Effects of delayed BCG vaccination on cellular immune responses in HIV-exposed infants

By CHRISTOPHE TOUKAM TCHAKOUTE
TKMCHR001

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Supervisor: Dr Heather Jaspan
Co-supervisor: Professor Clive Gray

Department of Clinical Laboratory Sciences
Division of Immunology
Faculty of Health Sciences

University of Cape Town

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List of Abbreviations

List of abbreviations	
°C	Degrees Celsius
aP	Acellular Pertussis Vaccine
APC	Antigen-Presenting Cell
ART	Antiretroviral Therapy
BCG	Bacille Calmette Guerin
BCR	B Cell Receptor
BP	Bordetella Pertussis
BrdU	5-Bromo-2-Deoxyuridine
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTaP-IPV-Hib	Diphtheria, Tetanus, acellular Pertussis, Polio and Infant Haemophilus type B vaccine
EPI	Expanded Programme on Immunisation
HIV	Human Immunodeficiency Disease
ICS	Intracellular Cytokine Staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
IRIS	Immune Reconstitution Inflammatory Syndrome
MAD	Median Absolute Deviation
MHC	Major Histocompatibility Complex
ml	Millilitres
M.TB	*Mycobacterium Tuberculosis*
NALP3	NACHT, LRR and PYD domains-containing protein 3
NLRP3	NOD-like receptor family, pyrin domain containing 3
NK	Natural Killer
PAMP	Pathogen-Associated-Molecular-Pattern
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PCV7	7-valent Pneumococcal Conjugate Vaccine
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern Recognition Receptor
RPMI	Roswell Park Memorial Institute
RV	Rotavirus Vaccine

SEB	Staphylococcal enterotoxin B
TB	Tuberculosis
T_{CM}	Central memory T-cell
TCR	T-cell Receptor
TD	T cell-dependent
T_{EM}	Effector memory T-cell
Th	T-helper
TI	T cell independent
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory T cell
TT	Tetanus Toxoid
µl	Microlitres
Vivid	Violet Fluorescent Reactive Dye
WBA	Whole blood assay
wP	Whole Cell Pertussis Vaccine

Abstract

Background: Bacille Calmette-Guerin (BCG) vaccination prevents disseminated tuberculosis in children, and induces strong Th1 responses in neonates. BCG may also improve immune responses to other vaccines and antigens. However, BCG vaccination can result in severe disease in the context of HIV-infection, and is therefore contraindicated. In tuberculosis endemic regions, BCG is administered soon after birth, before in utero and perinatal HIV infection is ruled out. Therefore determining the effect of delaying BCG vaccination on adaptive immune functions in HIV-exposed infants is a central aim of this dissertation. T cell proliferation and cytokine responses to BCG, and other vaccines and antigens including pertussis, tetanus, and SEB were assessed in HIV-exposed infants who received BCG at birth and compared to responses in infants who received the vaccine at 8 weeks of age.

Methods: HIV-exposed, uninfected infants were randomized to receive BCG vaccination at birth (early BCG arm) or 8 weeks of age (delayed BCG arm), after testing HIV deoxyribonucleic acid (DNA) PCR negative at birth and six weeks respectively. Between 1- 3mL whole blood was collected from 28 participants in each arm at 6, 8 and 14 weeks of age and stimulated with Mycobacterium bovis bacillus Calmette-Guérin (BCG), Tetanus toxoid (TT), Bordetella pertussis (BP) antigens, a negative control (medium alone) and a positive control, Staphylococcal enterotoxin B (SEB). Cells were fixed and upon thawing, permeabilized and stained for intracellular INF-γ, IL-2, IL-13 and IL-17 cytokines

(ICS) and Ki67, CD3 and CD8 with fluorescence-conjugated antibodies and measured using multiparameter flow cytometry. Data analysis was performed using FlowJo V9.4, GraphPad prism V5, STATA V11, Pestle V1.7 and Spice V5.22 software packages.

Results: There was no difference in BCG-specific T-cell proliferation between infants in the two arms when measured at weeks 6, 8 and 14. However, in those infants who received delayed BCG, there were significantly higher frequencies of IFN-γ-expressing CD4+ T cells and multifunctional BCG-specific responses at week 14. There were no differences in CD4+ and CD8+ T cell proliferative responses as well as IFNγ-expressing CD4+ T cells in response to BCG six weeks post-vaccination.

When compared with the delayed BCG arm, there were significantly higher CD4+ T cell proliferative responses to SEB at week 14 in the early BCG arm, but no differences in proliferative response to TT or pertussis. Higher frequencies of pertussis-specific IL-13-expressing CD4+ and CD8+ T cells at week 14 were evident in the early BCG arm, but there were no differences in IL-2, IFN-y or IL-17 expression to pertussis. There were no significant differences in cytokine responses to TT or SEB at any time point.

Conclusion: HIV-exposed infants in the delayed BCG arm (BCG vaccinated at 8 weeks of age) had signifcantly higher Th1 responses to BCG when compared to infants in the early BCG arm. This implies that delaying BCG vaccination from birth to 8 weeks in HIV-exposed, uninfected infants does not impinge on vaccine immunogenicity and may even be beneficial to infants.

When assessing how delaying BCG vaccination affected T cell responses to other unrelated vaccines (pertussis and TT) and antigens (SEB), BCG timing did not have any remarkable effect on T cell proliferative responses, except for higher CD4+ T cell proliferative responses to SEB. This suggests that early BCG vaccination may have minimal non-specific effects on the infant immune system. Compared to infants in the early BCG arm, infants in the delayed BCG arm had higher frequencies of pertussis-specific IL-13-expressing T cells at week 14. Thus, even though the non-specific effects of BCG are limited, the vaccine may have an adjuvant-like effect on immune responses to other unrelated vaccines. The results in this dissertation have important implications for the design of optimal BCG vaccination and TB prevention strategies in HIV-exposed, uninfected infants.

Presentations and Publications

Conference presentations

Christophe Toukam, Elvis Kidzeru, Anneke Hesselling, Jo-Ann Passmore, Hoyam Gamieldien, Clive Gray, Donald Sodora[4], Heather Jaspan. **Effects of delayed BCG vaccination on immune responses to other antigens in South African HIV-exposed infants.** Oral presentation **at** 8th world congress of the World Society for Paediatric Infectious Disease (WSPID). Cape Town, South Africa, November 2013.

Christophe Toukam, Elvis Kidzeru, Anneke Hesselling, Jo-Ann Passmore, Hoyam Gamieldien, Clive Gray, Donald Sodora[4], Heather Jaspan. **Effects of delayed BCG vaccination on cellular immune responses to other antigens in the HIV-exposed infants.** Oral presentation at the 6TH SAAIDS CONFERENCE Durban, South Africa, June 2013.

Publications

Christophe Toukam Tchakoute, Anneke C. Hesseling, Elvis B. Kidzeru, Hoyam Gamieldien, Jo-Ann S. Passmore, Christine E Jones, Clive M. Gray, Donald L. Sodora, and Heather B. Jaspan. **Delaying BCG vaccination until 8 weeks of age results in robust BCG-specific T cell responses in HIV-exposed infants.** Manuscript submitted to Journal of Infectious Disease, February 2014 (under review).

Chapter 1. Literature Review

1.1. The immune system

Throughout a lifetime, an individual is continuously exposed to pathogens that can cause infections and diseases. The immune system is the organized assembly of cells and molecules that have evolved to protect the host from these invading pathogens (Janeway et al., 2008). In order to do so, the immune system must accomplish four functions: immunological recognition, immune effector function, immune regulation and immunological memory (Janeway et al., 2008).It can be divided into two arms that are characterized by different features and play diverse functions: the innate immune system and the adaptive immune system (Iwasaki et al., 2010). However, these systems are tightly linked.

1.1.1. Innate immunity

Innate immunity is generally non-specific and made up of immune cells that are activated shortly after pathogen exposure (Janeway et al., 2008). The cells that make up the innate immune system are derived from white blood cells or leucocytes and originate in the bone marrow (Janeway et al., 2008). They recognize pathogen-associated molecular patterns (PAMPs) on pathogens via their pathogen recognition receptors (PRR) (Iwasaki et al., 2010). However, these cells largely lack immunological memory and remain unchanged regardless of the number of encounters with an antigen over time (Janeway et al., 2008). Innate cells provide the first line of defense against pathogens and defects in their mechanisms can be harmful to the host (Iwasaki et al., 2010, Akira et al., 2006). The innate immune system is made up of different types of cells playing distinct roles in protecting the host (Janeway et al., 2008).

Macrophages are innate cells which are long-lived, reside in almost all tissues, and are the differentiated form of monocytes that circulate in the blood (Gordon et al., 2010; Murray et al., 2011). Macrophages are phagocytes, therefore one of their main functions is to engulf pathogens at the sites of infection and present antigens to T-cells (Gordon et al., 2010; Murray et al., 2011). Macrophages are antigen-presenting cells (APCs: a group of a immunocompetent cells that mediate immune response by processing and presenting antigenic peptides to T cells (adaptive immunity) and subsequently lead to the activation of these T cells (Gordon et al., 2010; Murray et al., 2011). In addition, they can trigger inflammation and recruit other macrophages to the site of infection (Gordon et al., 2010; Murray et al., 2011).

Granulocytes are also innate cells characterized by the presence of dense granules inside their cytoplasm. There are three types of granulocytes and each type performs different functions:

Neutrophils are phagocytes that play an important role in the activation of bactericidal mechanisms and the clearance of extracellular pathogens (Mantovani et al., 2011).Recent evidence suggests that neutrophils can regulate the effector functions of innate and adaptive immune cells via the cytokines and other secreted effector molecules (Mantovani et al., 2011). In addition, neutrophils have been shown to play an important role in autoimmune diseases and chronic inflammation (Lande et al., 2011). Eosinophils and basophils are less studied. They are involved in allergic inflammatory responses (Cheung et al., 2006; Obata et al., 2007).

Dendritic cells (DCs) are also APCs of the innate immune system present in peripheral blood and tissues (Geissmann et al., 2010; Sixt et al., 2005). They present antigens to T-cells in the lymph nodes and express co-stimulatory molecules that bind to co-receptors on T-cells' surface and stimulate them (Geissmann et al., 2010; Sixt et al., 2005). DCs play a critical role in the activation and regulation of adaptive immunity and are important in the

establishment of immunologic responses (Geissmann et al., 2010). DCs have been shown to involved in the induction of antibody responses via T helpers (Boscardin et al., 2006).

Other innate cells include mast cells. Although they are part of the innate immune system, a mast cell's origin is not well established but they are believed to be involved in the activation of allergic response in tissues. As described above, innate immune cells recognize pathogens through their PRRs that can distinguish self from non–self (Janeway et al., 2008). Toll-like receptors (TLRs) are a group of PRRs that recognize distinct PAMPs and are important for innate immune responses (Takeuchi et al., 2010). They are involved in the first line of defense against invading pathogens and can induce inflammatory responses and cytokines production (Kollmann et al., 2009). There are 11 members of the TLR family recognizing different pathogens patterns (Takeuchi et al., 2010). Upon antigen recognition, innate cells, especially phagocytes, release cytokines and chemokines that play various functions (Iwasaki et al., 2010). For example, they can induce inflammation at the site of infection (Iwasaki et al., 2010). This inflammation subsequently leads to an increase in the number of effector cells at the site of infection through chemotaxis (Iwasaki et al., 2010). It also promotes rapid repair of infected tissues and provides and additional barrier to infection. In addition, these cytokines and chemokines bind to T-cells and lead to the activation and migration of these T-cells (Janeway et al., 2008). Therefore, innate immunity constitutes the first line of defense in an infection through a rapid, non-specific response. Innate cells bind to pathogens through their recognition receptors and induce adaptive immunity through antigen presentation and cytokine release (Mogensen, 2009).

1.1.2. Adaptive Immune system

The adaptive immune system is activated by the innate immunity and the two act synergistically to clear pathogen infection (Iwasaki et al., 2010). Unlike innate immunity, the adaptive immune system (adaptive immunity) is slowly

induced, acquired, highly specific, and capable of generating immunological memory (Iwasaki et al., 2010). The adaptive immune system is characterized by a primary immune response several days after pathogen invasion and a more rapid memory response that can be established after a second encounter with a specific pathogen (Janeway et al., 2008). The adaptive immune system can be divided into two parts that have different mechanisms but play complementary roles: the humoral immune system and cell-mediated immunity (Janeway et al., 2008).

1.1.2.1. Humoral immune system

Humoral responses are initiated in the lymph nodes by naïve B cells, which recognize soluble antigens and bind to them via their surface immunoglobulins or B cell receptors (BCR) (Pape et al., 2007). Each naive B cell is characterized by a unique surface antigen-specific receptor and can only be activated after encountering its specific antigen. B-cell activation leads to their differentiation into plasma cells and induction of antibody production (Janeway et al., 2008). B cell activation occurs via two different mechanisms. In T-cell dependent (TD) B cell activation, B cells are activated after recognition of their specific antigen coupled with helper T-cell signalling (Pape et al., 2007). First, the B cell receptor recognizes and binds to its specific antigen (Pape et al., 2007). This leads to an internalization and degradation of the antigen into small peptides (Pape et al., 2007). The antigenic peptide is then presented on the B-cell surface MHCII complex to a specific helper T-cell in the germinal centers (Coffey et al., 2009). Upon binding, the T-cell releases cytokines that activates the B cell and leads to its differentiation into plasma cells (Pape et al., 2007).

On the other hand, in T-cell independent (TI) activation, the B cell binds to its specific antigen and is activated by secondary binding to TLRs such as TLR-4 or TLR9 (Pasare et al., 2005). The resulting activated B cell is restricted to IgM antibodies (Pasare et al., 2005). T-cell independent activation also occurs when the B cell receptor-binds to its specific antigen and massive cross-linking of B

cell receptors subsequently takes place (Defrance et al., 2011), which in turn leads to B cell activation and antibody secretion.

In the primary B-cell response, activated cells present in the lymph nodes move to follicles where they undergo somatic hypermutation. Somatic hypermutation improves B cells antigen specificity (Janeway et al., 2008). Following somatic hypermutation, B cells move to the germinal centers where they present antigens to T-cells and differentiate into memory B-cells or effector plasma cells (Janeway et al., 2008).

Once differentiated, plasma cells secrete antibodies, which are also referred to as immunoglobulins. They are characterized by two heavy chains and two light chains. There are different classes depending on the isotypes of their heavy chains. Each class plays a function and is localized at a specific site within the host (Janeway et al., 2008).

IgM is the first antibody to be produced in a humoral immune response (Foote et al., 1995). IgM is generally low affinity as it is produced before hypersomatic mutation of B-cells and are particularly important in the activation of complement (Schroeder et al., 2010). In addition, IgMs are also present in the mucosa where they prevent pathogen entry (Brandtzaeg et al., 1999; Schroeder et al., 2010). IgG, IgA, IgE are released later in the response. IgG is the main class of antibody. It has high affinity and binds antigen for macrophage ingestion, and plays an important role in natural killer cell (NK) neutralization (Schroeder et al., 2010). IgG plays an important role in vaccine-induced immunity due to its high blood concentration. On the other hand, the principal role of IgA is to transfer antigenic proteins across the mucosal epithelium (Schroeder et al., 2010). IgE is secreted by mast cells and is involved in allergic responses (Schroeder et al., 2010). Antibodies have short half-lives, the average half-life of IgG being three weeks (Foote et al., 1995). Vaccines help sustain long-term antibody responses by maintaining memory B cells that will later differentiate into antibody secreting plasma cells (Amanna et al., 2007).These memory B cell responses can

be regulated by Helper T cells of the cellular immunity, which in return can recognize antigens presented by B cell surface MHCII molecules (Mauri et al., 2012).

1.1.2.2. T-cell mediated immunity

The second component of adaptive immunity is T-cell mediated immunity. T cells originate in the bone marrow and develop in the thymus (Janeway et al., 2008). Once they have completed their development and selection in the thymus, naïve T cells move to blood stream and peripheral lymphoid organs (Burgdorf et al., 2007). They become activated and differentiate after their T cell receptors (TCRs) encounter specific antigens presented on MHC complex molecules on antigen-presenting cell surfaces (Burgdorf et al., 2007). Unlike B cells, T cellular main targets are infected cells can only recognize antigenic peptides presented by Major Histocompatibility Complex (MHC) molecules (Burgdorf et al., 2007). There are two types of TCRs: the first type is composed of an alpha and beta polypeptide chain and represent approximately 95% of the TCR population (Guy et al., 2009). These T-cells are referred to as conventional T-cells. The second type is made up of a gamma and delta polypeptide chain, and are called gamma-delta or non-conventional T cells (Pennington et al., 2005). CD3 is a multimeric protein complex composed of four distinct polypeptide chains (Guy et al., 2009; Malissen, 2003). There are two main CD3+ T cells: helper T cells (CD4+) and CD8 T-cells (Smith-Garvin et al., 2009).The CD3 complex interacts with the T cell receptor (TCR) at the T cell membrane surface and is important for intracellular signalling (Guy et al., 2009). Both TCRs and the CD3 protein complex are characteristics of the T cell lineage and can be used as T cell markers (Guy et al., 2009).

Non-conventional (γδ) and NK T cells are also present in whole blood in smaller proportions (Bonneville et al., 2010). Non-conventional T cells characterized by their γδ TCR recognize non-peptide antigens such as phosphor-antigens

(Bonneville et al., 2010). On the other hand, NK T -cells recognize glycolipids presented by the CD1d molecule on APC (Parekh et al., 2005).

1.1.1.2.1. CD8 T cells

After completing thymic maturation, naïve CD8 T cells bear CD8 co-receptors on their surface (Janeway et al., 2008). In the event of an invasion of the host immune system by a virus, naïve CD8 T-cells express the adhesion molecule L-selectin and the chemokine receptor CCR7 which help them migrate into lymph nodes where they recognize viral peptides presented on MHC I molecules by APCs (Burgdorf et al., 2007). Upon this recognition, CD8 T-cells generally differentiate either into effector Cytotoxic T Lymphocytes (CTL) or memory CD8 T cells (Burgdorf et al., 2007). These two types are characterized by different surface markers and secrete different molecules.

Upon differentiation, effector CTL undergo some changes on their surface, and start expressing markers such as CD57 and CD11d (Hamann et al, 1999; Williams et al., 2007). These allow them to kill virus-infected cells mediated by effector molecules such as granzymes and perforins (Hamann et al, 1999; Williams et al., 2007). In addition, CTLs secrete high levels of IFN-g and TNF-alpha important for control of intracellular pathogens such as Human Immunodeficiency Virus (HIV) and *Mycobacterium Tuberculosis* (M.TB) (Hamann et al, 1999; Williams et al., 2007). Effector T cells, characterized by high expression of CD57, lack the ability to proliferate and die by programmed cell death after long-term antigenic stimulation (Hamann et al, 1999; Williams et al., 2007).

On the other hand, memory CD8 T-cells are long-lived cells with a strong proliferative ability upon antigen re stimulation (Williams et al., 2007). As shown in table 1, unlike effector CD8 T cells, memory CD8 T cells are CD45RA- CD57- cells and they do not secrete as much perforin and granzyme B (Hamann et al, 1999; Williams et al., 2007). Memory CD8 T cells proliferation is mediated by their co-stimulatory molecules CD28 and CD27 (Hamann et al, 1999; Williams

et al., 2007). They are particularly important in vaccine-induced immunity against certain pathogens and can also express antiviral cytokines such as IFN-g (Hamann et al, 1999; Williams et al., 2007). Memory CD8-T cell activity is regulated by CD4+ T-cells. Memory T cells have been generally classified as central memory (T_{CM}) and effector memory (T_{EM}) (Kaech et al., 2007; Wherry et al., 2003).T_{CM} are CD62L^{hi}CCR7^{+} cells located in lymphoid organs and are thought to be involved in secondary memory responses (Kaech et al., 2007; Wherry et al., 2003). T_{EM} are CD62L^{lo}CCR7^{-} found in non-lymphoid tissues and they exhibit express lytic activity (Kaech et al., 2007; Wherry et al., 2003). CD8^{+} T_{CM} cells have been shown to induce greater protective immunity than T_{EM} cells in mice (Kaech et al., 2007; Wherry et al., 2003). In addition, CD8^{+} T_{CM} cells exhibit a greater proliferative ability than cells greater proliferative burst of T_{CM} cell-derived secondary effector T cells (Kaech et al., 2007; Wherry et al., 2003). However, the division of CD8+ T cells into T_{CM} and T_{EM} cells may be an oversimplification (Badovinac et al., 2007; Wherry et al., 2003).

Table 1.1: Properties of.CD8.T-cell subsets (Reproduced from Hamman et al., 1999 (Hamann et al., 1999).

Table I. Properties of CD8^{+} T-cell subsets[a]

	Naive	Memory	Effector
Surface markers			
CD45RA	+	−	+
CD27	+	+	−
CD28	+	+	−
CD11a	−/+	+	++
CD11b	−	−	++
CD49d	−	+	++
CD57	−	−	++
CD95	−	++	+
Cytokines			
IL-2	+	++	−
IL-4	−	+	−
IFN-γ	−	++	++
TNF-α	−	++	++
Mediators of cytotoxicity			
CD95 ligand	−	−/+	++
Perforin	−	−/+	++
Granzyme B	−	−/+	++
Functions			
Cytoxicity	−	−/+	++
Ag-induced proliferation	+	+++	−

Abbreviations: IFN-γ, interferon γ; IL-2, interleukin 2; TNF-α, tumour necrosis factor α.
[a]Adapted from Ref. 2.

1.1.1.2.2. CD4+ T-cells

CD4+ T-cells complete their development in a similar fashion to CD8+ T cells, and are characterized by their CD4 co-receptor. After completing their development, naïve CD4+ T-cells move to lymphoid tissues where they recognize antigenic peptides presented by APCs on MHCII molecules (Janeway et al.,2008). During their TCR activation, in particular cytokine environments,

naive CD4 T cells differentiate into one of several lineages of T helper (Th) cells, including Th1, Th2, Th17 and Tregs (Wan et al., 2009; Zhu et al., 2010).Th1 cells are important in the control of intracellular pathogens such as HIV and *Mycobacterium Tuberculosis.* They play an important role in phagocyte-dependent host responses (Romagnani, 1997). Th1 cells release cytokines such as IFN-g and IL-2, which activate infected macrophages to promote the killing of pathogens (Wan et al., 2009; Zhu et al., 2010).In addition, studies have shown that Th1 cells can lead to class switching of activated B cells (Smith et al., 2000; Zhu et al., 2010).It has also been demonstrated that Th1 cells are involved in certain autoimmune diseases such as Crohn's disease (Romagnani, 1997).Unlike Th1 cells, Th2 cells are primarily involved in phagocyte-independent host responses and the control of extracellular pathogens such as helminthes, toxins (Janeway et al., 2008; Zhu et al., 2006). They release cytokines such as IL-4 and IL-5, which induce activation of B-cells and the production of antibodies (Janeway et al., 2008; Zhu et al., 2006).In addition, Th2 cells release the cytokine IL-13 which is important in allergic responses (Walter et al., 2001).Th2 and Th1 cells are mutually inhibitory (Romagnani, 1997). IFN-g produced by Th1 cells was shown to inhibit the proliferation of Th2 cells (Wan et al., 2009; Zhu et al., 2010). Similarly, IL-10, cytokine produced by Th2 cells has the ability to downregulate Th1 cell cytokine production (Couper et al., 2008). This suggests that the Th1 and Th2 responses should be well balanced to enable optimum host responses to different types of pathogens (Wan et al., 2009; Zhu et al., 2010). This balance is particularly important for vaccine responses, especially for individuals whose immune systems are still developing or deficient (infants or HIV-infected individuals (Poland et al., 2007; Siegrist, 2007).

A third CD4+ T-cell subset that has recently been identified and is referred to as Th17. Th17 cells release cytokines such as IL-17, IL-21, and IL-22 (Korn et al., 2009; Liang et al., 2006). IL-17 can induce inflammatory responses and may play a protective role against bacterial infections at the mucosa (Lin et al., 2010; Witowski et al., 2004). In addition, mice studies have demonstrated a strong

correlation between a high expression of IL-17 and better protection against mycobacteria.

Regulatory T-cells often referred to as Tregs, represent another subsets of CD4 T-cells. These are CD4+ CD25+ T cells that do not proliferate upon their receptor activation and are suppressive in nature (Mills, 2004). They are thought to be important in the prevention of autoimmune diseases (Sakaguchi et al., 2006). In fact, studies have shown they can release IL-10 and inhibit the activity of self-reactive CD4+ and CD8+ T-cells (Beissert et al., 2006; Kuhn et al., 2009).

Figure 1.1 shows the differentiation of effector CD4 T-cells and the types of cytokines they secrete and figure 1.2 shows these effector T-cells interact with other cells of the immune system.

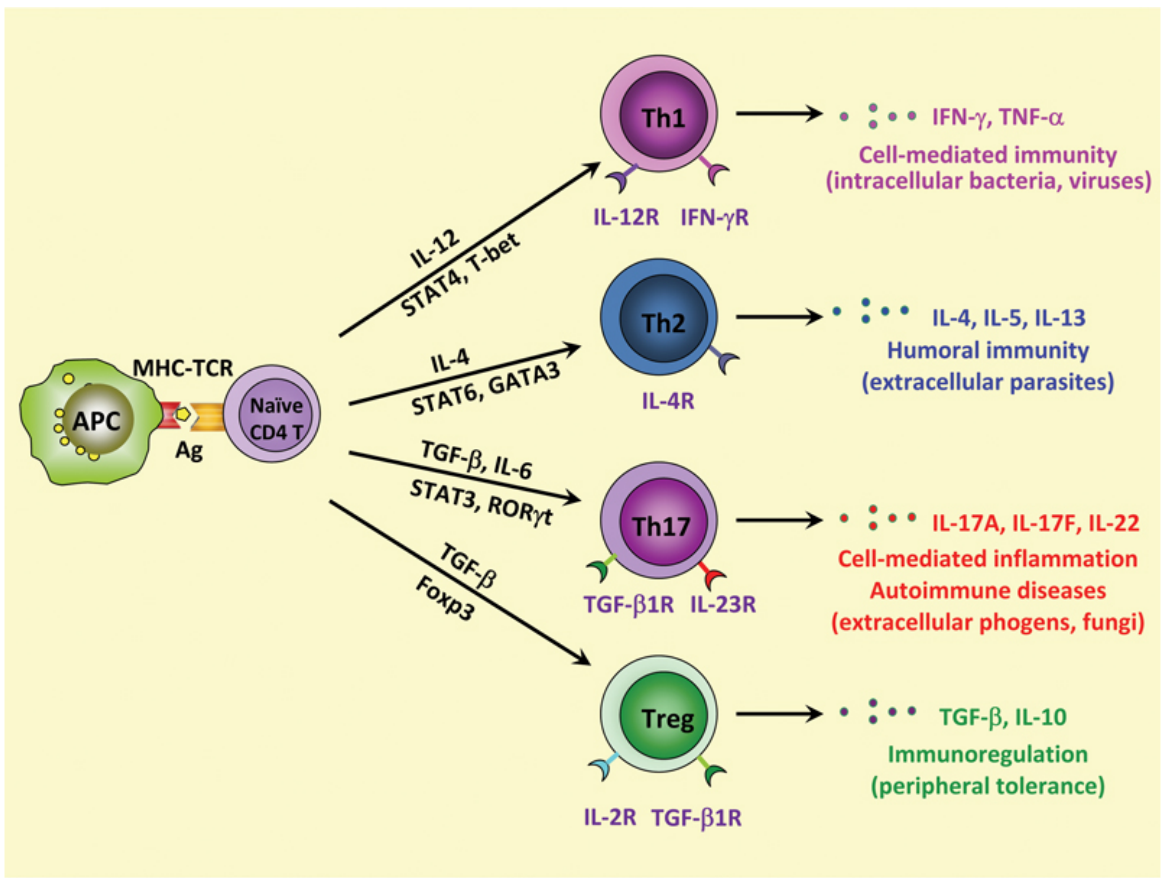


Figure 1.1: Naive CD4+ T differentiation into Th1, Th2, Th17, and Treg cells. Upon antigen presentation by the APC-MHC complex, naive CD4 T cells differentiate into Th1 cells through the activation of the regulatory transcription factor T-bet via the STAT1 and STAT4 pathways after secretion f IL-12 that plays a vital role in the development of Th1 cells. Th1 cells secrete IFN-γ which is important for protection against intracellular bacteria and viruses. IL-4 promotes the activation of STAT6 and GATA3, responsible for Th2 cell differentiation. Th2 cells are important in humoral immunity against parasites, and their activity is mediated through cytokines of IL-4, IL-5 and IL-13 that they secrete. TGF-β and pro-inflammatory cytokines, such as IL-6 and IL-23, induce differentiation of naive CD4+T cells differentiation into IL-17-producing Th cells (Th17) through transcription factors STAT3 and RORγt. Th17 cells induce inflammatory responses and may play a protective role against bacterial infections. In addition, TGF-β can also induce differentiation of naive CD4+T cells into Foxp3+Treg cells, which produce TGF-β and IL-10 and act as modulators of immune responses (Leung et al., 2010).

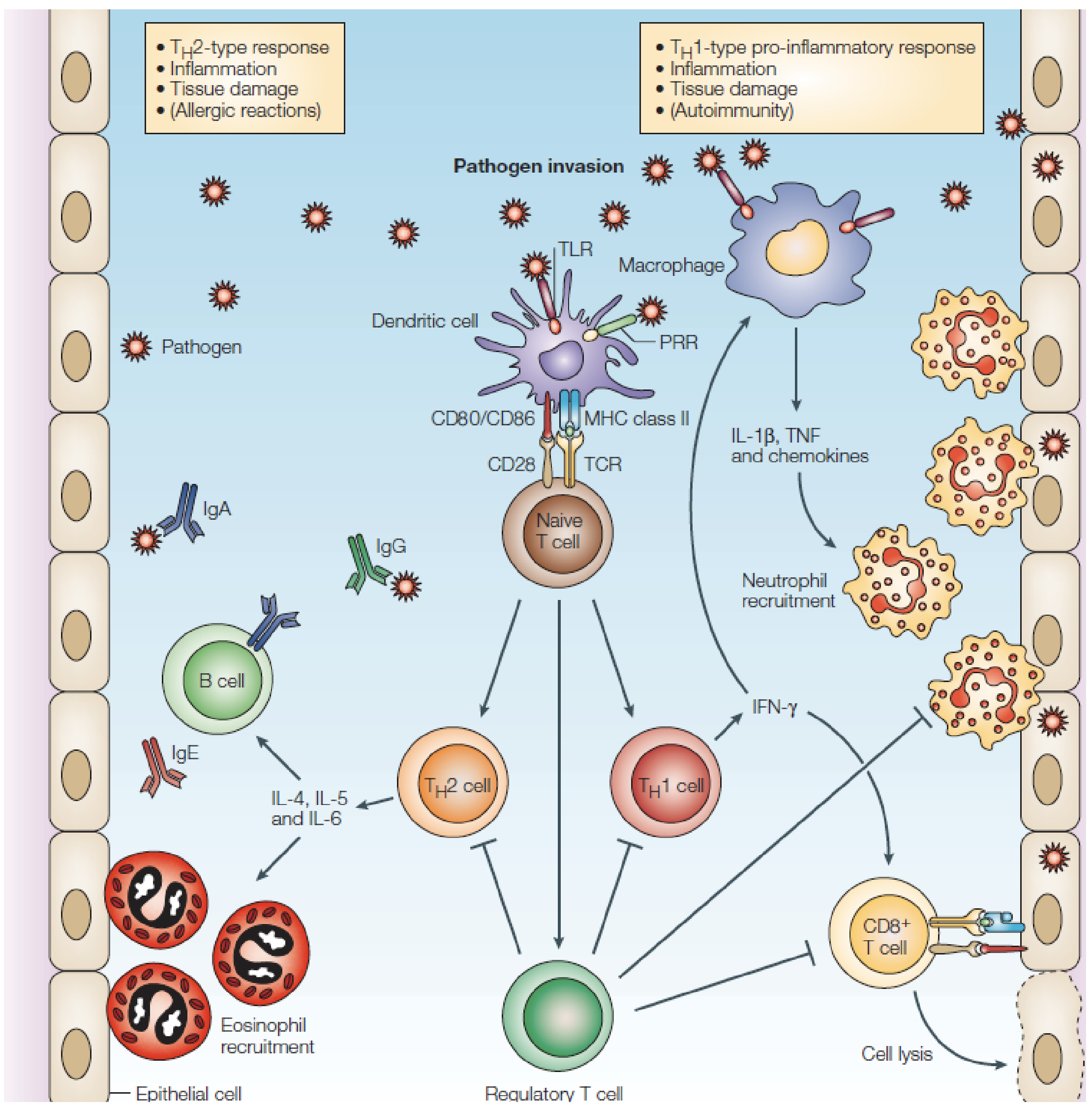


Figure 1.2: Interaction between CD4 T-cells and other immune cells. Upon host infection by a pathogen, Macrophages and Dendritic Cells (APCs) recognize pathogens through their Pathogen Recognition Receptors (PRRs). Dendritic cells present antigen to naive CD4+ T cell via MHCII molecules. Upon antigenic presentation to their TCRs, naïve CD4+ T cells differentiate into Th1, Th2, Th17 (not shown) and regulatory T cells (T regs). Th2 cells release cytokines such as IL-4, IL-6 that induce eosinophil recruitment and induce antibody production by B cells. Th1 cells secrete mostly IFN-γ that enhances CD8+ T cell activity. Regulatory T cells downregulate the activity of Th1, Th2 and CD8+ T cells (Mills, 2004).

Similar to memory CD8+ T cells, memory CD4+ T-cells are long-lived cells that proliferate after a second encounter with an antigen (Seder et al., 2003). Their establishment is particularly important for protection against a pathogen after vaccination (Seder et al., 2008).

As described above, cells of the immune system, particularly T-cells, regulate the activity of other immune cells via the different cytokines that they secrete (Figure 1.2).

1.2. Cytokines

Cytokines are soluble effector molecules secreted by immune system's cells, especially T-cells (Janeway et al., 2008). These cytokines can trigger inflammatory responses at different sites of infection locally or at a distance (Gouwy et al., 2005). In addition, they can regulate activity of some immune cells by binding to specific receptors on the surface on these cells (Gouwy et al., 2005; Kelso, 1998).There are different groups of cytokines secreted by different cells and each plays a different function. Here, we will focus only on the main cytokines secreted by T-cells.

1.2.1. Interferons

There are two families of interferon cytokines also referred to as IFNs. Type I IFNs (for example IFN-α, IFN-ß, and IFN-ῴ) are encoded by multiple genes, and type II IFN-γ encoded by a single gene (Honda et al., 2006; Theofilopoulos et al., 2005). Even though they are not structurally related, they all have been shown to have antiviral activity (Randall et al., 2008). In fact, mouse studies have revealed that mice lacking Type I and II IFN genes are highly susceptible to viral infections (Stetson et al., 2006). Type I IFNs are mainly produced by macrophages, bind to specific receptors on macrophages, and lead to their activation (Frucht et al., 2001; Randall et al., 2008). Besides their antiviral activity, Type I IFNs affect the release of pro-inflammatory cytokines or nitric oxide by dendritic cells and the activity of type II interferon (IFN- γ) (Bogdan, 2000; Le Bon et al., 2001).

On the other hand, IFN-γ is secreted by a few types of cells: NK cells, NK T-cells, CD4-T cells and CTLs (Randall et al., 2008; Theofilopoulos et al., 2005). IFN-γ binds to a trimeric membrane receptor on some immune cell surfaces and

subsequently induces signals within these cells that modify their activity and their function (Theofilopoulos et al., 2005). IFN-γ has been demonstrated to enhance macrophage activity against viruses and bacteria (Randall et al., 2008; Theofilopoulos et al., 2005). In fact, IFN-γ binding to macrophages leads to secretion of IL-12, important for control of *mycobacteria Tuberculosis* (Demissie et al., 2004; Newport, 1996; Pai et al., 2003). Furthermore, IFN-γ upregulates the expression of MHC I and MHC II by APCs and therefore enhances antigen presentation to T-cells (Janeway et al., 2008; Theofilopoulos et al., 2005). IFN-γ can modulate the permeability of epithelial barriers (Theofilopoulos et al., 2005). IFN-γ also inhibits Th2 cells activity and promote the secretion of Ig2a by B –cells (Janeway et al., 2008; Theofilopoulos et al., 2005).

As depicted above, interferons modulate the activity of innate and adaptive immunity and therefore are essential for protection against pathogens especially viruses and bacteria. However, their overexpression can be detrimental to the host. Thus, in some cases they are inhibited by other cytokines secreted by T-cells such as IL-1 and IL-4 (Dinarello, 2009).

1.2.2. Interleukins

Interleukins represent another family of cytokines secreted by effector T-cells. Within this family, they can be divided into subfamilies depending on their functions, structures and the receptor they bind to (Dinarello, 2009; Janeway et al., 2008). Here we will only discuss the main interleukins subfamilies. The IL-1 subfamily is made up of 18 cytokines which bind to IL-1 Types I and II receptors and are mostly secreted by macrophages and T-cells (Akdis et al., 2011).IL-1ß and IL-1α are the most studied cytokines of the subfamily and have been shown to induce systemic and local inflammation (Akdis et al., 2011; Dinarello, 2009). Furthermore, IL-1 cytokines mostly act on T-cells and epithelial cells and have been associated with autoimmune and pro-inflammatory diseases such as psoriasis and IBD (Akdis et al., 2011; Dinarello, 2009). Such diseases can be

treated by the administration of IL-1RA, an IL-1 receptor antagonist (Akdis et al., 2011).

IL-2 is a trimeric cytokine mainly secreted by Th1, CD8+ and NKT-cells (Akdis et al., 2011). IL-2 has been shown to promote B-cell growth and to enhance their antibody production (Akdis et al., 2011). Furthermore, it enhances the growth and cytolytic activity of NK cells (Akdis et al., 2011). IL-2 plays a critical role in the function and development of Tregs (Furtado et al., 2002; Nelson, 2004). In fact, Furtado et al demonstrated that IL-2 knockout mice could not develop functioning Tregs (Furtado et al., 2002).IL-2 has been associated with T-cell mediated autoimmune diseases (Akdis et al., 2011; Setoguchi et al., 2005; Waldmann, 2006)and some T-cell lymphomas and leukemias have been shown to express an IL-2 receptor on their surface (Akdis et al., 2011; Waldmann, 2006). Anti–IL-2Ra antibodies are used in immunotherapy to inhibit immune response in patients with autoimmune diseases and prevent transplant rejection (Akdis et al., 2011; Waldmann, 2006). In addition, they are currently being investigated as a potential therapeutic for certain cancers (Akdis et al., 2011; Waldmann, 2006).

On the other hand, IL-13, IL-4, and IL-5 bind to closely related receptors and belong to the same subfamily (IL-4 subfamily) (Akdis et al., 2011).They are produced by Th2 cells and play similar functions (Akdis et al 2009). They are associated with the activation and recruitment of mast cells (Akdis et al 2009). In addition, IL-13 and IL-4 have been shown to activate B-cells (Akdis et al., 2011; Janeway et al., 2008). Furthermore, they upregulate the expression of MHC II by macrophages and B-cells and mediate class switching to IgG and IgE (Akdis et al., 2011; Janeway et al., 2008). Roussel *et al* also demonstrated that IL-13 inhibits pro-inflammatory cytokines and is required for maintenance of IgE-mediated allergic responses (Roussel et al., 1998).

IL-17, IL-21 and IL-22 secreted by Th17 cells represent an emerging subfamily of Interleukins. They are induced by IL-23 and thought to be pro-inflammatory

cytokines (Stockinger et al., 2007). IL-21 and IL-22's other functions are still being elucidated (Stockinger et al., 2007). It has been shown that IL-17 can stimulate macrophages and epithelial cells to secrete pro-inflammatory cytokines such as IL-1 and TNF-α (Iwakura et al.,2006; Stockinger et al., 2007). Due to its pro-inflammatory properties, IL-17 has been associated with many autoimmune diseases (Komiyama et al., 2006; Nakae et al., 2003). Recent mice studies showed that mice lacking IL-27, a known IL-17 inhibitor, were more susceptible to autoimmune diseases (Batten et al.,.2006). Despite these negative effects, IL-17 may play an important role in host defense. In fact, it induces recruitment of neutrophils at the sites of infection and therefore links innate immunity to adaptive immunity (Wu et al., 2007).

In summary, cytokines are important effector molecules. They help in the recruitment of specific immune cells and play a critical role against some pathogens. However, in some cases they can trigger inflammation and be detrimental to the host (Gouwy et al., 2005). Because of their interplay on many immune cells, cytokines contribute greatly to T cell homeostasis of the immune system especially in the mucosa and the gut (Boyman et al., 2007), i.e. to preserve normal T-cell counts following T-cell depletion or expansion (Boyman et al., 2007). IL-15 and IL-7 are the main cytokines influencing T cell homeostasis due to their effects on T cell proliferation (Stockinger et al., 2004). Figure 1.3 shows the functions of the main cytokines produced by T cells.

It is important to note that cytokine function and activity greatly depend on the maturity of the immune system. The properties and mechanisms discussed above are mostly observed in adults. Neonates have different immunological characteristics.

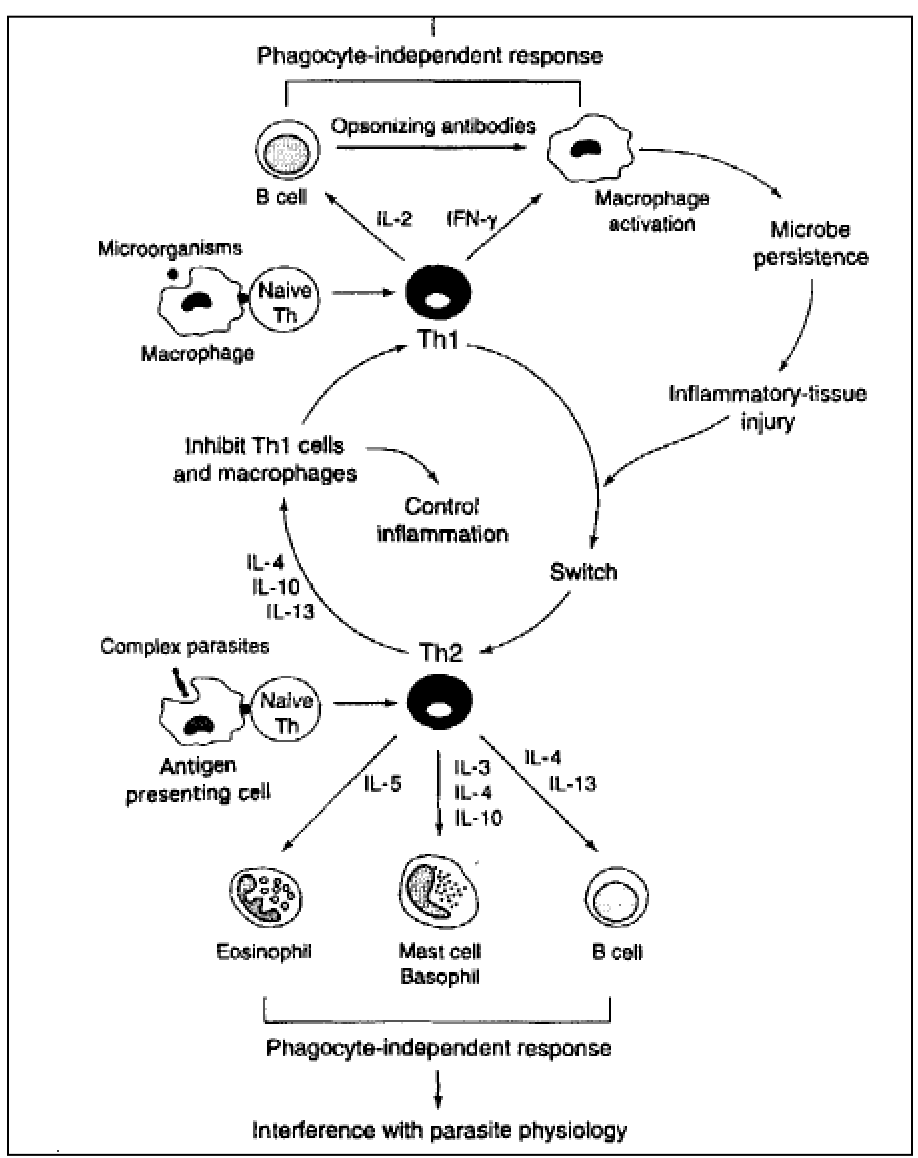


Figure 1.3: Function of main cytokines produced by T-cells. Interleukins and Interferons are the main cytokines produced by CD4+ T cells, especially Th1 and Th2 cells. Th2 secrete IL-5 that has been shown to be important for eosinophil recruitment at the site of infection. In addition, Th2 cells also produce IL-4, IL-10 and IL-13.Thse cytokines activate mast cells and also enhance B cell activity. In addition, IL-4, IL-10 and IL-13 inhibit Th1 cells and macrophages and favor Th2 differentiation. On the other hand, Th1 cells release IL-2 and IFN-γ. IL-2 facilitates B cell activity and phagocytose-dependent mechanisms. IFN-γ activates macrophages (Romagnani, 1997)

1.3. Immunity in early infancy

During the first months of life, an individual is more susceptible to pathogens. This is partially due to the "immaturity" of the immune system in early life (Newburg et al., 2007). In some respects, the neonatal immune system is still developing and does not function as well as an adult immune system (Kovarik et

al., 1997) (Chappuis et al 1998, Kovarik et al 1998). Both neonatal innate and adaptive immunity are different from their adult counterparts (Maródi, 2006; Zaghouani et al., 2009).

1.3.1. Innate immunity in infants

Neonates have greatly impaired neutrophil adherence and activity (Carr, 2000). Since neutrophils are the first line of defense when the host immune system is invaded by pathogens, impairment of their function contributes to the rapid spread of infections (Kovarik et al., 1997; Maródi, 2006; Petty et al., 1998). In addition to poor neutrophil function, Petty et al demonstrated that neonatal dendritic cells have a limited ability to induce T-cell proliferation in response to different mitogens compared to adult dendritic cells (Petty et al., 1998). This could partly be due to the reduced MHC expression observed in neonates (Maródi, 2006). Neonates demonstrate reduced production of some pro-inflammatory cytokines such as IL-12, IL-23 and IFN-γ (Kovarik et al., 1997). These cytokines are important for the activation of mononuclear phagocytes especially macrophages (Maródi, 2006). In fact, macrophage responses are often sub-optimal in neonates (Maródi, 2006). Many studies reveal that TLR signaling pathways are greatly impaired in neonates (De Wit et al., 2003; Kollmann et al., 2009). Kollmann et al recently demonstrated that neonatal TLR-mediated innate responses are different from those in adults (Kollmann et al., 2009; Kollmann et al., 2012). Neonatal innate immune cells (DCs, macrophages) secrete very small quantities of IFN-γ and IL-12p70 in response to TLR ligands compared to adult innate immune cells (Kollmann et al., 2009; Kollmann et al., 2012). On the other hand, neonatal innate immune cells secrete more Th2 (IL-4) and Th17 (IL-17, IL-23) cytokines in response to TLR ligands than adult innate immune cells (Kollmann et al., 2009). These differences explain why neonates are more vulnerable to both viruses such as Cytomegalovirus (Kollmann et al., 2009; Maródi, 2006)or bacterial sepsis (Lavoie et al., 2010). Thus, their impaired TLR-mediated responses suggest that neonatal T-cell immunity may also be

distinct from that of adults (Kollmann et al., 2009) and these differences will be addressed in the following section.

1.3.2. Neonatal T-cell immunity

Table 1.2 shows the main characteristics of neonatal immunity. In most cases, neonatal T cells preferentially make type 2 cytokines such as IL-4, IL-5, and IL-13, and therefore their immune responses are biased toward Th2 responses (Garcia et al., 2000; Maródi, 2006; Siegrist, 2007). In some cases, Th1 responses are practically undetectable in neonates (Garcia et al., 2000; Zaghouani et al., 2009). However, under some circumstances, neonates can produce Th1 cytokines, such as to BCG and Cytomegalovirus (CMV) (Marchant et al., 1999; Park et al., 2006).

The Th1/Th2 imbalance can be partially explained by the predominance of Th2 cytokines during pregnancy (Kovarik et al., 1997), and partly to the fact that infants are deficient in production of IL-12. It has been demonstrated that the presence of IL-4 during T-cell priming can inhibit IL-12Rβ2 chain expression on naive T cells and therefore direct cells towards Th2 responses in humans and mice (Li et al., 2004; Ohshima et al., 1997). Lee et al showed that delayed maturation of IL-12 producing dendritic cells results in a Th2 bias in neonates (Lee et al., 2008). In addition, as described above, neonatal deficiency in Th1 responses can also be attributed to the impaired TLR-mediated innate responses of APCs especially dendritic cells, which play an important role in the activation of Th1 cells (Kollmann et al., 2009). The immaturity of neonatal immunity has many implications for neonatal immunization and their immune responses to vaccines.

Table 1. 2: Main characteristics of neonatal immunity.

Innate Immunity	T-cell Immunity
Impairment of DC and macrophage activity.	
Reduction of MHC molecules expression	
Impairment of TLR–mediated immune responses	
Lack of pro-inflammatory cytokines secreted by innate cells	Downregulation of IFN-g

1.4. Vaccination in neonates

1.4.1. Vaccines, antigens and adjuvants

"A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles the disease-causing microorganism, and is often made from weakened or killed forms of the microbe, its toxins or one of its surface proteins."(Stern et al., 2005). A vaccine induces the host immune system to recognize foreign antigen, and mount an immune response against it (Ada et al, 2001). This leads to the establishment of immunological memory that will potentially protect the host after later encounters with the virulent form of the pathogen (Castellino et al.,.2009). The concept of vaccination was first described by Edward Jenner who noted that an 8-year-old boy who he had previously inoculated with pus from a cowpox lesion on a milkmaid's hand was immune to variola (Stern et al., 2005).

Most vaccines are prophylactic and help prevent future infections. Prophylactic vaccines play an important role in protection against infectious diseases such as Tuberculosis and Diphtheria and have helped eradicate others such as smallpox (Stern et al., 2005). A vaccine could also be therapeutic, as is the case of cancer and HIV vaccines that are currently being developed (Lu et al., 2004; Simon et al., 2001), but this thesis will focus on preventative vaccines.

There are many types of vaccines; each type has different properties and elicits different types of responses (Ada et al., 2001; Castellino et al., 2009; Liu, 2003). The main types of vaccines are: live attenuated vaccines, whole inactivated vaccines, toxoid vaccines, subunit vaccines, conjugate vaccines, recombinant vaccines and DNA vaccines (Ada et al., 2001; Castellino et al., 2009; Liu, 2003). Live attenuated and whole cell inactivated vaccines induce long-lived immunity and generally have higher efficacy (Belshe et al., 2007; Linhares et al., 2008).However, attenuating mutations present in live vaccine strains such as oral polio vaccine can revert to wild type and cause disease (Minor, 2009). Whole cell inactivated vaccines such as whole-cell pertussis vaccines may also be potentially harmful due to the presence of specific antigens in the vaccine strains (Gustafsson et al., 1996). Due to these safety concerns, synthetic vaccines such as recombinant vaccines and subunit vaccines are often preferred. However, these vaccines are often less immunogenic. Thus, in order to enhance immune responses to those vaccines, adjuvants are often used (Morefield et al., 2005; Sokolovska et al., 2007).These chemical agents can increase DC activity and antigen presentation (Morefield et al., 2005; Sokolovska et al., 2007). Adjuvants function through the induction of relevant cytokines and the regulation of co-stimulatory molecules (Salerno-Gonçalves et al., 2006). Different adjuvants are chosen according to the type of response that is desired. Various TLR ligands including CpG-containing oligonucleotides are used to induce CTL responses. CpG-containing oligonucleotides, which signal through TLR9, can induce strong CD8+ CTL response and might allow a reduction in the dose of antigen (Salerno-Gonçalves et al., 2006). Reports from animal and human studies indicate that they are generally safe even at high doses (Salerno-Gonçalves et al., 2006). In addition, in a recent clinical trial, CpG was found to enhance antigen-specific cell-mediated immunity when administered with hepatitis B vaccine in HIV infection (Angel et al., 2008). Another example of an adjuvant is Aluminium salts which are used in many vaccines where the desired protective response is antibody-mediated such as in some tetanus vaccines (Marrack et al., 2009). Figure 1.4 describes the mechanism of action of Aluminium salts.

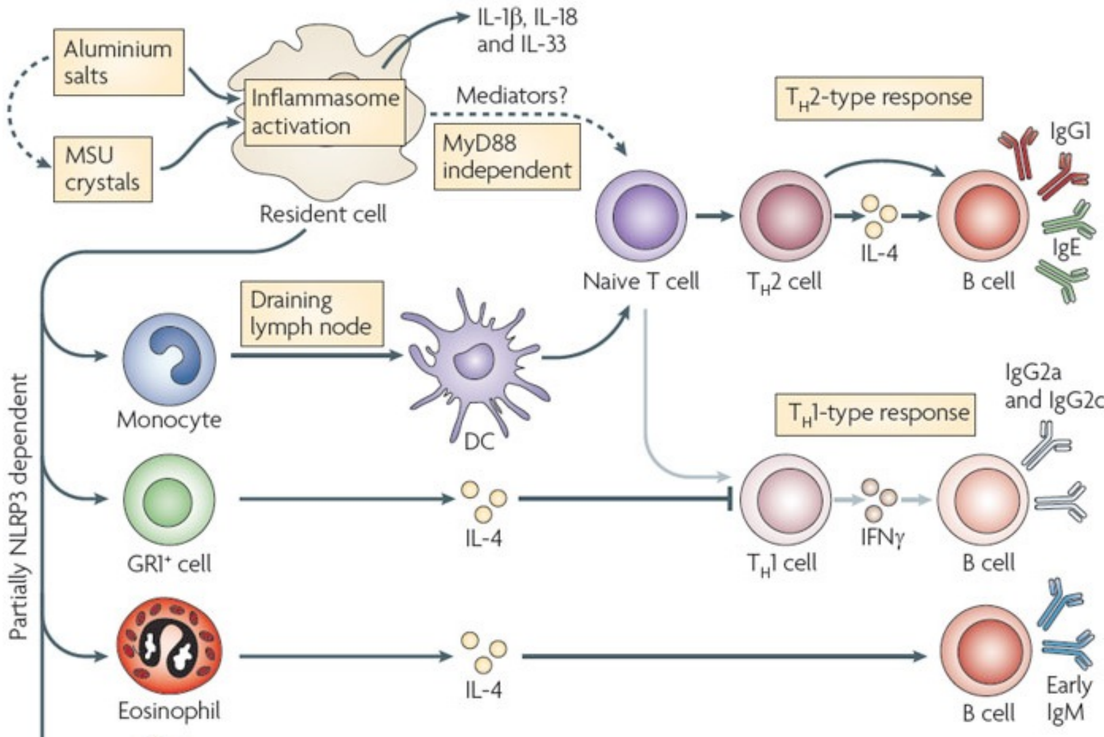
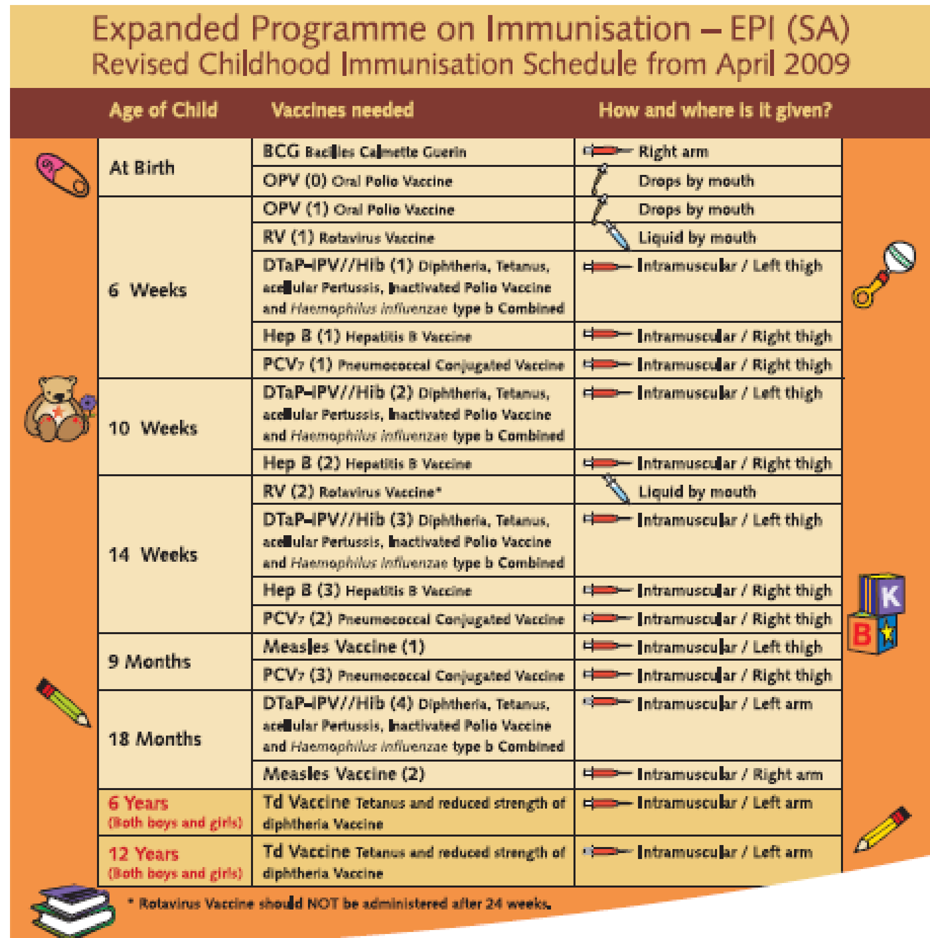


Figure 1.4: Aluminium salt mechanism of action as a vaccine adjuvant. Aluminium salts crystals activate resident phagocytes partly through the NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3). NLRP3 inflammasome induces adaptive Th2.cytokine production such as interleukin-4 (IL-4) which enhances antibody production. IL-4A Th2 cytokine downregulates IFN-γ secretion and Th1 responses (Marrack et al., 2009).

1.4.1.1. Vaccination

Vaccination is regarded as one of the greatest public health achievements of the 20th century (André, 2003). Since the implementation of the Expanded Program on Immunization (EPI) worldwide in 1974, vaccines have saved over 20 million young lives and greatly reduced the burden of major infectious diseases in children such as Tuberculosis, Diphtheria, Tetanus, and Measles (UNICEF, 2012). Some countries have their own adapted immunization programs based on their diseases burden and their economic resources. Table 1.3 shows the South African EPI vaccination schedule.

Table 1.3: **South African Expanded Program on immunization** (Department of Health, 2009)



Expanded Programme on Immunisation – EPI (SA)
Revised Childhood Immunisation Schedule from April 2009

Age of Child	Vaccines needed	How and where is it given?
At Birth	BCG Bacilles Calmette Guerin	Right arm
	OPV (0) Oral Polio Vaccine	Drops by mouth
6 Weeks	OPV (1) Oral Polio Vaccine	Drops by mouth
	RV (1) Rotavirus Vaccine	Liquid by mouth
	DTaP-IPV//Hib (1) Diphtheria, Tetanus, acellular Pertussis, Inactivated Polio Vaccine and *Haemophilus influenzae* type b Combined	Intramuscular / Left thigh
	Hep B (1) Hepatitis B Vaccine	Intramuscular / Right thigh
	PCV7 (1) Pneumococcal Conjugated Vaccine	Intramuscular / Right thigh
10 Weeks	DTaP-IPV//Hib (2) Diphtheria, Tetanus, acellular Pertussis, Inactivated Polio Vaccine and *Haemophilus influenzae* type b Combined	Intramuscular / Left thigh
	Hep B (2) Hepatitis B Vaccine	Intramuscular / Right thigh
14 Weeks	RV (2) Rotavirus Vaccine*	Liquid by mouth
	DTaP-IPV//Hib (3) Diphtheria, Tetanus, acellular Pertussis, Inactivated Polio Vaccine and *Haemophilus influenzae* type b Combined	Intramuscular / Left thigh
	Hep B (3) Hepatitis B Vaccine	Intramuscular / Right thigh
	PCV7 (2) Pneumococcal Conjugated Vaccine	Intramuscular / Right thigh
9 Months	Measles Vaccine (1)	Intramuscular / Left thigh
	PCV7 (3) Pneumococcal Conjugated Vaccine	Intramuscular / Right thigh
18 Months	DTaP-IPV//Hib (4) Diphtheria, Tetanus, acellular Pertussis, Inactivated Polio Vaccine and *Haemophilus influenzae* type b Combined	Intramuscular / Left arm
	Measles Vaccine (2)	Intramuscular / Right arm
6 Years (Both boys and girls)	Td Vaccine Tetanus and reduced strength of diphtheria Vaccine	Intramuscular / Left arm
12 Years (Both boys and girls)	Td Vaccine Tetanus and reduced strength of diphtheria Vaccine	Intramuscular / Left arm

* Rotavirus Vaccine should NOT be administered after 24 weeks.

In the next sections pertussis, Tetanus and BCG vaccines are discussed because assessing infants' immune responses to those vaccines is the main objective of this dissertation.

1.4.1.1.1. Tetanus toxoid (TT) vaccine

Tetanus is caused by a bacterium *Clostridium tetani*. This bacterium is found in cultivated soils and other places such as animal excrement and dust. The bacterium penetrates the body through injuries from contaminated objects

(Hassel, 2013)or inhalation in neonates. Once inside the host, it releases a toxin called tetanospasmin into the blood stream (Hassel, 2013). This toxin affects nerve signaling and causes severe sustained muscle contractions, especially "lockjaw", with which adult tetanus is mostly associated (Hassel, 2013).Even though the disease is not spread from person to person, tetanus claims the lives of 73 000 children under five annually (UNICEF, 2012).

The main prevention against tetanus is the tetanus toxoid (TT) vaccine. The vaccine can be administered alone as TT, or in combination with other vaccines (CDC, 2013). There are many formulations based on age of individual being immunized. One formulation is DTaP, which is a combination TT with the diphtheria and pertussis vaccines in infants (CDC, 2013). During their first year of life, infants receive three doses of DTaP at 2, 4 and 6 months of age in the USA (CDC, 2013) and 6, 10 and 14 weeks in South Africa (Department of Health, 2009). DTaP coverage worldwide is estimated at 83% and the vaccine prevents 700 00 deaths of children under five annually (UNICEF, 2012). TT induces a Th2 response and antibodies titers are good correlates protection for the vaccine (Plotkin, 2008). The vaccine is often administered with adjuvants such as aluminium hydroxide to enhance T cell-dependent antibody responses (Gupta, 1998). Neonatal tetanus can also arise in rural areas when deliveries are carried out without sterile procedures. However, neonates can be protected from tetanus by the TT antibodies they receive from their vaccinated mothers via the placenta (Gall et al., 2011).

1.4.1.1.2. Pertussis vaccines.

Pertussis is a contagious respiratory disease caused by the bacterium *Bordetella pertussis* (BP). These bacteria release toxins which damage and inflame the respiratory system (CDC, 2013). The disease is also referred to as whooping cough due to the severe spasmic coughing that it causes (CDC, 2013). Pertussis mostly affects children and is very lethal in neonates who can be contaminated by parents or caregivers (Wendelboe et al., 2007). 360 000 deaths per year in

children under five are due to whooping cough worldwide (UNICEF, 2012). Immunization is the best prevention against pertussis, especially in children. Similar to tetanus vaccines, pertussis vaccines are given in combination with diphtheria and Tetanus vaccines in DTaP in children and Tdap in adults (CDC, 2013). As described above for tetanus, 3 doses of the vaccines are administered during the first year of life (CDC, 2013). However, unlike tetanus, two different pertussis vaccines have been developed to this day (WHO, 2010).

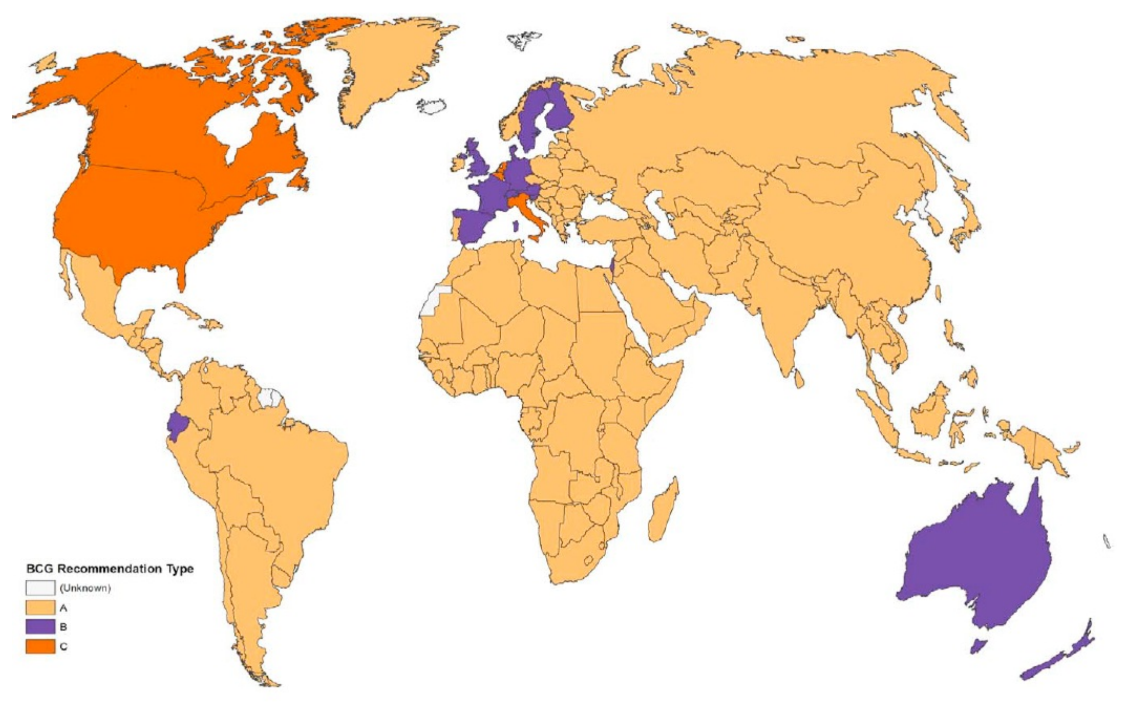
The whole cell pertussis vaccine (wP) is a suspension of whole *Bordetella pertussis* bacteria that have been inactivated and have been implemented in WHO expanded immunization program since for routine vaccines since the 1940s (Crowcroft et al., 2006). It has been associated with some mild adverse effects such as swelling, pain at the site of infection, drowsiness (Crowcroft et al., 2006; Long et al., 1990). In addition, the vaccine can increase the risk of seizures in children (Cody et al., 1981; Crowcroft et al., 2006). In order to address these safety concerns, an acelluar pertussis vaccine (aP) was developed.

The acellular pertussis vaccine (aP) is made up of one or many purified components of *Bordetella pertussis* such as inactivated pertusiss toxin or filamentous heamagglutinin (Crowcroft et al., 2006). There are different types on aP vaccines depending on the number of purified components present in the vaccine. Several studies confirmed that adverse events were less frequent after administration of aP vaccines than with wP (Cody et al., 1981; Wintermeyer et al., 1994). Studies have shown that aP and wP induce different T cell subtypes in children(Higgins, Jarnicki, Lavelle, & Mills, 2006; Ryan et al., 1998) Ryan et al. demonstrated that immunization of children with wP vaccines leads to secretion of IFN-γ by T cells whereas T cells from children immunized with aP vaccines secreted IFN-γ and/or IL-5 (Higgins et al., 2006; Ryan et al., 1998). This suggests Wp vaccine-induced immunity is mediated by Th1 cells while aP vaccine-induced immunity involves Th1 and Th2 cells (Ross et al., 2013; Ryan et al., 1998). In addition, it has also been revealed that Th17 cells may play important role in protection following immunization of children with wP vaccine

(Higgins et al., 2006; Ross et al., 2013). While Th2 responses may be important for the induction of antibodies in the respiratory tract, many studies suggest that Th1 cells are more important for long term protection against pertussis and its spread throughout the body (Mills et al.,.1993; Mills et al., 2001; Warfel et al., 2013). Warfel et al. recently demonstrated that aP vaccines protect against the disease but failed to prevent transmission of *Bordetella pertussis* in non-primate models (Warfel et al., 2013). Thus, despite the adverse effects associated with it, wP vaccine induces more optimal T cells responses against efficient against pertussis than aP vaccines (Warfel et al., 2013) Ever since their implementation in different immunization programs worldwide, aP and wP vaccines have played considerable role in pertussis control. They prevent 600 000 thousands deaths every year (UNICEF, 2012).

1.5. BCG vaccination

Tuberculosis is caused by *Mycobacterium tuberculosis,* a slowly growing bacterium which attacks the lungs and other parts of the body (WHO report on TB, 2013). The disease spreads through the air and can be lethal if not treated properly (WHO report on TB, 2013). Tuberculosis (TB) constitutes a major public health problem affecting mostly Sub-Saharan Africa and Asia. According to the World Health Organization (WHO) report on Tuberculosis 2013, there were 9 million new TB cases in 2012 and 1.4 million of TB-related deaths (WHO report on TB, 2013). Bacille Calmette-Guerin (BCG) is currently the only vaccine used for TB prevention (WHO report on TB, 2013). It is a live attenuated *Mycobacterium bovis* strain that is administered to almost 100 million children each year (Hesseling et al., 2006). Figure 1.5 is a map of the global BCG coverage.



BCG Recommendation Type	
Unknown	
A	
B	
C	

Figure 1.5: Global coverage of BCG vaccination. A: Countries with universal BCG vaccination programs. B: Countries that no longer recommend BCG vaccination for everyone. C: Countries that never had universal BCG vaccination programs (Zwerling et al., 2011).

1.5.1. Effects of BCG vaccination in neonates

In TB-endemic regions, BCG is generally given at birth. It induces a strong Th1 response even in neonates, who are notorious for their Th2 biased immune system (Marchant et al., 1999).In addition, because the neonatal immune system is still immature at birth, BCG efficacy may increase with vaccination age in infants (Hussey et al 2012). In fact, Kagina et al showed in a longitudinal study that delaying BCG vaccination from birth to 10 weeks resulted in enhanced memory CD4 T-cells in South African HIV-unexposed infants (Kagina et al.,

2009). On the other hand, two studies carried out in the Gambia revealed that delaying BCG vaccination until 2 and 4 months led to a decrease in Th1 responses (Burl et al., 2010; Marchant et al., 1999). Therefore, while evidence suggests that the timing of vaccination may have some effects on BCG efficacy and immunogenicity in infants, it is not clear what these effects are.

1.5.2. Effects of BCG vaccine on other vaccines and all-cause mortality

BCG is cost effective and particularly efficacious against severe forms of childhood TB such as TB meningitis and military TB in immunocompetent infants (Trunz et al., 2006).BCG may also improve immune responses to other unrelated vaccines and antigens (Ota et al., 2002). Ota et al showed that BCG vaccination can boost antibody responses to OPV and HBV and increase the production of both Th1 and Th2 in response to Tetanus toxoid (TT) and Hepatitis B vaccines in HIV-uninfected Gambian infants (Ota et al., 2002).However, these effects depend mostly on the timing of the vaccination and the age of the infant (Ota et al., 2002). Thus, BCG may improve infants' protection from other infections.

In fact, some studies also show that BCG can reduce all-cause mortality in infants (Aaby et al., 2011; Garly et al., 2003; Roth et al., 2006). In a randomized, controlled trial assessing the effects of BCG vaccination in Low birth weight children in Guinea-Bissau, Roth et al showed that infants with a BCG scar had better survival (Roth et al., 2004).Moulton et al also demonstrated that BCG was associated with a significant reduction in mortality hazards in Indian infants (Moulton et al., 2005). However, other studies have shown the opposite and the overall non-specific effects of BCG vaccination on all cause mortality are subject to debate and may depend on other factors such as environment and the implementation of other health interventions such vitamin A administration (Benn et al., 2008; Roth et al., 2010). However, many of these studies did not take place in high HIV-prevalent populations.

1.5.3. BCG and HIV

As shown in figure 1.6, many TB-endemic endemic regions such as South Africa are also affected by HIV. Hesseling et al recently reported a TB incidence rate of 1596 cases per 100 000 population among HIV –infected infants versus 65.9 cases per 100,000 population among HIV-uninfected infants in South Africa (Hesseling et al., 2009).Thus, many studies have investigated the immunogenicity of BCG in HIV-infected individuals. There is very little data on the efficacy on BCG in HIV-infected infants (Bhat et al.,1993; Fallo et al., 2005). A Study carried out in Zambia showed that HIV-infected infants were more likely to be tuberculin non-reactive and unable to develop a scar following BCG vaccination compared to HIV-uninfected infants (Bhat et al., 1993).Mansoor et al recently showed that HIV infections greatly impairs T cell responses to BCG in infants in their first year of life (Mansoor et al., 2009). In addition, BCG has also been associated with adverse effects such as such as immune reconstitution inflammatory syndrome (IRIS) and disseminated BCG disease (Hesseling et al., 2009). IRIS is caused by the recovery of pathogen-specific T-cell responses and disturbances of innate immune responses, which subsequently affect the tissues infected by the pathogen (French, 2012). BCG IRIS is very common among South African HIV-infected infants and can result in high morbidity (Rabie et al., 2011). Rabie et al showed that low CD4 counts and high viral load at antiretroviral therapy (ART) initiation are strong risk factors for BCG-IRIS in South African infants (Rabie et al., 2011).

In addition, T cell activation induced by early BCG vaccination of HIV-infected could theoretically lead to more rapid disease progression (Jaspan, AIDS 2012). Hesseling et al recently documented a considerable risk of disseminated BCG disease of approximately 1% in HIV-infected infants, associated with high mortality (Hesseling et al., 2009). Based partly on these data, the World Health Organization (WHO) changed their recommendations on the routine vaccination of HIV-infected infants, now making infant HIV infection, if known, a contraindication to BCG vaccination (WHO SAGE, 2007). However, since HIV status is not known at birth when BCG is administered, routine BCG vaccination

continues in most TB endemic countries including South Africa where nearly 260 00 neonates are born to HIV-infected mothers (Hesseling et al., 2006).Not only is there no evidence of any protective effect of the BCG vaccine in HIV-infected infants but very little is known about BCG immunogenicity and efficacy in HIV-exposed, uninfected infants (infants born to HIV-infected mothers but who are uninfected) (Hesseling et al., 2006). Recent evidence suggests HIV-exposed infants produce less IFN-Y and TNF-α in response to BCG (Mazzola et al.,.2011; Van Rie et al., 2006) Hence, to inform future policy, there is an urgent need to investigate the effects of delayed BCG vaccination in HIV –exposed infants. Since there is considerable risk associated with BCG vaccination at birth in HIV-exposed infants, it is important to assess how immune responses to BCG are affected when BCG vaccination is delayed in HIV-exposed infants.

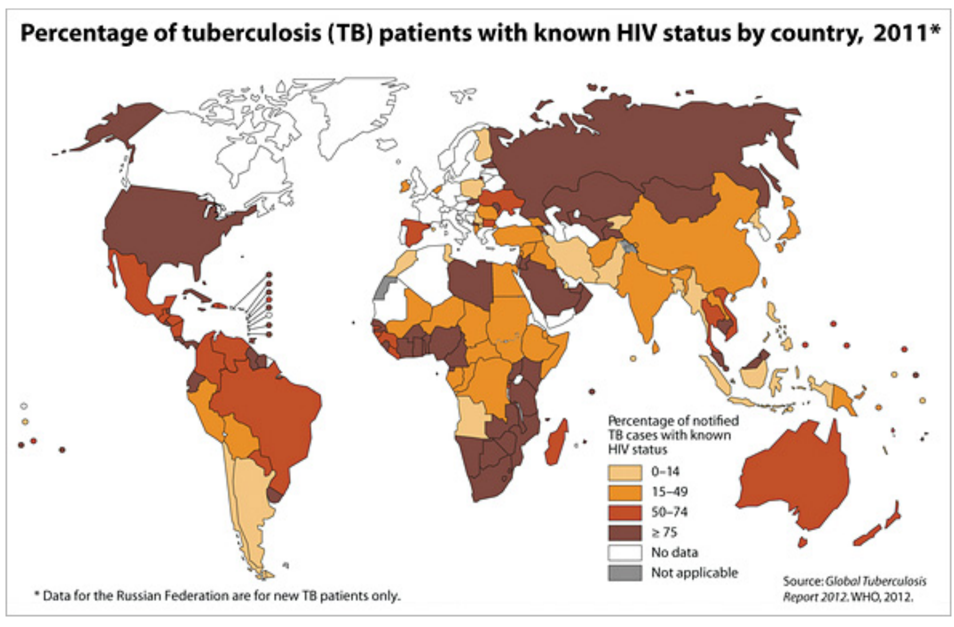


Figure 1.6: Prevalence rates of new TB cases in HIV infected individuals in 2011 (WHO Global TB report, 2012)

1.6. HIV prevalence and influence on the immune system

HIV remains a major public health problem with 2.7 million newly infected people in 2010 (WHO, 2011). Sub-Saharan Africa is particularly affected by the virus with almost 300 000 newly infected children in 2009 (UNAIDS, 2010).

Paediatric infections can occur vertically from mother to child *in utero*, perinatally or postpartum (from breastfeeding) (Newell et al., 2004; Thorne et al., 2004). The risk of pediatric infections can be considerably reduced with antiretroviral drugs (Newell et al., 2004). The improvement of mother to child transmission prevention has led to increased numbers of HIV-exposed, uninfected infants in Sub-Saharan Africa (Filteau, 2009). These are infants that are born to HIV-infected mothers but are not HIV-infected themselves. In sub-Saharan Africa, approximately 20% of infants are HIV-exposed and uninfected (Filteau, 2009). Hence, it is critical to know how HIV-exposure affects the health of these infants and their immune responses to other pathogens. Despite the limited data availability on HIV exposed uninfected infants, it is well documented that these infants are characterized by higher mortality than HIV-unexposed uninfected infants (Filteau, 2009; L. Kuhn et al., 2005).

Recent studies have shown that mortality in HIV-exposed uninfected infants was associated with risk factors such as low birth weight, maternal CD4 counts and gestational age (Filteau, 2009; Wei et al.,2004). HIV-exposed uninfected infant morbidity is also higher than HIV-unexposed infants (Filteau, 2009; L. Kuhn et al., 2005). In a study carried out in South Africa, HIV-exposed infants were found more susceptible to Pneumonia than HIV-unexposed infants, including *pneumocystis jiroveci*, an opportunistic pathogen (McNally et al., 2007). In addition, HIV-exposed uninfected infants are also more susceptible to other vaccine preventable diseases (Kuhn et al., 2005). There are many factors that could explain the increased susceptibility to diseases in HIV-exposed infants. It is probable that HIV exposure may influence the infant immune system and hence immune responses to some vaccines (Kuhn et al., 2005).

HIV-exposed infants generally have a large number of activated and memory T-cells (Clerici et al., 2000). Jones et al demonstrated that HIV-exposed infants have low levels of specific antibodies at birth, but a strong humoral response after vaccination (Jones et al., 2011). Some studies have also reported impairment in cytokine production in HIV-exposed uninfected infants in general

(Chougnet et al., 2000; Rich et al.,1997). These immunological anomalies may be due to infant exposure to HIV *in utero* and maternal placental immune responses (Clerici et al., 2000; Kuhn et al., 2002). Nonetheless, it is still unclear how these differences affect immune responses to other vaccines, and whether these changes are responsible for the vulnerability of HIV-exposed infants (Filteau, 2009).Hence, a better understanding of the immune system of HIV-exposed infants is critical to reduce their mortality and to inform new prevention strategies against tuberculosis and other infectious diseases.

1.7. Assays used to measure T cell vaccine responses in infants

In this section, the different assays used to measure vaccine responses in infants are discussed. Table 1.4 below summarizes the main studies that have assessed cellular immune responses to BCG in HIV-exposed infants and the types of assays they have used.

Table 1.4: Studies assessing immune responses to BCG in HIV-exposed infants

Authors and year	Study design	Infant age	Assay	Immune responses measured
(Van Rie et al., 2006)	Longitudinal	Birth-week 6	WBA (7 days)	IFN-γ production
(Mansoor et al., 2009)	Longitudinal	3-9 months	WBA (12 h)	T cell proliferation/ IFN-γ, IL-2 and TNF-α production
(Mazzola et al., 2011)	Cross sectional	6-26 months	PBMC	T cell proliferation/ IFN-γ, TNF-α production
(Borges-Almeida et al., 2011)	Cross-sectional	Cord blood	CBMC	T cell proliferation/ IFN-γ, IL-2, IL-4, IL-12, IL-7 and TNF-α production

1.7.1. Whole blood assay (WBA) and its importance

In a WBA, whole blood is stimulated with optimized antigen concentrations and incubated at 37°C as soon as possible after collection. There are long-term and short-term WBAs, ranging from 12 hours to weeks (Hanekom et al., 2004). Hanekom et al showed that in short-term assays, a delay in incubation was associated with a reduction in specific responses (Hanekom et al., 2004). One of the major advantages of the WBA is the smaller blood volume it requires (200-300 µl) compared to peripheral blood mononuclear cell (PBMC)-based assays (Hanekom et al., 2004). This is particularly important in paediatric studies in Tb-endemic countries where blood volume is often a limiting factor (Deenadayalan et al., 2013). Another advantage, particularly with assays of infant cells, is that no additional serum is needed for whole blood. The optimal antigen concentrations used in the WBA usually differ from those used in PBMC-based assays. Many types of antigens such as whole bacterial antigens, proteins and peptides antigens have been tested successfully in the WBA (Hanekom et al., 2004; Remick et al., 2000).

1.7.2. Proliferation and Intracellular cytokine staining assay (ICS)

Proliferation is the expansion of cells by the division of single cells into two daughter cells. T cell proliferation generally occurs before their differentiation into different effectors (Kaech et al., 2001). As depicted in table 1.5, there are many methods available to measure conventional T-cell proliferation and activation in response to antigen stimulation. Each of these methods has its advantages and disadvantages. All these methods are accurate and sensitive but except for Ki67 staining, they all require large blood volumes. In addition, strong correlation between Ki67 staining and CFSE staining have been shown (Soares et al., 2010).

.

Table 1.5: Different methods used to measure antigen-specific responses

Method	Immune response measured	Advantages	Disadvantages
^{3}H-thymidine incorporation (Soares et al., 2010)	T cell proliferation	Used with peripheral blood mononuclear cells (PBMC)	Cannot use flow cytometry Large blood volumes required
Oregon Green incorporation (Soares et al., 2010)	-T cell proliferation -Identification of cellular sub-populations	-Flow cytometry -Co-staining with other markers -Used with (PBMC)	Large blood volumes required Cannot use whole blood
Carboxyfluorescein Succinimidyl Ester (CFSE) staining (Soares et al., 2010)	-T cell proliferation -Identification of cellular sub-populations	-Flow cytometry -Co-staining with other markers -Used with PBMC	Large blood volumes are required. -Cannot use whole blood
5-bromo-2'-deoxyuridine (BrdU) (Soares et al. 2010)	-T cell proliferation -Identification of cellular sub-populations	Flow cytometry -Co-staining with other markers -Used with PBMC	Large blood Volumes are required Cannot use whole blood
Ki-67 staining (Soares et al., 2010) (Soares et al., 2013) (Hanekom et al., 2004)	-T cell proliferation -Identification of cellular sub-populations	-Flow cytometry -Co-staining with other markers -Used with PBMC -Use whole blood -sensitive. -Small blood volumes required	Does not help determine the number of proliferation cycles Low detection of Ki67+ in adults

1.8. Aims and objectives of the study

The aim of dissertation was to assess how delaying BCG vaccination from birth to 8 weeks affects immune responses to BCG, other vaccines, and superantigen in HIV-exposed infants. In order to achieve this, there are two specific aims:

1) To compare CD4+ and CD8+ T-cell proliferative responses and intracellular cytokine responses to BCG at 6, 8 and 14 weeks between infants who received BCG at birth and those who received it at 8 weeks of age. Our hypothesis is that delaying BCG vaccination from birth to 8 weeks results in an altered proliferative and cytokine CD4 T-cell response to BCG at 14 weeks of age

2) To compare CD4+ and CD8+ T-cell proliferative responses and intracellular cytokine responses to Pertussis, Tetanus (TT), and SEB at 6, 8 and 14 weeks between infants who received BCG at birth and those who received it at 8 weeks of age. The hypothesis is that delaying BCG vaccination from birth to 8 weeks results in an altered proliferative and Th2 or Tc2 skewed cytokine T-cell response to other antigens.

1.8.1. Rationale

There are few studies assessing how delayed BCG vaccination (from birth to 8 weeks) affects cellular responses to BCG in HIV-exposed infants, a highly relevant group for such a vaccine strategy. In addition, there are no studies assessing how delayed BCG vaccination affects cytokine and proliferative T-cell responses to tetanus and pertussis vaccination in HIV-exposed infants. Since there is a considerable risk associated with BCG vaccination in HIV-exposed infants at birth, understanding how delayed BCG vaccination would affect immune responses to EPI vaccines in HIV-exposed infants will help inform policies on the appropriate timing for BCG vaccination for these infants, and help in the implementation of new prevention strategies against tuberculosis and other infectious diseases in HIV–exposed infants.

Chapter 2. Cohort Characteristics, Materials and Methods

2.1. Cohort characteristics and recruitment

2.1.1. Study design

This thesis describes a substudy of a longitudinal, randomized controlled trial of BCG vaccination at birth versus at 8 weeks of age in HIV-exposed infants. The primary outcome measure for this substudy was the difference in CD4+ proliferation in response to BCG between the early and delayed arms at 6 weeks of age (baseline) and at 14 weeks of age. The sample size and power calculations were based on the comparison of the median number of CD4+ki67+ cells per µl in HIV-unexposed infants in response to TT at 14 weeks of age calculated using previously published data (Kidzeru et al 2014). This sample size (n = 28) allowed 80% power to detect a 5% difference in the frequency of CD4+ki67+ cells per µl (2-tailed test at P=0.05, assuming a 5% loss-to-follow up)

2.1.1.1. Recruitment of participants

HIV-exposed infants were recruited from the Midwife Obstetric Unit at Khayelitsha Site B, Western Cape, South Africa. Consent forms were provided in English, Xhosa and Afrikaans and the consent process was conducted in the preferred language of the participant according to Good Clinical Practice guidelines.

2.1.1.2. Participants and blood collection

Infants were recruited within 24 hours of delivery and randomized to receive BCG vaccine at birth (early arm) versus BCG vaccine at 8 weeks of age (delayed arm). Infants were enrolled at birth, and follow-up visits were conducted at 6, 8, and 14 weeks from February 2010 to June 2012. Infants were HIV DNA PCR

tested at birth and if negative; were eligible for randomization. For this study, a total of 91 infants were selected from the 122 that completed the study: 41 in the delayed arm and 50 in the early arm. Infants in the early arm received BCG at days 2-3, when the results of the HIV DNA PCR were available. The delayed arm received their BCG vaccination at week 8, after the results of the standard of care HIV DNA PCR at 6 weeks were available.

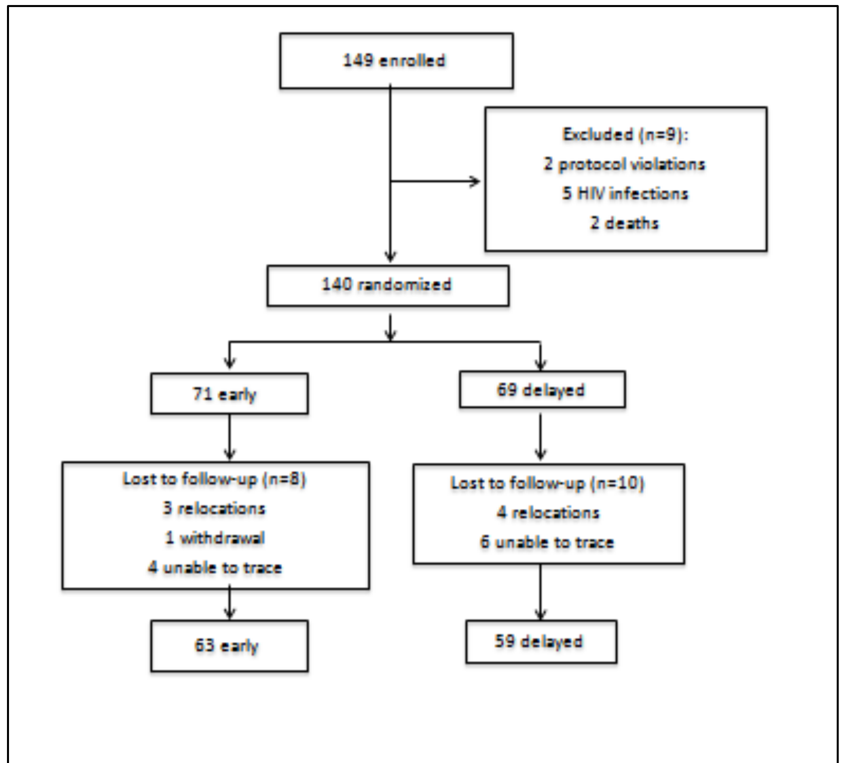


Figure 2.1: Study design.

2.1.1.3. Study Setting

Khayelitsha is an informal settlement with an antenatal HIV prevalence of 30.1% in 2011 (City of Cape Town Department of health, 2011). Site B has established pMTCT and pediatric ARV clinics. The Western Cape has one of the highest TB rates globally, with an infant TB rate estimated (culture-confirmed) at 1596 cases per 100,000 population among HIV-infected infants (95% CI, 1151-2132) and 66 cases per 100,000 population among HIV-uninfected infants (95% CI, 56-75) (Madhi et al., 2011). infants receive three doses of Diphtheria-Tetanus-aPertussis (DPT)-iPV-Hib, Hepatitis B, Rotavirus (RV), and pneumococcal

conjugate (PCV7) vaccinations at 6, 10 and 14 weeks of age (Department of Health, 2009) (Table 1.3 in chapter 1).

Exclusion criteria:

Infants were excluded if they met the following criteria:

1. Mother was not willing and able to give consent
2. Baby weighed less than 2.4kg at birth
3. Mother had cough for more than two weeks, fevers or weight loss, for which she was then refered to the TB Clinic
4. Baby unhealthy: sepsis/ convulsions/ asphyxia/ severe respiratory distress/ severe congenital abnormality
5. Mothers HIV test in pregnancy was negative
6. Mother or infant were planning to move away from Khayelitsha in the following 4 months
7. Prematurity (i.e. <36 weeks)
8. Mother was younger than 18 years
9. Infant tested HIV DNA PCR positive (for which immediate referral to the ARV clinic occurred.)

HIV testing

Blood collection for HIV DNA PCR testing was done at birth through the study and six weeks of age through the routine pMTCT programme. Infants were excluded if they tested positive for HIV infection.

2.1.2 Characteristics of the cohort

We compared the characteristics of the two groups. As shown in table 2.1, there were no significant differences in baseline characteristics between the early and delayed arms, including birth weight (3.12 kg vs. 3.05 kg p=0.407, maternal CD4 counts (388 cells/mm^3 vs. 358 cells/mm^3 p=0.107), breastfeeding (17% vs. 10% p=0.469), sex (48% versus 60%; p=0.140), and median gestational age (39 vs. 39 weeks p= 0.653).

Table 2.1: Baseline characteristics of the cohort.

Characteristic	Early (n=63)	Delayed (n=59)	P value
Median age of BCG vaccination (days) (IQR)	3 (2-4)	58 (56-62)	<0.001
Median maternal CD4 count, cells/mm^3 (IQR)	388 (276-477)	358 (254-548)	0.103
Mean birth weight, kg (s.d.)	3.12 (0.39)	3.05 (0.41)	0.407
Mean gestational age (weeks)	39	39	0.653
Male gender n (%)	24(48)	24 (60)	0.140
Breastfed (%)	17	10	0.467

2.2. Laboratory materials and methods

The main objective of this dissertation was to assess cellular immune responses to EPI vaccines as well as SEB in early (birth) versus delayed (8 weeks) vaccinated infants. To this end, we utilized several techniques and the overall process involved two main assays:

2.2.1. Whole blood assay (WBA).

The whole blood assay used was adapted from that previously described by Soares et al. Between 1-3mL of whole blood was collected from infants at 6, 8 and 14 weeks of age into a preservative-free heparinised tube and transported to the laboratory within 6 hours of blood draw. Whole blood was mixed in a 1:10 dilution with warm Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma Aldrich) without additives or antibiotics, and 1200ul/well was placed into a 24 well culture plate and incubated at 37°C with 5% CO2 with 1x105 cfu/ml Danish BCG and a negative control (media alone). The following antigens were added:.1x105 cfu/mL Mycobacterium bovis Bacille Calmette-Guérin (BCG).[Danish strain 1331;.SSI],.0.16.IU Tetanus toxoid (TT).[TETAVAX, Aventis Pharma (Pty) Ltd],.0.16.IU whole cell inactivated Pertussis antigen

(PERTUSSIS).[DifcoTM Bordetella Pertussis Antigen, BD] and a negative control (medium alone). The different antigens and vaccines were diluted to the specific concentration using warm RPMI 1640 culture medium (Sigma Aldrich) without additives or antibiotics.

After 24 hours, 100ul of supernatant was removed and 1 ug/ml Staphylococcal enterotoxin B (SEB) was added to its required positive control well and cells were placed back at 37°C for a further 5 days. On the 6th day, 1ug/ml of phorbol 12-myristate 13-acetate (PMA) and 5ug/ml Ionomycin was added to stimulate intracellular production of cytokines by cells non-specifically (Hanekom et al., 2004; Soares et al., 2013; Soares et al., 2010),along with 5ug/ml Brefeldin A, to prevent extracellular cytokine secretion, for the last 4 hours of stimulation. Negative control cells were stimulated in the same manner with media alone on day 0 and with Ionomycin and PMA on day 6. Cells were harvested in 20 Mm EDTA to dissociate them from the plate. Red blood cells were lysed and remaining white cells were stained with Pacific Blue Live/Dead stain [violet viability dye (VIVID)], fixed with FACS Lysing Solution (BD) and cryopreserved in a 10% dimethyl sulfoxide (DMSO) freezing solution for storage at -80°C until analysis.

2.2.2. Ki67 Proliferation and Intracellular cytokine staining assay (ICS)

The frozen fixed cells were then thawed and stained with a Ki67 Proliferation and Intracellular cytokine staining (ICS) panel. Ki67 was used to measure T cell proliferative responses to antigen stimulation. Ki67 is a human protein that is strictly associated with cell proliferation. Ki67 protein is present during all the division stages of the cell and absent in resting. This characteristic makes it an excellent marker for cell growth and proliferation (Scholzen et al., 2000). In addition Ki67 can be co-stained with other cellular markers, is highly sensitive and requires only small blood volumes (Soares et al 2010). Thereafter, ICS was used to quantify the proportions of cytokines expressed by these proliferating T cells. Fixed frozen cryopreserved cells were thawed rapidly (< 1 minute) in a

37°C water bath, permeabilized in 100 ul of Cytofix/Cytoperm™ solution (BD) and stained for cellular markers (CD3 and CD8), intracellular cytokines (ICS): INF-γ, IL-2, IL-13 and IL-17 and as well as for proliferation marker Ki67 (Soares et al., 2010) with optimized volumes of fluorescence-conjugated antibodies (Soares et al., 2013)(table 2.2). Samples were acquired on a BD LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA).

Table 2.2: Flow cytometry panel: 8-colour flow cytometry panel used with the different fluorochromes, markers and their functions in the immune system

FLUOROCHROME	MARKER	MARKER'S FUNCTION	CLONE	SUPPLIER
Pacific blue	**VIVID**	Live/Dead cell marker		Invitrogen
APC-Cy7	**CD3**	T-Lymphocytes marker	UCHT1	Biolegend
PerCP-Cy5.5.	**CD8**	Cytotoxic lymphocytes, kill infected cells	SK1	BD Biosciences
FITC.	**Ki67**	Marker of T cell proliferation	B56	BD Biosciences
AlexaFluor-700.	**IFN-γ.**	Th1 cytokine important for the control of intracellular pathogens	B27	Biolegend.
APC.	**IL-2**	Th1 cytokine important for the growth of T-cells	5344.111.	BD Biosciences.
PE	**IL-13.**	Th2 cytokine important for the control of extracellular pathogens and antibody production by B cells	JES10-5A2.	BD Biosciences
PE-Cy7.	**IL-17**	Th17 cytokine that helps in the recruitment of Th1 cells and neutrophils.	BL168	Biolegend

2.2.3. Flow cytometry data analysis

Following acquisition, colour compensation was performed using FlowJo v9.4 (Treestar, Ashland OR USA) to account for the spectral overlap, and perform a subtraction of the spillover of one fluorochrome from another. After gating for

singlets (single cells that are not aggregated with others), live CD3+ cells, CD8+ cells and CD8- cells were gated (considered as CD4+ cells as PMA-Ionomycin lead to CD4 down-regulation in T cells) (Rueggs et al., 1992)(Figure 2.2). Furthermore, Ki67+ proliferating cells were gated on CD8- (CD4+) and CD8+ populations (Figure 2.2). We then assessed IFN-γ and IL-2 (Th1/ Tc1), IL-17 (Th17, Tc17) and IL-13 (Th2/ Tc2) expression by gating these cytokines within CD8- Ki67+. (CD4+ Ki67+) and CD8+Ki67+ cells (Figure 2.3). Boolean gating was used to determine the proportion of CD4+ and CD8+ cells expressing none, a single or a combination of multiple cytokines. Boolean gates reflect any changes to the gates on which they depend. Figures 2.2 and 2.3 below describe how the gates were established, from the time gates for including consistent fluorescence over time and exclusion of doublets using forward scatter-area and forward scatter-height parameters in a singlets gate; to exclusion of dead cells [violet viability dye positive cells (VIVID+)] gating live CD3+ cells and gating CD8- (CD4+) and CD8+ T cells and finally Ki67-incorporated T cells.

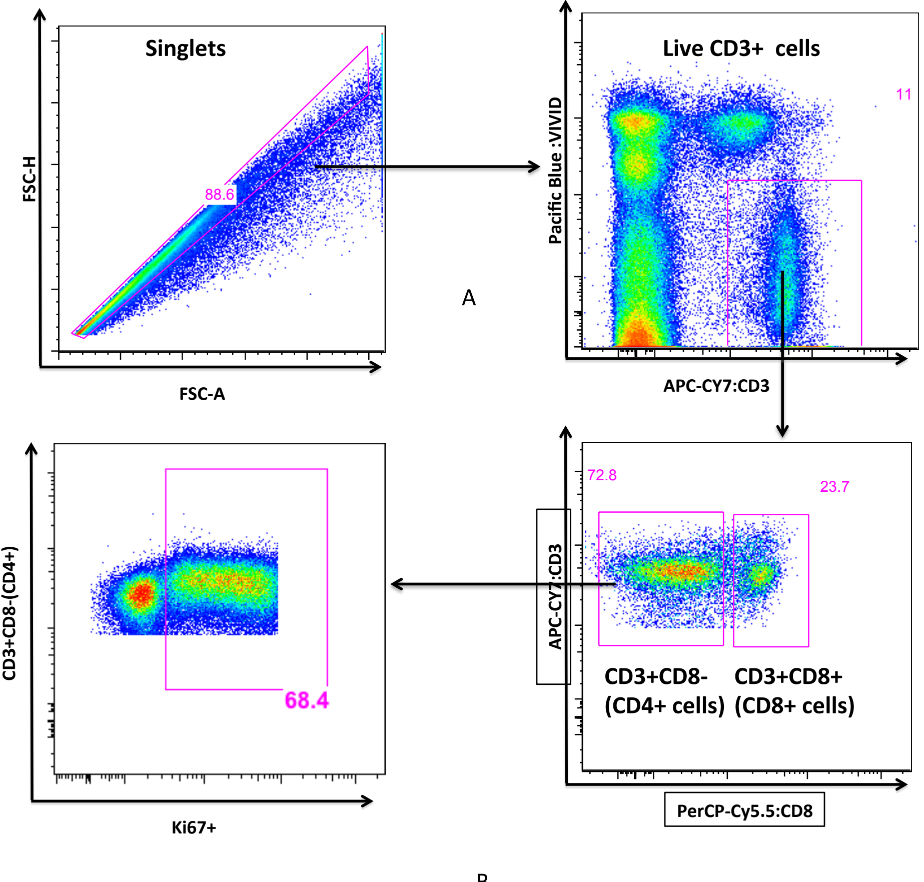


Figure 2.2a: Gating strategy, a representative flow cytometry plot of a response to Staphylococcal enterotoxin B.[SEB] (Positive control): After gating on singlets, live CD3+ cells, CD3+CD8- (CD4+) (A), CD3+CD8+(CD8+) T cells were gated on, and consequently Ki67+ proliferating T cells (CD4+Ki67+) (B). The values represent the percentage of T cells that fall within the gates in this representative experiment.

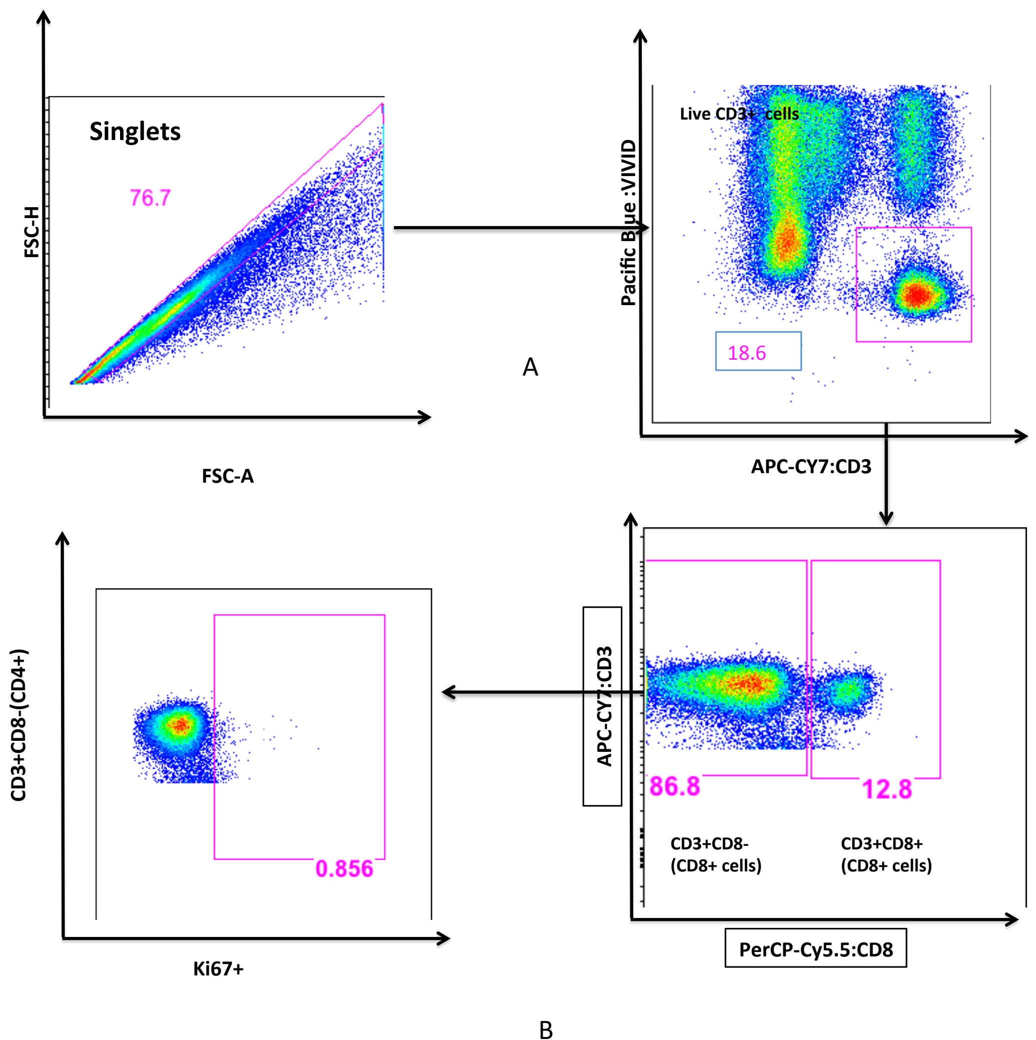


Figure 2.2b: Gating strategy, a representative flow cytometry plot of a response to negative control (medium only): After gating on singlets, live CD3+ cells, CD3+CD8-(CD4+) (A), CD3+CD8+(CD8+) T cells were gated on, and consequently Ki67+ proliferating T cells (CD4+Ki67+) (B). The values represent the percentage of T cells that fall within the gates in this representative experiment.

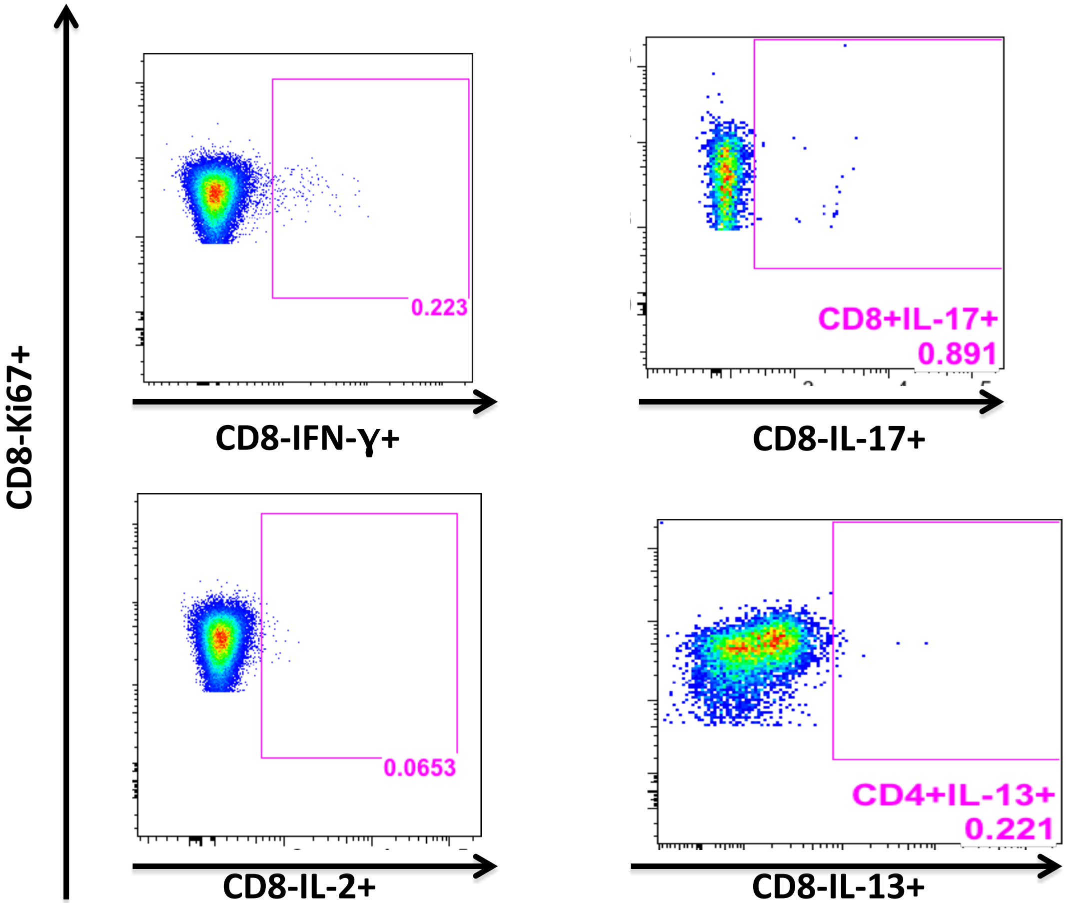


Figure 2.3a: Gating of CD8-Ki67+(CD4+Ki67+) cytokine producing T cells. A representative flow cytometry plot of CD4+Ki67+ proliferating cells expressing intracellular cytokines; IFN-Y, IL-2 (Th1), IL-17 (Th17) and IL-13 (Th2) in response to Staphylococcal enterotoxin B [SEB] (positive control). Similar gates were laced for CD8+ proliferating cells expressing cytokines.

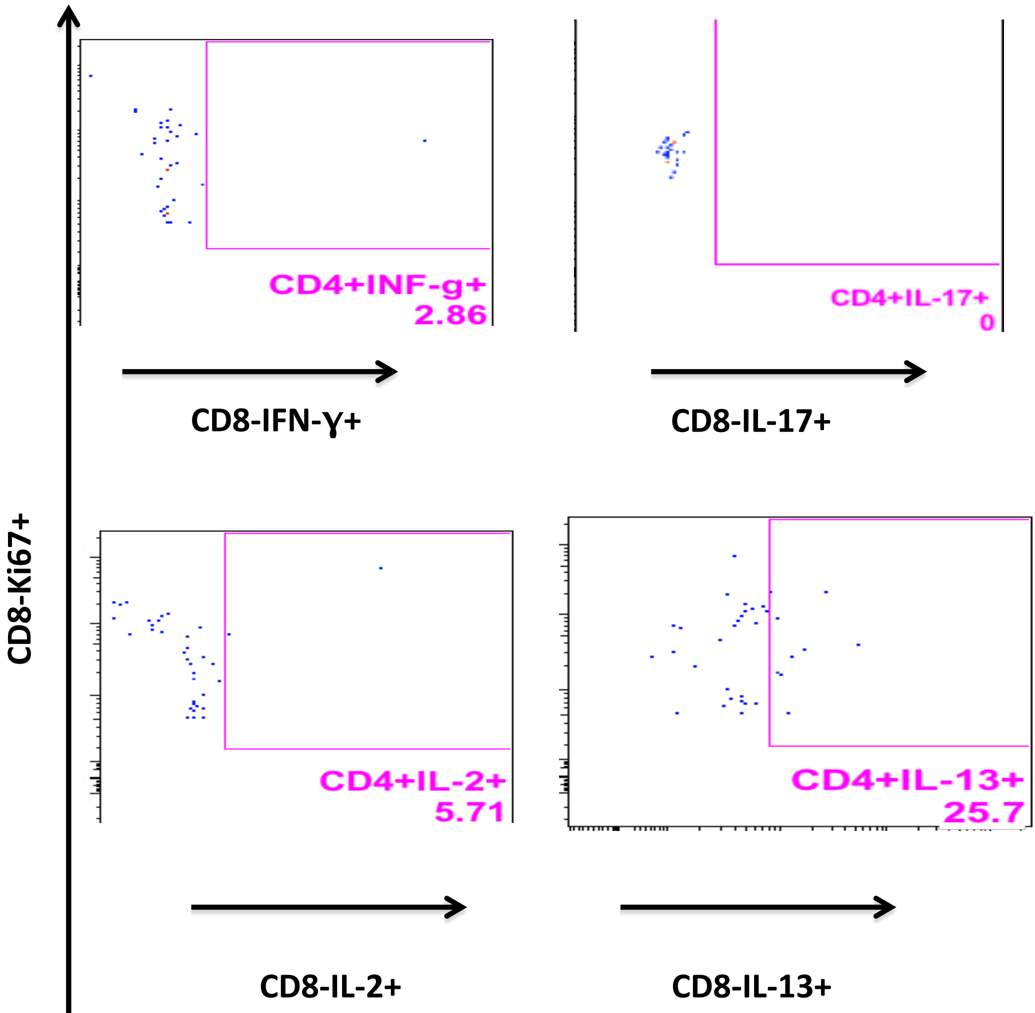


Figure 2.3b: Gating of CD8-Ki67+(CD4+Ki67+) cytokine producing T cells. A representative flow cytometry plot of CD4+Ki67+ proliferating cells expressing intracellular cytokines; IFN-Y, IL-2 (Th1), IL-17 (Th17) and IL-13 (Th2) in response to medium only (negative control). Similar gates were laced for CD8+ proliferating cells expressing cytokines.

2.2.3.1. Pass/ Fail criteria, cut-offs and controls for the flow cytometry data

Following analysis by FlowJo, the data was exported into spice and pestle in order to perform background subtraction. To ensure no bias, we applied the following criteria:

a.) Assay validity and positivity (before background subtraction)

The frequency of proliferating cells in response to SEB stimulation (positive control) had to be greater than the median plus three times the Median Absolute Deviation (MAD) of the negative control (Median alone) as indicated in formula below. If not, the sample was excluded. MAD is an estimator of the median defined as the deviations of each sample negative control frequency from the negative control's median, the MAD is the median of the absolute values of the deviation (Rousseeuwa et al., 1993).

<u>Assay validity formula: Frequency of CD4+ proliferating cells</u>

% Ki67+CD4+ [SEB] > % Ki67+CD4+ (Median + 3MAD) [Media]

b.) Proportion of proliferating cells (Ki67+)

The frequency of CD4+ and CD8+ T cells expressing Ki67 (i.e. CD4+Ki67+ and CD8+Ki67+ T cells) of the test antigens (BCG, pertussis, TT, and SEB) minus background (% of Ki67+CD4+ and CD8+ T cells in the media control)

i.e.: (i) [(BCG, pertussis, TT and SEB induced %Ki67+CD4+ cells) – (Induced %Ki67+CD4+ cells in the media)]

c.) Cut off for cytokine responses For single cytokine analyses data is reported as frequency of grandparent T cell population (as explained in figure 2.4) which

was the frequency of total CD4+ and CD8+ T cells that were proliferating and producing cytokines (the ratio of CD4+Ki67+ and CD8+Ki67+ T cells producing cytokines over the total CD4+ and CD8+ T cell population). For the multiple cytokine analysis, data is reported as the frequency of proliferating T cells producing cytokines and the denominator is proliferating cells only.

i) The test antigens (i.e. BCG, pertussis, TT, and SEB) cytokine responses were only quantified if the event counts of proliferating T cells (i.e. CD4+Ki67+ T cells) were ≥ 20. An average of 200 000 cells were acquired. Using this filter,20 samples out of the 168 analysed were excluded.

ii) In addition, background subtraction of the % of total CD4+ and CD8+ T cell cytokine production in the media from the % of total CD4+ and CD8+ T cell cytokine production due to the specific test antigens i.e. BCG, pertussis, TT, and SEB (Figure 3.3) was performed.

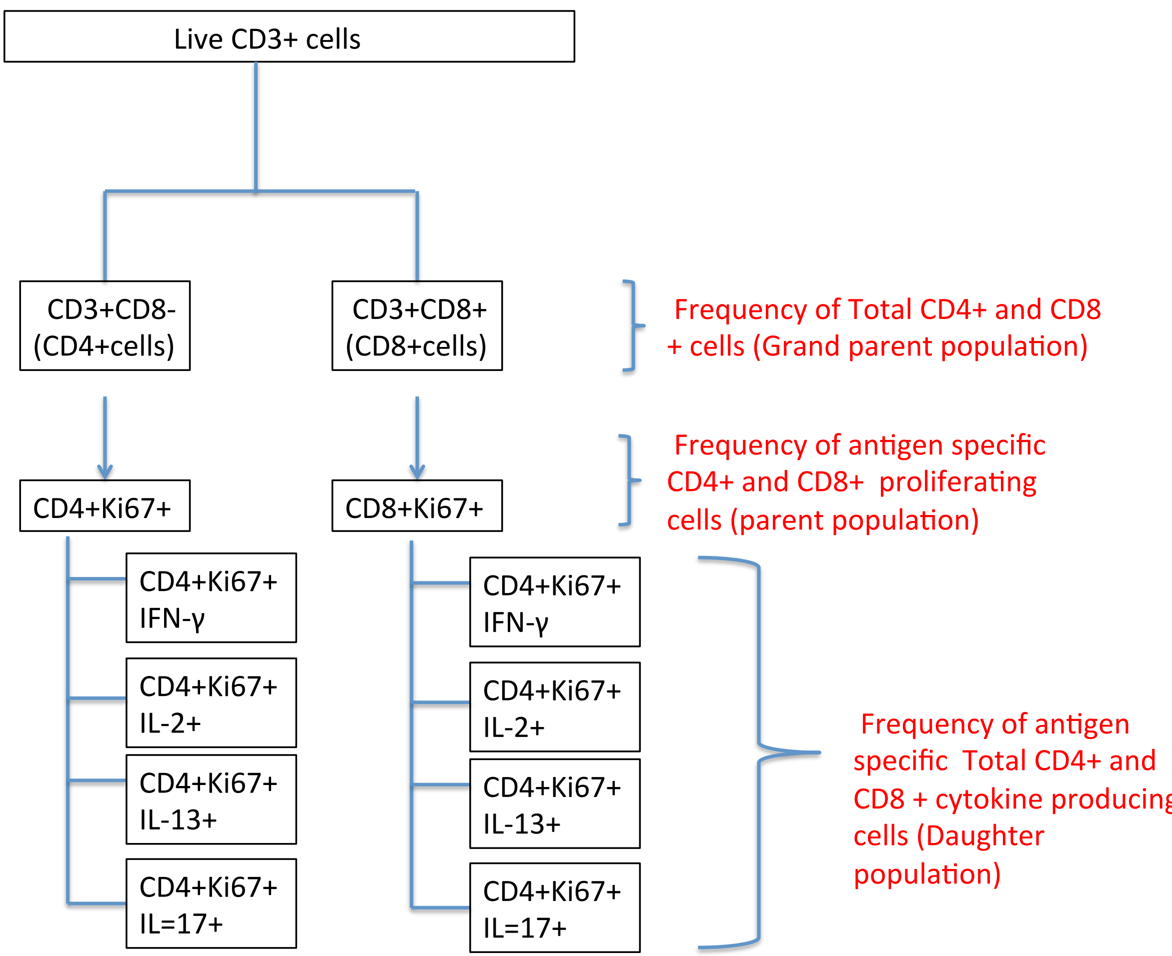


Figure 2.4: Schematic description of the frequencies of total CD4+ and CD8+ cells (grandparent population), antigen specific CD4+ and CD8+ proliferating cells (parent

population) and antigen specific total CD4+ and CD8+ cytokine producing cells (daughter population).

2.2.4. Statistical considerations

Different statistical software was used to perform data analysis after collecting data from flow cytometry. These software and their functions are listed in the table 2.3 below.

Table 2.3: Statistical software used for data analysis

Software Program	Type of analysis
FLOWJO v9.4.7 (for MAC, Stanford University, 1995-96;.TreeStar, Inc. 1997-2012)	Flow cytometric data analysis after acquisition
Microsoft Excel 2007	Data cleaning
Pestle v1.7 (for MAC, Mario Roederer, National Institute of Allergy & Infectious Diseases, 2004-2011)	To perform background subtraction on flow cytometry data and import them into **SPICE**
SPICE v5.22 (for MAC, Mario Roederer-Joshua Nozzi, National Institute of Allergy &Infectious Diseases,2004-2011)	Generate and analyze intracellular cytokine Polyfunctionality data between groups
GraphPad Prism v5 (for Windows, GraphPad Software, San Diego California USA)	To analyze and compare differences in proliferation and absolute cytokine expression by cells between groups
STATA v11 (for windows, StataCorp LP, College station, TX77845, USA)	To perform inferential statistics analyses and evaluate the validity of data

2.2.4.1. Statistical Tests

Many statistical tests were applied to complete the data analysis as listed in table 2.4:

Table 2.4: List of statistical tests applied for data analysis

Statistical Test	Rationale	Type of Data	Software
Shapiro-Wilk test	Confirm normality of. data distribution. If not normally distributed then data was log transformed for normal distribution	-Cohort description - T cell proliferation. -T cell cytokine production -T cell polyfunctional cytokine responses	STATA v11
Wilcoxon rank sum test (Mann-Whitney test)	Compare continuous data between two categories not normally distributed.	T cell proliferation. -T cell cytokine production -T cell polyfunctional cytokine responses (Early arm vs. Delayed arm)	STATA v11 GraphPad Prism v5 SPICE v5.22
ANOVA test	analyze the differences between group means and their associated procedures (such as "variation" among and between groups).	T cell polyfunctional cytokine responses	STATA v11
Spearman correlation	Affect the effects of one outcome on another	T cell proliferation	GraphPad Prism v5

2.3. Discussion

The similar baseline characteristics of the two groups confirmed that due to randomization there were no major differences between the two groups of infants other than the timing at which BCG was administered. Thus, although many factors could influence vaccine responses, these were well balanced between groups. No information on the socioeconomic status of the mothers was collected but we know overall 31.4% of mothers lived in brick structures, 60.8% in shacks and 7.8% lived in another form of habitat not specified. Additionally, viral load was not routinely available for all the mothers. This constitutes one of the limitations of our study. Nonetheless, CD4 counts are good indicators of the maternal health status and how they respond to ARV therapy. Although not without limitations such as the need for non-specific stimulation on Day 6, the assay used in this thesis have been validated previously (Soares et al., 2010) as an excellent method for obtaining large amounts of data from small blood volumes. Ki67+ proliferation is as good if not better than other proliferation markers such as 5-Bromo-2-Deoxyuridine (BrdU) and Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Soares et al., 2010). Ki67 can also be used in conjunction with other markers. Recent vaccine studies have used Ki67 in conjunction with anti-apoptic marker bcl-2 (Soares et al., 2013; Mc Elrath et al., 2008). Bcl-2 has been extensively used in mice studies to assess T cell activation and apoptosis following viral infection (Blattman et al., 2003; Sarkar et al., 2008). However, due to the small blood volumes collected in this study and large number of fluorochromes already being used in our panel, bcl-2 was not used in this panel. Thus, in future studies, it would be very useful to use bcl-2 and Ki-67 markers to assess T cell proliferation and apoptosis following vaccination.

In addition, T cell cytokine expression and polyfunctionality figures show two different types of data. The data reported in T cell cytokine expression figures is dependent on the proliferation response (antigen-specific) T cells reported as a frequency of total CD4+ and CD8+ T cells, whereas T cell polyfunctionality data is not, and is reported as proportions of cytokine producing cells.

Chapter 3. Effects of Delayed BCG Vaccination on Cellular Responses to BCG

3.1. Introduction

Recent studies have shown that delaying BCG vaccination may affect immune responses to BCG in **HIV–unexposed** infants (Burl et al., 2010; Kagina et al., 2009; Lutwama et al., 2013). However, very little is known about the effects of delayed BCG vaccination in **HIV-exposed** infants. In this chapter, we aimed to investigate how delaying BCG vaccination from birth to 8 weeks, when perinatally acquired HIV is ruled out, affects immune responses to BCG in HIV-exposed infants. In order to achieve this, we first compared CD4+ and CD8+ T cell proliferative responses to BCG between birth-vaccinated (early arm) and 8 week-vaccinated (delayed arm) HIV-exposed infants. This was done by measuring the frequency of Ki-67-expressing CD4+and CD8+ T-cells in 6-day whole blood assay cultures described in the methods (see section 2.2.1) with samples collected at six, eight and 14 weeks of age. Furthermore, using ICS, we assessed the effects of delayed BCG vaccination on T cellular cytokine expression and co-expression (polyfunctionality) at 6, 8 and 14 weeks of age. The cytokines of interest were IL-2, IFN-γ, IL-17 and IL-13. IL-2 and IFN-γ are Th1 cytokines and play an important role in the control of intracellular pathogens such as M.TB (Manca et al., 2001).IL-17 belongs to Th17 cytokines that has been shown to induce pro-inflammatory responses and relate to mycobacterial protection (refer to properties of IL-17 in chapter 1). IL-13 is a Th2 cytokine associated with mast cell activity and B cell production of antibodies against extracellular pathogens (Müller et al., 2007).

3.2 T-cell proliferative responses to BCG in early versus delayed-BCG-vaccinated infants at 6, 8 and 14 weeks of age

The proliferative responses to BCG were compared between HIV-exposed infants in the early arm (receiving BCG at birth) and those in the delayed arm (receiving BCG arm at 8 weeks) using Ki67, a cell cycle marker (refer to chapter 2). Table 3.1 describes the different time points at which blood was collected and at which infants received the main vaccines studied in this dissertation.

Table 3.1: Time points at which infants in the early and delayed arms received their vaccines

Time points	Early arm	Delayed arm
0 weeks	BCG administered and blood collected	Blood collected
6 weeks	Blood collected, pertussis and TT vaccines administered	Blood collected, pertussis and TT vaccines administered
8 weeks	Blood collected	BCG vaccine administered, blood collected
10 weeks	Pertussis and TT vaccines administered	Pertussis and TT vaccines administered
14 weeks	Blood collected	Blood collected

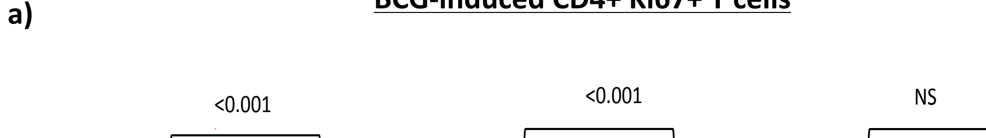
3.2.1. CD4+ proliferative responses to BCG at weeks 6, 8 and 14

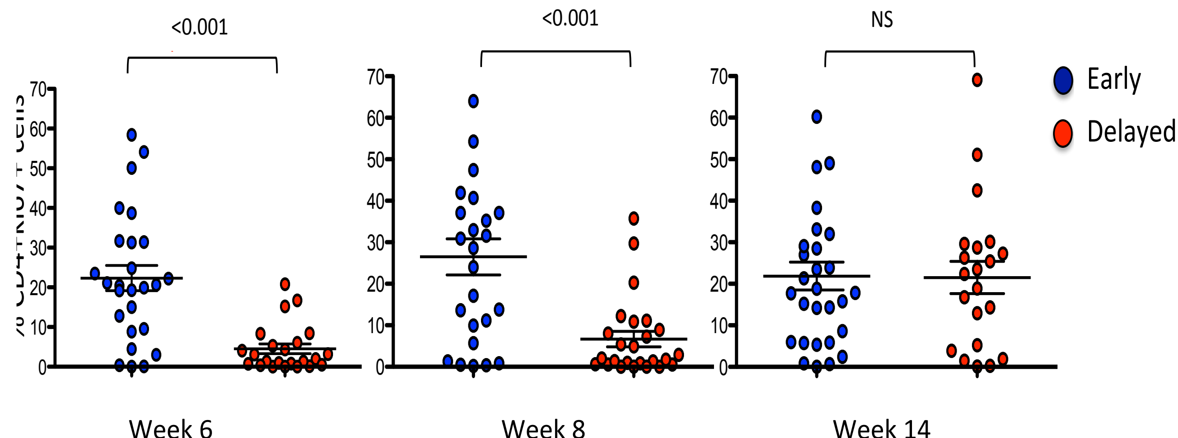
At 6 weeks of age, infants in the birth BCG vaccination group exhibited higher CD4+ T cell proliferation in response to BCG than those given delayed BCG (early arm median=20.5%) versus delayed arm (median=1.89%; p<0.001) (Figure 3.1a). Similarly, at 8 weeks of age, infants in the birth BCG vaccination group also had higher CD4+ T cell proliferation in response to BCG than those given delayed BCG (early arm median=28.6% versus delayed arm median=1.99% and p<0.001). These findings were expected given that the delayed BCG arm received BCG vaccination only at 8 weeks of age. On the other hand, at week 14, infants in both arms had similar proliferative CD4+ responses (early arm median=17.8% versus delayed arm median=22.4% and p=0.968). When CD4+ proliferative

responses to BCG were compared 6 weeks post vaccination (week 6 for infants vaccinated at birth and week 14 for infants vaccinated at week 8), no significant difference was found (20.5% versus 22.4%, p=0.881) (Figure 3.1b).

a)

BCG-induced CD4+ Ki67+ T cells





b)

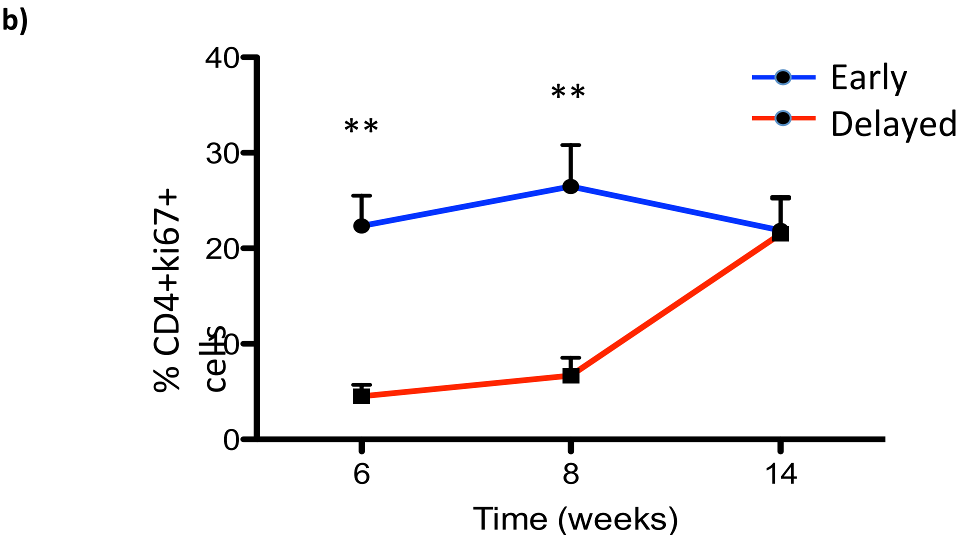


Figure 3.1: a). Frequency of CD4+ T cells expressing Ki67+ in response to BCG stimulation determined by flow cytometry in the early (blue) versus delayed (red) BCG vaccinees at 6, 8, and 14 weeks. Lines represent medians and whiskers interquartile ranges. p<0.05 significant level (unadjusted) and statistical significance was obtained using Mann-Whitney U test. **b). Longitudinal assessment of the median frequency of CD4+Ki67+ cells.** Whiskers represent interquartile ranges. *p<0.05; **p<0.01.

3.2.2. CD8+ proliferative responses to BCG at weeks 6, 8 and 14

Similar to CD4+ T cells, infants in the early arm had higher CD8+ T cell proliferation in response to BCG compared to infants in the delayed arm at 6 weeks of age (early arm median=18.5% versus delayed arm median =3.61% and p=0.0363)(figure 3.2.a). At 8 weeks of age, infants in the early arm also had higher CD8+ T cell proliferation in response to BCG compared to infants in the delayed arm (early arm median=21.5% versus delayed arm median=4.61% and p=0.0184) (figures 3.2a and 3.2.b). At week 14, there was no difference in proliferative CD8+ proliferative responses between infants in the two arms (early arm median=9.5% versus delayed arm median=8.61% and p=0.8241).

When CD8+ proliferative responses to BCG were compared at 6 weeks post vaccination (week 6 for early arm-infants and week 14 for delayed arm-infants vaccinated at week 8), no significant difference was found (figure 3.2.a).

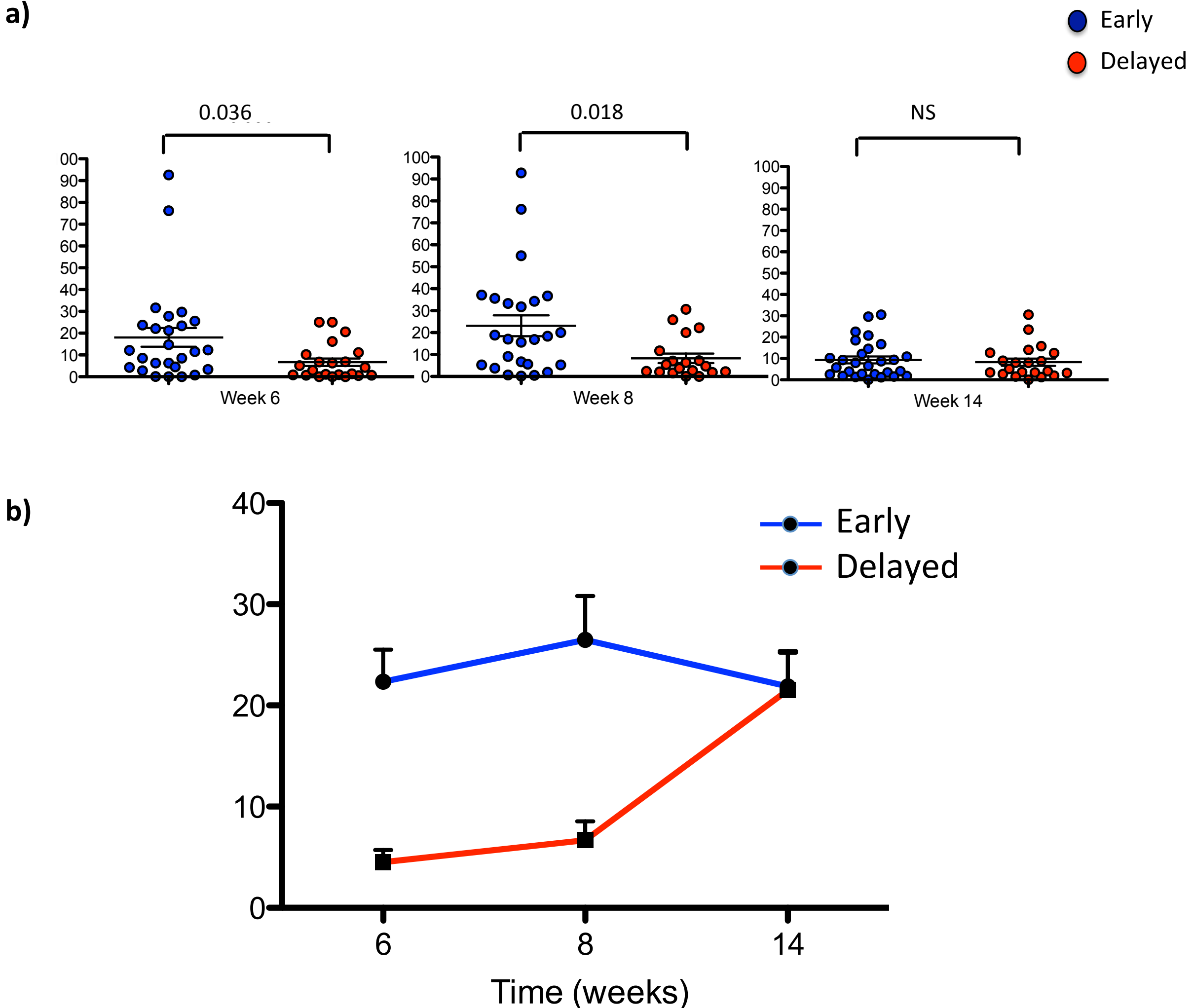


Figure 3.2: a). Frequency of CD8+ T cells expressing Ki67+ in response to BCG stimulation determined by flow cytometry in the early (blue) versus delayed (red) BCG vaccinees at 6, 8, and 14 weeks. Lines represent medians and whiskers interquartile ranges. $p<0.05$ significant level (unadjusted) and statistical significance was obtained using Mann-Whitney U test. **b). Longitudinal assessment of the median frequency of CD8+Ki67+ cells.** Whiskers represent interquartile ranges. *$p<0.05$; **$p<0.01$.

3.2.3. Effects of pre vaccination responses on post vaccination responses in the delayed arm

At 6 and 8 weeks of age, 3 of the 28 infants in the delayed group possessed high levels of BCG-specific CD4+ proliferation. (15-30%) despite not having yet been BCG vaccinated possibly a consequence of environmental Mycobacteria exposure. Since there is a potential for pre-existing BCG proliferative responses

to impact post-vaccination proliferative responses in infants who received delayed vaccination, the effects of pre-vaccination responses (weeks 6 and 8) on post vaccination proliferative responses (weeks 14) were assessed. There was no correlation between BCG-specific CD4 proliferative responses pre-vaccination in the delayed BCG arm at week 6 and their responses post-vaccination at week 14 (r=0.027; p=0.946) (figure 3.3.a). Similarly, CD4+ proliferative responses at week 8 did not correlate with CD4+ proliferative responses at week 14 (r=0.393; p=0.261) (figure 3.3.a)

In addition, there was no correlation between BCG-specific CD8+ T cell proliferative responses pre-vaccination in the delayed BCG arm at week 6 and their responses post-vaccination at week 14 (r=0.427; p=0.234). CD8+ proliferative responses at week 8 also did not correlate with CD4+ proliferative responses at week 14 (r=-0.259; p=0.4933) (figure 3.3.b)

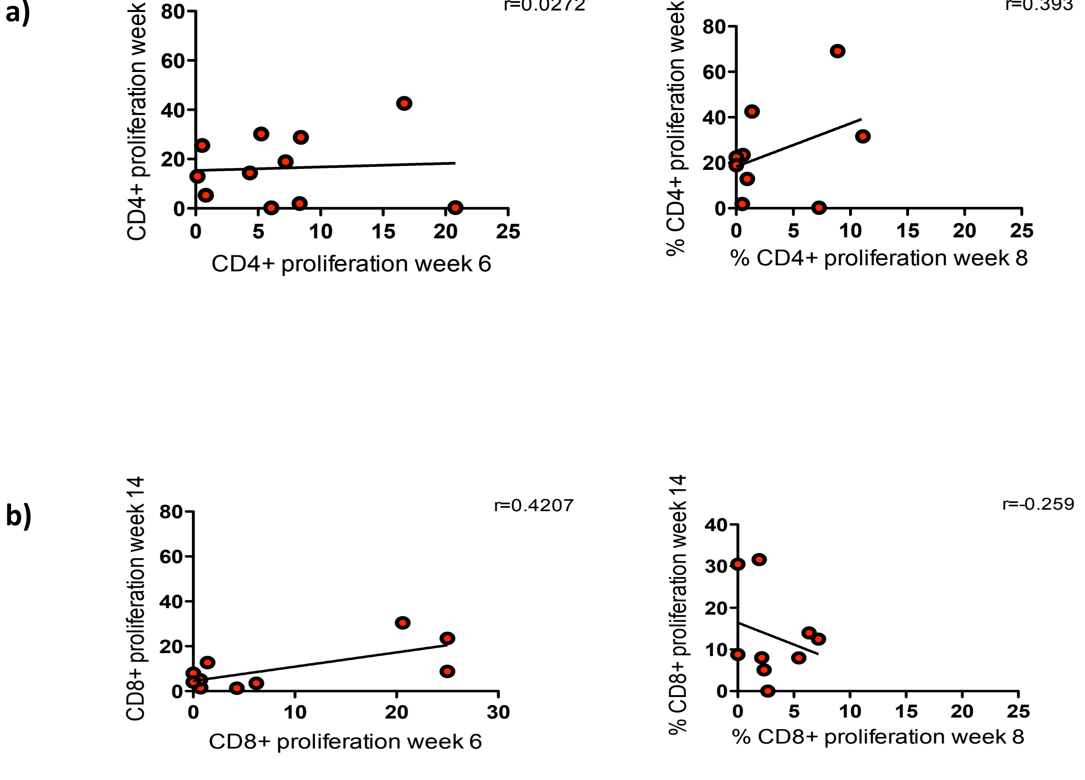


Figure 3.3: a). Spearman Correlation between CD4+ proliferative responses at week 6 (left), week.8 (right) and CD4 proliferative responses at week 14. p values were 0.946 and 0.261 respectively. **b). Spearman correlation between CD8+ proliferative responses at week 6 (left), week 8 (right) and CD8 proliferative responses at week 14.** p values were 0.259 and 0.493 respectively. A Spearman correlation analysis was performed using STATAv11 and Graph pad Prism V5.

3.3. T cell intracellular cytokine expression in response to BCG in HIV-exposed infants vaccinated with BCG in early-BCG vs. delayed-BCG vaccinated infants

After assessing T cell proliferative responses, T cell cytokine expression in response to BCG were compared between birth-vaccinated infants (early arm) and 8 week-vaccinated (Delayed arm) HIV-exposed infants.

3.3.1. IFN-g and IL-2 (Th1/ Tc1) expression in response to BCG in early vs. delayed arms at 6, 8 and 14 weeks of age

3.3.1.1. Frequency of CD4+ proliferating T cell expressing IL-2 or IFN-γ (Th1 cytokines) at weeks 6, 8 and 14.

Figure 3.4a shows that as hypothesized, infants in the early arm (receiving BCG at birth) had a higher proportion of CD4+ T cells secreting IFN- and IL-2 compared to infants in the delayed BCG arm (receiving BCG at week 8) at weeks 6 and 8 ($p<0.001$). At week 14, infants in the delayed arm (receiving BCG at 8 weeks) had a larger proportion of CD4+ T-cells expressing IFN-γ ($p<0.001$) when compared to infants in the early arm. There was no difference in IL-2 expression by CD4+ proliferating cells between the two groups at week 14.

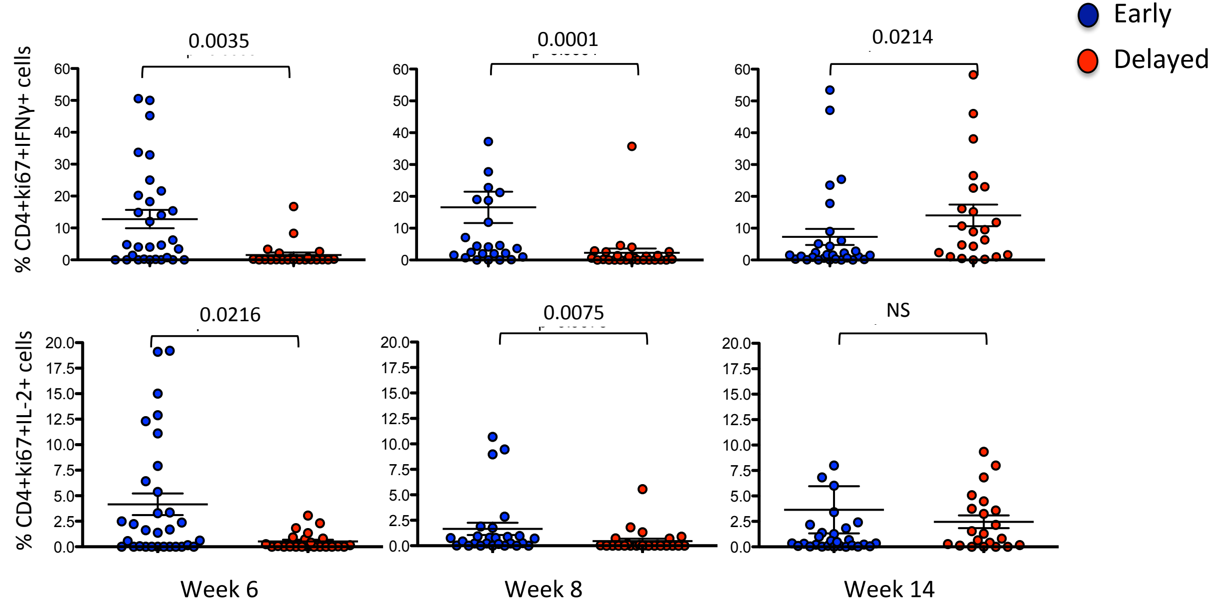


Figure 3.4a: Frequency of CD4+Ki67+ cells expressing IFN-γ+ and IL-2+ in the early (blue) versus delayed (red) HIV-exposed infants after BCG stimulation for six days in the whole blood assay measured by flow cytometry. Frequencies reported are of total CD4+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using Mann-Whitney U test.

3.3.1.1.Frequency of CD8+ proliferating T cell expressing IL-2 or IFN-γ (Tc1 cytokines) at weeks 6, 8 and 14.

Similarly to CD4+Ki67, infants in the early arm (receiving BCG at birth) had a higher proportion of CD8+ T cells secreting IFN-γ and IL-2 at weeks 6 ($p=0.049$

and p= 0.028 respectively) (figure 3.4b). At week 8, infants in the early arm (receiving BCG at birth) had higher frequencies of IFN-γ –expressing proliferating CD8 cells ($p<0.001$) and there was no difference in IL-2 expression by proliferating CD8+ cells. At week 14, infants in both arms had similar levels IL-2 and IFN-γ-expressing CD8+ cells (figure 3.4b).

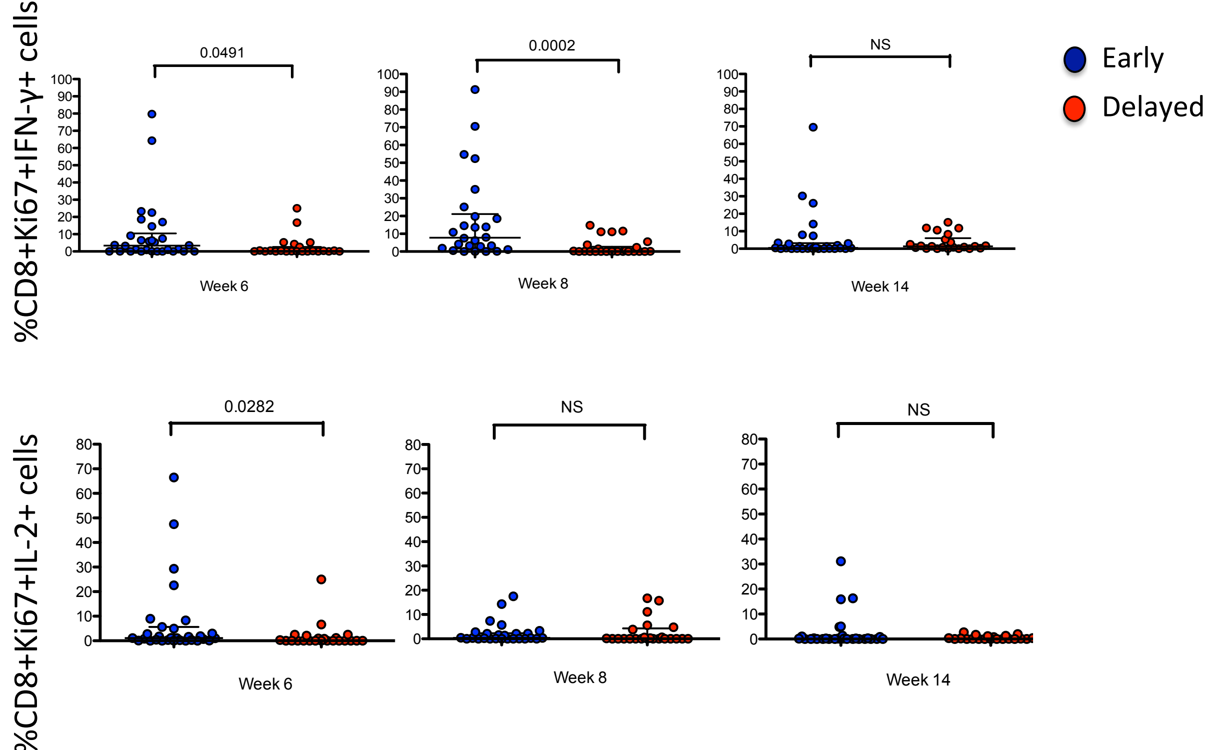


Figure 3.4b: Frequency of CD8+Ki67+ cells expressing IFN-γ+ and IL-2+ in the early (blue) versus delayed (red) HIV-exposed infants after BCG stimulation for six days in the whole blood assay measured by flow cytometry. Frequencies reported are of total CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using Mann-Whitney U test.

3.3.2. IL-17 (Th17) expression in response to BCG in early vs. delayed arm at 6, 8 and 14 weeks age.

3.3.2.1. Frequency of CD4+ proliferating T cell expressing IL-17 (Th17) at weeks 6, 8 and 14.

At 6 weeks of age, early-vaccinated infants had significantly higher frequencies o f IL-17-expressing CD4 proliferating cells in response to BCG at 6 weeks than those in the delayed arm (p=0.0428). However, at weeks 8 and 14, no

differences in IL-17 expression were observed between the two groups (figure 3.5a).

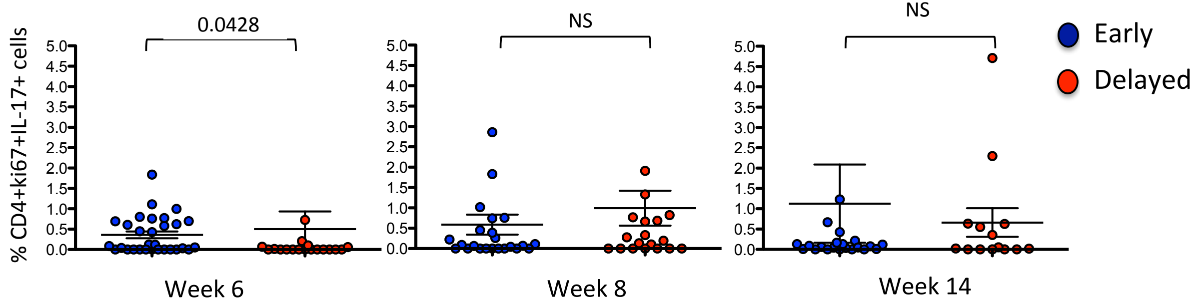


Figure 3.5a: Frequency of CD4+Ki67+ cells expressing IL-17+ in the early (blue) versus delayed (red) HIV-exposed infants after BCG stimulation for six days in the whole blood assay measured by flow cytometry. Frequencies reported are of total CD4+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using Mann-Whitney U test.

3.3.2.1. Frequency of CD8+ proliferating T cell expressing IL-17 (Tc17) at weeks 6, 8 and 14.

Early-vaccinated infants had significantly higher frequencies of IL-17-expressing CD8 proliferating cells in response to BCG at 6 weeks than those in the delayed arm (p=0.0089). On the other hand, at 8 and 14 weeks of age, infants in both arms had similar level of IL-17-expressing CD8+ proliferating cells in response to BCG (figure 3.5.b).

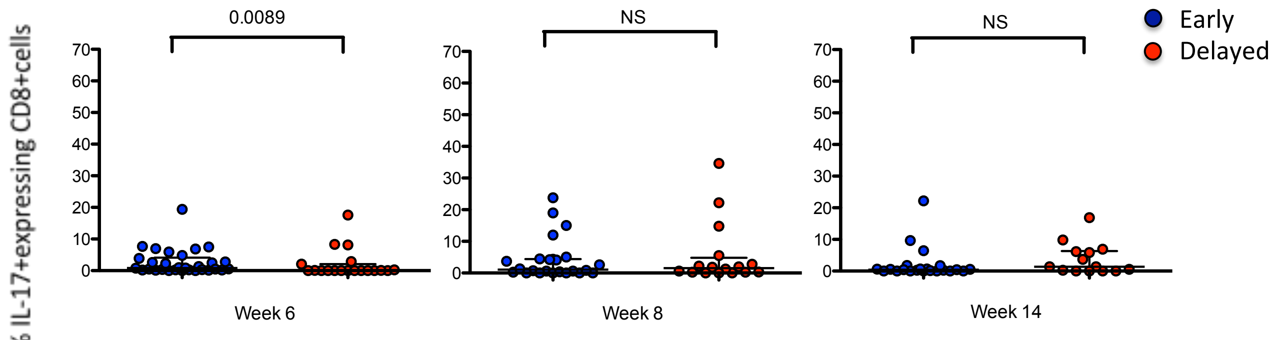


Figure 3.5b: Frequency of CD8+Ki67+ cells expressing IL-17+ in the early (blue) versus delayed (red) HIV-exposed infants after BCG stimulation for six days in the whole blood assay measured by flow cytometry. Frequencies reported are of total CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using Mann-Whitney U test.

4.3.2. IL-13 (Th2/ Tc2) expression in response to BCG in Birth- versus 8 week vaccinated infants at 6, 8 and 14 weeks age.

4.3.2.1. Frequency of CD4+ proliferating T cell expressing IL-13 (Th2) at weeks 6, 8 and 14.

Early-vaccinated infants at higher frequencies of IL-13-expressing CD4+ proliferating cells in response to BCG at week 6 than those in the delayed arm (p= 0.0091) (figure 3.6a). This was no longer the case at weeks 8 and 14 when infants in the two arms had similar frequencies of IL-13-expressing CD4 proliferating cells in response to BCG (figure 3.6a).

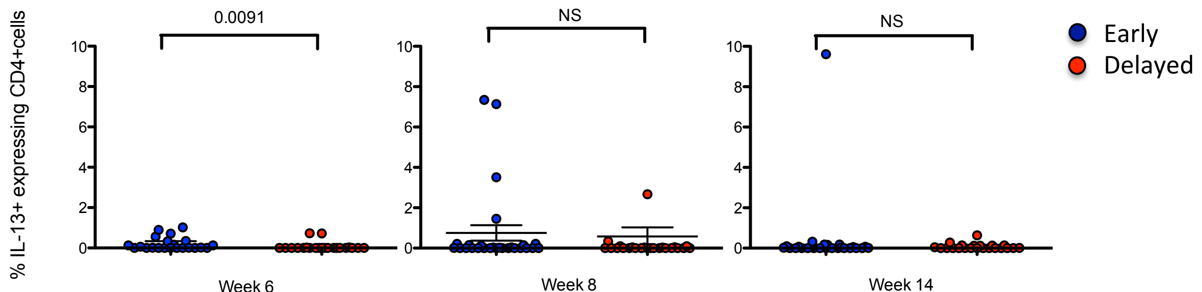


Figure 3.6a: Frequency of CD4+Ki67+ cells expressing IL-13+ in the early (blue) versus delayed (red) HIV-exposed infants after BCG stimulation for six days in the whole blood assay measured by flow cytometry. Frequencies reported are of total CD4+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using Mann-Whitney U test.

4.3.2.1. Frequency of CD8+ proliferating T cell expressing IL-13 (Tc2) at weeks 6, 8 and 14.

Early-vaccinated infants demonstrated higher frequencies of IL-13-expressing CD8+ proliferating cells in response to BCG at week 6 than those in the delayed arm (p=<0.001). At weeks 8 and 14, infants in the two arms had similar frequencies of IL-13-expressing CD4 proliferating cells in response to BCG (Figure 3.6.b).

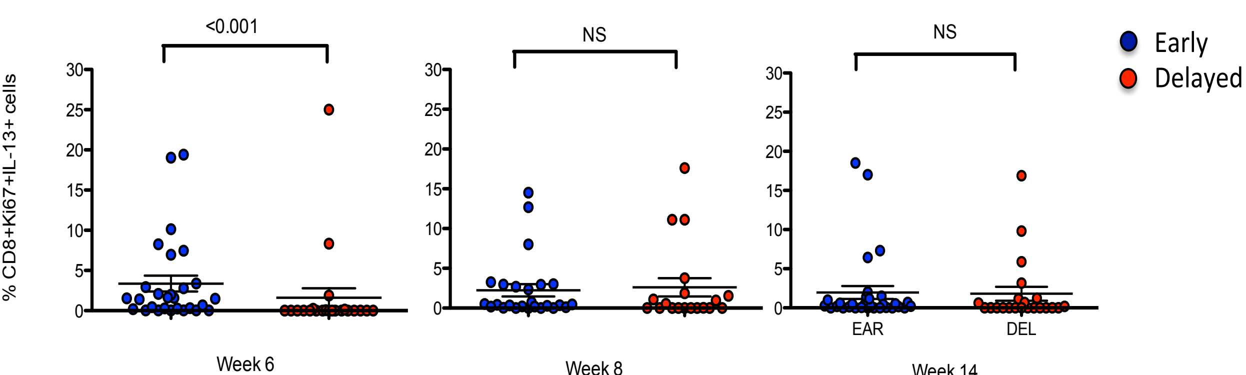


Figure 3.6b: Frequency of CD4+Ki67+ cells expressing IL-13+ in the early (blue) versus delayed (red) HIV-exposed infants after BCG stimulation for six days in the whole blood assay measured by flow cytometry. Frequencies reported are of total CD4+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using Mann-Whitney U test.

3.4. T-cell polyfunctional responses to BCG in early versus delayed-BCG vaccinated infants at 6, and 14 weeks of life

Co-expression of cytokines by specific T cells (polyfunctionaility) in response to antigen stimulation is often a measure of T cell function.

3.4.1. Polyfunctionality of T cell responses to BCG stimulation at 6 weeks post-vaccination

T cell polyfunctional responses to BCG between early and delayed BCG-vaccinated infants were compared after all the infants had received BCG i.e. at 14 weeks. Additionally, T cell polyfunctionality to BCG at 6 weeks in the early arm were compared to T cell polyfunctional responses to BCG at 14 weeks in the delayed arm. The aim was to determine whether T cell polyfunctional responses to BCG are the same between the two-arms 6 weeks post-vaccination.

3.4.1.1. Polyfunctionality of CD4+ T cell responses to BCG stimulation at 6 weeks post-vaccination

Figure 3.7 shows the breadth of CD4+ T cell cytokine responses to BCG at 6 weeks post-vaccination (6 weeks for early arm versus 14 weeks for delayed arm). The frequency of proliferating cells that were making none, any one, or any combination of cytokines, between infants in the delayed BCG arm versus the early BCG arm at 6 weeks post BCG vaccination were compared. As seen in the top graph, when comparing all the different combinations of cytokines, infants in the early and delayed arms had comparable frequencies of proliferating CD4+ cells expressing zero, one, two or three cytokines (figure 3.7.a). There was no significant difference for the expression of any particular cytokine combination between the early and delayed arms 6-weeks post-vaccination. Overall infants in the two arms had similar CD4+Ki67+ polyfunctionality 6 week post-vaccination (ANOVA p=0.729) (figure 3.7b).

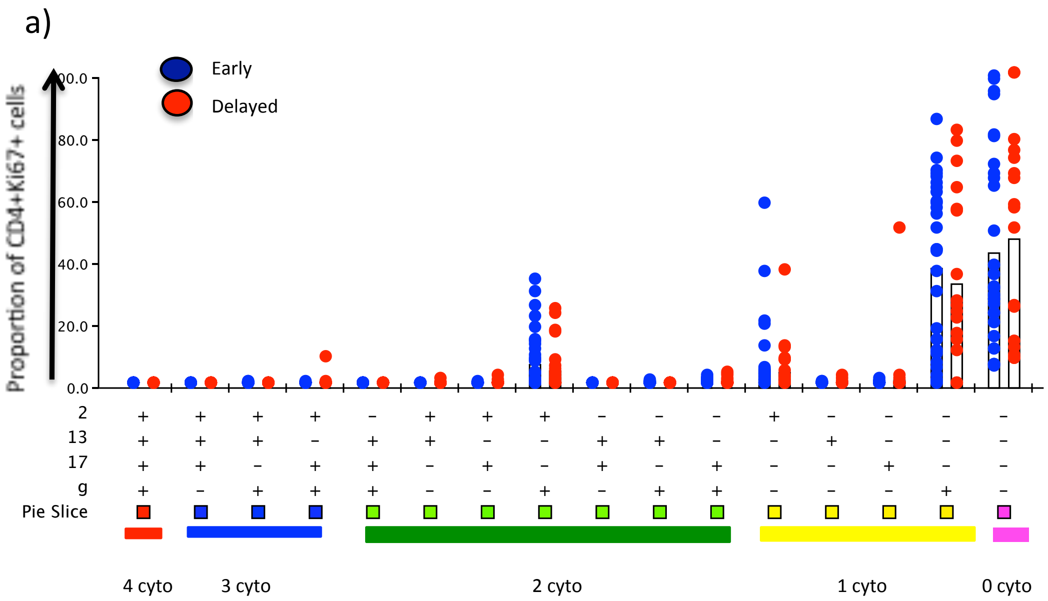
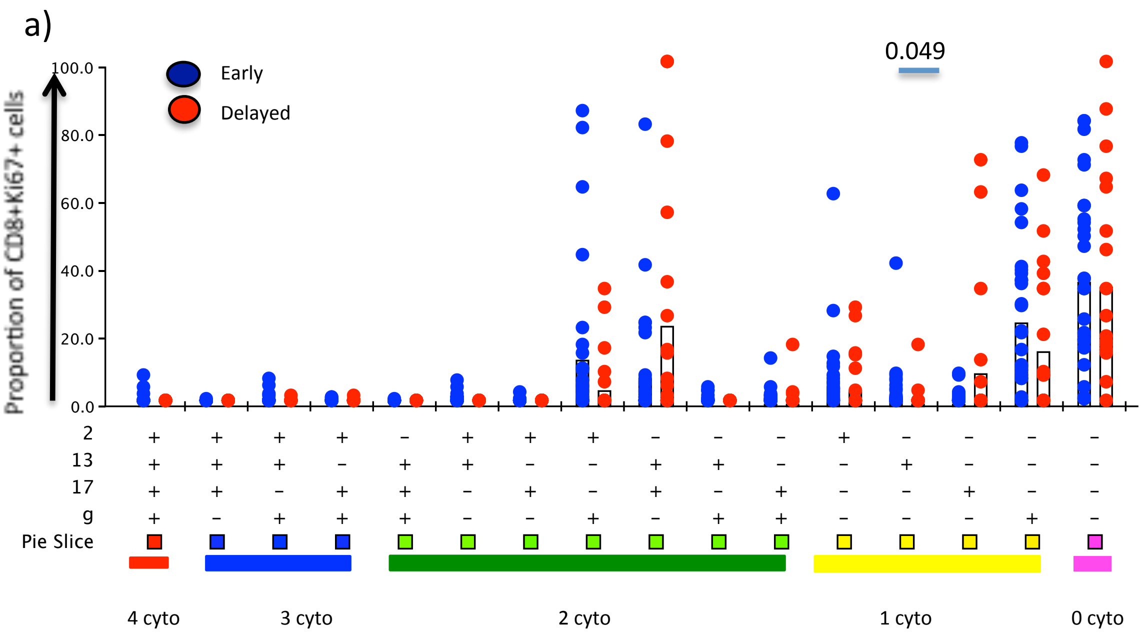


Figure 3.7: Proportions of BCG-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 6 weeks post vaccination (6 weeks for early arm and 14weeks for delayed arm) in birth-vaccinated (early group) infants versus delayed-vaccinated HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD4+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD4+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD4+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between the two groups. Statistical analysis was performed using Wilcoxon signed rank test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

3.4.1.2. Polyfunctionality of CD8+ T cell responses to BCG stimulation at 6 weeks post vaccination

At 6 weeks post vaccination, when comparing all the different combinations of cytokines, infants in the early BCG arm had significantly higher proliferating CD8+ T cells expressing IL-13 alone than infants in the delayed arm (p= 0.049). However, after adjusting for multiple comparisons, the difference was no longer significant (p=0.784 not shown). Other than IL-13 expression by proliferating CD8+ cells, there was no difference in the frequencies of CD8Ki67+ cells expressing zero, one, two or three cytokines and any other specific cytokine combination (figure 3.8a). Overall, there was no difference in proliferating CD8+ T cell polyfunctionality between infants in the early and those in the delayed arm (p=0.101) (figure 3.8.b).



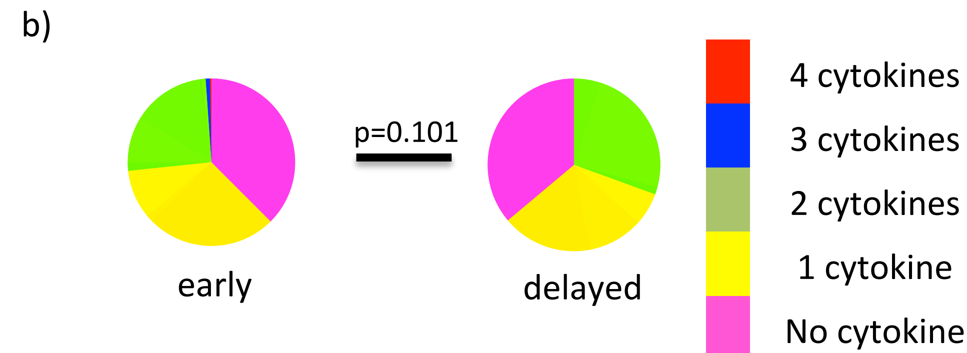
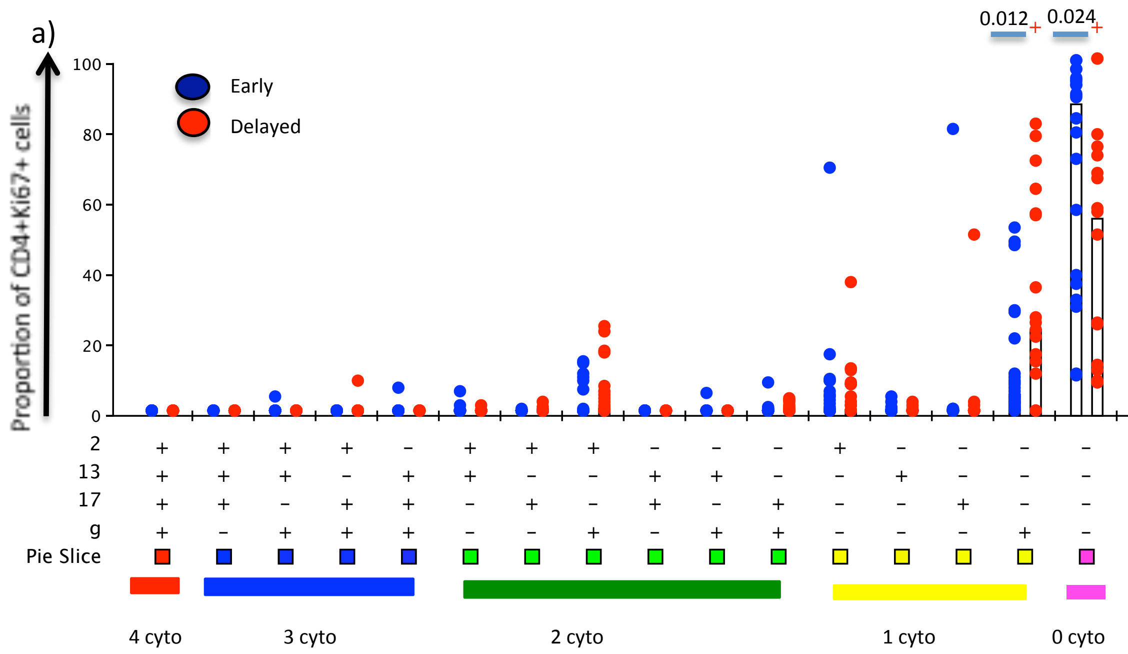


Figure 3.8: Proportions of BCG-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 6 weeks post vaccination (6 weeks for early arm and 14weeks for delayed arm) in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots) (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. bars correspond to medians and; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total_magnitude of the combinations of cytokine responses.

3.4.2. Polyfunctionality of T cell responses to BCG stimulation at 14 weeks of age

3.4.2.1. Polyfunctionality of CD4+ T cell responses to BCG stimulation at 14 weeks of age

At week 14, infants in the delayed BCG arm, who had received BCG 6 weeks prior, had significantly higher frequency of proliferating CD4+ T cells that produced at least one cytokine in response to BCG, and significantly higher frequency of cells making IFN-γ than those the early arm who had received BCG 14 weeks prior (p=0.024 and 0.012 respectively) (figure 3.9a). Neither of these differences remained significant after adjustment for multiple comparisons. Overall, CD4+ T cells from infants in the delayed arm were significantly more polyfunctional in response to BCG stimulation than those in the early arm (ANOVA p=0.018) (Figure 3.9b).



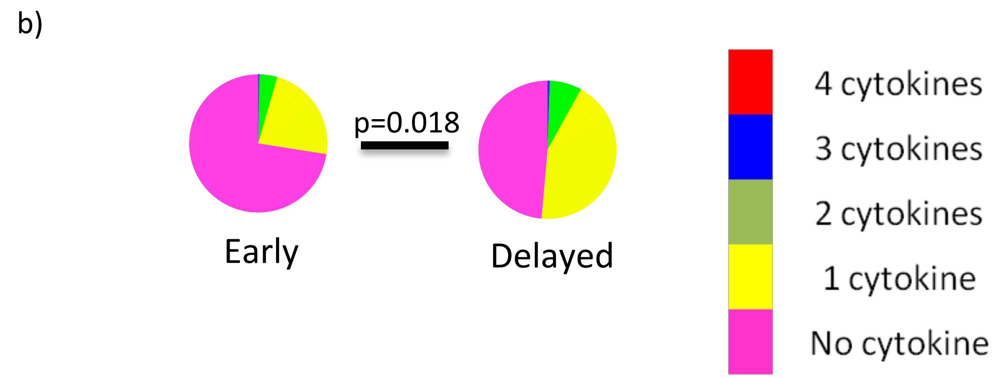
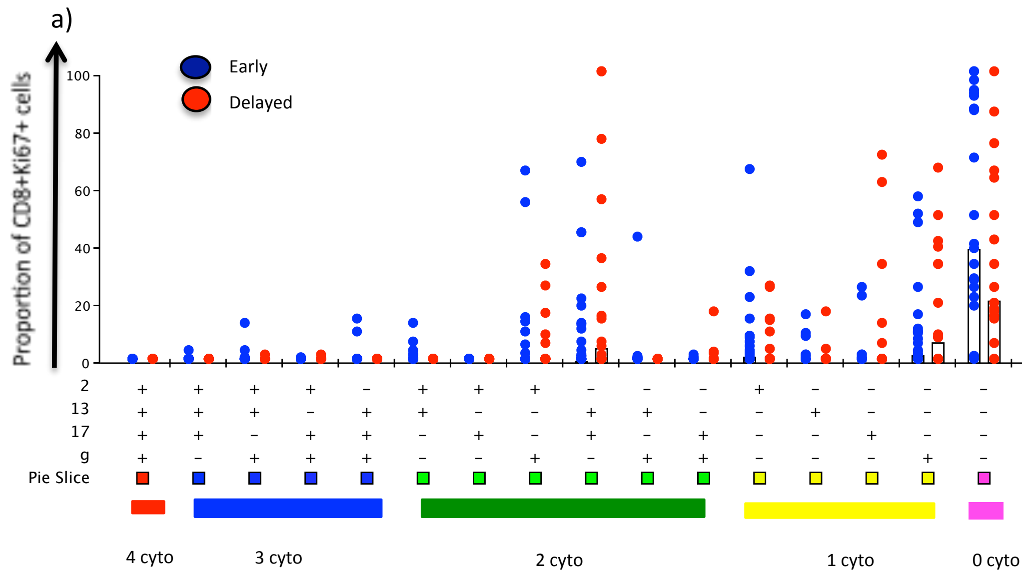


Figure 3.9: Proportions of BCG-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 14 weeks of age in birth-vaccinated (Early group) infants versus 8-week-vaccinated (Delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD4+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD4+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD4+ T cells. Bars correspond to medians ranges; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

3.4.2.2. Polyfunctionality of CD8+ T cell responses to BCG stimulation at 14 weeks of age

As shown in figure 3.10a, when comparing each combination of cytokines individually, there were no significant differences between infants in the early and delayed arms. There was also no difference in the overall CD8+ T-cell polyfunctionality between infants in the early and delayed arms at week 14 ($p=0.111$)(figure 3.10b).

a)



b)

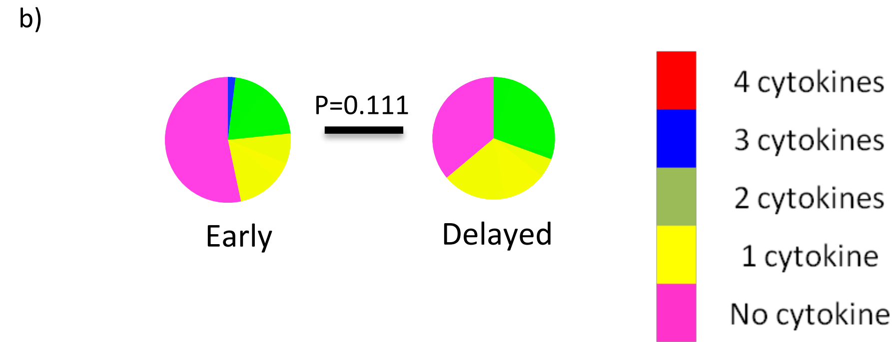


Figure 3.10: Proportions of BCG-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 6 weeks of age in birth-vaccinated (Early group) infants versus 8-week-vaccinated (Delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed ran*k test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

3.5. Discussion

In this chapter, the effects of delaying BCG vaccination from birth to 8 weeks on CD4+and CD8+T cell proliferative and cytokine responses to BCG in HIV-exposed infants was assessed. These responses would help us determine the impact of delaying BCG vaccination on the vaccine immunogenicity in HIV-exposed infants, a strategy that may allow for infant BCG vaccination in line with WHO guidelines. Few studies have investigated the effects of delayed BCG vaccination in infants (Burl et al., 2010; Kagina et al., 2009; Marchant et al., 1999). However, all this studies were carried out in HIV-unexposed infants. This is one of the first studies to examine the effects of delaying BCG vaccination on T cell responses in HIV-exposed, uninfected infants, a group highly relevant for such a vaccination strategy.

In this chapter, the main result showed that delaying BCG vaccination from birth to 8 weeks did not alter CD4+ and CD8+ T cell proliferation to BCG 6-weeks post vaccination. These findings are consistent with reports of proliferative in responses in HIV-unexposed infants following delayed BCG vaccination in South Africa, Uganda and The Gambia (Burl et al., 2010; Kagina et al., 2009; Lutwama et al., 2013). In all these previous studies, BCG vaccination was delayed from birth to 10, 6 and 8 weeks of age in HIV-unexposed infants respectively (Burl et al., 2010; Kagina et al., 2009; Lutwama et al., 2013). Thus, it seems as if delayed BCG vaccination does not have any effect on CD4+ and CD8+ T cell proliferation regardless of HIV exposure.

Furthermore, this chapter showed that at 14 weeks of age, HIV-exposed infants who were vaccinated with BCG at 8 weeks of age had significantly higher frequencies of IFN-γ–secreting CD4+ T cells compared to birth-vaccinated infants. Delaying BCG resulted in greater polyfunctionality of CD4+ and CD8+ T cell as measured by cytokine response at 14 weeks. Whilst the immune correlates of protection against TB are unknown, studies have shown that IFN-γ-secreting CD4+ T-cells may play an important role in protection against TB

(Flynn et al., 1993; Reed et al., 2009). In addition, higher frequencies of polyfunctional CD4+ T-cells expressing many Th1 cytokines such as IL-2 and IFN-γ+ are generally recognized as an increase in quality of response (Beveridge et al., 2007; Mueller et al., 2008).

Neonates are characterized by an "immature" immune system and defects in antigen presentation and IL-12 production (Marodi, 2002; Velilla et al., 2007). As a result, their immunity is generally skewed towards Th2 responses (Marodi, 2002; Rose et al., 2007; Velilla et al., 2007). However, it has been well established that Th1 responses improve with age (Marodi, 2002), which could explain the higher frequencies of BCG specific IFN-γ secreting CD4+ T-cells and polyfunctional CD4+ T-cells in infants given BCG a few weeks after birth.

Previous studies of the effects of delayed BCG on cytokine responses in HIV-unexposed infants have shown variable results. In a study from the Gambia, delaying BCG vaccination to two and four months resulted in lower IFN-γ and IL-17 responses in the delayed BCG vaccinated group compared to those vaccinated at birth, when assessed at two months post vaccination (Burl et al., 2010; Marchant et al., 1999).In a non-randomised, observational study carried out in Uganda, infants who inadvertently received delayed BCG vaccination at 6 weeks of age also produced lower IFN-γ responses when measured at 9 months (Lutwama et al., 2013). On the other hand, similar to our findings in HIV-exposed infants, Kagina et al. found that delaying BCG vaccination to 10 weeks of age resulted in higher frequencies of BCG-specific IFN-γ-secreting CD4 T-cells and IFN-γ+IL-2+ CD4 T cells subset at in the delayed vaccination group at 20 weeks of age (Kagina et al., 2009). Finally, we also observed low IL-17 expression by all the infants following BCG vaccination. This is consistent with a recent study carried out in South Africa that examined T cell cytokine production over time following BCG vaccination in HIV-unexposed infants (Soares et al., 2013).

One of the main limitations of this study was the short duration of follow-up, therefore the long-term effects of delaying BCG in HIV-exposed infants could not be assessed. This was due to the nature of the study design and the setting in which the samples were obtained. Another limitation is that cells were restimulated with mitogens on day 6 to measure intracellular cytokine production, and these cytokines may not be the same as the cells would have made on day 1. It is also possible that using a shorter 12-hour whole blood assay rather than a six-day whole blood assay would have led to different outcomes. Despite these limitations, we were able to assess the effect of delayed BCG vaccination on BCG immunogenicity in HIV exposed infants at an age when most infants begin to be exposed to TB. On the other hand, one of the strengths of this study was that it was randomized and the two group characteristics were balanced except for the age of BCG vaccination, it is very unlikely that the differences observed in the two groups be due to other confounders.

In settings like South Africa, where TB and HIV co-infection is high, BCG is still routinely administered at birth to infants born to HIV-infected mothers despite all the risks in HIV-infected individuals. For optimal safety reasons, BCG should be administered only after HIV status is confirmed negative and excluded. The findings of this chapter suggest that delaying BGG vaccination to 8 weeks of age in HIV-exposed uninfected infants does not impinge on vaccine immunogenicity and may even improve Th1 responses. Thus, delaying BCG vaccination in HIV-exposed infants appears to be a worth further exploration for TB prevention even in a setting with high TB burden.

Chapter 4. Effects of Delayed BCG Vaccination on Cellular Responses to Pertussis and Tetanus Vaccines

4.1. Introduction

Acellular pertussis and tetanus toxoid (TT) are antigens included in the combined vaccine DTap (CDC, 2013). DTap is made up of TT, acellular pertussis and diphtheria vaccines. In South Africa, this vaccine is first administered to infants at 6 weeks of age as opposed to BCG, which is administered at birth (Department of Health, 2009) and is boosted at 10 and 14 weeks. Pertussis uses extracellular and intracellular pathways to avoid immune responses especially in the respiratory tract (Mills, 2001). Th1 responses are important in the control of pertussis infection especially in the lungs (Mills, 2001). On the other hand, Th2 cytkoines have been shown to induce antibody responses to extracellular pertussis (Higgs et al.,2012). TT is the vaccine against tetanus and is made up of deactivated Tetanus toxin produced by *Clostridum tetani*, (CDC, 2013). Th2 and antibody responses are thought to be important for control of Tetanus (Poulsen et al., 2007).In this chapter, the effects of BCG, a strong Th1 response inducer, on T cell responses to pertussis and TT in HIV-exposed infants are investigated. We hypothesized that HIV-exposed infants who received BCG at birth (early arm) would have lower Th2 responses to TT and pertussis due to downregulation of Th2 by Th1 cytokine production enhanced by early BCG vaccination. To achieve this aim, we first compared CD4+ and CD8+ T cell proliferative responses to pertussis and TT between HIV–exposed infants in the early and delayed arms at weeks 8 and 14. This was done by measuring frequency of Ki67-expressing CD4+ and CD8+ T cell in a whole blood assay as described in chapter 3. Furthermore, we assessed the effects of delayed BCG vaccination on CD4+ and CD8+ T cell expression of Th1/ Tc1 (IFNγ and IL-2), Th17/ Tc17 (IL-17), and Th2/ Tc2 (IL-13) cytokines in response to pertussis and

TT stimulation in the two arms. These responses as well as T cell polyfunctional responses to TT and pertussis were also measured at 8 and 14 weeks.

4.2. T-cell proliferative responses to pertussis and TT in early versus delayed-BCG vaccinated infants at 8 and 14 weeks of age

4.2.1. CD4+ and CD8+ proliferative responses to pertussis in early versus delayed-BCG vaccinated infants at 8 and 14 weeks of age

The CD4+and CD8+ T cell proliferative responses to pertussis were compared between birth-vaccinated (early arm) and 8-week-vaccinated (delayed arm) HIV exposed infants at weeks 8 and 14.

As shown in figure 4.1, at 8 weeks of age, before the delayed BCG arm had been BCG vaccinated, there was no difference in CD4+ T cell proliferation in response to pertussis between HIV-exposed infants in the early arm and those in the delayed arm (3.4% vs. 3.1% p= 0.361). Similar to CD4+ T cells, there was no significant difference in the frequencies CD8+Ki67+ between HIV-exposed infants in the early arm and those in the delayed arm following pertussis stimulation (8.6% vs. 7.3%, p=0.555).

At 14 weeks of age, as indicated in figure 4.1, in response to pertussis stimulation, there was no difference in the frequencies of proliferating CD4+ cells between HIV-exposed infants in the early arm and those in the delayed am (2.72% vs. 0.929%, p= 0.161). Furthermore, as observed at week 8, there was no also no difference in CD8+ T cell proliferative responses to pertussis at week 14. Infants who received BCG at birth (early arm) had comparable frequencies of CD8+Ki67+ cells as infants who received BCG at 8 weeks of age (delayed arm) (4.94% vs. 6.03%, p=1.00) (figure 4.1).

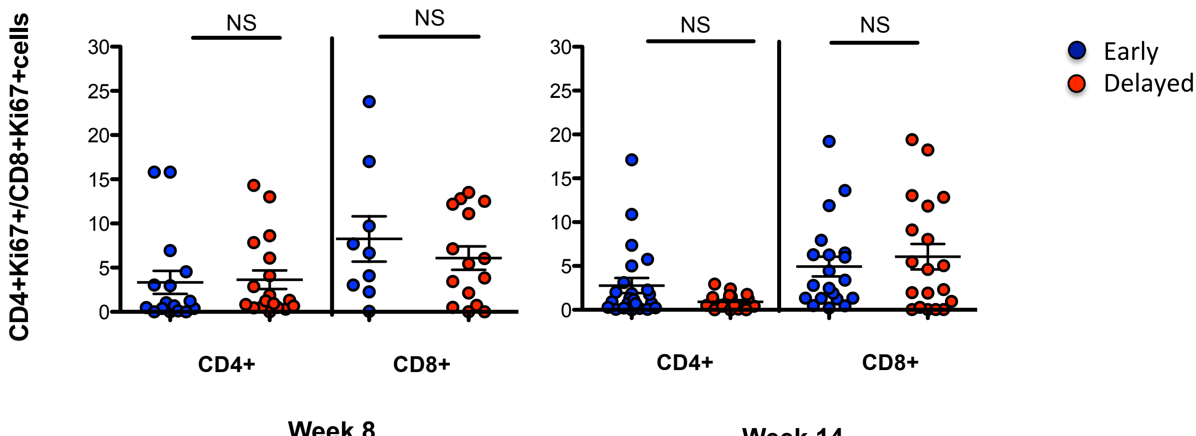


Figure 4.1: Frequency of CD4 and CD8+T cells expressing Ki67+ in response to pertussis stimulation determined by flow cytometry in the early (blue) versus delayed (red) BCG vaccinees at 8 and 14 weeks. Lines represent medians and whiskers interquartile ranges. p<0.05 significant level (unadjusted) and statistical significance was obtained using Mann-Whitney U test.

4.2.1.CD4+ and CD8+ proliferative responses to TT

T cell proliferative responses to TT in birth BCG-vaccinated and 8-week BCG-vaccinated HIV-exposed infants were compared at 8 and 14 weeks of age. At week 8, in response to TT stimulation, there was no significance difference in frequencies of CD4+Ki67+ cells between infants in the early arm and delayed BCG arms (1.04% vs. 1.03%, p= 0.793) as shown in figure 4.2. We also noted very comparable levels of CD8+Ki67+ cells between birth and 8 week-vaccinated HIV-exposed infants (1.03% vs. 1.09%, p=0.700) (figure 4.2).

At 14 weeks of age, infants in the early and delayed BCG arms had similar frequencies of proliferating CD4+ cells in response to TT stimulation (2.02% vs. 1.43%, p=0.488). In addition, there was no difference in CD8+ T cell proliferative responses to TT between birth-vaccinated (early arm) and 8 week-vaccinated (delayed arm) infants at 14 weeks of age as shown in figure 4.2 (1.30% vs. 3.13%, p=0.850). However, we only had three blood samples at 14 weeks of age from infants in the early BCG arm that were stimulated with TT.

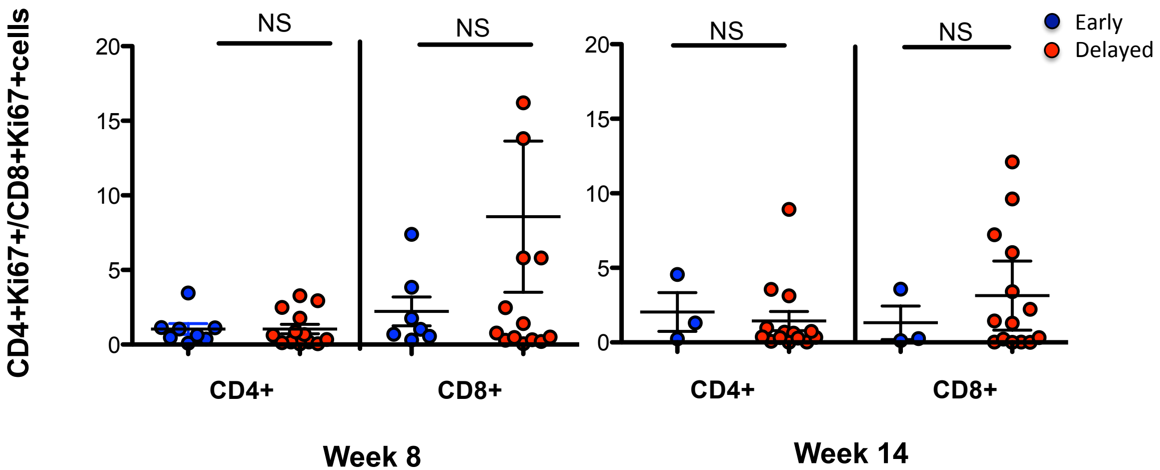


Figure 4.2: Frequency of CD4 and CD8+T cells expressing Ki67+ in response to TT stimulation determined by flow cytometry in the early (blue) versus delayed (red) BCG vaccinees at 8 (left) and 14 (right) weeks. Lines represent medians and whiskers interquartile ranges. $p<0.05$ significant level (unadjusted) and statistical significance was obtained using Mann-Whitney U test.

4.3. T cell cytokine expression in response to pertussis and TT in early-BCG vs. delayed-BCG vaccinated infants at 8 and 14 weeks of age

4.3.1. IFNγ and IL-2 (Th1/Tc1) expression in response to pertussis and TT in early versus delayed-BCG vaccinated infants at 8 and 14 weeks of age

4.3.1.1. IFNγ and IL-2 (Th1/Tc1) expression in response to pertussis in early versus delayed-BCG vaccinated infants at 8 and 14 weeks of age

Using ICS, we first measured the levels of IFN-γ and IL-2 expression by proliferating CD4+ and CD8+ cells following pertussis stimulation at weeks 8 and 14. At week 8, there was no significant difference in the frequency of IFN-γ-expressing CD4+Ki67+ cells in response to pertussis between birth-and 8 week BCG-vaccinated infants (0.278% vs. 0.687% respectively, p=0.639) (figure 6.3). In addition, there was also no difference IFN-γ expression by proliferating CD8+ cells between infants in the early arm and those in the delayed arm (3.92% vs. 3.24% respectively, p=0.987) (figure 6.3).

Infants the in the early and delayed BCG arms also had similar levels of IL-2 expression by CD4+ proliferating cells following pertussis stimulation at week 8 (0.274% vs. 0.096% respectively, p=0.541). On the other hand, as shown in figure 4.3, infants vaccinated with BCG at birth (early BCG arm) had higher frequencies of IL-2 expressing CD8+Ki67+ cells compared to infants in the delayed BCG arm (who had not yet received BCG vaccination) (4.012% vs. 0.244% respectively, p=0.044). This difference was no longer statistically significant after adjusting for multiple comparisons.

At 14 weeks of age, following pertussis stimulation, infants in the early and delayed BCG arms had similar levels of IFN-γ –expressing CD4+Ki67+ cells (0.504% vs. 0.378% respectively, p=0.254). A similar outcome was also observed for IFN-γ expression by CD8+ proliferating cells between birth-and 8-week BCG-vaccinated infants (1.50% vs. 1.54% respectively, p=0.275). Furthermore, no difference was found in IL-2 expression in response to pertussis at week 14, (CD4+Ki67+ 0.119% vs. 0.105%, p=0.401 and CD8+Ki67+ cells 2.39% vs. 0.394%, p=0.252) (figure 4.3).

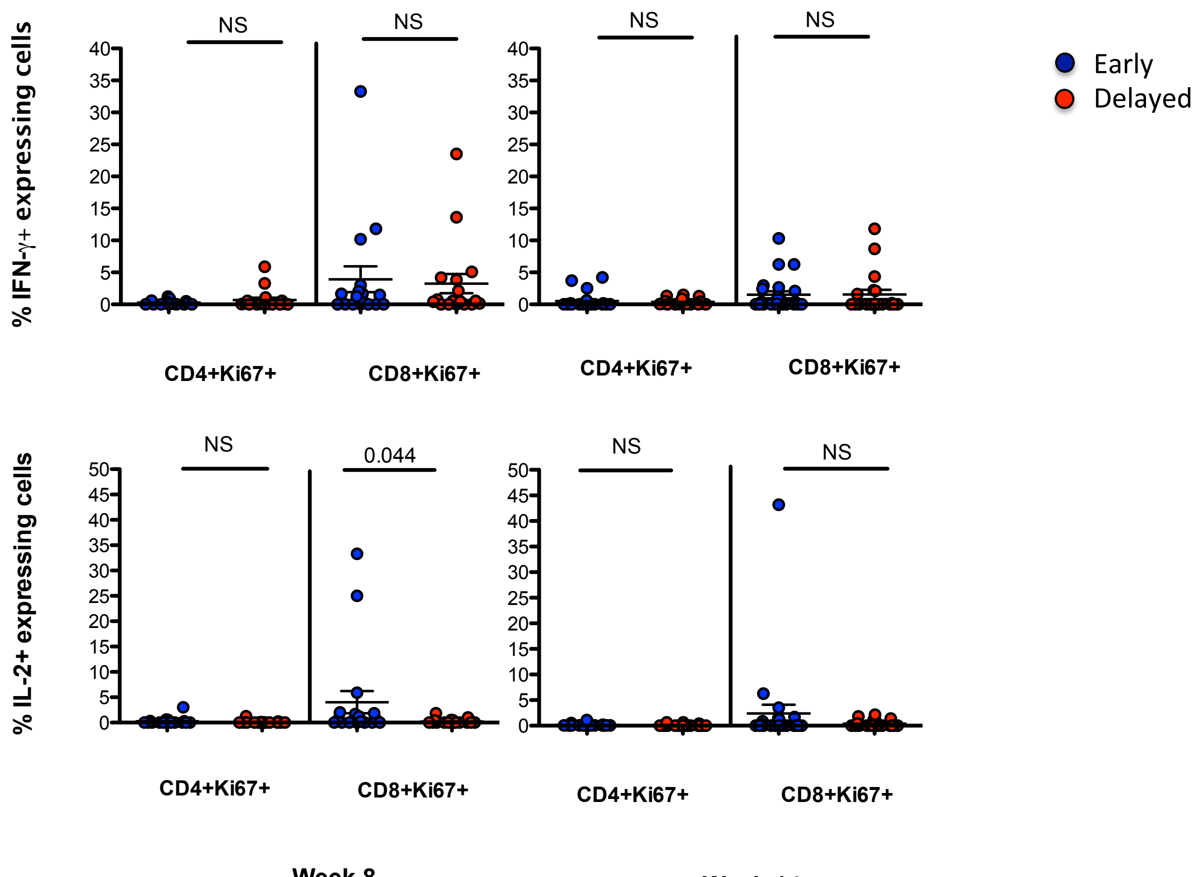


Figure 4.3: The frequency of T cells producing IFNγ and IL-2 at 8 and 14 weeks of life after pertussis stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IFN-γ and IL-2 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of pertussis-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported as frequency of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

4.3.1.2. IFNγ and IL-2 (Th1/Tc1) expression in response to TT in early-BCG vs. delayed-BCG vaccinated infants 8 and 14 weeks of age

At week 8, in response to TT, there was no difference in the frequencies of IFNγ-expressing-CD4+Ki67+ between infants in the early BCG arm and those in the delayed BCG arm as shown in figure 4.4 (0.187% vs. 0.343%, p=0.928). In addition, infants in early and delayed arms also had similar levels of IFNγ-expressing-CD8+Ki67+ cells following pertussis stimulation (0.223% vs. 0.514%,

p=0.479) (figure 4.4). Furthermore, at week 8, infants in the early and delayed exhibited similar levels of IL-2 expression by proliferating CD4+ (0.027% vs. 0.82%, p=0.109) and CD8+-cells (0.223% vs.0.544%, p=0.889) (figure 4.4).

At week 14, following TT stimulation, birth BCG-vaccinated and 8 week-vaccinated infants had similar frequencies of IFNγ-expressing-CD4Ki67+cells (0.03% vs. 0.02%, p=0.506) and IFNγ-expressing-CD8+Ki67+cells (0.05% vs. 0.16%, p=0.867) (figure 4.4). Like with IFN-γ expression, infants in the two arms also had similar levels of IL-2 expression by CD4+Ki67+ (0.436% vs. 0.141%, p=0.897) and CD8+Ki67+ (0.015% vs. 0.207%, p=0.605) cells in response to TT (figure 4.4.) However, due to the very small sample size of samples stimulated with TT at week 14 in the early arm, it is possible that this does not reflect the true effects of TT stimulation on cytokine responses.

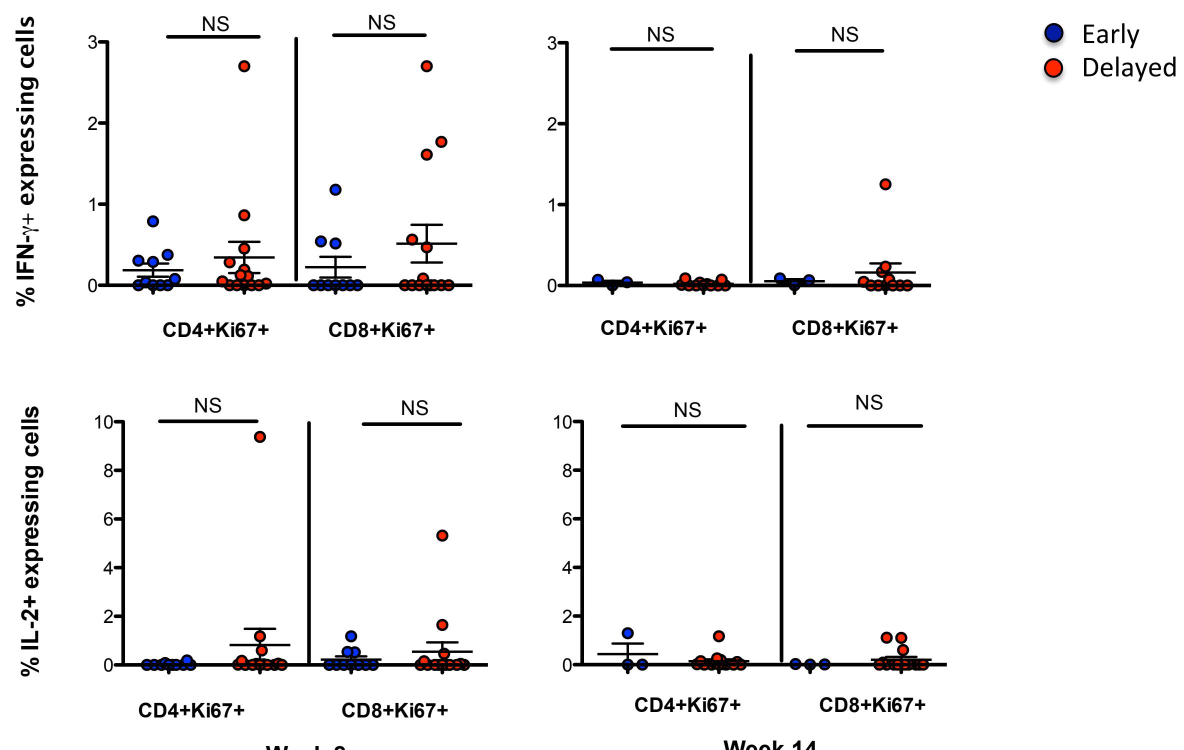


Figure 4.4: The frequency of T cells producing IFNγ and IL-2 at 8 and 14 weeks of life after TT stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IFNγ and IL-2 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of TT-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported as the frequency of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

4.3.2. IL-17 (Th17/ Tc17) expression in response to pertussis and TT in early-BCG vs. delayed-BCG vaccinated infants 8 and 14 weeks of age

After assessing Th1/ Tc1 (IFNγ and IL-2) cytokine expression by proliferative CD4+ and CD8+ cells, IL-17 (Th17/ Tc17 cytokine) expression by CD4+ and CD8+ proliferating cells in response to pertussis and TT at weeks 8 and 14 was assessed.

4.3.2.1. IL-17 (Th17/Tc17) expression in response to pertussis in early-BCG vs. delayed-BCG vaccinated infants 8 and 14 weeks of age

At week 8, following pertussis stimulation, there was no difference in IL-17 expression by CD4+ proliferating cells between birth-vaccinated infants (early arm) and 8 week-vaccinated infants (delayed arm) (1.03% vs. 2.03% respectively, p=0.755). Similar to CD4+ cells, there was also no difference IL-17 expressing-CD8+Ki67+ cells between birth- and 8 week-vaccinated infants at 8 weeks of age (22,7% vs. 16.4% p=0.255) (figure 4.5).

At week 14, following pertussis stimulation, infants in the early and delayed arms had similar levels of IL-17-expressing CD4+Ki67+ (0.115% vs. 0.112% respectively, p=0.069) and CD8+Ki67+ cells (1.85% vs. 1.98% respectively, p=0.782) (figure 4.5).

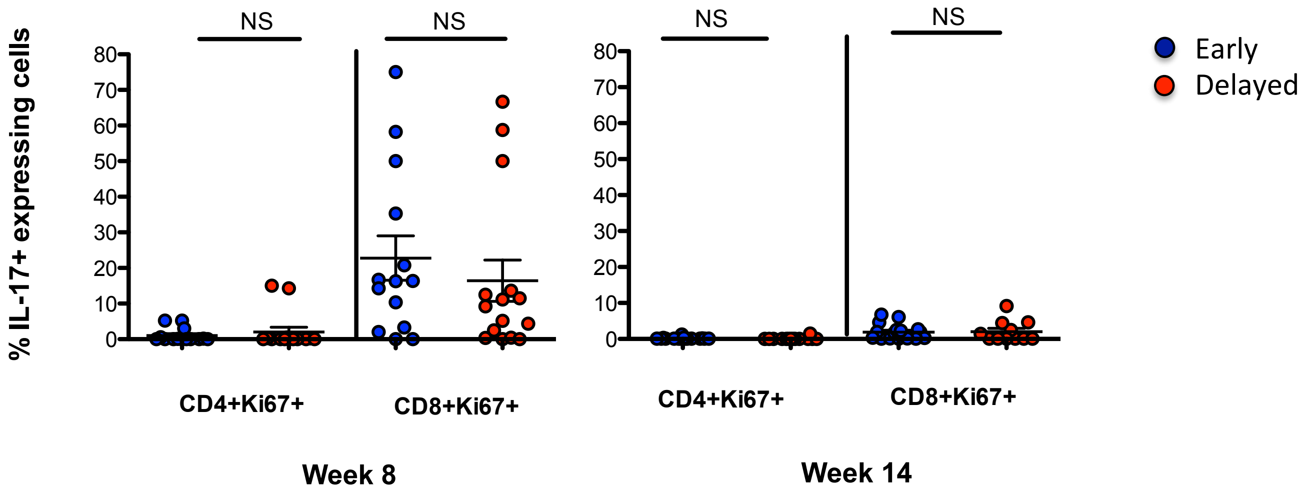


Figure 4.5: The frequency of T cells producing IL-17 at 8 and 14 weeks of life after CpG stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-17 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of pertussis-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported as the frequency of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

4.3.2.2. IL-17 (Th17) expression in response to TT in early-BCG vs. delayed-BCG vaccinated infants 8 and 14 weeks of age

In response to TT stimulation at week 8, birth BCG-vaccinated (early arm) and 8 week BCG-vaccinated infants had similar levels of IL-17 expression by CD4+Ki67+ cells (0.156% vs. 0.252%, p=0.875) (figure 4.6). Levels of IL-17 expression by CD8+ proliferating cells were also comparable between birth- and 8-week BCG-vaccinated infants (1.45& vs.3.29%, p=0.332) (figure 4.6).

At week 14, in response to TT stimulation, birth- and 8 week BCG-vaccinated infants exhibited comparable frequencies of IL-17-expressing CD4+ proliferating cells (p=0.394). They also had similar frequencies of IL-17 expressing-CD8+ proliferating cells (p=0.721) (figure 4.6).

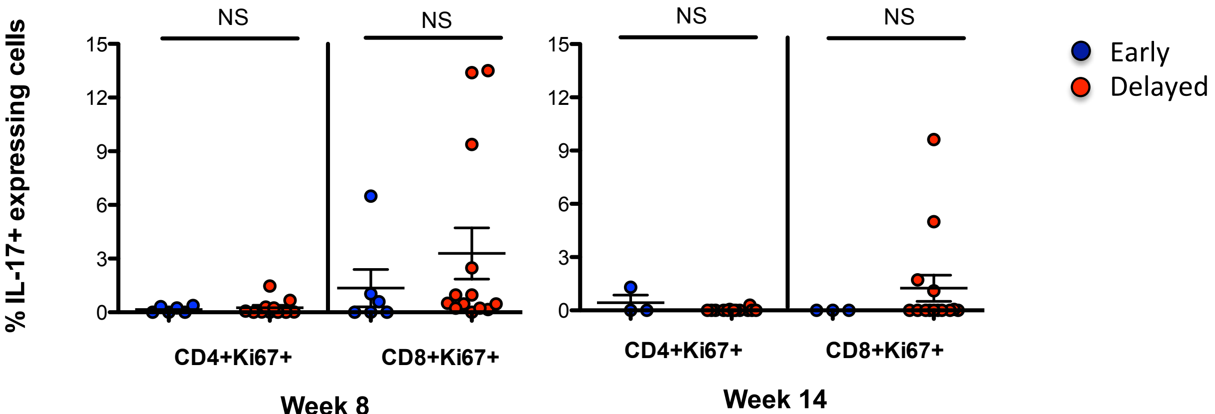


Figure 4.6: The frequency of T cells producing IL-17 at 8 and 14 weeks of life after TT stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-17 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of TT-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported as the frequency of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

4.3.3. IL-13 (Th2/Tc2) expression in response to pertussis and TT in early-BCG vs. delayed-BCG vaccinated infants at 8 and 14 weeks of age

Finally, IL-13 (Th2/Tc2) expression by proliferating CD4+ and CD8+ cells in response to pertussis and TT was assessed at weeks 8 and 14 stimulation using ICS.

4.3.3.1. IL-13 (Th2/Tc2) expression in response to pertussis in early-BCG vs. delayed-BCG vaccinated infants at 8 and 14 weeks of age

At week 8, in response to pertussis stimulation, no difference was found in the frequencies of IL-13-expressing-CD4+Ki67+ cells between infants the early arm and infants in the delayed arm (0.01% vs. 0.081% respectively, p=0.984) as shown in figure 4.7. We also found no difference in the frequencies of and IL-13 expressing-CD8+Ki67+ cells between the early and delayed arms (4.4% vs. 2.69% respectively, p=0.446).

On the other hand, at week 14, infants in the early BCG arm had significantly higher IL-13 expression by CD4+ proliferating cells in response to pertussis compared to those in the delayed BCG arm (0.158% vs.0.024% respectively, p=0.038). This difference was no longer significant after adjustment for multiple comparisons. In addition, birth-vaccinated infants exhibited higher frequencies of IL-13-expressing-CD8+Ki67+ in response to pertussis than 8 week=vaccinated infants (2.11% vs. 0.879% respectively, p=0.0035) (figure 4.7). This difference remained significant after adjustment for multiple comparisons.

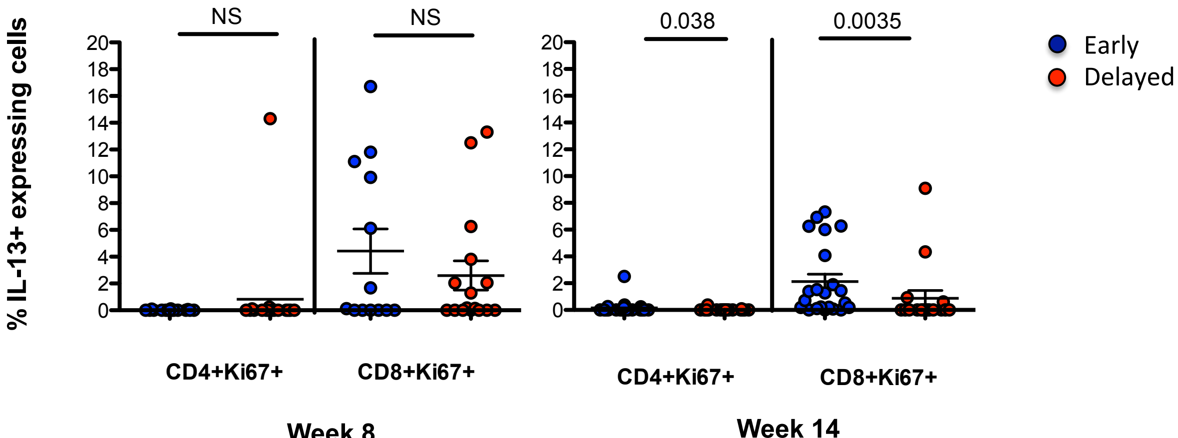


Figure 4.7: The frequency of T cells producing IL-13 at 8 and 14 weeks of life after pertussis stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-13 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of pertussis-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported as the frequency of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

4.3.3.2. IL-13 (Th2/Tc2) expression in response to TT in early-BCG vs. delayed-BCG vaccinated infants at 8 and 14 weeks of age

Infants in the early and delayed arms had similar levels of IL-13-expressing CD4+ proliferating cells following TT stimulation at week 8 (0.352% vs. 0.999%, p=0.488). In addition, at week 8, we observed no difference in the levels of IL-13-expressing-CD8+Ki67+ cells between birth- and 8 week-vaccinated infants (0.627% vs.2.37%, p=0.185) (figure 4.8).

At week 14, infants in the two arms (early and delayed BCG) also had comparable levels of IL-13-expressing-CD4+Ki67+ cells following TT stimulation (0.005% vs. 0.08%, p=0.945). We observed a similar outcome for CD8+ proliferating cells (0.014% VS. 0.141%, p=0.635) (figure 4.8).

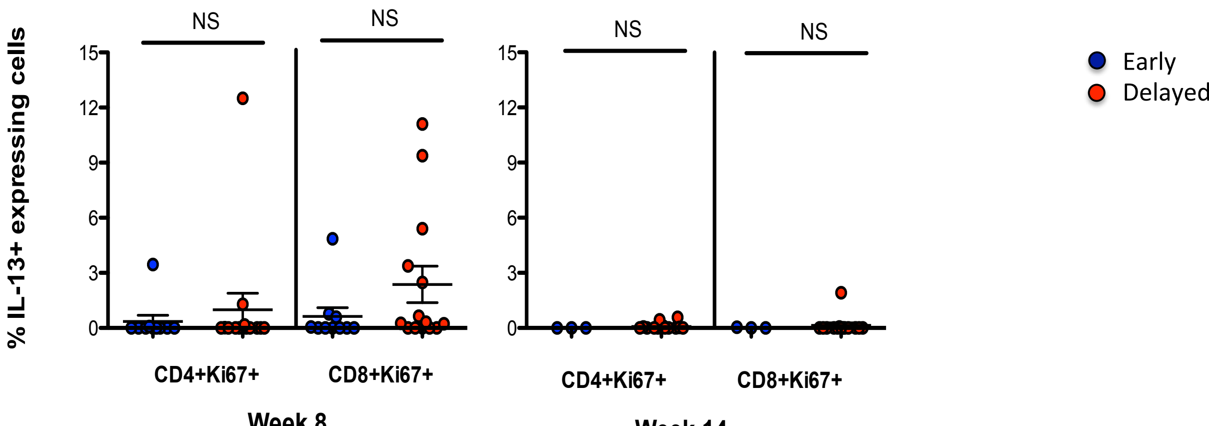


Figure 4.8: The frequency of T cells producing IL-13 at 6,8 and 14 weeks of life after TT stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-13 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of TT-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported as the frequency of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

4.4. T-cell polyfunctional responses to pertussis and TT in early-BCG vs. delayed-BCG vaccinated infants at 8 and 14 weeks of age

In this chapter, we assessed the frequencies of CD4+ and CD8+ proliferating cells expressing any zero, one, two, three or more cytokines simultaneously in early-BCG vs. delayed-BCG vaccinated infants at 8 and 14 weeks in response to pertussis and TT.

4.4.1. T-cell polyfunctional responses to pertussis in early-BCG vs. delayed-BCG vaccinated infants at week 8

4.4.1.1. CD4+ T cell polyfunctional responses to pertussis in early-BCG vs. delayed-BCG vaccinated infants at week 8.

The ability of proliferating T cells to make at least one, a combination of any two, three, or all four cytokines in response to pertussis at week 8 was compared between infants in the early BCG arm (birth vaccinated) and those in

the delayed BCG arm (not yet vaccinated). There was no difference in the frequencies of CD4+ T cells expressing zero, one, two, three cytokines between infants in the two arms at 8 weeks of age. Overall, infants in the early and delayed arms had similar CD4+ T cell polyfunctionality (ANOVA p=0.269) (figures 4.9a and 4.9b).

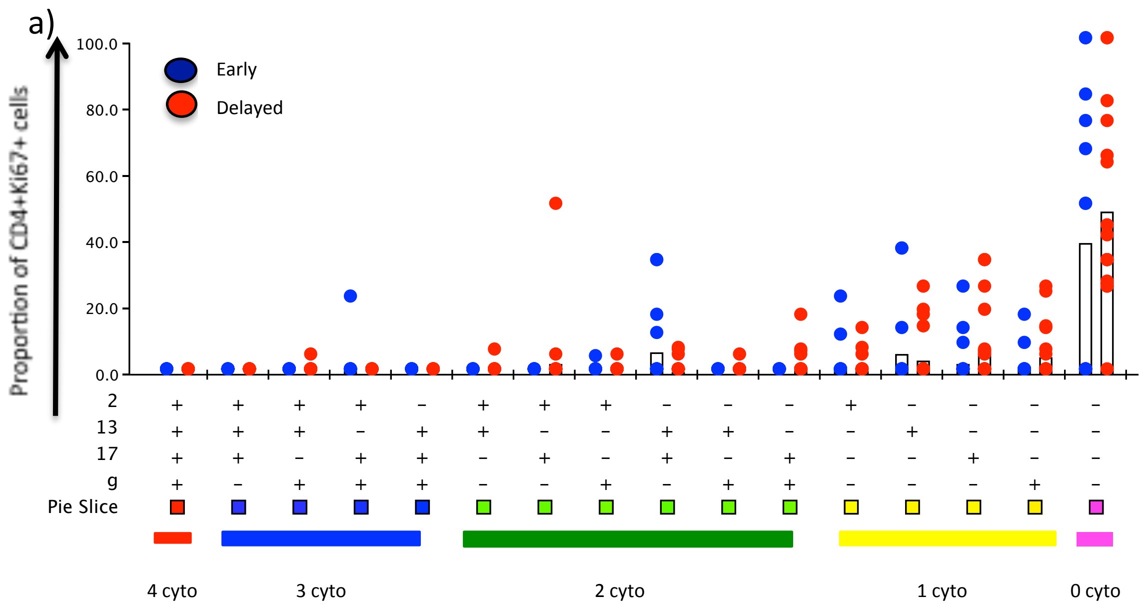
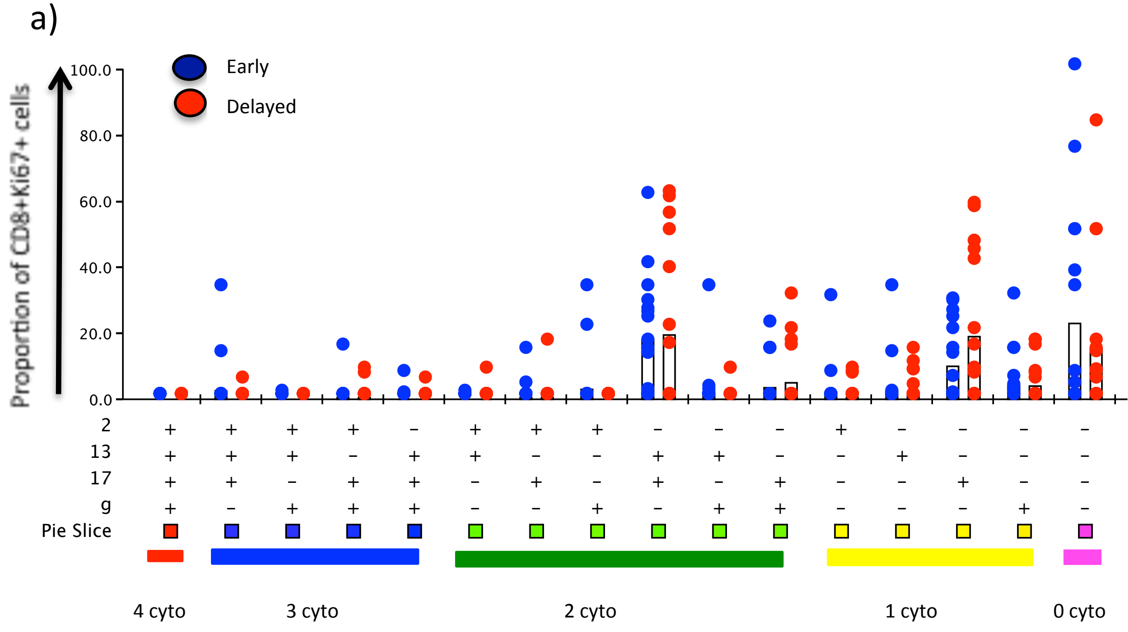


Figure 4.9: Proportions of pertussis-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 8 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD4+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD4+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD4+ T cells. Bars whiskers correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

4.4.1.2. CD8+ T cell polyfunctional responses to pertussis in early versus delayed-BCG vaccinated infants at week 8.

Similar to CD4+ T cells, infants in the early and delayed had comparable levels of CD8+ T cells expressing zero, one, any combination of two or three cytokines simultaneously (figure 4.10a). Overall, there was no difference CD8+ T cell polyfunctionality between birth- (early arm) and 8 week-vaccinated infants at week 8 (ANOVA $p=0.769$) (figure 4.10.b)

a)



b)

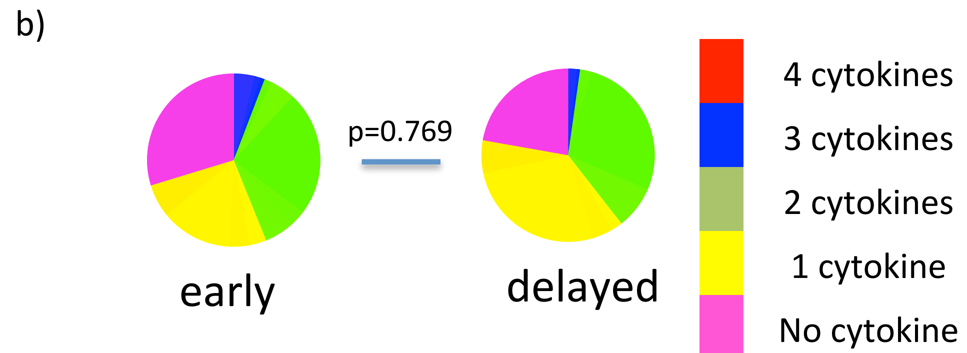


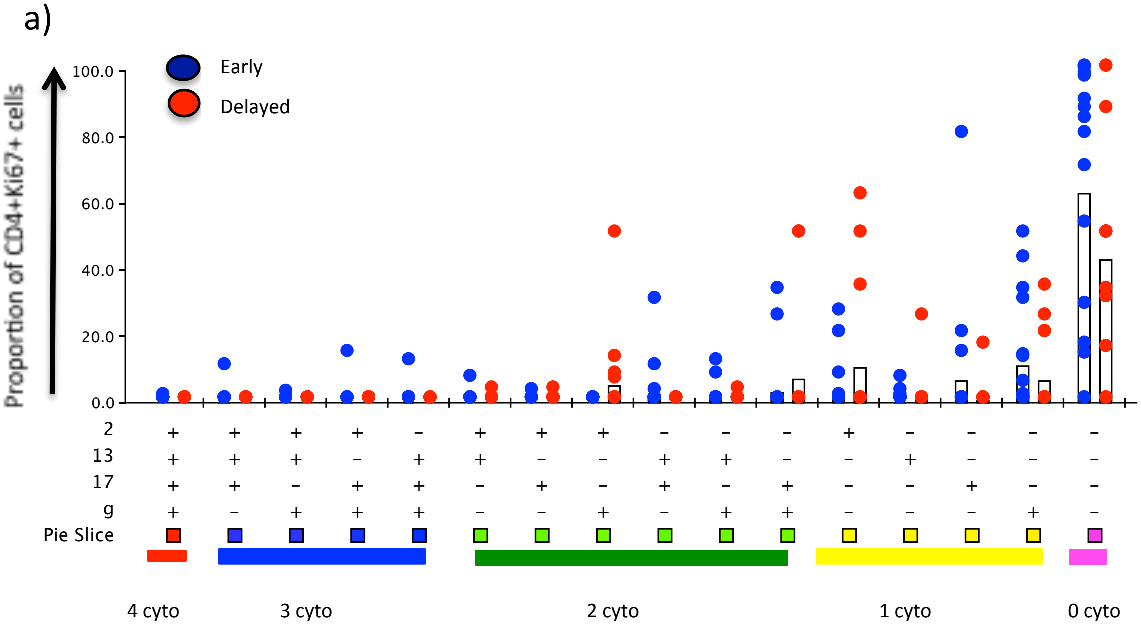
Figure 4.10: Proportions of pertussis-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at.8 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed ran*k test (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

4.4.2. T-cell polyfunctional responses to pertussis in early-BCG vs. delayed-BCG vaccinated infants at week 14

4.4.2.1. CD4+ T cell polyfunctional responses to pertussis in early-BCG vs. delayed-BCG vaccinated infants at week 14.

At 14 weeks of age, in response to pertussis stimulation, infants in the early BCG arm and those in the delayed BCG arm had comparable frequencies of CD8+ cells expressing zero, one, two or three cytokines as shown in figure 4.11.a. In addition, there was no difference between the two arms for any particular cytokine and the overall levels CD4+ T cell polyfunctionality were very similar between the two arms (ANOVA p=0.127) (figure 4.11b).

a)



b)

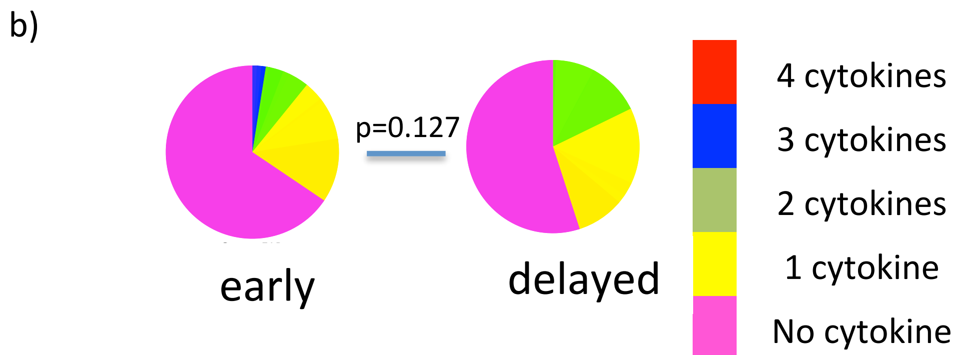
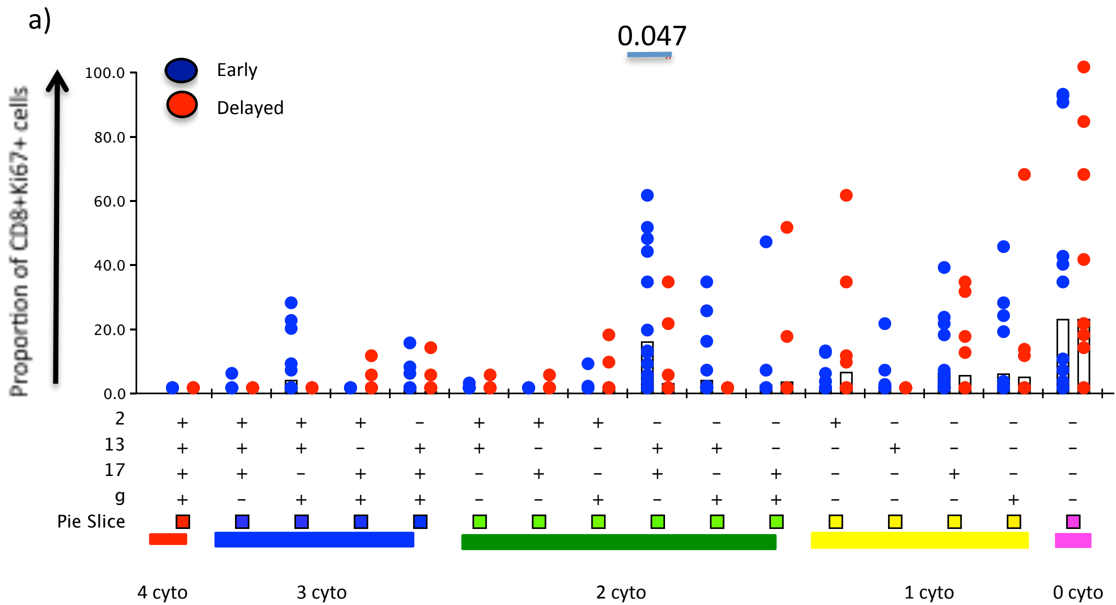


Figure 4.11: Proportions of pertussis-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 14 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD4+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD4+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD4+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed ran*k test (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses

4.4.2.2. CD8+ T cell polyfunctional responses to pertussis in early-BCG vs. delayed-BCG vaccinated infants at week 14.

At week 14, infants in the two arms also had similar levels of CD8+ T cells expressing zero, one, two or three cytokines (figure 4.12a). However, infants in the early BCG arm had significantly higher frequencies of CD8+Ki67+ cells expressing IL-17 and IL-13 simultaneously ($p=0.047$) (figure 4.12a). This difference was no longer significant after adjusting for multiple comparisons. Nonetheless, overall, infants in the two arms had similar levels of CD8+ T cell polyfunctionality in response to pertussis at week 14 (ANOVA $p=0.377$) (figure 4.12b).



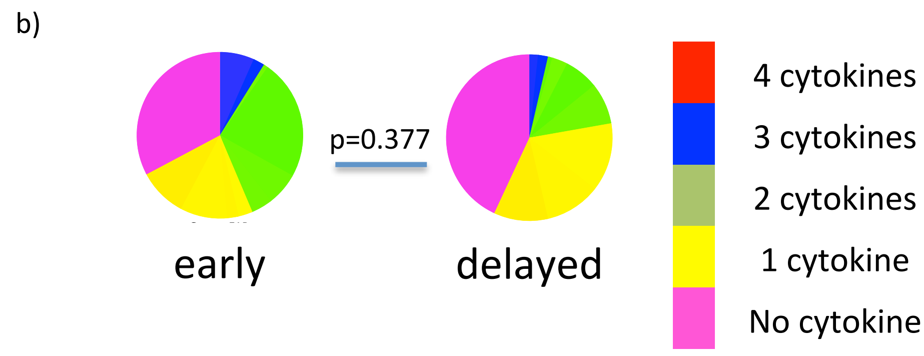


Figure 4 12: Proportions of pertussis-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at.14 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

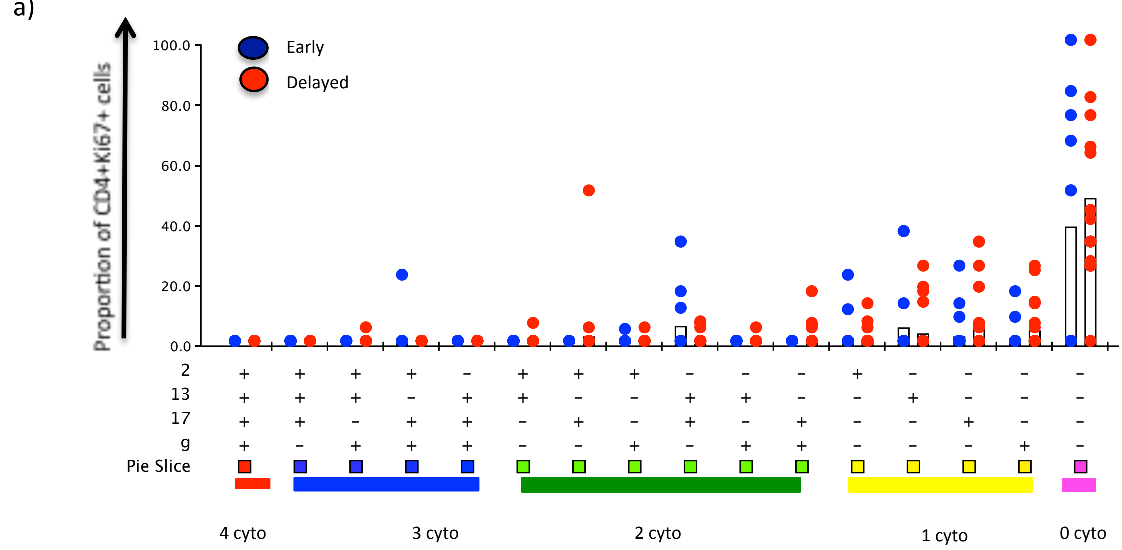
4.4.3. T-cell polyfunctional responses to TT in early-BCG vs. delayed-BCG vaccinated infants at week 8

The ability of proliferating cells to make at least one, a combination of any two, three, or all four cytokines in response to TT at week 8 between infants in the early BCG arm and those in the delayed BCG arm

4.4.3.1. CD4+ T cell polyfunctional responses to TT in early-BCG vs. delayed-BCG vaccinated infants at week 8.

When we assessed the ability of proliferating CD4+ T cells to make at least one, a combination of any two, three, or all four cytokines in response to TT at week 8, infants in the early and delayed arms had comparable levels of CD4+ T cells expressing one, two and three cytokines (figure 4.13a). In addition, Infants in the two arms also had comparable frequencies of proliferating CD4+ T cells not expressing any cytokine (figure 4.13a). Overall, there was no difference in CD8+ T cell polyfunctionality between the two arms at week 8 (ANOVA p=0.272) (figure 4.13b).

a)



b)

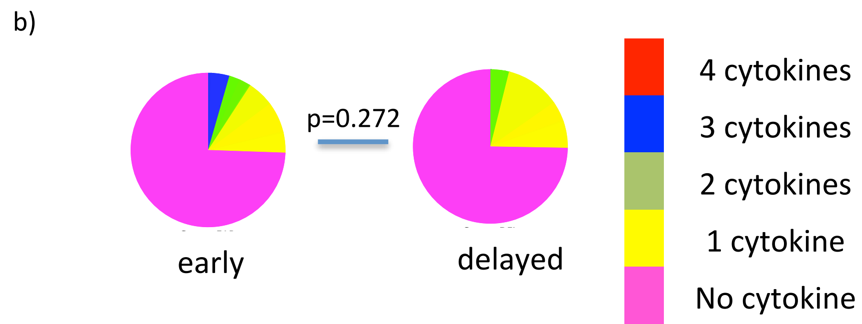
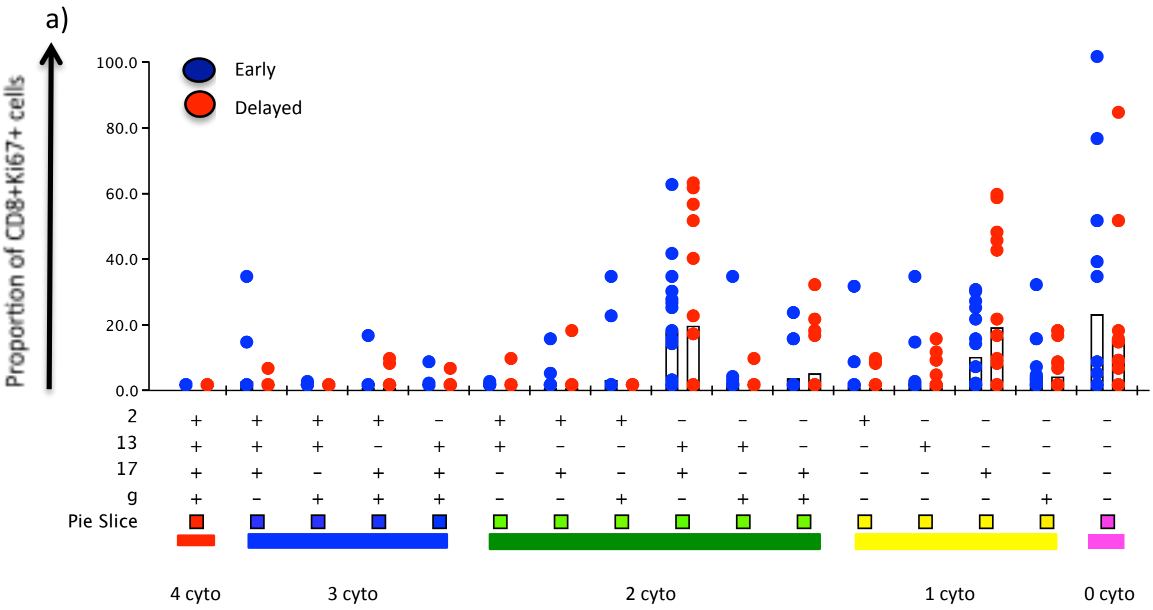


Figure 4.13: Proportions of TT-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 8 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD4+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD4+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD4+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed ran*k test (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

4.4.3.2. CD8+ T cell polyfunctional responses to TT in early-BCG versus delayed-BCG vaccinated infants at week 8.

At week 8, infants in the two arms had similar proportions of CD8+ T cells expressing one, any two or three cytokines following pertussis stimulation (figure 4.14.a). Infants in the early BCG arm and those in the delayed BCG arm also had similar levels of CD8+ proliferating cells not expressing any cytokine (p=1.00 not shown) (figure 4.14a). Finally, overall, there was no difference in CD8+ T cell polyfunctionality between the two arms (ANOVA p=0.771) (figure 4.14b).



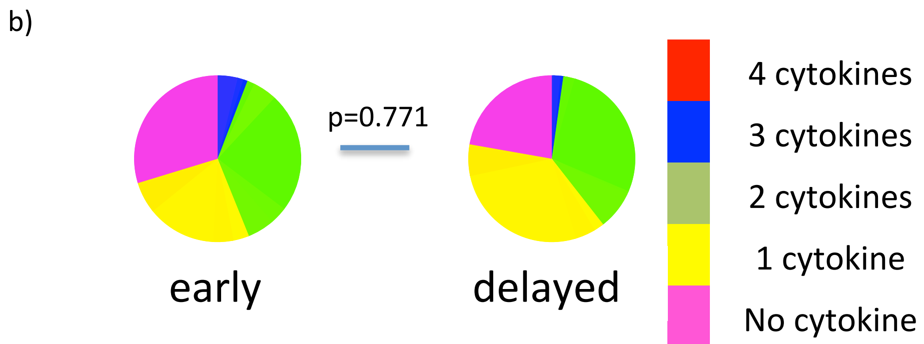


Figure 4.14: Proportions of TT-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 8 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

4.4.4. T-cell polyfunctional responses to TT in birth vs. 8-week vaccinated infants at week 14

Due to the very few number of samples of birth-vaccinated infants available for TT stimulation at week 14, we were unable to compare CD4+ and CD8+ T cell polyfunctional responses to TT between the two arms at week 14.

4.5. Discussion

In this chapter, we aimed to investigate the effects of delayed BCG vaccination on T cell responses to acellular pertussis and TT, two vaccine antigens known to elicit Th2 responses in infants (Ennis et al., 2005; Mills et al.,2001; Rowe et al., 2000). Few studies have assessed how BCG affects cellular responses to TT or pertussis (Ota et al., 2002). This is the first study investigating the effects of delayed BCG vaccination on T cell responses to pertussis and TT in HIV-exposed infants. Our findings demonstrate that delaying BCG vaccination from birth to eight weeks of age does not affect pertussis- and TT-specific CD4+ and CD8+ T-cell polyfunctionality and proliferation measured at 8 and 14 weeks of age in HIV-exposed, uninfected infants. However, birth-vaccinated infants had higher frequencies of pertussis-specific IL-13+-expressing CD4+ and CD8+ cells at 14 weeks than infants vaccinated with BCG at 8 weeks. It is well documented that during HIV infection, there is a change in the Th1/Th2 balance with a gradual shift towards Th2 cytokines (Becker, 2004; Clerici et al., 1994). Thus, HIV-exposed, uninfected infants (particularly those that are predominantly non-breastfed) may have different Th2 cytokine profiles to pertussis and TT antigens compared with unexposed infants. IL-13 is a Th2 cytokine involved in anti-inflammatory responses and IgE-mediated allergic responses (Roussel et al., 1998; Walter et al., 2001) and aP (acellular pertussis) vaccination has been shown to induce IL-13 production (Ennis et al., 2005; Rowe et al., 2000). While there is debate about which arms of the cellular immune response to pertussis are the best correlate of protection (Ausiello et al., 1997; De Gouw et al., 2011; Ross et al., 2013), it is widely accepted that cellular immunity to pertussis is vital for bacterial clearance (Mills et al., 1993; Mills et al., 2001). Some studies have

demonstrated that aP vaccine induces protection against exacerbation of allergic asthma in bordetella pertussis infection through IL-13 production (Ennis et al., 2005). Our findings in this chapter suggest that early BCG vaccination may improve IL-13 T cellular responses to acellular pertussis at week 14.

BCG has been shown to enhance DC production of IL-12 in response to many antigens (Henderson et al.,1997). This subsequently induces strong Th1 responses in neonates (Marchant et al., 1999). It is well documented that and that Th1 and Th2 cells reciprocally inhibit their function (Romagnani, 1997). Thus, observing that earlier BCG could improve Th2 responses to pertussis was unexpected and contrary to our initial hypothesis. However, recent data suggest that neonatal DCs have a defective expression of IL-12 gene and other co-stimulatory molecules (Goriely et al., 2001; Ota et al., 2002). This leads neonatal DCs to induce the secretion of Th1 and Th2 cytokines by neonatal CD4+ T cells upon BCG stimulation (Ohshima et al., 1997). In addition, the observed difference was at 14 weeks after infants in the two arms had received BCG. It is also possible that the Th1 skewing effects of BCG may have worn off in the early BCG arm and as a result higher Th2 cytokine expression was observed.

Ota el al showed that early BCG vaccination enhances Th2 and antibody responses to HBV in HIV-unexposed infants (Ota et al., 2002). They also observed enhanced IL-13 secretion in response to TT at 4 months of age (Ota et al., 2002). However, this increase in Th2 cytokine responses did not affect antibody responses to TT (Ota et al., 2002).In our study, we did not observe any difference in IL-13 expression in response to TT stimulation between birth- and 8-vaccinated infants at 14 weeks. This could be due to the short duration of follow up (14 weeks) and the small sample size at week 14. In addition, contrary to Ota el al who only assessed the levels of soluble cytokines secreted in the plasma, we measured the proportions of cytokines expressed by CD4+ and CD8+ T cells.

Pertussiss and TT vaccines are administered at 6 weeks of age in neonates. The peak immune response to TT vaccine in neonates generally occurs around 27 weeks of age (Soares et al., 2013) and it is not well known when immune responses to pertussis peak (Rowe et al., 2000). Thus, it is possible that giving BCG two weeks only after Pertussis and TT could affect immune responses to these vaccines in those infants. Therefore it is very possible that we missed the possible differences in immune responses to pertussis and TT between the groups due to BCG timing. Thus, in future delayed BCG studies, it would be important to take into consideration the potential effects that BCG could have on pertussis, TT and any other vaccine.

Chapter 5. Effects of Delayed BCG Vaccination on Cellular Responses to SEB

5.1. Introduction

BCG has been reported to improve all cause mortality in Gambian infants (Aaby et al., 2011; Garly et al., 2003) and Ota et al have found that BCG can improve immune responses to certain other antigens and propose that BCG enhances the maturation of the neonatal immune system (Ota et al., 2002). Kleinnijenhuis propose that BCG may have these effects through inducing epigenetic changes in the NOD-2 receptor present in monocytes (Kleinnijenhuis et al., 2012). Thus, in this chapter, using the same assays as described in chapter 3, we aim to determine whether BCG improves T cell proliferative and cytokine responses to the non-recall antigen SEB in HIV-exposed infants. SEB is a super antigen that binds to MHC class II α-chain and the TCR β-chain and leads to massive T cell proliferation (Fraser, 2011).CD4+ and CD8+ T cell responses to SEB can help determine their quality and maturity level of an immune response (Proft et al., 2003). If delaying BCG vaccination for HIV-exposed infants is to be considered an option, it is important to understand the non-specific effects of BCG, and whether BCG vaccination does enhance T cell responses to unrelated antigens, including non-recall antigens, such as SEB in HIV-exposed infants.

5.2. T-cell proliferative responses to SEB in early-BCG versus delayed-BCG vaccinated infants at 6, 8 and 14 weeks of age

At 6 and 8 weeks of age, there was no difference in CD4+ and CD8 T cell proliferative responses to SEB between HIV exposed infants in the early BCG arm versus infants in the delayed BCG arm (figure 5.1). However, at 14 weeks of age, 14 weeks after the early arm infants received BCG, and 6 weeks after the delayed arm was BCG vaccinated, infants vaccinated at with BCG at birth had higher frequency of proliferating CD4+ cells to SEB than infants vaccinated with BCG at 8 weeks of age (early arm median=29,6% vs. delayed arm median=17 %, p=0.018).

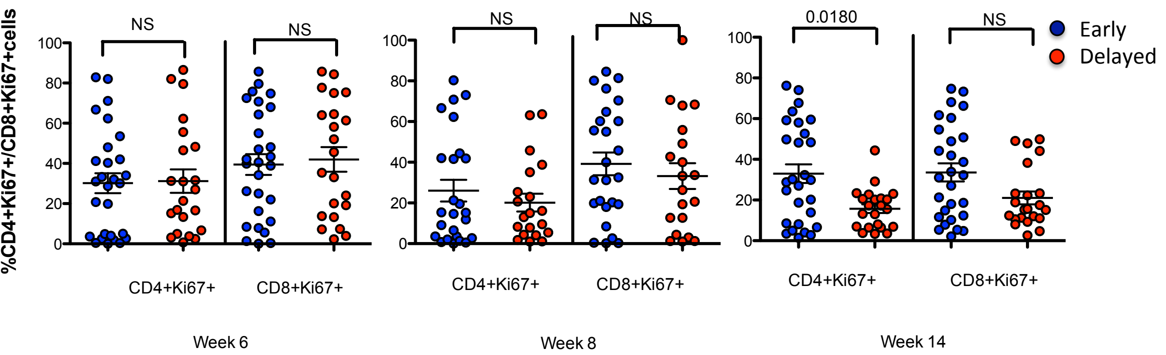


Figure 5.1: Frequency of CD4 and CD8+T cells expressing Ki67+ in response to SEB stimulation determined by flow cytometry in the early (blue) versus delayed (red) BCG vaccinees at 8 and 14 weeks. Lines represent medians and whiskers interquartile ranges. p<0.05 significant level (unadjusted) and statistical significance was obtained using Mann-Whitney U test.

5.3. T cell cytokine expression in response to SEB in early-BCG vs. delayed-BCG vaccinated infants at 6, 8 and 14 weeks of age

5.3.1. IFNγ and IL-2 (Th1/Tc1) expression in response to SEB in early-BCG versus delayed-BCG vaccinated infants at 6, 8 and 14 weeks of age

As shown in figure 5.2, infants in the early BCG arm had a higher proportion of CD8+ T cells expressing IL-2 in response to SEB stimulation at weeks 6 compared

to infants in the delayed BCG arm at 8 weeks (p = 0.035); this difference was not significant after adjustment for multiple comparisons. At 8 and 14 weeks of age, infants in the early and delayed BCG arms had similar frequencies of CD4+Ki67+ and CD8+Ki67+ cells expressing IL-2 (figure 5.2). There was no difference in the frequencies of CD4+Ki67+and CD8+Ki67+ cells expressing IFN-γ in response to SEB stimulation at 6, 8 and 14 weeks between the two arms (figure 5.2).

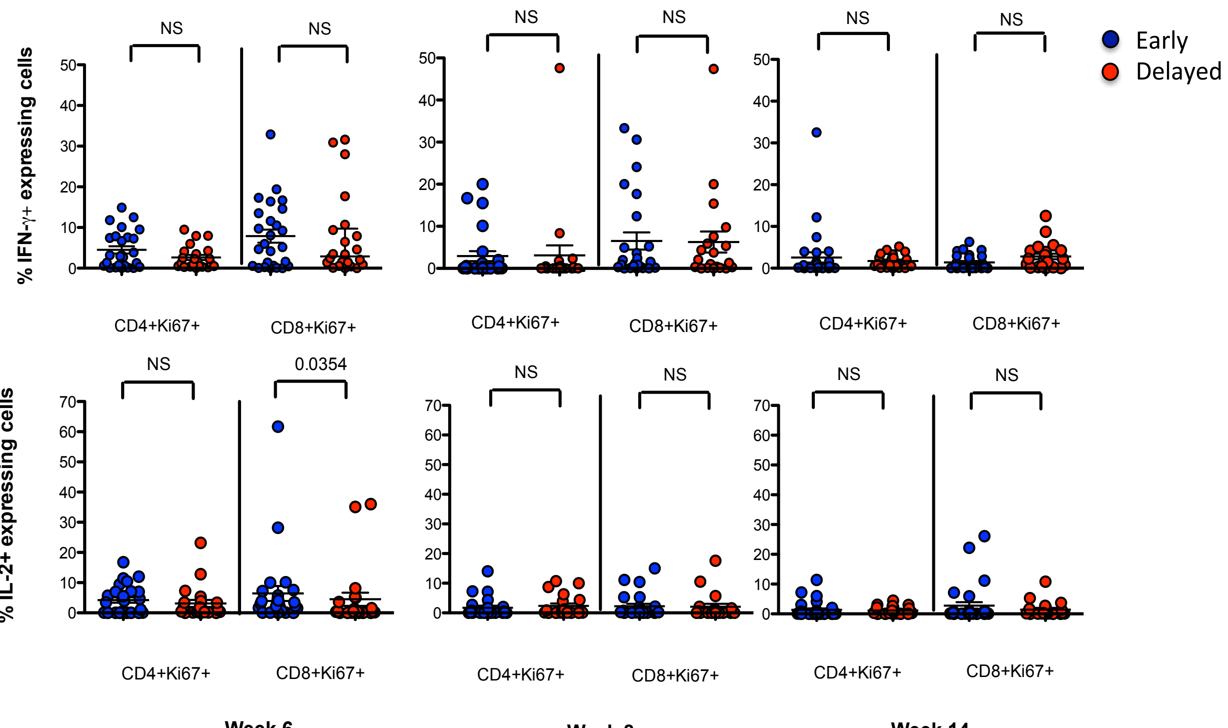


Figure 5.2: The frequency of T cells producing IFNγ and IL-2 at 6,8 and 14 weeks of life after SEB stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IFNγ and IL-2 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of SEB-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported are the frequencies of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

5.3.2. IL-17 (Th17/ Tc17) expression in response to SEB in early-BCG versus delayed-BCG vaccinated infants at 6, 8 and 14 weeks of age

When compared, there was no difference in the frequencies of CD4+Ki67+ and CD8+Ki67+ expressing IL-17 in response to SEB between infants in the early BCG arm and those in the delayed BCG arm at any of the three time points. Except for CD8+Ki67+ cells at 8 weeks of age, as it was previously observed for BCG stimulation (section 4.3.2), infants in the two arms exhibited very low levels of IL-17 expression in response to SEB stimulation (Figure 5.3).

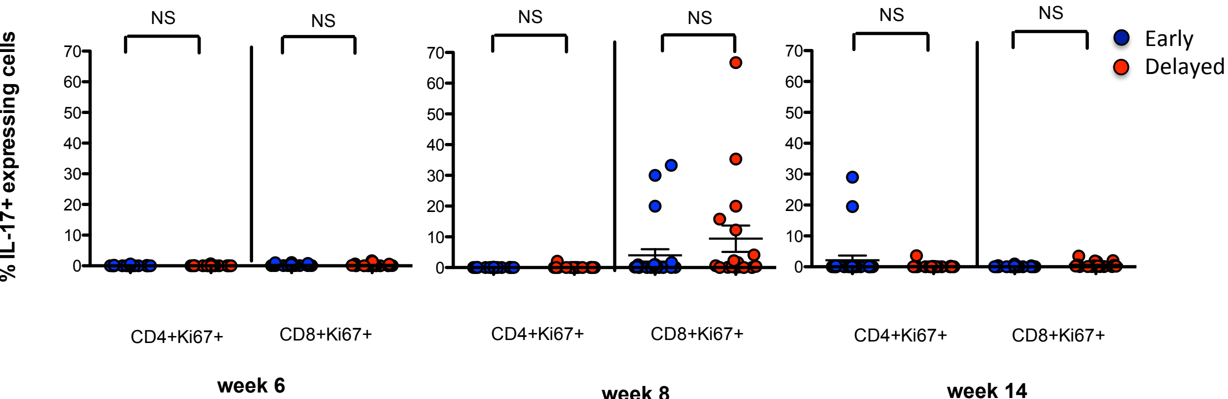


Figure 5.3: The frequency of T cells producing IL-17 at 6,8 and 14 weeks of life after SEB stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-17 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of SEB-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported are the frequencies of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

5.3.3 IL-13 (Th2/Tc2) expression in response to SEB in early-BCG versus delayed-BCG vaccinated infants at 6, 8 and 14 weeks of age

As indicated in figure 5.4, at 6, 8 and 14 weeks, infants in the early and delayed BCG arms had similar levels of CD4+Ki67+ and CD8+Ki67+ cells expressing IL-13 in response to SEB stimulation.

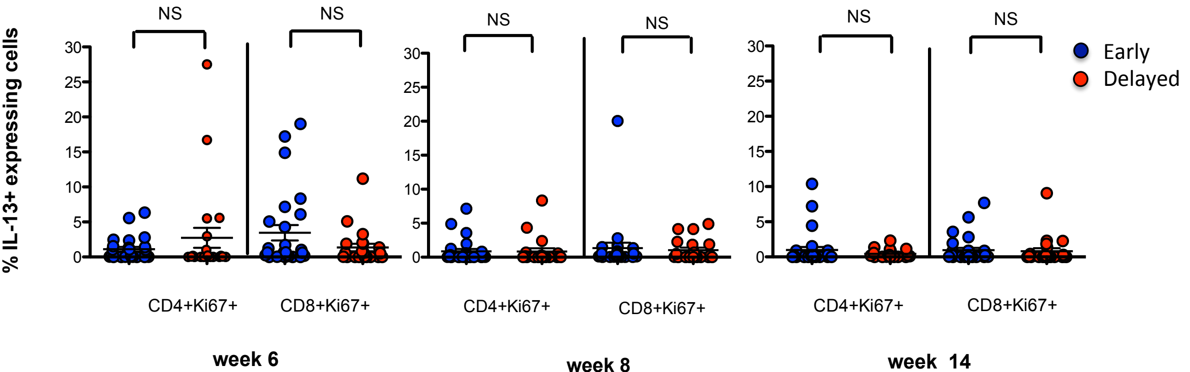


Figure 5.4: The frequency of T cells producing IL-13 at 6, 8 and 14 weeks of life after SEB stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-13 producing T cells out of total CD4+ (on the left side of each graph) and CD8+ (on the right side of each graph) T cells, determined by flow cytometry of SEB-stimulated whole blood of HIV-exposed infants vaccinated with BCG at birth (early arm) (blue dots) compared to HIV-exposed infants vaccinated with BCG at 8 weeks of age (delayed arm) (red dots). Frequencies reported are the frequencies of proliferating cytokine producing cells out of total CD4+ or CD8+ T cells. Bars indicate medians; alpha=0.05 (unadjusted); statistical significance was tested using *Mann-Whitney U* test.

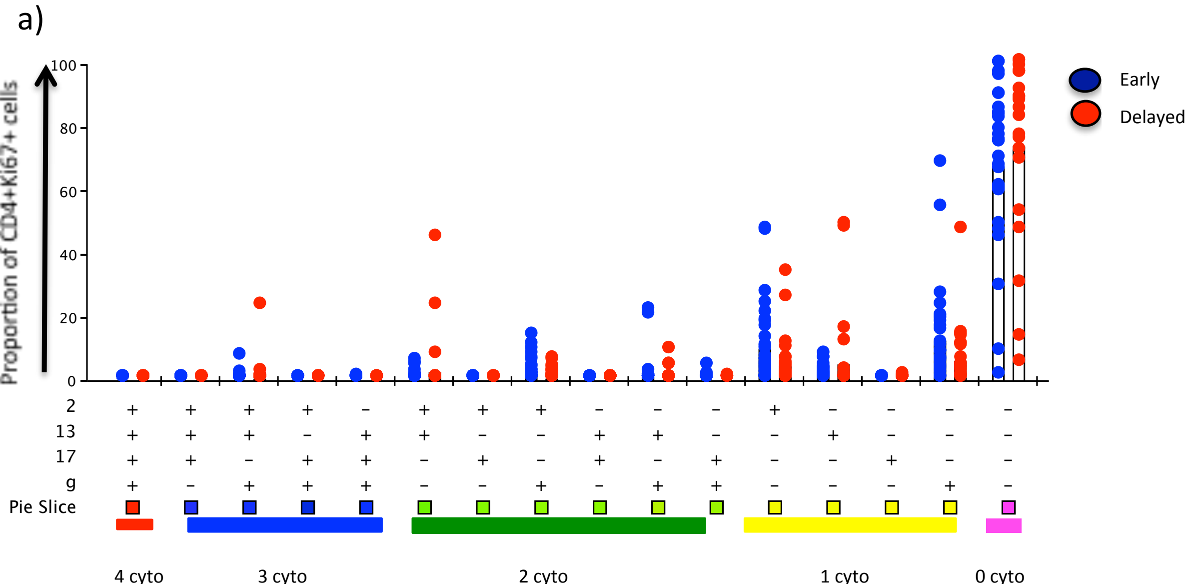
5.4. T-cell functional responses to SEB in in early-BCG vs. delayed-BCG vaccinated infants at 6, 8 and 14 weeks of life

5.4.1.1 CD4+ T-cell polyfunctional responses to SEB in in early-BCG vs. delayed-BCG vaccinated infants at week 6

The ability of proliferating cells to make at least one, a combination of any two, three, or all four cytokines in response to SEB at week 6 between infants in the early arm versus those in the delayed arm were compared. There were no significant differences in the expression of the different combinations of

cytokines (figure 5.5a). Overall, there was also no significant difference in CD4+ cell polyfunctionality as indicated in figure 5.5b (overall ANOVA p = 0.216).

a)



b)

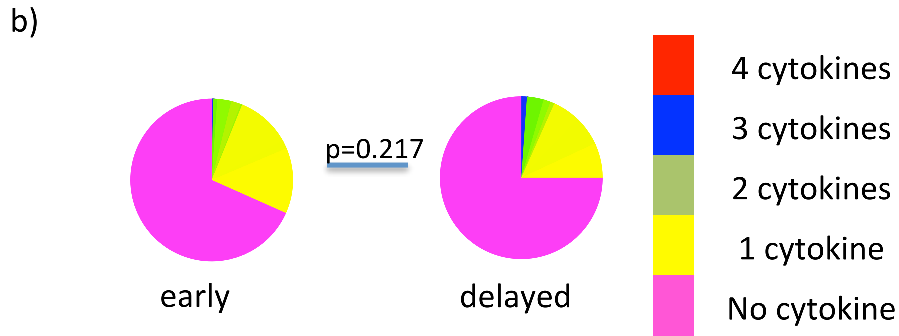
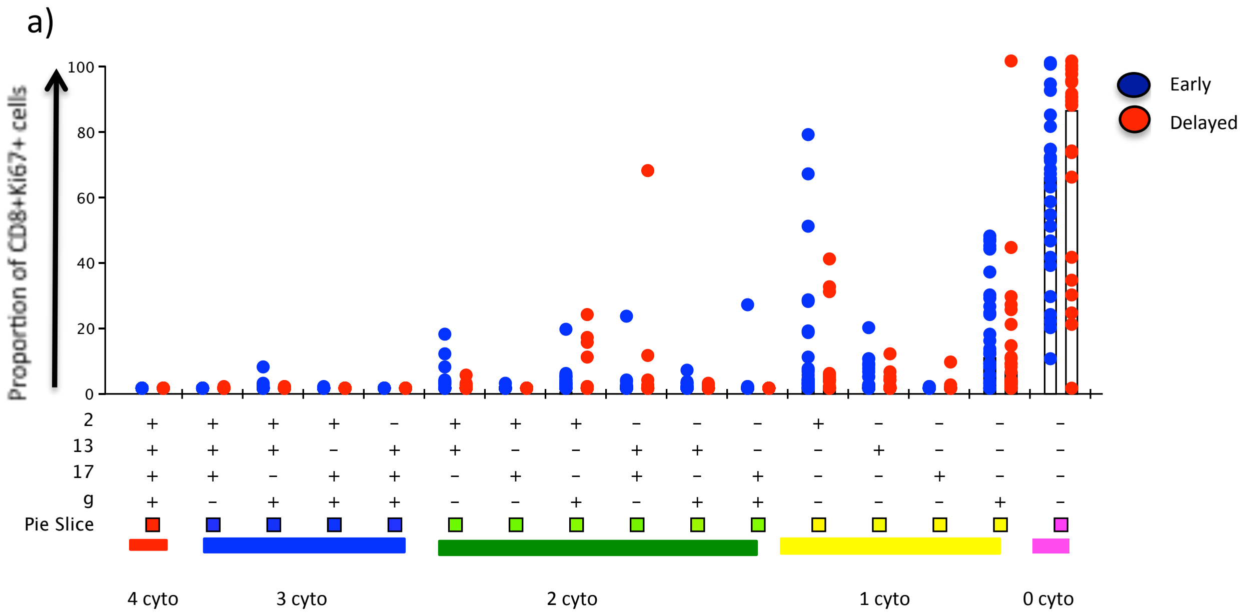


Figure 5.5: Proportions of SEB-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 6 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD4+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD4+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD4+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

5.4.1.2. CD8 T-cell polyfunctional responses to SEB in early-BCG vs. delayed-BCG vaccinated infants at week 6

Similar to CD4+ T cell cytokine responses, at week 6, no difference in CD8+ polyfunctional cytokine responses was observed between early BCG and delayed BCG-vaccinated infants. There was no difference in the frequencies of all the different combinations of cytokines expressed by CD8+ cells in response to SEB (figure 5.6a) between infants in the early BCG arm and those in the delayed BCG arm. In addition, as indicated in figure 5.6b, there was also difference in the overall CD8+ cell polyfunctionality between infants in the two arms (ANOVA p = 0.264).

a)



b)

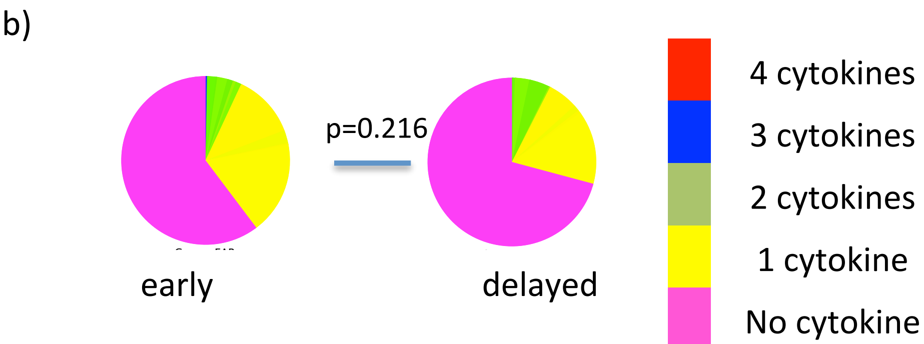
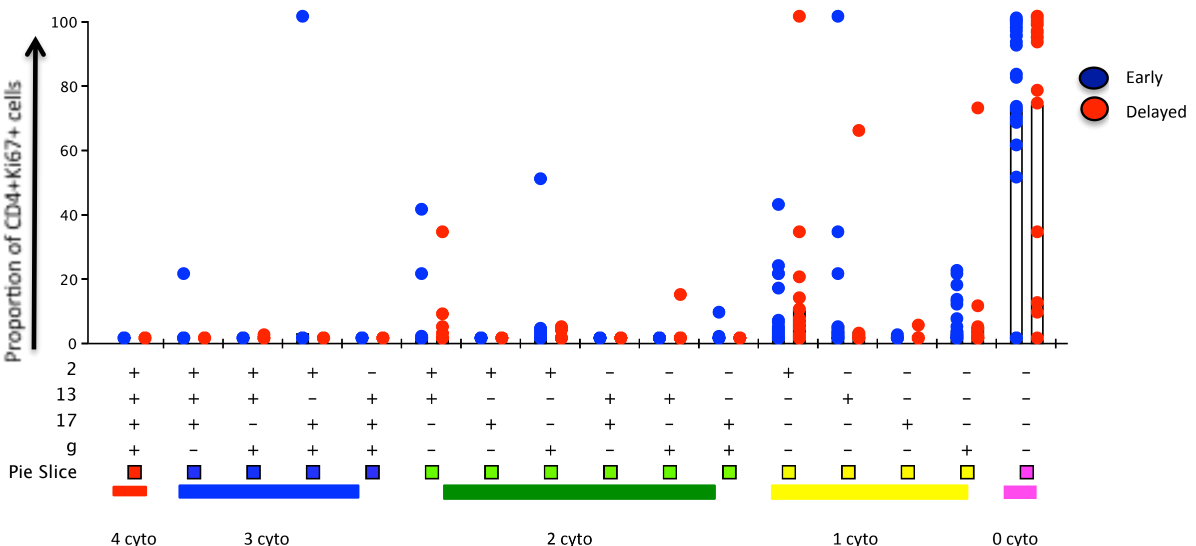


Figure 5.6: Proportions of SEB-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 6 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Bars correspond to medians respectively; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed ran*k test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

5.4.2.T-cell polyfunctional responses to SEB in in early-BCG vs. delayed-BCG vaccinated infants at week 8

5.4.2.1 CD4 T-cell polyfunctional responses to SEB in in early-BCG vs. delayed-BCG vaccinated infants at week 8

The polyfunctionality of proliferating CD4+ cells following SEB stimulation was compared between the two BCG vaccination arms by assessing the frequencies of cells making zero, any one, or any combination of two or three cytokines at week 8 (Figure 5.7). There was no difference in CD4+ cytokine responses between infants in the early BCG arm and those in the delayed arm (figure 5.7a). Overall, CD4+ cell polyfunctionality was similar between the two arms at week 8 (ANOVA p =0.951) (Figure 5.7b).



b)

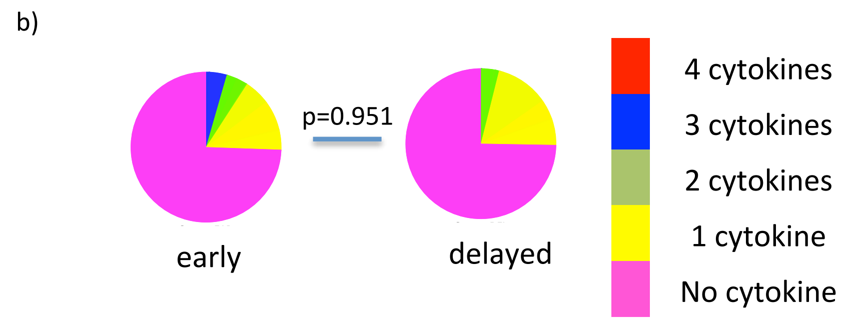
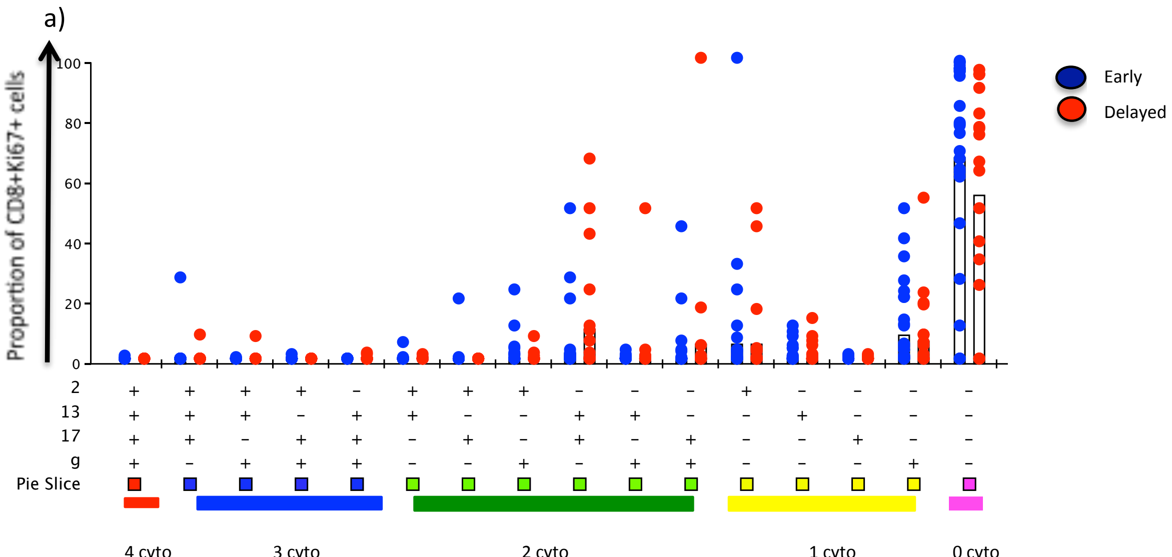


Figure 5.7: Proportions of SEB-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 8 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD4+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD4+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD4+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed ran*k test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

5.4.2.2 CD8 T-cell polyfunctional responses to SEB in in early-BCG vs. delayed-BCG vaccinated infants at week 8

When the polyfunctionality of proliferating CD8+cells following SEB stimulation was assessed in infants in the early and delayed BCG arms at 8 weeks of age, no difference was found in CD8+ polyfunctional cytokine responses after SEB stimulation between infants who received BCG at birth and those who received BCG at 8 weeks of age (ANOVA p = 0.269) (figure 5.8b).



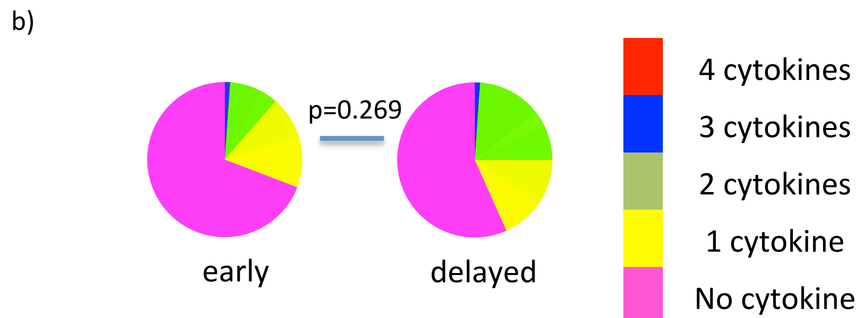
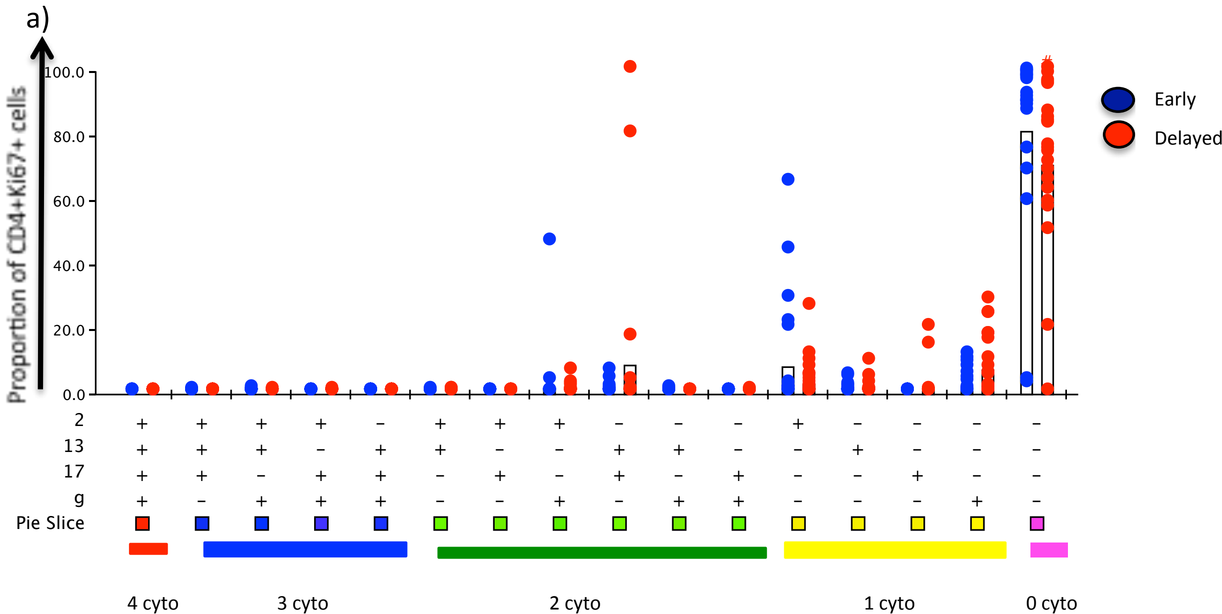


Figure 5.8: Proportions of SEB-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 8 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

5.4.3.T-cell polyfunctional response to SEB in in early-BCG vs. delayed-BCG vaccinated infants at week 14

5.4.3.1 CD4 T-cell polyfunctional responses to SEB in in early-BCG vs. delayed-BCG vaccinated infants at week 14

At week 14, 14 weeks after birth vaccination and 6 weeks after delayed vaccination, infants in the early and delayed arms had similar levels of CD4+ cells expressing one, two and three cytokines in response to SEB as shown in figure 5.9a. In addition, frequencies of CD4+ cells expressing zero cytokine were also similar between the two arms. Overall, there was also no difference in CD4+ polyfunctionality between infants in the two arms (ANOVA $p = 0.676$) (see figure 5.9b below).



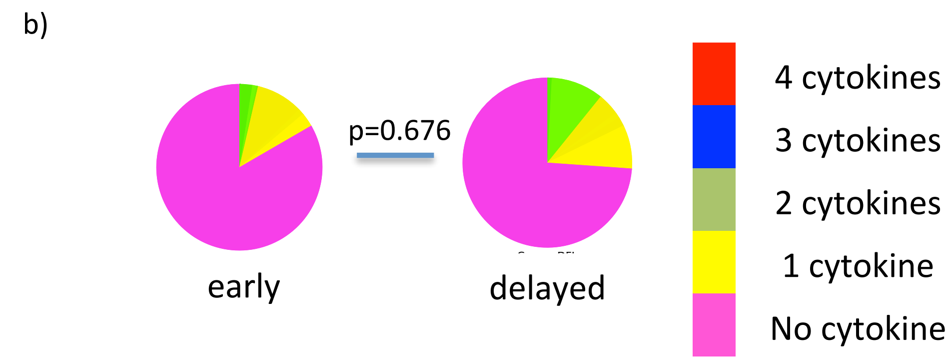
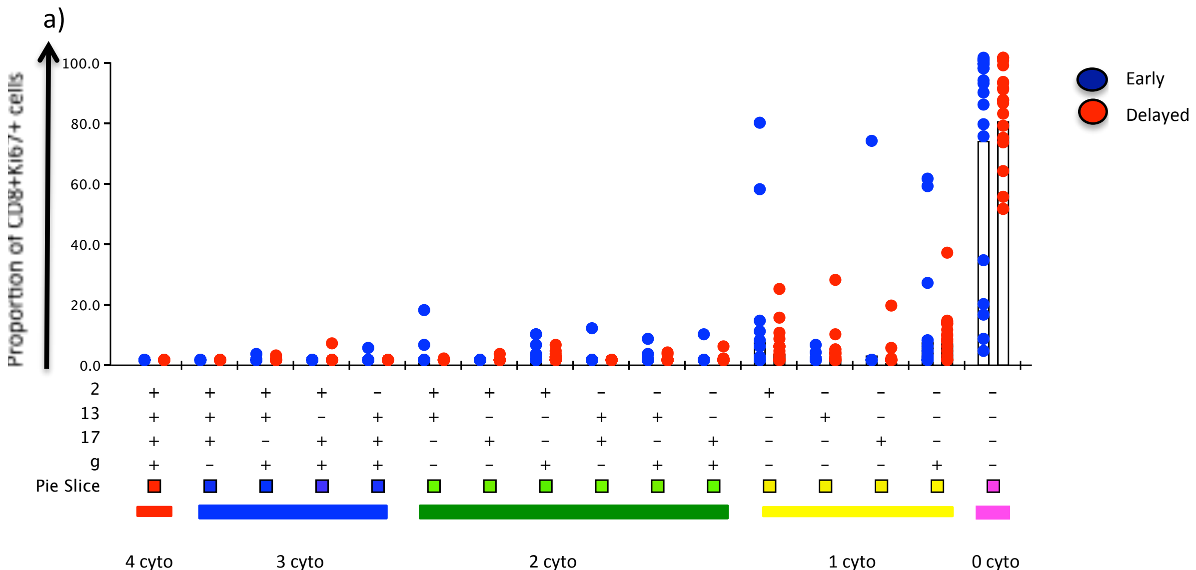


Figure 5.9: Proportions of SEB-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 14 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD4+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD4+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD4+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

5.4.3.2 CD8+ T-cell polyfunctional responses to SEB in birth vs. 8-week vaccinated infants at week 14

Similar to CD4+ cells, frequencies of CD8+ cells expressing one, two and three cytokines in response to SEB were similar between infants in the two BCG vaccination arms at 14 weeks of age (figure 5.10a). In addition, as noted in figure 5.10a, frequencies of CD8+ cells expressing zero measurable cytokine were also similar. Overall infants in the two BCG arms exhibited similar CD8+ T cell polyfunctionality (ANOVA p =0.144).



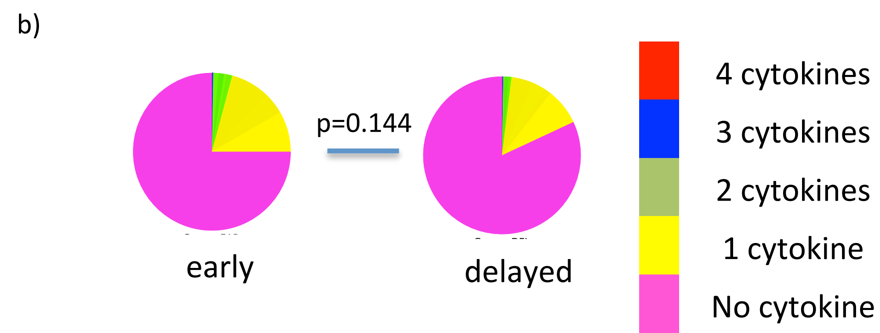


Figure 5.10: Proportions of SEB-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 14 weeks of age in birth-vaccinated (early group) infants versus 8-week-vaccinated (delayed group) HIV-exposed infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in infants in the early arm (blue dots) versus infants in the delayed arm (red dots). (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Bars correspond to medians; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between the two groups. Statistical analysis was performed using *Wilcoxon signed rank* test. (b) The pie charts at the bottom summarise the data with the pie slice legend on the graph showing the colours of the various cytokine combinations and the p-value showing the overall significance of the total magnitude of the combinations of cytokine responses.

5.5 Discussion

This chapter aimed to investigate the effects of BCG vaccination on immune responses to non-vaccine antigens in HIV-exposed infants, in particular, the superantigen SEB. Few studies have investigated neonatal T cell responses to SEB in HIV-exposed infants. Kagina et al assessed the effects of delayed BCG vaccination on CD4+ T cell cytokine response to SEB in HIV-unexposed neonates (Kagina et al., 2009). Assessing the affect of BCG vaccination in non-recall antigens can help to understand the non-specific effects of BCG on neonatal T cell response to unrelated antigens and the overall adjuvant effects of BCG on the immune system. Also, responses to SEB represent the full potential of cells to respond to stimulation. In order to do this, the effects of delayed BCG vaccination (from birth to 8 weeks) on T cell proliferative and cytokine responses to SEB were assessed in HIV-exposed, uninfected infants at weeks 6, 8 and 14 using intracellular staining as described in chapter 3. Delaying BCG vaccination from birth to 8 weeks does not seem to affect CD4+ and CD8+ T cell proliferative responses to SEB at 6 and 8 weeks of age. However, infants in the early BCG arm had higher CD4 T cell proliferative responses to SEB compared to infants in the delayed BCG arm at week 14, after both groups had received BCG vaccination. Finally, no differences in CD4+ and CD8+ cytokine responses to SEB were found between infants in the two BCG arms. In addition, infants in the early and delayed arms exhibited similar CD4+ and CD8+ polyfunctional cytokine responses following SEB stimulation at weeks 6, 8 and 14.

Since it was observed that HIV-exposed infants vaccinated with BCG at birth (early arm) had higher CD4 T cell proliferative response to SEB at 14 weeks of age than infants who received BCG at 8 weeks of age (i.e. 14 weeks prior versus 6 weeks prior), it is possible that early BCG vaccination subtly enhances the maturation of the neonatal immune system. Studies in adults and mice suggest that mycobacteria such as BCG can enhance DC maturation and production of IL-12 that subsequently leads to an increase in T cell activity (Henderson et al., 1997). More recently, Larsen et al also showed that DC maturation was

enhanced in the presence of BCG (Larsen et al., 2007). In addition, DCs are not the only APCs affected by BCG. It has also been demonstrated that BCG vaccination increases monocytes responses to unrelated antigens in healthy individuals through a mechanism that involves epigenetic changes in the monocytes through their NOD2 receptor (Kleinnijenhuis et al., 2012).This trained immunity could explain the increased non-specific immune responses to other antigens induced by BCG. Thus, it is plausible that trained immunity induced by BCG could enhance non-specific T cells responses to SEB as observed in this chapter. However, we observed very minor alterations in SEB responses in this study. All these previous observations were made in HIV-unexposed individuals, and it is unclear whether the same phenomena are likely to be true also in HIV-exposed individuals.

Finally, BCG timing did not seem to have any effect on CD4+ and CD8+ cytokine responses and polyfunctionality. These findings were consistent with previous reports of CD4+ T cell cytokine responses to SEB following delayed BCG vaccination in HIV-unexposed infants (Kagina et al 2009). Kagina et al found that at 20 weeks, there was no difference in the frequencies of IFNγ-expressing CD4+ cells in response to SEB stimulation in HIV-unexposed infants who received BCG at birth and those who received it at 10 weeks of age (Kagina et al., 2009). In addition, they also observed no difference in CD4 T cell polyfunctionality in response to SEB between early and delayed arms at 20 weeks of age (Kagina et al., 2009). Thus, BCG does not seem to affect CD4 or CD8 T cell cytokine responses to SEB.

The low cytokine responses to SEB in general in these infants may be specific to superantigen responses in the neonatal period. In fact, Hayward et al reported that Th1 and Th2 cytokine responses to SEB were seven times lower in human neonates than in adults (Hayward et al., 1994).Cusumano et al also showed that neonatal mice exhibited very low levels of IFNγ and IL-2 in response to SEB stimulation (Cusumano et al., 1997). However, since SEB is a strong stimulus, it may be that by day 6, the cytokine production potential of the cells in our

experiments was exhausted. The results in this chapter are also consistent with Islander et al who demonstrated that neonatal T cells express very low IL-17 levels following SEB stimulation (Islander et al., 2010). A large proportion of neonatal CD4+ T cells are naïve T cells characterized by the high expression of the CD45RA receptor (Storek J et al., 1995) and CD45RA has been associated with low T cell responses to SEB (Hayward et al., 1994). In addition, low cytokine responses to SEB may also be due to the need for additional stimulation other than just cross-linkage of the TCR and MHC II in infancy. Neonatal dendritic cells and other APCs are characterized by their low expression of MHCII molecules (Velilla et al., 2007) and optimal cross-linkage of the MHCII complex and the TCR is necessary to induce good cytokine responses to SEB (Fraser, 2011).

Consistent with previous studies carried out in neonates, low Th1, Th17 and Th2 cytokine responses to SEB were found in these young infants (Islander et al., 2010; Hayward et al., 1994). A longer duration of follow up would therefore be informative to assess the longitudinal effects of delayed BCG vaccination on cytokine responses to SEB. Despite these limitations, the findings in this chapter show that early BCG vaccination may have some limited non-specific effects on the neonatal immune system. As a result, it is important to weigh the risks and benefits of delayed BCG vaccination in neonates.

Chapter 6. Conclusion

Bacille Calmette-Guerin (BCG), a live attenuated *Mycobacterium bovis* strain, is administered to almost 100 million children at birth annually and remains the only licensed vaccine for TB prevention (WHO epidemiologic report, 2004).. BCG induces strong Th-1 responses even in neonates, who have a Th-2 biased immune system, and protects against severe forms of childhood TB such as TB meningitis and miliary TB in immunocompetent children (Marchant et al., 1999). In addition BCG immunogenicity may increase with vaccination age as the neonatal immune system develops (Hussey et al., 2002). In fact, delaying BCG vaccination from birth to 10 weeks has been shown to enhance memory CD4 T-cells in South African HIV-unexposed, uninfected infants (Kagina et al., 2009). Furthermore, data suggest that early BCG vaccination may improve immune responses to other antigens and unrelated vaccines such as OPV and HBV (Ota et al., 2002).

Many TB-endemic endemic regions such as South Africa are also affected by HIV. BCG has been associated with serious adverse events due to BCG immune reconstitution inflammatory syndrome (BCG IRIS) and disseminated BCG disease in HIV-infected infants. Although, HIV-infection is a contraindication to BCG vaccination, the vaccine continues to be administered to HIV-exposed, uninfected neonates. Very little is known about BCG immunogenicity in HIV-exposed infants (Hesseling et al., 2006; Rabie et al., 2011).Recent studies demonstrate reduced IFN-γ and TNF-α production by HIV-exposed infants in response to BCG (Mazzola et al., 2011; Van Rie et al., 2006).In addition, to the previously observed altered immune features of the HEU infants in response to BCG vaccination, this dissertation has shown that:

(i) Delaying BCG vaccination from birth to 8 weeks of age results in improved T cell functionality and higher frequencies of IFN-γ-expressing CD4+ T cells in

response to BCG in HIV-exposed infants at 14 weeks of age.

(ii) Early BCG vaccination induces higher CD4+ T cell proliferation in response to SEB in HIV-exposed infants at 14 weeks of age.

(iii) Early BCG vaccination leads to higher frequencies of IL-13-expressing CD4+ and CD8+ T cells in response to pertussis at 14 weeks of age. Thus, early BCG vaccination may alter immune responses to acellular pertussis in HIV-exposed infants.

In chapter 3, when the effects of delayed BCG vaccination on CD4+ and CD8+ proliferative and cytokine responses to BCG were assessed, delayed BCG vaccination did not seem to have any effect on T cell proliferation. This finding was in close agreement with other infant studies which show that delaying BCG vaccination from birth to six or ten weeks does not affect T cell proliferative responses in HIV-unexposed infants (Kagina et al., 2009; Lutwama et al., 2013). On the other hand, still in chapter 3, following BCG stimulation, we observed that infants in the delayed BCG arm (receiving BCG at 8 weeks) had higher frequencies of IFN-γ-expressing CD4+ T cells compared to infants in the early BCG arm at 14 weeks of age. Kagina et al. also observed increased in frequencies of IFN-γ-expressing CD4+ T cells at 20 weeks in South African HIV-unexposed infants who received BCG at 10 weeks (Kagina et al., 2009). As discussed in chapter 3, it is well documented that Th1 responses increase with time (Marodi, 2002). Therefore, it is possible that the increase in Th1 cytokines (especially IFN-γ-) be due to the more developed immune system of infants in the delayed BCG arm at the time they received their BCG vaccine. This could also explain why infants in the delayed BCG arm exhibited higher CD4+ and CD8+ T cell polyfunctionality in response to BCG at 14 weeks of age. Furthermore, the effects observed at week 14 could simply be due to the waning of the response to BCG in the early BCG arm. The immune correlates of protection against TB are unknown, therefore, it is not clear how increased Th1

cytokines and T cell polyfunctionality in the delayed BCG arm affect BCG efficacy but it is thought that increased CD4+ T cell polyfunctionality and Th1 cytokines are important for TB control (Beveridge et al., 2007; Mueller et al., 2008).

In chapters 4 and 5, the non-specific effects of BCG were determined by assessing T cells responses to pertussis and TT vaccines and the superantigen SEB. At week 14, infants in the early BCG arm had higher CD4+ proliferative responses to SEB. SEB is a super antigen that binds to MHC class II α-chain and the TCR β-chain and leads to massive T cell proliferation (Fraser, 2011). Responses to SEB can be used to determine the maturity of the immune system. Thus, the higher CD4+ proliferative responses to SEB observed in infants in the early BCG arm may imply that BCG vaccine can enhance the neonatal immune system. It is not clear how BCG enhances the immune system maturity but in a recent study Kleinnijenhuis et al., showed that early BCG vaccination leads to the reprogramming and increased activity in monocytes (Kleinnijenhuis et al., 2012). Thus, BCG can enhance APCs activity and subsequently lead to improved T cell proliferative responses. These BCG non-specific effects could lead to improved mortality in infants as shown in a randomized controlled cohort in the Gambia (Roth et al., 2006) as well as enhanced immune responses to other unrelated vaccines (Ota et al., 2002). When assessing whether early BCG vaccination could affect T cell responses to pertussis and TT vaccines, except for IL-13-expressing CD4+ cells, BCG timing had very little effect on immune responses to pertussis and TT. The higher frequencies of IL-13-expressing CD4+ cells observed at week 14 in response to pertussis stimulation could be due to the immaturity of the neonatal immune system or simply due to the waning effects of BCG in the early arm (Ota et al., 2002).Ota et al., also observed frequencies IL-13-expressing CD4+ cells in BCG vaccinated infants at two months of age (Ota et al., 2002). IL-13 is a Th2 cytokine especially important in IgE-mediated allergic responses (Roussel et al.,1998; Walter et al., 2001) and protection against exacerbation of allergic asthma following pertussis vaccination (Ennis et al., 2005). However, Ota et al., also showed that increased

IL-13 expression does not always translate into higher IgE responses (Ota et al., 2002).

The low level responsiveness to TT observed at 14 weeks compared to BCG and SEB may be due to the time intervals between immunization and blood sampling, or even the low sample size for this antigen and not due to intrinsic characteristics of the neonatal immune system. The low TT-stimulated samples sizes at week 14 make it difficult to assess how BCG timing affects T cell responses to TT. However, recent data shows that T cell responses to TT increase after 14 weeks and are sustained for a long period (Soares et al., 2013).

Thus, even though the findings in this dissertation suggest that BCG does affect immune responses to other vaccines and antigens, it is not clear what these non-specific effects are and how they affect pathogen susceptibility. The remarkable changes induced by delayed BCG vaccination seen by Ota et al (Ota et al., 2002) were certainly not recapitulated here, however the assays and readouts used were different. It is possible that ex vivo stimulation followed by a six day-whole blood culture. In fact many activated cells may undergo apoptosis when restimulated and not be reported in the final read out. It is also possible that different cytokine profiles could have been obtained if blood was stimulated and cultured for a shorter period. In order to better interpret these results, it would be necessary to assess how blood culture length affects cytokine profiles in future studies. Nonetheless it is important to note that the cytokine profiles obtained in response to BCG in this study were very similar to Kagina et al who had a 12 h culture time(Kagina et al., 2009).

As stated in section 2.3, viral load was not routinely available for all the mothers. Thus, it was not possible to compare the levels of HIV exposure among infants and assess how viral load can affect immune responses to the different vaccines. Nonetheless, because this study was randomized, we expect the levels

of exposure between the two groups of infants to be similar. In addition, CD4 counts are good indicators of the maternal health status and how they respond to ARV therapy. In this study, infants in the groups had similar maternal CD4 counts and maternal CD4 count had almost no effect on T cell proliferative and cytokine responses. However, for future analyses, in order to gain more insight on the effects of HIV exposure on immune responses, it would be important to assess how viral load affects immune responses to different vaccines.

The effects incurred by BCG vaccination of adults and mice in Kleinnijenhuis et al's experiments were also not evident here. In their study, BCG vaccination of mice protected them against subsequent candidal and bacterial challenges (Kleinnijenhuis et al., 2012). It is possible that more effects of delayed BCG vaccination on cellular immune responses in HIV-exposed infants would have been evident if we had lengthened the follow-up period, but also possible that the effects of BCG on infants and adults or mice differ.

Assessing how BCG timing affects T cell responses to BCG and other vaccines in in HIV-exposed infants may contribute to developing more effective vaccination strategies for such a vulnerable group. In addition, determining the effects of BCG on other immune cells such as dendritic cells, monocytes, or Tregs in these infants would help understand how BCG and HIV-exposure affect the neonatal immune system maturation. Although neonatal and adult immune systems are different, HIV-exposed, uninfected infants represent a formidable controlled model to study the immune system of other highly exposed individuals who are at high risk of acquiring HIV such as commercial sex workers and injection drug users. This is particularly important in the development of a future HIV vaccine.
The knowledge gained in this study will assist in designing future studies that address how BCG timing affects the vaccine efficacy in HIV-exposed infants and the non-specific effects of BCG on the neonatal immune system.

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