facilitating differentiation of naïve $CD4^+$ T cells into mature Th1 effector cells, and stimulates both NK and $CD8^+$ T cells to produce IFN-γ (Bastos *et al.*, 2004; Hunter, 2005; Kastelein *et al.*, 2007). IL-12 production would,

therefore, be a pre-requisite for induction of T cell mediated protective immunity (Kastelein *et al.*, 2007).

It is possible that NK cell activation and the subsequent production of IFN-γ might be directly induced by interaction of these cells with BCG. Two recent studies have reported direct binding of BCG to NK cells; firstly via NKp44 and secondly via TLR2 (Esin *et al.*, 2008; Marcenaro *et al.*, 2008). Once the neonatal immune system is exposed to antigen i.e. following BCG vaccination at birth, both $CD4^+$ and $CD8^+$ T cells would be primed to produce higher levels of IFN-γ. This would cause a more sustained response than that of NK cells exposed to BCG. These findings are in agreement with others who reported a robust Th1 memory response in infants vaccinated with BCG at birth (Marchant *et al.*, 1999; Vekemans *et al.*, 2001; Hussey *et al.*, 2002). Vekemans *et al* reported similar levels of IFN-γ production by $CD4^+$ T cells in infants and adults following BCG vaccination, and re-stimulation of the cells with PPD (Vekemans *et al.*, 2001). Murray *et al* reported a specific $CD8^+$ T cell proliferation, up-regulation of cytotoxic molecules and IFN-γ expression at 10 weeks of age following *ex vivo* stimulation of blood with BCG; this $CD8^+$ T cell response was lower than the BCG-induced $CD4^+$ T cell response (Murray *et al.*, 2006). The slightly higher response in BCG-specific $CD8^+$ T cells in the study shown in this chapter could be due to a smaller sample size.

The mode of birth has been reported to be associated with differences in levels of cytokine production; cortisol, prostaglandins or cytokines which are secreted during labour could account for differences in experimentally induced cytokine production (Brown *et al.*, 2003; Protonotariou *et al.*, 2003). During labour the cytokine environment switches to Th1, to accelerate the inflammatory process which is required for successful labour and delivery; cytokines that are increased during labour include IL-6, IL-8 (induces cervical ripening), IL-1β, IL-2, IL-15, IL-18, TNF-α and IFN-γ, whereas IL-10 levels are reduced (Athanassakis and Vassiliadis, 2002; Keelan *et al.*, 2003; Malamitsi-Puchner *et al.*, 2005; Wilczynski, 2005). The pro-inflammatory cytokines such as IL-1β stimulate prostaglandins which are responsible for the commencement of uterine contractions (Wilczynski, 2005). In addition, labour has been found to cause an increase in the neonatal circulation of neutrophils,

monocytes and NK cells. Following normal labour neutrophils and T cells have been shown to be activated compared to infants born by elective caesarean (Thornton *et al.*, 2003; Malamitsi-Puchner *et al.*, 2005). A cortisol surge is also associated with labour. Cortisol is immunosuppressive and may cause inhibition of both cytokine production and T cell function in cord blood (Thornton *et al.*, 2003). In addition, during normal delivery, the foetus is exposed to vaginal flora and LPS stimulation, whereas a caesarean section is conducted in a sterile environment and no such stimulation exists (Malamitsi-Puchner *et al.*, 2005). Thus, the fact that I used cord blood from elective caesareans probably provides an explanation for any differences between this study and those reported by other investigators.

In conclusion, in this chapter investigating cytokines before and after BCG vaccination, I found that the cytokine IFN-γ was indeed produced by leukocytes before BCG vaccination. However, the quantity of IFN-γ measured was low, as the only cells producing this cytokine in cord blood appeared to be the NK cell. The mean frequency of NK cells producing IFN-γ in cord blood was 0.64% (± 0.75%). In contrast post BCG vaccination both $CD4^+$ (0.21% ± 0.16%) and $CD8^+$ T cells (0.49% ± 0.47%) produced this cytokine. In young infants 0-6 months of age, lymphocytes account for 63.1% (50.1-70.1%) of the leukocytes of which 43.2% (37.6-46.4%) are $CD4^+$ T cells and 14.9% (7.7-22.0%) $CD8^+$ T cells (van Gent *et al.*, 2009). In addition, a Turkish study reported that in the 0-1 year age group, only 11% of lymphocytes were $CD3^-CD16^+CD56^+$ NK cells (Ikinciogullari *et al.*, 2004). Although in this study I was unable to determine the percentage of NK cells expressing ICC IFN-γ in the **post BCG vaccination** group, it can be seen that given the percentage of T cells in the peripheral blood, T cells ($CD4^+$ and $CD8^+$ T cells) would be the major cell responsible for the production of IFN-γ. My results appear to confirm the hypothesis that cytokine mediated **protection** against Mtb engendered by BCG vaccination is **quantitatively different** (i.e higher amounts of these soluble mediators are measured after vaccination), but **not qualitatively** different (i.e. a similar profile of cytokines is detected pre and post BCG vaccination) from the pre-vaccination innate cytokine protective response of neonates induced by exposure to mycobacteria.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The findings in this thesis indicate that the neonatal innate immune response to mycobacterial antigen, as expressed in cells obtained from cord blood, does not appear to be impaired. Four novel findings support this assertion: 1. Naïve cord blood mononuclear cells stimulated with BCG produced IFN-γ. This cytokine was produced by NK cells in lieu of T cell activation. 2. Cord blood derived DC attained similar levels of maturation and activation as cells obtained from adult blood. 3. Monocyte-derived cord blood cells were fully capable of differentiating into either classically activated or alternatively activated macrophages depending on the specific *in vitro* culture conditions. 4. These polarised macrophages were comparable phenotypically and functionally to similarly derived macrophages from TST^+ adults.

The observation that both monocyte derived macrophage and DC activation and function were similar to those observed in the blood of adults after exposure to mycobacterial antigen is striking. A central macrophage function studied in cord blood cells, that was comparable to that of peripheral blood monocyte derived macrophage obtained from TST^+ adults, was the bactericidal capabilities of the cells against virulent Mtb. This observation suggests that even later in life, adult mononuclear phagocytes do not acquire the capacity to kill Mtb *in vitro*. Regarding cord blood DC, an important observation was that when compared to adult DC these cells upregulated costimulatory and antigen presenting surface molecules similarly. Once sufficiently matured and activated, DC are reported to be capable of promoting primary activation of naïve T cells (Banchereau and Steinman, 1998; Randolph *et al.*, 2008). This activity was demonstrated by the ability of cord blood derived DC to stimulate allogeneic T cell proliferation and production of IFN-γ. In addition, the profile of cytokines produced following exposure of cord blood cells to BCG was similar to the cytokine profile seen in the peripheral blood cells of infants post BCG vaccination, although the cells producing these cytokines differed. Since cord blood

derived T cells are naïve and do not respond to mycobacterial antigens, the IFN-γ produced in these experiments was produced by NK cells and not by $CD4^+$ or $CD8^+$ T cells. This observation suggests that NK cells are a very important source of IFN-γ production, especially in the absence of an acquired T cell immune response (Trinchieri, 2003). NK cells have been shown to contribute to directing the host response towards a type 1 cytokine profile (Trinchieri, 2003). Thus, my results suggest that NK-cell derived IFN-γ could serve both to enhance macrophage activation and to prime lymphoid cells for the subsequent adaptive Th1 cytokine response (Flynn and Chan, 2001; Lieberman and Hunter, 2002; Raja, 2004; Wang *et al.*, 2004; Feng *et al.*, 2006).

The finding of a comparable profile of cytokine responses in unvaccinated infants compared to that of BCG vaccinated infants, does not imply that BCG vaccination does not induce profound changes in the neonatal immune system. Indeed, the added immune activation, as seen by the induction of antigen-specific T cells (Marchant *et al.*, 1999; Soares *et al.*, 2008), provides protection against the more severe forms of TB even though this response is incapable of providing significantly improved protection against TB per se (WHO, 2004; Mahomed *et al.*, 2006; Walker *et al.*, 2006; Moyo *et al.*, 2010). The ability of BCG vaccination to protect against severe disease is clinically very important as demonstrated in South Africa, where it has been shown that BCG vaccination reduces disseminated disease by 87% compared with those not vaccinated (Mahomed *et al.*, 2006). BCG vaccination appears to accelerate the formation of Mtb induced granulomas and thereby assists in containing the infection and preventing miliary TB or systemic TB infection (Hanekom *et al.*, 2007; Russell, 2007). Furthermore it prevents Mtb from crossing the blood brain barrier and causing TBM (Kumar *et al.*, 2005). TBM, which has the highest incidence rate in young children, is a severe complication of TB with high morbidity and mortality. The neurological outcome following successful treatment can vary from symptoms such as vision and hearing impairment to blindness, severe intellectual impairment and neurologic sequelae (van den Bos *et al.*, 2004; van Well *et al.*, 2009). Thus, BCG vaccination plays a vital role in protection from more serious forms of TB infection in young children (Kumar *et al.*, 2005; Moyo *et al.*, 2010). Because vaccination protects against severe forms of the disease, the WHO recommends that BCG vaccination

should be given in high TB prevalence countries, such as South Africa. (Colditz *et al.*, 1994; Colditz *et al.*, 1995; Fine *et al.*, 1999; WHO, 2004; Walker *et al.*, 2006).

Although BCG vaccination of neonates induces a robust immune response in all individuals, it clearly provides only limited protection against *M. tuberculosis* infection and progression to disease (WHO, 2004; Mahomed *et al.,* 2006; Walker *et al.,* 2006; Moyo *et al.,* 2010). Thus even though mycobacterial stimulation of innate immunity combined with acquired antigen specific T cell immunity involve the activation of multiple components of the host immune response one must assume not all of these mediators protect against Mtb (Kagina *et al.*, 2010). Rather, the level of immune activation may be a measure of the extent of mycobacterial antigens the host is exposed to. The best example of this in TB is the fact that anti-Mtb specific antibodies as well as T cell activation are induced in patients with active disease; thus neither has the ability to fully protect against the infection and progression to disease or against a recurrent episode of TB (Glatman-Freedman and Casadevall, 1998; Kagina *et al.*, 2010). Consequently, it is important to distinguish between an immune response to Mtb infection and a protective response against TB. Clearly the immune response that is generated provides some protection, but not all components of the immune response predict or correlate with protection. To date there is no definitive *in vitro* correlate of immune protection against TB that identifies a certain immune response as an indicator of protection against active TB infection (Fletcher, 2007). The consequence of this is that we do not know which of the many components of the immune response that can be measured, actually protects against TB. Extensive additional studies will be required to address this gap in our knowledge.

The results reported in this thesis support my hypothesis that **protection** against Mtb engendered by BCG vaccination is **quantitatively different**, but **not qualitatively** different from the prevaccination innate **protective response** of neonates against this pathogen. Thus, although the quantity of Th1 cytokines produced by cells of the adaptive response to TB was increased, compared to the innate response, BCG vaccination did not reduce the 1-2% incidence of TB during the first 2 years of life seen in communities in South Africa (Moyo *et al.*, 2010). Is it possible that a newly developed improved vaccine will provide enhanced protection than is currently afforded by BCG against TB per se? Not only must this new candidate vaccine

provide the same protection as BCG, but in addition it would need to boost the BCG response to protect against active pulmonary TB (Colditz *et al.*, 1994; Colditz *et al.*, 1995; Fine *et al.*, 1999; WHO, 2004; Walker *et al.*, 2006). A better understanding of the contribution of the innate immune response in the neonate against vaccination with BCG and/or exposure to TB would help in the design of better vaccination strategies. The fact that in this study, neonatal DC had the ability to mature and become activated and functional as APC, has important implications for the development of vaccine strategies for neonates. My results suggest that by targeting vaccines to DC, a robust protective immune response against Mtb may be induced. DC-based vaccines for TB have been developed and tested in animal models and it was found that if DC are targeted, the immunogenicity of a vaccine is vastly improved (Collins and Kaufmann, 2001 A; Gupta *et al.*, 2007). The role of DC in priming Th1 cytokine polarisation is well established (Banchereau and Steinman, 1998; Mellman and Steinman, 2001; Savina and Amigorena, 2007; Sinha *et al.*, 2007). In addition, DC can reportedly be used as adjuvants in vaccines for the regulation of antigen specific immunity (Steinman and Pope, 2002). A vaccine strategy that either delivers antigen-pulsed DC, or a vaccine that targets DC may be beneficial against Mtb, as it might induce optimal activation of T cells (Collins and Kaufmann, 2001 A; Steinman and Pope, 2002; Franco-Paredes *et al.*, 2006).

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