The Effect of HIV-exposure on Immune Responses to Expanded Programme on Immunization Vaccines and Antigens

By

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DECLARATION

I **Elvis Banboye Kidzeru**, hereby declare that this dissertation is my own, unaided work (except where acknowledgements indicate otherwise). It is being submitted for the Degree of Master of Science in Medicine at the University of the Cape Town, Cape Town. It has not been submitted before for any degree or examination at any other University.

Signature: Signed by candidate

Date: day of 7 June, 2013

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen presenting cells
BaP	Bordetella acellular Pertussis
BCG	Bacillus Calmette-Guérin
BP	*Bordetella Pertussis*
CD	Cluster of differentiation
CMI	Cell mediated immunity
CpG-ODN	Cytidine-phosphate-Guanosine oligodeoxynucleotide
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DTaP	Diphtheria-Tetanus-acellular Pertussis vaccine (Paediatric formulation)
dTap	Diphtheria-Tetanus-acellular Pertussis vaccine (adult formulation)
DTP	Diphtheria-Tetanus-Pertussis vaccine
EBF	Exclusive breast feeding
EPI	Expanded Programme on Immunization
FDA	Food and Drug Administration
GAVI	Global Alliance for Vaccine Initiative
GEE	Generalised estimation equation
HAART	Highly active antiretroviral therapy
HEU	HIV-exposed uninfected
HIV	Human immunodeficiency virus
HU	HIV-unexposed
ICS	Intracellular cytokine staining
IFN-Ɣ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1β	Interleukin-1beta
Ki67-PA	Ki67 proliferation assay
MF	Mix feeding
MHC	Major histocompatibility complex
NK	Natural killer
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PCV_7	Pneumococcal Conjugate Vaccine

pDC	Plasmacytoid dendritic cells
PMA	Phorbol 12-myristate 13-acetate
PMTCT	Prevention of mother-to-child transmission
PRR	Pattern-recognition receptors
RPMI	Roswell Park Memorial Institute
RV	Rotavirus vaccine
Sags	Superantigens
SEB	Staphylococcal enterotoxin B
SIV	Simian immunodeficiency virus
SSA	Sub-Saharan Africa
TB	Tuberculosis
TcR	T cell receptors
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrotic factor-alpha
Treg	T regulatory
TT	Tetanus toxoid
WBA	Whole blood assay
WHO	World Health Organisation

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ABSTRACT

The Effect of HIV-exposure on Cellular Immune Responses to EPI Vaccines and Antigens

Background

Immunization against vaccine-preventable infections is essential to reducing childhood morbidity and mortality. The immaturity and tolerogenicity of the immune system of infants renders them susceptible to infectious diseases and makes induction of protective immunity via vaccines a challenge. HIV-exposed infants are HIV uninfected and born to HIV-infected mothers and have increased morbidity and mortality of unknown aetiology. We hypothesise that T cells of HIV-exposed uninfected (HEU) infants have impaired proliferative ability and cytokine production in response to vaccine antigens than HIV unexposed (HU) infants.

Methods

We recruited HEU and HU infants at birth and followed them until 14 weeks of life. Between 1-3mL whole blood was collected from 46 participants in each group of infants at 6 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. Multiparameter flow cytometry (BD LSR Fortessa) was used to measure expression of these markers. Data analysis was performed by FlowJo V9.4, GraphPad prism V5, STATA V11, Pestle V1.7 and Spice V5.22 software packages.

Results

HIV-exposure was found to enhance the proliferative responses to BCG vaccination and SEB, but had no effect on BP and TT responses. After adjusting for potential confounders, HEU infants had higher CD4+ T cell proliferation to BCG through time from 6 to 14 weeks of life. HIV-exposed uninfected infants possessed significantly lower frequencies of CD8-expressing IL-17 and IL-13 compared with HU infants in response to BCG at 14 weeks of life. Proportions of polyfunctional cytokine responses were significantly lower in the HEU compared to the HU

infants irrespective of the time points or antigen stimulation. Generally, HIV-exposed infants had similar BCG, BP and TT vaccine-induced intracellular cytokine responses over time from 6 to 14 weeks of life.

Conclusion

The findings may have important implications for the optimal timing and vaccination strategies for HIV-exposed infants and careful immunological evaluation of future HIV and TB vaccines in this group.

HEU infants had significantly lower frequencies of IL-13 production by CD8+ T cells compared to HU infants in response to BCG at 6 and 14 weeks of life, and of IL-17 production by CD8+ T cells in response to BCG at 14 weeks of age. The presence of these cytokine producing cells at a significantly lower frequency may be sub-optimal to protect HEU infants from infections and other viral infections as a significantly higher frequency of these infants presented with flu like symptoms compared to their un-exposed counterparts. However, HEU infants produced a significantly higher frequency of IL-13 and IL-17 by CD4+ T cells at 14 weeks of life in response to BCG.

No significant differences were observed between HEU versus HU infants frequency of cytokine production by CD4+ and CD8+ T cells following BP and TT stimulation at 14 weeks of life, illustrating equal levels of protection between the groups of infants. HIV exposure was a major predictor of IL-17 production by CD8+ T cells in response to BCG longitudinally through time to 14 weeks after adjusting for other confounders presented by the cohort in the multivariate model, this shows that CD8 lymphocytes may be primed in *utero in* HEU infants.

HIV-exposure can alter immune responses to some antigens at 14 weeks (lower CD8+ cytokine production to BCG) but not 6 and HIV-exposure has no effect on frequency of cytokine production by CD4+ and CD8+ T cells following BP and TT stimulation at 14 weeks of life. The proportion of vaccine antigen stimulated CD4+ and CD8+ T cells that responded to *in vitro* culture were significantly less polyfunctional in the HEU compared to the HU infants at both time points when comparing all the cytokine combinations.

PRESENTATIONS AND PUBLICATIONS

ABSTRACTS

Elvis Kidzeru, Anneke Hesselling, Jo-Ann Passmore, Hoyam Gamieldien, Clive Gray, Donald Sodora, Heather Jaspan. The effect of HIV-exposure on cellular immune responses to EPI vaccines and other relevant antigens. Published at the International African Vaccinology Conference (IAVC) November **2012** in Cape Town, South Africa, oral presentation.

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CHAPTER ONE

Literature review

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1.1 The immune system (General)

The immune system is an organization of cells and molecules with specialized roles to defend against infections. There are two different types of responses to invading microbes; the innate response which is rapid and non-specific (Delves, 2000), and the acquired or adaptive response, which is specific, and takes several days to weeks to develop, has immunological memory and provides the host with a state of protective immunity against re-infection with the same pathogen (Janeway, 2001).

Functionally, the innate immune response is the body's first line of defence and involves phagocytic cells (neutrophils, monocytes, macrophages) and immune factors (complement, cytokines and acute phase proteins), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), natural killer (NK) cells, natural killer T cells (NK T-cells) and Gamma Delta T-cells (γδ T-cells) ((Delves, 2000)(Beck *et al.*, 1996). Another major cellular component of the innate immunity is the interdigitating dendritic cell (e.g. langerhans' cells in the skin) that continuously endocytose extracellular antigens and become activated as antigen-presenting cells (APC) when pattern-recognition receptors (PRR) on their surface recognise distinctive pathogens-associated molecular patterns (PAMPs) on the surface of microorganisms(Medzhitov *et al.*, 1997).

Despite these innate immune mechanisms, pathogens often pass these defences and adaptive immunity is then required. Adaptive immunity is further divided into the humoral and cell mediated immune systems that involve the proliferation of antigen-specific B and T cells after antigenic peptides are displayed to specific surface receptors for binding. While B cells secrete immunoglobulins for antigen-specific antibodies in charge of eliminating extracellular pathogens and molecules (toxins, bacteria), T cells take care of intracellular microorganisms i.e. cell mediated immunity (CMI) (viruses, mycobacteria, some fungi, protozoa or other facultative intracellular pathogens) and also provide help for B cells to produce antibodies (Beck *et al.*, 1996).

During CMI responses, cells that can destroy other cells become activated, with their destructive activity limited to cells that are either infected with, or producing a specific antigen. CMI

responses may also destroy cells making mutated forms of normal molecules, as in some cancers(Anthony, 2008).

1.1.1 Innate response to pathogens

Innate immunity covers many areas of host defense against pathogenic microbes, including the recognition of PAMPs(Janeway *et al.*, 2002), which enable the immune system to distinguish between infectious (non-self) and non-infectious (self) molecular structures by its specific PRRs. PRRs are highly diverse; some recognise PAMPs directly, while others (e.g. Toll like receptors [TLR]) recognise the product generated by PAMPs (e.g. bacterial CpG-ODN)(Mogensen, 2009). However, the nature of the PRR ligand determines the receptor for signalling the presence of the specific pathogen. Cellular PRRs are expressed on effector cells including professional APC and cells on the surface of epithelia and some expressed intracellularly(Inohara *et al*., 2005).

PRRs directly induce innate effector mechanisms following recognition of a PRR ligand, for example, viral nucleic acids that are recognised by cells in which they replicate. This interaction induces expression of inflammatory cytokines and chemokines by the cell (Medzhitov *et al*., 1997). This lead to the maturation, differentiation, and proliferation of multiple immune cells, including B and T lymphocytes after the antigen is presented to them as peptides by major histocompatibility complex (MHC) molecules. Including, NK cells, monocytes, macrophages, and dendritic cells (DCs). Together, these cells secrete cytokines and chemokines that create a pro-inflammatory [interleukin-1beta (IL-1β), IL-6, IL-18, and tumor necrosis factor-alpha (TNF-α)] and T helper 1 (Th1)-biased (IFN-ϒ and IL-12) immune milieu (Bendelac, 1997)(Klinman *et al*., 2004).

1.1.2 T cell-mediated immunity

T and B cells predominantly mediate the adaptive arm of immunity.

The cell-mediated compartment of the immune system involves the professional APC notably by DCs, macrophages and B cells, that up-regulate costimulatory molecules on their surface for lymphocyte activation(Delves, 2000)(Nyland, 2009). Small antigen peptides are presented by

MHC molecules on the surface of APC in form of short peptides to T cells after processing (or proteolytic cleavage).

There are many classes of MHC molecules, two of which are involved in classical T cell; MHC classes I and II that present the peptides to T-cell receptors (TcRs) on the surface of cytotoxic (CTL) and helper (Th) T lymphocytes respectively, thus stimulating an immune response specific to that peptide antigen(Fraser, 2011). DCs are particularly efficient at initiating (priming) immune responses for which immunologic memory has not been established, by activating naïve T cells to expand into a population of antigen-specific effector T cells(Delves, 2000). MHC class I molecules bearing peptides activate CTLs which kill infected target cells, whereas, MHC class II molecules bearing peptides from pathogens in intracellular vesicles activate either T helper-type 1 (Th1) or T helper-type 2 (Th2) cells. Each naïve CD4 T cell has the potential to differentiate into either of the functionally distinct T helper effector cell subsets, Th1 or Th2, Th17 and T regulatory cells (Szabo *et al.*, 2003).

CD4+ T helper cells are important mediators of adaptive cellular immune responses. Based on their cytokine production profile, they have until recently been classified into Th1 and Th2 subsets. Th1 effectors produce predominantly Interferon-gamma (IFN-γ) and IL-2 and regulate cellular immunity against intracellular infections, whereas Th2 cells produce predominantly Interleukin (IL)-4, IL-5 and IL-13 and mediate humoral immunity activating the naïve antigen-specific B cells to produce IgM antibodies against parasitic infections. Moreover, Th2 cells can subsequently stimulate the production of different immunoglobulin isotypes, including IgA and IgE, as well as neutralizing and/or weakly opsonizing subtypes of IgG(Chu *et al.*, 2000).

Recently, a CD4+ Th cell subset known as T helper 17 (Th17) cells was described(Weaver *et al.*, 2006)(Lin, 2011). Th17 cells produce the cytokines IL-17A (IL-17) and IL-17F, as well as the cytokines IL-21, and IL-22. This new Th cell lineage fills in some of the missing gaps in host immunity not fully explained by the Th1/Th2 paradigm(Wozniak *et al.*, 2006)(Lin *et al.*, 2011).

Though humoral and cell-mediated immunity have many distinctive features, they are not completely independent. Macrophages, NK cells, neutrophils and eosinophils can use antibodies

as receptors for killing target cells. Activation of complement system by antigen-antibody complexes gives rise to chemotactic peptides which help in assembling the cell types required for a cell-mediated response(Rus *et al*., 2005). In addition, the immune system regulates its function by several ways including regulatory T cells often measured by levels of IL-10 and CD4+CD25+FoxP3+ regulatory T cells. These cells are present to dampen immune responses to micro organisms(Jiang *et al.*, 2006).

During T cell development and maturation, cells pass through three different subsets; naïve, central and effector memory subsets. These subsets can be differentiated using flow cytometry by identifying several receptors on T cells for example CD45RA/RO, and CCR7. CD45RA/RO consists of different isoforms of a protein tyrosine phosphatase expressed at different stages of differentiations that regulate Src (sarc) kinases required for T cell receptor signal transduction. CCR7 is a chemokine receptor that regulates homing and trafficking of cells to secondary lymphoid organs. (Sallusto *et al*., 1999)(Janeway, 2008).

Immunological memory is defined as the ability of the immune system to respond more rapidly and effectively to pathogens that have been previously encountered. Naïve T cell subsets are characterized by CD45RA+CCR7+, central memory (CM) by CD45RA-CCR7+ and the effector memory (EM) CD45RA-CCR7- (Sallusto *et al*., 1999)(Janeway, 2008). These subsets have different functional properties from localization, proliferation capacities, cytokine expression and cytotoxic functions. While naïve and CM T cells are found predominantly in the lymph nodes, the EM T cells are in the skin and mucosa (sites of infection) ready to fight pathogens. The proliferation capacity after antigenic stimulation of CM T cells is highest compared to naïve T cells and lowest in EM T cells that are more specialized to fight pathogens(Sallusto *et al*., 1999)(Janeway, 2008). Based on expression of cytotoxic molecules, high frequencies of EM T cells expressed perforin, granzyme A and granzym B compared to CM T cells, while naïve T cells expressed none of the cytotoxic molecules(Takata *et al*., 2006)(Romero *et al.*, 2007). The cytokine secretion profiles of these cell sub sets vary, naïve and CM T cells secrete more IL-2 and less IFN-γ, IL-4 and IL-5 compared to EM T cells(Sallusto *et al*., 1999)(Janeway, 2008).

1.1.3 Development of the immune system

Evidence suggests that foetal and adult T cells arise from different pluripotent haematopoietic stem cells (HSC) present at different stages of development. This suggests that the foetal T cell HSC lineage is biased towards immune tolerance, an observation that offers a mechanistic explanation for the tolerogenic properties of the developing foetus and for variable degrees of immune responsiveness at birth(Mold *et al.*, 2012).

HSC are early precursor cells which give rise to all the blood cell types including the myeloid lineage (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, and dendritic cells) and lymphoid lineages (T cells, B cells and NK cells)(Moss, 2001)(Abramson *et al.*, 1977). The HSC are found within the bone marrow (of the femurs, humeri, hip, ribs, sternum and other bones). Therefore, the bone marrow is the primary site and origin of lymphocytes. Lymphoid stem cells differentiate into three major populations of mature lymphocytes: T cells, B cells and NK cells. These subsets can be distinguished by their surface phenotypes(Chaplin, 2003). T cells are defined by their unique cell surface expression of the T cell receptor (TCR) while B cells are phenotypically defined by their production of the immunoglobulin antigen-binding proteins(Chaplin, 2006).

1.2 Immunity in the neonate (Innate and adaptive responses)

The immune system of neonates is said to be relatively underdeveloped and depends largely on maternal antibodies transmitted *in-utero* and in some cases *post partum.* The complexity of the immune system of infants renders them susceptible to infectious diseases and makes induction of protective immunity via vaccines a challenge(Ota *et al.*, 2012). Neonatal innate, humoral, and cellular immunity is different to that of adults(Jaspan *et al.*, 2007)(Jaspan, *et al.*, 2006). At the single-cell level, neonatal innate cells have been found to be generally less capable of producing multiple cytokines simultaneously, i.e. less polyfunctional(Kollmann *et al.*, 2012). Infants can produce small amounts of immunoglobulin G (IgG) during the first few months of life following routine vaccinations; their ability to make IgA lags behind even more.

Maternal IgG and IgA may reach infants through the placenta and breast milk, respectively. These immunoglobulins may interfere with the infant's humoral response to certain vaccines(Lambert *et al.*, 2005). Although T cell immunity is less affected by maternal antibody, certain aspects of T cell immunity of the neonate are immature. Neonates are thought to predominantly make Th2 cytokines, which include; interleukins (IL) IL-4, IL-5, and IL-13, important for B cell function and control of extracellular pathogens and helminths(Lambert *et al.*, 2005). In contrast, Th1 secrete interferons-gamma (IFN-γ), IL-2 and tumour necrosis factors (TNF), which are important for control of intracellular pathogens such as viruses(Jaythoon *et al.*, 2000).

Infant macaques have higher fractions of CD4+CD25+CD127+^{low}FoxP3+ T regulatory cells in the peripheral blood and in lymphoid tissues, and these cells showed greater *in vitro* suppressive activity on a per cell basis(Connor *et al.*, 2007). Contrary to the thinking that neonatal immunity is immature, evidence suggests that the development of CD4+CD25^{high}FoxP3+ T regulatory cells that suppress fetal antimaternal immunity persist at least until early adulthood(Mold *et al.*, 2009). These cells represent a form of antigen-specific tolerance in humans, induced *in utero* and probably active in regulating immune responses after birth(Mold *et al.*, 2009).

Little is known about the neonate's and infant's ability to mount Th17 responses in humans. Most of the data on IL-17 has been from mouse models. It has been shown in mice that activation of innate immune cells through TLR4 supports the direction and induction of Th1 and Th-17 cells, which mediate protective cellular immunity to *Bordetella pertussis*(Higgins *et al.*, 2012). IL-10 signalling blockade during BCG vaccination of mice resulted in a sustained Th17 (IL-17) response that increased protection against Mycobacterium tuberculosis infection(Pitt *et al.*, 2012). Further, BCG vaccination induces lung IL-17- expressing CD4+ T cells in mice. The IL-17 expressing CD4+ T cells are said to trigger the production of chemokines that recruit CD4+ T cells producing interferon-γ, which ultimately restrict bacterial growth(Khader *et al.*, 2007).

However, in humans, delaying BCG vaccination from birth to 4.5 months of life lowers IL-17 and other responses after vaccination but produces a comparable Mycobacterial response at 9 months of life(Burl *et al.*, 2012).

Maternal factors and *in utero* exposure to various microorganisms may play a role in neonatal immunity. Hepatitis C virus (HCV)-exposed neonates have a relatively suppressed immune activation, proinflammatory marker production, however, they have an increased production capacity for IFN-γ (Babik *et al.*, 2011). This suggests that when exposed to HCV *in utero*, the balance between suppressive and pro-inflammatory responses is altered. Also, exposure to malarial antigens *in utero* caused a predominant regulatory response in newborns born to mothers with ongoing chronic placental malaria (PM), while those with resolved infection produced both regulatory and inflammatory responses(Flanagan *et al.*, 2010).

1.2.1 Breastfeeding and immune responses in infants

Maternal antibody protects infants against many infectious diseases and maternal immunization augment this protection in young infant against diseases(Englund *et al.*, 1998)(JACOB *et al.*, 1977). Moreover, breast milk reduces the frequency and severity of gastrointestinal and respiratory infections in infants(ELLESTAD-SAYED, 1979)(Downham *et al.*, 1976) and in addition to these specific antibodies to pathogens and other factors within breast milk enhance the immune responses of the neonatal immune system(Van Rie *et al.*, 2005).

However, there is limited data on effect of HIV-exposure and more data is needed on the effect of breastfeeding on T-cell mediated immune responses to neonatal vaccines. Exclusive breastfeeding enhances vaccine responses in infants(Rennels, 1996)(Pabst *et al.*, 1988). Some studies demonstrated a trend toward a decreased seroresponsiveness in breastfed compared with non-breastfed children(Moon *et al.*, 2010). The inhibitory influence of maternal antibodies on infants' immune responses to vaccines (e.g. whole cell pertussis) is thought to be B-cell specific and leaves the infant T-cell responses largely unaffected(Van Rie *et al.*, 2005). In general, the evidence that breastfeeding enhances the functional protection of the neonatal immune system against pathogens surpasses that for lack of enhancement, however, more evidence for this is needed (Stephens *et al.*, 1985).

1.3 Cytokines

Cytokines are small, nonstructural proteins with molecular weights ranging from 8 to 40,000 Daltons (d) that nearly all nucleated cells are capable of synthesizing and, in turn, responding to(Dinarello, 2000). Cells that can respond to cytokines include; the same cell that secreted cytokine (autocrine), a nearby cell (paracrine), or a distant cell reached through the circulation (endocrine)(Bowers, 2012). Cytokines can be grouped according to function; (a) Mediators and regulators of Natural Immunity (pro-inflammatory/innate) [e.g. Tumor Necrosis Factor alpha (TNF-α), Interleukin-1 (IL-1), IL-6 and IL-12 (b) Adaptive cytokines {Th1 [Interferon-gamma (IFN-γ) and IL-2], Th2 [IL-4 and IL-13] and Th17 [IL-17] (c) Mediators and regulators of specific immunity (regulatory) [IL-10](Bowers, 2012). Below is the description of the various cytokines that we investigated this dissertation and their interactions.

1.3.1 Adaptive cytokines

1.3.1.1 T helper type 1 (Th1) cytokines

Interferons (IFN) are best known for interfering with viral replication(Isaacs, 1989), hence termed virus inhibitory proteins. The major source of IFN is mitogen activated T lymphocytes. Three types of IFN have been described; IFN-alpha (α) and IFN-beta (β) that belong to the type I super family and IFN–gamma (γ) that belong to the type II family of interferons. T lymphocytes and NK cells are the main sources of IFN-γ(DeMaeyer, 1988). It is induced naturally by antigens to which T cells have been pre-sensitized. However, non-specific T cell activators (PMA-Ionomycin) *in vitro* can also induce IFN-γ synthesis in T cells and NK cells(Hardy, 1989)(Perussia *et al.*, 1992).

IFN-γ production increases with age in infants(Van Rie *et al.*, 2006). Neonates have limited capacity to produce IFN-γ and reduced cell-mediated immune responses involving cytotoxicity against intracellular pathogens(Siegrist, 2007)(Ota *et al.*, 2012).

IL-2 stimulates resting T cells and supports long term growth, activation, and proliferation of T cells (Smith, 1988). IL-2 is also a potent modulator of T and NK cell function, and it is the most extensively investigated cytokine to date. It also is used as a vaccine adjuvant to boost Th1 responses against intracellular pathogens in infants(Smith *et al.*, 1979). The major source of IL-2

is mature T cells (both T helper and cytotoxic) within 4-12 hours following activation by antigen or mitogen(Smith *et al.*, 1979). Vaccination at birth with BCG has been shown to significantly enhance Th1 responses to mycobacterial antigens(Burl *et al.*, 2012).

1.3.1.2 T helper type 2 (Th2) cytokines

IL-13 is a very important cytokine that possesses a number of anti-inflammatory and immunomodulatory activities. IL-13 is a member of the Th2 group of cytokines together with IL-4 and IL-5. Neonates produce predominantly Th2 responses instead of the much needed Th1 responses against intracellular pathogens(Jaspan *et al.*, 2007)(Siegrist, 2007). In addition, African neonates mount significantly higher Th2 cytokines compared to Europeans, possibly reflecting continuous presence (stimulations) of antigens that preferentially drive Th2 responses(Wilfing *et al.*, 2001).

1.3.1.3 T helper type 17 (Th17) cytokines

Members of the Th17 cytokines include IL-17A (IL-17) and IL-17F, as well as the cytokines IL-21, and IL-22 (Wozniak *et al.*, 2006)(Lin *et al.*, 2011). Th17 cells may influence vaccine-induced memory immune responses mostly to bacterial antigens. IL-17 has been shown to populate the lungs, the primary site of *Mycobacteria tuberculosis* infections, and in turn recruit IFN-γ producing antigen-specific T cells persistent in the central lymphoid organs(Lin *et al.*, 2011). T helper cells that mostly IL-17 or IL-22 have been described, and are known for their involvement in chronic inflammation, wound healing and skin diseases, respectively(Sala *et al.*, 2012).

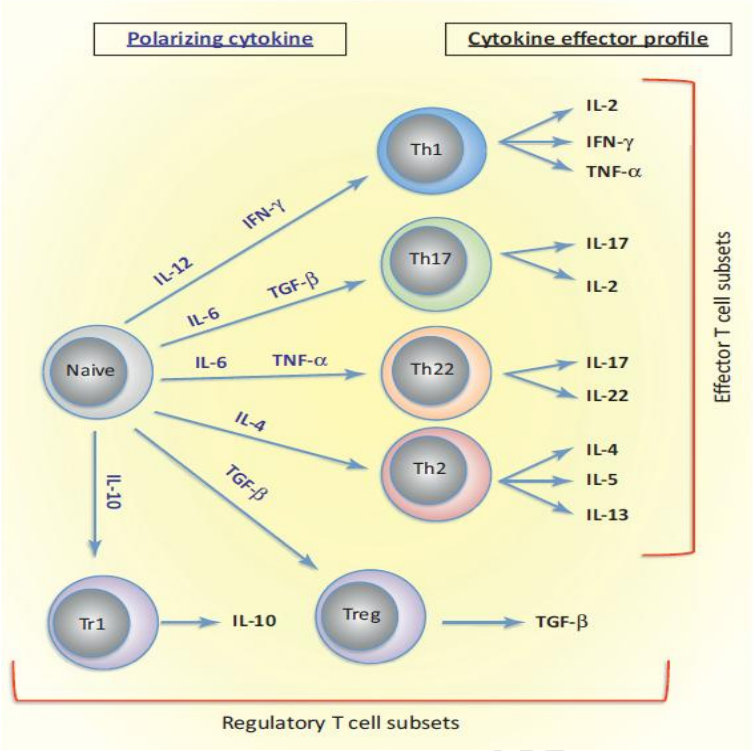


Figure 1.1: **Functional CD4+ T cell subsets**. Naïve CD4+ T cells differentiate into functional subsets under the influence of different combinations of cytokines and acquire stable cytokine expression profiles and functions. Effector CD4+ T cells may differentiate into T helper 1 (Th1), Th17, Th22 and Th2 cells expressing predominantly interferon-gamma (IFN-γ), interleukin-17 (IL-17), IL-22, and IL-4 respectively. Regulatory T cell subsets with immunosuppressive function are classified into T regulatory (Treg) cells producing IL-10 and CD4+CD25+ Foxp3+ cells that express transforming growth factor-b (TGF-b) [Source: Modified from (Sala *et al*., 2012)].

1.4 HIV prevalence and maternal-infant HIV transmission

Human Immunodeficiency virus (HIV) causes acquired Immunodeficiency syndrome (AIDS) and is one of the world's most serious health and development challenges(UNAIDS, 2012)(UNAIDS, 2011a). Currently, approximately 34 million people have died of AIDS-related causes since the first cases were reported in 1981 (The Henry J. Kaiser Family Foundation/UNAIDS, 2012)(UNAIDS, 2011b). Almost all cases of HIV (97%) reside in low- and middle-income countries, particularly in sub-Saharan Africa (SSA)(The Henry J. Kaiser Family Foundation/UNAIDS, 2012).

Over 3.5 million women of childbearing age are infected annually with HIV in SSA and large numbers of children are born to HIV-positive women(UNAIDS, 2012).

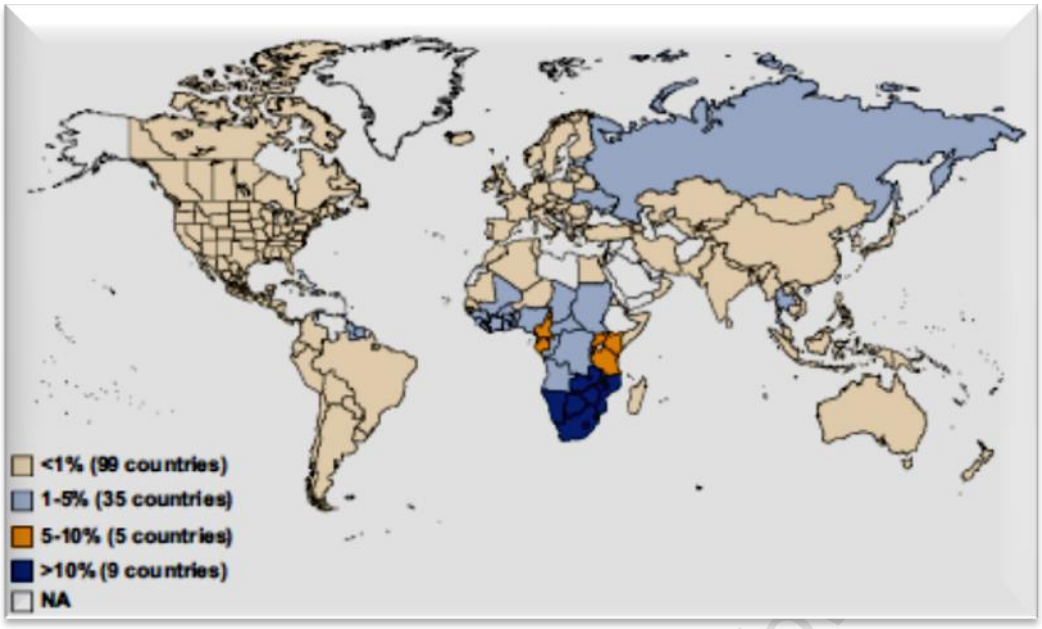


Figure 1.2: **Adult HIV Prevalence Rate (Ages 15–49),** [Source: (UNAIDS, 2010)(UNAIDS, 2009)**]**

Paediatric HIV-1 infection is still a major pandemic, it is estimated that there are more than 2 million children infected with HIV-1 worldwide and that more than 1800 new HIV infections are transmitted daily from mothers to infants(UNAIDS/WHO, 2006). Transmission rate can be reduced to as low as 1-2% with the use of HAART or dual therapy(UNAIDS, 2012).

1.5 The HIV-exposed uninfected (HEU) infant

HIV-exposed but uninfected (HEU) infants are HIV-uninfected infants born to HIV-infected mothers(Jones *et al*., 2011). An estimated two million infants are exposed *in utero* to HIV of which an approximately 1.5 million are HIV uninfected. Moreover, due to the high maternal HIV prevalence mostly in SSA, and the introduction of prophylactic interventions to prevent vertical transmission rate of HIV coupled with the successful and ever improving prevention of mother-to-child transmission (PMTCT) programs in many parts of the world(Mbori-ngacha *et al*., 2001), this leaves a growing proportion of HEU infants in regions where antenatal HIV prevalence is high(Filteau, 2009).

1.5.1 Immunological abnormalities in HEU infants

There are several reports of increase morbidity and mortality in HEU compared to the HIV-unexposed (HU) infants(Epalza *et al.*, 2011)(Kuhn *et al.*, 2006)(Mussi-pinhata, 2006)(Marinda *et al.*, 2007). Maternal immunosuppression due HIV infection tends to have adverse influence on the health of infants in addition to the risks of vertical HIV transmission(Kuhn *et al.*, 2006). However, Zambian HIV exposed infants who remained uninfected after perinatal or early breastfeeding-related HIV exposure were of high risk of mortality and morbidity (pneumonia and/or sepsis) during the first few months of life regardless of their maternal disease stage or Socioeconomic status(Kuhn *et al.*, 2006).

Skin/mucous membrane infections and respiratory tract infections are common infections observed in HEU infants more often than HIV-unexposed infants(Mussi-pinhata, 2006). Also, the incidence of Group B Streptococcal infection is significantly higher in HEU than in the un-exposed infants(Epalza, 2010).

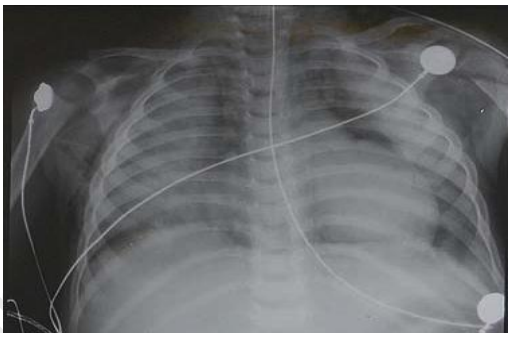


Figure 1.3: *Pneumocystis jeroveci* pneumonia in HIV-exposed infants (Source: HIV.VA.gov)

Immunological abnormalities caused by HIV exposure in infants born to HIV infected mothers are still to be defined(Mazzola *et al.*, 2011). There are data documenting different responses to vaccine immunogens(Van Rie *et al.*, 2006)(Jones, 2011)(Mazzola *et al.*, 2011), and an increased risk of *Pneumocystis jeroveci* pneumonia, an opportunistic infection, (Marinda *et al.*, 2007) in HEU infants.

HEU infants have Increased immune activation, possibly as a result of *in utero* exposure to HIV antigens and/or antigens related to other infections in HIV-infected mothers(Rich *et al.*, 1997). Also, elevated levels of pro-inflammatory cytokines are present in HEU infants(Hygino *et al.*, 2008). Both immune activation and a proinflammatory cytokine profile is a major hallmark of HIV-infection and reported to correlate with many immune functional disturbances(Davenport *et al.*, 2007)(Lieberman *et al.*, 2010)(Pw, 2007).

Finally, the immunological characteristics of very young HEU infants in SSA may be different to elsewhere in the developed world. To improve the morbidity of this immunologically vulnerable

group of infants, we need to understand the nature of their immune responsiveness to determine optimal vaccination and disease prevention strategies. More information is needed on the influence of intrauterine HIV exposure on cellular immune responses to mycobacterial and other antigens.

1.5.2 Specific (acquired) immune responses in HEU Infants

1.5.2.1 Antibody production in HEU infants

Humoral immunity refers to antibody production and the accessory processes that accompany it, including Th2 activation and cytokine production, germinal centre formation and isotype switching, affinity maturation and memory cell generation(Rao, 2010). It also refers to the effector functions of antibody, which include pathogen and toxin neutralization, classical complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC) and opsonisation and consequently phagocytosis and pathogen elimination(Nyland, 2009).

More information about vaccine protective response for infants born to HIV-infected mothers still needs to be known. A study by Abramczuk *et al*., found that although HEU infants had protective titers for diphtheria and tetanus, their geometric mean anti-tetanus titers were lower than those of the HU infants(Abramczuk *et al*., 2011). Jones *et al.* (2011) demonstrated that HEU infants have lower specific antibody to tetanus and pertussis at birth, but robust responses following vaccination(Jones *et al*., 2011). This may be due to decreased quality or quantity of passively acquired maternal antibody.

1.5.2.2 Cellular immune responses in HEU infants

There are few data on the cellular immune response to vaccines in HEU, except for BCG. Bacille Calmette Guerin (BCG), a live attenuated *Mycobacterium bovis* vaccine, is almost universally given in SSA, where the brunt of the global paediatric HIV burden is concentrated(Hesseling *et al*., 2009). BCG is usually given at birth; its coverage worldwide is estimated at over 100 million doses per year, resulting in vaccination of 75% of infants born in 2002. In HIV-uninfected children, BCG is safe, efficacious and cost-effective against disseminated tuberculosis(Trunz *et al*., 2006).

BCG can elicit potent type 1 responses and effective CTL responses in infants when given at birth(Ota *et al.*, 2012), although these type I responses may not be the correlate of vaccine-induced protection. We do not know, however, how efficacious BCG is in HEU infants, although some evidence suggests that it may not be as immunogenic as in HU infants(Rie *et al.*, 2006)(Mazzola *et al.*, 2011). In a study by Mazzola *et al*, the level of IFN-γ produced in response to BCG increased with age in HEU infants. But at an early age, HEU infants produced less IFN-γ and TNF-α in response to BCG than HIV-unexposed infants suggesting there may be defects in immune responses(Mazzola *et al.*, 2011).

1.6 Vaccines and super antigens

A vaccine is a preparation of a weakened or killed pathogen, such as a bacterium or virus, or of a portion of the pathogen's structure or DNA that upon administration stimulates an immune response against the pathogen but is incapable of causing severe infection(Griffin *et al.*, 2002)(Siegrist, 2008). Diseases caused by bacteria and some other pathogens can be prevented by vaccines licensed for use in several parts of the world(CDC, 2005). Hence, vaccines are an important part of disease prevention and control(Griffin *et al.*, 2002).

Vaccines developed thus far can be classified as follows; live attenuated vaccines, inactivated vaccines, subunit vaccines, toxoid vaccines, conjugate vaccines, virus-like particles, DNA vaccines and/or recombinant vector vaccines(The US department of health and human services). The problem with pure recombinant or synthetic antigens used in modern day vaccines is that they are generally far less immunogenic than older style live or killed whole organism vaccines which are in turn often more risky. This has lead to the use of immunological agents (adjuvants) that enhance immune responses to a vaccine but are not immunogenic on their own(Petrovsky *et al.*, 2004).

For a successful assessment of immune response to various vaccine antigens using *in vitro* assays, super antigens [e.g. staphylococcal enterotoxin B (SEB)] are often used as positive controls for their powerful immune response generated(Rajagopalan *et al.*, 2002). Superantigens

act through widespread activation of certain Vβ TCRs and therefore are also interesting mechanisms to assess when evaluating immune responses in HEU infants.

1.6.1 Vaccination

The Expanded Programme on Immunization (EPI) was launched in 1974 and less than 5% of the world's children were immunized during their first year of life against 6 killer diseases in the same year; polio, diphtheria, tuberculosis, pertussis (whooping cough), measles and tetanus(UNICF/WHO, 2011). Today, immunization is been carried out effectively throughout the world, with some vaccines been prioritized in some areas where the disease burden is of major public health concern(CDC, 2005). Below is the EPI vaccination schedule used in South Africa (Table 1.1).

Table 1.1: **The EPI vaccination schedule used in South Africa**;(1) BCG given at birth, (2) DTaP-IPV/Hib primed at 6 weeks, first, second boosters at 10, 14 weeks and third booster at 18 months of life and further at 6 and 12 years of age (Source: http://www.savic.ac.za)

1.6.1.1 Bacillus Calmette-Guérin (BCG)

After 13 years and 230 sub-cultures, in 1921, Calmette and Guerin together announced the discovery of a new strain of bovine tubercle bacilli which grew luxuriantly, was completely avirulent in animals and cause antibody production against tuberculosis, and showed no change in virulence if grown on standard egg based medium(Rajasekaran, 1961). BCG vaccine is the only vaccine currently available for immunization against tuberculosis (TB) infections and has been used since the 1920s. During this time numerous sub-strains have evolved from the original strain and have been used for vaccine production. Not surprisingly, the diversity of sub-strains, manufacturing processes, route of administrations, immunization schedules and levels of exposure to environmental mycobacteria and virulent *Mycobacterium tuberculosis* infection can cause variation in the immunogenicity of the vaccine(WHO, 2004).

BCG is a live attenuated strain of *Mycobacterium bovis*, is almost universally given in sub-Saharan African countries where the brunt of the global burden of paediatric infection including tuberculosis and HIV-1 is concentrated(Hesseling *et al.*, 2009)(Organon, 2009). BCG vaccination has been part of the EPI since 1974, with coverage in infants exceeding 80%. The vaccine's safety record after more than 4 billion administrations is as impressive as its low cost of US$0.10-0.20 per dose of product(Kaufmann *et al.*, 2010). BCG vaccination is usually given at birth or in the perinatal period. In 2002, an estimated 75% of the 130 million children born worldwide were vaccinated with BCG(Hesseling *et al.*, 2009). BCG has consistent efficacy for the prevention of tuberculosis meningitis and miliary tuberculosis in young children without HIV infection, but affords only variable and incomplete protection against pulmonary TB at any age(CDC, 2011)(WHO, 2009)(Trunz *et al.*, 2006).

1.6.1.2 acellular Bordetella Pertussis (aBP)

Pertussis, or whooping cough, is an acute infectious disease caused by the bacterium *Bordetella pertussis*. Outbreaks of pertussis were first described in the 16th century, and the organism was first isolated in 1906(Gebhart, 1980)(States, 2008). Pertussis was one of the most common childhood diseases in the 20th century and a major cause of childhood mortality in the United

States. Before the availability of pertussis vaccine in the 1940s, more than 200,000 cases of pertussis were reported annually. Since the widespread use of vaccine began, incidence has decreased more than 80%(Mooi *et al.*, 2009)(States, 2008). Two forms of vaccines have been used (i) Whole-cell pertussis vaccine (wP) and (ii) acellular Pertussis vaccine (aP). The wP vaccine is composed of a suspension of formalin-inactivated *Bordetella pertussis* cells. It was developed in the 1930s and used widely in clinical practice by the mid-1940s(Mooi *et al.*, 2001).

Whole-cell Pertussis vaccine is 70%-90% effective in preventing serious pertussis disease. However, its protection decreased with time, resulting in little or no protection 5 to 10 years following the last dose. Local reactions such as redness, swelling, and pain at the injection site occurred following up to half of doses of whole-cell DTP vaccines. Fever and other systemic events were also common (seizures) (CDC, 2005)(Demirjian *et al.*, 2009). This further raised concerns about its safety and led to the development of more purified (acellular) Pertussis vaccines that are associated with a lower frequency of adverse reactions.

Acellular Pertussis vaccines are subunit vaccines that contain purified, inactivated components of *Bordetella pertussis* cells(Sato, 1999). Several acellular Pertussis vaccines have been developed for different age groups; these contain different Pertussis components in varying concentrations which include; the paediatric formulation (DTaP) often available in combination with other antigens [Infanrix hexa™ (DTaP-hepB-IPV-Hib); Infanrix-IPV™ (DTaP-IPV); Infanrix Penta™ (DTaP-hepB-IPV)] and the adolescent and adult formulation (dTap) of which four types are available containing reduced Pertussis antigen content in comparison to the vaccines for young children [Boostrix™ (dTap) and Boostrix-IPV™ (dTap -IPV), and Adacel™ (dTap) and Adacel Polio™ (dTap-IPV) (available since 2005)](NCIRS, 2009). Pertussis vaccine is available on the EPI schedule in many countries for children at 6, 10 and 14 weeks and at 18 months of age(Gebhart, 1980). An adolescent booster dose is available via school-based programs at 12-17 years of age which vary by countries(CDPH, 2010)(NCIRS, 2009).

1.6.1.3 Tetanus toxoid (TT)

Tetanus is a deadly infectious disease for which immunization is available in EPI schedules of most countries worldwide at both infant level and for females of reproductive age(Aziz, 2010).

Tetanus results from infection with *Clostridium tetani*, a commensal bacterium in the gut of humans and domestic animals which is found also in soil. It is not the presence of the bacteria which causes disease, but the toxins that are produced by the bacteria under anaerobic conditions(CDC, 2005). These toxins can be spread through the blood vessels and finally affect the nervous system causing tetanic muscle contraction and pain. The condition is extremely painful and potentially lethal(Aventis Pasteur, 1989)(Gaublomme, 2007).

Immunization against tetanus is carried out by the administration of DTaP in infants and dTap in adults, both containing Tetanus toxoid with aluminium hydroxide as an adjuvant, and the vaccine is available in combination with other antigens as well as alone. The immunization schedule is similar to that of pertussis as they are administered in conjunction(CDC, 2012)(Gaublomme, 2007). Following routine use of tetanus toxoid in the United States, the occurrence of tetanus decreased dramatically from 560 reported cases in 1974 to an average of 50-100 cases reported annually from the mid 1970s through the late 1990s. Neonatal tetanus occurs among infants born under unhygienic conditions to inadequately vaccinated mothers. Vaccinated mothers confer protection to their infants through transplacental transfer of maternal antibody(Sanofi Pasteur, 1974).

1.6.2 Superantigens [Staphylococcal enterotoxin b (SEB)]

Superantigens are bacterial proteins that bind to the external portion of MHC class II and interact with restricted but large number of Vβ TCR's, so that a single superantigen can activate up to 20% or more of the total T cells in the body. In contrast, conventional antigenic peptides bind only to a subset of MHC molecules and to a very small fraction of the huge array of TCRs, thereby activating only a very small fraction of the total pool of T cells(Chaplin, 2006). Superantigens bind directly to the MHC molecule outside of the antigen-binding groove, and to TCR proteins outside of their antigen-MHC binding site without proteolytic processing(Chaplin, 2010)(Fraser, 2011) (Figure 1.4). Superantigens cause non-specific activation of T-cells resulting in polyclonal T cell activation and massive cytokine release. For example, the toxic shock syndrome toxin-1 (TSST-1) produced by *Staphylococcus aureus* binds to and activates all T cells with TCRs using the Vβ2 and Vβ5.1 chains(Chaplin, 2003)(Chaplin, 2010). It is important to note that bacterial Superantigens (Sags) constitute a family of very stable bacterial proteins that

are the most potent known activators of the immune system. Some member of Sags include toxins of *Staphylococcus aureus* and *Streptococcus pyogenes* (Fraser, 2011).

At least one of the Superantigens, staphylococcal enterotoxin B (SEB), also binds to the costimulatory molecule CD28, suggesting that a much larger and potentially more stable complex is formed at the immunological synapse than was previously thought(Rajagopalan *et al.*, 2002)(Fraser, 2011). SEB is the most extensively used and investigated superantigen today. It is unknown if HIV-exposure affects the responsiveness to Sags. HIV-exposure as well as infection may affect responses to Superantigens. Following SEB stimulation, HIV-infected children on HAART had significantly lower IL-2+ CD8 T cells and IFN-g+ CD4 T cells compared to the control uninfected infants(Mccloskey *et al.*, 2004).

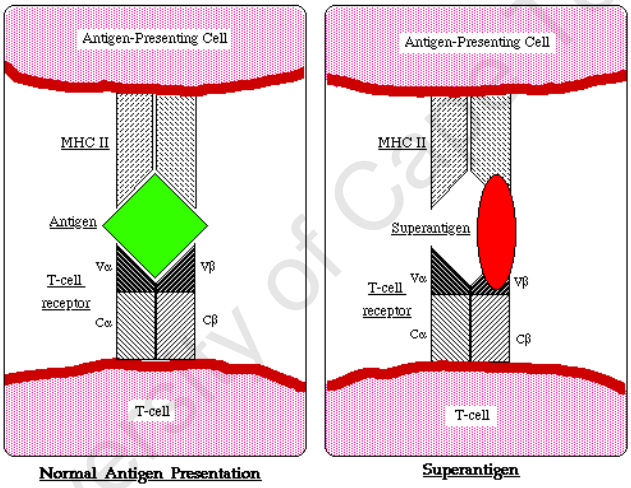


Figure 1.4: **The superantigen Theory** (source:http://www.mcb.uct.ac.za/cann/335/AIDSI.html)

1.7 Aims and objectives of the thesis

We aim to evaluate the cellular immune responses to certain EPI vaccines as well as SEB in HEU versus HU infants. To this end we have two specific aims:

(1) To compare CD4+ and CD8+ T cell proliferative responses to vaccines and SEB in HEU versus HU infants and to evaluate the evolution of the T cell proliferation to BCG and SEB from 6 to 14 weeks of life.

(2) To compare the quality and breadth of CD4+ and CD8+ T cell intracellular cytokine production to vaccines and SEB in HEU versus HU infants and to evaluate the evolution of cytokine production to BCG and SEB from 6 to 14 weeks of life.

Rationale

(i) There are few studies comparing the proliferative ability of HEU infants' lymphocytes in response to any vaccination to HU infant's lymphocytes.

(ii) No study comparing the intracellular cytokine response to tetanus and pertussis vaccination between HEU and HU infants.

(iii) Understanding the immune responses of HIV-exposed infants will help with design of neonatal HIV vaccines and other vaccines for this vulnerable group of infants

In this study we will look at the cellular immune responses to vaccines critical for providing protection for infants with the *ultimate goal to design interventions to improve the health of HEU infants, and to inform the design of a neonatal HIV vaccine.*

Hypothesis

Generally, we hypothesise that HEU infants have less proliferation and less cytokine production by T-cells to vaccine antigens compared to HU infants.

1.7.1 Specific objectives

Specific objective 1

To compare CD4+ and CD8+ T cell proliferative responses to BCG, BP and TT vaccinations and SEB in HEU versus HU infants and to evaluate the evolution of the T cell proliferation to BCG and SEB from 6 to 14 weeks of life.

Hypothesis

HEU infants have lower lymphoproliferative ability at 6 and 14 weeks of life in response to vaccination [i.e. BCG administered at birth, Pertussis and Tetanus toxoid vaccine at 6 and 10 weeks] and SEB.

Specific objective 2

To compare the quality and breadth of total CD4+ and CD8+ T cell intracellular cytokine production to BCG, BP and TT vaccine and SEB antigen in HEU versus HU infants and to evaluate the evolution of cytokine production to BCG and SEB from 6 to 14 weeks of life.

Hypothesis

HIV-exposed uninfected infants express lower quantity and breadth of cytokine producing cell responses to vaccination and SEB.

CHAPTER 2

COHORT CHARACTERISTICS

2.1 Study design

This was an observational cohort study.

The primary outcome measure was proliferation in response to Bacille Calmette Guerin (BCG), tetanus toxoid (TT), Bordetella pertussis (BP) and Staphylococcal enterotoxin B (SEB) between HIV-exposed uninfected (HEU) and HIV-unexposed (HU) infants at 6 and 14 weeks of age. The sample size and power calculations were based on the comparison of the primary outcome measure in an independent cohort i.e. the median number of CD4+ki67+ frequency in the HEU infants in response to BCG vaccination at birth measured at 8 weeks of age. **This sample size (n=46) allowed an 80% power to detect a 10% difference in the frequency of CD4+ki67+ cells (2-tailed test at p=0.05)**.

2.1.1 Participants and blood collection

This was a longitudinal observational study of 46 HEU and an equal number of HU infants. Cellular immune responses were compared in whole blood of 6-week and 14-weeks old HEU infants to their unexposed counterparts after 6 days of incubation. Infants were recruited for two large studies conducted by Dr Heather Jaspan:

Group 1. HIV-exposed uninfected (HEU) infants were studied to evaluate the effects of delaying BCG vaccination at either birth or given at 8 weeks of age. 46 infants who were randomised to the early BCG arm (BCG adminisered at 2-3 days after birth) were included in the current study. Infants were enrolled at birth, and followed-up at 2-3 days, 2 weeks, 6, 8, and 14 weeks from February 2010 to June 2012.

Group 2. HIV-unexposed infants (HU) were recruited at birth for an observational infant feeding study from June 2011 to August 2012. Forty six of these infants were used as controls in the current study. Infants were followed up at 6 and 14 weeks only.

2.1.2 Recruitment of participants

Both studies were recruited in Khayelitsha Site B, Western Cape, South Africa and consent forms were provided in English, Xhosa and Afrikaans. Consent for both studies included vaccine responses and was obtaineded from the mother in her preferred language and according to Good Clinical Practice guidelines. Questionnaires were similar for both cohorts.

Study setting

Khayelitsha site B is an informal settlement with an antenatal HIV prevalence of 30.1% in 2011(City of Cape Town, 2011). It has good existing research capacity for prospective studies and ongoing recruitment and with established prevention of mother-to-child transmission (PMTCT) and pediatric antiretroviral (ARV) clinics. All deliveries at Site B are vaginal.

BCG and oral polio vaccine (oPV) are routinely given at birth. Since April 2009, infants receive three doses of Diphtheria-Tetanus-aPertussis(DaPT)-iPV/Hib, Hepatitis B, Rotavirus (RV), and pneumococcal conjugate (PCV_7) vaccinations at 6, 10 and 14 weeks of age (DOH, 2009) (See table 1.1).

Since HIV infected infants have a high risk of BCG adverse events, in 2007 WHO made HIV infection a full contraindication for BCG vaccination(WHO-SAGE, 2007). BCG was administered to HEU infants at a median age of two days (either at day 2 or 3 after birth) after HIV infection was ruled out. HIV-infected infants were not BCG vaccinated and referred immediately for antiretrovirals. BCG was administered at the morning after delivery for HU infants.

2.1.3 Exclusion criteria and HIV testing

For both groups of infants, ineligibility was based on the following;

1. Mother was not willing and able to give consent
2. Baby weighed less than 2.4 kg
3. Baby was not healthy (presented with sepsis/ convulsions/ asphyxia/ severe respiratory distress/ severe congenital abnormality)

4. Mother or anyone in her house had TB or a cough for longer than two weeks, and the baby was then refered for isoniazid therapy(INH)
5. Mother was planning to move away from Khayelitsha in the next 4 months
6. Prematurity (i.e. <36 weeks)
7. Mother was younger than 18 years

Additionally for the HIV-exposed infants, eligibility was based on the following;

8. Mother's HIV test in pregnancy was positive
9. Infant tested HIV DNA PCR negative either at 2-3 days or later at 6 weeks after birth

And for the HIV-unexposed infants, eligibility is based on the following;

1. Mother's HIV test in pregnancy was negative

HIV testing

For the HIV-exposed infants, blood collection for HIV DNA PCR testing was done at birth and six weeks of age.

2.2 Characteristics of the cohort

We compared the characteristics of 46 HEU versus the 46 HU infants. Among all the parameters recorded, the birth weight ($p=0.009$), exclusive breast feeding (EBF) at birth ($p<0.0001$), the age at which BCG was given ($p<0.001$), and percentage morbidity ($p<0.0001$) were significantly different between HIV-exposed uninfected (HEU) and HIV-unexposed (HU) infants (Table 2.1).

The median and the interquatile range (IQR) birth weight of the HEU versus HU infants [i.e. 3.1 (2.8-3.4) Kg versus 3.3 (3.1-3.6) Kg] and the percentage (%) of infants exclusively breastfed (EBF) at birth between HEU versus HU infants [i.e. 23.8% versus 100%] were significantly lower in the HEU compared to HU infants. However, by 6 weeks and 14 weeks, the weight of the HEU was not significantly different to the HU infants, and the proportion of EBF infants was also not significantly different between the two groups of infants. The median and the IQR age at which BCG was given to HEU versus HU infants [i.e. 3 (2-4) days versus 0 (0-1) day] and

overall percentage morbidity in HEU versus HU infants [i.e. Skin rash (30.9% versus 2.2%), Runny nose (35.7% versus 0%) and cough/fever (26.2% versus 13.0%)] were significantly higher in the HEU compared to the HU infants. The age in days at the 6- and 14-week visits, as well as the gestational age of the HEU infants was not significantly different to the HU infants (Table 2.1).

Based on the exclusion criteria, mothers were not recruited if they were younger than 18 years. No information was taken based on parity of the mothers..There was little information collected on the socioeconomic status. Housing, electricity, and running water data was collected the HEU infant's mothers, 31.4% lived in brick/formal structures, 60.8% in shacks, and a small proportion about 7.8% that stayed in any other form of housing not mentioned. There was no information on HU infant's mothers. This is one of the limitations of the study. However, we believe they would be similar based on a recent cross-sectional study we performed.

Table 2.1: **Characteristics of the cohort between HEU versus HU infants at 6 and 14 weeks of life**; the read outs for each parameter is indicated as medians and interquatile ranges (IQR) as well as in percentages (%) and p-values with red fonts illustrate parameters that were significantly different between HEU vs. HU infant groups i.e. p-values<0.05, statistical significance was obtained using **Rank sum, *Chi square or $Fisher's exact tests.

Parameters	HIV-Unexposed (HU) Infants n=46	HIV-exposed Uninfected (HEU) Infants n=46	p-value
Median birth weight (Kg) (IQR)	3.3 (3.1-3.6)	3.1 (2.8-3.4)	0.009**
Median weight at 6 weeks (Kg) (IQR)	4.9 (4.6-5.2)	4.8 (4.2-5.2)	0.097**
Median weight at 14 weeks (Kg) (IQR)	6.6 (6.0-6.9)	6.6 (6.2-7.2)	0.596**
Exclusive breastfed (EBF) at birth (%)	100%	23.8%	<0.001$
Exclusive breastfed (EBF) at 6 weeks (%)	39.5%	26.2%	0.191*
Exclusive breastfed (EBF) at 14 weeks (%)	26.5%	24.3%	0.835*
Median age at BCG given in days (IQR)	0 (0-1)	3 (2-4)	<0.001**
Median age at 6 weeks visit in days (IQR)	43.5 (43-48)	43 (42-44)	0.254**
Median age at 14 weeks visit in days (IQR)	100 (98-112)	106 (98-113)	0.078**
Median gestation age in weeks (IQR)	39 (38-40)	39 (37-40)	0.205**
Morbidity (%) (6 Wks): - Skin rash	1 (2.2%)	13 (30.9%)	<0.001$
- Runny nose	0 (0%)	15 (35.7%)	
- Cough/fever	6 (13.0%)	11 (26.2%)	

2.3 Discussion

The results show that HIV-exposed infants have lower median birth weight than HU infants ($p<0.001$) and also an overall increase in morbidity ($p<0.001$), emphasizing the vulnerability of HEU infants. There were some other differences evident in the cohort noted when analyzing the data and interpreting the results. Some of these, including birth breastfeeding practices and birth weight, would need to be accounted for as possible confounders when interpreting results.

Data collection on morbidity and mortality were almost identical in the two cohorts. Both groups of infants were recruited in the same community, presented to the same exposure and followed up similarly through the four seasons of the year in South Africa.

Finally, Zambian HEU infants who remained uninfected after perinatal or early breastfeeding-related HIV exposure were of high risk of mortality and morbidity during the first few months of life regardless of their maternal disease stage or Socioeconomic status(Kuhn *et al*., 2006). It is important to note that, maternal viral loads at delivery for the HEU infant's mothers were not measured as it is not routine to collect baseline viral loads and most mothers in our study were not diagnosed during pregnancy. No information was taken on antiretroviral regimens placed for PMTCT at the time of delivery. However, at the time the study was performed, SA PMTCT guidelines recommended dual therapy for women with CD4 above 200 cells/mm^3, and HAART for women with lower CD4 counts(South African Department of Health, 2010).

CHAPTER 3

METHODOLOGY

3.1 Materials and methods

Based on the main aim of this dissertation; to evaluate cellular immune responses to EPI vaccines as well as SEB in HEU versus HU infants, we employed several techniques to achieve our goal. The overall process involved two assays:

1. Whole blood assay (WBA) ant its importance

Optimized antigen concentrations were used. It has been shown that, it is important for whole blood to be incubated at 37^0C with antigens as soon as possible after collection(Hanekom *et al.*, 2004). The WBA allowed immediate incubation of blood with antigens that would need to be processed (e.g. BCG and whole cell pertussis), and hence, produce optimal results(Soares *et al.*, 2010)(Soares *et al.*, 2013). Table 3.1 below shows studies that have looked at cellular immune responses in HEU infants to date. Optimal antigen concentrations for WBA may significantly differ from that used in PBMC-based assays. Whole bacterial antigens, recombinant vaccinia virus-based antigens, proteins, and peptide antigens have been used successfully in the WBA(Hanekom *et al.*, 2004)(Remick, 1999).

Table 3.1: Published studies assessing vaccine cellular immune responses in HIV-exposed uninfected (HEU) infants

Parameters	Study Design	Vaccine type	Infant age	Assay	Proliferation/Cytokine
Mazzola *et al.*, 2011	Cross-sectional	BCG	6.1mths - 26.3mths	PBMC	CD4+,CD8+/IL-10,IFN-Ɣ and TNF-α[a]
Rie *et al.*, 2006	Longitudinal	BCG	Birth - wk6	WBA[1]	ND/IFN-Ɣ[a]
Mansoor *et al.*, 2010	Longitudinal	BCG	3mth - 9mth	WBA[2]	CD4+,CD8+/IFN-Ɣ , IL-2 and TNF-α[a and b]
Borges-almeida *et al.*, 2011	Cross-sectional	BCG	Cord blood	CBMC	CD4+,CD8+/IL-2, IL-4, IL-7, IL-10, IL- 12, TNF-α and IFN-Ɣ[a]

1 = 7 day whole blood assay
2 = 12 hour whole blood assay
a = ELISA
b = Flow cytometry

2. Flow cytometry [Proliferation and intracellular cytokine staining assay (ICS)]

Conventional T cell (CD4 and CD8) that have the αβ T cell receptor (TCR) were the cells of interest. Using the WBA, antigen presenting cells (APC) would present the antigens *in vitro* to T cells via the MHC-peptide complex. Optimum stimulation of T cell was aided by other signals mediated by membrane receptors; B7.1/7.2 on APC and CD28 on T cells, endogenous IL-2 to it receptor on T cells, as well as other pro-inflammatory cytokines (IL-6 and IL-12) by APC. These would lead to signal transduction by the CD3 complex and proliferation of T cells and further cytokine production(Janeway, 2008). Table 3.2 below shows the available methods to measure antigen specific responses and their importance.

Also, non-conventional T cells [gamma-delta (ϒδ) and Natural killer (NK) T cells] would also be present in whole blood. They are a minority of the peripheral CD3+ T cell population and with limited TCR usage(Janeway, 2008). ϒδ T cells can recognize non-peptide antigens such as microbial metabolites and phosphor-antigens via their ϒδ TCR directly(Bonneville *et al.*, 2010), and NK T cells recognize glycolipids via their simi-invarian (Vα14-Jα18) TCR presented by the CD1d molecule on APC(Kaer, 2005)(Godfrey *et al.*, 2004). NK T cells can act directly and indirectly to modulate function of other cell types. They directly activate lytic function by NK cells via IFN-ϒ production; indirectly activate proliferation, lytic function and cytokine production by CD4 and CD8 T cells via direct activation of dendritic cell(Cerundolo *et al.*, 2009).

Table 3.2: Available methods to measure antigen specific responses, their pros and cons

Method	Response type	Pros	Cons
Oregon Green incorporation -(Soares *et al*., 2010)	-T cell proliferation -Identification of cellular sub-populations (phenotype and functional characteristics)	-Flow cytometry -Co-staining with other markers -Used with peripheral blood mono nuclear cells (PBMC)	-Cannot use whole blood -Less robust (in terms of culture manipulation) -Large blood volumes
Carboxyfluorescein Succinimidyl Ester (CFSE) staining -(Soares *et al*., 2010)	-T cell proliferation -Identification of cellular sub-populations (phenotype and functional characteristics)	-Flow cytometry -Co-staining with other markers -Used with PBMC	-Cannot use whole blood -Less robust -Large blood volumes
5-bromo-2'-deoxyuridine (BrdU) -(Soares *et al*., 2010)	-T cell proliferation -Identification of cellular sub-populations (phenotype and functional characteristics)	-Flow cytometry -Co-staining with other markers -Used with PBMC	-Cannot use whole blood -Less robust -Large blood volumes
^{3}H-thymidine incorporation -(Soares *et al*., 2010)	T cell proliferation	-Used with PBMC	-Cannot use flow cytometry -Cannot use whole blood -Less robust -Large blood volumes
Ki-67 staining - (Soares *et al*., 2010) - (Soares *et al*., 2013) - (Hanekom *et al*., 2004) - (Remick, 1999)	-T cell proliferation -Identification of cellular sub-populations (phenotype and functional characteristics)	-Flow cytometry -Co-staining with other markers -Used with PBMC **-Use whole blood** **-Robust** **-Small blood volumes**	

3.1.1 Whole blood assay (WBA)

Between 1-3mL whole blood was collected from the two groups of infants at 6 and 14 weeks of age into a heparinised tube and transported to the laboratory within 6 hours. Samples were in culture within 8 hours of blood draw. The blood collections from HEU infants were performed from February 2010 to June 2012, and from the HU infants from June 2011 to August 2012.

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 36^{o}C with 5%CO_2 without antibiotics. The

following antigens were added: $1x10^5$ cfu/mL *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) [Danish strain 1331; SSI], 0.16 IU Tetanus toxoid (TT) [TETAVAX, Aventis Pharma (Pty) Ltd], 0.16 IU Pertussis antigens (BP) [Difco™ Bordetella Pertussis Antigen, BD] and a negative control (medium alone) (Figure 3.1a).

After 24 hours of incubation 1ug/ml Staphylococcal enterotoxin b (SEB) was added to its required well and then cells were placed back at 36°C for a further 6 days(Soares *et al.*, 2010). On the 6th day, 0.001mg/ml of phorbol 12-myristate 13-acetate (PMA) and 0.005mg/ml Ionomycin was added which work synergistically to stimulate cells [PMA and Ionomycin stimulate intracellular production of cytokines by cells non-specifically and have been shown to work synergistically(Hanekom *et al.*, 2004)(Soares *et al.*, 2010)(Soares *et al.*, 2013)], along with 0.05mg/ml Brefeldin A which captures cytokines intracellularly, for the last 4 hours of incubation and the cells were harvested in 20mM EDTA.

Negative control cells were stimulated with media alone on day 0 with Ionomycin and PMA on day 6. Positive controls cells were stimulated with SEB on day 1 and with Ionomycin and PMA on day 6. After culture, red blood cells were lysed and white cells were stained with Pacific Blue Live/Dead stain [violet viability dye (VIVID)] before fixing with FACS Lysing Solution and cryopreserving in a 10% DMSO-containing solution for storage at -80°C until analysis. This assay was chosen due to the high yield of information obtainable from very small volumes of blood (Remick, 1999) (Figure 3.1b).

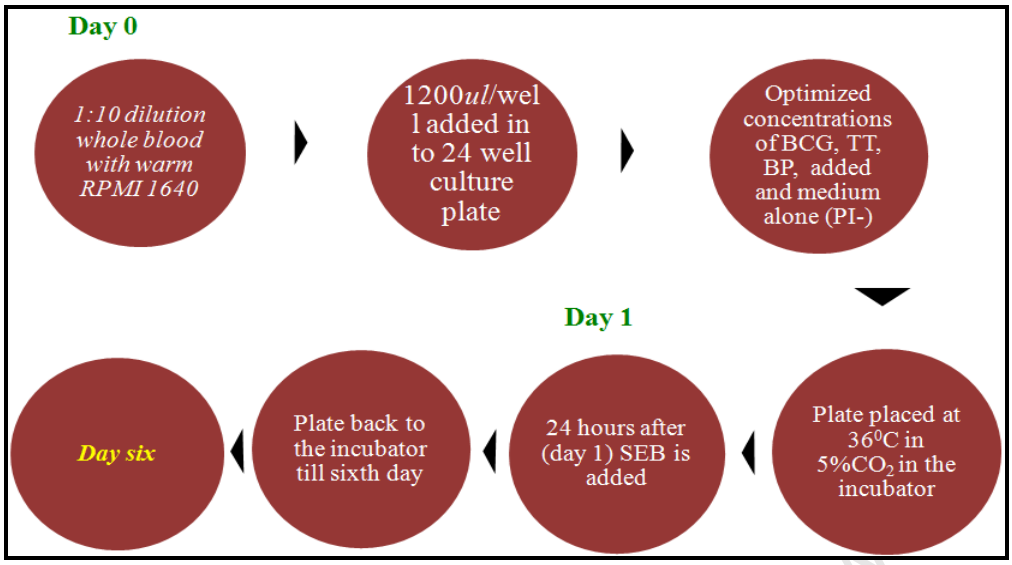


Figure 3.1a: The whole blood assay procedure: On day 0, whole blood diluted in RPMI was incubated with antigens (BCG, BP, TT and medium alone). SEB was added on day-1 (24 hours after incubation) to its required well, and the culture plate was placed back in the incubator till day 6.

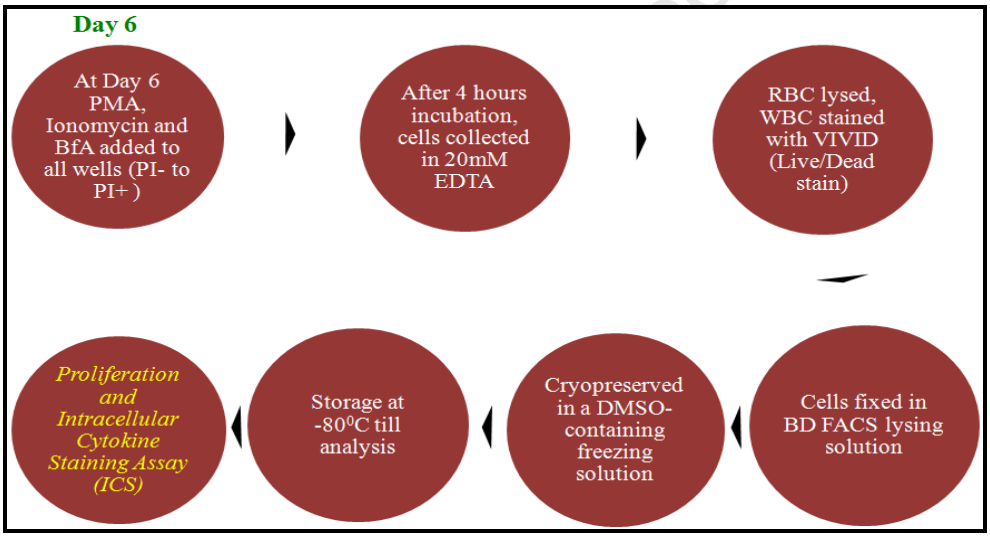


Figure 3.1b: The whole blood assay procedure day-6: PMA and Ionomycin and Brefeldin A were added to all wells for the last four hours of stimulation, after which the cells were harvested in 20mM EDTA, red blood cells (RBC) were lysed and white blood cells (WBC) stained with live/dead stain. Cells were further fixed in BD FACS Lysing solution, cryopreserved and stored at -80^{0}C till analysis (ICS and flow cytometry).

3.1.2 Proliferation measured by Ki67 incorporation and Intracellular cytokine staining assay (ICS)

To answer the first, second and third specific objectives (section 1.7.1), fixed cryopreserved cells were thawed, permeabilized and stained for intracellular INF-γ, IL-2, IL-13 and IL-17 (ICS) and for cellular markers (CD3 and CD8) and for proliferation marker (Ki67) with optimised volumes of fluorescence-conjugated antibodies. Expression was measured by multiparameter flow cytometry (LSR Fortessa) using an eight colour panel (Table 3.3).

Table 3.3: Eight colour flow cytometry panel: The eight colour flow cytometry panel employed with the various antibodies markers, fluorochrome, their suppliers, rational and their importance for inclusion in the panel.

MARKER	FLUOROCHROME	CLONE	SUPPLIER	MARKER / FUNCTION
Vivid	Pacific Blue	-	Invitrogen	Live/Dead / Dead cell marker
CD3	APC-Cy7	UCHT1	Biolegend	T-Lymphocytes / T lymphocyte marker
CD8	PerCP-Cy5.5	SK1	BD Biosciences	Cytotoxic T-Lymphocytes / Kill infected cell
Ki67	FITC	B56	BD Biosciences	Proliferation / T cell proliferation marker
IFN-γ	Alexa Fluor 700	B27	Biolegend	TH1 / Control of intracellular pathogens
IL-2	APC	5344.111	BD Biosciences	TH1 / Growth and function of T-cells
IL-13	PE	JES10-5A2	BD Biosciences	TH2 / Directs antibody production by B cells
IL-17	PE-Cy7	BL168	Biolegend	TH17 / Recruits CD4 Th1 cells, Neutrophils and enhanced phagocytic killing

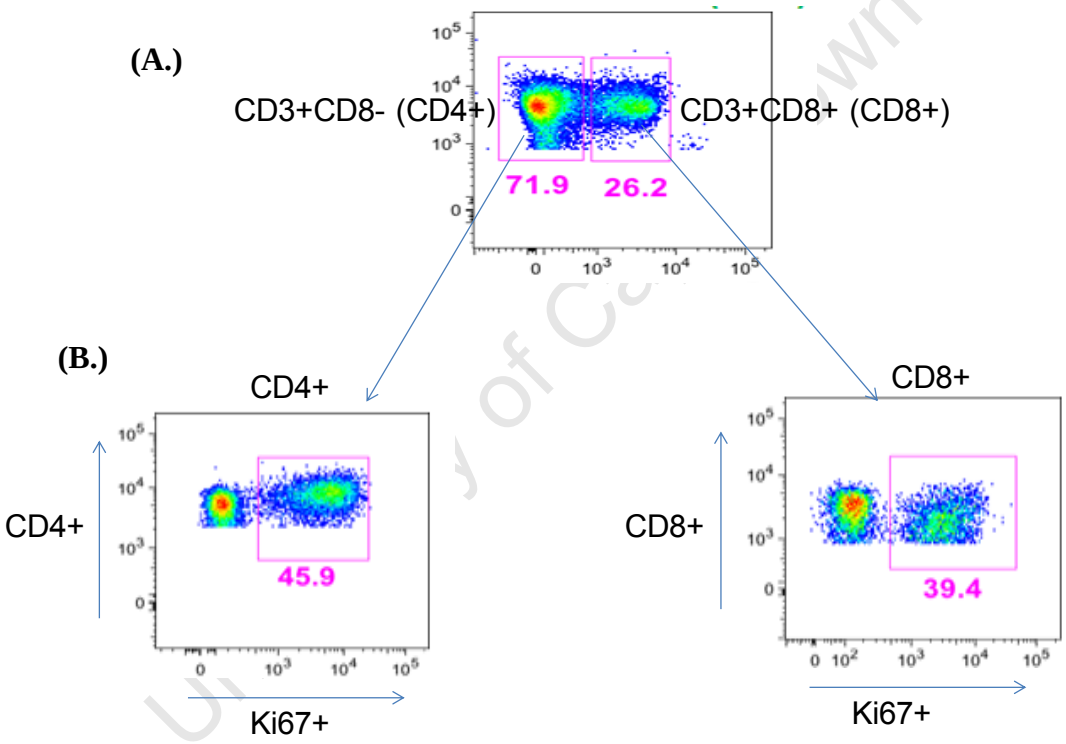
3.2 Data analysis

After flow cytometry acquisition, data was extracted for analysis. Below is a summary of the data analysis strategies.

3.2.1 Flow cytometry data analysis

After acquisition by flow cytometry, the files were first analyzed using FLOWJO v9.4.7 (Tree Star). After gating for singlets, live CD3+ cells, CD8+ cells and CD8- cells were gated (and regarded as CD4+ cells since PMA-Ionomycin causes CD4 degradation in T cells)(Rueggs *et al.*, 1992). CD4+Ki67+ and CD8+Ki67+ proliferating cells were then gated on CD8- (CD4+) and CD8+ populations respectively (Figure 3.2a). IFN-γ and IL-2 (Th1), IL-17 (Th17) and IL-13

(Th2) were then gated on within Ki67+ CD8- (CD4+) and CD8+ cells (Figure 3.2b). Boolean gating was used to quantify cells expressing a single or a combination of multiple cytokines. Appendix C below shows details on how the gates were determined, 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. Appendix F below shows the median background response to evaluate the level of non-specific stimulation in the assay.



Gating of Cytokine production from proliferating T cells (Next)

Figure 3.2a: Gating of CD4+, CD8+ and Ki67+ proliferating T cells. (A) A representative flow cytometry plot of CD3+CD8- (CD4+) (left) and CD3+CD8+ (CD8+) (right) T cells and (B) CD4+Ki67+ (left) and CD8+Ki67+ (right) proliferating cells in response to Staphylococcal enterotoxin B [SEB] (Positive control). The values represent the percentage of T cells that fall within the gates in this representative experiment.

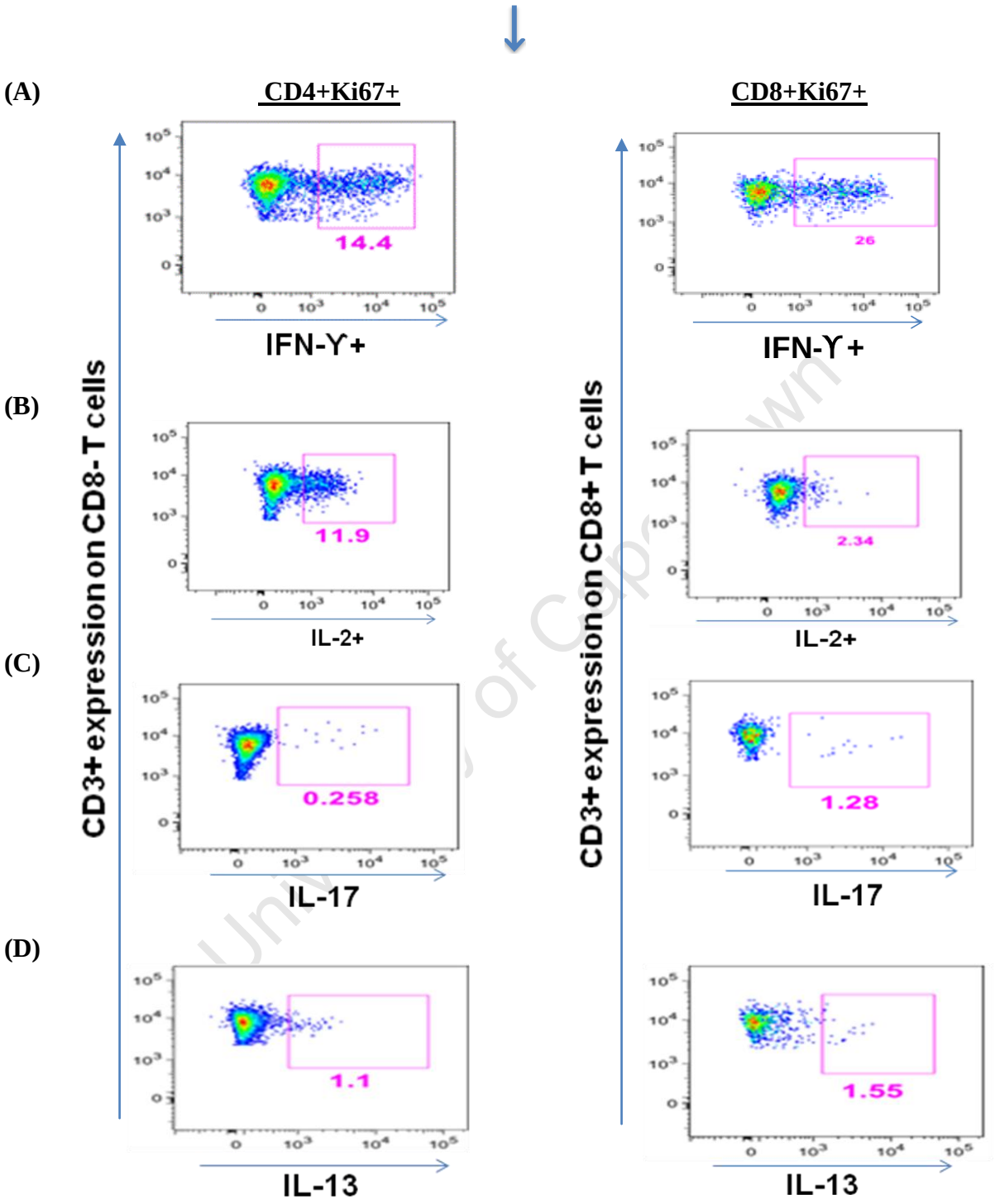


Figure 3.2b: Gating of CD4+Ki67+ and CD8+Ki67+ cytokine producing T cells. A representative flow cytometry plot of CD4+Ki67+ (left) and CD8+Ki67+ (right) proliferating cells expressing intracellular cytokines; (A) and (B) IFN-Y and IL-2 (Th1), (C) IL-17 (Th17) and (D) IL-13 (Th2) in response to

Staphylococcal enterotoxin B [SEB] – Positive control. The values represent the percentage of T cells that fall within the gates in this representative experiment.

3.2.1.1 Standard rules for cut-offs and controls for the flow cytometry data

Following analysis by FlowJo, the data was exported and organized for further analysis. In order to ensure no data bias and to perform background subtraction, the following rules were put in place:

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

The frequency of proliferating cells in response to SEB had to be greater than the median plus 3 times the Median Absolute Deviation (MAD) of the negative control, or the sample was excluded**. MAD** is a robust scale estimator about 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(Rouss *et al*., 1993).

i.e. Frequency of CD4+ proliferating cells

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

2.) 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, BP, TT and SEB) minus background (% of Ki67+ CD4+ and CD8+ T cells in the media control)

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

(ii) [(BCG, BP and SEB induced %Ki67+CD8+cells) – (Induced %Ki67+CD8+ cells in the media)] (Figure 3.3).

3.) Cut off for cytokine responses

For single cytokine analyses (Chapter 5), data is reported as frequency of grandparent T cell population which was the frequency of total CD4+ and CD8+ T cells producing cytokines (i.e. 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 (Chapter

6) data is reported as the frequency of proliferating T cells producing cytokines i.e. the denominator is proliferating cells only.

(a.) The test antigens (i.e. BCG, BP, TT and SEB) cytokine responses were only quantified if the event counts of proliferating T cells (i.e. CD4+Ki67+ T cells) were ≥ 20.

(b.) Further, we performed 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, BP, TT and SEB (Figure 3.3).

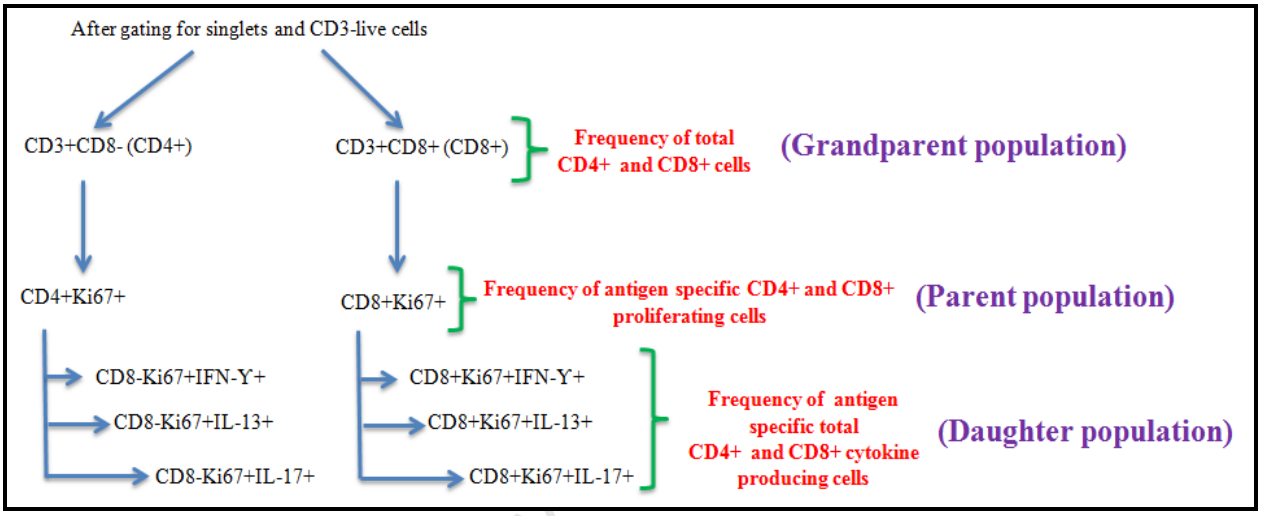


Figure 3.3: Schematic description of the frequencies of total CD4+ and CD8+ cells (grandparent population), antigen specific CD4+ and CD8+ proliferating cells (parent population) and antigen specific total CD4+ and CD8+ cytokine producing cells (daughter population).

3.3 Statistical considerations

Different statistical packages were used to complete the data analysis after obtaining data from flow cytometry as listed in the table below (Table 3.4).

Table 3.4: **Statistical packages used for data analysis**

Parameters	SOFTWARE PROGRAM	ANALYSIS TYPE	DATA TYPE
1	**FLOWJO v9.4.7** (for MAC, Stanford University, 1995-96; Tree Star, Inc. 1997-2012)	To analyze flow cytometric data after acquisition	Flow cytometry data analysis
2	**Microsoft Excel** 2007	To clean and organize data	
3	**Pestle v1.7** (for MAC, Mario Roederer, National Institute of Allergy & Infectious Diseases, 2004-2011)	To perform background subtraction and organize data for SPICE	
4	**SPICE v5.22** (for MAC, Mario Roederer-Joshua Nozzi, National Institute of Allergy & Infectious Diseases, 2004-2011)	To analyze intracellular cytokine polyfunctionality between groups	
5	**GraphPad Prism v5** (for Windows, GraphPad Software, San Diego California USA, www.graphpad.com").	To analyze differences in proliferation and absolute cytokine expression by cells between groups.	Graphics
6	**STATA v11** (for windows, StataCorp LP, College station, TX77845, USA).	To perform inferential statistical analyses.	Statistical evaluation of derived data

3.3.1 Statistical data analysis

Data analysis was performed by applying the following statistical tests;

Table 3.5: **Statistical tests used to complete data analysis**

Statistical test	Rationale	Statistical package	Data type
Shapiro-Wilk test	Confirm normality or non-normality of data distribution. If not normally distributed then data was log transformed for normal distribution	STATA v11	-Cohort description - T cell Proliferation -T cell cytokine production -T cell polyfunctional cytokine responses
Chi-square test	Relationship between two categorical variables	STATA v11	Cohort description (e.g. EBF vs. MF)
Fisher's exact test	Relationship between two categorical	STATA v11	Cohort description

	variables (one or more of the values in the cells has an expected frequency ≤5)		
Wilcoxon rank sum test (Mann-Whitney test)	Compare continuous data between two categories that is not normally distributed.	-STATA v11 - GraphPad Prism v5 - SPICE v5.22	-T cell Proliferation -T cell cytokine production -T cell polyfunctional cytokine responses (HEU vs. HU)
Linear regression (univariate and multivariate)	Compute comparisons between variables of interest at particular time points when adjusting for confounders	- STATA v11	-T cell Proliferation -T cell cytokine production (At 6 and 14 weeks of age)
Generalized Estimation Equation (GEE) population average models	Compute comparisons between variables of interest through time from one time point to another	- STATA v11	-T cell Proliferation -T cell cytokine production (From 6 to 14 weeks of age)
Holm step-down adjustment for multiple comparisons approach (based on the Bonferroni inequality P'), (Columb *et al*., 2006).	(i) the p-values for the each parameter measured for all conditions (m) were arranged in ascending order, (ii) starting with the smallest p-value, P'=(m)p, (iii) if significant, we proceeded to the next smallest p-value and calculated P'=(m-1)p, (iv) the step-wise process was continued (…m-2,…) until P'>0.05, which we continued and tested all the hypotheses for all p-values<0.05 [P'=(…,…m-(m-1))p<0.05]	Not applicable	Adjusted p-values

3.3.1.1 Univariate analyses

Data was first described through univariate analyses (primary variable being HIV-exposure) between HIV-exposed uninfected (HEU) and HIV-unexposed (HU) infants at two separate time points six and 14 weeks. Comparisons of categorical and continuous data between the two groups were performed using different statistical tests (Table 3.5).

3.3.1.2 Multivariate analyses

Multiple regression models were constructed by examining *a priori* variables based conceptual knowledge and not just on statically significance between the groups at baseline. HIV-exposure was the primary variable (Table 3.5).

Generalized Estimation Equation (GEE) population average models were also constructed by examining variables of interest (Table 3.5) to evaluate the evolution of the T cell proliferation and intracellular cytokine production to BCG (administered at birth) and SEB from 6 to 14

weeks of life. GEE could not be performed to BP and TT induced T cell proliferation and intracellular cytokine production since *Bordetella pertussis* and Tetanus toxoids vaccines were only first administered at 6 weeks of life (see section 2.1.2).

CHAPTER 4

ANTIGEN SPECIFIC T-CELL PROLIFERATIVE RESPONSES IN HIV-EXPOSED UNINFECTED (HEU) VERSUS HIV-UNEXPOSED (HU) INFANTS AT 6 AND 14 WEEKS OF LIFE

4.1 Introduction

We compared CD4+ and CD8+ T cell proliferative responses to vaccine antigens [Bacillus Calmette-Guérin (BCG), *Bordetella pertussis* (BP) and Tetanus toxoid (TT)] and to Staphylococcal enterotoxin B (SEB) and the consequences of HIV-1 exposure on the development of antigen specific cellular proliferative immune responses in early life between HEU versus HU 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 3.1.1) with samples collected at six and at 14 weeks of age from both HEU and HU infants.

4.2 T-cell proliferative responses in HEU versus HU infants at 6 and at 14 weeks of life

The proliferative responses to BCG, BP, TT and SEB were compared between HEU versus HU infants.

4.2.1 BCG-, BP- and TT-induced T-cell proliferative responses

The p-values shown in Figure 4 are unadjusted. For BCG, there was no significant difference at 6 weeks, however, at 14 weeks, after adjustment for multiple comparisons, the frequency of both CD4+ (median of HEU=21.9% versus HU 8.7% and p=0.2035) was not significant and CD8+ (median of HEU=16.0% versus HU 6.7% and p=0.009) remained significant. [Figure 4.1(a)](Columb *et al.*, 2006). For BP and TT, no significant differences were observed between the two infant groups in the frequency of CD4+ and CD8+ proliferating T cells at 14 weeks of life [Figure 4.1 b and c). However, the number of samples with proliferating cells from which we could measure T cells proliferation in the HEU infants in response to TT was small. Appendix D below shows the number of HEU and HU infants whose CD4+ and CD8+ T cell proliferation responses to the different antigens measured by Ki67 incorporation was performed.

(a) **BCG-induced Ki67+ T cells**

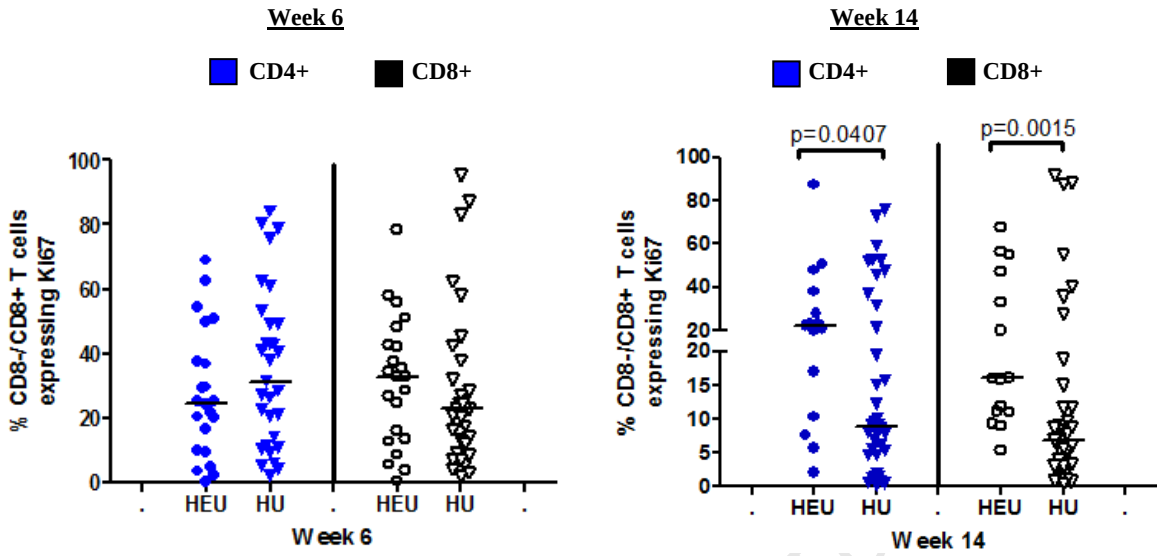
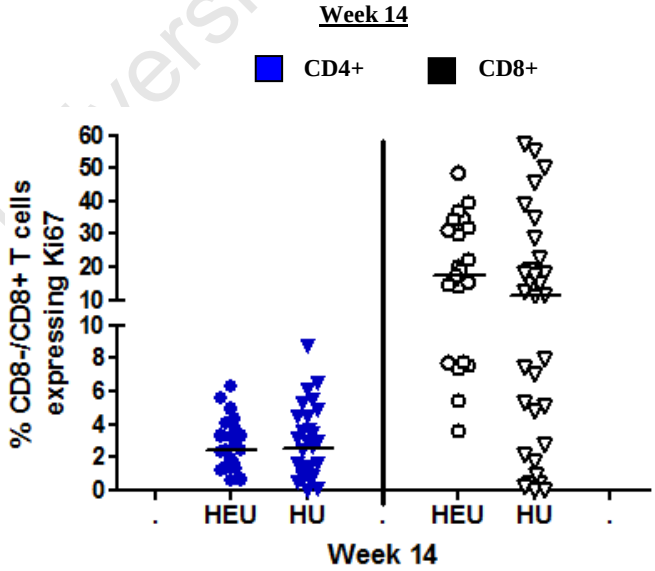


Figure 4.1a: Frequency of proliferating T cells after Bacillus Calmette-Guérin (BCG) stimulation at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level (unadjusted) and statistical significance was obtained using *Mann-Whitney U* test.

(b) **BP-induced Ki67+ T cells**



(c) **TT-induced Ki67+ T cells**

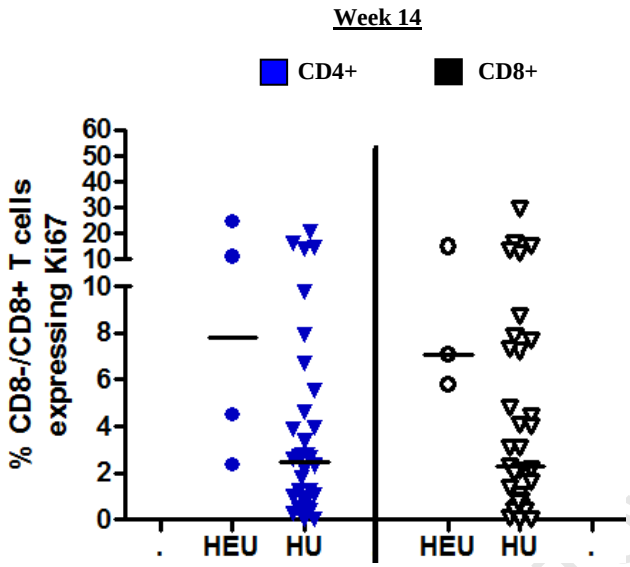


Figure 4.1b-c: Frequency of proliferating T cells after *Bordetella pertussis* (BP) and Tetanus toxoid (TT) stimulation at 14 weeks of life measured by flow cytometry. Frequency of CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) proliferating T cells determined by flow cytometry in **(b)** BP- and **(c)** TT-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level (unadjusted) and statistical significance was obtained using *Mann-Whitney U* test.

4.2.2 SEB-induced T-cell proliferative responses

The p-values shown on the graph are the unadjusted ones. A significantly higher frequency of both the CD4+ (HEU median=42.9% versus HU median=12.6 and p=0.0264) and CD8+ (HEU median=62.3% versus HU median=20.2 and p=0.008) proliferating T cells after SEB stimulation was observed in the HEU versus their unexposed counterparts at 14 weeks after adjustment for multiple comparisons, but not at 6 weeks of life [Figure 4.2].

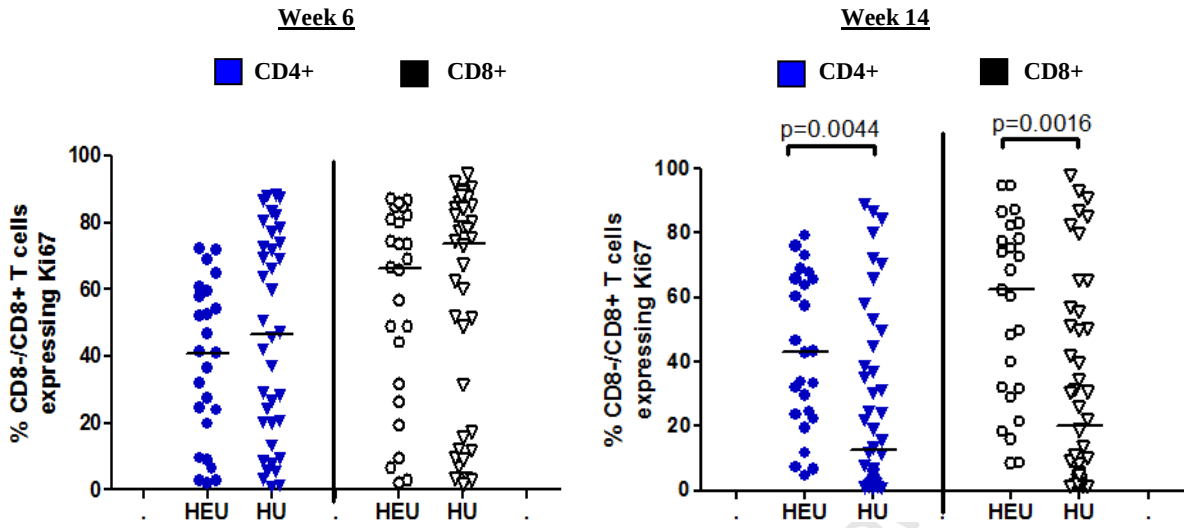


Figure 4.2: Frequency of proliferating T cell after Staphylococcal enterotoxin B (SEB) stimulation at 6 and 14 weeks of life measured by flow cytometry. Frequency CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) proliferating T cells determined by flow cytometry in SEB-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level (unadjusted) and statistical significance was obtained using *Mann-Whitney U* test.

4.3 Factors influencing antigen specific T cell proliferation

Based on the observational nature of our cohort (see table 2.1), we took into consideration factors that could confound T cell proliferation between HEU versus HU infant groups in response to vaccine antigens (BCG, BP and TT) and SEB.

4.3.1 Factors predicting BP- and TT- induced T cell proliferation at 14 weeks of life.

A priori factors that could influence BP- and TT- induced CD4+ and CD8+ T cell proliferation at 14 weeks of age were assessed in regression analysis (Table 4.1), because following the Expanded Program on Immunization (EPI) schedule in South Africa, the first dose of these vaccines are administered only at 6 weeks of life. The results were presented and interpreted as etiological models assessing the strength of the association between HIV-exposure and T cell proliferation when taking other covariate into consideration.

The following *a priori* predictive variables were studied based on conceptual knowledge and not just on statistical association with the exposure (see table 2.1); (i) HIV-exposure, (ii) gestation age, (iii) birth weight, (iv) weight at 6 weeks, (v) exclusive breast feeding (EBF) at birth, (vi) EBF at 6 weeks, (vii) age at 6 weeks, and (viii) age at 14 weeks.

We went further to correct for these factors using linear regression models and a backward elimination process was used. Only the results of the exposure (HEU using HU as reference population) were reported, with note of which (if any) other covariates adjusted for created a difference in the exposure variable. We performed multivariate analysis with only three predictive variables where we had less than 5 children per response (Appendix D, Table D1), as a model with 5 variables is unlikely to converge if there are less than 5 children in one of the two exposure groups (i.e. for TT at 14 weeks CD4+ and CD8+ T cell proliferation).

In Model 1, HIV-exposure alone was not significantly associated with CD4+ or CD8+ T cell proliferation in response to either BP or TT at 14 weeks of life (Table 4.1). Model 2 demonstrate the predictive effect of gestational age on T cell proliferation. However, the modified coefficient (95% CI) of the HIV-exposure variable was still not significant and in subsequent models. Model 3 accounts for the weight at birth and at 6 weeks. Additional potential confounders were introduced in Model 4 and 5. However, the number of samples for TT in the HEU infants was small.

This result was opposite to that for BCG where proliferation was positively associated with HIV-exposure alone unconfounded. This may be due to the type of antigen, suggesting that HIV-exposed uninfected infant T cells were less able to proliferate in response to protein antigens.

In these models, none of the other potential confounders were significant predictors of CD4+ or CD8+ T cell proliferation (Table 4.1), except for birth weight, when adjusting for HIV-exposure, gestational age, and weight at 6 weeks in Model 3. HIV exposure did not predicted T cell proliferation in responses to BP, but birth weight directly predicted CD4+Ki67+ responses to BP (Table 4.1).

Table 4.1: Factors predicting CD4+ and CD8+ antigen specific T cell proliferation at 14 weeks of life after stimulation with *Bordetella pertussis* (BP) and tetanus toxoid (TT) antigens, determined by linear regression models. Frequency of proliferating T cells (CD4+Ki67+ or CD8+Ki67+) after adjusting *a priori* confounders (variables) following BP and TT stimulation at 14 weeks of age. Condition = cond., Week = wk, exclusively breast feeding = EBF, coefficient = coef., and 95% confidence interval = 95% CI. Bold text represents variables that remain significant after multivariate adjustments for possible confounders. Not done = ND (where there was less than 5 children in one of the exposure groups).

Cond.	Variable	Model 1 Coef. (95% CI)	Model 2 Coef. (95% CI)	Model 3 Coef. (95% CI)	Model 4 Coef. (95% CI)	Model 5 Coef. (95% CI)
				CD4+Ki67+		
BP WK 14	HIV-exposure	2.86 (-4.11-9.83)	3.02 (-4.08-10.11)	3.92 (-3.33-11.17)	3.91 (-7.71-15.53)	1.43 (-12.51-15.36)
	Gestation age		0.32 (-1.50-2.14)	-0.14 (-2.09-1.81)	-0.05 (-2.04-1.93)	0.02 (-2.27-2.30)
	Birth weight			**0.02 (0.00-0.03)**	0.01 (-0.00-0.03)	0.02 (-0.00-0.03)
	Weight at 6 wk			-0.01 (-0.02-0.00)	-0.01 (-0.02-0.00)	-0.01 (-0.02-0.00)
	EBF at birth				-1.08 (-14.75-12.58)	-2.95 (-17.50-11.61)
	EBF at 6 wk				3.19 (-5.59-11.97)	6.72 (-3.75-17.19)
	Age at 6 wk					-0.54 (-1.78-0.69)
	Age at 14 wk					-0.55 (-1.28-0.18)
TT WK 14	HIV-exposure	-4.21 (-18.29-9.88)	-4.84 (-19.72-10.05)	-5.23 (-21.35-10.89)	**ND**	**ND**
	Gestation age		0.33 (-1.90-2.56)	0.35 (-1.93-2.62)		
	Birth weight			0.00 (-0.01-0.01)		
	Weight at 6 wk					
	EBF at birth					
	EBF at 6 wk					
	Age at 6 wk					
	Age at 14 wk					
				CD8+Ki67+		
BP WK 14	HIV-exposure	-1.43 (-14.22-11.36)	-2.44 (-15.18-10.30)	-0.49 (-13.91-12.92)	3.15 (-17.79-24.09)	-8.62 (-31.06-13.82)
	Gestation age		1.96 (-1.25-5.16)	1.96 (-1.56-5.49)	1.76 (-1.83-5.34)	1.01 (-2.67-4.69)
	Birth weight			-0.00 (-0.03-0.02)	0.00 (-0.03-0.03)	0.00 (-0.03-0.03)
	Weight at 6 wk			-0.00 (-0.02-0.01)	-0.01 (-0.02-0.01)	-0.00 (-0.02-0.01)
	EBF at birth				7.87 (-17.17-32.91)	5.36 (-18.45-29.17)
	EBF at 6 wk				-8.26 (-24.18-7.66)	-7.21 (-24.04-9.62)
	Age at 6 wk					1.39 (-0.60-3.39)
	Age at 14 wk					-0.59 (-1.78-0.59)
TT WK 14	HIV-exposure	-16.32 (-37.05-4.42)	-18.52 (-40.32-3.28)	-16.29 (-39.82-7.25)	**ND**	**ND**
	Gestation age		1.14 (-2.12-4.41)	1.04 (-2.28-4.36)		
	Birth weight			-0.00 (-0.02-0.01)		
	Weight at 6 wk					
	EBF at birth					
	EBF at 6 wk					
	Age at 6 wk					
	Age at 14 wk					

4.3.2 Factors predicting BCG- and SEB- induced T cell proliferation longitudinally through time from 6 to 14 weeks of life

Generalized Estimation Equation (GEE) population averaged model was used to predict the evolution of T cell immune responses longitudinally from 6 to 14 weeks of life in response to BCG vaccination and SEB stimulation over time; and to adjust for *a priori* factors (every

potential confounder) that could influence BCG- and SEB- induced CD4+ and CD8+ T cell proliferation over time from 6 to 14 weeks of age (Table 4.2). The results were presented and interpreted as etiological models assessing the strength of the association between HIV-exposure and T cell proliferation when taking other covariate into consideration.

The following *a priori* predictive variables were studied based on conceptual knowledge and not just on statistical association with the exposure (see table 2.1); (i) HIV-exposure, (ii) gestation age, (iii) birth weight, (iv) weight at 6 weeks, (v) exclusive breast feeding (EBF) at birth, (vi) EBF at 6 weeks, (vii) age at 6 weeks, and (viii) age at 14 weeks.

We went further to correct for these factors using GEE models and a backward elimination process was used. Only the results of the exposure (HEU using HU as reference population) were reported, with note of which (if any) other covariates adjusted for created a difference in the exposure variable. We performed multivariate analysis with only three predictive variables where we had less than 5 children per response (Appendix D, Table D1), as a model with 5 variables is unlikely to converge if there are less than 5 children in one of the two exposure groups (i.e. For TT at 14 weeks CD4+ and CD8+ T cell proliferation).

In Model 1, HIV-exposure alone unconfounded associated positively with CD4+ T cell proliferation longitudinally (Coefficient:11.83 and 95% CI: 1.64-22.02) (Table 4.2) and was the most important determinant of CD4+ T cell proliferation to BCG longitudinally when adjusting for other potential confounders including gestation age in Model 2 (Coefficient:12.47 and 95% CI: 2.46-22.49), birth weight and weight at 6 weeks of age in Model 3 (Coefficient:11.49 and 95% CI: 1.26-21.72) (Table 4.2). Model 4 and 5 include additional potential confounders EBF at birth and at 6 weeks, and the age at 6 and 14 weeks. However, the association of CD4+ T cell proliferation with HIV-exposure was no longer significant (Table 4.2).

In Model 1, HIV-exposure alone unconfounded did not predict CD4+ T cell proliferation to SEB longitudinally (Table 4.2) and did not predict CD4+ T cell proliferation to SEB when adjusting for potential confounders including gestational age in Model 2, weight at birth and weight at 6 weeks of age in Model 3. Model 4 and 5 include additional potential confounders EBF at birth and at 6 weeks, and the age at 6 and 14 weeks (Table 4.2).

In Model 1, HIV-exposure alone unconfounded did not predict CD8+ T cell proliferation to both BCG and SEB longitudinally (Table 4.2) and did not predict CD8+ T cell proliferation to both BCG and SEB when adjusting for potential confounders including gestational age in Model 2, weight at birth and weight at 6 weeks of age in Model 3. Model 4 and 5 include additional potential confounders EBF at birth and at 6 weeks, and the age at 6 and 14 weeks (Table 4.2).

HIV-exposure may not be very important for SEB responses when adjusting for other confounders in Model 2 to 5. Also, the association between CD4+ T cell proliferations to BCG vaccination were evident as HIV-exposure positively predicted CD4+ T cell proliferation over time from 6 to 14 weeks of age in response to BCG. The association between CD8+ T cell proliferation and HIV-exposure was not evident, but gestation age in Model 2 positively predicted CD8+ proliferation (Table 4.2).

Exposure *in utero* to HIV may stimulate T cells non-specifically to respond to other unrelated antigens which may be the cause of the increased lymphoproliferation to BCG and SEB.

Table 4.2: Generalized Estimation Equation (GEE) population averaged model predicting CD4+ and CD8+ antigen specific T cell proliferation after stimulation with Bacillus Calmette-Guérin (BCG) and Staphylococcal enterotoxin B (SEB) antigens longitudinally from 6 to 14 weeks of life. Frequency of proliferating T cells (CD4+Ki67+ or CD8+Ki67+) after adjusting *a priori* confounders (variables) following BCG and SEB stimulation at 6 and 14 weeks of age. Condition = cond., Week = wk, exclusively breast feeding = EBF, coefficient = coef., and 95% confidence interval = 95% CI. Red bold text represents the HIV-exposed variable that remains significant in a model and black bold text represents other variables that remain significant after multivariate adjustments for possible confounders.

Cond.	Variable	Model 1 Coef. (95% CI)	Model 2 Coef. (95% CI)	Model 3 Coef. (95% CI)	Model 4 Coef. (95% CI)	Model 5 Coef. (95% CI)
			CD4+Ki67+			
BCG	HIV-exposure	**11.83 (1.64-22.02)**	**12.47 (2.46-22.49)**	**11.49 (1.26-21.72)**	7.94 (-7.92-23.80)	9.14 (-8.22-26.52)
	Gestation age		-1.15 (-3.20-0.91)	-1.71 (-3.92-0.51)	-1.97 (-4.21-0.26)	-1.85 (-4.62-0.92)
	Birth weight			0.01 (-0.01-0.02)	0.01 (-0.01-0.02)	0.01 (-0.00-0.03)
	Weight at 6 wk			-0.00 (-0.01-0.01)	-0.00 (-0.01-0.01)	-0.00 (-0.02-0.01)
	EBF at birth				-3.71 (-20.31-12.89)	-4.18 (-22.19-13.82)
	EBF at 6 wk				-4.37 (-13.57-4.83)	-4.02 (-15.18-7.13)
	Age at 6 wk					-0.46 (-1.68-0.76)
	Age at 14 wk					0.36 (-0.49-1.19)
SEB	HIV-exposure	6.59 (-5.62-18.79)	7.30 (-5.13-19.74)	5.97 (-6.54-18.49)	3.13 (-16.99-23.24)	12.09 (-7.05-31.24)
	Gestation age		-0.09 (-2.54-2.35)	-0.52 (-3.16-2.13)	-0.48 (-3.13-2.16)	-0.30 (-3.13-2.53)
	Birth weight			0.00 (-0.01-0.02)	0.00 (-0.01-0.02)	0.01 (-0.01-0.03)
	Weight at 6 wk			0.00 (-0.01-0.01)	0.00 (-0.01-0.01)	-0.00 (-0.01-0.01)
	EBF at birth				-4.78 (-26.09-16.53)	6.33 (-13.63-26.29)
	EBF at 6 wk				4.19 (-7.11-15.50)	-2.25 (-13.81-9.31)
	Age at 6 wk					1.00 (-0.29-2.31)
	Age at 14 wk					1.22 (0.44-1.99)
			CD8+Ki67+			
BCG	HIV-exposure	3.02 (-7.57-13.61)	2.22 (-7.49-11.93)	1.64 (-8.23-11.50)	-4.74 (-19.83-10.35)	-1.75 (-16.75-13.25)
	Gestation age		**2.01 (0.05-3.98)**	1.98 (-0.15-4.11)	1.79 (-0.38-3.97)	2.43 (-0.07-4.93)
	Birth weight			0.00 (-0.01-0.02)	0.01 (-0.01-0.02)	0.00 (-0.01-0.02)
	Weight at 6 wk			-0.00 (-0.01-0.01)	0.00 (-0.01-0.01)	0.00 (-0.01-0.02)
	EBF at birth				-7.59 (-23.46-8.27)	-9.77 (-25.21-5.67)
	EBF at 6 wk				-1.72 (-10.70-7.26)	2.84 (-7.30-12.99)
	Age at 6 wk					-0.91 (-1.99-0.17)
	Age at 14 wk					0.44 (-0.31-1.19)
SEB	HIV-exposure	2.37 (-11.03-15.77)	2.13 (-11.39-15.65)	2.03 (-11.72-15.78)	6.48 (-15.74-28.69)	12.82 (-8.71-34.35)
	Gestation age		1.83 (-0.89-4.56)	1.89 (-1.05-4.83)	1.89 (-1.04-4.83)	2.31 (-0.93-5.54)
	Birth weight			-0.00 (-0.02-0.02)	-0.00 (-0.02-0.02)	0.00 (-0.01-0.02)
	Weight at 6 wk			0.00 (-0.01-0.01)	0.00 (-0.01-0.01)	-0.003 (-0.02-0.01)
	EBF at birth				6.36 (-17.20-29.92)	13.22 (-9.18-35.62)
	EBF at 6 wk				-3.05 (-15.61-9.49)	-5.89 (-19.10-7.32)
	Age at 6 wk					0.64 (-0.82-2.09)
	Age at 14 wk					1.54 (0.65-2.42)

4.3 Discussion

T cell proliferation is an important measure of vaccine immunogenicity and T cell function; and few studies have examined the effect of HIV-exposure on this parameter in infants (Mazzola *et al.*, 2011). This study was partly designed to investigate the proliferative ability of CD4+ and CD8+ T lymphocytes in response to vaccine antigens [Bacillus Calmette-Guérin (BCG), *Bordetella pertussis* (BP) and Tetanus toxoids (TT)]; including Staphylococcal enterotoxin B (SEB) among infants exposed to HIV-1 *in-utero* compared with control infants not exposed to HIV.

Ki-67 expression has been validated for measurement of in vitro proliferation after 6-day whole blood culture with antigens (Soares *et al.*, 2010). The advantage of this marker is that it can be used with whole blood, whereas Oregon Green and CFSE cannot. It is robust compared to CFSE in terms of culture manipulability. Small blood volumes prohibited the use of the BrdU assay.

In this chapter, we found that HIV-exposure enhance the proliferative response to BCG vaccination and to SEB. These findings were opposite to our original hypothesis, but consistent with reports of proliferative response in cord blood mononuclear cells (CBMC) from Brazilian HEU infants(Hygino *et al.*, 2008). However, in contrast, Mazzola *et al.* found HEU infants had a reduced BCG-specific T cell proliferation(Mazzola *et al.*, 2011).

In support of our findings, Mazzola *et al*. have shown that T cell lymphoproliferation increased significantly with age in HEU infants in response to BCG reflecting the establishment of an immune response following BCG vaccination(Mazzola *et al.*, 2011). SEB is used in many assays as a positive control, but biologically it can reveal important attributes of T cell functionality. SEB signals without the need for co-stimulatory molecules, but only through certain and not all Vβ TCR such as V beta 8.1/8.2 T cells(Herz *et al.*, 1999)(Renz *et al.*, 1993). Hygino *et al.* also found an increased response to non-specific stimulation (anti-CD3 anti-CD28) in CBMC of HIV-exposed infants born to HIV-infected mothers who had a high plasma viral load compared to CBMC of infants born to HIV-infected mothers who controlled their plasma viral load(Hygino *et al.*, 2008). Significant increase in lymphoproliferation in response to non-specific stimulations with age could reflect maturation of the infant's immune response (as seen in response to SEB).

Further, looking at the GEE analyses, HIV-exposure was the most important predictor of CD4+ T cell proliferation longitudinally over time to 14 weeks of life in response to BCG after adjusting for other confounders. HIV-exposure did not predict CD8+ T cell proliferation longitudinally over time to 14 weeks of life in response to either BCG or SEB stimulation but gestation age was the most important determinate of CD8+ T cell proliferation longitudinally over time to 14 weeks in response to BCG, further suggesting that the immune response in these infants matures with age.

The difference between the findings in this chapter and that of Mazzola *et al*. could be due to a wide number of factors, including the age range of their cohorts: the age of the cohort used for this thesis was from birth to 14 weeks and Hygino *et al.* used CBMC, whereas, Mazzola *et al*'s cohort was between 6.1 to 26.3 months infants, older than the infants in the other two. In addition, Mazzola *et al*. used the peripheral blood mononuclear cells (PBMCs) proliferation assay different from the whole blood assay (WBA) we used. The difference in assay type would require different storage methods that could affect the results. Another likely factor influencing the difference in results is geographical location that would entail exposure to different environment clades, as they included HIV-exposed infants in Sao Paulo, Brazil while we had a South African cohort. It has been shown that immunological characteristics between African and European infants are different(Wilfing *et al*., 2001).

This is also the first study comparing *Bordetella pertussis* and tetanus toxoid specific T cell proliferation in HEU versus HU infants. No significant difference in T cell proliferation was observed in response to these antigens between groups of infants at 14 weeks of life using regression analyses, although there were few numbers of TT samples. The infants showed a similar median CD4+ and CD8+ T cell proliferation between HEU versus HU groups at 14 weeks of age.

Therefore, the effect of HIV-exposure on vaccine immune responses may depend on the type of antigen. BCG is a live-attenuated organism that elicits strong cellular and antibody responses and often confers lifelong immunity with only one dose. Since it is a whole organism, it has many TLR ligands and other adjuvant-like proteins. Tetanus toxoid, a protein antigen and a bacterial

toxin, has alum as an adjuvant, and needs to be processed in order to be presented by antigen presenting cells to T cells as well as BCG. *Bordetella pertussis* is a killed antigen that stimulates a weaker immune response than live vaccines, and requires booster doses to maintain a person's immunity. This weak antigenicity may also determine how T cells respond in the assay. SEB is a superantigen that does not require antigen presentation and only results in certain CD4 or CD8 T cells to expand.

It was hypothesised that HIV-exposure would decrease proliferative responses to vaccine antigens and SEB, but we found instead that T cell proliferation was significantly improved after BCG and SEB stimulation. This surprising finding may be due to *in-utero* perturbation of the T cell receptor (TCR) repertoire(Kou *et al.*, 2000)(Halapi *et al.*, 1999)(Alfani *et al.*, 2000), as observed in individuals with HIV and after vaccination(Kou *et al.*, 2000)(Soroosh *et al.*, 2003); chronic immune activation and inflammation that might be encountered in the fetus of an HIV-infected woman due to exposure to HIV antigens, and/or exposure to other maternal antigens such as opportunistic pathogens(Rich *et al.*, 1997), or an immune dysfunction which could results in a lack of BCG containment in HEU infants that may allow more antigen exposure and at a later stage (e.g. 14 weeks of age) in life, and hyper-responsiveness to Superantigen, but would also cause weakened response to protein antigens.

It may also be that BCG *in vivo* selects for specific Vβ TCR families, which expand and then respond better to SEB in the whole blood assay. V beta 8 gene family products have been found to respond to BCG(Wilson *et al.*, 1993). Also, HIV-exposure, may delete (or expand) specific T cell clones (i.e. Vβ TCR) that are further expanded by BCG and then respond *in vitro* to SEB.

The study as described has a few limitations; namely, the follow-up period was only 14 weeks, with time points at 6 and 14 weeks only. It would have been interesting to determine the peak response by the evolution of T cell proliferation through 8 and 10 weeks for the HEU infants in response to BCG for better kinetics. However, Kagina *et al*. demonstrated that 10 week old infants vaccinated with BCG at birth showed peak memory responses at 10 weeks following BCG vaccination (Kagina *et al.*, 2009). Also, it was recently showed that CD4+ T cell responses to BCG after vaccination peaked at 6-10 weeks, but gradually waned over the first year of

life(Soares *et al*., 2013). It has been shown that *Bordetella pertussis* (BP)(Esposito *et al*., 2001) and tetanus toxoid (TT) T cell responses after vaccination peak at 10 weeks after priming at 6 weeks of age and did not wane after the last booster dose at 14 weeks of age at any time to 52 weeks(Soares *et al*., 2013).

Since this was an observational study, potential for bias could not be completely avoided by randomization. Hence the regression and GEE analyses were performed.

These findings may have important implications for the optimal timing and vaccination strategies for HIV-exposed infants and may require careful immunological evaluation of future HIV and TB vaccines in this group. The clinical importance of these findings remains unknown. Further investigation is required as the observed T cell proliferative changes may result in alteration of HEU infant's susceptibility to infections in addition to their already vulnerable state at later time points in life.

CHAPTER 5

CYTOKINE PRODUCTION BY ANTIGEN-SPECIFIC T CELLS IN HEU VERSUS HU INFANTS AT 6 AND 14 WEEKS OF LIFE

5.1 Introduction

We aimed to compare the quality of CD4+ and CD8+ T cell intracellular cytokine production to vaccine antigens [Bacillus Calmette-Guérin (BCG), *Bordetella pertussis* (BP) and Tetanus toxoids (TT)] and *in vitro* to Staphylococcal enterotoxin B (SEB) in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants, to address the hypothesis that HEU infants produce lower quantity of cytokines. This was achieved by measuring the frequency of Th1 (IFN-γ and IL-2), Th2 (IL-13) and Th17 (IL-17) cytokines expression by proliferating antigen-specific T cells in HEU versus HU infants at 6 and at 14 weeks of life.

Neonates have limited capacity to produce IFN-γ and cell-mediated immune responses involving cytotoxicity against intracellular pathogens(Siegrist, 2007)(Ota *et al.*, 2012). IL-2 stimulates resting T cells and supports long term growth, activation, and proliferation of T cells (Smith, 1988). IL-2 is also a potent modulator of T and NK cell function, and it is the most extensively investigated cytokine to date. Vaccination at birth with BCG has been shown to significantly enhance Th1 responses to mycobacterial antigens(Burl *et al.*, 2012). IFN-γ production increases with age in infants(Mazzola *et al.*, 2011).

IL-13 possesses a number of anti-inflammatory and immunomodulatory activities. African neonates mount significantly higher IL-13 responses to mitogens compared to Europeans, possibly reflecting continuous stimulation of antigens that preferentially drive its responses(Wilfing *et al.*, 2001).

Th17 cells may be important in vaccine-induced memory immune responses to bacterial antigens. IL-17 has been shown to populate the lungs, the primary site of *Mycobacteria tuberculosis* infections, and in turn recruit IFN-γ producing antigen-specific T cells persistent in the central lymphoid organs(Lin *et al.*, 2011).

In this chapter, the frequency of cytokine expression by proliferating antigen-specific T cells was reported as a frequency of total CD4+ and CD8+ lymphocytes. The intracellular cytokine staining (ICS) assay was performed and analyzed as described in the methods (see section 3.1.2).

It is generally known that antigen-induced T cell proliferation measures the expansion of antigen-specific T cells, this further provides for the measurement of cytokine production by the proliferating cells(Anthony *et al*., 2012). In general, measuring cytokine production by proliferating T cells permits a distinction between naïve and memory T cells. Memory cells are capable of the production of cytokines within a short while, for instance within 12 hours after antigen challenge, while naïve T cells must first undergo proliferation and differentiation before they can express cytokines(Mempel *et al.*, 2004)(MATESIC *et al*., 1998)(Sallusto *et al*., 2004).

The data in this chapter for the cytokine producing cells is presented in the following order; BCG, BP, TT and SEB and then IFN-γ, IL-2, IL-17 and IL-13 cytokine producing T cells. A summary presenting the data on the graphs is show on Table 5.1 followed by univariate and multivariate analysis adjusting for potential confounders. Statistical analysis was performed using GraphPad prism v5 and linear regression analyses, with alpha=0.05 and statistical significance was tested using *Mann-Whitney U* test (see table 3.3).

5.2 Cytokine production by antigen specific T cells at 6 and 14 weeks of life

The p-values shown on the graph are the unadjusted ones. At 6 weeks of age, significant differences in the frequencies of CD4+ cytokine producing cells between the two groups of infants in response to TT, BP, and SEB were not evident. After adjustment for multiple comparisons by using the Holm step-down approach, there was no significant in the frequency of CD8+ T cells producing IL-13 in HEU compared to HU infants in response to BCG (p=0.1270) at 6 weeks of age. Please see Table 5.1 ahead for a summary of the results(Columb, 2006).

At 14 weeks, after adjustment for multiple comparisons, there were no significant differences in the frequency of IL-13 (p=0.1830) and IL-17 (p=0.0615) production by CD4+ T cells in response to BCG between HEU versus HU infants. After adjustment for multiple comparisons, there was no significant differences in the frequency of IL-17 (p=0.1432) production by CD4+ T cells in response to TT between groups (Table 5.1).

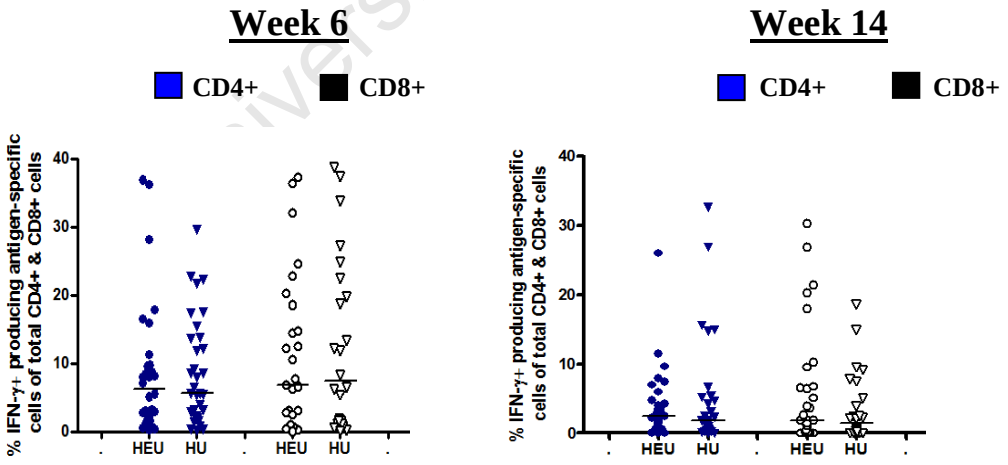
Also at 14 weeks, after adjustment for multiple comparisons HEU infants' CD8+ T cells produced a significantly lower frequency of IL-13 (p=0.0485) and IL-17 (p=0.0375) compared to HU infants in response to BCG (Table 5.1). Appendix D below shows the number of HEU and HU infants for whom measurement of CD4+ and CD8+ T cell cytokine production responses to the different antigens was performed.

5.2.1 BCG-induced cytokine producing T-cell responses in HEU versus HU infants at 6 and 14 weeks of life

5.2.1.1 IFN-γ and IL-2 cytokine producing cells induced by BCG in HEU versus HU infants at 6 and 14 weeks of life

Figure 5.1 shows that no significant differences in the frequency of IFN-γ and IL-2 production by antigen-specific CD4+ and CD8+ T cells out of total CD4+ and CD8+ T cells in response to BCG observed between HEU versus HU infants at 6 and at 14 weeks of age.

(a) BCG-induced IFN-γ production by T cells



(b) BCG-induced IL-2 production by T cells

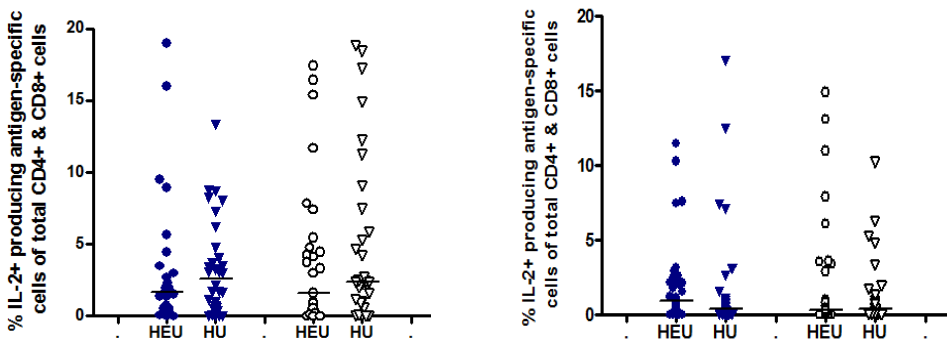


Figure 5.1: The frequency of T cells producing IFN-γ and IL-2 after BCG stimulation for 6 days in whole blood assay at 6 and 14 weeks of life. Frequency of antigen-specific (a) IFN-γ and (b) IL-2 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Frequencies reported are 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.

5.2.1.2 IL-17 cytokine producing cells induced by BCG in HEU versus HU infants at 6 and 14 weeks of life

Figure 5.2 shows that at 14 weeks, in response to BCG, there was a significantly higher frequency of IL-17 (p=0.0123) production by CD4+ T cells in HEU infants and a significantly lower frequency of IL-17 (p=0.0075) production by CD8+ T cells in HEU compared to HU infants, but no significant differences were observed at 6 weeks of life.

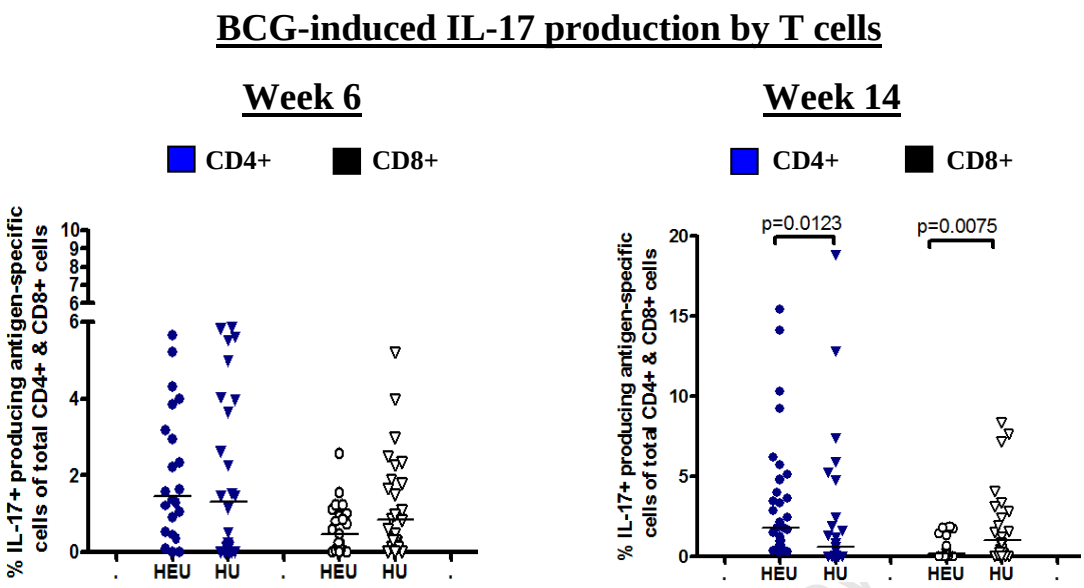


Figure 5.2: The frequency of IL-17 producing T cells at 14 weeks after BCG stimulation for 6 days in whole blood but not at 6 weeks of life. Frequency of antigen specific IL-17 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Frequencies reported are 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.

5.2.1.3 IL-13 cytokine producing cells induced by BCG in HEU versus HU infants at 6 and 14 weeks of life

HIV-exposure affected the IL-13 response to BCG markedly. At 6 weeks of age, HEU infants had a significantly lower IL-13 (p=0.0254) production by CD8+ T cells compared to HU infants in response to BCG; and at 14 weeks, HEU infants had a significantly higher frequency of IL-13 (p=0.0366) production by CD4+ T cells in response to BCG and a significantly lower IL-13 (p=0.0097) production by CD8+ T cells in response to BCG (Figure 5.3).

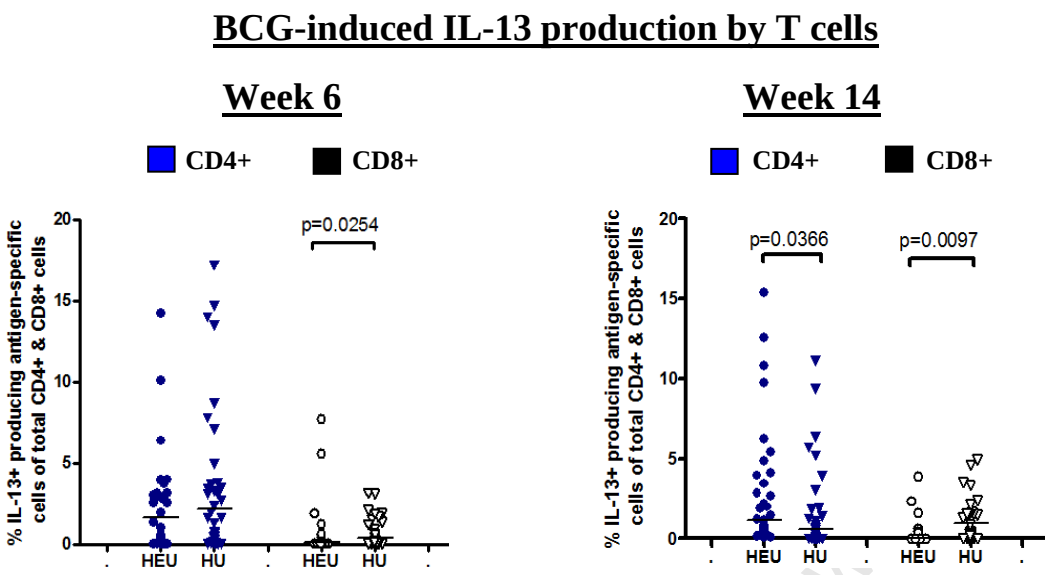


Figure 5.3: The frequency of IL-13 producing T cells after BCG stimulation for 6 days in whole blood assay at 6 and at 14 weeks of life. Frequency of antigen specific IL-13 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Frequencies reported are 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.

5.2.2 BP-induced cytokine producing T-cell responses in HEU versus HU infants at 14 weeks of life

5.2.2.1 IFN-γ and IL-2 cytokine producing cells induced by BP in HEU versus HU infants at 14 weeks of life

We assessed BP- induced cytokine production by CD4+ and CD8+ T cells at 14 weeks of age only, because the first dose of these vaccines is administered at 6 weeks of life. Figure 5.4 shows that no significant differences in the frequency of IFN-γ and IL-2 production by CD4+ and CD8+ T cells in response to BP were observed between HEU versus HU infants at 14 weeks of age. However, the number of samples with proliferating cells from which we could measure IFN-γ and IL-2 production by T cells in response to BP was small.

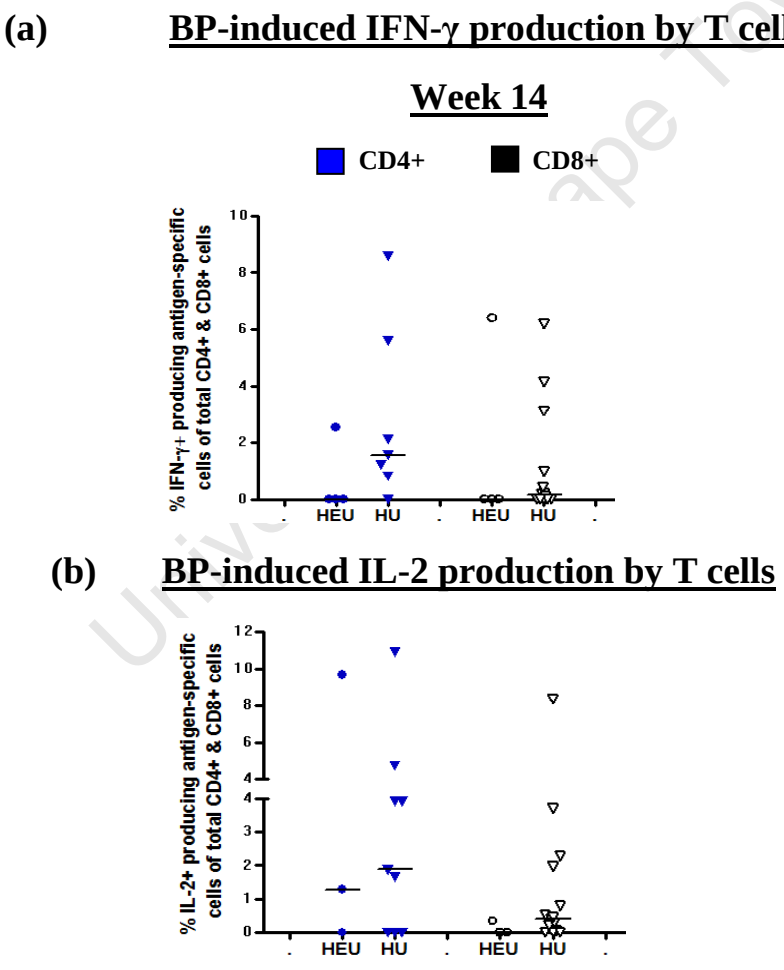


Figure 5.4: The frequency of T cells producing IFN-γ and IL-2 after BP stimulation for 6 days in whole blood assay at 14 weeks of life. Frequency of antigen-specific (a) IFN-γ and (b) IL-2 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by

flow cytometry of BP-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 14 weeks of life. Frequencies reported are 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.

5.2.2.2 IL-17 producing cells induced by BP in HEU versus HU infants at 14 weeks of life

Figure 5.5 shows that no significant differences in the frequency of IL-17 production by CD4+ and CD8+ T cells in response to BP were observed between HEU versus HU infants at 14 weeks of age, but the number of samples with proliferating cells from which we could measure IL-17 production by T cells in response to BP was small.

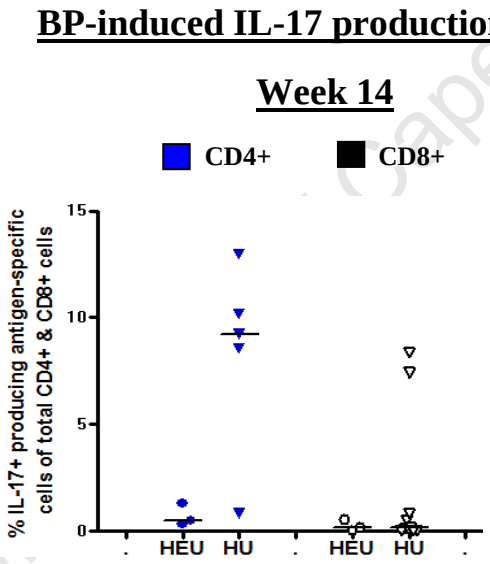


Figure 5.5: The frequency of T cells producing IL-17 after BP stimulation for 6 days in whole blood assay at 14 weeks of life. Frequency of antigen specific IL-17 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of BP-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 14 weeks of life. Frequencies reported are 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.

5.2.2.3 IL-13 cytokine producing cells induced by BP in HEU versus HU infants at 14 weeks of life

Figure 5.6 shows that no significant differences in the frequency of IL-13 production by CD4+ and CD8+ T cells in response to BP was observed between HEU versus HU infants at 14 weeks of age, but the number of samples with proliferating cells from which we could measure IL-13 production by T cells in response to BP in the HEU infants was small.

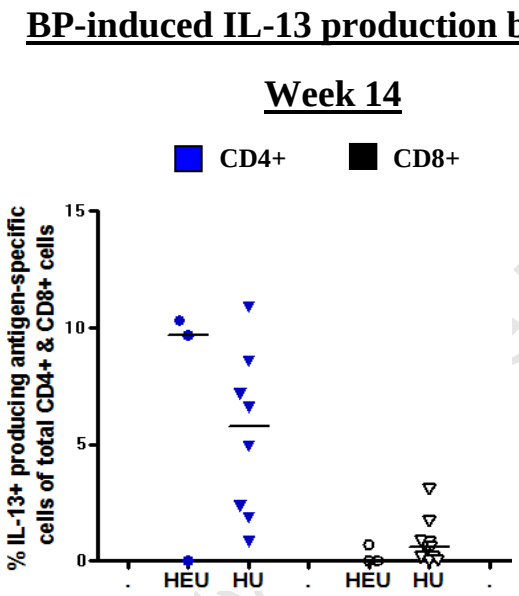


Figure 5.6: The frequency of T cells producing IL-13 after BP stimulation for 6 days in whole blood assay at 14 weeks of life. Frequency of antigen specific IL-13 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of BP-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 14 weeks of life. Frequencies reported are 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.

5.2.3 TT-induced cytokine producing T-cell responses in HEU versus HU infants at 6 and 14 weeks of life

5.2.3.1 IFN-γ and IL-2 cytokine producing cells induced by TT in HEU versus HU infants at 14 weeks of life

Figure 5.7 shows that no significant differences in the frequency of IFN-γ and IL-2 production by CD4+ and CD8+ T cells in response to TT were observed between HEU versus HU infants at 14 weeks of age, but the number of samples with proliferating cells from which we could measure IFN-γ and IL-2 production by T cells in response to TT was small.

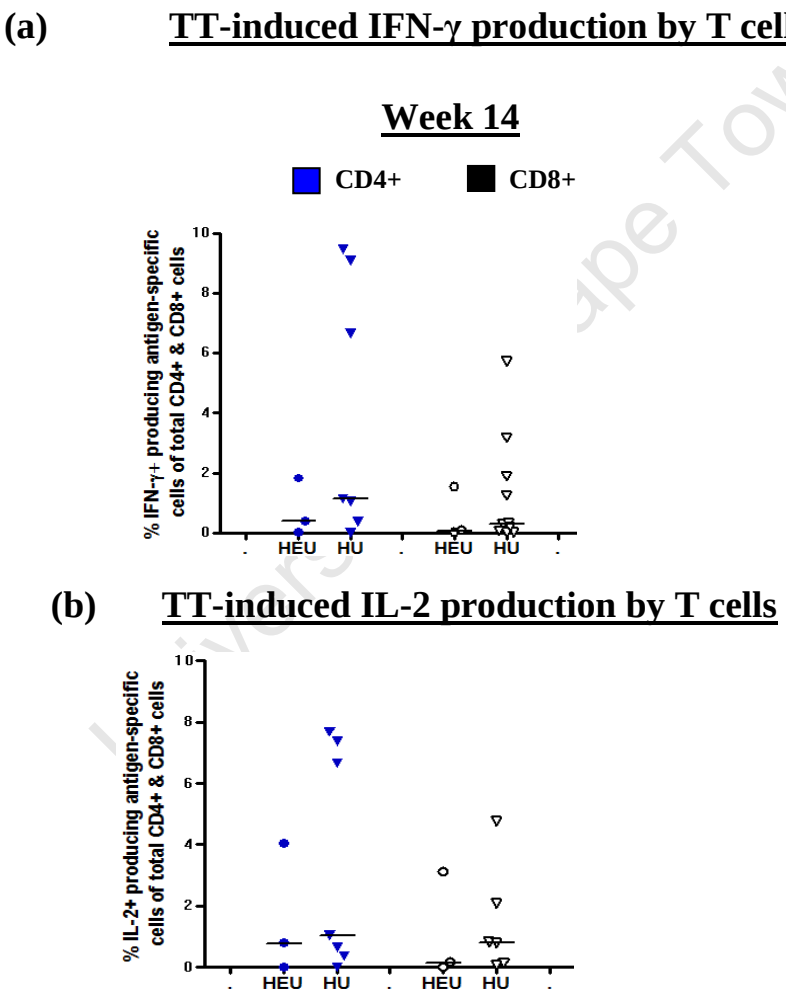


Figure 5.7: The frequency of T cells producing IFN-γ and IL-2 at 14 weeks of life after TT stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific (a) IFN-γ and (b) IL-2 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of TT-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU:

triangles) presented on the right of each side of the plot at 14 weeks of life. Frequencies reported are 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.

5.2.3.2 IL-17 cytokine producing cells induced by TT in HEU versus HU infants at 14 weeks of life

Figure 5.8 shows that at 14 weeks, after TT stimulation a significantly lower frequency of IL-17 (p=0.0358) production by CD4+ T cells was observed in HEU compared to HU infants, despite that the number of samples with proliferating cells from which we could measure IL-17 production in response to TT was small.

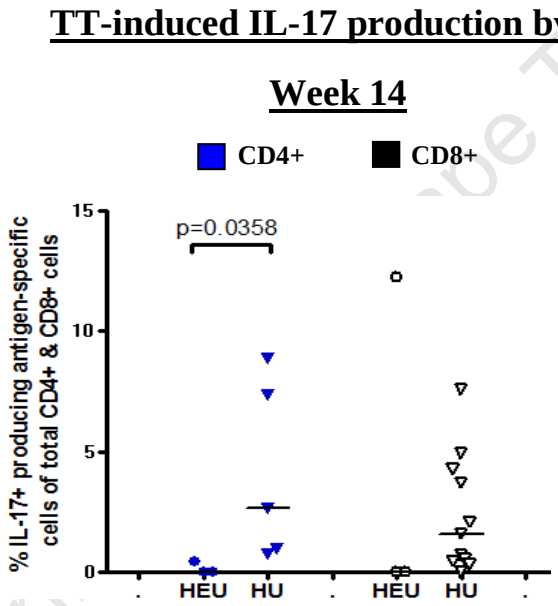


Figure 5.8: The frequency of IL-17 producing T cells at 14 weeks of life after TT stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-17 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of TT-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 14 weeks of life. Frequencies reported are 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.

5.2.3.3 IL-13 cytokine producing cells induced by TT in HEU versus HU infants at 14 weeks of life

Figure 5.9 shows that no significant differences in the frequency of IL-13 production by CD4+ and CD8+ T cells in response to TT were observed between HEU versus HU infants at 6 and at 14 weeks of age, but the number of samples with proliferating cells from which we could measure IL-13 production in response to TT was small.

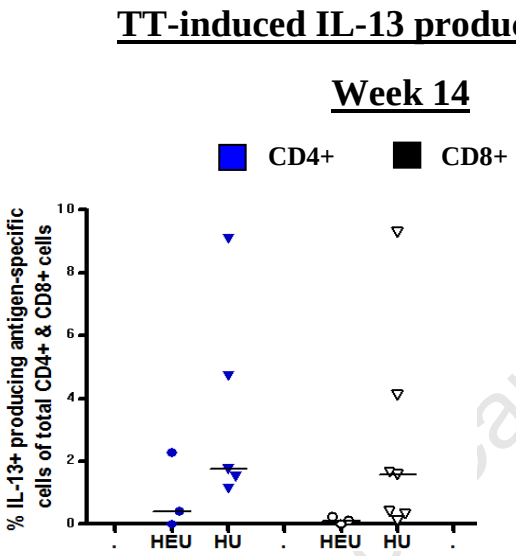


Figure 5.9: The frequency of T cells producing IL-13 at 14 weeks of life after TT stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-13 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of TT-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 14 weeks of life. Frequencies reported are 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.

5.2.4 SEB-induced cytokine producing T-cell responses in HEU versus HU infants at 6 and 14 weeks of life

5.2.4.1 IFN-γ and IL-2 cytokine producing cells induced by SEB in HEU versus HU infants at 6 and 14 weeks of life

Figure 5.10 shows that no significant differences in the frequency of IFN-γ and IL-2 production by CD4+ and CD8+ T cells in response to SEB were observed between HEU versus HU infants at 6 and at 14 weeks of age.

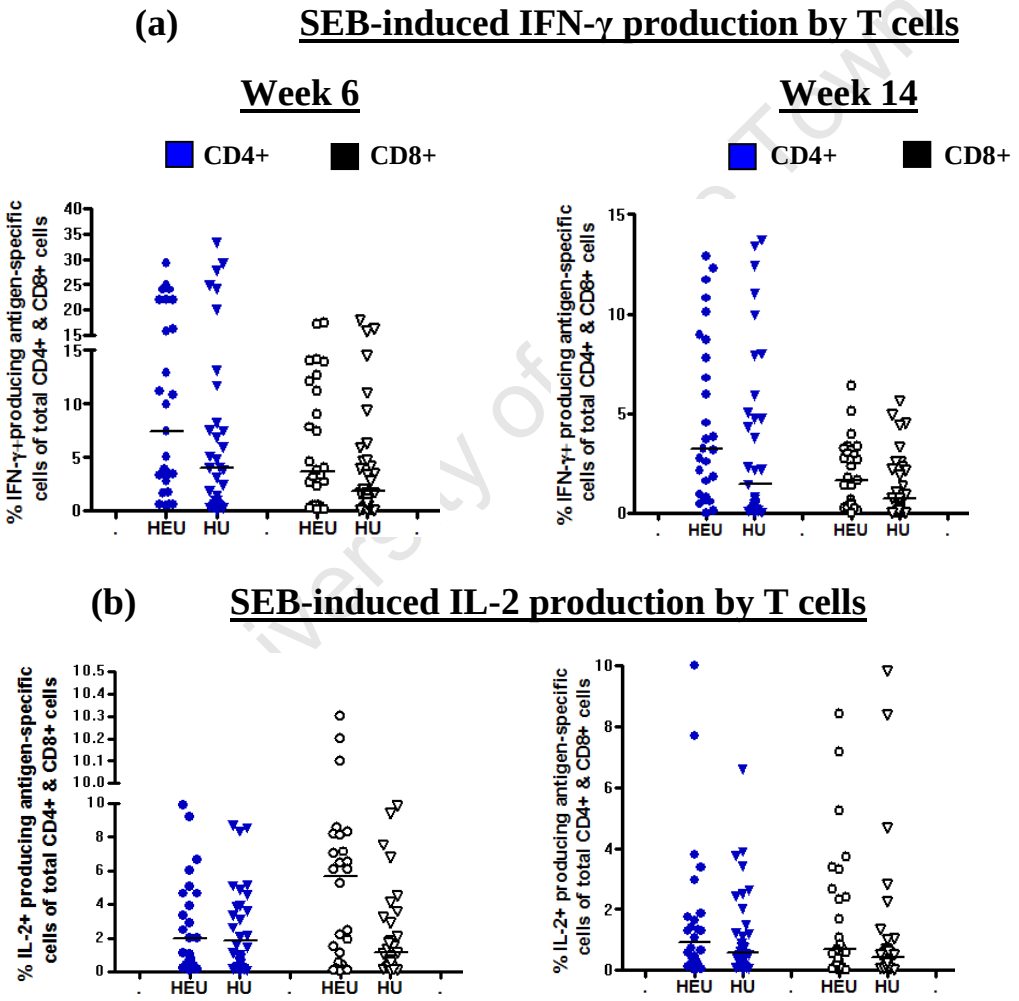


Figure 5.10: The frequency of T cells producing IFN-γ and IL-2 at 6 and 14 weeks of life after SEB stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific (a) IFN-γ and (b) IL-2 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of SEB-stimulated whole blood of HIV-exposed

uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Frequencies reported are 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.

5.2.4.2 IL-17 cytokine producing cells induced by SEB in HEU versus HU infants at 6 and 14 weeks of life

Figure 5.11 shows that no significant differences in the frequency of IL-17 production by total CD4+ and CD8+ T cells in response to SEB were observed between HEU versus HU infants at 6 and at 14 weeks of age.

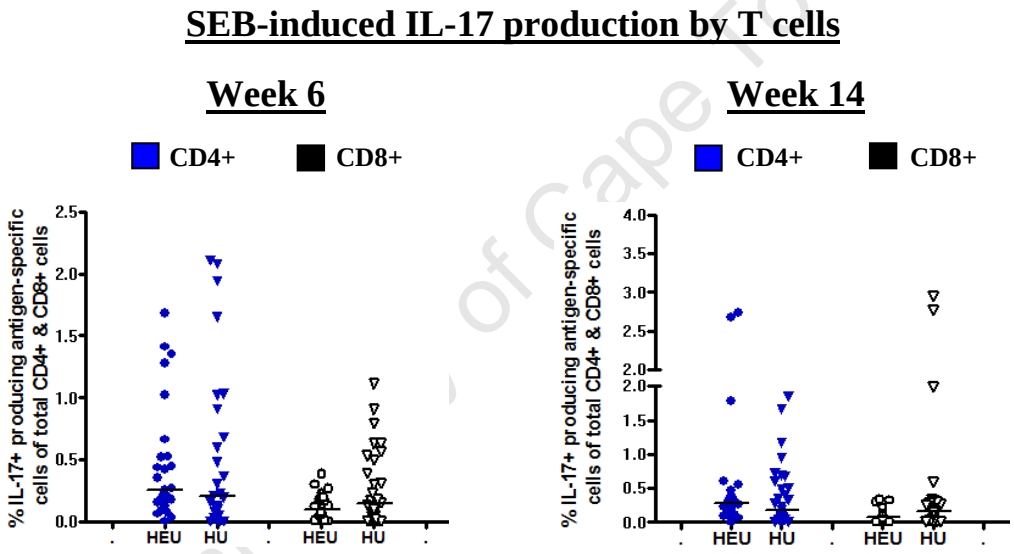


Figure 5.11: The frequency of T cells producing IL-17 at 6 and 14 weeks of life after SEB stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-17 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of SEB-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Frequencies reported are 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.

5.2.4.3 IL-13 cytokine producing cells induced by SEB in HEU versus HU infants at 6 and 14 weeks of life

Figure 5.12 shows that no significant differences in the frequency of IL-13 production by CD4+ and CD8+ T cells in response to SEB were observed between HEU versus HU infants at 6 and at 14 weeks of age.

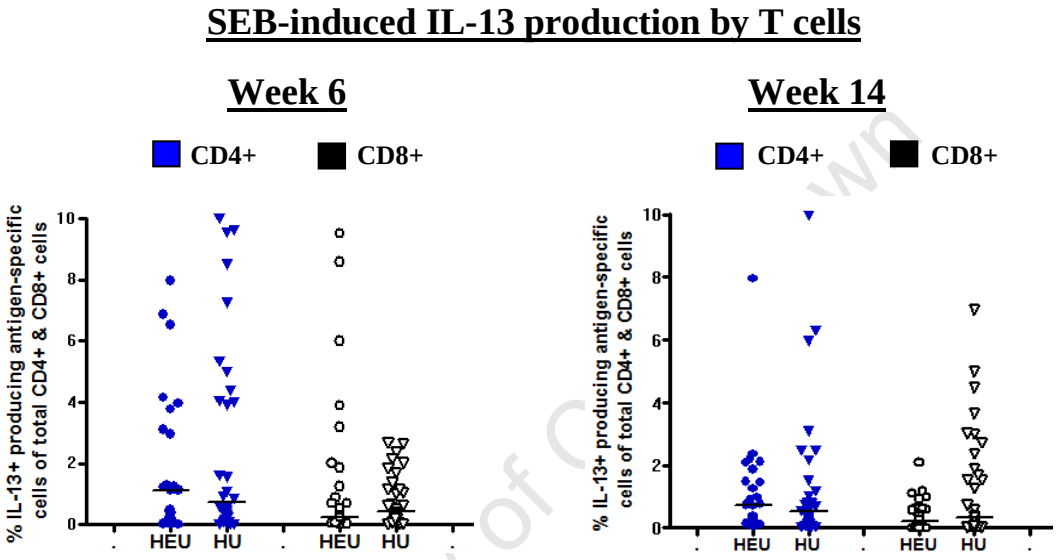


Figure 5.12: The frequency of T cells producing IL-13 at 6 and 14 weeks of life after SEB stimulation for 6 days in whole blood assay measured by flow cytometry. Frequency of antigen specific IL-13 producing T cells out of total CD4+ (in blue on the left side of each graph) and CD8+ (in black on the right side of each graph) T cells, determined by flow cytometry of SEB-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Frequencies reported are 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.

5.2.5 Summary table of all the cytokines producing T cells measured

Table 5.1 below presents the summary of results as shown on the graphs on Figures 5.1 – 5.12 above. After adjusting p-values for multiple comparisons only the frequencies of IL-13 and IL-17 cytokine production by CD8+ T cells remain significant.

Table 5.1: The frequency of CD4+ and CD8+ cytokine producing T cells, in HEU versus HU infants. Antigen specific [Bacillus Calmette-Guérin (BCG)**,** *Bordetella pertussis* (BP), Tetanus toxoid (TT) and Staphylococcal enterotoxin B (SEB)] CD4+ and CD8+ cytokine producing T cells at 6 and 14 weeks of life. Read outs for each condition, time point (TP), and cytokine include the median frequency of CD4+ and CD8+ cytokine producing cells and the significant difference indicated by the p-value unadjusted (and adjusted for multiple comparisons in parentheses if significant before adjusted) between HEU vs. HU infants. Adjustment for multiple comparisons was done using the Holm step-down approach(Columb *et al.*,, 2006) (see table 3.5). Cells indicated N/A are not applicable, since BP* and TT* are administered to infants in DTaP vaccine for the first time at 6 weeks only. Red highlights; represent p-values (and adjusted p-values) that remain significant after adjustment for multiple comparisons. TP= time point,

Condition (TP)	Cytokine	Median frequency (% of total CD4+ or CD8+ cells) of cytokine producing cells					
		CD4+			CD8+		
		HEU	HU	p-value (adjusted)	HEU	HU	p-value (adjusted)
BCG (Week 6)	IFN-ϒ	6.3	5.7	0.7000	6.7	7.4	0.7771
	IL-2	1.6	2.5	0.6755	1.6	2.4	0.4006
	IL-13	1.6	2.2	0.3044	0.09	0.4	**0.0254 (0.1270)**
	IL-17	1.5	1.3	0.5407	0.5	0.8	0.0774
BCG (Week 14)	IFN-ϒ	2.4	1.8	0.4982	1.8	1.4	0.3085
	IL-2	0.9	0.4	0.1071	0.3	0.4	0.8517
	IL-13	1.2	0.6	**0.0366 (0.1830)**	0.05	1.0	**0.0097 (0.0485)**
	IL-17	1.8	0.6	**0.0123 (0.0615)**	0.2	1.0	**0.0075 (0.0375)**
BP* (Week 14)	IFN-ϒ	0.0	1.6	0.1757	0.0	0.2	0.5500
	IL-2	1.3	1.9	0.9250	0.0	0.4	0.1424
	IL-13	9.7	5.8	0.7758	0.0	0.6	0.2704
	IL-17	0.5	9.2	0.0714	0.1	0.1	0.9371
TT* (Week 14)	IFN-ϒ	0.4	1.1	0.4236	0.08	0.3	0.5519
	IL-2	0.8	1.0	0.5676	0.2	0.8	0.7143
	IL-13	0.4	1.8	0.2500	0.1	1.6	0.0667
	IL-17	0.0	2.6	**0.0358 (0.1432)**	0.0	1.6	0.5316
SEB (Week 6)	IFN-ϒ	7.42	4.0	0.2094	3.7	1.8	0.2052
	IL-2	1.9	1.8	0.8791	5.6	1.1	0.0590
	IL-13	0.1	0.7	0.6124	0.2	0.4	0.7727
	IL-17	0.3	0.2	0.6	0.09	0.1	0.2099
SEB (Week14)	IFN-ϒ	3.2	1.4	0.1135	1.6	0.7	0.0693
	IL-2	0.9	0.6	0.4379	0.7	0.4	0.1008
	IL-13	0.7	0.5	0.5567	0.2	0.3	0.1769
	IL-17	0.3	0.2	0.2902	0.07	0.2	0.0828

5.3 Factors influencing antigen specific cytokine production by T cells at 6 and 14 weeks of life

Due to the observational nature of this cohort, there were *a priori* factors (every potential confounder) other than HIV-exposure (see table 2.1) which could also affect CD4+ and CD8+ T cell responses. We therefore aimed to adjust for these factors in regression and Generalized Estimation Equation (GEE) population averaged models.

5.3.1 Factors predicting BP- and TT- induced cytokine production by T cells at 14 weeks of life

We aimed to predict cytokine production by T cells, correcting for *a priori* factors (every potential confounder) that could influence BP- and TT- induced CD4+ and CD8+ T cell cytokine production at 14 weeks of age. However, the production of none of the cytokines assessed (IFN-γ, IL-2, IL-13 and IL-17) was significantly different between the two groups of infant in the bivariate analysis after stimulation with BP and TT; hence regression analysis were not performed.

5.3.2 Factors predicting BCG- and SEB- induced cytokine production by T cells longitudinally through time from 6 to 14 weeks of life

We aimed to use Generalized Estimation Equation (GEE) population averaged models to predict the evolution of T cell cytokine responses longitudinally from 6 to 14 weeks of life in response to BCG vaccination and SEB stimulation over time; and to adjust for *a priori* factors (every potential confounder) that could influence BCG- and SEB- induced CD4+ and CD8+ T cell cytokine production over time from 6 to 14 weeks of age (Table 5.2). GEE analysis was only performed for significantly different cytokine responses observed in the bivariate analysis (i.e. IL-13 and IL-17 production by CD4+ and CD8+ T cells found following BCG stimulation at week 14). The results were presented and interpreted as etiological models assessing the strength of the association between HIV-exposure and T cell proliferation when taking other covariate into consideration.

The following *a priori* predictive variables were studied based on conceptual knowledge and not just on statistical association with the exposure (see table 2.1); (i) HIV-exposure, (ii) gestation age, (iii) birth weight, (iv) weight at 6 weeks, (v) exclusive breast feeding (EBF) at birth, (vi) EBF at 6 weeks, (vii) age at 6 weeks, and (viii) age at 14 weeks.

We went further to correct for these factors using GEE models and a backward elimination process was used. Only the results of the exposure (HEU using HU as reference population) were reported, with note of which (if any) other covariates adjusted for created a difference in the exposure variable. We performed multivariate analysis with only three predictive variables where we had less than 5 children per response in any of the group (Appendix D, Table D1), as a model with 5 variables is unlikely to converge if there are less than 5 children in one of the two exposure groups (i.e. For TT at 14 weeks CD4 and CD8 proliferation).

In Model 1, HIV-exposure alone unconfounded did not associate with IL-13+ production by either CD4+ or CD8+ and with IL-17+ production by CD8+ T cells; but predicted IL-17+ production by CD8+ T cells (Coefficient:-11.36 and 95% CI:-18.7 - -3.9) negatively following BCG stimulation longitudinally (Table 5.2). After adjusting for other confounders, Model 2 gestation age, HIV-exposure did not predict production of any of the cytokines, except negative association with IL-17+ production by CD8+ T cells that became (Coefficient:-10.98 and 95% CI:-18.3 - -3.6) in response to BCG longitudinally through time to 14 weeks of age (Table 5.2). In Model 3 and 4 additional potential confounders such as weight at birth and at 6 weeks and exclusively breast feeding at birth and 6 weeks respectively. HIV-exposure did not still predict cytokine production except for IL-17+ by CD8+ T cells that associated negatively that became (Coefficient:-10.56 and 95% CI:-18.1 - -3.1) in Model 3, and (Coefficient:-11.32 and 95% CI:-21.9 - -0.7) in Model 4 (Table 5.2). In Model 5, the age at 6 and 14 weeks was added, HIV-exposure did not still associate to the production of any of the cytokines and the association observed with IL-17+ by CD8+ T cells disappeared (Table 5.2).

This analysis showed that HIV exposure was an important predictor of IL-17+ production by CD8+ T cells to BCG through time alone unconfounded and after adjustment for potential confounders.

Table 5.2: Generalized Estimation Equation (GEE) population averaged model predicting total CD4+ and CD8+ antigen specific cytokine producing T cells after 6 day whole blood assay stimulation with Bacillus Calmette-Guérin (BCG) over time, from 6 to 14 weeks of life. Frequency of CD4+ and CD8+ cytokine producing T cells after adjusting *a priori* confounders (variables) following BCG and SEB stimulation at 6 and 14 weeks of age. Condition = cond., Week = wk, exclusively breast feeding = EBF, coefficient = coef., and 95% confidence interval = 95% CI. Red bold text represents the HIV-exposed variable that remains significant in a model and black bold text represents other variables that remain significant after multivariate adjustments for possible confounders.

Cond.	Variable	Model 1 Coef. (95% CI)	Model 2 Coef. (95% CI)	Model 3 Coef. (95% CI)	Model 4 Coef. (95% CI)	Model 5 Coef. (95% CI)
			CD4+IL-13+			
BCG	HIV-exposure	1.05 (-2.78-4.89)	1.04 (-2.77-4.85)	0.96 (-2.95-4.87)	2.35 (-2.99-7.69)	1.28 (-4.93-7.49)
	Gestation age		0.66 (-0.09-1.41)	0.62 (-0.17-1.41)	0.61 (-0.19-1.41)	0.81 (-0.11-1.73)
	Birth weight			0.00 (-0.00-0.01)	0.001 (-0.01-0.01)	0.00 (-0.01-0.01)
	Weight at 6 wk			-0.001 (-0.004-0.00)	0.001 (-0.004-0.01)	0.00 (-0.004-0.01)
	EBF at birth				2.45 (-3.02-7.93)	1.95 (-4.14-8.05)
	EBF at 6 wk				-1.95 (-5.78-1.87)	-1.81 (-6.30-2.68)
	Age at 6 wk					-0.13 (-0.59-0.35)
	Age at 14 wk					-0.16 (-0.44-0.12)
			CD4+IL-17+			
BCG	HIV-exposure	-0.28 (-1.97-1.41)	-0.25 (-1.92-1.42)	-0.29 (-1.98-1.41)	-0.67 (-3.18-1.83)	0.67 (-2.28-3.62)
	Gestation age		0.33 (-0.03-0.68)	0.29 (-0.09-0.68)	0.26 (-0.12-0.65)	0.39 (-0.05-0.84)
	Birth weight			-0.001 (-0.01-0.01)	0.001 (-0.002-0.01)	0.001 (-0.003-0.01)
	Weight at 6 wk			0.001 (-0.01-0.01)	0.001 (-0.002-0.01)	-0.001 (-0.002-0.01)
	EBF at birth				-0.42 (-3.06-2.23)	-0.09 (-2.92-2.73)
	EBF at 6 wk				-0.74 (-2.59-1.11)	-1.33 (-3.49-0.83)
	Age at 6 wk					-0.06 (-0.29-0.17)
	Age at 14 wk					0.08 (-0.05-0.22)
			CD8+IL-13+			
BCG	HIV-exposure	-5.75 (-11.57-0.07)	-5.63 (-11.37-0.12)	-5.01 (-10.90-0.88)	-3.69 (-11.68-4.29)	-2.48 (-11.36-6.39)
	Gestation age		1.14 (-0.09-2.37)	1.29 (0.03-2.56)	1.27 (0.01-2.54)	**1.46 (0.07-2.85)**
	Birth weight			-0.004 (-0.01-0.01)	-0.004 (-0.01-0.01)	-0.005 (-0.01-0.01)
	Weight at 6 wk			0.01 (-0.005-0.01)	0.001 (-0.01-0.01)	0.002 (-0.006-0.009)
	EBF at birth				2.33 (-5.73-10.39)	2.73 (-6.17-11.63)
	EBF at 6 wk				-2.97 (-9.04-3.09)	-2.98 (-9.88-3.91)
	Age at 6 wk					-0.42 (-1.12-0.28)
	Age at 14 wk					0.12 (-0.29-0.52)
			CD8+IL-17+			
BCG	HIV-exposure	**-11.36 (-18.7- -3.9)**	**-10.98 (-18.3- -3.6)**	**-10.56 (-18.1- -3.1)**	**-11.32 (-21.9- -0.7)**	-7.36 (-18.97-4.25)
	Gestation age		1.02 (-0.63-2.68)	1.14 (-0.58- -2.89)	1.16 (-0.59-2.91)	1.34 (-0.51-3.18)
	Birth weight			-0.004 (-0.02-0.01)	-0.004 (-0.02-0.01)	-0.01 (-0.02-0.01)
	Weight at 6 wk			0.001 (-0.01-0.01)	0.001 (-0.01-0.01)	0.001 (-0.01-0.01)
	EBF at birth				-1.11 (-11.84-9.61)	-0.09 (-10.96-10.78)
	EBF at 6 wk				0.94 (-7.06-8.93)	2.36 (-6.15-10.87)
	Age at 6 wk					-1.05 (-1.99- -0.09)
	Age at 14 wk					0.57 (0.03-1.11)

5.4 Discussion

Generating vaccine-mediated protection in infants is a complex challenge(Siegrist, 2007). There is limited understanding of the cellular immune responses currently available vaccines elicit in infants and less known about HIV-exposed uninfected (HEU) infants' responses. In Chapter 4, we showed that HIV-1 influences both BCG- and SEB- CD4+ and CD8+ T cell proliferation at 14 weeks significantly higher in the HEU versus HU infants but not at 6 weeks, and has no influence on BP-, and TT-induced CD4+ and CD8+ T cell proliferation. However, after adjusting for other confounders in a multivariate regression analysis in a model including the age BCG was administered in days, birth weight, weight at 6 weeks and the age at 14 week visit, HIV-exposure was the most important determinate of CD8+ T cell proliferation in response to TT at 14 weeks of life.

Cytokines are also important factors to measure in immunogenicity, as cytokines are the molecules that regulate the function of immune cells and little is known about the effect of HIV-exposure on this parameter. In this chapter the hypothesis was that HEU infants would have lower cytokine producing T cells to vaccine antigens and *in vitro* to SEB compared to HU infants.

Some studies have documented findings when measuring cellular immunogenicity of mycobacterial and other antigens in this vulnerable group of infants(Mansoor *et al*., 2010)(Van Rie *et al*., 2006)(Mazzola *et al*., 2011)(Borges-almeida *et al*., 2011)(Hygino *et al*., 2008). The aims of this chapter were to compare the quality and magnitude of CD4+ and CD8+ T cell intracellular cytokine production to vaccine antigens [Bacillus Calmette-Guérin (BCG), *Bordetella pertussis* (BP) and Tetanus toxoids (TT)], and a mitogen Staphylococcal enterotoxin B (SEB) in HEU versus HU infants.

It is important to note that cytokine expression was quantified in the actively proliferating, Ki67+ cells. The whole blood assay (WBA) was the method applied to quantify CD4+Ki67+ and CD8+Ki67+ T cells that produced intracellular cytokines (i.e. IFN-γ, IL-2, IL-13 and IL-17) as described in the methods (See sections 3.1.1 and 3.1.2). The ability to measure cytokine production at a cellular level using ICS staining and Flow Cytometry has proved to be a very

powerful tool , but since we looked at proliferating T cells that expressed cytokines, we could have missed some cytokine producing cells that did not proliferate.

In this chapter it was found that:

(i) HEU infants produced a significantly higher frequency of IL-13 and IL-17 by CD4+ T cells at 14 weeks of life in response to BCG. IL-17 is important and has been shown to populate the lungs, the primary site of *Mycobacteria tuberculosis* infections, and in turn recruit IFN-γ producing antigen-specific T cells persistent in the central lymphoid organs(Lin *et al.*, 2011). HEU infants had significantly lower frequency of IL-13 production by CD8+ T cells compared to HU infants in response to BCG at 6 and 14 weeks of life, and of IL-17 production by CD8+ T cells in response to BCG at 14 weeks of age. The presence of these IL-13 producing cells at a significantly lower frequency may represent sub-optimal responses for protection from other viral infections, although the significance of IL-13 production by CD8 cells is not clear. Indeed, a significantly higher frequency of these infants presented with viral upper respiratory tract infections compared to their un-exposed counterparts (see table 2.1). However, it is not clear what these cytokines do in CD8+ T cells. (ii) No significant differences were observed between HEU and HU infants in the frequency of cytokine production by CD4+ and CD8+ T cells following BP and TT stimulation at 14 weeks of life, suggesting that cytokine responses to protein antigens are no different between the groups of infants (iii) HIV exposure was a major predictor of IL-17 production by CD8+ T cells in response to BCG longitudinally through time to 14 weeks after adjusting for other confounders presented by the cohort in the multivariate model, suggesting that cytokine response to live-attenuated bacterial antigens may be impaired.

Consistent with findings by Mansoor *et al*., BCG vaccination of HIV-exposed infants induced a similar frequency of IFN-γ and IL-2 production by T cells compared to unexposed infants (Mansoor *et al*., 2010). The frequency of IFN-γ and IL-2 production by CD4+ T cells in response to BCG was similar in HEU versus HU infants at 6 and at 14 weeks of life in our study. HIV exposure as well as other confounders influenced cytokine production by T cells in response to BCG longitudinally from 6 to 14 weeks of life as described above (Table 5.2).

This is the first study to compare the intracellular cytokine response to tetanus and pertussis vaccinations in HEU versus HU infants. HIV exposure did not influence BP- and TT- induced cytokine production by T cells at 14 weeks of life.

One limitation of this study is that we used PMA and Ionomycin as the intracellular cytokine staining (ICS) stimulation in the whole blood assay (WBA), so the cytokine producing T cells are derived from antigen specific responses, but were re-stimulated using PMA and Ionomycin which reflects the potential of the T cell to make cytokine, which has already expanded to the antigen in culture.

In summary, the findings in this chapter suggest that HIV-exposure has subtle effects on cytokine responses to some antigens at 14 weeks but not at 6. The effect of HIV-exposure on cytokines may be age-dependant due to the ontogeny of the infants immune system generally and cytokine production in infancy.

CHAPTER 6

BREADTH OF ANTIGEN INDUCED T CELL CYTOKINE EXPRESSION IN HIV-EXPOSED UNINFECTED (HEU) VERSUS HIV-UNEXPOSED (HU) INFANTS AT 6 AND 14 WEEKS OF LIFE

6.1 Introduction

This chapter aims to assess the proportions of proliferating CD4+ and CD8+ T lymphocytes in response to vaccine antigens [Bacillus Calmette-Guérin (BCG), *Bordetella pertussis* (BP) and Tetanus toxoids (TT)], and to SEB, expressing combinations of the following cytokines: IFN-γ, IL-2, IL-13 and/or IL-17, in HIV-exposed uninfected (HEU) versus HIV-unexposed (HU) infants at 6 and at 14 weeks of life.

The frequency of cytokine production by antigen specific CD4+ and CD8+ T cells in HEU versus HU infants at 6 and at 14 weeks of life was measured after the ICS assay (described in the methods). Data after statistical analysis were presented as medians and interquatile ranges (IQR), alpha=0.05; statistical analysis was performed using *Wilcoxon signed rank* test (see table 3.3).

This chapter addresses part of specific objective two, to compare the breadth of antigen specific CD4 and CD8 T cell intracellular cytokine production to BCG, BP and TT vaccine and SEB in HEU versus HU infants at 6 and 14 weeks of life. In Chapter 5 we compared the quality of antigen specific CD4 and CD8 T cell intracellular cytokine production to BCG, BP and TT vaccine and SEB antigen in HEU versus HU infants and the evolution of cytokine production to BCG and SEB from 6 to 14 weeks of life. In this chapter, proportions of cytokine producing cells of total proliferating cells are reported, whereas in the previous chapter, proliferating, cytokine-producing cells were reported as the proportion of total T cells (see Figure 3.3).

The different cytokines assessed have been described in section 1.3 and the role of these cytokine expressing T cells to provide insight into immunity to the vaccines has been described in sections 1.5.2.2 and 5.1.

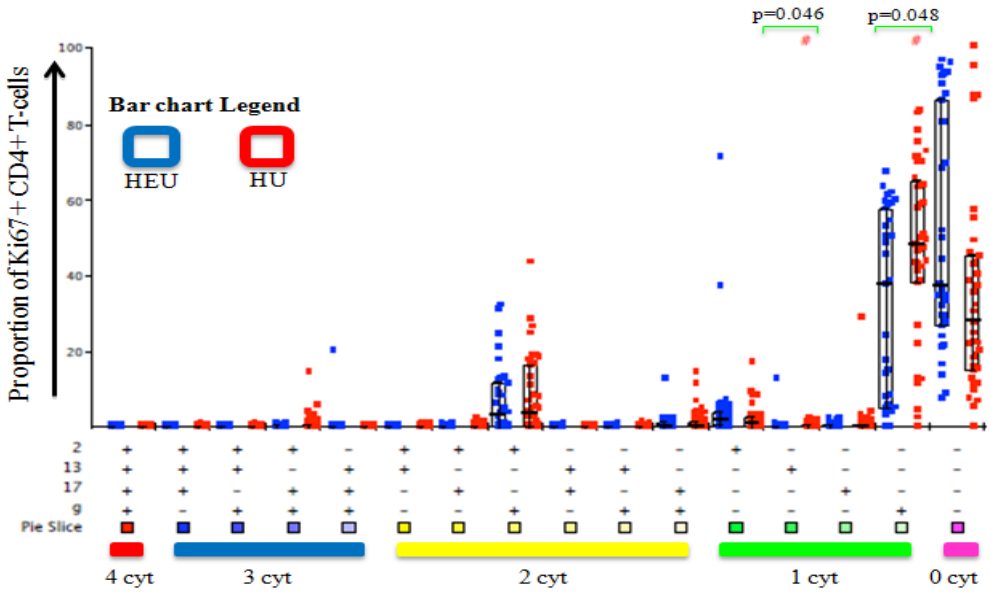
6.2 Breadth of BCG-induced proliferating CD4+ and CD8+ T cell cytokine expression at 6 weeks of life in HEU versus HU infants

(i) BCG-CD4+ at week 6

Figure 6.1 shows CD4+ T cell cytokine responses to BCG at 6 weeks. As seen in the graph, proportions of single cytokine producing CD4+ T cells, namely IL-13 (p=0.046) and IFN-γ (p=0.048), at 6 weeks of life were significantly lower in HEU infants (Figure 6.1a). The bottom

pies show that the overall cytokine production by CD4+ T cells was significantly lower in HEU compared to HU infants (p=0.0183) (Figure 6.1b). The difference in the proportion of CD4+ T cells that express no cytokines was evident and higher in HEU (Figure 6.1b).

(a)



(b)

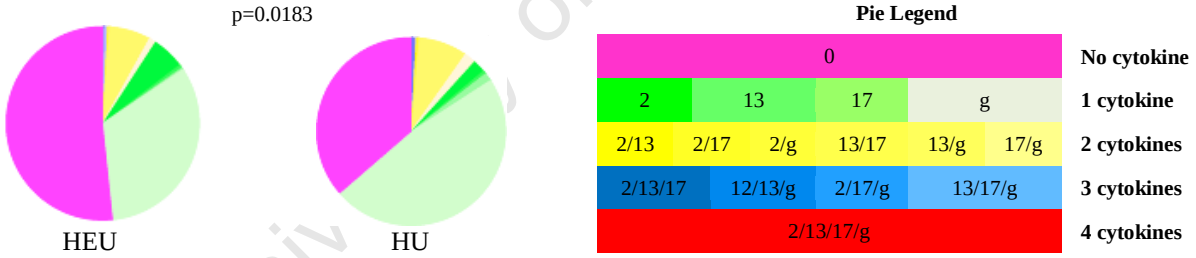
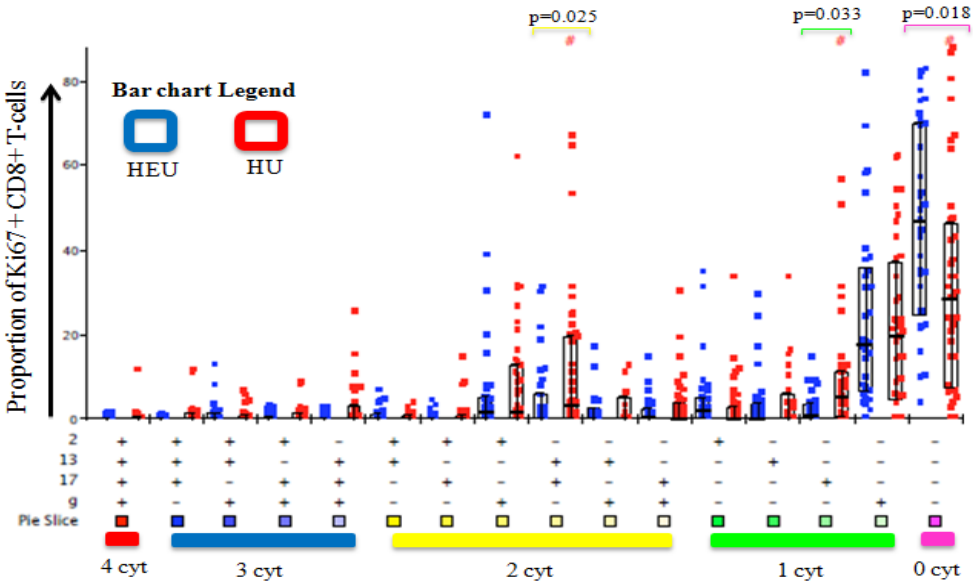


Figure 6.1: Breadth of BCG-induced proliferating CD4+ T cell cytokine production at 6 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of IL-2, IFN-γ, IL-13 and IL-17 cytokine(s) by proliferating CD4+ T cells in HEU versus HU infants. (a) Represents the possible combination of the responses 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 proportion of functionally distinct T cell populations within the proliferating CD4+ T cells (Each spot represents one infant). Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

(ii) BCG-CD8+ at week 6

Figure 6.2 shows the breadth of the CD8+ T cell responses to BCG at 6 weeks. The top graph shows that the proportions of the dual cytokine IL-13/IL-17 (p=0.025) and the single cytokine IL-17 (p=0.033), production by CD8+ T cells at 6 weeks of age were significantly lower in HEU compared to HU infants. Conversely, the proportion of cells with no cytokine expression was significantly (p=0.018) higher in HEU infants (Figure 6.2a). The bottom pies show that the overall cytokine production by CD8+ T cells was significantly lower in HEU infants (p=0.0132) (Figure 6.2b). The difference of the proportion of CD8+ T cells that produce three cytokines was evident and lower in HEU infants (Figure 6.2b).

(a)



(b)

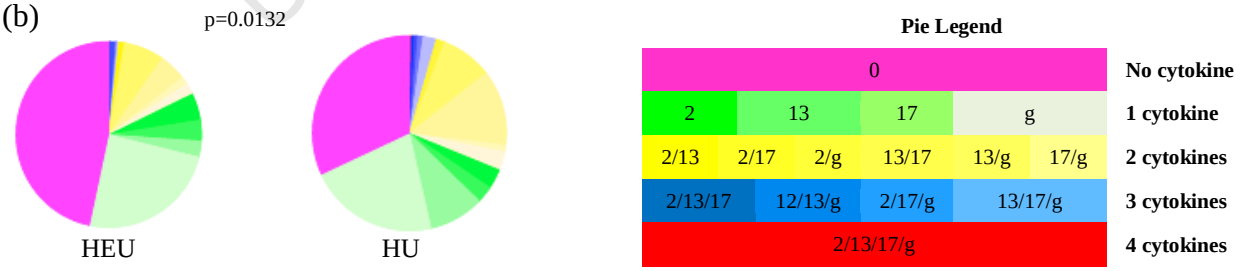


Figure 6.2: Proportions of BCG-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 6 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in HEU versus HU infants. (a) Represents the possible combination of the responses shown versus the proportions of the functionally distinct cell populations within the

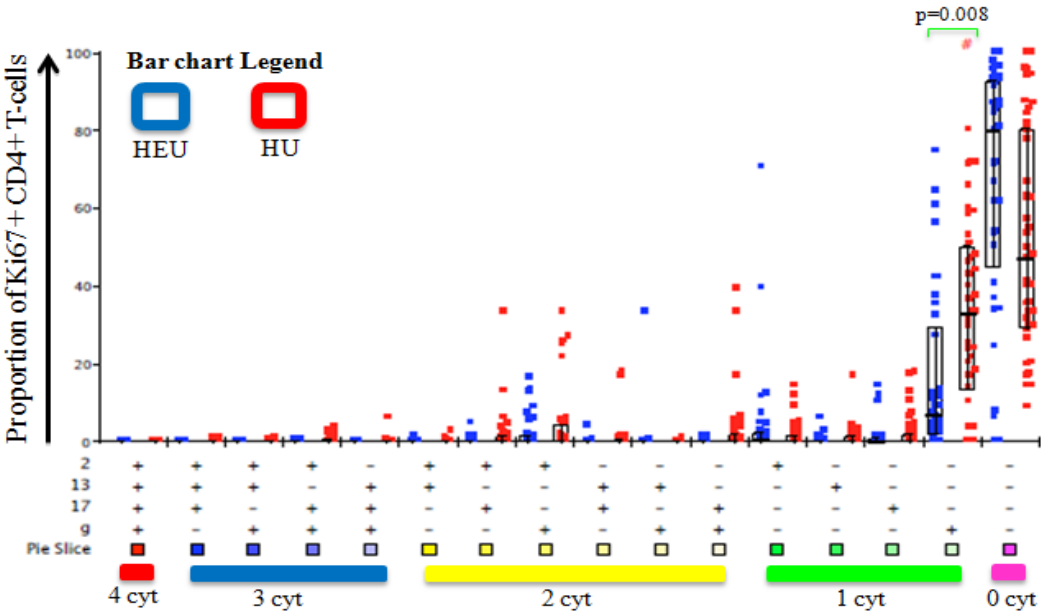
responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

6.3 Breadth of BCG-induced proliferating CD4+ and CD8+ T cell cytokine expression at 14 weeks of life in HEU versus HU infants

(i) BCG-CD4+ at week 14

Figure 6.3 shows the breadth of CD4+ T cell cytokine responses to BCG at 14 weeks. As seen in the top graph, the proportions of CD4+ T cells producing IFN-γ alone at 14 weeks of age was significantly lower in HEU infants (p=0.008) (Figure 6.3a). The bottom pies show that the overall breadth of cytokine production by CD4+ T cells at 14 weeks was significantly lower in HEU infants (p=0.0076) (Figure 6.3b).

(a)



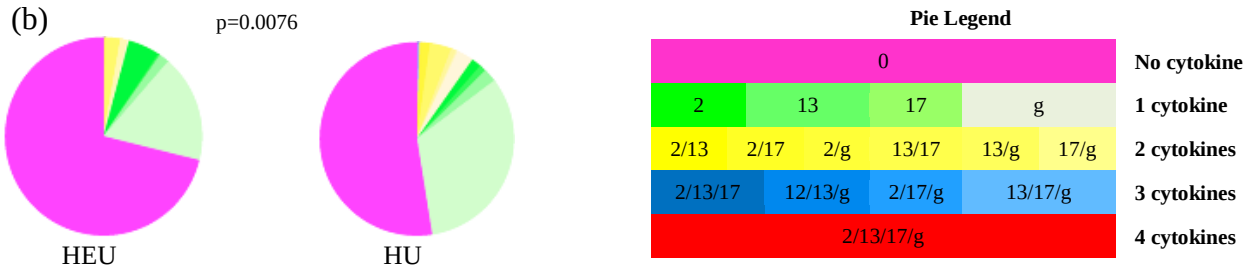
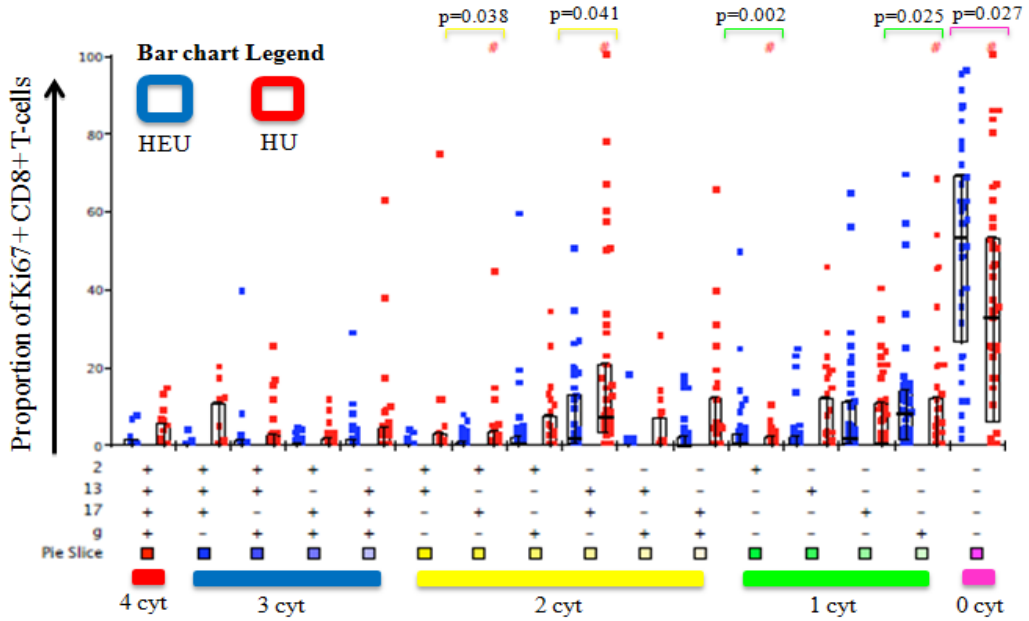


Figure 6.3: Breadth of BCG-induced proliferating CD4+ T cell cytokine production at 14 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of IL-2, IFN-γ, IL-13 and IL-17 cytokine(s) by proliferating CD4+ T cells in HEU versus HU infants. (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. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

(ii) BCG-CD8+ at week 14

Figure 6.4 shows the breadth of CD8+ T cell cytokine responses to BCG at 14 weeks. The top graph shows that the proportions of CD8+ T cells producing combinations of IL-2/IL-17 (p=0.038) and IL-13/IL-17 (p=0.041) at 14 weeks were significantly lower in HEU infants at 14 weeks of life (Figure 6.4a). The proportions of CD8+ T cells expressing single cytokines; IL-2 (p=0.002) and IFN-γ (p=0.025) were significantly higher in HEU infants (Figure 6.4a). Also, the proportion of CD8+ T cells that express no cytokine was significantly higher in HEU infants after BCG stimulation at 14 weeks of life (p=0.027) (Figure 6.4a). The bottom pies show that the overall proportion of cytokine production by CD8+ T cells was significantly lower in HEU infants (p=0.0106) (Figure 6.4b).

(a)



(b)

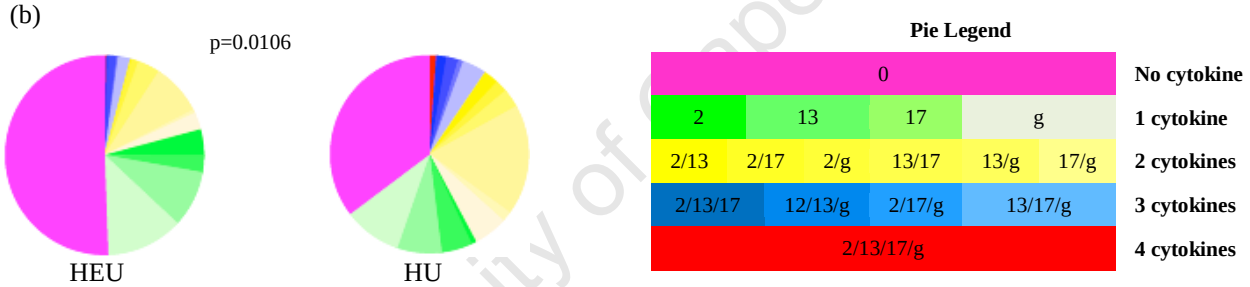


Figure 6.4: Proportions of BCG-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 14 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in HEU versus HU infants. (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. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

These data shows that the breadth of cytokine expression by BCG specific CD4+ and CD8+ T cells was significantly lower in the HEU infants irrespective of the time point. The proportion of cytokine production by CD4+ T cells was mostly single cytokines, while the production of two or more cytokines did not show any significant difference between groups. Differences in the proportions of CD8+ T cells producing one or a combination of cytokine(s) responses were evident between the two infant groups. Single cytokine expressions by CD8+ T cells were significantly higher in HEU at 14 weeks with significantly lower dual cytokine expression. HIV-exposure appears to markedly diminish the breath of cytokine production by T cells in response to BCG vaccination.

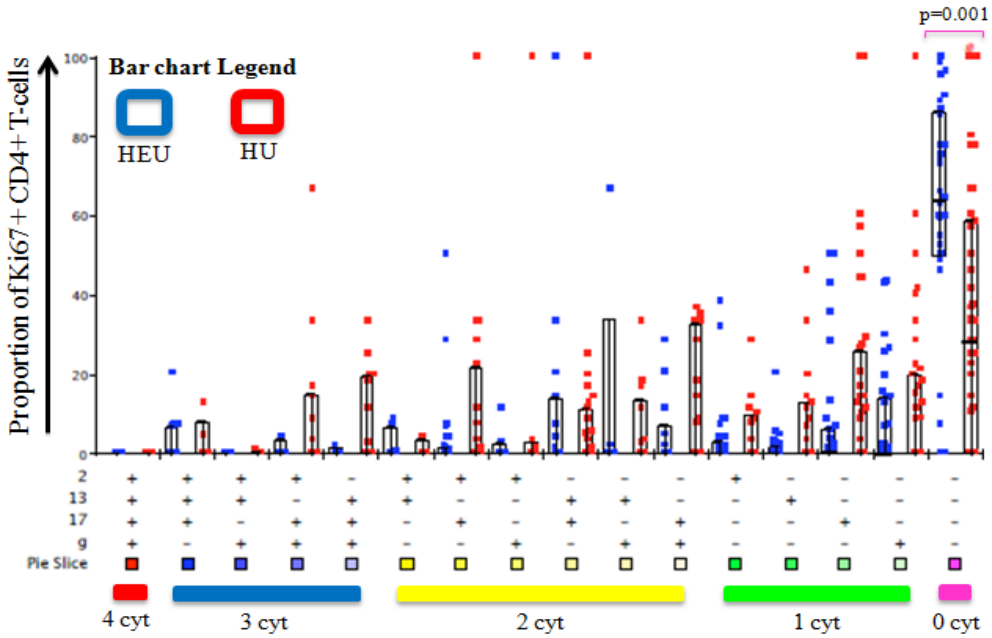
6.4 Breadth of BP-induced proliferating CD4+ and CD8+ T cell cytokine production at 14 weeks of life in HEU versus HU infants

(i) BP-CD4+ at week 14

Bordetella pertussis vaccination is first administered at six weeks of life and boosted at 10 weeks. Therefore, proportions of proliferating CD4+ and CD8+ T cells that produced cytokines alone or in combination at 14 weeks of life were compared between HEU versus HU infants (Figure 6.5 and 6.6).

Figure 6.5 shows the breadth of CD4+ T cell cytokine responses to BP at 14 weeks. The top graph shows that at 14 weeks of age, no significant difference in the proportion of CD4+ T cells expressing one or a combination of cytokine(s) between HEU versus HU infants in response to BP was observed (Figure 6.5a). However, the proportion of CD4+ T cells that did not express any cytokine was significantly higher in HEU infants (p=0.001) (Figure 6.5a). The bottom pies show the breadth of proliferating CD4+ T cells cytokine expression was lower in HEU infants (p=0.0008) (Figure 6.5b).

(a)



(b)

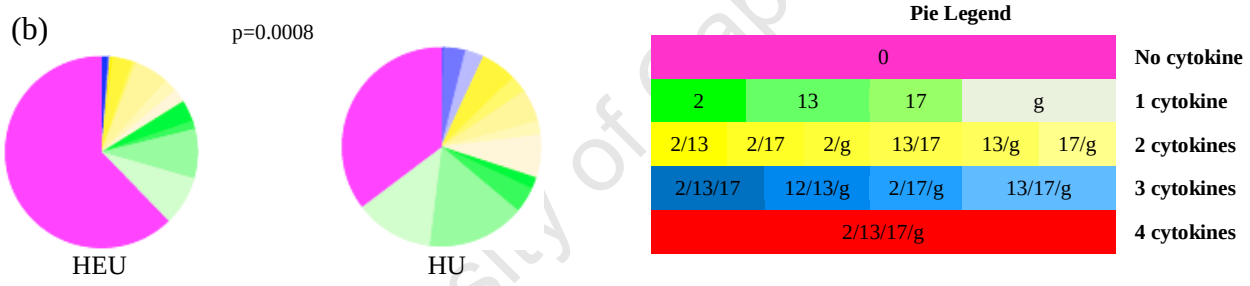
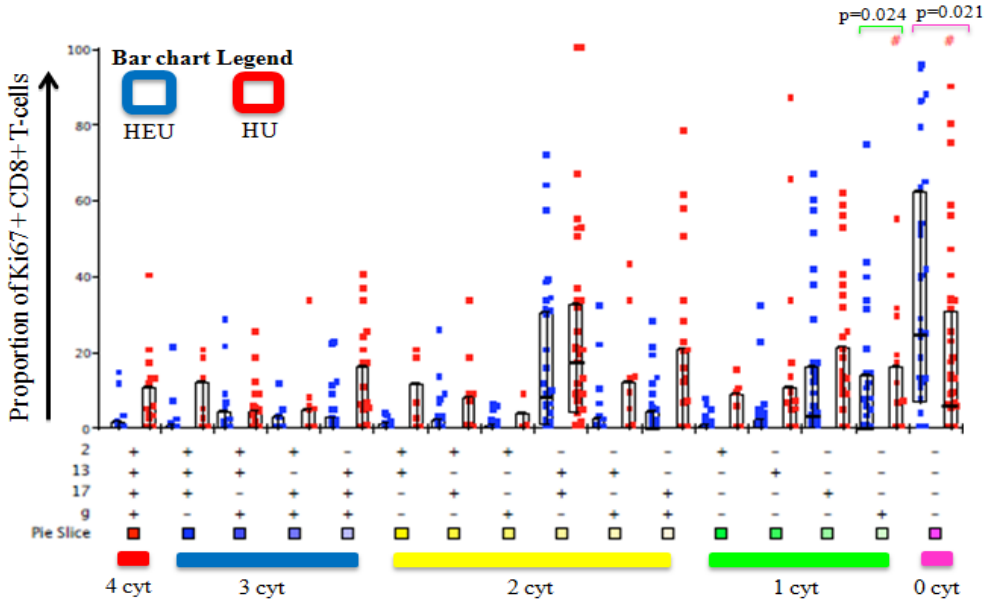


Figure 6.5: Proportions of BP-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 14 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of IL-2, IFN-γ, IL-13 and IL-17 cytokine(s) by proliferating CD4+ T cells in HEU versus HU infants. (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 proportions of functionally distinct T cell populations within the proliferating CD4+ T cells. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

(ii) BP-CD8+ at week 14

Figure 6.6 shows the breadth of CD8+ T cell cytokine responses to BP at 14 weeks. It is evident from the top graph that the proportion of CD8+ T cells that expressed the cytokine IFN-γ alone was significantly lower in HEU infants in response to BP at 14 weeks of age (p=0.024) (Figure 6.6a). No significant differences were observed in the proportions of CD8+ T cells that expressed more than one cytokine combination between HEU versus HU infants (Figure 6.6a). Also, the proportion of CD8+ T cells that expressed no cytokines was significantly higher in HEU infants (p=0.021) (Figure 6.6a). The bottom pies show that the difference in the overall proportion of cytokine expression by CD8+ T cells was significantly lower in HEU infants (p=0.0133) (Figure 6.6b).

(a)



(b)

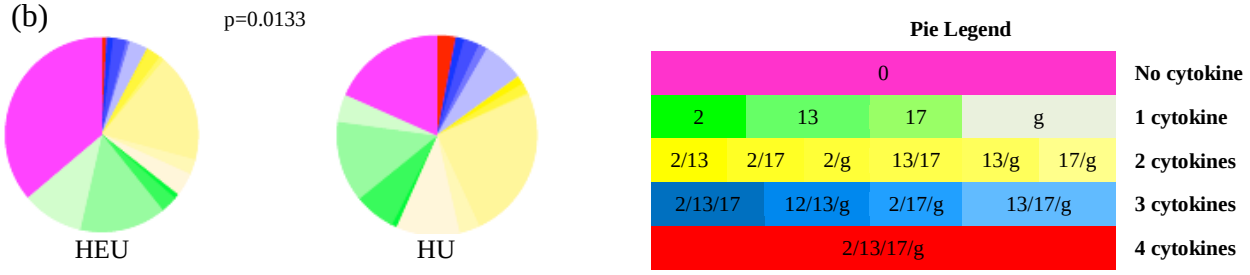


Figure 6.6: Breadth of BP-induced proliferating CD8+ T cell cytokine production at 14 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination

of intracellular cytokine(s) by proliferating CD8+ T cells in HEU versus HU infants. (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. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed ran*k test.

These data shows that in response to *Bordetella pertussis* at 14 weeks, there were no significant differences in the proportion of CD4+ T cells expressing one or a combination of cytokine(s) between HEU versus HU infants. However, a significant difference in the proportion of a mono-functional cytokine production by CD8+ T cells was evident and significantly lower in HEU infants. HIV-exposure has a profound effect on breadth of cytokine production in response to BP vaccination.

6.5 Breadth of TT-induced proliferating CD4+ and CD8+ T cell cytokine production at 14 weeks of life in HEU versus HU infants

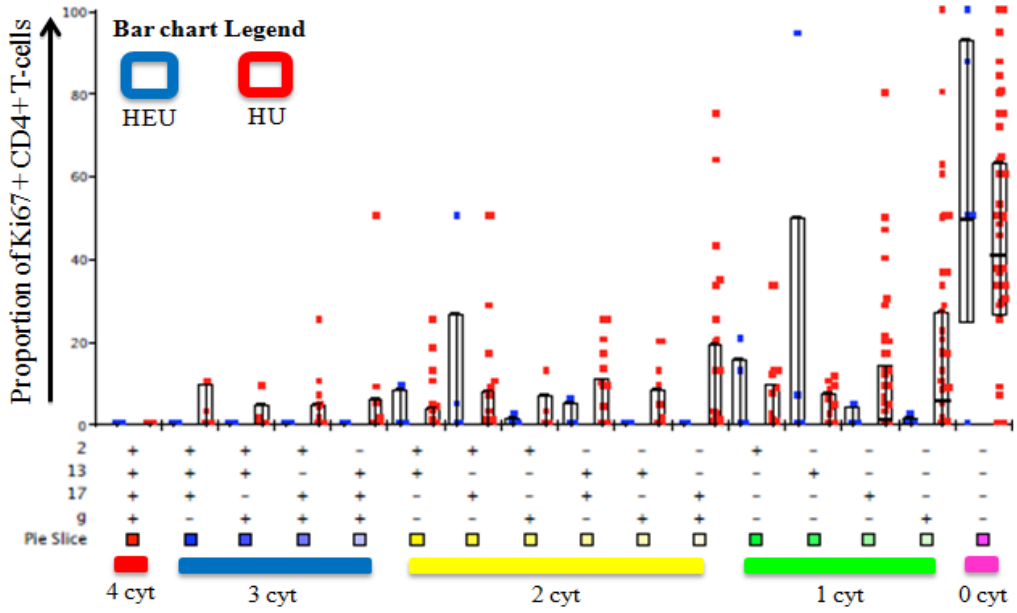
(i) TT-CD4+ at week 14

The administration of *Bordetella pertussis* vaccination is in combination with Tetanus toxoid vaccination to infants in South Africa, first at six weeks of life and boosted at 10 weeks, so we measured antigen specific CD4+ and CD8+ T cells expression of no cytokines, one or a combination of cytokine(s) by CD4+ and CD8+ lymphocytes at 14 weeks of life between HEU versus HU infant (Figure 6.7 and 6.8).

Figure 6.7 shows the breadth of the CD4+ T cell cytokine responses to TT at 14 weeks. The top graph shows that at 14 weeks of age, significant differences in the proportion of polyfunctional cytokine expressing cells by TT between HEU versus HU infants were not evident (Figure 6.7a). No significant difference was observed in the proportion of one or more cytokine expression by CD4+ T cells in response to TT between the two groups of infants (Figure 6.7a). The proportion of CD4+ T cells that could not produce a single cytokine did not differ significantly between

groups as observed from the graph (Figure 6.7a). Comparing the bottom pies, the overall cytokine expression by CD4+ T cells did not differ significantly between HEU and HU infants (p=0.1128) (Figure 6.7b).

(a)



(b)

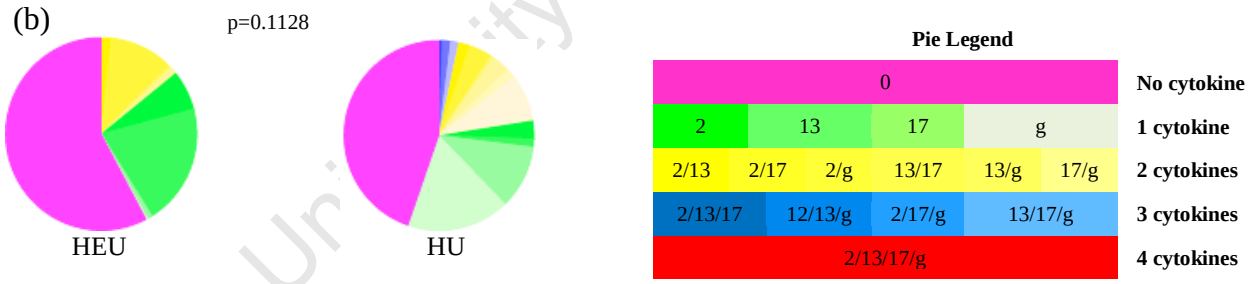
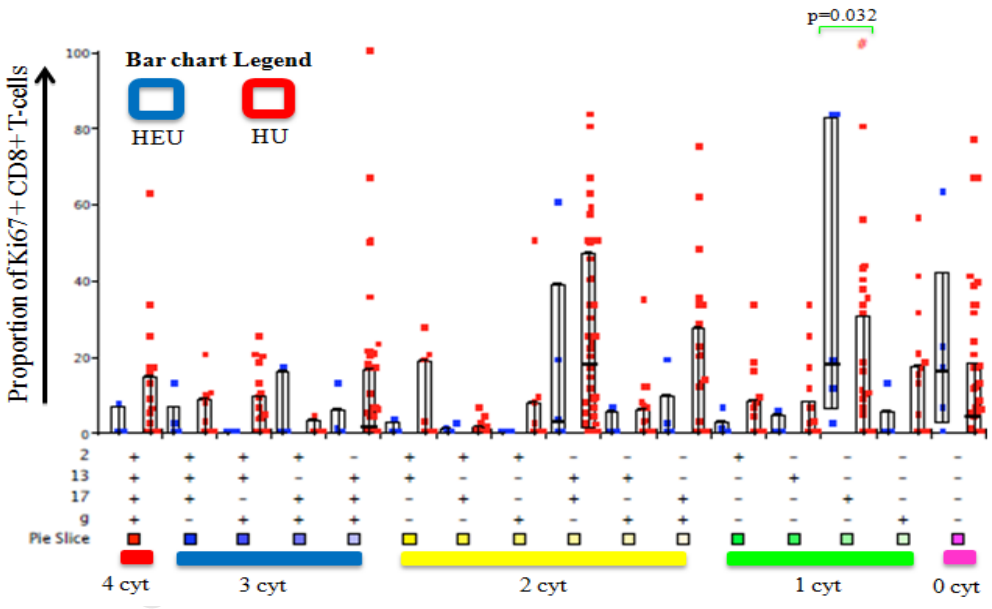


Figure 6.7: Proportions of TT-specific proliferating CD4+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 14 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of IL-2, IFN-γ, IL-13 and IL-17 cytokine(s) by proliferating CD4+ T cells in HEU versus HU infants. (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. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

(ii) TT-CD8+ at week 14

Figure 6.8 shows the breadth of the CD8+ T cell responses to TT at 14 weeks. The top graph shows that the proportion of CD8+ T cells that expressed the single cytokine IL-17 was significantly higher in HEU infants (p=0.032) (Figure 6.8a). The proportion of CD8+ T cells that expressed more than one cytokine or no cytokine at all did not differ significantly between the two groups in response to TT at 14 weeks (Figure 6.8a). The pies at the bottom show that the proportion of cytokine expression by CD8+ T cells did not differ significantly between HEU versus HU infants (p=0.0759) (Figure 6.8b). However, a trend toward lower proportions of any two or more combination of cytokines expressed by CD8+ T cells, evident in HEU compared to their unexposed counterparts (Figure 6.8b).

(a)



(b)

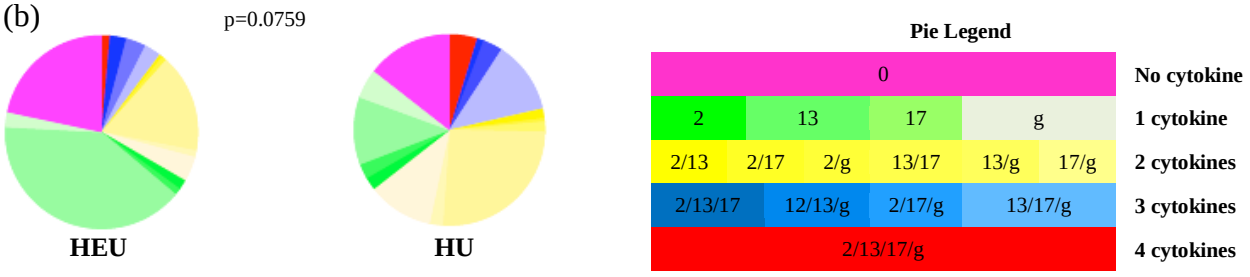


Figure 6.8: Breadth of TT-induced proliferating CD8+ T cell cytokine production at 14 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in HEU versus HU infants. (a) Represents 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. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

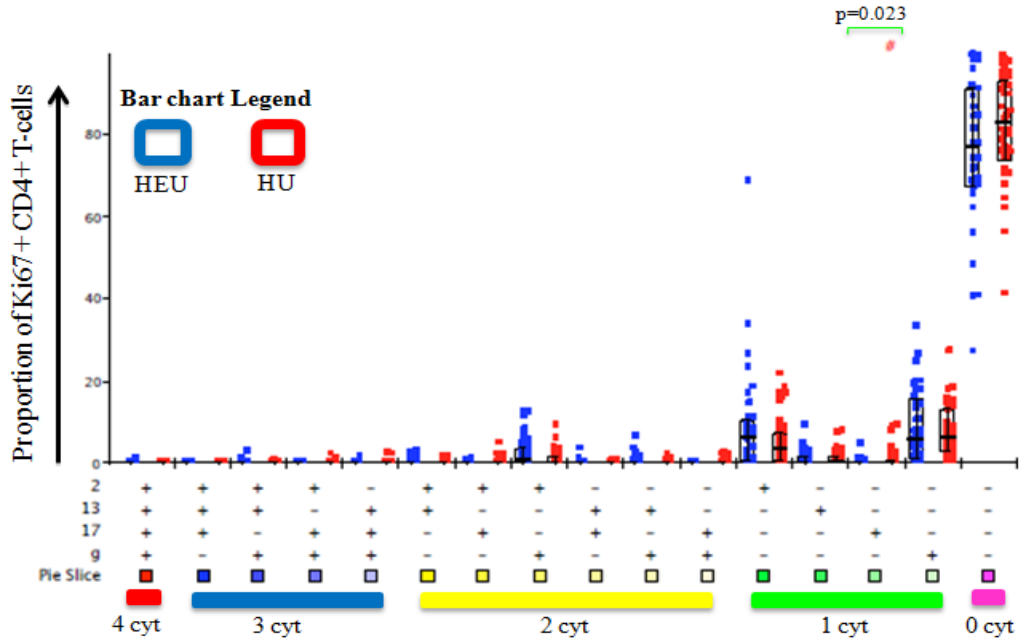
These results show that significant differences in response to TT were limited to the level of a single cytokine expression by CD8+ T cells,. It appears that the breadth of cytokine response to TT is less affected by HIV-exposure than the other vaccinations.

6.6 Breadth of SEB-induced proliferating CD4+ and CD8+ T cell cytokine expression at 6 weeks of life in HEU versus HU infants

(i) SEB-CD4+ at week 14

Figure 6.9 shows the breadth of the CD4+ T cell responses to SEB at 6 weeks. The top graph shows that the proportion of CD4+ T cells that expressed the single cytokine IL-17 was significantly lower in HEU compared to HU infants (p=0.023) (Figure 6.9a). The proportion of CD4+ T cells that expressed more than one cytokine or no cytokine at all did not differ significantly between the two groups in response to SEB at 6 weeks (Figure 6.9a). The pies at the bottom show that cytokine expression by CD4+ T cells did not differ significantly between HEU versus HU infants (p=0.1357) (Figure 6.9b) and there was a predominantly no cytokine expression regardless of group.

(a)



(b)

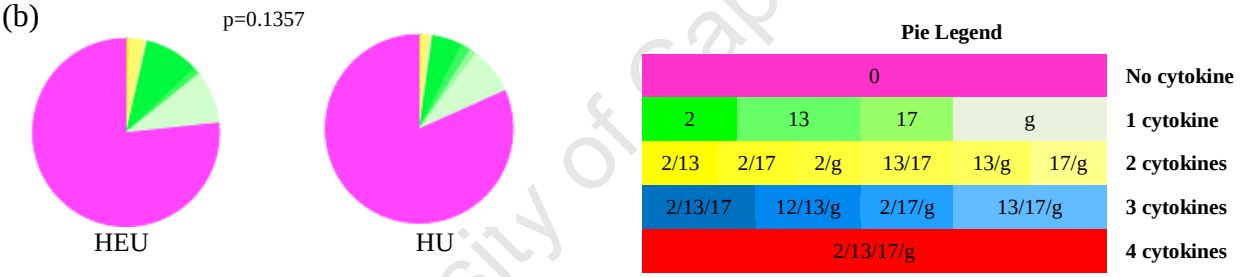
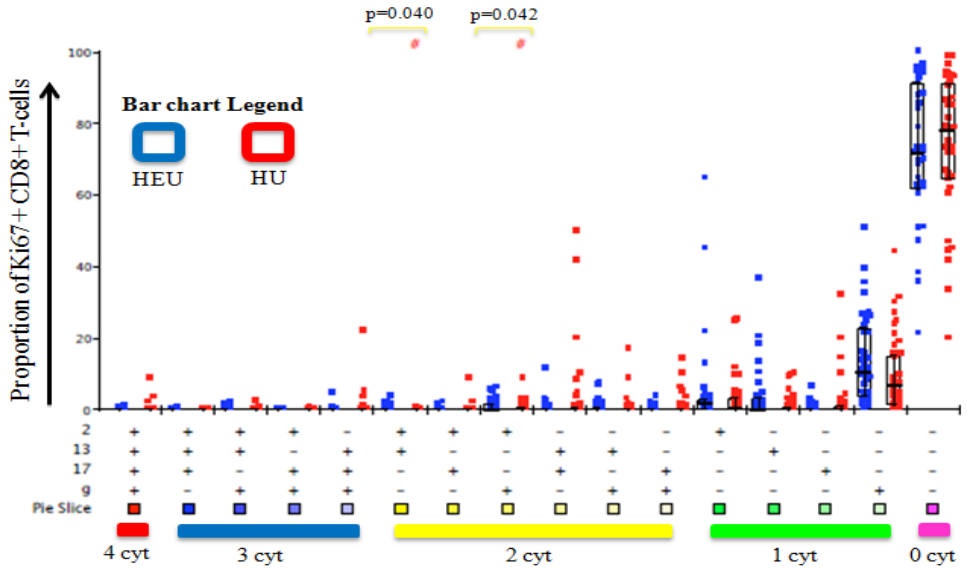


Figure 6.9: Breadth of SEB-induced proliferating CD4+ T cell cytokine production at 6 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of IL-2, IFN-γ, IL-13 and IL-17 cytokine(s) by proliferating CD4+ T cells in HEU versus HU infants. (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. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed ran*k test.

(ii) SEB-CD8+ at week 6

Figure 6.10 shows the breadth of CD8+ T cell cytokine responses to SEB at 6 weeks. The top graph shows that the proportions of CD8+ T cells expressing combinations of IL-2/IL-13 (p=0.040) and IL-2/IFN-γ (p=0.042) at 6 weeks were significantly higher in HEU infants (Figure 6.10a). The proportion of CD8+ T cells that expressed four cytokines, any combination of three cytokines, one cytokine or no cytokine at all did not differ significantly between the two groups in response to SEB at 6 weeks (Figure 6.10a). The pies show that the overall proportion of cytokine production by CD8+ T cells did not differ significantly between HEU versus HU infants (p=0.2570) (Figure 6.10b) with many cells unable to respond to SEB. As with CD4+ cells, the majority of CD8+ T cells at six weeks made no cytokine, regardless of group.

(a)



(b)

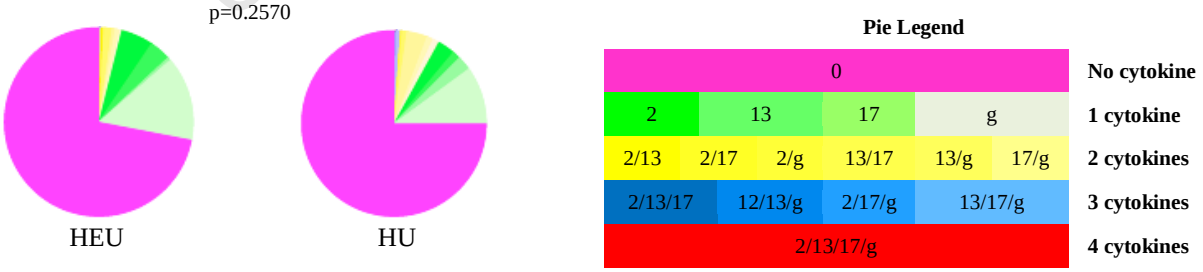


Figure 6.10: Proportions of SEB-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 6 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in HEU versus HU infants. (a) Represents the possible

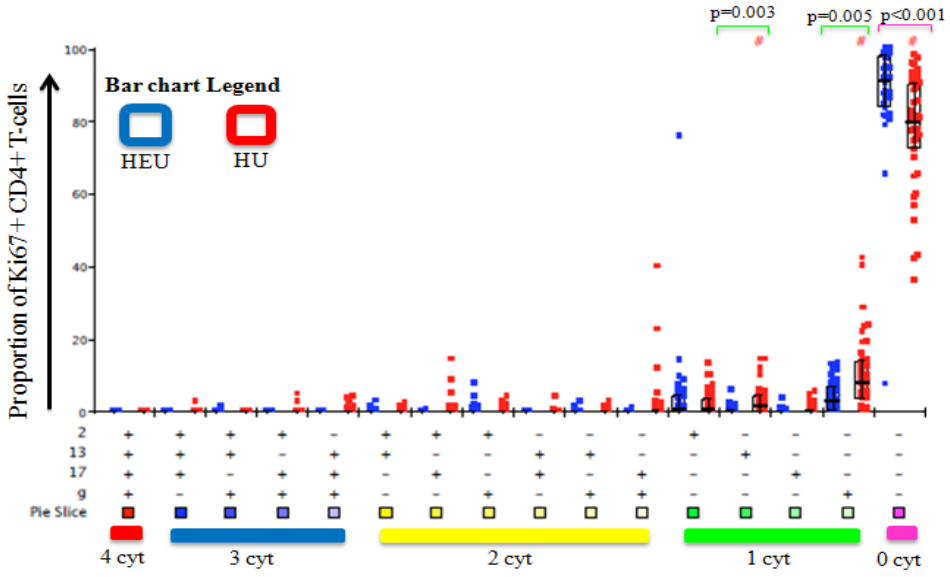
combination of the responses shown versus the proportions of the functionally distinct cell populations within the responding CD8+ T cells shown. Responses are grouped and colour-coded on the basis of the combinations of the cytokines produced. Spots correspond to the fractions of functionally distinct T cell populations within the proliferating CD8+ T cells. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

6.7 Breadth of SEB-induced proliferating CD4+ and CD8+ T cell cytokine expression at 14 weeks of life in HEU versus HU infants

(i) SEB-CD4+ at week 14

The proportion of CD4+ T cells that expressed the single cytokines IL-13 (p=0.003) and IFN-γ (p=0.005) were significantly lower in HEU infants (Figure 6.11a). The proportion of CD4+ T cells that expressed more than one cytokine did not differ significantly between the two groups in response to SEB at 14 weeks (Figure 6.11a). The proportion of CD4+ T cells that did not express any cytokine was significantly higher in HEU infants after SEB stimulation at 14 weeks of life (p<0.001) (Figure 6.11a). Similar to the findings at 6 weeks of age, the breadth of the CD4+ T cell responses to SEB at 14 weeks regardless of group was low and mostly made no cytokine at all, however HEU infants had significantly lower breadth (p=0.0015) (Figure 6.11b).

(a)



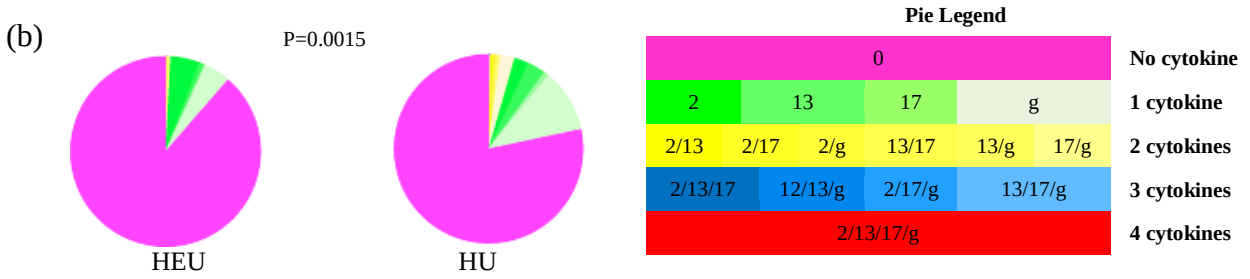
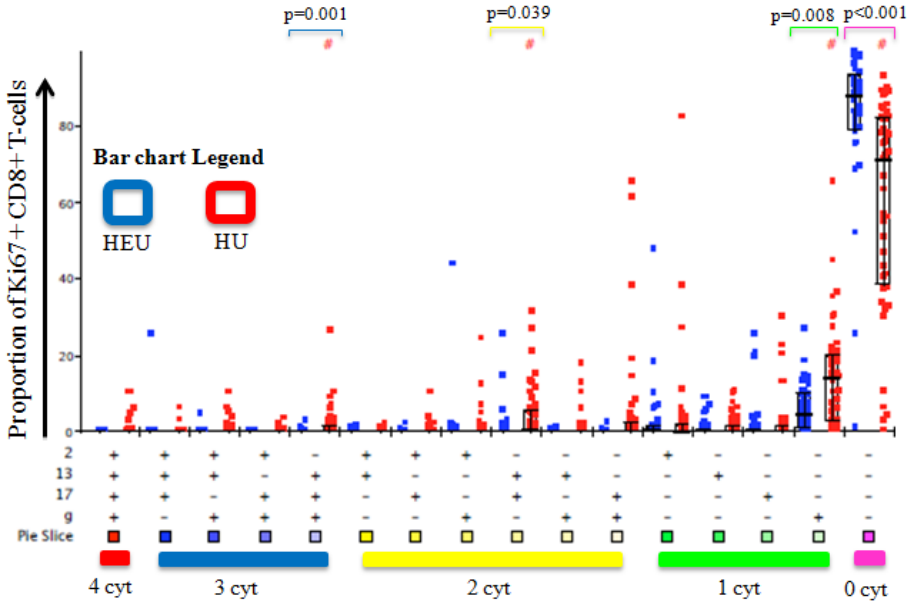


Figure 6.11: Breadth of SEB-induced proliferating CD4+ T cell cytokine production at 14 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of IL-2, IFN-γ, IL-13 and IL-17 cytokine(s) by proliferating CD4+ T cells in HEU versus HU infants. (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. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD4+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

(ii) SEB-CD8+ at week 14

Figure 6.12 shows the breadth of CD8+ T cell cytokine responses to SEB at 14 weeks. The top graph shows that the proportions of CD8+ T cells expressing combinations of IL-13/IL-17/IFN-γ at 14 weeks of life were significantly lower in the HEU compared to their unexposed counterparts (p=0.001) (Figures 12a). The proportions of CD8+ T cells expressing two combinations of IL-13/IL-17 (p=0.039) and one cytokine IFN-γ (p=0.008) at 14 weeks were significantly lower in HEU infants (Figure 6.12a). Conversely, the proportion of CD8+ T cells that could not express any cytokine was significantly higher in HEU infants after SEB stimulation at 14 weeks of life (p<0.001) (Figure 6.12a). The bottom pies show that the overall proportion of cytokine expression by CD8+ T cells in HU was higher than at 6 weeks, but HEU infants had significantly lower breadth than HU infants at this time point (p<0.0001) (Figure 6.12b).

(a)



(b)

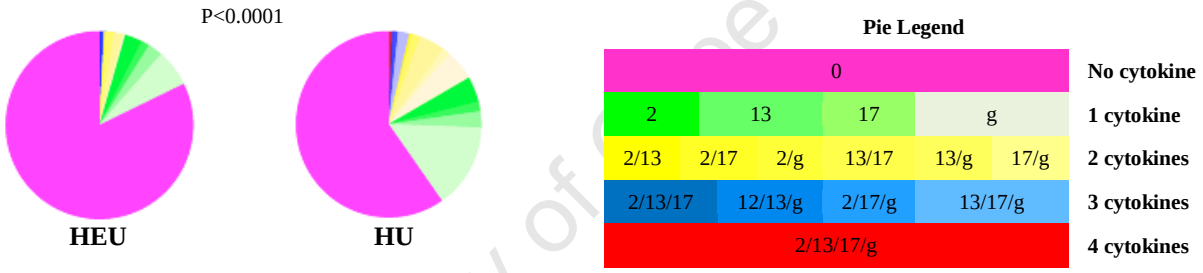


Figure 6.12: Proportions of SEB-specific proliferating CD8+ T cells producing no cytokine, one, or a combination of cytokine(s) based on expression of IL-2, IFN-γ, IL-13 and/or IL-17, at 14 weeks of life in HEU versus HU infants. Comparison of the proportion of cells expressing no cytokine, one or a combination of intracellular cytokine(s) by proliferating CD8+ T cells in HEU versus HU infants. (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. Error bars and whiskers correspond to medians and interquatile ranges (IQR) respectively; p-value indicates significant difference in the combination of cytokine production by CD8+ T cells between HEU versus HU infants. (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. (#) Alpha=0.05 and Statistical analysis was performed using *Wilcoxon signed rank* test.

These data show that, the breadth of cytokine production by proliferating T cells in response to SEB was significantly lower in the HEU infants irrespective of the time points. Significant differences in the proportion of cytokine production by CD4+ T cells was mostly single cytokines, while the production of two or more cytokines was evident by CD8+ T cells. Many

cells were unable to respond to SEB, the lack of responsiveness could indicate cell anergy due to an over whelming stimulation by SEB on infants cells. These data mean that *in utero* HIV-exposure could affect responses to *in vitro* stimulation as well as recall antigens.

6.8 Result summary

Table 6.1 illustrates that in general, the breadth of cytokine production by proliferating CD4+ T cells was significantly lower in the HEU infants irrespective of the time points or vaccine antigen stimulation. The proportion of cytokine production by CD4+ T cells was mostly single cytokines, while the production of two or more cytokines did not show any significant difference between groups.

Differences in the proportions of proliferating CD8+ T cells producing one or a combination of cytokine(s) responses were evident between the two infant groups. The proportion of CD8+ T cells producing two or more cytokines in response to BCG was significantly lower in the HEU infants, at 6 and 14 weeks, (Table 6.1). However, in response to SEB the production of dual cytokine combinations of IL-2/IL-13 and IL-2/IFN-γ by CD8+ T cells at 6 weeks was significantly higher in HEU compared to HU infants (Table 6.1).

In response to *Bordetella pertussis* and Tetanus toxoids at 14 weeks, significant differences in the proportion of proliferating CD4+ T cells producing one or a combination of cytokine(s) between HEU versus HU infants were not evident. However, a significant difference in the proportion of mono-functional cytokine production by CD8+ T cells was evident (Table 6.1). Significant differences were limited to the level of single cytokine production by CD8+ T cells, while significantly lower in HEU infants in response to BP; it was significantly higher in HEU infants in response to TT (Table 6.1).

Also, proportions of CD4+ and CD8+ T cells that could not produce cytokine upon antigen stimulation were significantly higher in the HEU infant group irrespective of the antigen stimulation or time point (Table 6.1).

These results are somewhat consistent with that observed in chapter five. There was a mixed effect in the quality of cytokine production in response to BCG. HEU infants had significantly lower frequencies of IL-13 production by CD8+ T cells compared to HU infants at 6 and 14 weeks of life, and of IL-17 production by CD8+ T cells at 14 weeks of age. However, HEU infants produced a significantly higher frequency of IL-13 and IL-17 by CD4+ T cells at 14 weeks of life. Similar findings were also observed in the breadth of cytokine production. Differences in the proportions of CD8+ T cells producing one or a combination of cytokine(s) responses were evident between the two infant groups. Single cytokine expressions by CD8+ T cells were significantly higher in HEU at 14 weeks but significantly lower dual cytokine combinations.

Table 6.1: Significant differences in the proportion of proliferating CD4+ and CD8+ T cells expressing no cytokine, one or a combination of cytokine(s) in HEU versus HU infants at 6 and 14 weeks of life. Vaccine antigen i.e. Bacillus Calmette-Guérin (BCG), *Bordetella pertussis* (BP) and Tetanus toxoid (TT) proportion of T cells expressing no cytokine, one or a combination of cytokine(s) i.e. IFN-γ, IL-2, IL-13 and/or IL-17 in HEU versus HU infants at 6 and 14 weeks of life. Parameters include read outs for each condition and time point (TP), p values between pie charts and points on the graph of HEU versus HU infants proportions of proliferating CD4+ and CD8+ T cells expressing no cytokine, one or a combination of cytokine(s). Cells on the table indicated N/A represents not applicable, since BP* and TT* are administered to infants in DTaP vaccine, with the first dose given (primed) at 6 weeks and NS represents no significant difference(s).

CYTOKINE COMBINATIONS SIGNIFICANTLY LOWER IN HEU INFANTS			
Parameters		**p-values HEU vs. HU bars**	
Condition	**TP**	**CD4+**	**CD8+**
BCG	**Week 6**	-IL-13 -IFN-γ	-IL-13/IL-17 -IL-17
	Week 14	-IFN-γ	-IL-2/IL-17 -IL-13/IL-17
BP	**Week 6**	-N/A	-N/A
	Week 14		-IFN-γ
TT	**Week 6**	-N/A	-N/A
	Week 14	-(NS)	
SEB	**Week 6**	-IL-17	
	Week 14	-IL-13 -IFN-γ	-IL-13/IL-17/IFN-γ -IL-13/IL-17 -IFN-γ
CYTOKINE COMBINATIONS SIGNIFICANTLY HIGHER IN HEU INFANTS			
Parameters		**HEU vs. HU bars**	
Condition	**TP**	**CD4+**	**CD8+**
BCG	**Week 6**		-No cytokine
	Week 14		-IL-2 -IFN-γ -No cytokine
BP	**Week 6**	-N/A	-N/A
	Week 14	-No cytokine	-No cytokine
TT	**Week 6**	-N/A	-N/A
	Week 14	-(NS)	IL-17
SEB	**Week 6**		-IL-2/IL-13 -IL-2/IFN-γ
	Week 14	-No cytokine	-No cytokine

6.9 Discussion

Overall, the proportion of vaccine antigen stimulated CD4+ and CD8+ T cells that respond to *in vitro* culture were significantly less polyfunctional in the HEU compared to the HU infants at both time points when comparing all the cytokine combinations.

The presence of polyfunctional T cells may be a representative of the quality of immune responses to vaccines and has been described to be associated with better clinical prognosis(John-stewart *et al.*, 2009)(Kagina *et al.*, 2009)(Burl *et al.*, 2012). Darrah *et al.*, showed that polyfunctional T cells expressing IFN-γ/IL-2/TNF-α correlate with vaccine mediated protection against Leishmania major(Darrah *et al.*, 2007). Polyfunctional T cells have been associated with long term control of HIV infection (Betts *et al.*, 2008). Polyfunctional responses to MTB distinguish latent infection versus active disease(Day *et al.*, 2012). Hence, HIV-exposure may negatively affect the response to certain antigens and vaccinations and may contribute to the increased morbidity and increased risk of TB disease(Hesseling *et al.*, 2010) of this vulnerable group.

On the other hand, after *ex vivo* stimulation with SEB, combinations of dual cytokine production by CD8+ cells were significantly higher in HEU infants at 6 weeks, but not observed in response to BCG. This may be due to the nature of the assay and antigens. The assay measured recall responses to BCG, a live replicating bacterial vaccine, whereas SEB responses were merely reflective of *in vitro* rather than *in vivo* TCR stimulation. Also, as suggested in chapter 4 (see discussion), it may be that BCG *in vivo* after vaccination selects for specific Vβ TCR families which expand and respond better to SEB in the whole blood assay. Also, HIV-exposure may expand (or delete) specific T cell clones i.e. Vβ TCR that are further expanded by SEB at a later time point (i.e. 14 weeks not 6) but respond *in vivo* to BCG in the assay.

It is vital to identify deficiencies of the immune system and their contribution to the increased morbidity seen in HEU infants, both to improve their morbidity and to inform vaccine design. It will also be important to measure the breadth of other cytokines that were not examined in this dissertation. It is possible that cells that appeared to not make any cytokine were in fact making other cytokines that we did not include in our panel. Deficiencies of cytokine breadth in response

to vaccines at an early age in the HEU infant may be due to exposure to maternal HIV and may influence the development of subsequent cellular as well as innate and adaptive immune responses to HIV itself.

CHAPTER 7

CONCLUDING REMARKS

Infectious diseases account for nearly six million deaths annually worldwide in children younger than five years. Immunization against vaccine-preventable infections is essential and cost effective in reducing childhood morbidity and mortality. Deficiencies of both innate and adaptive immunity in neonates and infants render them more susceptible to infectious diseases.

Due to the tolerant and complex nature of T cell immunity in neonates and infants, priming neonatal T cells is challenging and neonatal and infants T cell responses are biased towards T helper type-2 (Th2), thus complicating the induction of protective immune responses by vaccines(Jaspan *et al*., 2007)(Siegrist, 2007).

An estimated two million infants are exposed *in utero* to Human Immuno Deficiency Virus (HIV) world wide of which about a third acquires HIV and an estimated two thirds are HIV-uninfected, known as HIV-exposed uninfected (HEU) infants. These infants have higher morbidity and mortality despite being HIV-uninfected, and independent of their maternal disease stage(Mbori-ngacha *et al.*, 2001). It is unclear whether this increased vulnerability is due to dysfunctional immunological responses, rendering the infant open to underlying pathogen insult.

There are some documented immunological differences in HEU infants (section 1.5.1). This susceptible group of infants are exposed to low-dose of HIV-1 associated with the development of T helper type-1 responses(Kuhn *et al*., 2002). Some evidence suggests that they may have altered vaccine and mitogen responses(Van Rie *et al*., 2006)(Jones, 2011)(Mazzola *et al.*, 2011)

In addition, to the already observed altered immune features of the HEU infants, this dissertation has shown that;

(i) HIV-exposure can alter proliferative immune responses to some antigens at 14 weeks but not 6. These include significantly higher CD8+ T cell proliferation to BCG and both CD4+ and CD8+ T cell proliferation to SEB, and not *Bordetella pertussis* (BP) or tetanus toxoid (TT),

(ii) HIV-exposure diminishes the frequency of CD8+ T cell cytokines (IL-17 and IL-13) responses to BCG at 14 weeks (not at 6 weeks), but no significant difference to other antigens (BP, TT, and SEB).

(iii) Overall, magnitude and breadth of cytokine response to vaccines antigens were significantly lower in HEU infants irrespective of age examined.

Some differences were evident in the finding obtained between chapter 5 and chapter 6 in the single cytokine results. In response to BP, expression of IFN-γ by CD8+ T cells was significantly lower in HEU infants, and BCG-induced IFN-γ and IL-2 at 14 weeks were significantly higher in HEU infants in the breadth by proliferating T cells in Chapter 6, but not different in the quality of cytokine production reported in Chapter 5. Both Chapters are reporting different data, because the denominators differ. The data reported in Chapter 5 was dependant on the proliferation response (antigen-specific) T cells reported as a frequency of total CD4+ and CD8+ lymphocytes. Whereas Chapter 6 was not, and the data was reported as proportions of cytokine producing cells of total proliferating cells.

The importance of reporting the data as in Chapter 5 is that, when just looking at the frequency of Ki67+ cells that are IFN-γ+, the numbers were misleading, where the negative control sample (medium) would have for instance 10% positive but when it was only a single cell out of 10. So reporting the data as a frequency of total CD4+ and CD8+ T cells was a more appropriate comparison, since the denominators will be more consistent. Otherwise, when looking at the frequency of Ki67+ cells secreting IFN-γ, not only the numerator (i.e. the number of Ki67+ cells secreting IFN-γ) was dependent on the antigen stimulations, but also was the denominator (i.e. the number of Ki67+ cells).

Also, another reason why Chapter 6 was presenting with more significant differences as compared with Chapter 5 was because cells that were making no cytokine were also reported, which provided a very important piece of information. It seemed that HIV-exposure profoundly affected the ability to make any cytokine at all in response to many of the antigens.

This was an observational study, the effect on immune responses by potential confounders such as birth weight, weight and age at the time of visit, in addition to HIV-exposure, was considered. HIV-exposure inversely predicted CD8+ T cell proliferation in response to Tetanus toxoid stimulation at 14 weeks of life after correcting for these other confounders (Table 4.2), and did not predict single cytokine production by T cells in response to any vaccine antigen.

However, over time from 6 to 14 weeks of age, HIV-exposure positively predicted and was the most important determinant of CD4+ T cell proliferation in response to BCG and SEB stimulations (Table 4.2); and positively predicted IFN-γ production by CD4+ T cells in response to BCG longitudinally over time to 14 weeks of life after correcting for other confounders in multivariate analyses.

The results from this dissertation show that HIV-exposure significantly affects CD4+ T cell proliferation to live, attenuated bacterial antigen (BCG) from 6 to 14 weeks of life over time. It would be very important to conduct future studies on cellular immune responses over prolonged follow-up periods greater than 14 weeks of life to determine the evolution of T cell functions (i.e. proliferation and cytokine production). Comparisons such as these could inform timing and design of vaccines for HEU infants.

Further, the lack of or low level responsiveness to BP and TT at 14 weeks compared to BCG may be due to the time intervals between immunization and blood sampling, not due to intrinsic factors related to the immune system of infants. Studies are currently going on looking at further time points and with a larger sample size. A series of *ex vivo* stimulations and the 6 days culture period may also not reflect *in vivo* responses. Many activated cells, when re-stimulated, may undergo activation induced cell death (apoptosis), and thus will not be reported for the final read out. Lastly, the potential effects of antiretroviral (ARV) for prevention of mother to child transmission (PMTCT) of HIV on the HEU arm, not present in the HU arm is strongly acknowledged. ARVs can affect the infants *in utero* and postpartum and are an uncontrolled factor of unknown significance. As a limitation of the study, we did not have data for complete blood count (CBC) at these time points to record variation in infant WBC count. One might expect differences in CBC in HEU versus HU infants, since a fixed volume of whole blood was

added to the cultures, rather than a fixed cell count. This would be a factor to correct for in future studies. However, we also acquired a fix amount of cells with an average of 200,000 cells per acquisition, and all data was presented as frequency of lymphocytes not as absolute numbers.

Understanding T cell proliferation, cytokine milieu, dose and nature of the antigen (systemic or mucosal pathogen) as well as other neonatal immunological characteristics of HEU infants [impact of Regulatory T Cells (Tregs) and other suppressor cell populations such as Myeloid Derived Suppressor Cells (MDSCs) on vaccine immunity], may contribute to developing more effective vaccination strategies.

The additional advantage to studying the immune response of HEU infants is the knowledge gained as a controlled model for highly exposed uninfected individuals. A number of cohorts exist among populations at high risk of acquiring HIV, including HIV serodiscordant couples, commercial sex workers and injection drug users. With the absence of an effective HIV vaccine, the HEU infants provide a unique model for studying not only the consequences of early antigenic exposure in infants, but also provide very important insights for the study of immune correlates of protection to HIV and HIV-vaccine development.

The knowledge gained in this study will assist in designing studies in future to address the effect of HIV-exposure on mucosal vaccines [oral polio vaccine (OPV) and rotavirus vaccine (RV)] and further help to define the effect of HIV-exposure on other specific immune cells.

APPENDICES

Appendix A: Analysis within feeding and HIV-exposure mode to assess the effect of birth feeding mode on T cell responses.

Due to the fact that birth feeding mode was highly collinear with HIV-exposure status, subgroup analysis (i.e. analyses within groups of similar infants only) were also performed. Because the sample sizes of these groups were then very small, p-values were ignored and instead, 2-fold differences between medians (i.e. log order) was considered to reflect a meaningful difference in T cell proliferation. See table A1 in a few pages ahead that presents a summary of the graphs below.

It was found that;

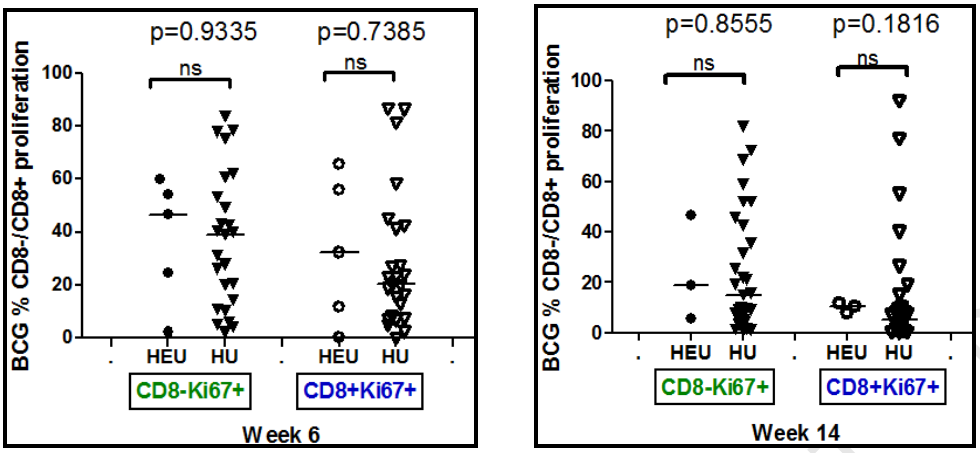
(i) Exclusively breastfed (EBF) HU show better T cell proliferation at 6 weeks compared to the EBF HEU infants but vice versa at 14 weeks in response to SEB (Figure A1). Also, EBF HEU at 6 weeks had better T cell proliferation than HU infants in response to BCG but similar levels at 14 weeks (Figure A1). (ii) All mixed fed (MF) HEU show better T cell proliferation at both 6 and 14 weeks compared to the MF HU infants at both 6 and 14 weeks of life in response to BCG and SEB (Figure A2). (iii) EBF infants in both the HEU and HU groups show better T cell proliferation compared to the MF infants in response to BCG at 6 weeks of age (Figure A3). (iv) EBF infants in both the HEU and HU groups show better T cell proliferation at 6 compared to 14 weeks of life in response to BCG and SEB (Figure A4). (v) MF HU infants show better T cell proliferation at 6 compared to 14 weeks of life in response to SEB (Figure A5).

Therefore, feeding mode could confound the relationship between HIV-exposure and vaccine responsiveness.

Similar to the observation by Pabst *et al*. where they described the positive influence of breast feeding on cell mediated immune responses to BCG vaccine given at birth by measuring antigen-induced blastogenesis in infants lymphocytes in response to purified protein derivative of Mycobacterium tuberculosis (Pabst *et al.*, 1988).

(i) Exclusively breastfed (EBF) HU show better T cell proliferation at 6 weeks compared to the EBF HEU infants but vice versa at 14 weeks.

A. BCG Stimulation



B. SEB Stimulation

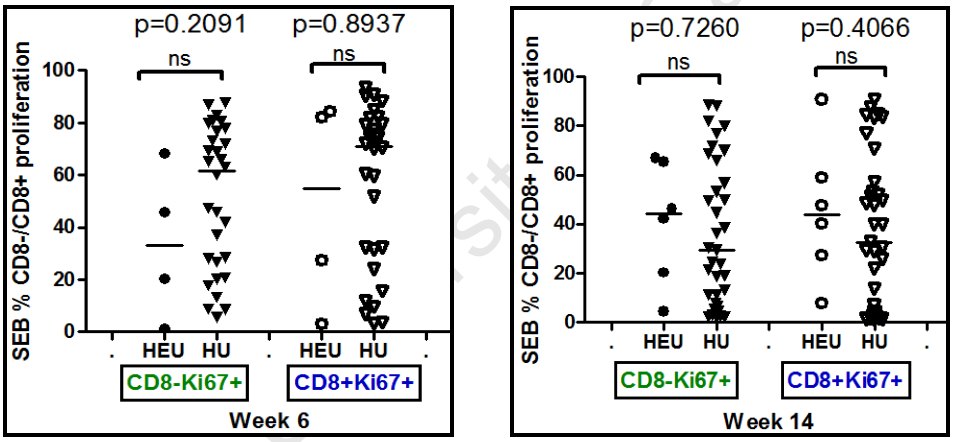
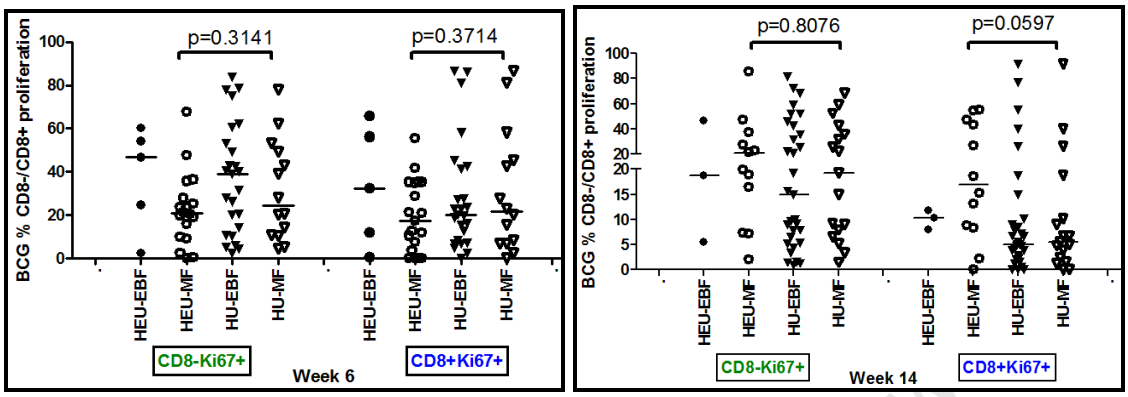


Figure A1: Frequency of proliferating T cells after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of exclusively breastfed (EBF) HU infants compared to the EBF HEU infants for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

(ii) All mixed fed (MF) HEU show better T cell proliferation at both 6 and 14 weeks compared to the MF HU infants at both 6 and 14 weeks of life.

A. BCG Stimulation



B. SEB Stimulation

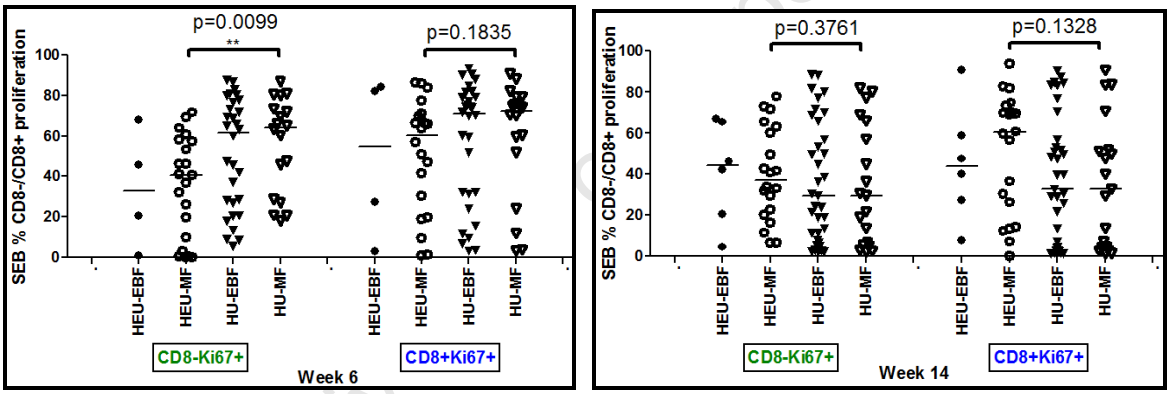
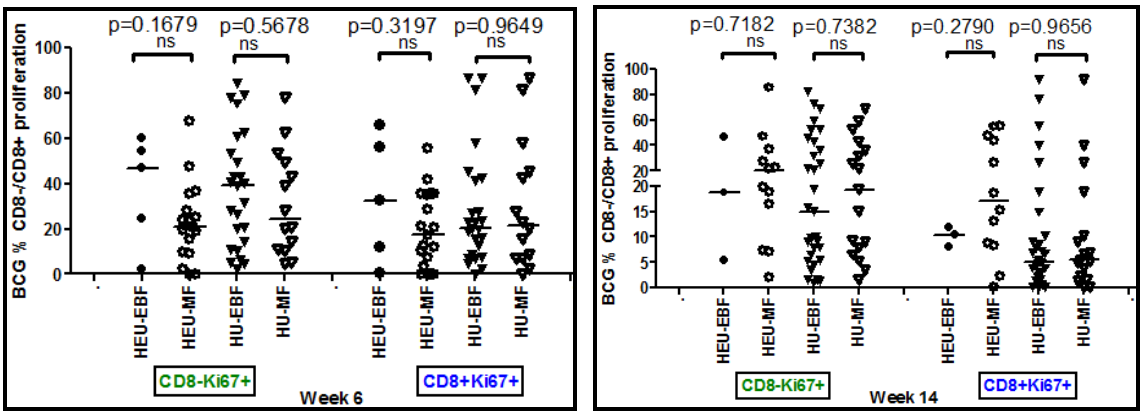


Figure A2: Frequency of proliferating T cells after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of mixed fed (MF) HU infants compared to the MF HEU infants for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

(iii) EBF infants in both the HEU and HU groups show better T cell proliferation compared to the MF infants.

A. BCG Stimulation



B. SEB Stimulation

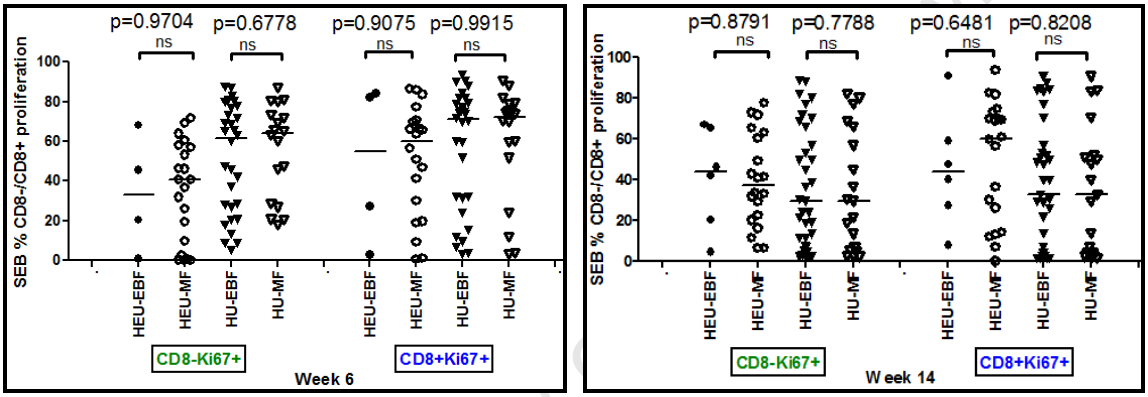
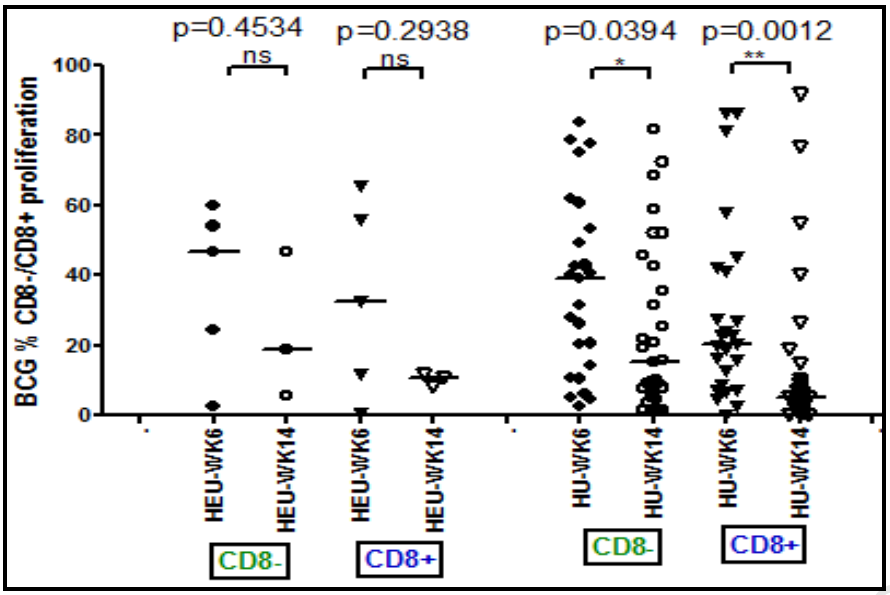


Figure A3: Frequency of proliferating T cells after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of HU and HEU infants T cells proliferation in exclusively breastfed (EBF) compared to mixed fed (MF) infants of the same group for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

(iv) EBF infants in both the HEU and HU groups show better T cell proliferation at 6 compared to 14 weeks of life.

A. BCG Stimulation



B. SEB Stimulation

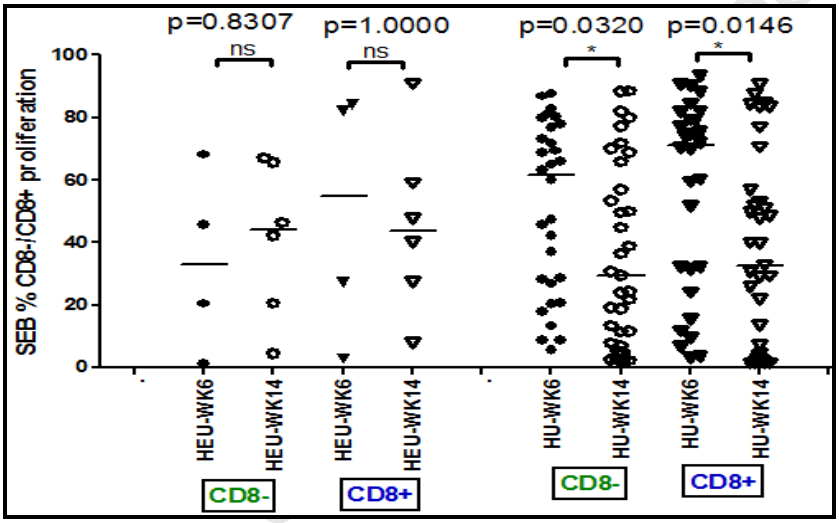
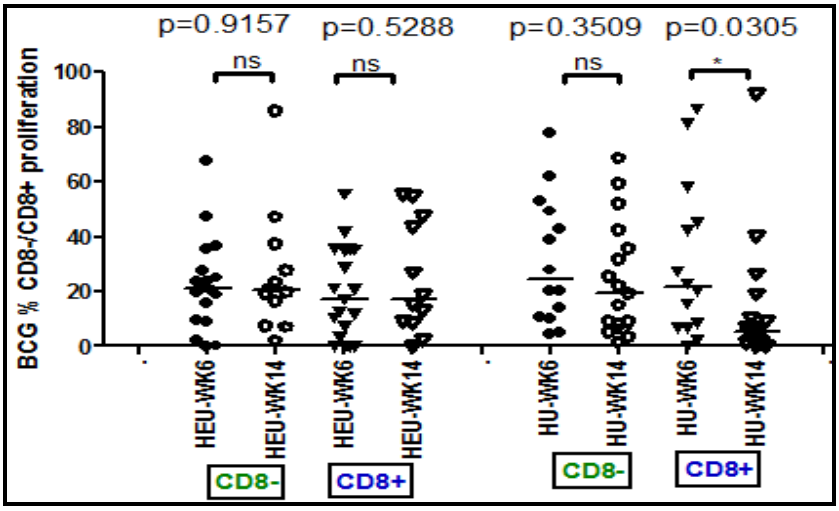


Figure A4: Frequency of proliferating T cells after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of exclusively breastfed (EBF) HU and HEU infants for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

(v) MF HU infants show better T cell proliferation at 6 compared to 14 weeks of life.

A. BCG Stimulation



B. SEB Stimulation

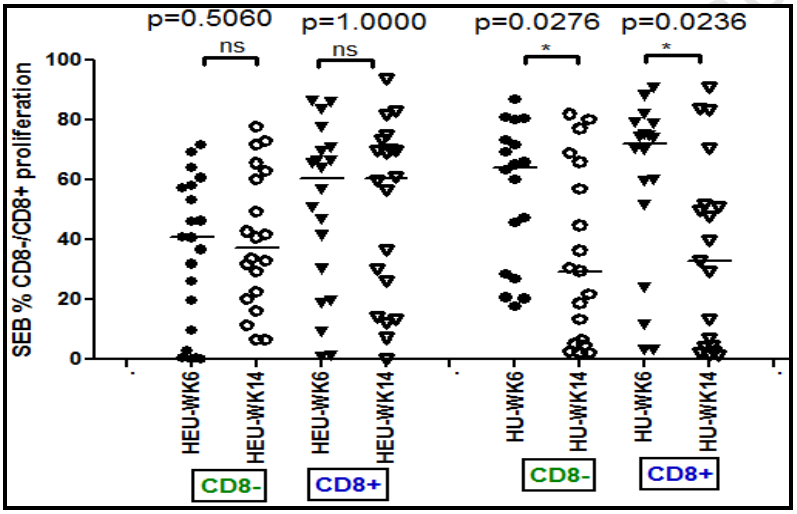


Figure A5: Frequency of proliferating T cells after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of mixed fed (MF) HU and HEU infants for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

Table A1 below shows a summary of the results presented on the graphs above.

Table A1: Ignoring p-values considering 2-fold differences (i.e. log order) to reflect a meaningful difference in CD4 and CD8 T cell proliferation.

Aspect	Parameter	Condition		CD4+	CD8+
(i) All EBF (HEU vs. HU)	Week 6	A.	SEB	HU > HEU	
	Week 14	A.	SEB	HEU > HU	HEU > HU
(ii) All MF (HEU vs. HU)	Week 6	A.	SEB	HEU > HU	
	Week 14	A.	BCG		HEU > HU
		B.	BP		HEU > HU
		C.	SEB		HEU > HU
(iii) HEU (EBF vs. MF)	Week 6	A.	BCG	EBF > MF	EBF > MF
HU (EBF vs. MF)		A.	BCG	EBF > MF	

Red highlights indicate week 6
Blue highlights indicate week 14
Green highlights indicate evolution of CD4 and CD8 responses from 6 to 14 weeks of life.

- **HEU**=HIV exposed uninfected; **HU**= HIV unexposed
- Comparing all Exclusively Breastfed **(EBF) only**; HEU vs. HU
- Comparing all Mixed fed **(MF) only**; HEU vs. HU
- Comparing **EBF vs. MF considering HEU & HU Infants separately**
- Within **EBF only** comparing 6 vs. 14 Weeks
- Within **MF only**; comparing 6 vs. 14 weeks

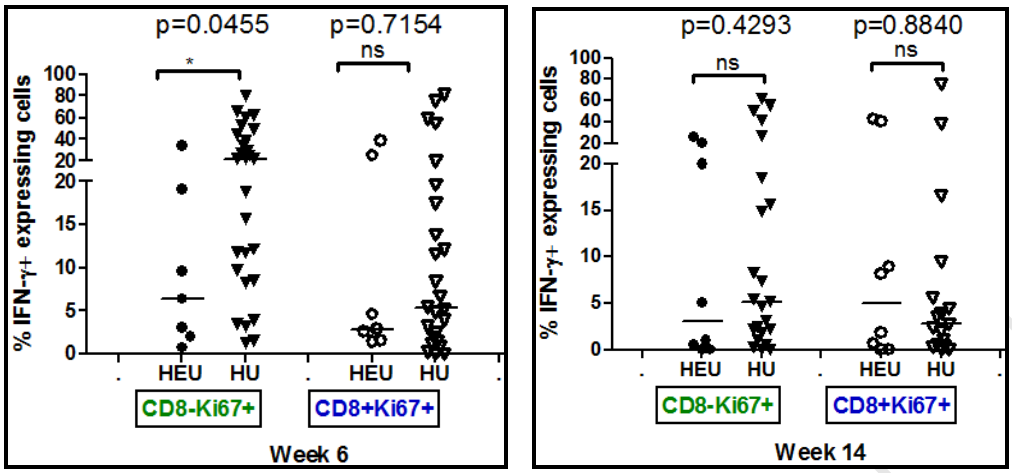
Appendix B: Ignoring p-values considering 2-fold differences (i.e. log order) to reflect a meaningful difference in T cell IFN-γ secreting cells.

Also, analysis where p-values were assessed based on 2-fold differences between medians (i.e. log order) to reflect a meaningful difference in T cell IFN-γ secreting cells was done.

(i) Exclusively breastfed (EBF) HU had better IFN-γ expression by T cells at 6 weeks compared to the EBF HEU infants (Figure B1). (ii) However, most mixed fed (MF) HEU had better IFN-γ expression by T cells at both 6 and 14 weeks compared to the MF HU infants at both 6 and 14 weeks of life to BCG, except for SEB stimulation at 14 weeks of life (Figure B2). (iii) MF infants in the HEU groups had better IFN-γ expression by T cells compared to the EBF infants at 6 weeks of life but not at 14 weeks in response to BCG (Figure B3); but EBF infants in the HU group showed better IFN-γ expression by T cells compared to the MF infants at 6 weeks of life (Figure B3). In response to SEB the levels of IFN-γ production did not differ (Figure B3). (iv) EBF infants in both the HEU and HU groups had better IFN-γ expression by T cells at 6 compared to 14 weeks of life in response to BCG and SEB (Figure B4). (v) MF HU infants had better IFN-γ expression by CD4+ T cells at 6 compared to 14 weeks of life and by CD8+ T cells in response to SEB, but better IFN-γ expression by CD8+ T cells at 14 compared to 6 weeks of life in response to BCG (Figure B5). See table B1 in a few pages ahead that presents a summary of the results presented on the graphs below.

(i) Exclusively breastfed (EBF) HU show better IFN-γ expression by T cells at 6 weeks compared to the EBF HEU infants.

A. BCG Stimulation (IFN-γ)



B. SEB Stimulation (IFN-Ɣ)

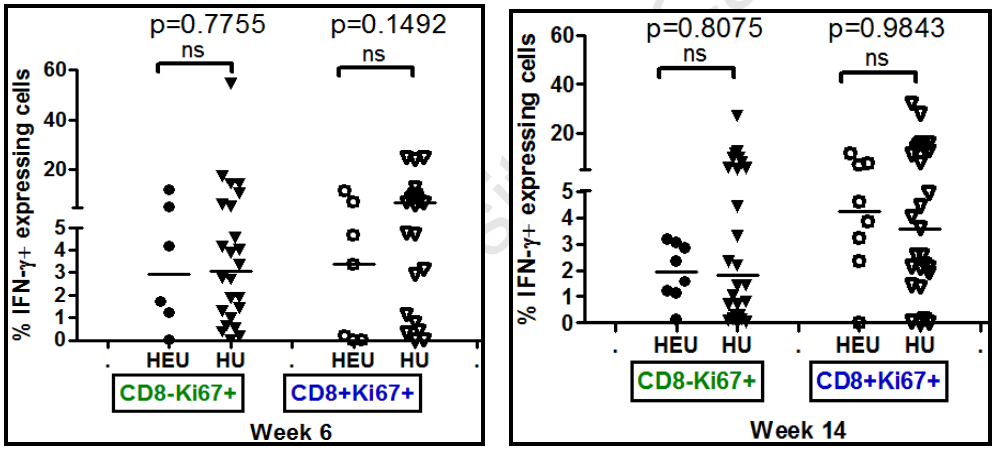
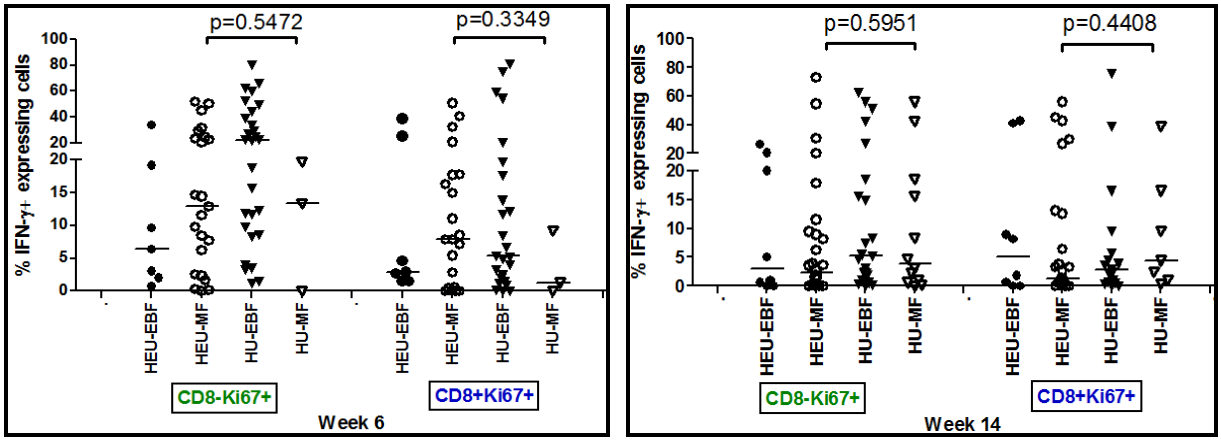


Figure B1: The frequency of T cells producing IFN-γ after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of exclusively breastfed (EBF) HU infants compared to the EBF HEU infants for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

(ii) All mixed fed (MF) HEU show better IFN-γ expression by T cells at both 6 and 14 weeks compared to the MF HU infants at both 6 and 14 weeks of life, except for SEB stimulation at 14 weeks of life.

A. BCG Stimulation (IFN-γ)



B. SEB Stimulation (IFN-γ)

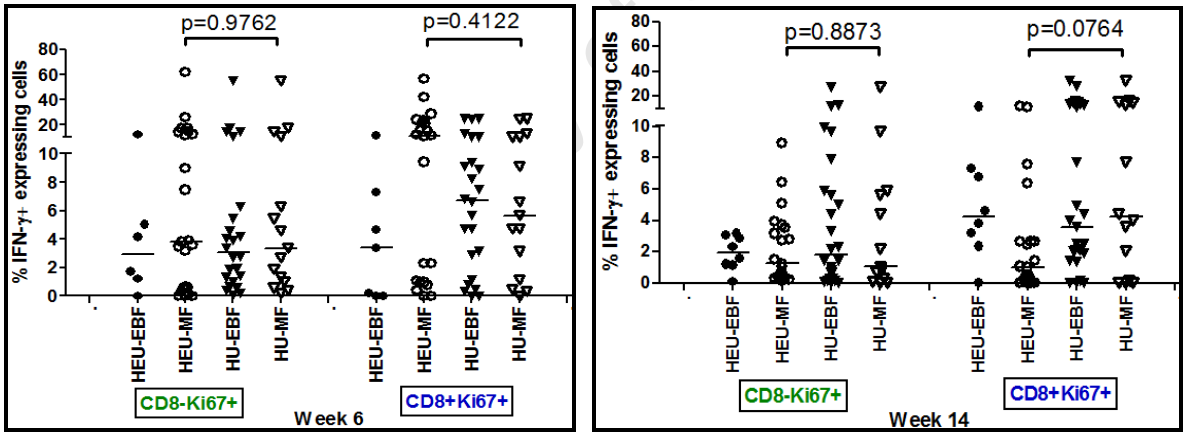
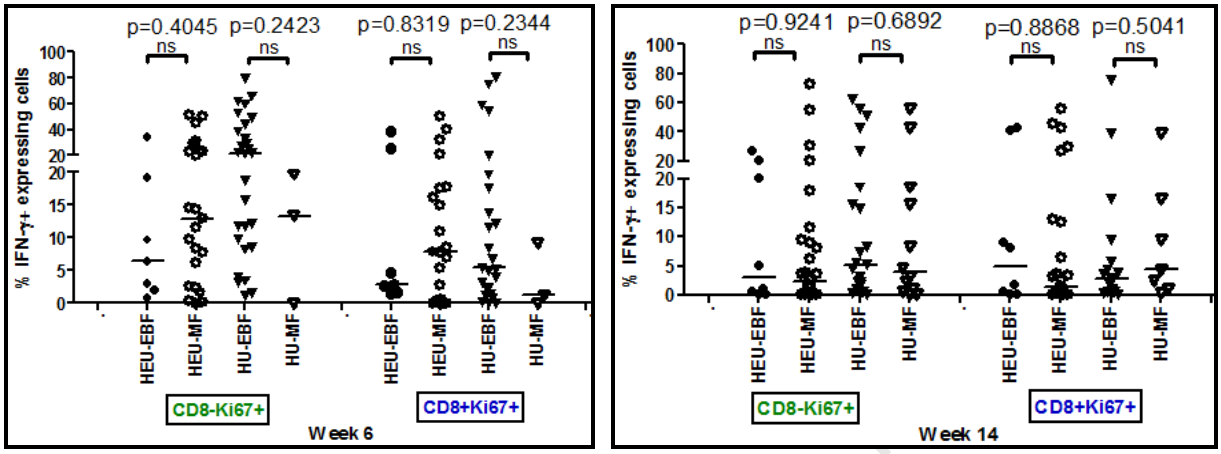


Figure B2: The frequency of T cells producing IFN-γ after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of mixed fed (MF) HU infants compared to the MF HEU infants for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

(iii) MF infants in the HEU groups show better IFN-γ expression by T cells compared to the EBF infants at 6 weeks of life but not at 14 weeks; but EBF infants in the HU group showed better IFN-γ expression by T cells compared to the MF infants at 6 weeks of life.

A. BCG Stimulation (IFN-γ)



B. SEB Stimulation (IFN-γ)

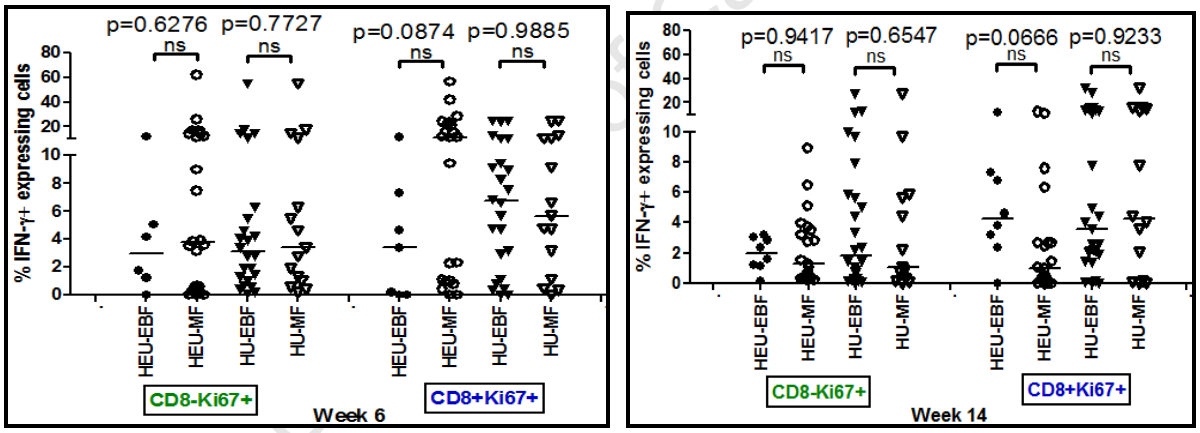
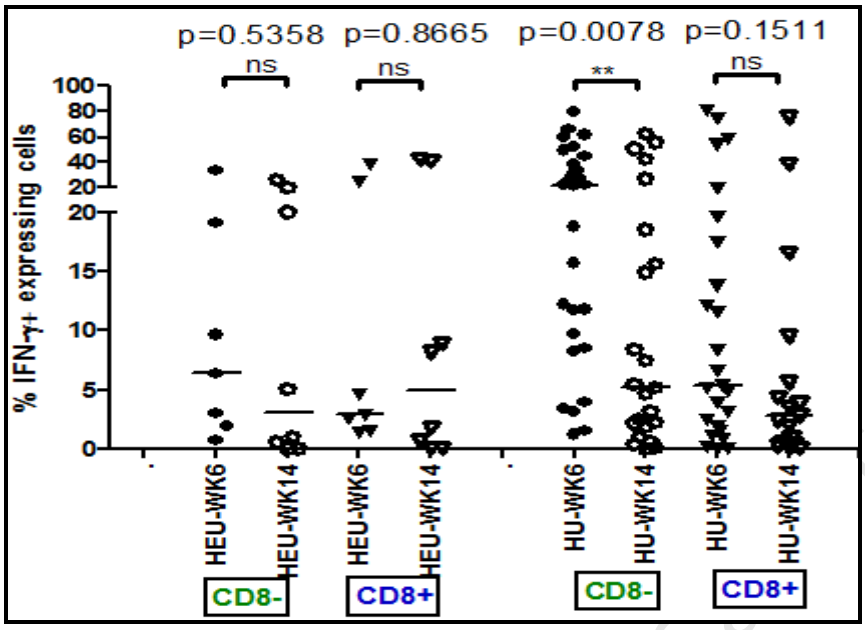


Figure B3: The frequency of T cells producing IFN-γ after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of HU and HEU infants T cells IFN-Ɣ production in exclusively breastfed (EBF) compared to mixed fed (MF) infants of the same group for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

(iv) EBF infants in both the HEU and HU groups show better IFN-γ expression by T cells at 6 compared to 14 weeks of life.

A. BCG Stimulation (IFN-γ)



B. SEB Stimulation (IFN-γ)

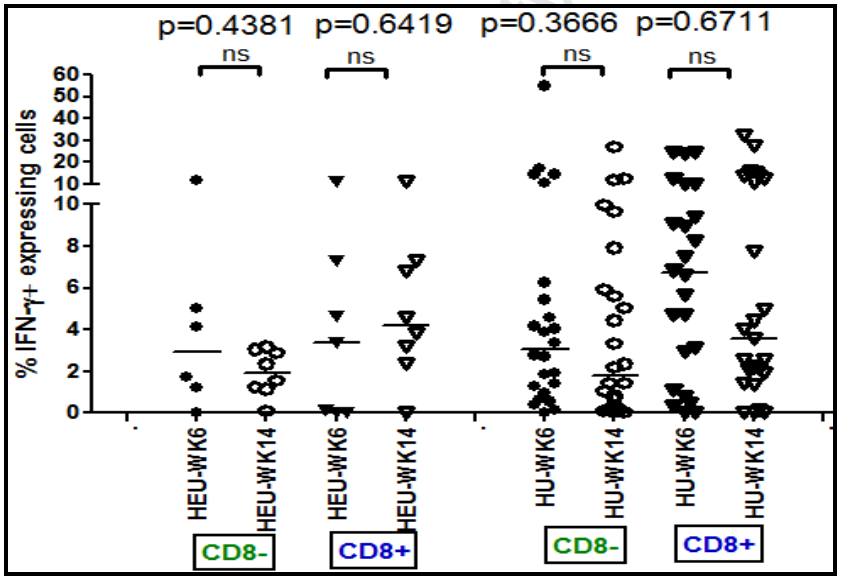
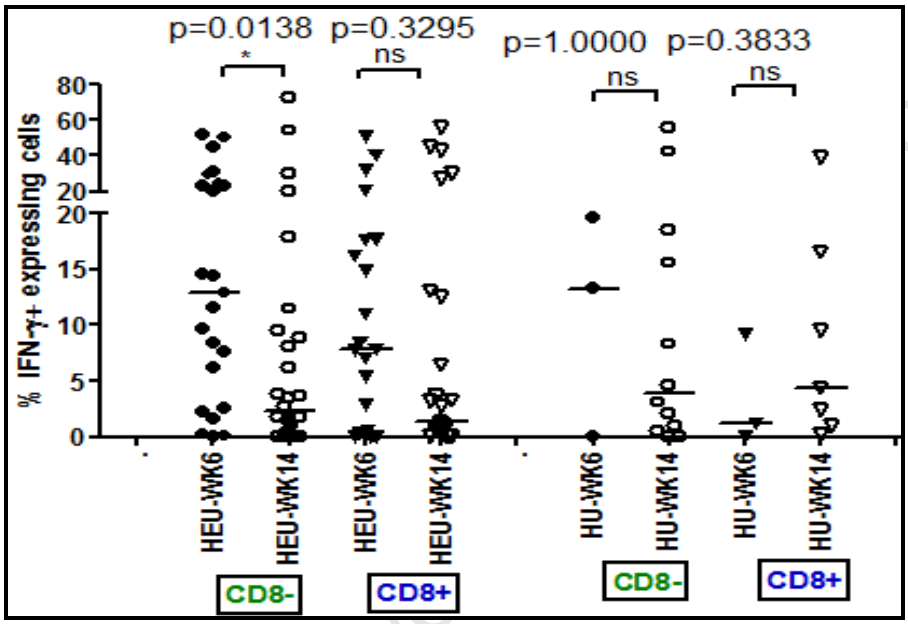


Figure B4: The frequency of T cells producing IFN-γ after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of exclusively breastfed (EBF) HU and HEU infants for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+

(CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

(v) MF HU infants show better IFN-γ expression by CD4+ T cells at 6 compared to 14 weeks of life and by CD8+ T cells in response to SEB, but better IFN-γ expression by CD8+ T cells at 14 compared to 6 weeks of life in response to BCG.

A. BCG Stimulation (IFN-γ)



B. SEB Stimulation (IFN-γ)

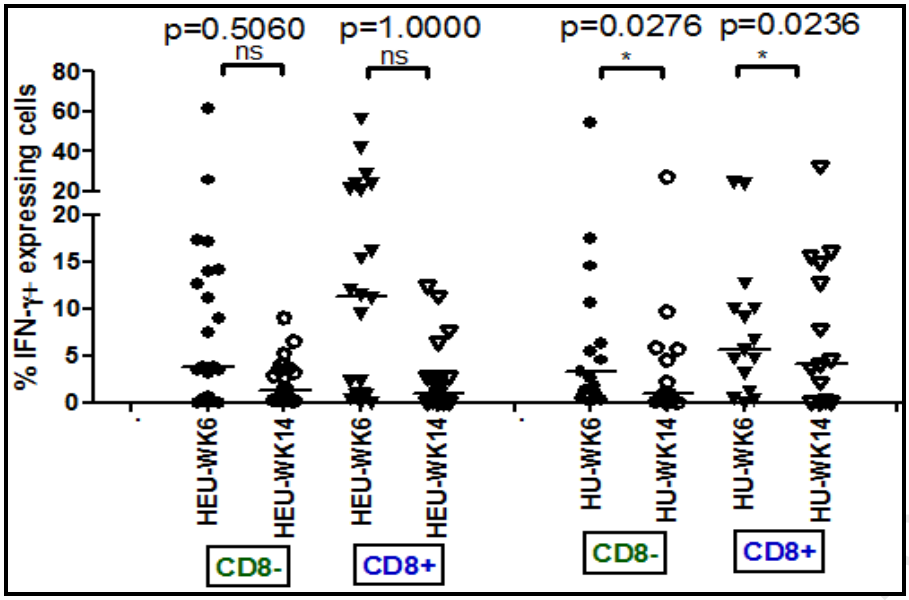


Figure B5: The frequency of T cells producing IFN-γ after (A) Bacillus Calmette-Guérin [BCG] and (B) Staphylococcal enterotoxin B [SEB] stimulation of mixed fed (MF) HU and HEU infants for 6 days in a whole blood assay at 6 and 14 weeks of life measured by flow cytometry. Frequency of CD4+ (CD8-) (filled ovals and triangles on the left side of each graph) and CD8+ (open ovals and triangles on the right side of each graph) proliferating T cells determined by flow cytometry in BCG-stimulated whole blood of HIV-exposed uninfected infants (HEU: ovals) presented on the left of each side of the plot compared to HIV-unexposed infants (HU: triangles) presented on the right of each side of the plot at 6 and at 14 weeks of life. Bars indicate medians; $p<0.05$ significant level and statistical significance was obtained using *Mann-Whitney U* test.

Table B1 below presents a summary of the data presented on the graphs above.

Table B1: Ignoring p-values considering 2-fold differences (i.e. log order) to reflect a meaningful difference in T cell IFN-γ secreting cells.

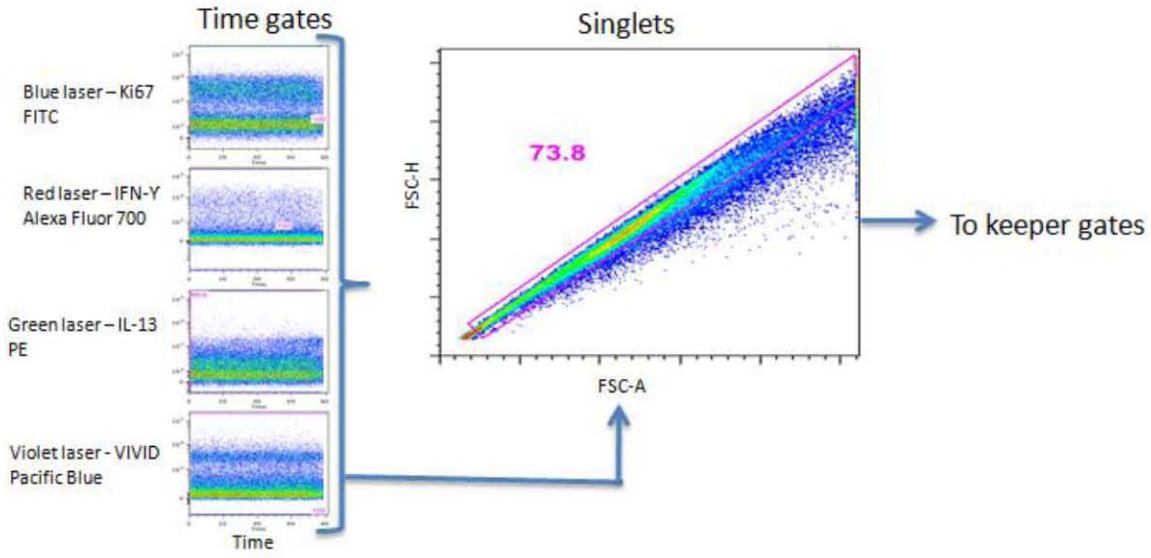
Aspect	Parameter	Condition	CD4+	CD8+
All EBF (HEU vs. HU)	**Week 6**	**A. BCG** **B. SEB**	**HU > HEU**	**HU > HEU**
All MF (HEU vs. HU)	**Week 6**	**A. BCG** **B. BP** **C. SEB**	**HEU > HU**	**HEU > HU** **HEU > HU**
	Week 14	**A. BP** **B. SEB**		**HEU > HU** **HU > HEU**
HEU (EBF vs. MF)	**Week 6**	**A. BCG** **B. BP** **C. SEB**	**MF > EBF**	**MF > EBF** **MF > EBF** **MF > EBF**
HU (EBF vs. MF)		**A. BCG**	**EBF > MF**	**EBF > MF**
HEU (EBF vs. MF)	**Week 14**	**A. SEB**		**EBF > MF**
All EBF (6 vs. 14 Weeks)	**HEU**	**A. BCG**	**6 > 14 Weeks**	
	HU	**A. BCG**	**6 > 14 Weeks**	
All MF (6 vs. 14 Weeks)	**HEU**	**A. BCG** **B. SEB**	**6 > 14 Weeks** **6 > 14 Weeks**	**6 > 14 Weeks** **6 > 14 Weeks**
	HU	**A. BCG** **B. SEB**	**6 > 14 Weeks** **6 > 14 Weeks**	**14 > 6 Weeks** **6 > 14 Weeks**

Red highlights indicate week 6
Blue highlights indicate week 14
Green highlights indicate evolution of CD4 and CD8 responses from 6 to 14 weeks of life.
HEU=HIV exposed uninfected; **HU**= HIV unexposed

- Comparing all Exclusively Breastfed **(EBF) only**; HEU vs. HU
- Comparing all Mixed fed **(MF) only**; HEU vs. HU
- Comparing **EBF vs. MF considering HEU & HU Infants separately**
- Within **EBF only** comparing 6 vs. 14 Weeks
- Within **MF only**; comparing 6 vs. 14 weeks

Appendix C: Flow cytometric analysis of CD8- (CD4+) and CD8+ T cells proliferation measured by Ki67 incorporation in a whole blood intracellular staining assay.

A.



B.

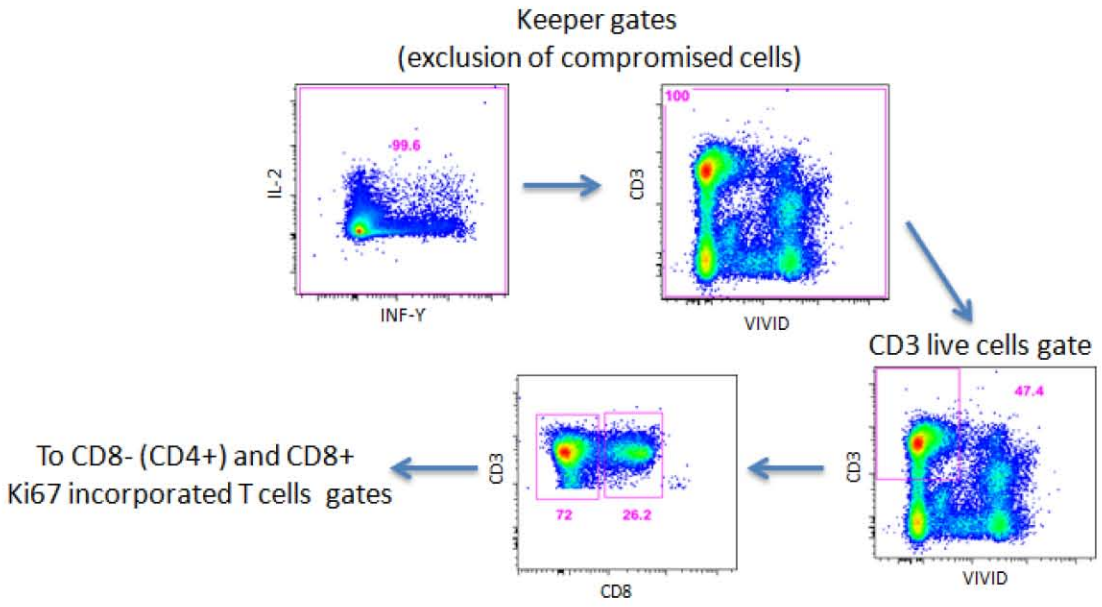


Figure C1. Flow cytometric analysis of CD8- (CD4+) and CD8+ T cells proliferation measured by Ki67 incorporation and cytokine in a whole blood intracellular staining assay. Gating strategy from a representative HEU infant whole blood stimulated with SEB for 6 days. A. From left to right, time gates for including consistent fluorescence over acquisition time and exclusion of doublets using forward scatter-area and forward scatter-height

parameters in a singlets gate. B. From left following arrow, exclusion of compromised cells using keeper gates, 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.

Appendix D: The number of HEU and HU infants whose CD4+ and CD8+ T cell responses to the different antigens was performed.

Out of the 46 participants analyzed per group (i.e. HEU versus HU infants), 3 HEU versus 1 HU at six weeks and 6 HEU versus 4 HU at fourteen weeks infant samples were rejected by the rule (section 3.2.1.1 sub 1) (i.e. back ground responses were greater than those of the positive control) and recorded as negative. The remaining positive samples spread out within the different antigens studied (Table D1) were further analyzed as in section 3.2.1.1 above.

Table D1: The number of HEU and HU infants whose CD4+ and CD8+ T cell responses to the different antigens was performed. Parameters include the different vaccine antigens *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), *Bordetella pertussis* (BP) and tetanus toxoid (TT), and a superantigen Staphylococcal enterotoxin B (SEB), the time points 6 and 14 weeks and CD4+ and CD8+ T cells types for which responses (proliferation and cytokine production) were measured in HEU versus HU infants with their respective p-values. Adjustment for multiple comparisons was done using the Holm step-down approach(Columb *et al.*,, 2006) (see table 2.2). Red highlights; represent p-values (and adjusted p-values) that remain significant after adjustment for multiple comparisons.

Antigen	Time point	T Cell type	HEU	HU
BCG	**6 Weeks**	**CD4+**	22	31
		CD8+	22	31
	14 Weeks	**CD4+**	15	39
		CD8+	15	39
BP	**14 Weeks**	**CD4+**	23	30
		CD8+	23	33
TT	**14 Weeks**	**CD4+**	4	34
		CD8+	3	33
SEB	**6 Weeks**	**CD4+**	25	38
		CD8+	25	38
	14 Weeks	**CD4+**	25	44
		CD8+	25	44
BCG	**6 Weeks**	**CD4+**	30	33
		CD8+	27	24
	14 Weeks	**CD4+**	33	29
		CD8+	34	25
BP	**14 Weeks**	**CD4+**	4	7
		CD8+	4	13
TT	**14 Weeks**	**CD4+**	3	7
		CD8+	3	11
SEB	**6 Weeks**	**CD4+**	27	33
		CD8+	32	32
	14 Weeks	**CD4+**	27	33
		CD8+	28	32
BCG	**6 Weeks**	**CD4+**	27	32
		CD8+	29	29
	14 Weeks	**CD4+**	35	28
		CD8+	37	25
BP	**14 Weeks**	**CD4+**	3	9
		CD8+	3	14
TT	**14 Weeks**	**CD4+**	3	7

		CD8+	3	6
SEB	**6 Weeks**	**CD4+**	25	30
		CD8+	26	29
	14 Weeks	**CD4+**	26	34
		CD8+	28	34
BCG	**6 Weeks**	**CD4+**	28	34
		CD8+	29	32
	14 Weeks	**CD4+**	35	29
		CD8+	37	26
BP	**14 Weeks**	**CD4+**	3	8
		CD8+	3	11
TT	**14 Weeks**	**CD4+**	3	5
		CD8+	3	7
SEB	**6 Weeks**	**CD4+**	25	32
		CD8+	29	32
	14 Weeks	**CD4+**	26	33
		CD8+	27	34
BCG	**6 Weeks**	**CD4+**	22	26
		CD8+	23	25
	14 Weeks	**CD4+**	32	27
		CD8+	34	24
BP	**14 Weeks**	**CD4+**	3	5
		CD8+	3	10
TT	**14 Weeks**	**CD4+**	3	6
		CD8+	3	11
SEB	**6 Weeks**	**CD4+**	27	28
		CD8+	28	30
	14 Weeks	**CD4+**	26	31
		CD8+	27	30

Appendix E: The median background response to evaluate the level of non-specific stimulation in the assay.

We had to determine the validity of our assay and ensure background responses were not high. The results showed that the whole blood assay (WBA) was valid for the measurement of immune responses to other antigens, and that there was little background.

SEB- (positive control) and BCG- (vaccine with known immunogenicity) induced T cell proliferation was valid as observed in the significantly higher CD4+ [Both SEB and BCG: 6 and 14 weeks ($p<0.0001$)] (Figure E1 and E2) and CD8+ [Both SEB and BCG: 6 and 14 weeks ($p<0.0001$)] (Figure E1 and E2) compared with the low back ground media alone T-cell responses after antigenic stimulation at 6 and 14 weeks of age.

A. SEB causes T-cell proliferation

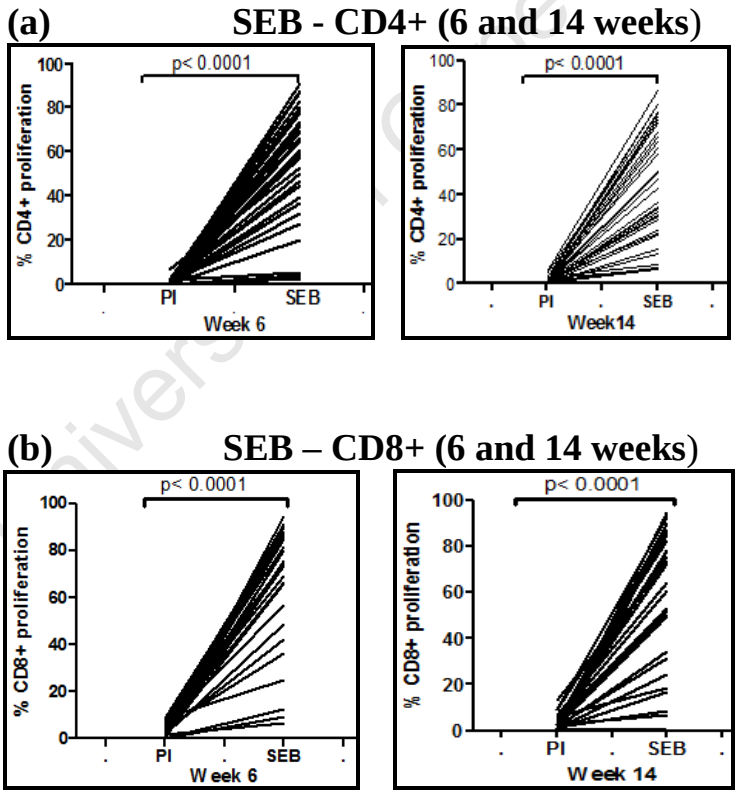


Figure E1: Magnitude of SEB induced CD4+/CD8+ T cells proliferation at 6 and 14 weeks. Infants express a significantly higher SEB-induced **(a)** CD4+ and **(b)** CD8+ proliferation at 6 weeks and 14 weeks of life; $p<0.05$ significant level.

B. BCG causes T-cell proliferation

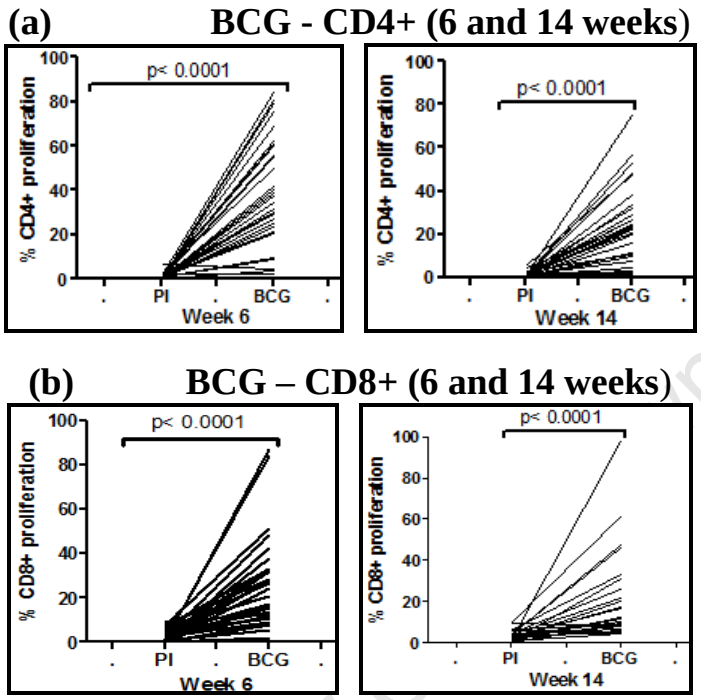


Figure E2: Magnitude of BCG induced CD4+/CD8+ T cells proliferation at 6 and 14 weeks. Infants have a significantly higher BCG-induced **(a)** CD4+ and **(b)** CD8+ proliferation at 6 and 14 weeks of life; $p<0.05$ significant level.

Appendix F: Co-linearity of birth weight and weight at 6 weeks.

From the Kruskal Wallis test below, it is clear that weight at birth and at 6 weeks are not collinear and are significantly different (p=0.0001).

```
. kwallis  birthweight, by(week6weight)

Kruskal-Wallis equality-of-populations rank test

+---------------------------+
week6w~t  Obs  Rank Sum
----------+-----+----------
3230    9   3073.50
3500    4   1822.00
3650    4    300.00
3670    4     58.00
3730    5     15.00
----------+-----+----------
3810    2     18.00
3860    2    167.00
3870    2    330.00
3880    7    875.00
3910    7   1029.00
----------+-----+----------
3940    5   1570.00
3960    8    876.00
4010    9    961.00
4020    5    497.50
4060    4    500.00
----------+-----+----------
4120   11   1548.50
4130    4    106.00
4150    7    696.50
4190    9    238.50
4210    5    375.00
----------+-----+----------
4230    7    385.00
4240    9   4563.00
4250    4   2214.00
4270    2    458.00
4290    4    966.00
----------+-----+----------
4320   11   1610.00
4340    8   2904.00
4360    5   3015.00
4390    8   1576.00
4400   10   2079.00
----------+-----+----------
4420    6   1374.00
4450    8   3876.00
4460    9   2087.00
4480   13   4423.00
4490    8    440.00
----------+-----+----------
```

4510	2	110.00
4520	8	1576.00
4530	12	3404.00
4550	6	1884.00
4560	8	3644.00
4580	6	2601.00
4600	19	7364.00
4630	11	1435.00
4640	15	6010.00
4650	7	1029.00
4660	7	1029.00
4670	9	5427.50
4680	11	1609.50
4700	18	8153.00
4720	3	1213.50
4730	5	2022.50
4740	2	53.00
4750	5	2867.50
4800	10	5229.00
4810	15	8655.50
4820	12	6990.00
4830	5	3142.50
4850	5	1707.50
4860	26	10342.50
4870	9	2304.00
4880	7	1921.50
4890	5	2022.50
4900	5	2990.00
4910	15	8130.00
4930	16	5273.00
4960	18	10231.00
5000	2	628.00
5010	24	11839.00
5020	2	1014.00
5030	13	7802.50
5050	6	3042.00
5060	4	660.00
5080	2	585.00
5100	16	2988.00
5130	13	8320.00
5170	5	3515.00
5200	7	5187.00
5210	5	2867.50
5230	10	6910.00
5250	4	2374.00
5270	20	7212.50
5280	6	3891.00
5290	8	4588.00
5300	6	1755.00
5360	2	1280.00
5460	4	2456.00

```
5470    5   3200.00
5500   25  17349.50
5550    7   4585.00
5560    8   6388.00
----------+-----+----------
5590    8   3236.00
5600    4   1366.00
5610    5   1570.00
5620    8   5384.00
5630    6   4335.00
----------+-----+----------
5650    6   3276.00
5670    4   2890.00
5680    5   3365.00
5770    8   5384.00
6200    9   7038.00
----------+-----+----------
6760    8   6324.00
+---------------------------+

chi-squared =   669.260 with 100 d.f.
probability =     0.0001

chi-squared with ties =   669.886 with 100 d.f.
probability =     0.0001
```

but that they are correlated:

```
spearman week6weight birthweight
 Number of obs =     802
Spearman's rho =       0.6546
Test of Ho: week6weight and birthweight are independent
    Prob > |t| =       0.0000
```

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