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INTERFERON GAMMA PRODUCTION IN HIV-1 EXPOSED UNINFECTED INFANTS

Fiona Sharon Anthony

A dissertation submitted to the Faculty of Health Sciences,
University of the Witwatersrand,
in fulfilment of the requirements for the degree

of

Master of Science in Medicine

Johannesburg, 2008

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

---- day of ---**January**---, 2008

Abstract

The immaturity of the neonatal immune system places infants at an increased risk of infections and also affects the effective induction of protective immune responses by vaccines. *In utero* sensitisation to infectious pathogens results in immune activation and can establish immunological memory and may influence the immune response to unrelated antigens. In this study, we investigated in early life (birth to 6-10 weeks) the development of interferon-gamma (IFN-γ) responses in uninfected infants born to HIV-1 infected mothers (exposed uninfected (EU) infants).

Whole blood cell cultures stimulated with phytohaemagglutinin (PHA) or *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) showed that EU infants have a greater ability to produce IFN-γ in response to PHA and BCG at birth compared to control infants (born to HIV-1 uninfected mothers). However, by six weeks of age control infants produced significantly more IFN-γ in response to PHA only. These results suggest that responses amongst EU infants establish and mature earlier than in control infants. In fact, over time a greater number of EU infants have a reduced ability or an inability to respond to stimuli such as PHA or BCG compared to control infants (when comparing responses at six weeks of age to responses of the matched birth samples).

Full blood counts (FBC) counts were carried out using an automated AcT 5 diff haematology analyser which measures proportions and absolute counts of the five groups of white blood cells, namely, lymphocytes, monocytes, neutrophils,

eosinophils and basophils. Importantly, there were no significant differences in the absolute lymphocyte counts of control infants and EU infants either at birth or at six weeks which could account for the IFN-γ production differences noted between these infant groups, although the EU infants did exhibit a trend of higher absolute lymphocyte counts at six weeks than control infants. Age-dependent maturational changes in cell counts were observed in both control and EU infant groups, with neutrophils predominating at birth and lymphocytes predominating by six weeks, indicative of immune development with age in both infant groups. Short-course antiretroviral exposure increased basophil counts of infants at birth but did not affect counts by six weeks.

Flow Cytometry studies using an Intracellular Cytokine (ICC) assay were conducted to establish which mononuclear cell types are predominantly responsible for producing IFN-γ in infants. ICC assays done on whole blood revealed that natural killer cells (NK) were predominantly responsible for the IFN-γ produced by 10 week old EU infants, and also at birth (control and EU infants). At birth whole blood cultures of 48% of EU infants already showed BCG-induced IFN-γ responses (prior to BCG vaccination); this could be explained by a non-specific response (NK cells) to the antigen but could also involve T cell responses ($CD4^+$ and/or $CD8^+$ T cells) as supported by ICC data obtained from control and EU newborn infants. The infant immune system was clearly unique from that of their mothers. In particular, mothers' demonstrated significant changes in blood counts from labour to six weeks postpartum indicative that immuno-modulation plays an essential role in a successful pregnancy. The single dose of nevirapine (sdNVP) taken by the mother at the onset of labour did not influence maternal blood counts significantly and maternal $CD4^+$ and

$CD8^+$ T cells, and NK cells produced significantly more IFN-γ than the $CD14^+$ monocytes and $CD19^+$ B cells, with $CD8^+$ T cells producing the most.

Our results have shown that EU infants are distinct from control infants with respect to immunological responses as measured by IFN-γ production, and that maturational differences do exist between control and HIV-1 exposed infants. While the clinical importance of these results remains undetermined it is important to establish whether such immunological changes may result in altered susceptibility to infectious diseases in this already vulnerable population, and how this may impact on the induction of protective immune responses by vaccines. It remains important to identify neonatal immune system deficiencies and understand the consequences of exposure to maternal HIV-1 (in the absence of acquiring HIV-1 infection), the understanding of which could contribute to the development of more effective vaccines to HIV-1 and other infectious diseases.

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Some of the data presented in this dissertation have been included in the following publication:

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CHAPTER 1

Introduction

1.1 The immune system - innate and adaptive immunity

A normal immune system is designed to keep potential environmental pathogenic microbes from gaining entrance and causing disease. This system comprises of two arms namely, the non-specific innate and the antigen-specific adaptive immune systems (Delves *et al*., 2000). Both are further divided into humoral and cellular components. Innate immunity is the body's first line of defence against all invaders. The barriers employed are physical, chemical and microbiological in nature and encompass elements of the immune system (neutrophils, monocytes, macrophages, complement, cytokines and acute phase proteins) which provide immediate host defence (Delves *et al*., 2000). This system is not able to detect intracellular organisms, notably viruses, mycobacteria, some fungi, protozoa or other facultative intracellular pathogens. The response is rapid, non-specific and often poorly targeted, and can lead to indiscriminate tissue damage (Parkin *et al., 2*001). These innate immune mechanisms hinder the entrance and spread of disease but can rarely prevent disease completely. If an invader gets past the first line of defence, specifically adapted defences come into play in the form of the adaptive arm of the response. Superficially the system functions quite simply. If the organism is capable of replicating outside of cells, the humoral immune response, mediated by antibody molecules, is of primary importance. By comparison if the microbe can live and replicate inside cells, as most viruses do, then they can evade the antibodies, and cell

mediated immunity (CMI) comes into play (http://encarta.msn.com/encyclopedia 761575681 /Immune_System.html). During humoral immune responses, antibodies which can adhere to and destroy antigens, appear in the blood and other bodily fluids. Humoral responses can also prevent viruses from entering cells. During CMI responses, cells that can destroy other cells become active. Their destructive activity is limited to cells that are either infected with, or producing a specific antigen. CMI responses may also destroy cells making mutated forms of normal molecules, as in some cancers (http://encarta.msn.com/text 761575681 /Immune_System.html). The adaptive response is precise but takes several days or weeks to develop. The adaptive response has memory, so that subsequent exposure leads to a more vigorous and rapid response. Ideally the adaptive response eliminates the infectious agent and provides the host with a state of protective immunity against re-infection with the same pathogen (Janeway *et al*., 2001).

1.1.1 The induced innate response

Innate immunity employs a variety of induced effector mechanisms to either clear an infection rapidly or to stave off infection while the adaptive response develops. These effector mechanisms are all regulated by the ligand binding abilities of pattern recognition molecules that are able to discriminate between non-infected self and infectious non-self ligands. Thus the phagocytes ability to discriminate between self and pathogen, controls its release of pro-inflammatory chemokines and cytokines that act together to recruit more phagocytic cells, especially neutrophils, (which can also recognize pathogens), to the site of infection (Aderem *et al*., 1999; DeVries *et al*., 1999; Janeway *et al*., 2001). Furthermore, cytokines released by the inflammatory

process, induce fever, the production of acute phase response proteins including the pathogen-binding mannan binding lectin and the C-reactive proteins, and the mobilization of antigen-presenting cells that induce the adaptive immune response. These cytokines include interleukin (IL)-6, IL-1 (beta), tumour necrosis factor (alpha) (TNF-α), interferon gamma (IFN-γ), transforming growth factor (beta) (TGF-β) and possibly IL-8 (Gabay *et al*., 1999;). Viral pathogens are recognised by the cells in which they replicate, leading to the production of interferons that serve to inhibit viral replication. They activate natural killer (NK) cells, which in turn can distinguish infected from non-infected cells. During the innate response the cytokines produced play a role in determining whether the response is T cell-mediated or predominantly humoral and subsequently shape and stimulate the development of the adaptive immune response (Bendelac *et al*., 1997; Hsieh *et al*., 1993; Moser *et al*., 2000; Janeway *et al*., 2001).

1.1.2 T cell-mediated immunity

Dendritic cells, macrophages and B cells are often known as professional antigen-presenting cells. Antigen presenting cells (APC) present antigens of the pathogen in the form of a short peptide in association with major histocompatibility (MHC) molecules to mature T cells (Wilson *et al*., 1992), that are constantly recirculating from the bloodstream through the lymphoid organs and scanning the environment for pathogens. T cell priming and the differentiation of armed effector T cells occur here, on the surface of the antigen-loaded dendritic cells, which result in the production of an expanded population of armed antigen-specific effector T cells (Nikolic-Zugic, 1991). Effector T cells fall into three functional classes that detect peptide antigens

derived from the different types of pathogen (i) MHC class I molecules bearing viral peptides are recognized by cytotoxic T cells (CTLs) which then kill infected target cells (ii) MHC class II molecules bearing peptide antigens from pathogens multiplying in intracellular vesicles, which are recognised by T helper-type 1 (Th1) or T helper type 2 (Th2) cells and (iii) those derived from ingested extracellular bacteria and toxins, are carried to the cell surface by the MHC class II molecules and presented to $CD4^+$ T cells (Cao *et al*., 1995; Wilson *et al*., 1992). These can differentiate into two types of effector T cells - Th1 and Th2 cells. Th1 cells activate the microbicidal properties of macrophages, and induce B cells to make IgG antibodies that are very effective at opsonizing extracellular pathogens for uptake by phagocytic cells. Th2 cells initiate the humoral response by activating the naïve antigen-specific B cells to produce IgM antibodies. These Th2 cells can subsequently stimulate the production of different isotypes, including IgA and IgE, as well as neutralizing and/or weakly opsonizing subtypes of IgG (Romagnani, 2006; Elson *et al*., 2000).

1.2 Development of cells of the immune system

Haematopoietic stem cells (HSC) are found in the bone marrow of adults, which includes the femurs, hip, ribs, sternum and other bones. HSC are progenitor or early precursor cells which give rise to all the blood cell types that include both the myeloid lineage (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, and dendritic cells) and lymphoid lineages (T cells, B cells and NK cells) (Abramson *et al*., 1977; Wu *et al*., 1967). Fig.1.1 schematically presents the development of the cells of the immune system. Common lymphoid progenitor cells that arise in the foetal liver and bone marrow give rise to both myeloid and lymphoid

cells including B and T lymphocytes (Abramson *et al*., 1977). There are also stem cells committed to differentiation only into T lymphocytes (Abramson *et al*., 1977). The thymus is the main site of T cell maturation whereas the B lymphocytes mature in the bone marrow. Precursor cells first colonise the thymus in the human at about the third week of gestation and continue to migrate into this organ throughout adult life, where they (thymocytes) undergo a series of differentiative events which includes gene rearrangement, phenotypic alteration and biochemical modification to yield the population of thymocytes that undergoes intrathymic selection (Wilson *et al*., 1992*)*. Once they have completed their maturation, naïve T cells recirculate continually from the bloodstream from which they migrate to the peripheral lymphoid organs, namely the lymph nodes, the spleen and the mucosal lymphoid organs (Janeway, 2001). Lymphoid stem cells differentiate into three major populations of mature lymphocytes: T cells, B cells and NK cells. These lymphocyte subsets can be discriminated by surface phenotype. T cells are defined by their cell surface expression of the cell receptor, namely the T cell receptor (TCR), a transmembrane heterodimeric protein that binds processed antigen displayed by APC; B cells are phenotypically defined by their expression of the B cell receptor for antigen, membrane anchored immunoglobulin (Chaplin, 2003). NK cells are defined morphologically as large granular lymphocytes. They recognise their virus-infected or tumour cell targets through the use of a complex collection of activating and inhibitory cell surface receptors (Chaplin, 2003).

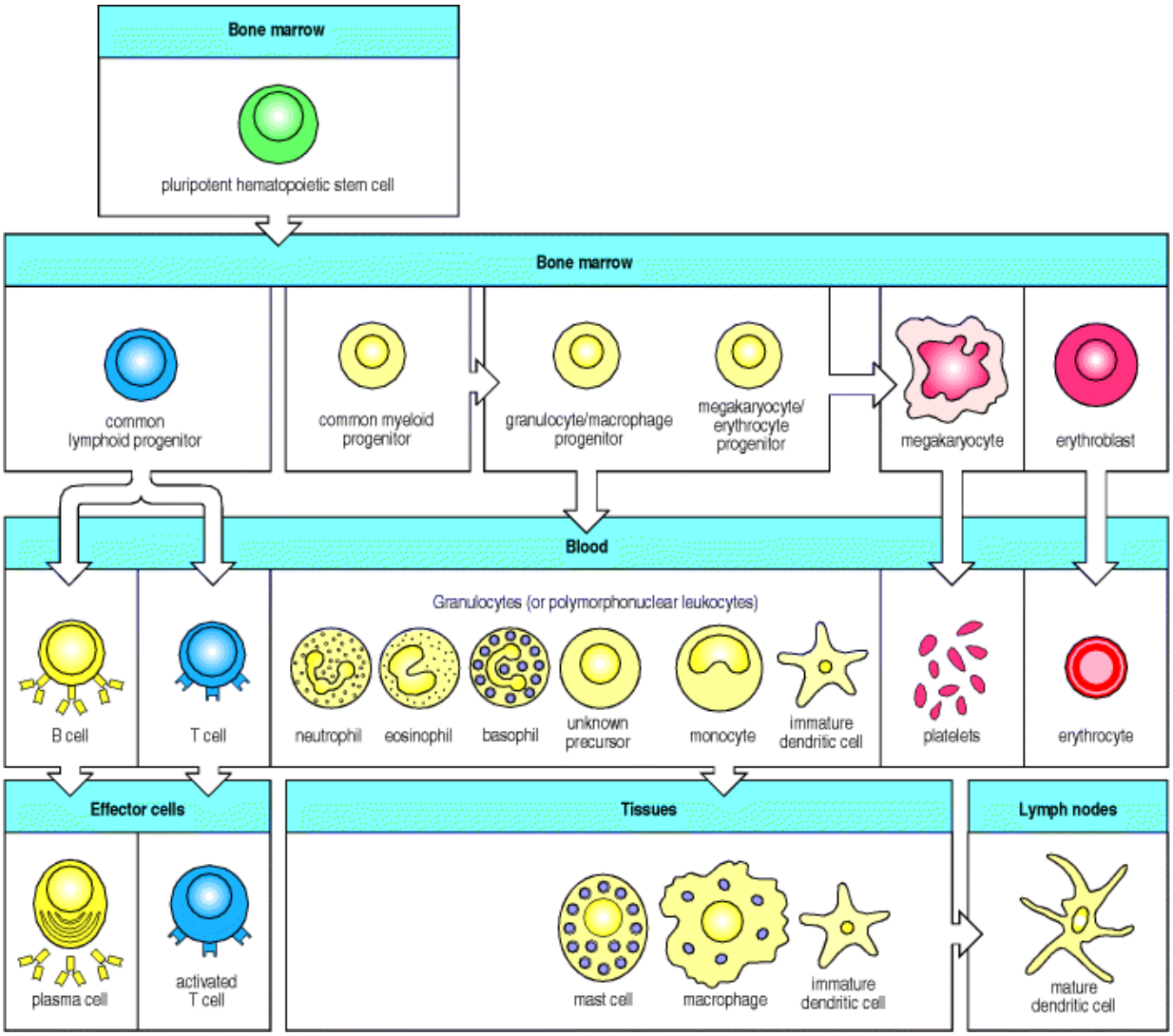


Fig. 1.1 Haematopoietic stem cells derived cell lineages. Pluripotent haematopoietic stem cells differentiate in bone marrow into lymphoid or myeloid stem cells. Lymphoid stem cells give rise to B cell, T cell and NK cell lineages. Myeloid stem cells give rise to a second level of lineage-specific colony forming unit (CFU) cells that go on to produce neutrophils, monocytes, eosinophils, basophils, mast cells, megakaryocytes, and erythrocytes. Monocytes differentiate further into macrophages in peripheral tissue compartments (Reproduced from Janeway *et al*., 2001)

1.2.1 Cellular components of the immune response

1.2.1.1 Neutrophils

Neutrophils are the most abundant type of white blood cells and form an integral part of the innate immune system. During the very early stages of infection, activated macrophages, endothelium and mast cells release cytokines. Two of these, granulocyte and granulocyte macrophage stimulating factors, stimulate division of myeloid precursors in the bone marrow, releasing millions of cells into the circulation and causing a characteristic neutrophil leukocytosis. Neutrophils normally flow freely in the blood. They undergo a process called chemotaxis that allows them to migrate towards sites of infection or inflammation (von Adrian *et al*., 2000; Parkin *et al*., 2001). Cell surface receptors are able to detect chemical gradients of molecules such as IL-8, IFN-γ and complement 5a (C5a) which these cells use to direct the path of their migration (Parkin *et al.,* 2001). Neutrophils are active phagocytes, capable of ingesting microorganisms or particles. They can only execute one phagocytic event, expending all glucose reserves in an extremely vigorous respiratory burst. The respiratory burst involves the activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme, which produces large quantities of superoxide and is sequentially reduced to singlet oxygen (Garred *et al*., 1995; Parkin et al., 2001). Neutrophils also release an assortment of proteins in three types of granules: (i) specific granules - these help kill the ingested microbe by a variety of oxygen-dependent mechanisms, (ii) azurophilic granules e.g. myeloperoxidase, (iii) tertiary granules, namely cathepsin and gelatinase. Ingestion and killing of organisms is 100-

fold more effective if the particle is first opsonised with specific antibody or complement (Borregaard *et al*., 2007).

1.2.1.2 Eosinophils, basophils and mast cells

Eosinophils are not phagocytic. These cells are much rarer than neutrophils. In humans these cells comprise about 2-10% of peripheral leukocytes. Eosinophils are predominantly tissue cells and do not re-enter the circulation (Colley, 1973). They act by releasing components of their granules onto the surface of organisms. The cationic proteins and reactive oxygen metabolites released are cytotoxic to the organism. The main role of eosinophils is in protecting the host from parasitic infections. Such infections induce antigen-specific IgE production, the antibodies coating the organism. Eosinophils bind to the antibody using their low affinity receptors (FcR11) (Parkin *et al.,* 2001). Eosinophils and basophils are more immediately concerned with the production of mediators that shape the inflammatory milieu, and are responsive to cytokines elaborated by the adaptive immune system. Mast cells and basophils play a central role in inflammatory and immediate allergic reactions. They are able to release potent inflammatory mediators, such as histamine, proteases, chemotactic factors and cytokines that act on the vasculature, smooth muscle, connective tissue, mucous glands and inflammatory cells. There are at least two types of mast cells based on enzymes they contain and tissue location. T mast cells (mucosal mast cells) contain only trypsin whereas connective tissue mast cells contain both trypsin and chymotrypsin (Parkin *et al.,* 2001; Delves *et al.,* 2000).

1.2.1.3 Dendritic cells

Dendritic cells are derived from haematopoietic bone marrow progenitor cells. These progenitor cells initially transform into immature dendritic cells. Their main function is to process antigen material and present it on the surface to other cells of the immune system, thus functioning as APC (Banchereau *et al*., 1998). Dendritic cells are present in small quantities in tissues that are in contact with the external environment, mainly the skin (where they are often called langerhans cells) and the inner lining of the nose, lungs, stomach and intestines. They can also be found at an immature state in the blood. Once activated, they migrate to the lymphoid tissues where they interact with T cells and B cells to initiate and shape the adaptive immune response (Ingulli *et al*., 1997; Matsuno *et al*., 1996). At certain development stages they grow branched projections, the dentrites that give the cell its name. However, these do not have any special relation with neurons, which also possess similar appendages (Steinman *et al*., 1979). Immature dendritic cells constantly sample the surrounding environment for pathogens such as viruses and bacteria. This is done through pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) (Kabelitz *et al*., 2007). TLRs recognize specific chemical signatures found on subsets of pathogens. Once they have come into contact with such a pathogen, they become activated into mature dendritic cells. Immature dendritic cells phagocytose pathogens and degrade its proteins and upon maturation present those fragments at their cell surface using MHC molecules (Kabelitz *et al*., 2007). Simultaneously, they upregulate cell-surface receptors that act as co-receptors in T cell activation such as CD80, CD86, and CD40 greatly enhancing their ability to activate T cells (Pasare *et al*., 2004). The dendritic cell is induced to travel through the blood stream to the spleen or through the

lymphatic system to a lymph node (Pasare *et al*., 2004). Here they act as APCs: they activate helper T cells and killer T cells as well as B cells by presenting them with antigens derived from the pathogen, alongside non-antigen specific costimulatory signals (Janeway *et al*., 1994; Knight *et al*., 1993). Every helper T cell is specific to one particular antigen. Only professional APCs (macrophages, B lymphocytes, and dendritic cells) are able to activate a helper T cell which has never encountered its antigen before. Dendritic cells are the most potent of all the antigen-presenting cells. Myeloid DC probably form monocytes, white blood cells which circulate in the body and, depending on the right signal, can turn into either dendritic cells or macrophages (Janeway *et al*., 1994).

1.2.1.4 Natural killer cells

NK cells originate from the common lymphoid progenitor cells, continue development in the bone marrow and circulate in the blood. They are larger than T and B lymphocytes and have distinctive cytoplasmic granules. Without the need for prior immunization or activation they are able to infiltrate the sites of early tumours or infectious agents in the body. They recognise their targets in one of two ways. First they bear Fc receptors that bind antibody coated targets leading to antibody-dependent cellular cytotoxicity (ADCC). The second system relies on the killer activating receptors and killer inhibitory receptors of these cells. The killer activating receptors recognise a number of different molecules present on the surface of nucleated cells, whereas the killer inhibitory receptors recognise MHC class I molecules, which are usually present on all nucleated cells. NK cells are activated in response to interferons or macrophage-derived cytokines. When NK cells are

exposed to IFN-α, IFN-β or IL-12 the killing activity of these cells is increased (Parkin *et al*., 2001; Janeway *et al*., 2001).

1.3 Cytokines

Cytokines are small proteins that are produced by virtually all cells, usually in response to an activating stimulus and have a wide variety of functions. Cytokines send intracellular signals by binding to specific cell surface receptors. The term cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes), chemokine (cytokines with chemotactic activities), and interleukins (cytokines made by one leukocyte and acting on other leukocytes). They can act in an autocrine manner, affecting the behaviour of the cell that releases the cytokine, or in a paracrine manner, affecting the behaviour of adjacent cells. Some cytokines can act in an endocrine manner, affecting the behaviour of distant cells (http://www2.gsu.edu/-biopjd/Lecture20F99.html).

1.3.1 Interferons

Interferons are a major class of cytokine that have a particular role in immunity. They are divided into type 1 interferons (α and β) which are the classical interferons induced in response to viral infections and type 2 interferons (γ or immune interferon). Type 1 interferons have potent antiviral activity as a primary function (Boehm *et al*., 1997). Both α and β interferon bind to the same cellular receptor and protect uninfected cells by inducing the intracellular production of molecules that inhibit or

interfere with viral RNA and DNA production. They increase the expression of MHC class I molecules leading to enhanced recognition of virally infected cells by specific cytotoxic T lymphocytes. Type 1 interferons also have an antiproliferative function and have been used in combination with antiviral drugs to treat some viral infections (Belardelli, 1995; Durbin *et al*., 2000).

1.3.1.1 Interferon-γ

The antiviral effects of IFN-γ were first identified in PHA-activated lymphocyte supernatants (Wheelock, 1965). Since then a wealth of research has focused on this antiviral aspect (Revel *et al*, 1986; Staeheli, 1990; Kerr *et al*., 1992). It was initially thought that cells of the lymphoid system, namely, $CD4^+$, $CD8^+$, NK and γδ T cells were the only cells capable of producing IFN-γ however, macrophages and dendritic cells have also been shown to produce IFN-γ (Szabo *et al*., 2003). A number of cytokines (eg. IL-12, IL-18), microbial products (eg. LPS), and signalling pathways (eg CD40) are capable of inducing IFN-γ production from macrophages or dendritic cells. IL-12 and IL-18 together induce maximal IFN-γ from macrophages and dendritic cells, whereas IL-12 alone is capable of strongly inducing IFN-γ (Szabo *et al*., 2003). IFN-γ has a number of immunoregulatory functions in addition to its antiviral effects, acting directly on the immune system to activate macrophage and neutrophil intracellular killing, stimulate NK cell function, and enhance antigen presentation by increasing MHC class II expression on APC (Trienchieri, 1995; Young *et al*., 1995; Nathan *et al*., 1983). A necessary step in the functioning of the IFN-γ is the interaction of IFN-γ with

receptors located on the surface of the cells, to subsequent activation of gene transcription. Over 200 genes are known to be regulated by IFN-γ (Boehm *et al*., 1997). The primary method of IFN-γ signal transduction is via a Jak-Stat tyrosine kinase dependant pathway (Ellis *et al*., 2004).

1.4 Immunity in the neonate

Human neonates are highly susceptible to infections by bacteria, fungi, and viruses. Deficiencies of both innate and adaptive immunity contribute to the impaired neonatal host defence (Marodi, 2006). Many components of the immune system function less well in neonates compared with adults giving rise to a concept “of immunodeficiency or immaturity” (Schelonka *et al*., 1998). Table 1.1 lists some differences noted in infants when compared to adult immune systems.

Table 1.1 Features of infants' immune systems that are characteristically different from adult immune systems [Reproduced and modified from Jaspan *et al*., (2006)]

Neutrophils	Higher number of circulating neutrophils, but decreased response to chemokines (Tan *et al*., 1995)
DC and other APC	In the presence of endogenous APC, human cord blood T cells proliferate poorly, and are poor producers of cytokines, including IL-12, IFN-γ, IL-4, GM-CSF and IL-5 (Adkins, 1999). Immature neonatal DC have decreased antigen presenting ability (Salio *et al*., 2003)
Helper T cell	Th2 skewed response until ± 12 months (Hassan *et al*.,2000; Siegrest, 2001; Upham *et al*.,2002). Neonates and children produce less IL-2, IL-4, IL-6 and IL-10 in response to mitogens (Chheda *et al*., 1996; Lewis *et al*., 1988; Lilac *et al*., 1997). Neonatal $CD4^+$ cells are less able to provide help for immunoglobulin synthesis (Splawski *et al*., 1991; Jelinek *et al*., 1986).
Cytotoxic T cell	Full maturation at 9 to 12 months (Chiba *et al*., 1989)
IFN-γ	Reach adult levels at 1-5 years (Chelimo *et al*., 2003). However certain immunogens can stimulate better IFN-γ responses eg BCG (Mahon, 2001)
IgG	Maternal IgG is present until 12-18 months. Infant IgG production increases sharply after the first few months, then gradually increases to adult levels at 2-7 years. Relative nadir 3-6 months (Janeway et al., 1999).
IgA	Maternal IgA throughout breast feeding (Janeway et al., 1999). Infant serum IgA increases very slowly after birth; only 20% of adult levels by 12 months (Janeway et al., 1999). In the postnatal period secretory IgA antibodies are produced earlier than, and independently of serum IgA antibodies (South, 1971)
IL-12 (and related antigen presentation	Capacity for IL-12 secretion is deficient (Siegrist, 2001)
Thymic function	Response to thymic independent antigen by 18 months (Stein, 1992)

1.4.1 Innate immunity in the neonate

The systems mediating innate immunity have certain quantitative deficiencies that affect the newborns response to infections. The neutrophil storage pool in human neonatal bone marrow is considerably smaller than that of the adult (Christensen *et al*., 1982). Neonatal neutrophils ingest and kill bacteria as efficiently as their adult counterparts but adhesion, chemotaxis, lower enzymatic activity and signalling and subsequent migration of these cells to sites of infection is impaired (Wolach *et al*., 1992). Further limitations occur at the levels of monocytes, macrophages, the complement system, NK and lymphocyte activated killer cell cytotoxicity, which remain below adult levels even after induction with exogenous interleukin 12 (IL-12)

and IL-15 (Neutra *et al*., 1996; Berger *et al*., 1990). The major components of innate immunity thus appear weakened in newborn infants.

1.4.2 Neonatal T cell responses

T cell responses in early life also appear to be impaired as infants succumb to serious infection with certain viruses such as herpes simplex and rubella (Schelonka *et al*., 1998). Naive T cells require a much longer TCR triggering period compared with mature effector cells (Valitutti *et al*., 1997). TCR diversity is more limited during foetal and neonatal life than in adults (Schroeder *et al*., 1995; Gavin *et al*., 1995). Additionally, the functional capacity of T cells in the foetus and neonate is immature in that the neonatal T cells express a limited repertoire of lymphokines in response to activation (Wilson *et al*., 1992). The majority of human cord blood $CD4^+/CD8^+$ T cells present $CD45RA^+$ naïve phenotype, whereas both the $CD45RA^+$ and the memory/mature $CD45RO^+$ phenotypes are represented in equal numbers in adult peripheral blood mononuclear cells (Kovarik *et al*., 1998). It is widely accepted that neonatal T cells are immunocompetent but that their differentiation is biased towards a Th2 profile under neutral conditions. It is not clear whether this Th2 bias can be attributed to the Th2 cytokine milieu of pregnancy, to altered interactions of APCs with T cells, or to both of these factors (Kovarik *et al*., 1998).

1.4.3 Major cytokine profiles of the foetus and neonatal infant

Neonatal monocytes and macrophages are qualitatively different from adult cells in that they are defective in the secretion of a variety of Th1-type cytokines. Nature

biased foetal immunity toward a Th2 polarisation which appears to be an evolutionary adaptation orchestrated via production of cytokines and other regulatory molecules (Marodi, 2006). The predominant production of Th2 cytokines in foetal and neonatal life appears to play a key role in damping the newborns innate immune responses (Trinchieri, 1995). Production of Th1 cytokines is reduced not only during foetal life but also after birth. Deficiency of IFN-γ production by neonatal T cells in response to mitogens or bacterial antigens has been well documented (Bryson *et al*., 1980; Wilson *et al*., 1986). A decreased production of IL-12 by cord mononuclear cells may be linked to IFN-γ deficiency in newborns (Lee *et al*., 1996). In early life there is diminished production of other cytokines as well such as IL-2, IL-4, IL-6, IL-13 and IL-18, by neonatal cells which may result from the abundance of Th2 cytokines (Ribeiro-do-Couto *et al*., 2001). The responsiveness of human neonatal monocytes and macrophages to individual cytokines differs significantly from that of adult cells and the defect in neonatal macrophage activation involves pathways downstream from ligand-binding events and includes signal transduction pathways (Marodi *et al*., 2001). An overall deficiency of certain cytokines may also explain why neonatal $CD4^+$ cells have diminished capacity to provide help for immunoglobulin synthesis (Splawski *et al*., 1991).

1.4.4 Antibody production in the neonate

The humoral immune system remains relatively underdeveloped, with the neonate being almost entirely dependent upon passively acquired maternal antibody (Jaspan *et al*., 2006). IgG and IgA responses to pathogens are weak during the first year of life. Acquisition of full antigen responsiveness proceeds with the diversification of the

antibody repertoire, which reaches adult-like patterns between two to six months of life for IgM and possibly later for IgG (Kovarik *et al*., 1998). Immune responses to polysaccharide antigens are thymus independent (TI) and these responses to TI antigens develop late in infancy by about 18 months of age (Stein *et al*., 1992).

1.4.5 IFN-γ production in the neonate

The production of IFN-γ is impaired in early life (Wilson *et al*., 1986). Decreased production of IFN-γ by neonatal cells appears to be due to differences in their intrinsic capacity to produce IFN-γ and to differences in regulatory mechanisms (Wilson *et al*., 1986). IFN-γ is a much more potent inducer of macrophages than IFN-α or β. In contrast to IFN-γ, IFN-α, and -β production by neonatal cells is similar to that by adult cells. Thus, decreased production of IFN-γ may be an important factor that predisposes the neonate to severe infection with intracellular pathogens (Wilson, 1986). According to Burchett *et al*. (1998) a lag in IFN-γ production occurs in the neonate for at least the first two months of age. The capacity of T cells to produce IFN-γ and the number of memory T cells both increases over time (Sanders, 1988). The IFN-γ production of T cells is low because of limited exposure to foreign antigens. IFN-γ is produced in larger amounts by T cells that appear to have been previously activated *in vivo* by their interaction with suitably presented antigens. Lewis *et al*. (1990) concluded that reduced IFN-γ production by neonatal T cells was a functional phenotype established during intrathymic development and was primarily transcription

mediated. In previous reports, reduced IFN-γ production by mitogen activated leukocytes or T cells have been variously attributed to suppression mediated by $CD4^+$ or $CD8^+$ T cell populations (Seki *et al.,* 1986; Haas *et al*., 1987). There is increasing evidence that memory T cells, defined as those that have been activated or primed by antigen are capable of increased levels of IFN-γ production, compared to antigenically naïve, virgin T cells (Wilson *et al*., 1986). The results of Taylor *et al*. (1985) showed that the neonatal macrophage is primarily responsible for the impaired IFN-γ by newborn cells. Research by Wilson *et al*. (1992) highlighted that the difference in expression of the IFN-γ gene could be due to differences in the structure of the chromatin or in methylation of critical sequences of the gene.

1.5 HIV prevalence and maternal infant HIV-1 transmission

At the end of 2006, a global total average of 39.5 million people, were living with HIV/AIDS (type 1 and 2). Of that total, an average of 4.3 million were newly infected in 2006. Although this data shows that HIV/AIDS remains a global problem, Southern Africa remains the epicentre of this epidemic: 32% of people with HIV worldwide live in Southern Africa and 34% of AIDS deaths globally occur here (http://www.mrc.ac.za/bod/DemographicImpactHIVIndicators.pdf). The latest data shows a 35% increase in HIV infection levels among pregnant women attending antenatal clinics. Even with an increase in the rollout of antiretrovirals and other preventative measures in place, worldwide current figures indicate that 26% of infants born to HIV (type 1 and 2) infected mothers are still becoming infected (http://data.unaids.org/pub/EpiReport/2006/04Sub_Saharan_Africa_2006_EpiUpdate

_eng.pdf). This would then mean that 74% of the infants born to these HIV (type 1 and 2) infected mothers, make up the exposed uninfected (EU) group of the population.

1.6 HIV-1 infection and innate and specific cellular immunity

Children infected with HIV-1 display a variety of immune abnormalities which includes defects in the microbicidal response of phagocytic cells (Lazzarin *et al*., 1986 and Roilides *et al*., 1993), impaired neutrophil antifungal and bactericidal neutrophil activity . Quantitative depletion and impaired function of $CD4^+$ T cells is a major factor contributing to HIV-1 associated immunosuppression (Fauci, 1998). Functional studies by Shearer and Clerici (1991), show a progressive loss in T cell proliferative responsiveness with disease progression, firstly to recall antigens, then alloantigen and mitogen respectively. Antiretroviral interventions in preventing mother-to-child transmissions were introduced as early as 1994. Chougnet *et al*. (1998, 2001) conducted two different studies to assess whether treatment of HIV-1 positive children by antiretroviral drugs for a six month period and a two year period respectively would improve immune function. It was determined that (i) there was a marked increase in the proliferative response and skin reactivity to recall antigens, (ii) viral loads decrease significantly, (iii) $CD4^+$ T-lymphocytes counts increased but (iv) restoration of defective cellular immune responses did not occur.

1.7 The HIV-1 exposed uninfected infant

Children born to HIV-1 infected mothers are at risk of acquiring infection *in utero* (while in the womb), intrapartum (during the birthing process) or postpartum (through breastfeeding) (Magder *et al*., 2005; Ahmed, 1996; Douglas *et al*., 1992). Significant immunological changes associated with intrauterine HIV-1 exposure have been described in HIV-1 uninfected infants born to HIV-1 infected mothers (Kuhn *et al*., 2002) such as sensitisation to HIV-1 recombinant antigens resulting in cell–mediated immune responses (Borkowsky *et al*., 1990) and the presence of CTL effector responses in the peripheral blood of these children (Cheynier *et al*., 1992). Factors that can influence the outcome of HIV-1 exposure in children born to HIV-1 infected mothers have been reviewed by Tiemessen and Kuhn (2006) and are presented in Fig.1.2. These authors further mention that (i) the integrity of innate immune functions could contribute towards the development of HIV-1 specific immunity, (ii) CC chemokines contribute to protective immunity against HIV-1 and to the attenuation of disease progression once infection is established, and (iii) children by virtue of their genetic similarity to their mothers can inherit a virus that has evolved to evade HLA-mediated responses.

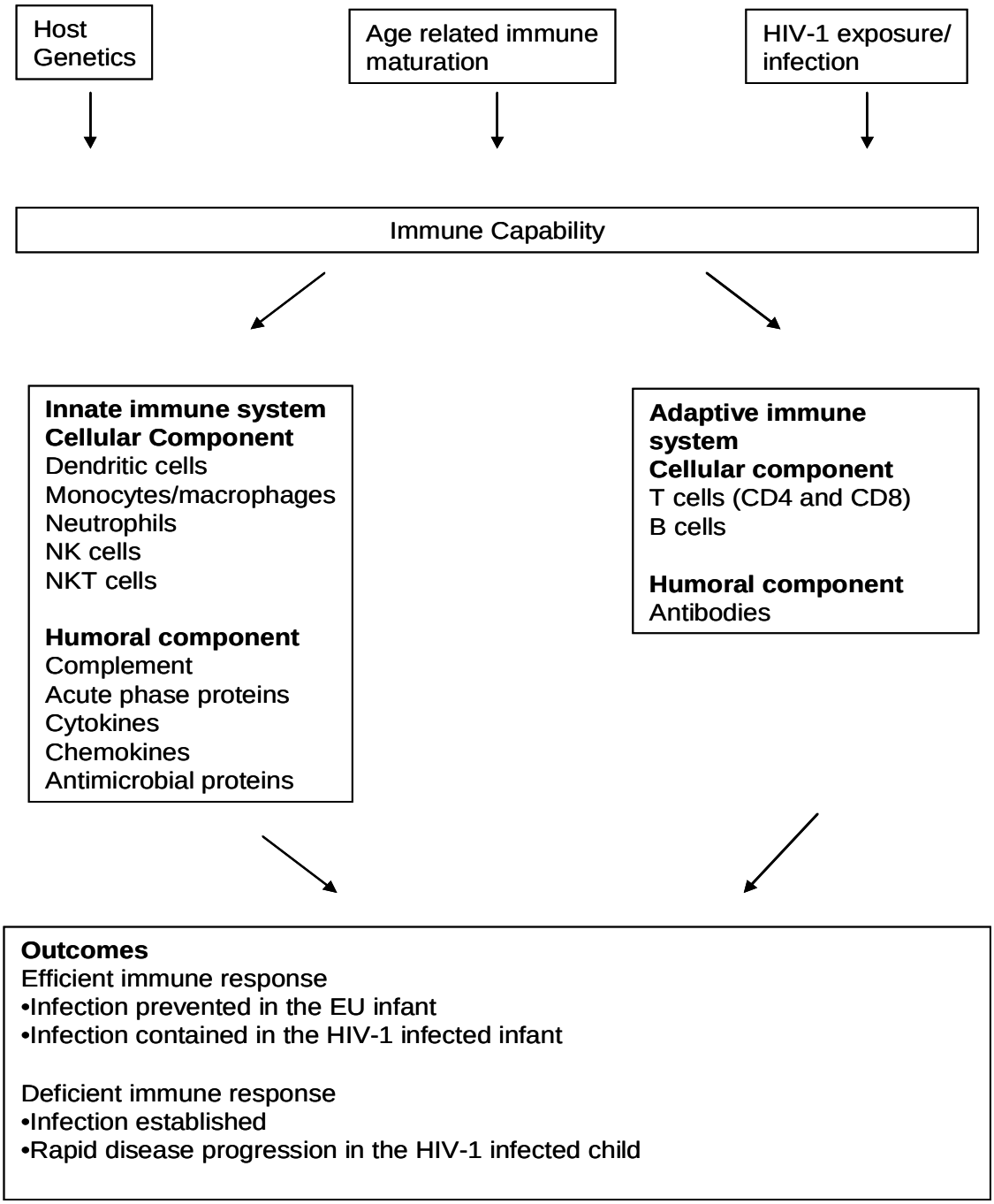


Figure 1.2 Factors (host genetics, maturational immune development and HIV-1 exposure/infection) influencing the immune capability and subsequent outcomes of HIV-1 exposure/infection in children. Cellular and humoral components of innate and adaptive immune systems are shown. NK-natural killer cells; NKT-natural killer T cells (Reproduced from Tiemessen and Kuhn, 2006)

With the increasing use of ART drugs to prevent mother-to-child transmission of HIV-1, large numbers of infants are exposed, however there is the consequent risk of ART toxicity. Studies by Pacheco *et al*. (2006), Le Chenadec *et al*. (2003) and El Beitune *et al*. (2004) show that (i) there is a persistent inhibition of haematopoietic cells up to the age of 18 months (ii) haemoglobin concentrations, neutrophil, lymphocyte, and $CD4^+$ T cells are lower at age 0-2 months in infants exposed to ART than those who are not. At age 6-24 months differences in lymphocyte and $CD4^+$ T cell counts persisted whereas $CD8^+$ T cell counts were significantly decreased (iii) combinations of ART drugs were associated with larger such decreases than occurs with monotherapy up to 15 months of age. These immunological contraints as well as the responses mentioned in section 1.7.1 and 1.7.2 are especially noteworthy given the immaturity of the neonatal cellular immune response.

1.7.1 HIV-1 specific (acquired) immune responses among EU infants

HIV-1 specific responses have been demonstrated in cord blood and peripheral blood of uninfected infants (1-18 months of age), and include measures of antigen-specific $CD4^+$ T helper cell activity (lymphoproliferative and IL-2 production studies), $CD8^+$ cytotoxic T-lymphocyte (CTL) responses, and $CD8^+$ noncytolytic HIV-1 suppressor activity. Collectively these findings demonstrate immunological memory suggestive of prior exposure to HIV (Kuhn *et al*., 2002). At least two studies have observed associations between the detection of responses to HIV-1 peptides at birth and lack of infection (Clerici *et al*., 1993; Wasik *et al.,* 1999). Confirming and strengthening these studies was one other prospective, epidemiological study conducted in a breastfed population (which can be likened to a natural challenge study) which

directly tested in a longitudinal manner, whether or not HIV-1 specific responses are associated with protection given subsequent viral exposure (Kuhn *et al*., 2001). All these studies have utilized some of the same gp160 peptides (T1, T2, TH4 of conserved regions and P18 MN and P18 111B of variable regions) (Berzofsky *et al*., 1991; Hale *et al*., 1989; Cease *et al*., 1987) to elicit T helper cell responses. Detection of an HIV-1 specific immune response to these gp160 peptides at birth therefore provides an important marker of protective immunity to HIV-1 in the infant.

1.7.2 Non-specific (innate) immune responses in EU infants

In further support of greater antigenic stimulation occurring in HIV-exposed uninfected infants compared to their unexposed uninfected counterparts is the fact there are notable alterations in a number of non-specific immune parameters. Examples include elevations in populations of activated helper T cells ($CD4^+$ HLA^-DR^+ $CD38^+$) and memory helper T cells ($CD4^+$ $CD45RA^-$ RO^+) (Rich *et al*., 1997), a trend towards increased levels of soluble L-selectin in the first two days of life, shed after lymphocyte activation by antigen-presenting cells (Kourtis *et al*., 2000), transient expansions in the T cell receptor beta-chain variable region (Soudenys *et al*., 2000), reduced IL-12 production in cord blood (Chougnet *et al*., 1996), reduced production of IL-2 in phytohaemagglutin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) (Rich *et al*., 1997), elevated PHA-stimulated IFN-γ and IL-10 in cord blood leukocytes (Nicastri *et al*., 1999), and raised serum levels of IL-7, a cytokine important in thymopoeisis, in newborn and older uninfected children of HIV-infected mothers (Clerici *et al*., 2000). This together with evidence of alterations of various cell subsets (increased memory cells, altered $CD4^+/CD8^+$ ratios, elevated numbers of activated

$CD38^+CD8^+$cells and $CD3^+/4^-/8^-$ cells) in the same study suggest that T cell homeostasis is impaired in these children. Some of these abnormalities persist with age and raise the question as to whether these children, through exposure to HIV-1, may be susceptible to other diseases (Clerici *et al*., 1993), or may have altered disease courses with other organisms, or even altered responses to some vaccines.

1.8 Aims and objectives of the study

It was the purpose of this study to contribute towards understanding immune responsiveness, as measured by IFN-γ production, in uninfected infants born to HIV-1-seropositive mothers. The study as designed, addresses consequences of HIV-1 exposure on the development of IFN-γ responses in early life. This is important as prenatal exposure to HIV-1 may affect the development of cell mediated immune responses by priming T cells, a factor that could influence the outcome of subsequent vaccine responses or of subsequent infectious events.

Specific objectives:

1. To determine whether exposure to HIV-1 *in utero* and at birth influences IFN-γ production in the infant as measured at birth and at six weeks of age.
2. To assess whether *in utero* HIV-1 exposure or exposure to maternal nevirapine influences alterations in numbers of different leukocyte cell subtypes.
3. To identify the cell types responsible for producing IFN-γ (using PHA and BCG as stimuli).

Comparisons have been drawn that take into account two scenarios in the context of mother-child relationship: (1) no exposure to HIV-1 (uninfected control - mother and infant) and (2) maternal HIV-1 infection and the EU infant.

CHAPTER 2

IFN-γ production in whole blood of mothers and their infants using PHA and BCG as stimuli

2.1 Introduction

Clinical trials conducted in developing countries have proven that short-course antiretroviral therapy (ART) can substantially reduce MTCT of HIV-1 (Connor *et al*., 1994; Guay *et al*., 1999; Taylor *et al*., 2000; Gray *et al* 2005). Despite the availability of such short-course prophylactic interventions it is estimated that during 2006 64 000 babies became infected through MTCT in South Africa (Dorrington *et al*., 2006). Even in the absence of ART drugs, most infants (65%-85%) of HIV-1 infected mothers do not become infected (Kuhn *et al*., 2002). Infants that remain uninfected despite exposure to maternal HIV-1 (intrauterine, intrapartum or through breastmilk) have been referred to as “exposed uninfected” (EU) infants. These infants exhibit many HIV-1-specific responses and non-specific/innate immune responses elicited through *in utero* priming of cells and remain uninfected despite challenge again at the time of delivery and ongoing exposure to HIV-1 during breastfeeding (Kuhn *et al*., 2002). Chougnet *et al*. (2000) suggest that the maternal environment and/or exposure *in utero* to HIV-1 products influence the newborns immune response and that the differences between infants born to HIV-1 positive and negative women may persist. In fact, HIV-1-specific T cell responses have been reported following low dose exposure to HIV-1 with a decrease in this T cell function being reported in subsequent

months following termination of the exposure to HIV-1 from the mother (Shearer and Clerici, 1996). Given the immaturity of the neonatal cellular immune response, the immunological differences observed in HIV-1 exposed uninfected infants are especially noteworthy.

We aimed, in this section of the study, to contribute towards understanding immune responsiveness, as measured by IFN-γ production, in uninfected infants born to HIV-1 seropositive mothers. Furthermore, we aimed to address consequences of HIV-1 exposure on the development of IFN-γ responses in early life. This was done by measuring IFN-γ responses in whole blood cultures that were set up with samples collected at birth and at six weeks of age from infants born to HIV-1 infected and uninfected mothers.

2.2 Materials and Methods

2.2.1 Study subjects and samples

The collection of samples was done at Chris Hani Baragwanath Hospital (CHBH), Soweto, South Africa. The study population recruited included women participating in the Perinatal Mother-to-child Transmission (PMTCT) program and included 13 HIV-1 uninfected mothers and their newborns and 120 HIV-1 infected women and their newborns. The women were enrolled between January 2003 and March 2003 and their HIV-1 infection status was determined prior to enrolment. All mothers and their infants received a standard regimen of one dose each of nevirapine (sdNVP) (mothers being given a single dose of NVP (200 mg) at the time of delivery and infants being administered a single dose of NVP (2 mg/kg) within 48 hours (hrs) of birth). Infants with a birth weight of less than 2500 g or with substantial medical problems at birth were excluded from the study. Infants that did not report for the six week follow-up were also excluded. Samples were only available for this study for 11 HIV-1 uninfected (control) mothers and their infants and 91 HIV-1 EU infants and their HIV-1 infected mothers.

Peripheral blood was collected in preservative-free ethylenediamine tetra-acetic acid (EDTA) vacutainer tubes (Becton Dickinson) from the mother (10 ml) at delivery and from the infants (5 ml) within 48 hrs of birth. The blood was transported to the Cell Biology Laboratory at the National Institute for Communicable Diseases, and processed within three hrs of collection. A further blood sample was collected from the infant at six weeks of age. HIV-1 DNA PCR tests were carried out to establish

infection status using the Roche Amplicor HIV-1 DNA PCR assay version 1.5 (Roche Diagnostic Systems, Inc, New Jersey, USA). For the purpose of this study all infected infants were excluded. Written informed consent was obtained for participation of the mothers and their infants. Ethical approval was obtained from the Committee for Research on Human Subjects of the University of the Witwatersrand (Appendix F).

2.2.2 *Ex vivo* whole blood stimulation assay

Maternal and infant whole blood, collected in section 2.2.1, was diluted 1:10 in sterile RPMI 1640 tissue culture medium supplemented with 2 mM L-glutamine (Sigma Aldrich, St Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich, St Louis, MO, USA) and 100ug/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA). Diluted blood (180 µl per well) was dispensed in triplicate into 96-well flat-bottomed plates for each stimulant as well as for control samples, to which only medium was added. Cultures were stimulated with 20 µl of either phytohaemagglutinin (PHA; final concentration 5 µg/ml) or live attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG: Danish strain 331, Statens serum Institute, Denmark; final concentration 15 µg/ml). PHA cultures were incubated at 37^{o}C in a humidified 5% CO_2 incubator for 3 days, and BCG cultures for 7 days. Culture supernatants were harvested at these time points and stored at -70°C prior to testing by enzyme linked immunosorbent assay (ELISA).

2.2.3. Quantitation of IFN-γ from culture supernatants using ELISA

The amount of IFN-γ produced in control (medium only), PHA and BCG stimulated cultures was determined from the culture supernatants using the commercially available IFN-γ ELISA kit (BD Biosciences Pharmingen, San Diego, California), using pairs of monoclonal antibodies, as described by the manufacturer. The optical density of each well was determined within 30 minutes of addition of the stop solution using a microplate plate reader (Organon Teknika Microwell system 230s reader, Labotec GMBH, Austria) set at 450 nm with a correction wavelength of 570 nm. The mean absorbance for each set of duplicate standards was calculated. The mean zero standard absorbance was subtracted from each of the samples. The concentration of each unknown sample was determined by calculating the concentration of IFN-γ corresponding to the mean absorbance from the standard curve run with each assay. Where samples required a two - a 100-fold dilution the IFN-γ concentration was determined by multiplying the concentration read from the standard curve by the dilution factor. Results for PHA and BCG were obtained by subtracting the medium control result. Results reflected the total amount of IFN-γ produced and were expressed in pg/ml. The detection limit for the IFN-γ ELISA was 5 pg/ml.

2.2.4 Statistical tests

A logarithmic transformation of the concentrations was used to normalize the distribution. The non-parametric Mann-Whitney test was used to determine whether

there were any significant differences in IFN-γ production between the different study groups. Statistical analyses were performed using SPSS software (version 11.0), SPSS Inc., Chicago, IL). All statistical tests were two-tailed and considered significant at $P<0.05$.

2.3 Results

2.3.1 IFN-γ production in the EU infant

The impaired protection of newborn babies against infections has been attributed to a deficient secretion of cytokines such as IFN-γ. To determine whether exposure to HIV-1 *in utero* and at birth has any effect on IFN-γ production, whole blood cultures of newborn infants and their corresponding mothers were set up according to the procedure described in section 2.2.2, that is, cultures were either unstimulated or stimulated with PHA and BCG and the amount of IFN-γ produced measured in culture supernatants by ELISA (section 2.2.3).

Newborn EU infants produced significantly higher levels of IFN-γ than the unexposed control group when stimulated with either PHA ($P<0.001$) (Fig. 2.1A) or BCG ($P=0.005$) (Fig. 2.1B). No significant differences in IFN-γ production were observed in either HIV-1 uninfected or HIV-1 infected mothers when PHA (Fig. 2.1C) or BCG (Fig. 2.1D) were used as a stimulus.

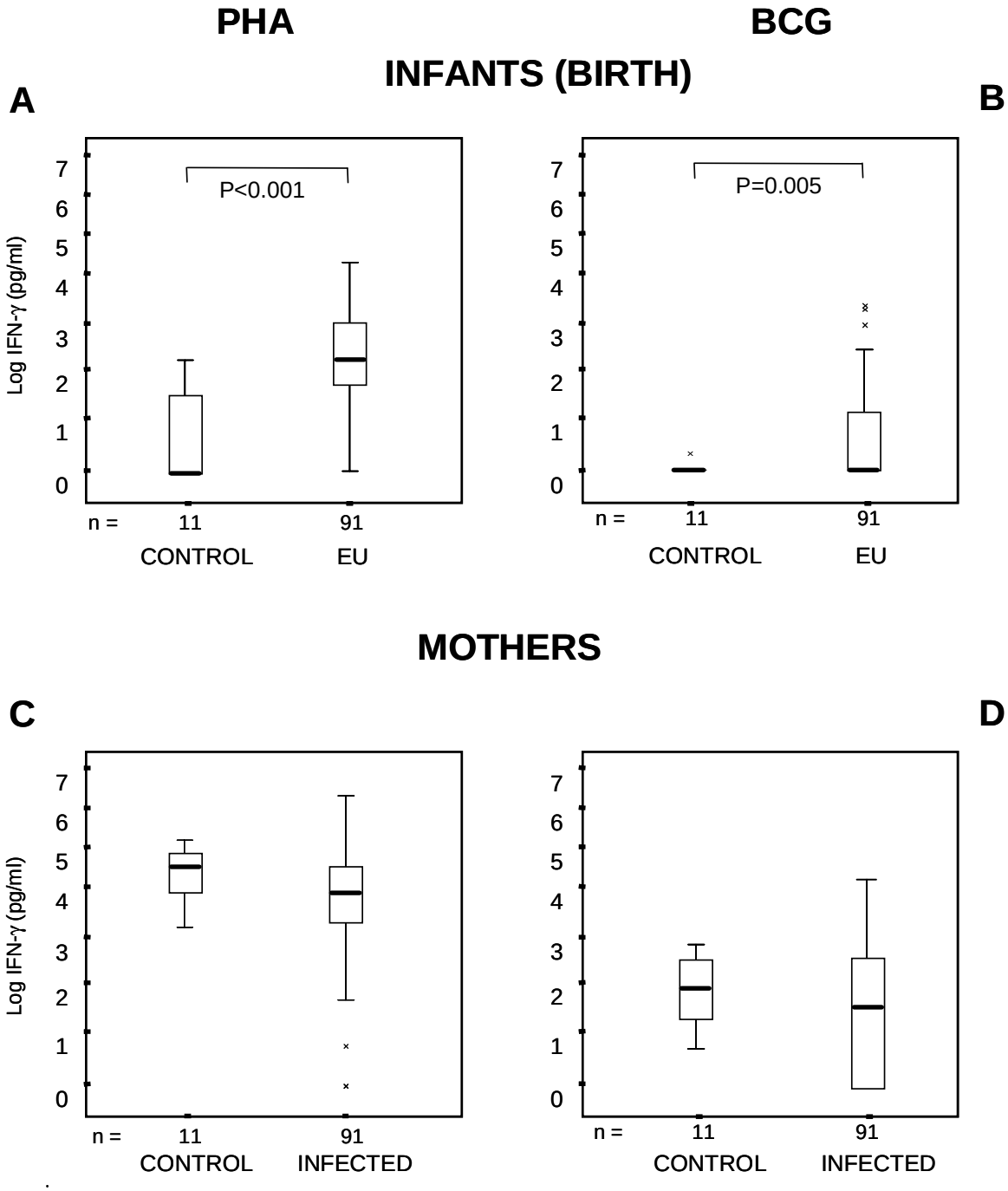


Fig. 2.1 IFN-γ levels measured from whole blood cultures of newborn control and EU infants in response to PHA (A) and BCG (B), and from their corresponding control and HIV-1 infected mothers in response to PHA (C) and BCG (D)

2.3.2 IFN-γ production of infants at birth and six weeks of age

To address the question whether IFN-γ production over time was different between uninfected control and EU infants, whole blood cultures (unstimulated and stimulated with PHA and BCG) were set up at birth and at 6 weeks of age. In addition, cultures were also set up for HIV-1 infected mothers and HIV-1 uninfected mothers at these same time points.

HIV-1 control (unexposed) infants produced significantly more IFN-γ at six weeks of age compared to birth when stimulated with either PHA ($P<0.001$) (Fig. 2.2A) or BCG ($P<0.001$) (Fig. 2.2B). Similarly, EU infants exhibited elevated levels of IFN-γ at six weeks compared to their birth levels to both stimuli (PHA, $P=0.001$, Fig. 2.2C; BCG, $P<0.001$, Fig. 2.2D). Interestingly, while we have shown that IFN-γ production in response to PHA and BCG is significantly higher in newborn (birth) EU than control (birth) infants (Fig. 2.1A and B), by six weeks, control infants produced significantly more IFN-γ in response to PHA only ($P=0.031$; BCG $P>0.05$) compared to EU infants (statistical data not shown in the Figures). These results suggest *in utero* exposure to HIV-1 influences the immune response of EU infants.

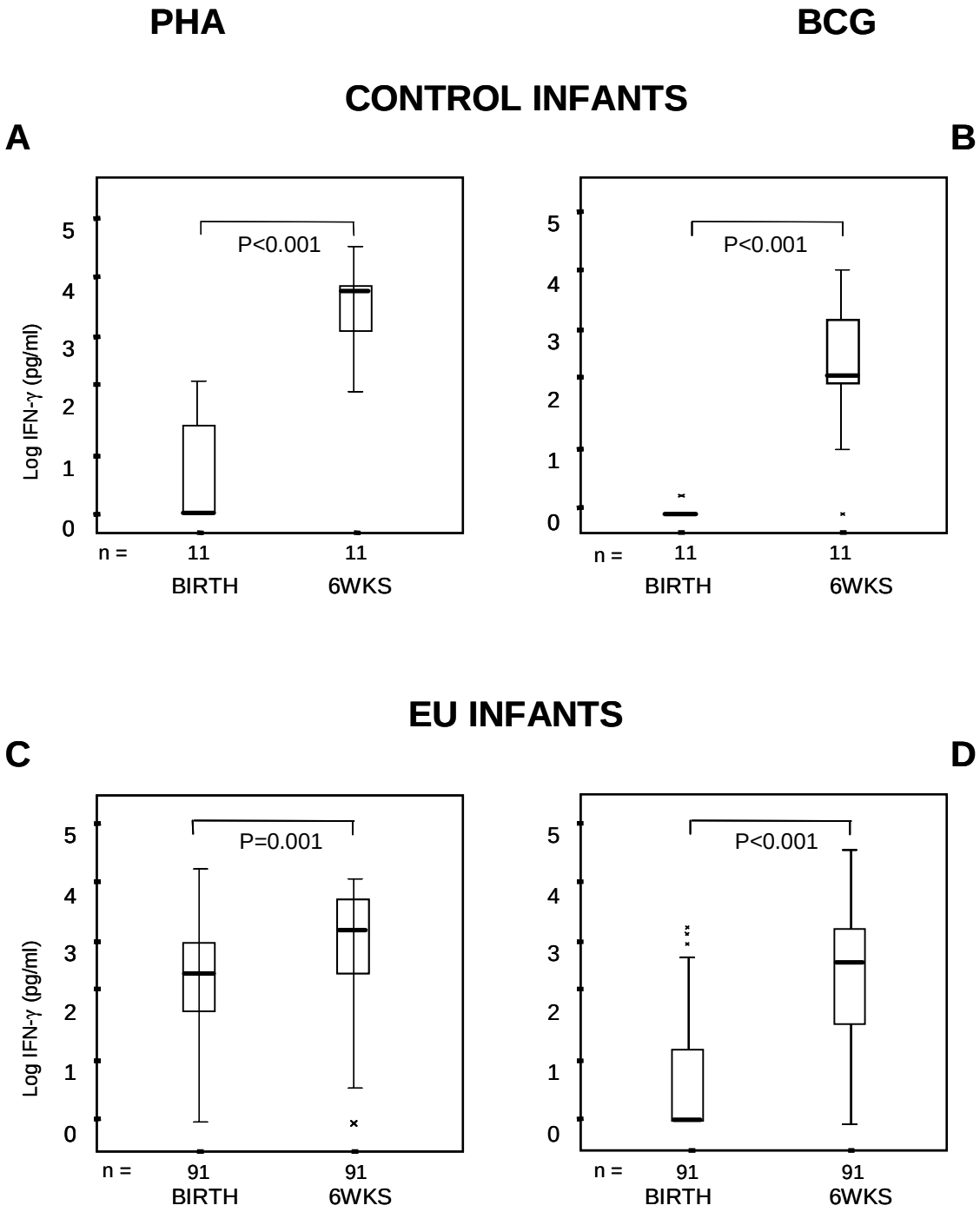
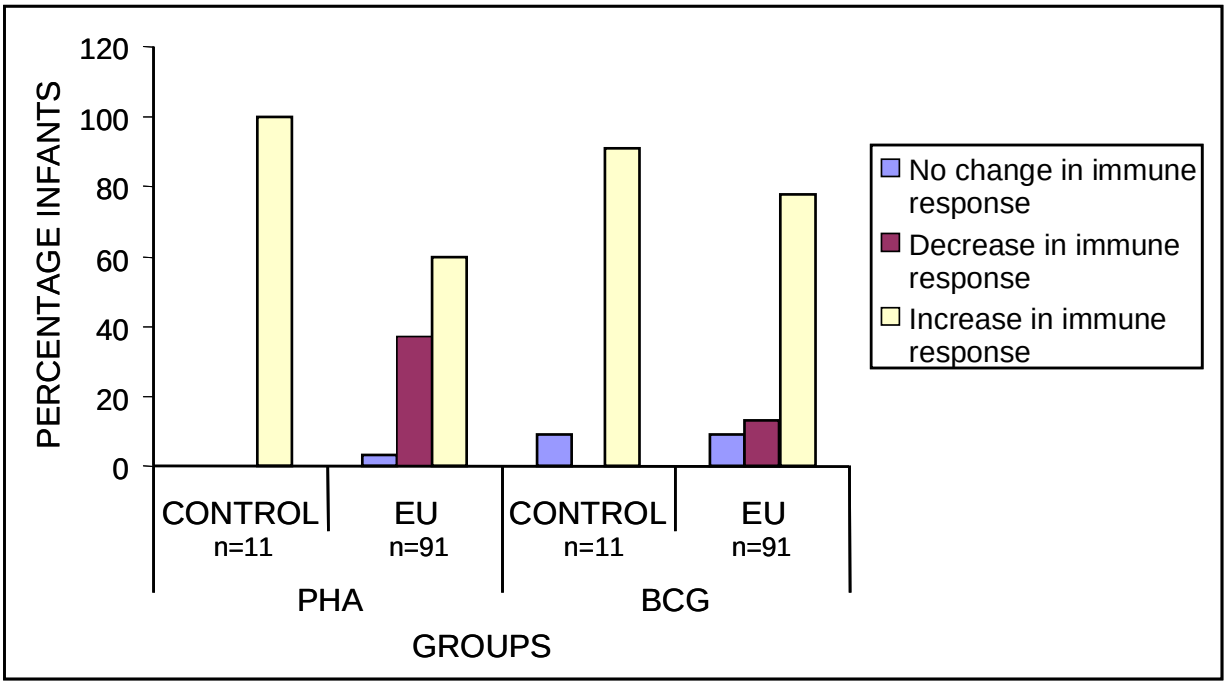


Fig. 2.2 IFN-γ from whole blood cultures of control infants at birth and six weeks (6 wks) in response to PHA (A) and BCG (B); and in EU infants at birth and six weeks in response to PHA (C) and BCG (D)

2.3.3 Maturation of immune responses from birth to six weeks in HIV-1 unexposed and EU infants using PHA and BCG as stimuli

Since the results in section 2.3.2 suggested that *in utero* and birth exposure to HIV-1 influences immune responses, it was interesting therefore to determine the extent of this effect by examining how immune responses developed from birth to six weeks of age in EU and control infants. This was achieved by measuring the change in producing capacity, in response to PHA and BCG in control and EU infants. All control infants (100%) responded with increased IFN-γ production when cultures were stimulated with PHA at 6 weeks relative to their corresponding birth sample, while only 60% of the EU infants demonstrated such an ability (Fig. 2.3). Furthermore, 91% of control infants exhibited increased immune response to BCG through vaccination given at birth while only 79% of EU infants could induce such responses (Fig. 2.3). EU infants tended to exhibit the widest range of patterns of responses with age viz. most infants showing either no change in response or an increased/decreased IFN-γ response when stimulated with either PHA or BCG (Fig. 2.3). A BCG response was already present in 48% (44/91) of EU infants at birth and 14% (13/44)) showed a decrease subsequent to vaccination whereas 34% (31/44) showed an increase subsequent to BCG vaccination (data not shown in Figures).



	PHA		BCG	
Change in immune response % (n/N)	CONTROL	EU	CONTROL	EU
No change	0 (0/11)	1 (1/91)	9 (1/11)	7 (6/91)
Decrease	0 (0/11)	31 (35/91)	0 (0/11)	14 (13/91)
Increase	100 (11/11)	60 (55/91)	91 (10/11)	79 (72/91)

Fig. 2.3 Ability over time (birth to six weeks) of control and HIV-1 EU infants to respond to PHA and BCG

2.4 Discussion

Newborns and infants are particularly susceptible to infections and this has been ascribed to deficient immunoresponsiveness. Th1 responses, characterized by the production of IFN-γ, have been considered to be required for protection against mycobacteria and viruses. Immaturity of T cell immunity and a bias to Th2 responses have been considered to be obstacles to successful neonatal vaccination. Specifically, T cell dependent responses have been shown to be biased towards a Th2 phenotype ascribed to particular conditions of antigen presentation at priming (Delespesse *et al*., 1998). Furthermore, the production of interferons, IL-12/IL-23 and IL-18 as well as other pro-inflammatory cytokines are reportedly deficient in neonates (Kovarik *et al*., 1998; Marodi, 2006). This study was designed to investigate the production of the immune cytokine, IFN-γ in response to PHA and BCG vaccine, among control infants and those exposed to HIV-1 *in utero* and to evaluate the maturation of the cellular immune response in the first six weeks of life in these two groups of infants.

A whole blood assay was the method of choice over other alternative assays e.g. the lymphocyte proliferation assay, cytokine detection with peripheral blood mononuclear cell cultures or the enzyme linked immunospot assay (ELISPOT) due to it (i) being simple for assessing immune cytokine profiles, (ii) showed little inter-assay variability, (iii) could be performed on small amounts of blood that did not require sophisticated processing or the purchase of expensive materials and equipment (Pai *et al*., 2004; Scholvinck *et al.,* 2004) and (iv) has been proven to reliably detect IFN-γ responses to

mitogenic stimuli and to more accurately reflect the *in vivo* situation (Hussain *et al*., 2002; Scholvinck *et al*., 2004).

Our results have shown that (i) newborn infants exposed *in utero* to HIV-1 produce significantly more IFN-γ than unexposed infants following PHA or BCG stimulation, (ii) overall an increase in IFN-γ producing capacity occurs within the first six weeks of life in both control and EU infants, (iii) BCG induced responses are more prevalent in newborn EU infants but by six weeks, maturation of responses of EU and control infants was similar, (iv) a lesser percentage of EU infants than control infants are able to respond with an increase in IFN-γ production at six weeks in response to PHA and BCG, (v) an interesting and unexpected observation between EU and control infants (data not shown) was that an IFN-γ response to BCG at birth is already present in 48% of the EU infants.

In support of our findings, Vekemans *et al*. (2001) have reported the median IFN-γ production to BCG and PHA to increase significantly in both HIV-1 exposed and unexposed children from birth to six weeks of age reflecting the establishment of an immune response (following BCG vaccination) and maturation of the infant's immune response (response to PHA). Importantly, these observations suggested that immunological differences observed at birth between HIV-1 exposed and HIV-1 unexposed infants may persist for at least the first six weeks of life.

Increased IFN-γ production may be accounted for by increased priming of the immune system as reflected by increased immune activation. The increased immune

activation, reported in HIV-1 exposed uninfected infants may be a consequence of *in utero* exposure to HIV-1 antigens and/or exposure to antigens related to other infections in their HIV-1 infected mothers (Rich *et al*., 1997). In fact, it has been reported that levels of immune activation markers, measured from cord blood plasma, are elevated in EU infants and that levels of the immune activation markers are further elevated in EU infants exposed to single-dose NVP when compared to their drug unexposed counterparts (Schramm *et al.,* 2006). Thus, exposure to drugs may be sufficient to drive an already activated immune system into a further anergic/immunodeficient state (Schramm *et al.,* 2006). The inability or decreased ability of some EU infants to produce IFN-γ at 6 weeks may indicate a lack of immune response to BCG, a lack of maturation of the cellular immune response or might well be a consequence of T cell anergy, an inability of the immune system to develop or a 'fast-tracked' immune response viz. the immune capability of newborn EU infants (as shown by higher IFN-γ production) compares to that of a six week old infant who has not similarly been primed through HIV-1 exposure. The already present BCG response in a substantial proportion of the EU infants at birth is interesting and most likely is due to an innate/non-specific response to BCG (which may be triggered by the greater state of immune activation in EU vs control infants), as an adaptive response would require previous exposure to BCG and would not be instantaneous, requiring time to develop.

The study as described has a few limitations, namely, (i) the follow-up period was at six weeks and no further longitudinal time points were assessed. It would have been interesting to determine, for EU infants that either showed no responses or decreased responses, the time points when immune responses could be measured or for infants

that did show responses the length of time that such responses would persist. The long term effect of *in utero* exposure definitely requires further research, (ii) a disadvantage of the whole blood assay is that the cell types responsible for the IFN-γ production could not be identified. In Chapter 4 this latter point is addressed using intracellular cytokine (ICC) staining to identify the predominant IFN-γ producing cell types.

Early immunization is required to protect infants from pathogens to which they are exposed after birth but this is limited by the immaturity of the neonatal immune system. Vaccination of newborns with BCG has been shown to result in the development of Th1-type responses (Marchant *et al*., 1999) however our findings indicate that prenatal exposure to HIV-1 influences the immune response to both PHA and BCG which could influence both the capacity to respond to early immunization or result in altered thresholds of responsiveness which could then influence the outcome of subsequent infectious events or vaccine responses. These findings may have important implications for the optimal timing and effectiveness of BCG vaccination in these infants and warrants careful immunological evaluation of Tuberculosis vaccines. While the clinical importance of these findings remains unknown, further investigation is warranted as the observed immunological changes may result in altered susceptibility to infectious diseases in this already vulnerable population.

CHAPTER 3

Haematological changes in infants born to HIV-1 uninfected and HIV-1 infected mothers

3.1 Introduction

It has long been shown that the human immune system is functionally less mature at birth and within the first years of life, undergoes a process of sequential development that is both programmed genetically and stimulated externally by antigen exposure (Erkeller-Yuksel *et al*., 1992). In particular, neonates are highly susceptible to bacterial, viral, and parasitic intracellular pathogens. This profound neonatal immunodeficiency can be attributed to immaturity and dysregulation of specific T cell mediated immunity, lack of memory T cells, and dysregulation of expression and production of specific lymphokines, cytokines and inflammatory mediators (Suen *et al*., 1998). Compared to adults, infants have increased numbers of lymphocytes as well as different distributions of the major lymphocyte subsets (Plaeger-Marshall *et al*., 1993). In Chapter 2, we looked at IFN-γ production from whole blood cultures of HIV-1 uninfected mothers and their infants as well as HIV-1 infected mothers and their EU infants. It was shown that EU infants produce significantly more IFN-γ at birth compared to controls indicative that T cell responsiveness may develop earlier due to *in utero* HIV-1 priming. It was interesting therefore to question, using a leukocyte haematology analyzer, whether differences in proportions or absolute lymphocyte blood counts (particularly since cells such as T cells and NK cells known to be major

producers of IFN-γ are in this cell compartment) might account for the disparities observed in the IFN-γ production between the infant groups or whether exposure to short course ART treatment (to prevent maternal HIV-1 transmission) may influence blood cell counts that could in turn affect IFN-γ production levels.

3.2 Materials and Methods

3.2.1 Study subjects and samples

The study subjects were recruited at CHBH, Soweto, South Africa and from Coronation Women and Children Hospital (CWCH), Johannesburg, South Africa. These participants formed part of the HIV Protective Immunity and Perinatal Exposure (PIPE) study. The participants were recruited from women identified as HIV-1 seropositive as part of a voluntary counselling and testing programme. Women were enrolled within 48 hrs of delivery and were given a single dose of NVP (sdNVP)(200 mg) to prevent maternal transmission of HIV-1 at the onset of labour. For various reasons some mothers did not take the dose of NVP. This nested case-controlled study comprised 83 HIV-1 infected mothers and their EU infants and 14 HIV-1 uninfected mother-child pairs. Of the HIV-1 infected mothers, only 72 mothers took NVP. Thus, only infants whose mothers had taken the dose of NVP at the onset of labour were exposed to NVP during labour and delivery. However, all infants were given a dose of NVP (2 mg/kg) within 48 hrs of birth to prevent postpartum transmission. Peripheral blood (10 ml) was collected by venipuncture from the mother at delivery and from the infants (5 ml) within 48 hrs of birth in preservative-free five ml EDTA vacutainer tubes, and again at six weeks. The blood was transported to the National Institute for Communicable Diseases Cell Biology laboratory and processed within three hrs of collection. Written informed consent was obtained from all participants and ethical approval was obtained from the Committee for Research on Human Subjects of the University of the Witwatersrand (Appendix F).

HIV-1 DNA PCR tests were carried out at to establish infection status and timing of infection using the Roche Amplicor HIV-1 DNA PCR assay version 1.5 (Roche Diagnostic Systems, Inc, New Jersey, USA). Maternal viral load was measured in plasma using the Roche Amplicor RNA Monitor assay (Roche Diagnostic Systems, Inc, New Jersey, USA) with a lower detection limit of 400 RNA copies/ml.

3.2.2 Full blood counts

A full blood count was performed on the maternal and infant blood samples within three hrs of blood having been drawn from patients, using a fully automated AcT 5 diff haematology analyser (Beckman Coulter Inc, Fullerton, CA) according to the manufacturer's protocol. The instrument is automated to draw up 52 µl of sample for analysis. The instrument differentiated monocyte, neutrophil, eosinophil and basophil populations based on absorbance patterns produced by differential cytochemical staining of their granules versus volume. Reference controls (low, medium and high; Beckman Coulter Inc, Fullerton, CA) were routinely used to confirm and monitor instrument accuracy and performance by providing measurements for counting, sizing, haemoglobin determination and white blood cell differentiation. For each sample a report was automatically printed and the data on the cell types was presented as a percentage as well as an absolute number per microlitre (µl). Specimen results were accepted when the control ranges fell within those specified for the reference controls. Specimens whose results fell out of the range of determination were diluted (1:2 or 1:4 in saline) and the dilution factor taken into account when leukocyte counts for these samples were analysed.

3.2.3 Statistical Tests

The non-parametric Mann-Whitney test was used to determine whether there were any significant differences in the proportions and absolute counts between the groups. The Wilcoxon test was used to determine whether there were any significant differences in the proportions and absolute counts of the different leukocyte populations of the groups at birth and six weeks. Statistical analyses were performed using SPSS software (version 11.0, SPSS Inc., Chicago, IL). All statistical tests were two-tailed and considered significant at $P<0.05$.

3.3 Results

3.3.1 Changes in infant leukocyte types at birth and at six weeks

The immune system of the young child undergoes maturation and development that is reflected in qualitative and quantitative changes in the peripheral blood (Jaspan *et al*., 2006). It was interesting therefore to compare the proportions and absolute counts of the different leukocyte types (neutrophils, lymphocytes, monocytes, eosinophils and basophils) of control and EU infants at birth and six weeks later (Table 3.1 and Fig. 3.1 A-D). In the control group of infants, lymphocyte and eosinophil proportions increased significantly over the first six weeks of life while neutrophil and basophil proportions decreased significantly. Proportions of monocytes remained unchanged over the first six weeks. Similarly, amongst EU infants, proportions of all leukocyte types, with the exception of monocytes, changed significantly (increased or decreased) over the first six weeks of life (Fig. 3.1 A-D). Particularly significant are the differences in the absolute counts of EU infants relative to control infants (Table 3.1). Whilst absolute counts of all leukocyte types are significantly altered from birth to six weeks amongst EU infants, only neutrophil, monocyte and basophil absolute counts of control infants change significantly from birth to six weeks. Importantly, no significant differences could be demonstrated between the absolute counts of the leukocyte types of birth infants (control and EU groups). At six weeks however, only the absolute eosinophil counts of EU infants were found to be significantly elevated compared to control infants. Notably, the absolute counts of the lymphocytes did not change significantly when comparing control and EU infants at birth ($P>0.05$) or control and EU infants at six weeks ($P>0.05$) (Fig. 3.2), although there was a trend

that EU infants have higher absolute lymphocyte counts at 6 weeks than control infants. Only the EU infants showed significantly increased absolute lymphocyte counts between birth and six weeks (P<0.001).

Table 3.1 Absolute leukocyte counts of birth and 6 week samples of mothers and their infants

Leukocyte type	**Infant**					
Median (IQR)	**Control**			**EU**		
n	**14**			**83**		
	Birth	**6 wks**	**P**	**Birth**	**6 wks**	**P**
Units ($x10^3$/µl)						
White blood cells	14.4 (12.0-20.7)	7.5 (6.8-9.6)	0.007	15.5 (13.1-18-3)	9.4 (7.0-11.8)	<0.001
Neutrophils	9.0 (6.0-11.3)	1.8 (1.4-2.3)	0.001	9.1 (7.0-10.9)	1.9 (1.4-2.6)	<0.001
Lymphocytes	4.5 (2.7-5.2)	4.8 (3.7-6.5)	>0.05	3.6 (3.0-4.4)	5.7 (4.5-7.7)	<0.001
Monocytes	1.3 (1.1-2.5)	0.7 (0.5-1.5)	0.016	1.5 (1.2-2.0)	1 (0.6-1.4)	<0.001
Eosinophils	0.2 (0.14-0.3)	0.3[1] (0.2-0.4)	>0.05	0.3 (0.2-0.4)	0.2[1] (0.1-0.4)	0.015
Basophils	0.4 (0.31-1.1)	0.1 (0.1-0.2)	0.001	0.5 (0.3-1.5)	0.1 (0.1-0.2)	<0.001
	Mother					
Median (IQR)	**Control**			**Infected**		
n	**14**			**83**		
	Birth	**6 wks**	**P**	**Birth**	**6 wks**	**P**
Units ($x10^3$/µl)						
White blood cells	12.7 (9.5-19.8)	5.6 (3.9-7.5)	0.004	10.3 (8.4-15.8)	4.9 (4.1-6.1)	<0.001
Neutrophils	9.5 (6-13.2)	2.5 (1.5-3.8)	0.002	6.9 (5.3-10.8)	2.3 (1.7-3.1)	<0.001
Lymphocytes	2.3 (1.8-2.9)	2.3 (1.6-2.7)	>0.05	2.3 (1.8-2.7)	1.9 (1.6-2.5)	0.001
Monocytes	0.8 (0.6-1.4)	0.3 (0.3-0.5)	0.009	0.8 (0.5-1.1)	0.4 (0.3-0.5)	<0.001
Eosinophils	0.2[2] (0.1-0.4)	0.2 (0.08-0.3)	>0.05	0.2[2] (0.1-0.3)	0.1 (0.07-0.2)	>0.05
Basophils	0.1[3] (0.07-0.2)	0.1 (0.03-0.07)	>0.05	0.1[3] (0.05-0.1)	0.03 (0.02-0.05)	<0.001

Statistical differences between birth and 6 wk samples are presented

[1] P=0.035, EU infants versus control infants at six wks
[2] P=0.018, infected mothers versus C mothers at delivery
[3] P=0.037, infected mothers versus C mothers at delivery

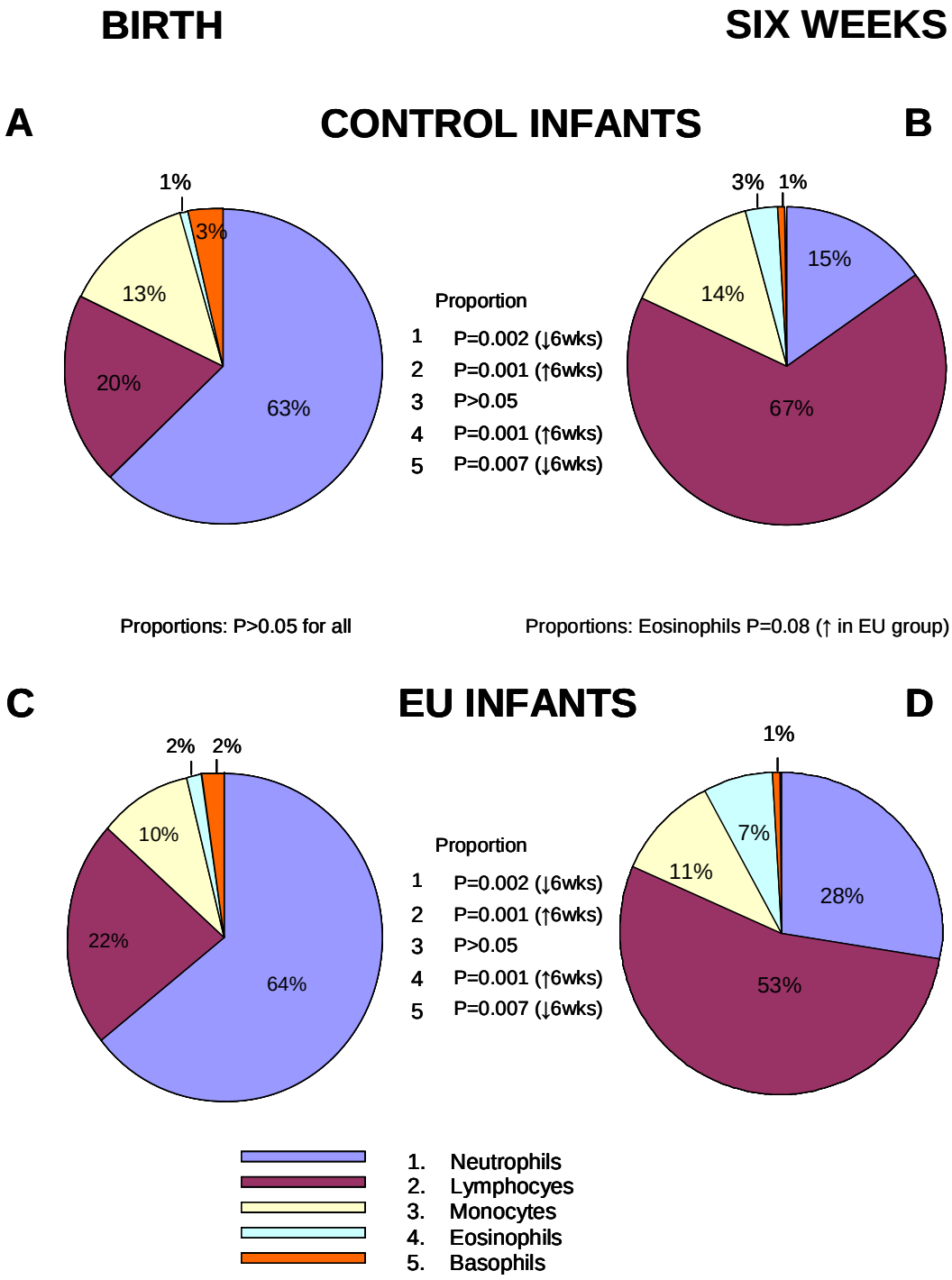


Fig. 3.1 Proportions of leukocyte types in control birth infants (A), control 6 week old infants (B), EU birth infants (C) and EU 6 week old infants (D). Significant changes (data presented between the pie charts) in the proportions of the leukocyte types are shown for the control and EU infants from birth to 6 weeks and across groups at similar time points

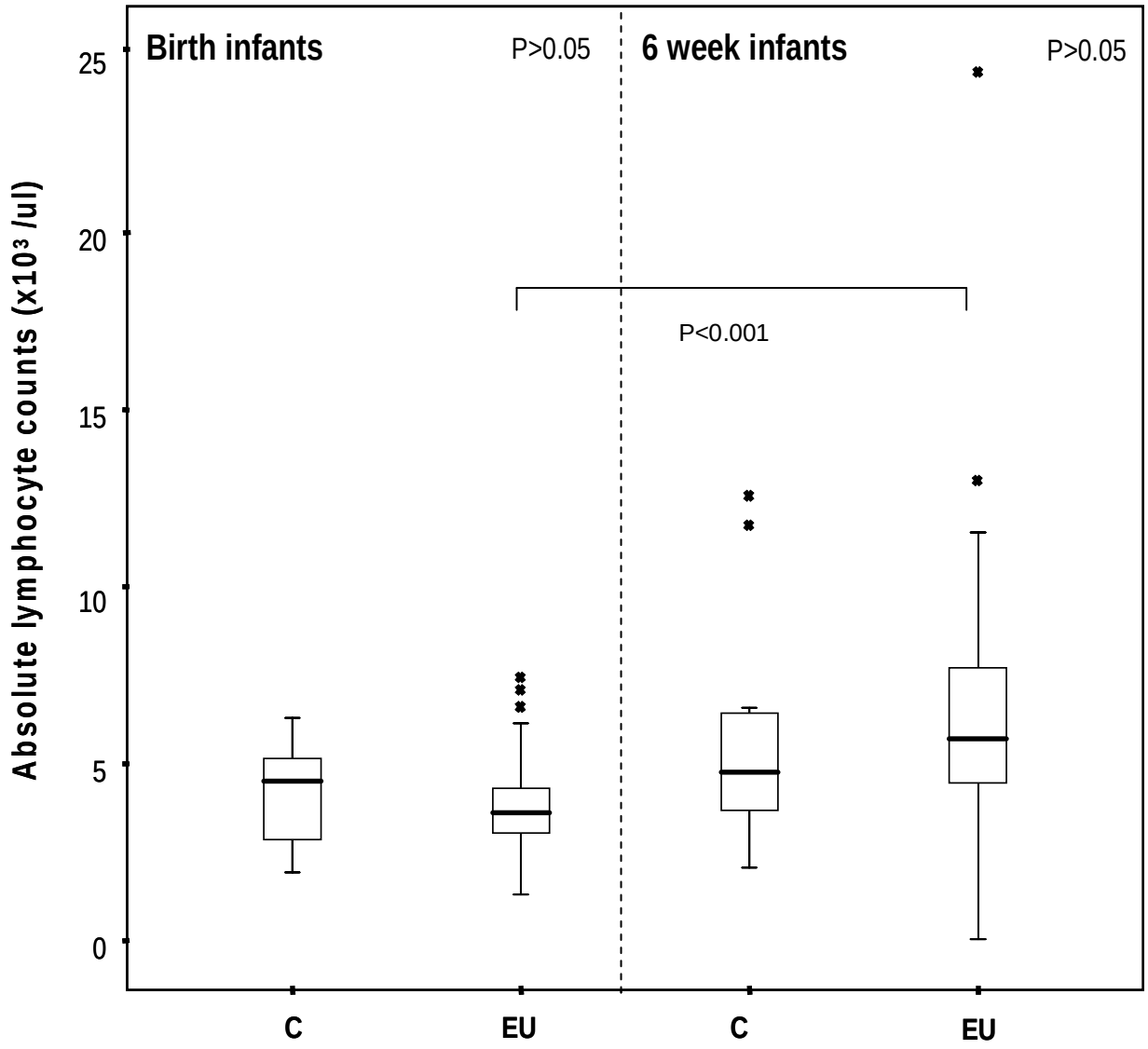


Fig. 3.2 Comparison of absolute lymphocyte counts of control (C) and EU infants at birth and at six weeks

3.3.2 Changes in maternal leukocyte counts in control and HIV-1 infected mothers following delivery and six weeks later

Immune responses in pregnant mothers are considered to be biased towards Th2 cytokine expression. It was therefore interesting to establish how leukocyte types change after delivery and to establish the extent of these changes by comparing HIV-1 uninfected mothers to those of HIV-1 seropositive mothers (Fig. 3.3 A-D and Table 3.1). In control mothers, six weeks after delivery, proportions of neutrophils were significantly decreased and lymphocyte and eosinophil proportions significantly increased. This was similarly observed in HIV-1 positive mothers. Within the first six weeks, absolute counts of neutrophils, lymphocytes, monocytes and basophils of HIV-1 infected mothers were significantly altered from their counts following delivery (Table 3.1). This was only true for the absolute neutrophil and monocyte counts of uninfected mothers. HIV-1 positive mothers had significantly reduced absolute counts of eosinophils and basophils compared to uninfected mothers at birth, however, these differences were resolved six weeks post partum.

3.3.3 Haematological changes in mothers who received single dose NVP at the onset of labour and their infants

In vitro studies using clinically relevant concentrations of antiretrovirals e.g. zidovudine (AZT) have reported reduced responsiveness of PBMC to mitogen and a decreased ability to proliferate (Heagy *et al*., 1991). It was therefore interesting to determine whether short exposure to NVP (maternal dosage given at the onset of labour; infants exposed to the dose given to the mother and a further dose given within 48 hrs of

birth), could significantly impact on the composition of maternal or infant leukocyte types at birth and if so whether these differences would persist over time.

When maternal leukocytes were compared between mothers that received sdNVP and those who did not (Table 3.2) the absolute blood counts did not significantly differ at either birth or six weeks later suggesting that sdNVP exposure does not impact on maternal blood counts. Infants exposed to NVP given to the mother at the onset of labour have significantly elevated basophil counts (P=0.043) at birth. By six weeks this effect was lost and leukocyte blood counts of infants exposed to maternal NVP were not significantly different from counts of infants given NVP within 48 hrs of birth.

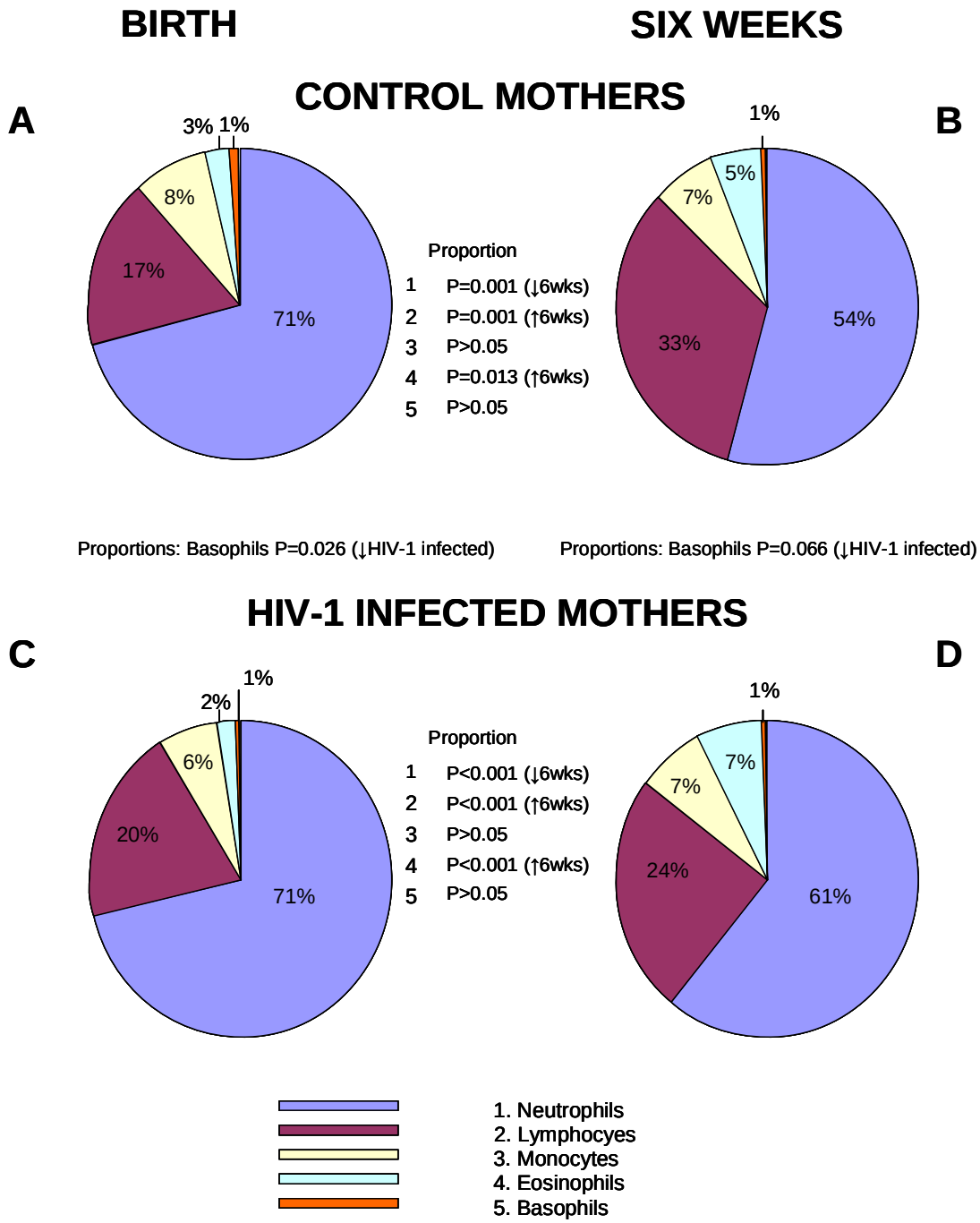


Fig. 3.3 Proportion of leukocyte types in control mothers at delivery (A), control mothers 6 weeks later (B), HIV-infected mothers at delivery (C) and HIV-1 infected mothers 6 weeks later (D). Significant changes (data presented between the pie charts) in the proportions of the leukocyte types are presented for the control and HIV-1 infected mothers from the time of labour to 6 weeks post partum and across the groups for these time points

Table 3.2 Significant differences in the absolute leukocyte counts of mothers and their infants distinguished on NVP versus no NVP exposure

Effects of NVP on leukocyte types of mothers and infants at birth and six wks				
	Mother[1]		EU infant[2]	
Leukocyte type	**NVP vs no NVP birth P**	**NVP vs no NVP 6 wks P**	**NVP vs no NVP birth P**	**NVP vs no NVP 6 wks P**
White blood cells	>0.5	>0.5	>0.5	>0.5
Neutrophils	>0.5	>0.5	>0.5	>0.5
Lymphocytes	>0.5	>0.5	>0.5	>0.5
Monocytes	>0.5	>0.5	>0.5	>0.5
Eosinophils	>0.5	>0.5	>0.5	>0.5
Basophils	>0.5	>0.5	0.043*	>0.5

[1] Mothers given sdNVP at onset of labour n=72 (birth and six wks)
Mothers not taking NVP at onset of labour n=12 (birth and six wks)
[2] Infants exposed to maternal sdNVP n=70 (birth and six wks); no blood counts were performed on two of the infants at six wks
Infants not exposed to any NVP n=12 (birth and six wks)
* Increased in the presence of NVP

3.4 Discussion

Progressive physiological maturation of the immune system results in age-dependent differences in immune function in infants, adolescents and adults (Jaspan *et al*., 2006). In Chapter 2, we showed that EU infants produce significantly more IFN-γ at birth. In this Chapter we therefore explored whether significant changes in the leukocyte counts, particularly lymphocytes, could account for the enhanced IFN-γ production of EU infants or whether the short exposure to sdNVP could influence leukocyte counts.

The full blood count (FBC) or complete blood count (CBC) automated assay was the method of choice over alternative assays like Flow Cytometry (Shahabuddin *et al.*,1998) and direct and indirect immunofluorescence (Solinger,1985) due to the method (i) being simple and easy to perform; (ii) did not require the use of expensive antibodies; (ii) could be performed on small amounts of blood that did not require sophisticated processing and (iv) did not require that the operator undergo an intensive training programme. Notably, the FBC provides information about the types and numbers of cells in the blood, especially red blood cells, white blood cells and platelets. For our purposes, a total WBC cell count was required and the FBC assay adequately fulfilled this purpose. Limitations of the method include the fact that the blood represents only about 2% of the total lymphocyte pool in the normal adult human body and it is therefore not possible to estimate the changes in the number of lymphocytes within the lymphoid tissues by using data derived only from blood (Westermann *et al*., 1990).

It is important to note that our results came from a cohort of healthy and HIV-1 infected adult women and their newborn infants of African descent. Aside from demonstrating time dependent maturational changes in the control and EU infants as well as the leukocyte changes influenced by pregnancy in the mothers (control and HIV-1 infected) we could establish reference limits corresponding to “normal values” for these groups since earlier data suggests that the leukocyte counts in people of African descent are significantly lower than in caucasians (Sadowitz *et al*., 1983). Aside from race differences, it has also been recognized that peripheral blood lymphocyte subsets of adults can also vary in accordance to age, and environmental factors (Chng *et al.,* 2004). Some of these same factors, including gender, have been shown to influence leukocyte counts of infants (Taha *et al*., 2002; European Collaborative Study, 2003; 2004) and children up to eight and 12 years (European Collaborative study, 2003) of age.

Using the Act5diff haematology analyzer therefore we demonstrated that amongst infants (i) there are no statistically significant differences in the proportions or absolute leukocyte types of control and EU infants at birth; (ii) six weeks after birth EU infants have significantly different proportions and absolute eosinophil counts only compared to control infants; (iii) absolute lymphocyte counts are not significantly different either at birth or at 6 weeks between control and EU infants; (iv) while the proportion of blood cell types changes over time for both control and EU infants (neutrophils and basophil proportions decrease and lymphocyte and eosinophil proportions increase significantly with time) this does not hold true for the absolute leukocyte counts of EU and control infants (neutrophil, monocyte, eosinophil and basophil counts decrease with only lymphocyte counts increasing with time for EU

infants and only decreases in neutrophil, monocyte and basophil counts being significant for control infants); (v) the predominant cell type (based on absolute counts) of infants (control and EU) at birth are neutrophils while at six weeks it is lymphocytes and (v) sd NVP increased basophil counts of infants at birth but at six weeks the effect was no longer present. For mothers (i) some leukocyte counts at delivery compared to the profiles found 6 weeks post partum; (ii) at the time of birth HIV-1 positive mothers have reduced eosinophil and basophil absolute levels compared to uninfected mothers and (iii) the single dose of NVP taken by the mother at the onset of labour does not affect the leukocyte counts significantly.

Neutrophil and basophil proportions decrease in the uninfected control infants during the first six weeks of life and lymphocyte counts increase within the first 6 weeks of life. The significant increase in the eosinophil population has not been previously reported. Interestingly, eosinophilia is a feature of parasitic infections, allergic disorders and some drug reactions (Zuo *et al*., 2007). The significant decrease over time of the absolute monocyte count noted in our study may reflect the significant changes in cell types characteristic of a maturing immune system i.e. change from innate to adaptive immune responses.

During pregnancy physiologic immune adaptations occur involving changes in cell counts, phenotypes, functions and soluble factors e.g. cytokines such that a successful pregnancy is established and maintained (Luppi, 2003). Thus, the changes observed amongst control and HIV-infected mothers from labour to 6 weeks post partum were not unexpected. In fact, a study by Thompson *et al.,* (1999) suggests that uninfected maternal peripheral blood shows an increase in the total

white blood cell count following labour. Variability in the blood counts of the mothers are likely to be indicative of inflammatory states produced by disease states e.g viral (HIV, as is the case in this study), bacterial (tuberculosis) and other protozoal or rickettsial infections.

Lymphocyte counts did not differ significantly between control and EU infants either at birth or at 6 weeks, thus by extrapolation the higher IFN-γ levels in EU infants in Chapter 2 are unlikely to be the result of differential counts between these two groups. Short term antiretrovirals did impact on cell counts in that basophil counts were increased at birth but these normalized by six weeks, therefore NVP was unlikely to have influenced IFNγ production in the EU infants in such a way that could account for differences in production of IFN-γ between control and EU infants. It should be noted that all the infants from the cohort in Chapter 2 received NVP so the question of NVP effects on IFN-γ production was not addressed directly.

In conclusion, the differences noted in IFN-γ production between control and EU infants in early life could not be attributed to altered distributions of the major leukocyte groupings.

CHAPTER 4

Identifying mononuclear cell types that produce IFN-γ in the HIV-1 exposed uninfected infant

4.1 Introduction

Early innate production of IFN-γ is a critical step in immunological defence against intracellular pathogens (Chougnet *et al*., 2000; Suen *et al*., 1998 and Trivedi *et al*., 1997), playing a role in determining whether cell mediated or humoral responses will predominate. IFN-γ is secreted mainly by T lymphocytes ($CD4^+$ and $CD8^+$) and by NK cells, modulating the function of a wide variety of cell types (Trinchieri, 1985) and possibly amplifying activation of these same cells in a variety of tissues (Lewis, 1990). IFN-γ has dramatic effects on monocyte and macrophage function and is probably the most important mediator of macrophage activation (Murray *et al.,* 1998*).*

The principle that the cytokine environment present at the time of priming influences responses and infection outcome also applies in neonates. Impaired immune protection of infants has been attributed to the predominant Th2 type responses (Marodi, 2006). Furthermore, the production of IFN-γ, a Th1 cytokine, is impaired in early life (Wilson *et al*., 1986), with a lag in the ability to produce IFN-γ occurring for at least the first two months of life (Burchett *et al.,* 1998). Over time, both the capacity of T cells to produce IFN-γ and the number of memory T cells increases (Sanders *et al*.,

1998). IFN-γ is produced in larger amounts by T cells that have been previously activated *in vivo* by their interaction with suitably presented antigens.

In Chapter 2 we established that whole blood cultures of newborn infants exposed *in utero* to HIV-1 produce significantly more IFN-γ than unexposed infants following PHA or BCG stimulation. In Chapter 3, we demonstrated that absolute lymphocyte counts did not differ significantly between control and EU infants at birth or at six weeks and that exposure to NVP administration did not influence the lymphocyte counts at either of these time points, suggesting that increased IFN-γ in EU infants was not likely to be due to greater numbers of lymphocytes in the cultures of this group. Lymphocytes comprise the two major IFN-γ producers, T cells ($CD4^+$ and $CD8^+$) and NK cells, as well as B cells. The remaining cell types in whole blood viz. polymorphonuclear neutrophils and monocytes are not known to produce IFN-γ when stimulated, however monocytes have been reported to have an accessory role in enhancing lymphocyte IFN-γ production (Mckenzie *et al*., 1993; Carson *et al*., 1995). What is not apparent from using whole blood cultures is which cells are contributing towards the total amount of IFN-γ secreted. We therefore wanted to establish which mononuclear cell types contributed significantly to IFN-γ produced in response to mitogen (PHA) or vaccine (BCG), in HIV-1 exposed uninfected infants. This was achieved using a whole blood ICC assay which allows the identification of IFN-γ producing cell types.

4.2 Materials and methods

4.2.1 Study subjects and samples

Participants used in this part of the study formed part of the HIV Protective Immunity and Perinatal Exposure study described in Chapter 3 section 3.2.1. Blood samples from eight HIV-1 infected mothers and their 10 week old EU infants were utilized for this study. In addition, blood samples from one HIV-1 seropositive mother and her EU newborn (DNA PCR-negative at six weeks and 10 weeks) and two controls (HIV-1 seronegative mothers and their newborn infants) were also included for ICC analysis. All HIV-1 infected mothers and their infants received a standard regimen of one dose each of NVP (200 mg for the mother and 2 mg/kg for the infant). Two mothers received three drugs NVP, AZT and 3TC for approximately four months prior to sample collection.

Venous blood was collected in seven millilitre sodium heparin (Na Hep) and five millilitre EDTA vacutainer tubes and was processed within three hrs of collection. Blood collected in the Na Hep tubes was used for ICC determination and EDTA blood tubes were used for DNA PCR or viral load and $CD4^+$ T cell count determinations. Blood from the one EU birth sample was drawn within 48 hrs of birth after being given a dose of NVP. Bloods from the eight EU infants were drawn 10 weeks after they had received NVP. For infants, HIV-1 DNA PCR tests were carried out to establish infection status and timing of infection using the Roche Amplicor HIV-1 DNA PCR assay version 1.5 (Roche Diagnostic Systems, Inc, New Jersey, USA).Maternal viral loads were measured from plasma using the Roche Amplicor RNA Monitor assay

(Roche Diagnostic Systems, Inc, New Jersey, USA) with a lower detection limit of 400 RNA copies/ml. Maternal $CD4^+$ T cell counts were determined using the commercially available FACSCount system (Becton Dickinson, San Jose, CA).

4.2.2 Intracellular cytokine assay

Blood samples collected in NaHep tubes were used for ICC analyses. Whole blood (200 µl) was either stimulated with PHA (final concentration 5 µg/ml) or BCG vaccine (final concentration 15 µg/ml) or unstimulated (samples were incubated with 4 µl of a 1:10 dilution of dimethylsulphoxide (DMSO)(Sigma-Aldrich Corp., St Louis, MI, USA). 2 µl of the co-stimulatory antibodies CD28 and CD49d (BD Biosciences, San Jose, CA, USA), diluted 1:10 in sterile phosphate buffered saline (PBS) (NICD media department, Sandringham, SA) as well as 4 µl of the secretory inhibitor Brefeldin A (BFA) (Sigma-Aldrich Corp., St Louis, MI, USA) were added to each sample. Samples were incubated for 12 hrs at 37°C, after which they were cooled to 18°C. To arrest further stimulation and to remove cells from the sides of the sample tubes 20 µl of EDTA (final concentration 20 mM)((Roche Diagnostic Systems, Inc, New Jersey, USA) was added to each sample. Samples were mixed vigorously on a vortex and incubated for 15 minutes at room temperature. Thereafter, 2 ml 1x FACS lysing solution (BD Biosciences, San Jose, CA, USA) was added to each tube and incubated for 10 minutes at room temperature in order to simultaneously lyse the erythrocytes and fix the leukocytes. The samples were then centrifuged at 2000 rpm for five minutes at room temperature and the supernatants decanted. Next, cells were permealized by adding 500 µl of FACSperm (BD Biosciences, San Jose, CA, USA) (diluted 1:10 in distilled water) and incubated for 10 minutes. at room temperature.

The cells were then washed twice with wash buffer (NICD media department, Sandringham SA). Three and four colour staining using antibodies for surface and for intracellular IFN-γ was carried out and distinguished NK cells ($CD16^+CD56^+CD8^-CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), monocytes ($CD14^+$) and B cells ($CD19^+$). The antibody combinations used are listed in Table 4.1. Samples, to which the relevant antibodies were added, were covered with foil, placed in a dark cupboard and subsequently incubated for one hour at room temperature then washed and resuspended in 150 µl of PBS containing 1% paraformaldehyde (Electron Microscopy Sciences, Pretoria, South Africa). Samples were stored at 4°C until acquisition on a Flow Cytometer (described in section 4.2.2.5). Samples were acquired within 24 hrs.

Table 4.1 Fluorescent antibody combinations used for staining whole blood samples

Tube number	FITC	PE	PERCP	APC
1	$CD16^+CD56^+$	IFN-γ	$CD8^+$	$CD3^+$
2		IFN-γ	$CD4^+$	$CD3^+$
3	$CD14^+$	IFN-γ	$CD19^+$	

NK cells: $C16^+CD56^+CD8^-CD3^-$
$CD4^+$ T- cells: $CD3^+CD4^+$
$CD8^+$ T cells: $CD3^+CD8^+$
Monocytes: $CD14^+$
B cells: $CD19^+$

4.2.3 Flow Cytometry

All samples were acquired on a Becton Dickinson FACScalibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose CA). BD Calibrite beads and BD FACSComp software version 6.3.2 (Becton Dickinson Immunocytometry Systems, San Jose CA) were used for assessing instrument performance before use.

Samples stained with a single fluorescent antibody were set up with each experiment and served as the compensation tubes. Cell populations were gated based on their forward scatter (FSC) and side scatter (SSC) properties, that is, cells were distinguished on the basis of size and granularity, respectively. Fig. 4.1 - 4.3 provides an example of the gating strategy used in this study. At least 20 000 cells were collected during acquisition. Data was analysed using Flowjo software (Tree Star, San Carlos, CA, USA) and results were expressed as the percentage of IFN-γ producing cells expressing specific cell type markers that identify $CD4^+$ and $CD8^+$ T lymphocytes, NK cells, monocytes and B cells. A positive response was considered as $\geq$0.1% IFN-γ producing cells after subtracting the background staining from cells stimulated with anti-CD28 and anti-CD49d in the absence of PHA and BCG.

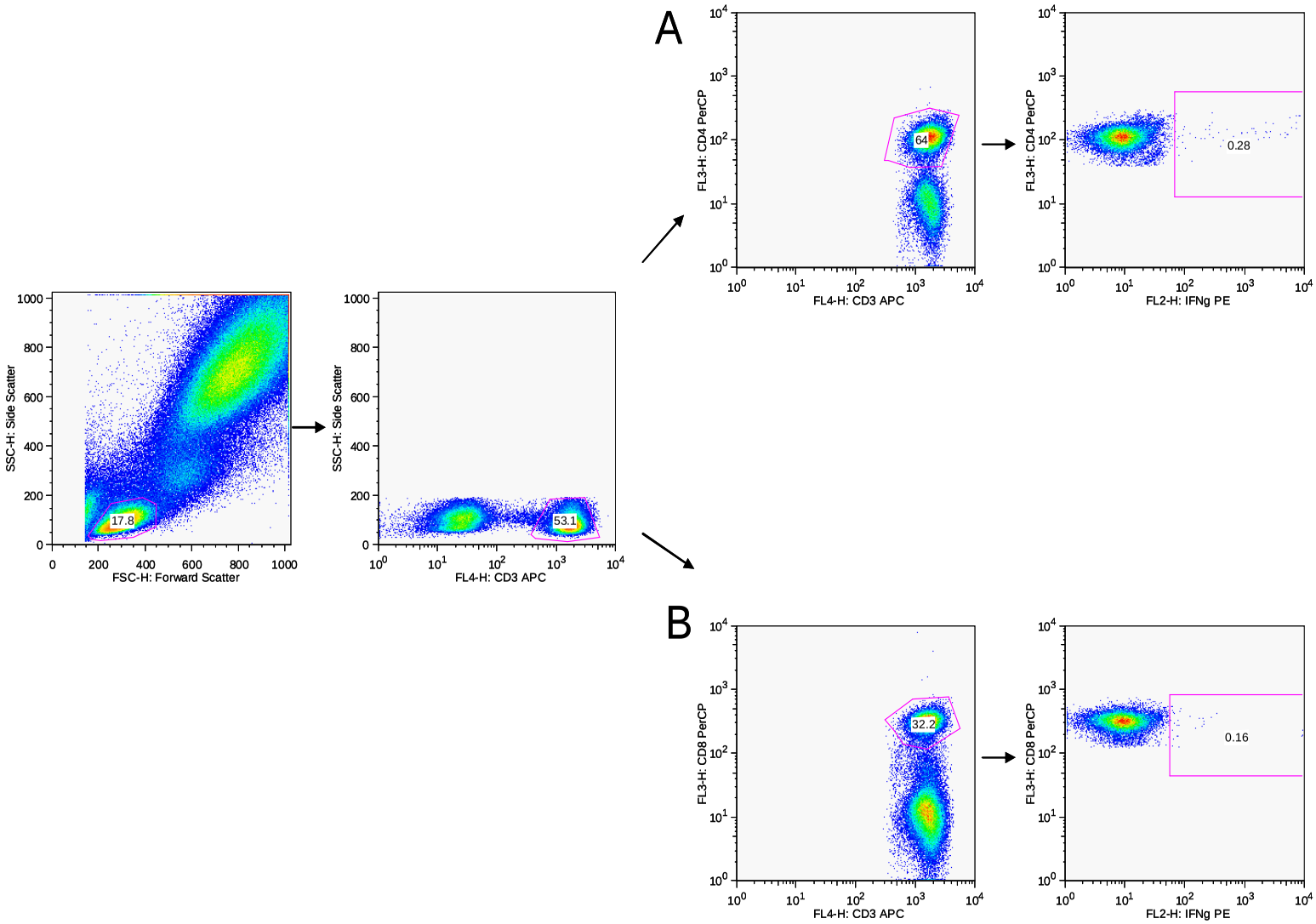


Fig. 4.1 Gating strategies used for (A) CD4+ (B) CD8+ cells. The first gate was placed around the lymphocyte population based on FSC vs SSC, followed by gating on the CD3+ population,and then gating was on the CD4+ (A) and CD8+ (B) cells and finally on the cells staining with IFN-γ

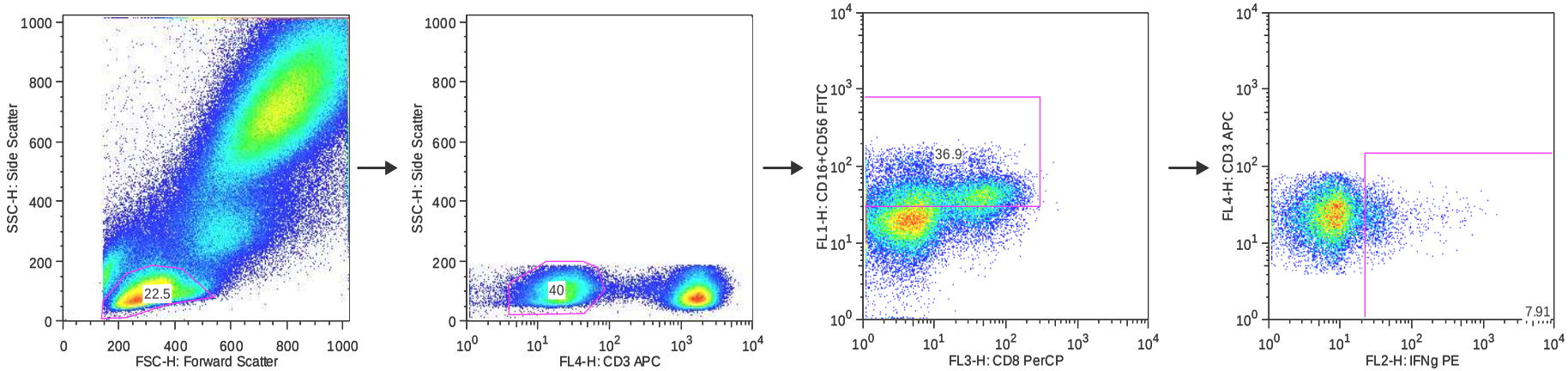


Fig. 4.2 Gating strategies used for $CD16^{+}CD56^{+}$ NK cells. The first gate was placed around the lymphocyte population based on FSC vs SSC, followed by gating on the $CD3^{-}$ population, and then gating was on the $CD16^{+}CD56^{+}$ cells and finally on the cells staining with IFN-γ

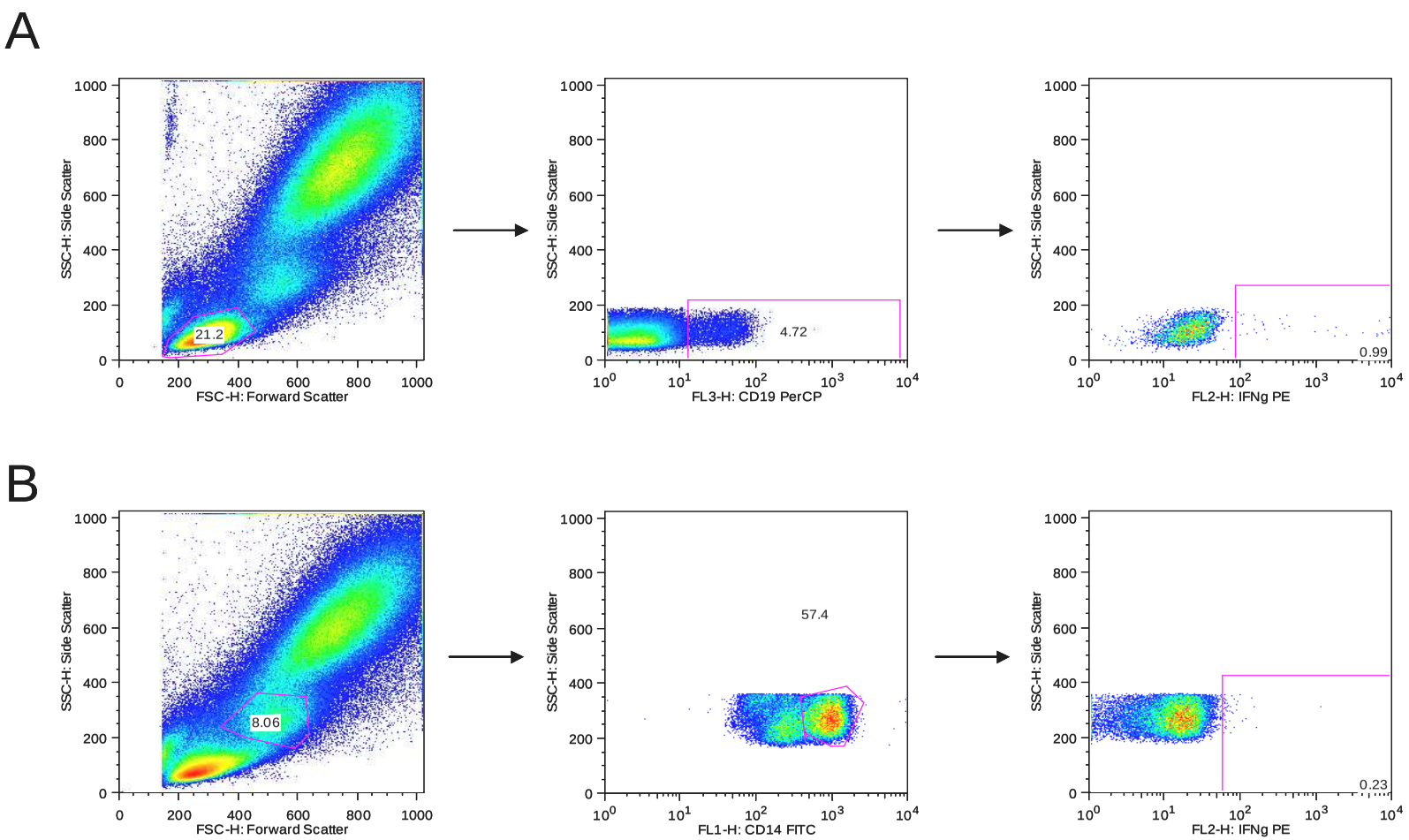


Fig. 4.3 Gating strategies used for (A) CD19+ B cells and (B) CD14+ monocytes. For CD19+ B cells (A) the first gate was placed around the lymphocyte population based on FSC vs SSC, followed by gating on the CD19+ population, and finally the cells staining with IFN-γ. For CD14+ monocytes (B) the first gate was placed around the monocyte population based on FSC vs SSC, followed by gating on the CD14+ population, and finally on the cells staining with IFN-γ

4.3 Statistical analyses

The Wilcoxon signed rank test was used to determine whether there were any significant differences in IFN-γ production between the different cell types and to establish if cell type specific production of IFN-γ differed for the different stimuli. Statistical analyses were performed using SPSS software (version 11.0, SPSS Inc., Chicago, IL). All statistical tests were two-tailed and considered significant at $P<0.05$.

4.4 Results

4.4.1 Samples for ICC analysis

Since IFN-γ production is poor in newborns, we selected to address the question of which cell types were more likely to produce IFN-γ in infants at 10 weeks of age, a time when their immune capability is much improved. We therefore focused this analysis on eight 10 week old EU infants (DNA PCR negative at six weeks and 10 weeks) and their HIV-1 infected mothers. It was interesting to include mothers in the ICC analyses to see how their IFN-γ producing capacity compared to their infants. We also included two HIV-1 negative mothers and their newborn infants and one HIV-1 seropositive mother and her newborn EU infant. Table 4.2 shows the characteristics for the mother-infant pairs used for this study.

Table 4.2 Characteristics of mothers and infants

	Infant blood taken after NVP	Mother-Infant ID*	Infant age at time of ICC	Maternal $CD4^+$ (cells/µl)	Maternal viral load (RNA copies/ml)
	UNINFECTED CONTROLS (MOTHERS AND INFANTS)				
		473	Birth	1473	ND
		481	Birth	ND	ND
HIV-1 INFECTED MOTHERS AND THEIR EU INFANTS					
NVP	yes	429	Birth	939	488
NVP	yes	667	10 wks	709	845
$NVP^+AZT^+3TC^+$	yes	670	10 wks	197	<400
NVP	yes	683	10wks	1033	<400
NVP	yes	685	10 wks	525	217 000
NVP	yes	742	10 wks	463	5 840
NVP	yes	743	10 wks	ND	4 180
$NVP^+AZT^+3TC^+$	yes	777	10 wks	191	<400
NVP	yes	778	10 wks	509	40 600

ND: not determined

* Samples were coded as the ID number shown preceded with an “M” for mother and a “C” for her matched child

4.4.2 IFN-γ production by different cell types in 10 week old EU infants

In order to determine which cells are the main producers of IFN-γ in EU infants, ICC analysis and Flow Cytometry was carried out on whole blood collected from eight 10 week-old EU infants. The percentage of $CD4^+$ and $CD8^+$ T cells, NK, $CD14^+$ and $CD19^+$ cells producing IFN-γ were compared for blood that was unstimulated (Fig. 4.4A) or stimulated with PHA (Fig. 4.4B) or BCG (Fig. 4.4C). Results were obtained for $CD19^+$ cells for only four of the eight infants. Notably, there are significant differences in the ability of different cell types to produce IFN-γ (Fig. 4.4 A, B and C). A significantly higher percentage of NK cells produced IFN-γ compared to $CD4^+$, $CD8^+$ and $CD14^+$ cells (P=0.012), irrespective of the stimulus. Although there was a trend towards reduced IFN-γ production in B cells ($CD19^+$) compared to NK cells and an increase relative to other cell types, this was not significant. No appreciable differences could be noted in the percentages of $CD4^+$, $CD8^+$ and $CD14^+$ cells (monocytes) involved in IFN-γ production.

We next questioned the extent to which IFN-γ could be induced by the two stimuli (PHA and BCG). Significant differences in the production of IFN-γ, between the different stimuli, for each of the cells are listed in Table 4.3. In general, PHA proved to be a more potent stimulus than BCG, although increases in IFN-γ production were mostly marginal. PHA significantly increased the proportions of IFN-γ-producing NK cells (P=0.05), proportions of $CD4^+$ (P=0.012) and $CD8^+$ (P=0.042) T cells when compared to unstimulated control cells. There were no significant differences in cell

type specific IFN-γ production for any other comparisons (PHA vs unstimulated, BCG vs unstimulated, or PHA vs BCG).

Table 4.3 Ability of cell types to produce IFN-γ in response to different stimuli. Significant differences are presented

Cell type	PHA vs Unstimulated	BCG vs Unstimulated	PHA vs BCG
$CD4^+$ T cells	0.012*	>0.05	>0.05
$CD8^+$ T cells	0.042*	>0.05	>0.05
$CD56^+$ $CD16^+$ (NK cells)	0.050*	>0.05	>0.05
CD19 (B cells)	>0.05	>0.05	>0.05
CD14 (monocytes)	>0.05	>0.05	>0.05

* increased with PHA stimulation

Fig. 4.5 shows the data for each individual infant in order to illustrate the relative changes in IFN-γ production between cell types when exposed to different stimuli. The magnitude of IFN-γ responses was quite variable between different individuals, but patterns of responses were similar for some. For example infants C670, C777, C778, C685 and C683 in general had similar response profiles when stimulated with PHA or BCG. However, the remaining three infants were either poorly or non-responsive to one or other stimulus (C667, C742 and C743).

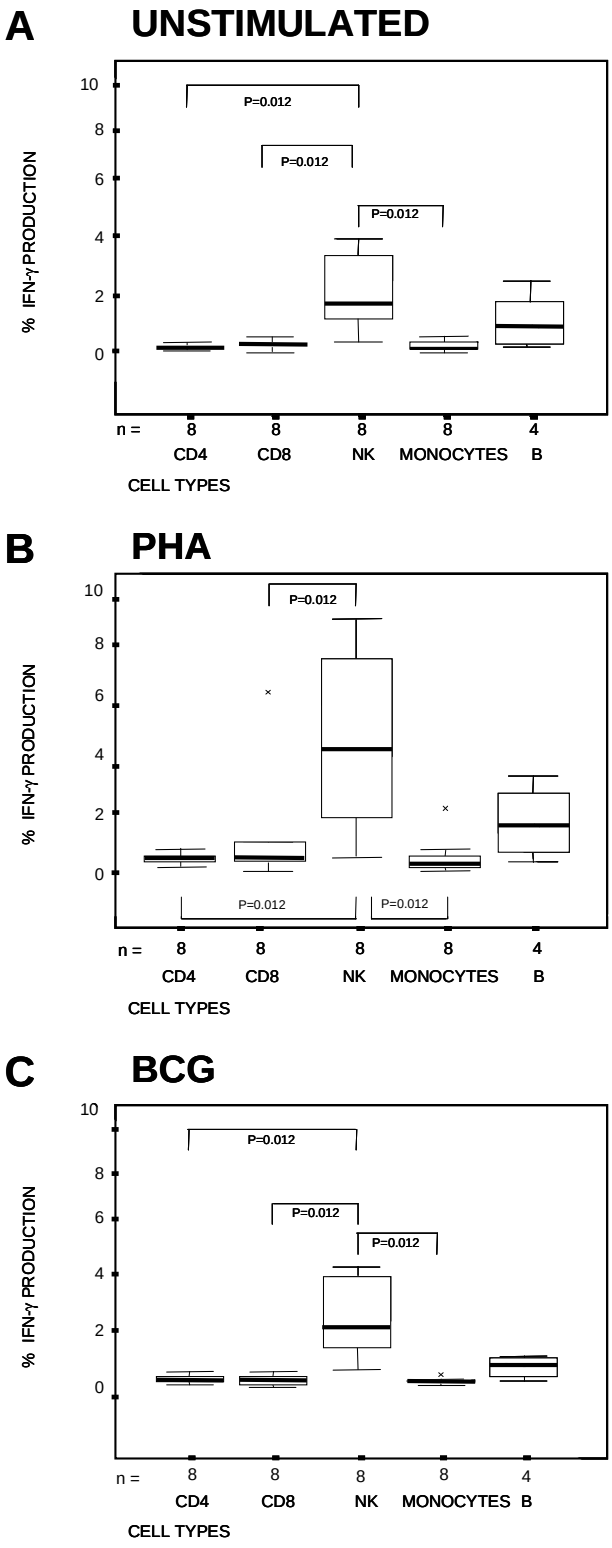


Fig. 4.4 IFN-γ production by different cells of EU infants at 10 weeks in response to (A) no stimulus (B) PHA (C) BCG

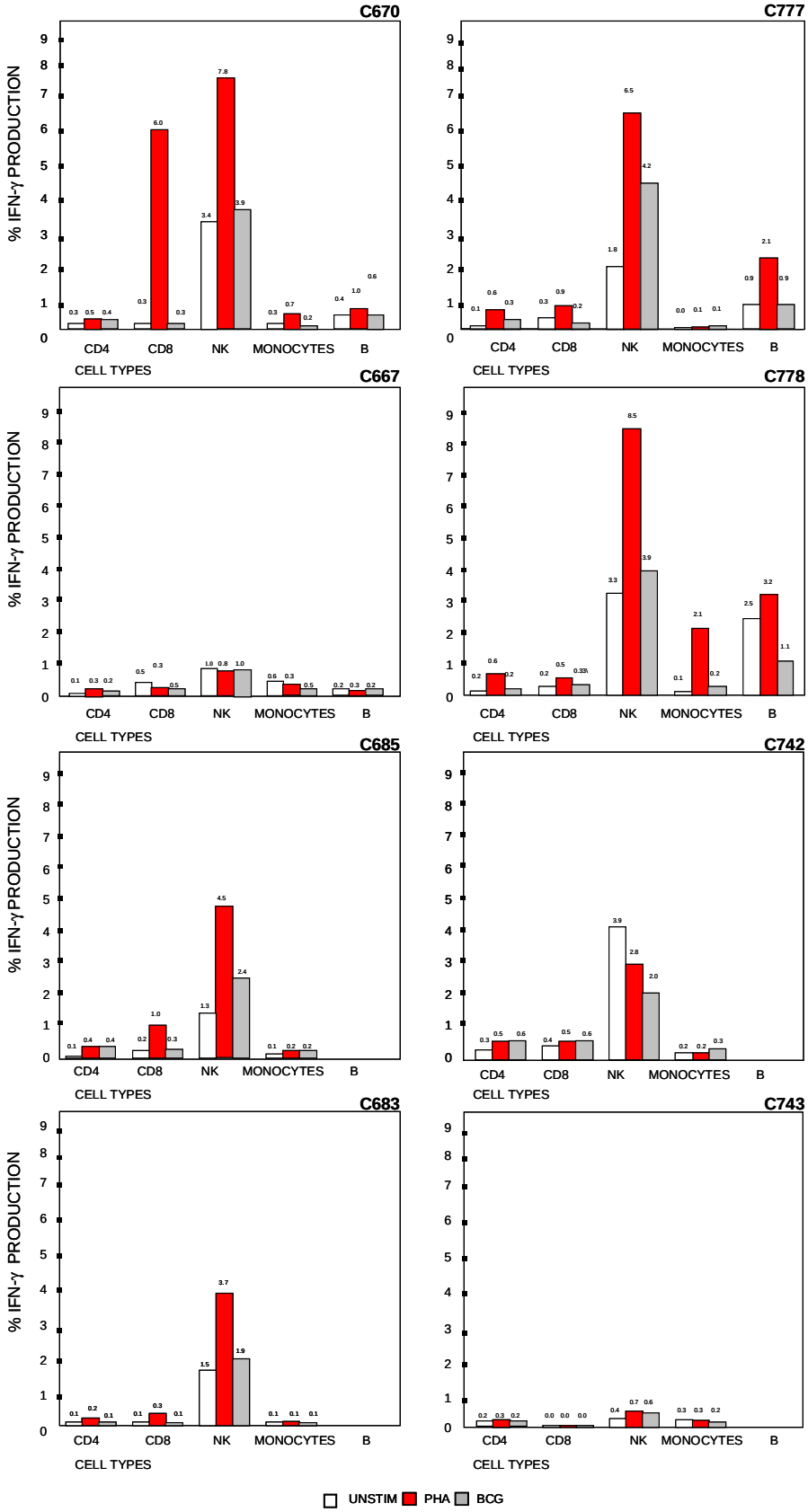


Fig. 4.5 IFN-γ production by different cell types of 8 individual EU infants (C670, C777 etc) at 10 weeks

4.4.3 IFN-γ production in mothers of EU infants

The distribution of cells, in peripheral blood, contributing to IFN-γ production in HIV-1 infected adults is influenced by stage of HIV-1 disease progression (Lieberman *et al*., 2001). Overall, the immune integrity of all the mothers was considered to be good as viral loads were almost undetectable (<400 copies/ml) with one mother having a viral load of 217 000 copies/ml (Table 4.2). Mothers with the higher viral loads had $CD4^+$ T cell counts greater than 400 cells/µl. Intriguingly, two mothers had low viral loads (<400 copies /ml), yet low $CD4^+$ T cell counts (<200 cells/µl). Fig. 4.6 shows the ICC assay data obtained for each HIV-1 infected mother, illustrating the relative abilities of the cell types to produce IFN-γ. Results were obtained for CD19 cells for only four of the eight mothers. Fig. 4.7. shows a comparison of IFN-γ production by $CD4^+$ and $CD8^+$ T cells, NK cells and $CD14^+$ cells in response to PHA (PHA in general being the more potent stimulus), for EU infants and their infected mothers. Not unexpectedly all four cell types from HIV-1 infected mothers produced substantially more IFN-γ than their infants. Interestingly, amongst the HIV-1 infected mothers, $CD8^+$ T cells were the better producers of IFN-γ however, in the EU infants the NK cells produced most of the IFN-γ.

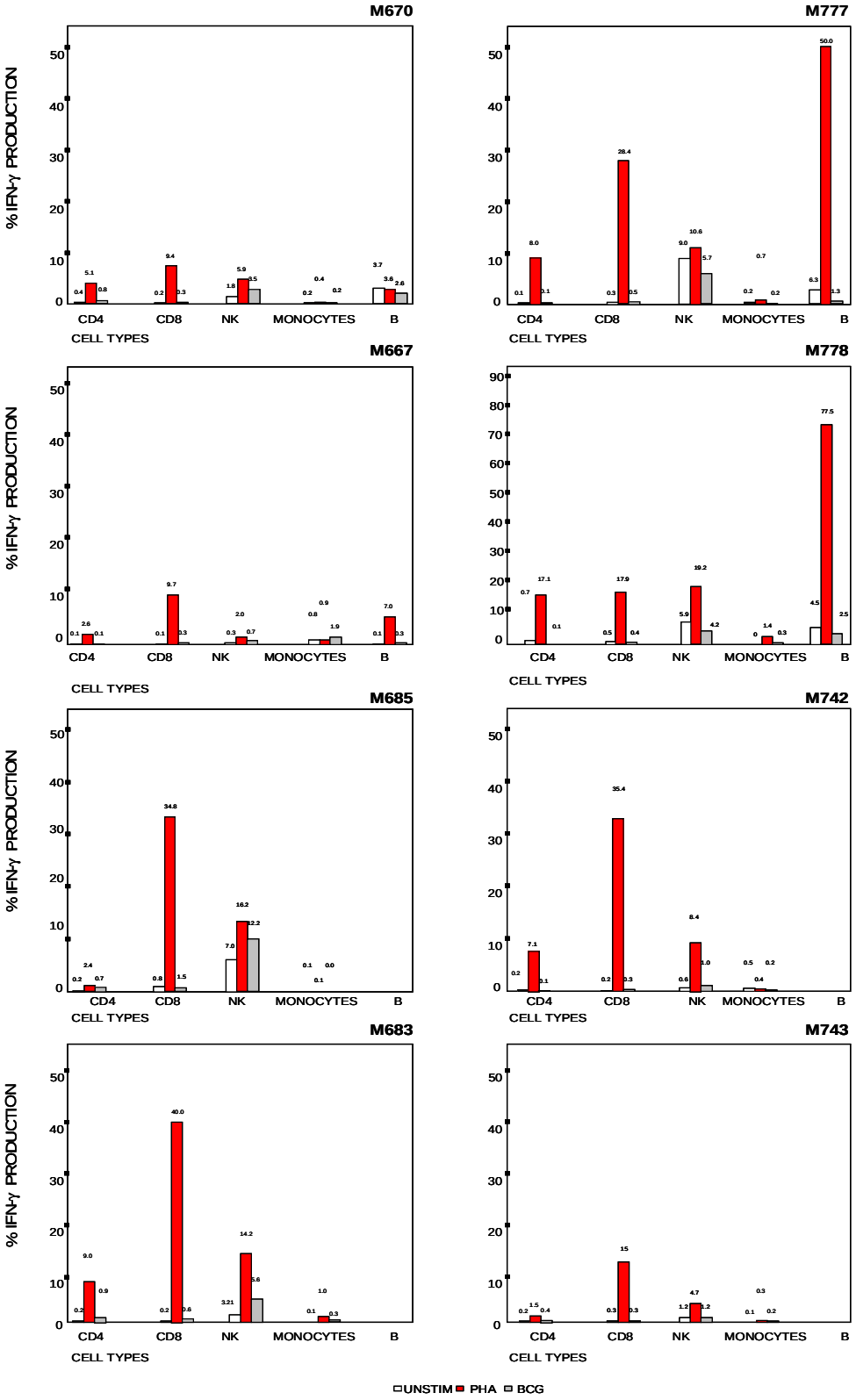
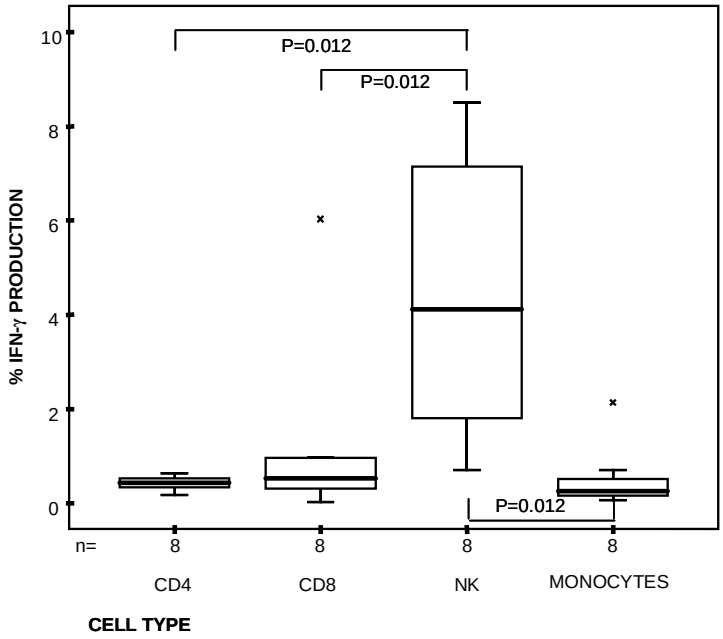


Fig. 4.6 IFN-γ production by the different cell types of all 8 HIV-1 infected mothers (M670, M777 etc) of 10 week old EU infants

A INFANTS



B MOTHERS

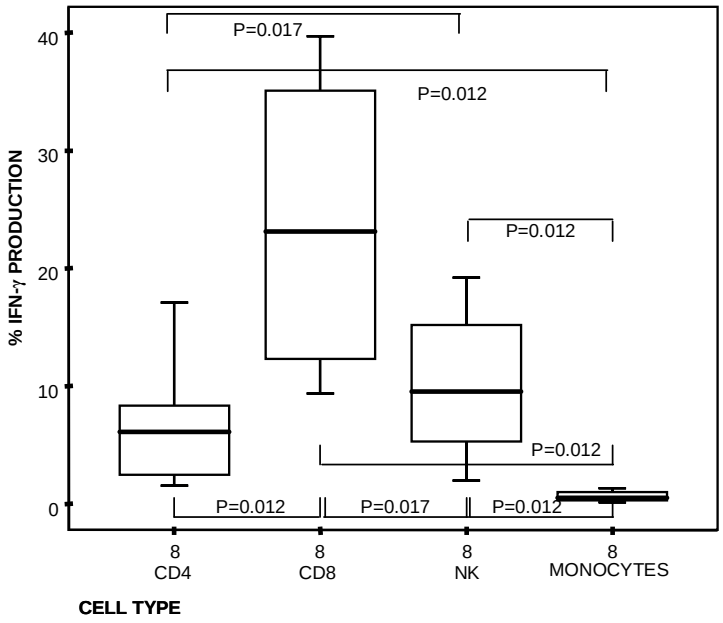


Fig. 4.7 IFN-γ production by PHA stimulated cells of EU infants at 10 weeks and their HIV-1 infected mothers

4.4.4 IFN-γ production at birth

Having shown that NK cells were the major cell type producing IFN-γ in infants at 10 weeks of age we wanted to determine whether there were any differences in the production of IFN-γ in newborns. ICC analysis was thus conducted on two control uninfected infants and their corresponding HIV-uninfected mothers, and one EU newborn and its corresponding mother (Fig. 4.8). As demonstrated earlier, NK cells proved again to be the major producers of IFN-γ in the control HIV-1 negative infants, followed by prominent IFN-γ production by B cells (Fig. 4.8 A and B). In the one EU newborn infant, responses by all of the cell types, with the exception of $CD4^+$ T cells, were largely undetectable (Fig. 4.8 C). It was interesting that all mothers of newborns (uninfected and HIV-1 infected) had in particular poor $CD8^+$ T cell production of IFN-γ, in contrast to that found amongst HIV-1 infected mothers tested at 10 weeks (Fig. 4.7), likely reflecting the altered immunological milieu associated with pregnancy.

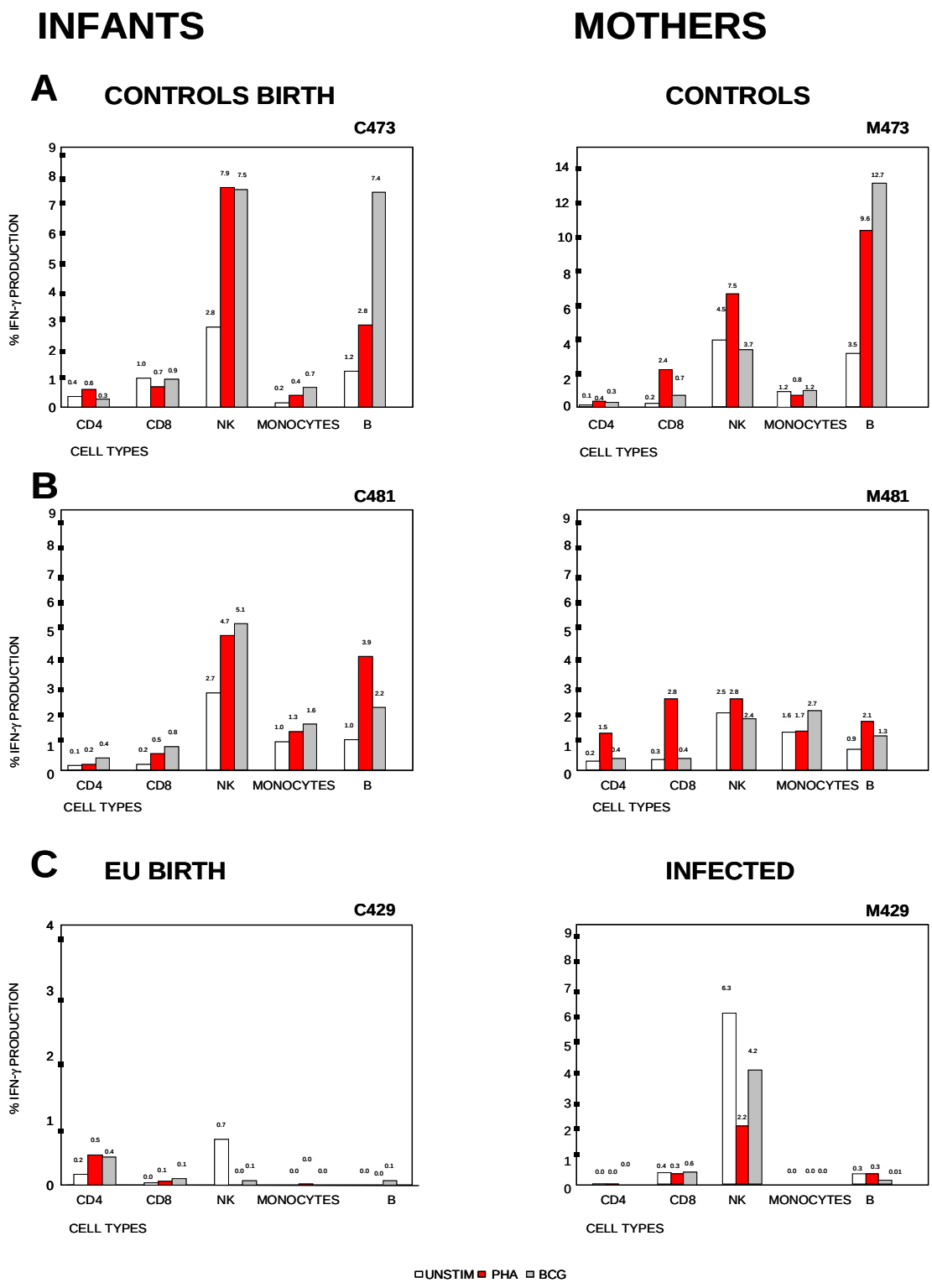


Fig. 4.8 IFN-γ production by the different cell types of infants at birth and their corresponding mothers. (A and B) control uninfected infants at birth (C473, C481) and their uninfected mothers (M473, M481) and (C) EU infant at birth (C429) and its HIV-1 infected mother (M429)

4.5 Discussion

Newborn infants are highly susceptible to infections and deficiencies in their immune system (innate and adaptive) have been thought to account for the failure of infants to mount protective responses against pathogens (Delespesse *et al*., 1998). IFN-γ production, which is impaired in newborns (Wilson *et al.,* 1986) is critical for the development of effective immune responses and is secreted by T cells and NK cells under appropriate stimuli during the immune response (Szabo *et al*., 2003). Since we had established, in Chapters 2 and 3, that EU infants produce significantly more IFN-γ than control infants and that the increased production was not due to increased lymphocyte counts in these infants, we wanted to determine which mononuclear cell types contributed to the IFN-γ produced in response to mitogen (PHA) or vaccine (BCG).

The ability to measure cytokine production at a cellular level using ICC staining and Flow Cytometry has proved to be a very powerful tool because (i) a given cell population can be analyzed since parameters such as size and granularity, as well as expression of surface and intracellular markers defined by fluorescent antibodies can be used for the separation, (ii) this approach permits the detection of functional populations that respond to specific soluble antigens, (iii) whole blood can be used without the need for the separation of specific cell populations – this is an important advantage when working with small sample volumes from infants, and (iv) the whole blood ICC assay allows the study of individual cells directly *ex vivo* minimizing artifacts due to long term culture.

In this study, we demonstrated that: (i) the NK cells of 10 week old EU infants produced significantly more IFN-γ than the T cells or B cells, (ii) induced IFN-γ responses were undetectable in the one exposed uninfected newborn examined at birth, however, in control birth infants NK cells remained the main producers of IFN-γ, (iii) maternal $CD4^+$ and $CD8^+$ T cells as well as the NK cells produced substantially more IFN-γ with the $CD8^+$ T cells producing the most.

Excessive IFN-γ production at the maternal-foetal interface has been shown to be deleterious to the placental integrity as well as being a major cause of foetal loss (Wegmann *et al*., 1993). The bias of foetal immunity to Th2 polarization therefore serves to protect against Th1 placental and foetal damage but plays a significant role in 'damping' the newborns innate immune responses. Since infants have a Th2 driven immune system plus the fact that they are essentially antigen-inexperienced and have virtually no development of specific/adaptive immunity it was not surprising that the NK cells were found to be the dominant IFN-γ producers. IFN-γ production by NK cells has been reported to play a central role in immunity, being the main cytokine produced by NK cells early in the immune response, and driving subsequent Th1 responses (Trinchieri, 1995). $CD4^+$ and $CD8^+$ T cells and NK cells of HIV-1 infected mothers, on the other hand, produced substantially more IFN-γ than their infants, with the $CD8^+$ T cells proving to be better producers of IFN-γ in the HIV-1 infected mothers. In contrast all mothers of newborns (uninfected and HIV-1 infected) had poor $CD8^+$ T cell production. This result may be explained by the fact that chronically HIV-1 infected mothers have expansions of $CD8^+$ T cells as a result of their infection

when compared to the uninfected adults where there is no such priming of cells, and that the immunological environment differs in HIV-1 infected mothers at delivery compared to 10 weeks later.

Importantly findings in infants have shown that the infant immune system is unique from that of the adult and that the innate immune response predominates in newborns and also by 10 weeks of age. This is particularly interesting when looking at the responses to BCG vaccine given at birth, as the response then measured at 10 weeks would be expected to be largely attributed by a memory $CD4^+$ T cell response. However, it was apparent that higher proportions of NK cells produce IFN-γ even when "unstimulated" (note these are not strictly unstimulated as the ICC assay control includes addition of CD49d and CD28 antibodies used to enhance responsiveness to a second stimulus of the test samples) in both uninfected controls (at birth) and EU infants (10 weeks), it is therefore very likely that the increased secreted levels of IFN-γ reported in six week old infants in whole blood cultures following BCG vaccination (Chapter 2) would be due to production of both innate (particularly NK cells) and/or acquired immune responses ($CD4^+$ and/or $CD8^+$ T cells).

Given the variation in IFN-γ response profiles obtained for the eight EU infants, it is likely this same extent of variation would be encountered in birth samples. Therefore, it would be important to increase the sample size for study of these responses at birth (control and EU infants) as the data shown in the small numbers here cannot be considered as representative. In Chapter 2 it was established that 48% of newborns had detectable levels of IFN-γ induced at birth in response to BCG, a response suggested to be non-specific in nature. However by ICC assay one control newborn

showed a $CD4^+$ and $CD8^+$ T cell response to BCG, and the one EU newborn had a positive $CD4^+$ T cell response to BCG, suggesting that memory T cell responses to BCG are present in some infants at birth. Whether these can be explained by maternal exposure to BCG, *Mycobacterium tuberculosis* or some other cross-reacting mycobacterium, needs to be established in future studies.

Innate immunity as the first line of defence against invading pathogens viz. bacterial and viral infections, is particularly important in warding off infections in the newborn infant and permits the development of the adaptive immune response over time. Numerous studies have however shown that under certain conditions, newborns are able to mount adult-like protective T cell responses (Legrand *et al*., 2006; Marchand *et al*., 2003). The ability of infants to induce strong lymphoproliferative responses and Th1 type cytokine responses is important as this impacts on the induction of protective immune responses by vaccines. It is recognized that immunological responses are governed by factors such as dose and route of vaccine administration (Goetch *et al*., 2000). For the development of more effective infant vaccines it is important therefore to identify the immune system deficiencies and how they contribute to the increased vulnerability seen in infants. Furthermore, immune dysregulation at an early age in the EU infant may through exposure to maternal HIV-1 influence the development of subsequent innate and adaptive immune responses to HIV-1 itself, other organisms and vaccines.

CHAPTER 5

Concluding remarks

Deficiencies of both innate and adaptive immunity contribute to the impaired neonatal host defence making infants more susceptible to infectious diseases. Immaturity of T cell immunity and thus difficulty in priming neonatal T cells and a bias to T helper type-2 responses are considered to be obstacles to inducing protective immune responses by vaccines in neonates.

The environment present at the time of immune cell priming influences the resulting response and therefore the outcome of infection. Interestingly, studies have shown that the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine given at birth induces potent Th1-type immune responses and this influences the immune response to unrelated antigens in early life (Marchand *et al*., 1999; Ota *et al*., 2002). Low-dose exposures to HIV-1 have also been associated with the development of T helper type-1 responses (Shearer and Clerici, 1996) and there is substantial evidence showing immune consequences of exposure to maternal HIV-1 (or by virtue of being born to an HIV-1 seropositive mother) in HIV-1 exposed infants (see examples of HIV-1 specific responses and innate immune responses in sections 1.7.1 and 1.7.2, respectively). Adding further to this growing list of altered immune features of the EU infant, findings from this study showed an enhanced capacity to produce IFN-γ among EU newborns of HIV-1 infected mothers. In addition, EU infants also showed different profiles of maturation when stimulated with PHA or BCG (over the first 6 weeks of

life), further distinguishing them from their uninfected counterparts. Findings revealed that IFN-γ produced in response to BCG, studied as a vaccine response (given at birth that is measured at 6-10 weeks of age), is in fact predominantly produced by innate immune cells (particularly NK cells) and comparatively only very small proportions of T cells produced IFN-γ (considered as part of an adaptive immune response or recall response). That 48% of newborns among EU infants already had demonstrable responses to BCG (prior to BCG vaccination) suggests that this is either non-specific in nature (i.e. innate immune cells such as NK cells are stimulated) or may involve T cell responses (as suggested by ICC data in Chapter 4) developed due to prior exposure *in utero* to mycobacterial organisms that may cross-react with BCG, or what seems unlikely, to BCG itself. Further studies are required to establish the true nature of the origin and specificity of these responses.

An important question that remains is how the altered immune environment of EU infants, because of earlier antigenic exposure than would occur in uninfected infants born to HIV-1 uninfected mothers, impacts on immune responses to other organisms and vaccines. Certainly, the development of HIV-1 specific responses in EU infants has been associated with protective immunity to HIV-1. It may be seen to be advantageous to an infant to have a "fast-tracked" immune development through greater antigenic exposure in early life. However, it is also likely that other aspects of early immune development may be compromised through having an HIV-1 infected mother, whose immune system and nutritional well-being is impaired to varying extents dependent on stage of disease. With lower maternal $CD4^+$ T cell counts, mothers would also become susceptible to secondary infections which then would also expose the child to a greater number of infections than would occur with a more

robust healthy immune system. Other factors like poor passive transfer of maternal antibodies in such women could also be another factor contributing to infant susceptibility to certain organisms.

It remains important in the future to conduct studies on immune response capability as described here for IFN-γ, but at the same time to follow-up these infants and monitor their immune responses to important childhood vaccines, and to assess their susceptibility to other infections in relation to immune response capacity (using clearly defined immune parameters of innate and adaptive immune responses). It will be comparisons such as these that will help us to understand if some of these altered immune responses are advantageous or disadvantageous to the developing infant, and whether susceptibility to other infections in the EU infant may be a consequence of their own immune inability to deal with an infectious event, maternal factors or due to the fact that their environmental exposure to infectious organisms is heightened.

Understanding the cytokine environment, dose and nature of the antigen as well as other neonatal immunological characteristics may contribute to developing more effective vaccination strategies. The EU infant represents an important model for studying not only the consequences of early antigenic exposure in infants, but also provides a very important model for the study of correlates of protective immunity to HIV-1, as these infants have escaped acquiring HIV-1 infection despite exposure to maternal HIV-1.

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APPENDIX A: Abbreviations

ADCC	antibody-dependant cellular cytotoxicity
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cells
ART	antiretroviral therapy
ARV	antiretrovirals
AZT	Azido-Thymidine, zidovudine
BCG	Bacillus Calmette-Guérin
BFA	brefeldin A
BSA	bovine serum albumen
CBC	complete blood count
$CD4^+$	T lymphocyte
$CD8^+$	T lymphocyte
$CD14^+$	monocytes
$CD16^+$ $CD56^+$	NK cells
$CD19^+$	B cells
CD80	The protein CD80 is a molecule which provides a costimulatory signal necessary for T cell activation
CD86	The protein CD86 is a molecule which provides a costimulatory signal necessary for T cell activation
CD40	A receptor molecule on the surface of mature B cells

$CD45RA^+$	naïve antigen expression in T-lineage neoplasm
$CD45RO^+$	mature antigen expression in T-lineage neoplasm
CFU	colony forming unit
CHBH	Chris Hani Baragwanath Hospital
C5a	complement 5a
CMI	cell mediated immunity
CTLs	cytotoxic T lymphocytes
CWCH	Coronation Women and Childrens hospital
DNA	deoxyribose nucleic acid
DMSO	dimethylsulphoxide
EDTA	ethylenediamine tetra-acetic acid
EU	exposed uninfected
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot assay
FcR11	low affinity receptors
FSC	forward scatter
HIV	human immunodeficiency virus
HSC	haematopoetic stem cells
hrs	hours
ICC	intracellular cytokine staining
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IgE	immunoglobulin E
IL-1	interleukin-1

IL-6	interleukin-6
IL-8	interleukin-8
IL-12	interleulin-12
IL-18	interleukin-18
IFN-α	interferon-alpha
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IQR	interquartile range
LPS	lipopolysaccharide
ml	millilitre
MHC	major histocompatibility complex
NK	natural killer cells
NADPH	nicotinamide adenine dinucleotide phosphate
Na Hep	sodium heparin
NICD	National Institute for Communicable Diseases
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PRRs	pattern recognition receptors
PIPE	protective immunity and perinatal exposure study
pg	picogram
PMTCT	perinatal mother to child transmission
RNA	ribose nucleic acid
rpm	revolutions per minute

SSC	side scatter
sdNVP	single dose nevirapine
TB	tuberculosis
Th1	T helper-type 1
Th2	T helper-type 2
TCR	T cell receptor
TLRs	toll-like receptors
TI	thymus independent
TNF-α	tumour necrosis factor alpha
TGF-β	transforming growth factor
3TC	Lamivudine (2',3'-dideoxy-3'-thiacytidine)
µg	microgram
µl	microlitre
µM	micromolar
week(s)	week(s)

APPENDIX B: Composition of buffers and media

Haematology diluent (wash buffer)

Dissolve	NaCl	8.12 g in 200 ml distilled water
	KCl	0.28 g in 100 ml distilled water
	KH_2PO_4	0.26 g in 100 ml distilled water
	LiCl	0.43 g in 200 ml distilled water

Combine and make up to 1 litre with distilled water

Add EDTA (0.36 g) and adjust pH to 7.2 with HCl

Fixer

1.5% Formaldehyde PBS, 2% BSA

Phosphate Buffered Saline (PBS)

NaCl	80.0 g
NA_2HPO	11.6 g
KH_2PO_4	2.0 g
KCl	2.0 g

Combine and make up to 1 litre with distilled water

Adjust pH to 7.0

Wash Buffer for ELISA

Add 0.05% Tween-20 to PBS buffer

APPENDIX C: List of Suppliers

Item	Manufacturer
Plasticware	
Falcon polypropylene tubes	Becton Dickinson, San Jose, CA
Sterile pipettes	Adcock Ingram scientific South Africa
Micro-centrifuge tubes, 1.5 ml	Eppendorf, Hamburg, Germany
96-well flat bottomed plates	Nunc A/S, Roskilde, Denmark
96 well microtitre plate round bottom	Corning, Costar Corp., Cambridge
96 well microwell plates Falcon	Becton Dickinson, San Jose, CA
3.6 ml storage vials	Nunc A/S, Roskilde, Denmark
ICC Antibodies	
FITC-conjugated mouse anti-human CD16	Becton Dickinson, San Jose, CA
FITC-conjugated mouse anti-human CD56	Becton Dickinson, San Jose, CA
FITC-conjugated mouse anti-human CD14	Becton Dickinson, San Jose, CA
PerCP CD8	Becton Dickinson, San Jose, CA
PerCP CD19	Becton Dickinson, San Jose, CA

Cytokines

IFN-γ	R and D systems, Oxon, England

Stimuli

PHA	Sigma Aldrich, St Louis, MO, USA
BCG	Statens Serum Institut, Denmark

Cell Culture Media

RPMI 1640	Sigma Aldrich, St Louis, MO, USA
Foetal Calf Serum	Adcock Ingram Scientific, South Africa

Chemicals

Bovine Serum albumin	Sigma-Aldrich, St Louis, MO, USA
Sodium Hydroxide	BDH, Poole, England
EDTA	Merck, Darmstadt, Germany ll
Hydrochloric Acid	Sigma-Aldrich, St Louis, MO, USA
Potassium Phosphate (KH_2PO_4	Merck, Darmstadt, Germany
Potassium chloride (KCl)	Merck, Darmstadt, Germany
Sodium Chloride (NaCl)	Merck, Darmstadt, Germany
Sodium Phosphate (NA_2HPO)	Merck, Darmstadt, Germany

Blood collection vacutainers

EDTA vacutainers	Becton Dickinson, San Jose, CA
Na Heparin vacutainers	Becton Dickinson, San Jose, CA

Reagents

IFN-γ ELISA kit	BD Biosciences Pharmingen San Diego California
Reference blood controls	Beckman Coulter Inc. Fullerton CA
Brefeldin A	Sigma-Aldrich St Louis, MO, USA
Histopaque Ficoll	Sigma-Aldrich, St Louis, MO, USA
Tween-20	Adcock Ingram Scientific, South Africa
FACS lysing Solution	Becton Dickinson, San Jose, CA
FACSperm solution	Becton Dickinson, San Jose, CA

APPENDIX D: Statistical evaluation of results

SPSS version 11.0 (SPSS Inc. Chicago, III, USA) was used in performing all the statistical analyses of the results obtained. All the statistical tests were two-tailed and considered significant at $P<0.05$

Comparison of two samples (Mann-whitney U-test)

The Mann-Whitney U-test is a non-parametric method which usually is used to compare medians of non-normal distributions. In this study the unpaired test (Mann-Whitney U-test) was used, which determines whether two medians differ. The Mann-Whitney U-test is used when data from two independent samples, possibly of different size are being compared.

Wilcoxin signed rank test

The Wilcoxin signed rank test is a non-parametric method that looks for differences between two related samples. In this study the Wilcoxin signed rank test was used to establish if cell type specific production of IFN-γ differed for the different stimuli.

APPENDIX E: Computer software packages

This dissertation was produced on Microsoft Word for Windows XP.

The references were created by Endnote version 9.

The databases were managed using Microsoft Office Excel 2003.

Statistical evaluation of results was performed using SPSS version 11.0.

APPENDIX F: Ethical clearance

This study was approved by the university of the Witwatersrand committee for Research on Human Subjects (Medical), protocol number…**M071161**……. Patients were recruited after informed consent was obtained and the confidentiality of all records ensured.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Anthony

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M071161**
PROJECT	Interferon-Gamma Production in HIV-1 Exposed Uninfected Infants
INVESTIGATORS	Ms FS Anthony
DEPARTMENT	Virology & Comm Diseases
DATE CONSIDERED	07.11.30
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

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

DATE 07.12.07 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof C Tiemessen

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES