T cell responses in Kenyan infants: impact on HIV-1 evolution during infection and an assessment of vaccine-induced memory responses in HIV-exposed uninfected infants

Thesis submitted for the degree of Doctor of Philosophy

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Trinity Term 2013

ABSTRACT

The past 10 years has seen mother to child transmission (MTCT) of HIV-1 shift from being one of the predominant forces in the global epidemic to a phenomenon that is largely preventable and envisioned as being on the path to elimination. This thesis is based on two cohorts of Kenyan infants recruited before and after the development of effective antiretroviral interventions to prevent MTCT. Two main lines of enquiry are pursued with the aim to contribute to improved health outcomes of infants affected by HIV-1. The first seeks to further our understanding of the capacity of the infant cytotoxic T lymphocyte (CTL) response to influence viral evolutionary dynamics in early infection. Chapter 3 presents a modern phylogenetic analysis of longitudinal viral sequences derived from infants following *in utero* or *peripartum* infection. The results indicate that despite high levels of viral replication, infant CTL selection pressure plays a significant role in shaping early viral evolution. The second stems from an accumulating body of evidence that suggests that infants born to HIV-1 infected mothers who themselves are free from infection, termed HIV-1 exposed uninfected (HEU) infants, nevertheless face significantly higher rates of infectious disease-associated morbidity and mortality than HIV-1 unexposed infants. This study therefore sought to characterise the immunological status of HEU infants with particular emphasis on the phenotypic and functional properties of the T cell compartment. Chapter 4 presents the immunological characterisation of a cohort of healthy Kenyan infants recruited as a control population at two time points in early life. Chapter 5 present a cross-sectional comparison of HEU and control infant cohorts. The results suggest a level of altered immunological reactivity with respect to the T helper type 1 (Th1) response to polyclonal stimulation. In addition a compromised memory Th1 response was observed following polyclonal stimulation and following stimulation with Bacillus Calmette-Guerin and tetanus toxoid vaccine antigens.

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ABBREVIATIONS

Å	Angstrom
AIDS	Acquired Immunodeficiency Syndrome
ANC	Antenatal Care
APC	Antigen Presenting Cell
ART	Antiretroviral Therapy
ARV	Antiretroviral
AZT	Azidothymidine
BCG	Bacillus Calmette-Guérin
Bcl	B Cell Lymphoma
CA	Capsid
CD	Cluster of Differentiation
cDNA	Complementary DNA
CMV	Cytomegalovirus
CRF	Circulating Recombinant Form
CTL	Cytotoxic T Lymphocyte
CTM	Cotrimoxazole
DC	Dendritic Cell
DC-SIGN	DC-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin
DNA	Deoxyribonucleic Acid
DPT	Diphtheria-Pertussis-Tetanus
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPI	Expanded Programme on Immunisation
FOXP3	Forkhead Box P3
Gp	Glycoprotein
HBV	Hepatitis B Virus
HEU	HIV-Exposed Uninfected
HIV-1	Human Immunodeficiency Virus-1
HLA	Human Leucocyte Antigen
HSC	Haematopoietic Stem Cell
HSV-2	Herpes Simplex Virus-2
ICS	Intracellular Cytokine Staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IN	Integrase
IRF	Interferon Regulatory Factor
KEMRI	Kenyan Medical Research Institute

LANL	Los Alamos National Laboratory
LANLDB	LANL Database
LPS	Lipopolysaccharide
LTR	Long-Terminal Repeat
MA	Matrix
mDC	Myeloid DC
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
ML	Maximum Likelihood
mRNA	Messenger RNA
MTCT	Mother-To-Child Transmission
Myd88	Myeloid Differentiation Primary Response Gene 88
NC	Nucelocapsid
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NOD	Nucleotide-Binding Oligomerization Domain
NRTI	Nucleoside Reverse Transcription Inhibotor
OPV	Oral Polio Vaccine
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
pDC	Plasmacytoid DC
PIC	Pre-Integration Complex
PMTCT	Prevention of MTCT
PolII	RNA Polymerase II
PPD	Purified Protein Derivative
PR	Protease
PRR	Pattern Recognition Receptor
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RORγt	RAR-Related Orphan Receptor Gamma Thymus
RRE	Rev Response Element
RT	Reverse Transcriptase
SEB	Staphylococcus Enterotoxin B
SIV	Simian Immunodeficiency Virus
STAT5	Signal Transducer and Activator Of Transcription *5*
STI	Sexually Transmitted Infection
TAR	Transactivation Response Element
TB	Tuberculosis
Tcm	Central Memory T Cell
TCR	T Cell Receptor
Tem	Effector Memory T Cell

Temra	Effector Memory T Cell re-Expressing CD45RA
TGF	Transforming Growth Factor
Th	Helper T Cell
Tim	T Cell Immunoglobulin Mucin
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
Treg	Regulatory T Cell
TT	Tetanus Toxoid
URF	Unique Recombinant Form
VL	Viral Load
WHO	World Health Organisation

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1   CHAPTER 1: INTRODUCTION

1.1   SECTION 1: INFANT IMMUNOLOGY

The immune system carries out a set of core functions that protect an individual from disease. These include differentiation of self from non-self, pathogen elimination, immune-regulation, and the generation of long-lasting immunological memory. The capacity to carry out these functions differs between infants and adults and is reflected in the heightened susceptibility to infection in early life: close to 4 million deaths occur globally each year in infants <6 months of age due to infectious diseases that are rarely fatal in children and adults (1). The relative immaturity of the developing immune system plays an important role in this vulnerability to infection. In addition, in early life there is a distinct requirement to maintain immunological tolerance and this influences the quality of inflammatory response in infancy and childhood. For instance, *in utero* maternal-foetal rejection is prevented by the inhibition of pro-inflammatory immune responses in both the mother and the foetus. Furthermore, during birth, the neonate transitions from a normally sterile environment to one with high levels of antigen exposure where survival is dependent on the prevention of harmful pro-inflammatory responses to skin and gut colonising microorganisms. In this context, the immune system in early life has evolved to afford certain level of active immune protection which is bolstered by passive immunity, and in which pro-inflammatory responses are carefully regulated. Deviations in the timing of developmental transitions (**fig. 1.1**), such as in preterm births, can further increase the risk of infection.

This section will discuss some of the distinctive features of infant immune responses and the role of vaccination in enhancing protective immunity in early life. Due to
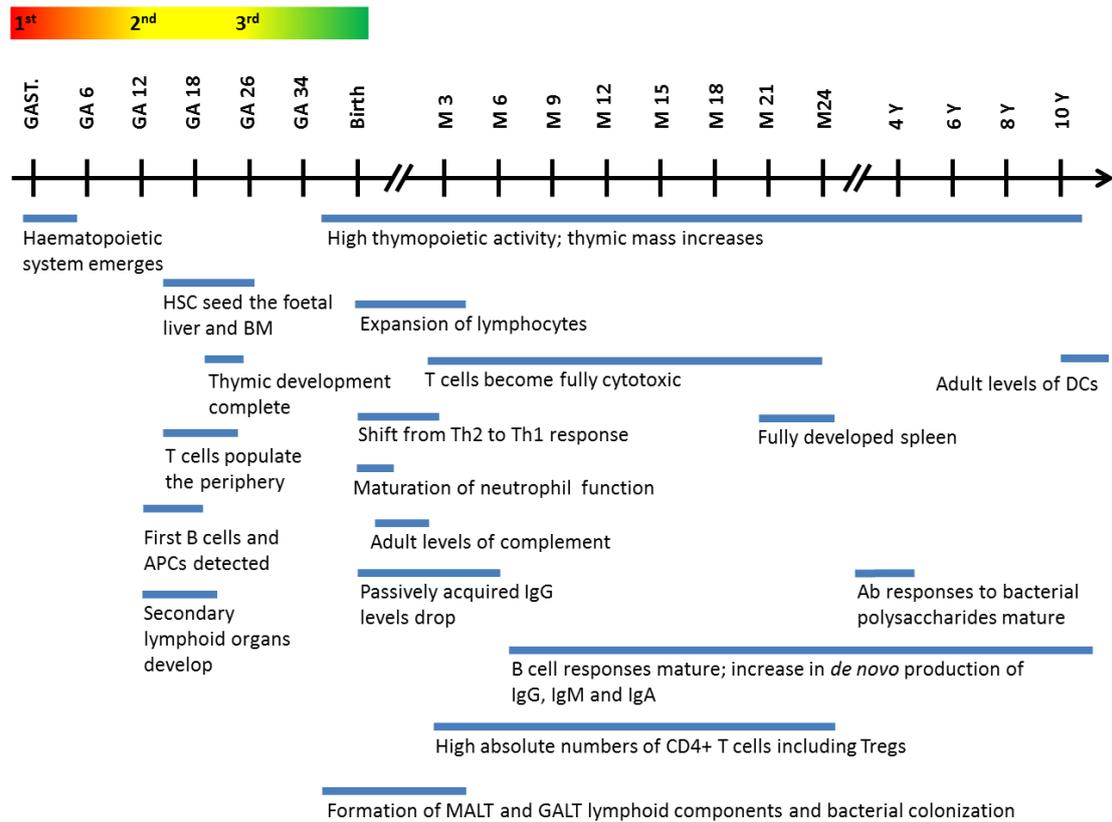
Figure 1.1 Key transitional stages in immune development before and after birth.
Pregnancy trimester periods are indicated in red, yellow and green. GAST.= gastrulation; GA= gestational age; M= months; Y= years; Ab= antibody

ethical considerations and the large volumes of blood needed to study many immunological processes, a lot of what is known about early life immunity comes from mouse models or from human cord blood cells. This discussion will focus on what is known from human studies and where possible indicate changes that occur in the post neo-natal period.

1.1.1 Innate immunity

Following a breach of mucosal and epithelial barriers, the innate immune system is the first line of defence against infection. It consists of soluble and cellular components

that conventionally are thought to lack the ability to adapt to antigenic challenge and therefore act through broad specificities that do not change upon antigen reencounter. These components identify potential infectious agents through recognition of pathogen-associated molecular patterns (PAMPs), conserved molecular motifs not present on healthy mammalian cells. PAMPs interact with host pattern recognition receptors (PRR) which can be either associated to the cell surface or bound to intracellular organelles and include toll-like receptors (TLRs), NOD-like receptors, C-type lectins and RIG-receptors. The soluble components of the innate immune response include cytokines and complement. Cytokines are signalling molecules with diverse immune-modulatory properties that bridge and form part of innate and adaptive immunity. The complement system is composed of approximately 30 soluble proteins that function in concert with cellular and other soluble components of the immune system (antibodies and lectins) to promote pathogen clearance through direct microbial killing and phagocytosis. The classical cellular components of innate immunity include monocytes/macrophages and granulocytes that are able to ingest and destroy extracellular microbes through phagocytosis, and dendritic cells, professional antigen presenting cells that elicit adaptive immune responses and influence the generation of immunological memory. Natural killer cells and invariant natural killer T cells are additional cellular subsets that share properties of innate and adaptive immunity and are able to mediate host cell lysis during infection or stress.

TLRs and cytokine responses

There are 10 human TLRs that have been identified. TLRs 2, 4 and 5 are expressed on the cell surface and recognise peptidoglycan, LPS and flagellin, respectively. TLRs 3, 7, 8 and 9 are expressed on endosomes and bind nucleic acids. Antigen-recognition by

TLRs triggers intracellular signalling cascades which are mediated by adaptor molecules such as MyD88. Depending on the TLR engaged, transcription factor complexes such as NF-kB or IRFs are activated, leading to an inflammatory response. Analysis of human cord monocytes has indicated that expression levels of all TLRs are equivalent to those found in adults (2). However, functionally, certain neonatal TLRs have been to found have reduced potential to induce Th1 polarizing and pro-inflammatory cytokines such as TNF-α (2), IFN-γ (3) and IL-12p70 (4). By contrast expression of cytokines with Th2-polarizing and anti-inflammatory properties such as IL-8 (5), IL-6 (6) and IL-10 (7) are maintained or enhanced. Mechanistically, reduced MyD88 expression in cord blood monocytes have been implicated in blunted TNF expression following TLR4 stimulation (8). In addition, high levels of inhibitory adenosine found in neonatal plasma have also been shown to inhibit TLR2-mediated production of TNF in a cAMP-dependent manner (9). MicroRNA-mediated down regulation of components of TLR4 signalling components has also been reported in infant monocytes (10). TLR-8 stands out as being able to activate pro-inflammatory responses in neonates: following ligand engagement neonatal cord monocytes and mDCs were able to trigger adult-levels of TNF (11). TLR-8 has therefore been proposed as an adjuvant target in vaccination strategies aimed at inducing Th1 responses. How do infant innate cytokine responses mature? There seems to be a non-linear maturation of cytokine responsiveness in the neonate. IL-10 production, for instance, in response to TLR-2 stimulation is found at high levels around birth, decreases at 2 years of age and an increase again in adulthood (12). The capacity of monocytes to produce TNF and IL-6 resembles that in adults by 3 years of age; IL-12 and IFN-γ responses however can remain low into the teenage years.

Granulocytes

In adults the majority of granular leucocytes in circulation are neutrophils. Neutrophil numbers increase very rapidly late in gestation to become the dominant leucocyte in circulation at birth (13). Neutrophils mediate their effector functions through phagocytosis, release of antimicrobial enzymes and peptides (degranulation) and the production of reactive oxygen species. Several of these features have been shown to be compromised in early life particularly in preterm infants. As a result, in the neonate, sepsis due to neutrophil dysfunction syndrome poses a high risk (14). Reduced cell surface adhesion molecule expression on neutrophils has been shown, which results in impaired chemotaxis and ability to extravasate into the sites of infection (15). Phagocytic capacity has also been shown to be reduced in neonatal neutrophils on account of reduced levels of complement receptor 3 expression, particularly in pre-term infants (16). In addition, a novel effector function, mediated by the formation of neutrophil extracellular traps, a scaffold for antimicrobial peptides, has been reported to be compromised (17). Despite these potentially severe defects, neutrophil function matures rapidly following birth.

Antigen presenting cells

DCs, monocytes/macrophages and B cells are professional antigen presenting cells (APC) that have evolved specialised functions to present cognate antigen to circulating T cells. Dendritic cells are distinguished among APCs in their ability to prime naïve T cells and will be the focus of this section, though many features of DC functionality in infancy apply to other APCs.

Phenotypically, DCs can be identified by a lack of the expression of the lineage markers (present on lymphocytes, monocytes and granulocytes) and the expression of

MHC class II. Two subsets, myeloid DC (mDC) and plasmacytoid DC (pDC) have been described. mDC can be distinguished by CD11c expression and play an important role in inducing IL-12-mediated Th1 T cell differentiation. pDCs can be distinguished through CD123 (IL-3α) expression and have been recognised as the main source of type 1 interferons, key antiviral cytokines.

In order to induce activation, APCs physically interact with T cells and provide 2 signals: signal 1, resulting from the interaction between the TCR and the cognate peptide MHC complex; and signal 2, resulting from antigen independent interaction between co-stimulatory molecules. Co-stimulation can involve interactions between *stimulatory* molecules on T cells (e.g. CD28) and APCs (e.g. CD86 and CD80) and *inhibitory* molecules on T cells (e.g. CTLA-4 and PD-1) and APCs (e.g. PD-L1 and PD-L2). The balance of these signals determines the outcome of T cell activation. Cytokines can further shape the outcomes of these interactions and influence, for instance, the development of different CD4+ T helper cell subtypes.

Fluctuations in DC frequencies in early life have been analysed in some detail. Bulk cord blood DCs have been shown to have lower frequencies compared to adults and reduced (18) or absent CD11c (19) expression levels, resulting in increased pDC/mDC ratios (20). Longitudinal analysis of circulating DC has shown significant drops in pDC numbers over the first years of life, eventually stabilising in children, and the establishment of a stable mDC population by the first year of life (21,22). However at 12 months, the number of circulating mDC and pDCs numbers have been shown to be reduced in comparison to adults(23) . Monocyte frequencies in cord blood have been reported to be similar (24) or increased (25) in comparison to those in adult circulation.

Ex vivo neonatal APCs have been shown to express low basal levels of MHC class II (26) and of co-stimulatory molecules CD40, CD80 and CD86 (18,19). In addition, up-regulation of co-stimulatory molecules following CpG stimulation of pDCs (27) and LPS stimulation of mDCs and has been shown to be blunted in comparison to adults- a reflection of the impaired TLR-mediated signalling discussed above.

Functionally, neonatal DCs have specific impairments. mDC produce lower levels of the Th1 polarising cytokine IL-12 and other pro-inflammatory cytokine such as TNF-α and IL-1β (28). Neonatal pDC also exhibit marked reduction in the production of their signature cytokine IFN-α (27). These features play a central role in early life polarisation of T helper cell responses that highlight the qualitative and quantitative differences between infant and adult DC that influence adaptive immune responses.

1.1.2 Adaptive immunity

The adaptive arm of the immune system is defined by its high degree of specificity in antigenic recognition and in the ability to induce enhanced immunological responses upon secondary encounter with antigen. Adaptive immunity is mainly composed of lymphocytes and the soluble products they produce such as cytokines and antibodies. Following priming of naïve lymphocytes, effector populations of antigen-specific cells undergo clonal expansion. Upon pathogen clearance, the clonal populations of effector lymphocytes contract leaving a pool of long-lived memory cell which can persist at low levels in circulation. Memory cells are the mediators for enhanced secondary responses and their induction is key to vaccine-induced immunological protection. Two main types of lymphocytes exist, B cells which mediate the humoral response, and T cells which mediate cellular effector responses and will be the main focus of this section.

Humoral Immunity

B cell are present in the developing foetus and in the neonate, though their functional capacity is greatly reduced: antibody production is mainly restricted to IgM (29). To compensate for the low level of protection afforded by the infant humoral immune response, the immunological memory imprinted on maternal antibodies is transferred to the infant. Immunological protection from maternally acquired antibodies can occur either through *in utero* transfer of IgG across the placenta, or through ingestion of IgA in breast milk that protects the intestinal mucosa and acts in the intestinal lumen. Infant humoral responses functionally develop throughout infancy and by 9 months of age maternal IgG of maternal origin is replaced by infant IgG; IgA levels remain at 30% of adult levels at 12 months of age (30).

T lymphocytes

T cells derive their name from their primary site of development and maturation, the thymus. During foetal development, the thymus is populated by developing lymphoid cells that generate CD2 bearing pro-thymocytes- T cell precursors derived from haematopoietic stem cells (HSC) that originate in the foetal liver and bone marrow. Pro-thymocytes are committed to the T cell lineage, a process which is regulated by Notch-1 signalling and the transcription factor GATA3. These cells undergo extensive proliferation in the thymus, mediated through interaction with thymic stromal cells and IL-7, and a large population of immature precursor cells is generated. A key event in T cell maturation is the rearrangement of antigen receptor genes that give rise to the diversity in the T cell receptor (TCR). A complex sequence of DNA recombination events results in the formation of αβ and γδ T cells with an extensive repertoire of antigen specificity through an analogous mechanism that mediates the generation of

diversity in immunoglobulin (Ig) molecules in B cells. In brief, a number of mechanisms contribute to TCR diversity which include a) random rearrangements of the V(D)J genetic segments b) use of distinct VD and J genetic segments in a particular TCR c) the pairing of distinct α and β chains of the TCR and d) the process of removal or addition of nucelotides at the junctions between TCR coding segments, a process mediated by the enzyme terminal deoxyribonuceotidyl transferase. During the recombination of TCR genes, DNA molecules are excised from the genome, and detection of these fragments, or T cell-excision circles (TRECs), has been used as a marker for quantifying the rate of *de novo* T cell production from the thymus. TCR gene recombination is a major check point in T cell development and cells that generate out of frame genetic rearrangements are deleted.

Subsequent to the successful rearrangement of TCR genes and TCR expression, T cell precursors, which express both CD4 and CD8 molecules on their cell surface (double positive cells), undergo the processes of positive and negative selection. Positive selection ensures selection of T cells that recognise self-MHC molecules, and is mediated by the generation of survival signals following TCR engagement; negative selection results in the deletion, by apoptosis, of T cells with high avidity for self MHC, thus eliminating potentially auto reactive cells. T cells selected on the basis of appropriate MHC II binding retain CD4 expression and switch off CD8 expression, and T cells selected on the basis of MHC I conversely retain CD8 expression and switch off CD4 expression. Mature CD4 and CD8 T cells are detected in foetal liver and spleen by 14 weeks of gestation and by the third trimester of pregnancy the TCR repertoire is similar to that seen in adults (31).

CD8 T cells

The two subsets of αβ T cells, CD4 and CD8 T cells carry out distinct immunological functions. The TCR ligand of CD8 cells is the MHC-I molecule which is expressed on the vast majority of nucleated cells in the body. MHC-I molecules can present peptides derived from viral proteins or from proteins induced through a dis-regulated cell cycle. Upon cognate antigen recognition, mature CD8+ cytotoxic T lymphocytes (CTLs) are able to induce cytotoxicity through the production of perforin and granzymes, and to induce apoptosis through Fas-mediated interactions. In addition, CD8 T cell are able to produce antiviral cytokines such as IFN-γ, other pro-inflammatory cytokines such as TNF-α and cytokines that promote their proliferation such as IL-2.

Early studies in mice indicated that, following allogeneic stimulation, neonatal CTL responses were defective (32). However, it has been shown in the mouse model that specific manipulations, particularly in respect to the induction of T helper type 1 promoting conditions such as IL-12 (33) or through use of DNA vaccines (34), that adult like CTL responses can be induced.

There is a surprising lack of human studies focusing on CD8 T cell responses in neonates and infants. An analysis of congenital *Trypanosoma cruzi* infection showed an expansion of activated cord blood CD8 T cells and strong cord blood IFN-γ responses following incubation with the *T. cruzi* parasite, though this was only apparent in cultures stimulated with IL-15, a cytokine which is involved in homeostatic CD8 T cell maintenance (35). In addition CMV-specific neonatal CD8 T cell responses were analysed following congenital infection revealing a phenotypic profile similar to that found in adult infection, with robust perforin and antiviral cytokine expression (36). By contrast analysis of infant CTL responses in the context of HIV infection has indicated

that these may be significantly less functional than those in adults. This is further discussed in section III. However, it is apparent that significant plasticity exists in the functional capacity of infants CTL responses, where under certain conditions mature adult–like responses can be induced.

CD4 T cells

Despite the potential for cytotoxicity, CD4 T cells are principally known as helper T (Th) cells. In mediating their functions CD4 T cells interact and stimulate additional cellular subsets including CD8 T cells, DCs and B cells thus acting as key intermediaries in the immune response. CD4 T cells are primed following interaction with professional APC which present antigens in the context of MHC class II molecules. Naive CD4 T cells have the capacity to differentiate into a number of helper T cell subsets with distinct functional roles. Classically, helper T cells were divided in to Th1 and Th2 subsets. Th1 are distinguished by the ability to express IFN-γ and by their capacity to induce antimicrobial inflammatory responses and cellular immunity. In addition Th1 cells signal through the STAT1 intermediary and express the transcription factor Tbet. Th2 cells are distinguished by their ability to express IL-4, IL-15 and IL-13, among other cytokines, their ability to support antibody production and their involvement in allergy. Th2 cells signal through STAT6 and express the transcription factor GATA3. In recent years, additional helper T cell subsets have been identified, including Th17 cells which secrete IL-17 and IL-22, among other cytokines, and expresses the transcription factor RORγt and regulatory T cells (Treg) which express the immunosuppressive cytokine IL-10 and the transcription factor FOXP3.

Early investigations in neonatal mice and humans reported a lack of responsiveness to allogeneic or microbial antigens that were otherwise immunogenic in adults(32). These

findings led to the perception that neonatal CD4 T cells were functionally deficient. With the identification of Th1 and Th2 subsets of CD4+ T helper cells, it was understood that the lack of response was primarily a lack of pro-inflammatory Th1 type responses, and that, particularly in mice, neonatal responses were biased towards Th2 type responses. However, it has been shown that under specific circumstances, for instance following vaccination with BCG (37), human neonates are able to induce Th1 responses that resemble those generated in adults. The nature of the immune response to BCG is discussed below, though this underscores the notion that similarly to the CD8 T cell compartment, naïve neonatal CD4 T cells have broad developmental potentials that are shaped during priming and are influenced by factors such as antigen dose, type of stimulus, quality of the APC priming and critically, immune regulation.

Immune regulation and regulatory T cells

During thymic development, the deletion of self-reactive T cells serves to limit autoimmune pathology. In addition, further mechanisms have evolved that regulate potentially harmful inflammatory T cell responses and inflammatory responses from other immune compartments. In the context of early life, these mechanisms are of particular importance and bear a strong influence on the outcomes following infection. Key mediators in immune regulation are Treg cells. A number of Treg subsets exist, though by and large, Tregs can be divided into naturally occurring Tregs (nTreg) and inducible Tregs (iTreg). nTregs develop from thymic precursors in the presence of TGF-β and IL-2, and express the α chain of the IL-2 receptor (CD25). Peripheral CD4 T cell can also develop regulatory phenotype in the presence of TGF-β through STAT5 signalling. This results in the expression of the Treg defining transcription factor FOXP3. Tregs mediate their function through secretion of immunosuppressive

cytokines such as IL-10, IL-35 and TGF-β. In addition, Tregs have been shown to mediate their functions through direct cell-cell contact with effector cells or APCs through non-specific bystander suppression (38).

In humans, CD4+ CD25+ FOXP3+ T cells with *in vitro* suppressive capacity have been shown to appear in the second trimester of pregnancy (39). Indeed it appears that Tregs play critical functions in the maintenance of foetal-maternal immune tolerance. Compared to adults, regulatory T cells are found in elevated frequencies in secondary foetal lymph nodes (40). In addition, foetal Tregs suppress foetal immune responses against maternal antigens that cross the placental barrier (41). Cord blood is also a rich source of Tregs, with a predominantly naïve phenotype and highly suppressive functions that have been investigated as potential immunosuppressive agents to be used in the context of HSC transplantation (42). In mice, the default differentiation of up to 70% of neonatal CD4+ CD8- Foxp3- thymocytes into Treg cells with regulatory capacities was reported following various TCR stimulation condition in the absence of TGF-β (43). Caution must be exercised when drawing parallels to humans, though it is apparent that the immunosuppressive properties of Treg develop at an early stage in development and greatly influence the tolerogenic intrauterine and post natal nature of the immune response.

Immune activation

Priming of naive T cells occurs in the secondary lymphoid organs through interactions with dendritic cells and results in immune activation. The process involves the engagement of the TCR with its cognate peptide presented in the context of MHC. This process leads to the formation of the immunological synapse and a signalling cascade that results in altered gene expression profiles associated with the development of

effector function. It is beyond the scope of this introduction to fully describe the molecular events that result in T cell activation; however it is of relevance to discuss the various molecular markers that are associated with T cell activation and their significance in assessing levels of *in vivo* immunological activity particularly in early life.

One of the earliest cell surface molecules to be unregulated following activation is the glycoprotein CD69. CD69 functions by decreasing expression of chemokine receptors that mediate the exit of T cells from secondary lymphoid organs. As a consequence, activated T cells persist at the sites of activation and undergo clonal expansion in response to local IL-2 production, which in turn result in the down regulation of CD69. Similarly CD25, the α chain of the IL-2 receptor, is expressed early following activation and enables T cells to proliferate in response to IL-2 stimulation. A number of markers persist on the cell surface following cellular activation. CD38, for instance, has diverse roles on activated T cells including activity as a hydrolase where it mediates the conversion of nicotinamide adenine dinucleotide (NAD+) into nicotinamide, adenosine diphosphate–ribose (ADPR), and cyclic ADPR. Immune activation levels in the context of chronic viral infections such as HIV-1 infection have been studied through the expression of CD38 where it has been associated with enhanced immunopathology and disease progression (44,45). In infants, CD38 expression has been found to be constitutive on lymphocytes and therefore may not be suited as a lone marker for activation. Combined expression of CD38 with the MCH-II molecule HLA-DR has therefore been used to assess immune activation levels in infants (46) and in adults (47). In the context of HIV-1 infection and other chronic viral infections such as hepatitis C virus, persistent immune activation of virus-specific T cells leads to the

process of immune exhaustion. This occurs when virus-specific T cells become progressively less competent at mounting antiviral responses and become locked in a terminally differentiated defective state characterised by decreased cytokine production and proliferative capacity, and increased susceptibility to apoptosis. A number of markers have been associated with T cell exhaustion including the B7/CD28 co-inhibitory receptors programmed death -1 (PD-1) and CTLA-4. These molecules are upregulated on activated T cells and convey inhibitory signals upon TCR engagement, which in the context of chronic antigenic stimulation results in exhaustion. Tim-3, an Ig superfamily member, is an additional exhaustion marker that has also recently been associated with immune exhaustion in HIV-1 infection (48). Few studies have assessed exhaustion marker expression in early life. A recent study indicated that chronic exposure to *Plasmodium falciparum* results in an expansion PD-1 expression on CD4 T cells (49), though the functional consequence of this effect is unclear.

Immunological memory

Priming of antigen-specific T cells leads to clonal expansion, acquisition of peripheral homing receptors, and licencing to carry out effector functions such as cytokine production and cytotoxicity. In addition, antigen-experienced cells replace expression of the RA isoform of the CD45 molecule (CD45RA) with the RO isoform. Following the clearance of an antigenic stimulus, a contraction phase ensues in which the majority of antigen-specific T cells die through apoptosis due to lack of survival signals such as growth factors. A small number of cells, however, survive the contraction phase as memory cells. Memory cells can survive in a resting state and are capable of undergoing self-renewal- indeed stem cell-like memory T cell populations have been recently described (50). The self-renewal properties of memory T cell have been

shown to be independent of antigen stimulation and survival is maintained through a homeostatic response to IL-7 (particularly in the case of memory CD4 T cells) and IL-15. The expression of the IL-7 receptor (CD127) on memory T cells is consistent with this process. Upon re-encounter with antigen, memory T cells are capable of undergoing rapid clonal expansion leading to a population of antigen-specific cells which possess enhanced functional properties. A number of subsets of memory T cells have been identified largely on the basis of the expression of cell surface markers, in particular markers that influence tissue homing (51,52). CCR7 and CD62L ligand for instance, are lymph node homing markers that are expressed on naïve and central memory (Tcm) T cells. Lack of expression of these markers is used to identify effector memory T (Tem) cells which home to diverse peripheral tissues through up-regulation of alternate tissue specific homing receptors. Additional markers have also been used to differentiate memory T cell populations based on properties such as differentiation status (CD27 and CD28) proliferation (Ki67) and resistance to apoptosis (Bcl-2).

Infant vaccination and memory

Due to the lack of antigenic exposure *in utero,* neonatal T cells have a mainly naïve phenotype (CD45RA+ CD45RO-). As a consequence neonates have limited immunological memory. This feature plays an important role in infant susceptibility to infection and as a result infants are a key target population for vaccination. Vaccination strategies exploit the immune response in order to induce protective immunological memory and infants are vaccinated against a range of pathogens as shown in **table 1.1** In addition, maternal immunisation is employed, as in the case of antenatal TT immunisation, as a means to enhance passive immunity to potentially deadly diseases. As with maternal TT immunisation, most of the protective responses elicited by infant

vaccines are dependent on B cell responses and antibody production. The infant B cell compartment, as discussed, is functionally immature, and as a result, multiple booster doses of vaccines are needed to induce protection. Diminished T cell help as well as defective APC function, may also mediate defective B cell responses in infants. In addition, maternal antibody interference plays an important role in restricting infant antibody responses as is the case of measles vaccination. As a result, measles vaccination is delayed to 12 months of age in low burden countries and 9 months of age in high burden countries following clearance of maternal IgG from infant circulation.

The only vaccine that is thought to mediate its protective effect through T cell responses is Bacillus Calmette-Guérin (BCG). BCG is a live attenuated strain of *Mycobacterium bovis* that was developed through serial passage and first used on large human populations in 1921 (53). Several different strains of BCG have been developed over the years, which may have varying immunogenic effects (54). The immunological correlates of protection of BCG vaccination, however, have not been identified. The capacity of BCG-to induce a strong Th1 response, even in neonates is well documented (37,55), and INF-γ production has been associated with protection from diverse intracellular pathogens (56–58). Detailed characterisations of the T cell response to BCG vaccination has been carried out in South African (59,60) and Gambian (61) infants where a complex cytokine response dominated by CD4 T cells producing the Th1 cytokines IFN-γ, IL-2 and TNF-α has been found. In addition, following the peak response to BCG vaccination memory Th1 cells have been shown to develop with Tem (62) or Tcm (59) phenotype in vaccinated neonates. In order to characterise antigen specific cellular responses to BCG, a number of mycobacterial antigens have been used. These include tuberculin purified protein derivative (PPD), which is an extract of

M. tuberculosis that is commonly used in the Tuberculin Skin Test. In addition, the BCG vaccine itself (55,59,62,63) is used to stimulate *in vitro* immunological responses, as are various *M. tubercuoslsis* proteins antigens such as ESAT6 (64) and novel candidate vaccine antigens such Ag85B and TB10.4 (63,65). Additional discussions relating to T cell responses to vaccination in infants, particularly to BCGs and TT vaccination are show in section III of this introduction, Chapter 4 and Chapter 5.

Table 1.1 Expanded Programme on Immunisation schedule as applied in Kenya

Vaccine	Age of administration	Route
Bacillus Calmette–Guérin	Birth	Intradermal
Oral polio vaccine	6, 10 and 14 weeks	Oral
Pentavalent	6, 10 and 14 weeks	Intramuscular
PCV 10	6, 10 and 14 weeks	Intramuscular
Measles	9 months	Subcutaneous

Pentavalent: Diptheria-Tetanus-Whole-cell pertussis-*Haemophilus influenza* type b-Hepatitis B
PCV 10: 10-valent pneumococcal conjugate vaccine.

1.2   SECTION II: THE HIV-1 VIRUS AND INFANT INFECTION

1.2.1   Taxonomic classification

HIV-1 is a retrovirus that belongs to the family *Retroviridae* (66). Retroviruses are unique among viruses in that they carry out reverse transcription, the process of DNA synthesis from an RNA template. HIV-1 further belongs to the genus of lentiviruses that derive their name from the slow rate of disease progression associated with infection. Lentiviruses have a complex genomic composition compared to other retroviruses and are capable of infecting non-diving mammalian cells.

1.2.2   HIV-1 Structure

Mature HIV-1 virions form enveloped spherical particles, approximately 1000-1500Å in diameter (67). The viral envelope consists of a host-derived phospholipid bilayer (and associated membrane bound host proteins such as MHC molecules) and the viral glycoproteins (gp) gp41 and gp120 (**fig. 1.2**). Heterotrimeric complexes of gp41 and gp120 form 4 to 35 spikes (68,69) on the virion envelope which are anchored thorough the trans-membrane domain of gp41. The viral matrix protein (p17, MA) associates with the inner surface of the plasma membrane through myristolated residues and stabilises the spherical virion (70). Within the core of the virion is the capsid structure, recently resolved to the single atom level (71), composed of capsid proteins (p24, CA) arranged in a characteristic conical shape. The capsid surrounds the genome which is in association with the nucleocapsid (p7, NC), a protein that mediates genomic stability and the incorporation and packaging of genomic RNA into nascent virions (66). Within the capsid are associated enzymes critical for the replication cycle of retroviruses: reverse transcriptase (RT), intergrase (IN) and protease (PR).
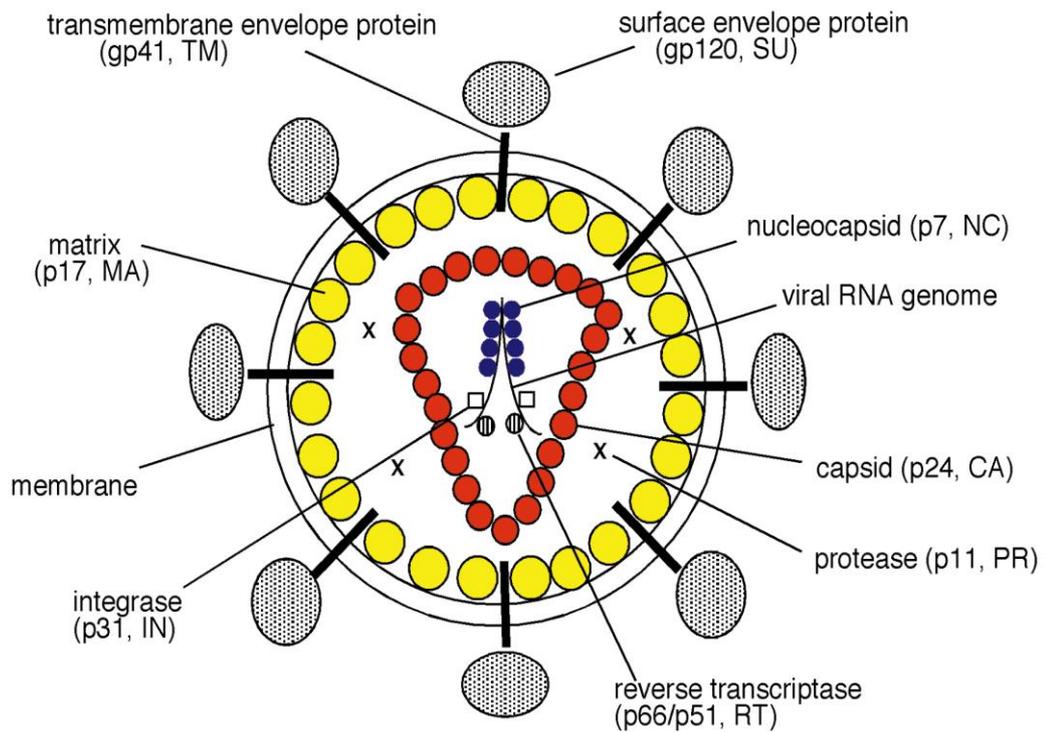
Figure 1.2 Structure of the mature HIV-1 virion. Major structural and enzymatic proteins are shown as well and the viral genome. For clarity, PR is only shown outside the capsid. Adapted with permission from (72).

1.2.3   HIV-1 genomic organisation

The HIV-1 genome consists of two copies of positive sense ssRNA molecules each approximately 10 kb in size. 9 genes are encoded in the genome within all 3 reading frames (**fig. 1.3**). In addition the protein coding segments of the genome are flanked by two long terminal repeats (LTRs) which contain *cis*-acting elements that mediate integration, gene expression and the packaging of the genome into virions.

The major structural proteins are encoded by *gag*, *pol* and *env* genes. The *gag* gene initially expresses a poly-protein precursor product (Pr55 ^{Gag}) which mediates virion assembly at the host cell membrane. Following viral budding, Pr55^{Gag} is proteolytically

cleaved into p17, p24 p7, p6 and the spacer proteins p2 and p1, triggering major structural rearrangements in the maturing virion (66). The *pol* gene encodes 4 enzymes: PR, RT, RNaseH and IN. A Gag-Pol poly-protein precursor (Pr160$^{Gag-Pol}$) is initially expressed that undergoes autocatalysis to form the mature enzymes. The *env* gene encodes the envelope precursor protein gp160 which is extensively glycosylated and proteolyltically cleaved by host cellular enzymes into gp41 and gp120.

The HIV-1 genome also encodes two essential regulators of viral gene expression, transactivator of transcription (Tat) and regulator of expression of virion proteins (Rev) and 4 accessory proteins viral infectivity factor (Vif), viral protein r (Vpr), viral protein u (Vpu) and negative factor (Nef). The accessory proteins have been shown to be dispensable for viral replication in certain *in vitro* systems (73,74). However, *in vivo*, these proteins play key roles in mediating host immune evasion and in counteracting a number of intrinsic host antiviral factors including a number of recently described host restriction factors (75,76). The main functions of the accessory molecules are summarised in **table 1.2**.

Table 1.2 HIV-1 accessory proteins and their functions

Protein	Size (kDa)	Functions
Nef	p27	CD4(77) and HLA-A and B downmodulation (78) T-cell receptor downmodulation (79) Enhanced FasL-medaited apoptosis of CTLs (80) Enhances viral transcription(81)
Vpr	p18	Role in nuclear import of the PIC (82) Cell cycle arrest at G2 (83)
Vpu	p16	CD4 downmodulation (84) Antagonise tetherin (85) Promotes virion release(86).
Vif	p23	Promotion of virion maturation (87) Counteracts ¥APOBEC-mediated G-A hypermutation (88)

* Tetherin: sequesters virions from budding sites ¥APOBEC3G: deaminates cytosines during reverse transcription resulting in accumulation of uracil nucleotides and G-A hypermyuatation during cDNA synthesis
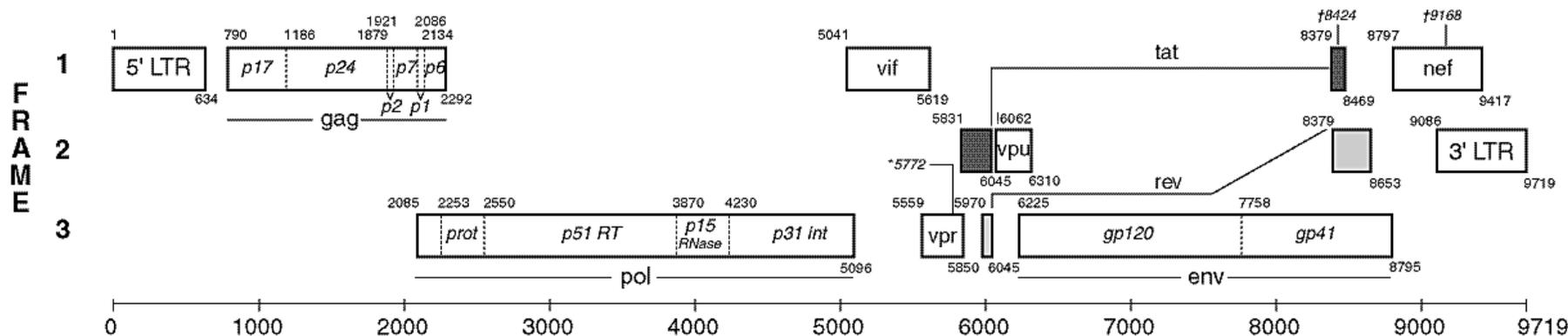
Figure 1.3 The organisation of the HIV-1 genome. Depicted is the HXB2 reference genome. Open reading frames are shown as rectangles. The number in the upper left corner of each rectangle indicates gene start, normally the position of the a in the ATG start codon for that gene, while the number in the lower right records the last position of the stop codon. The tat and rev spliced exons are shown as shaded rectangles. *5772 marks a frameshift in the vpr gene caused by an "extra" T relative to most other subtype B viruses; !6062 indicates a defective ACG start codon in vpu; †8424, and †9168 mark premature stop codons in tat and nef. Adapted with permission from (89).

1.2.4 Viral replication cycle

The HIV-1 replication cycle is depicted in **figure 1.4**. HIV-1 virions infect cells bearing the CD4 receptor and the co-receptor CCR4 or CXCR5. Gp120 initially mediates the interaction with the host CD4 receptor- this interaction induces a conformational change in Gp120, exposing the co-receptor binding sites (90). Co-receptor binding induces a conformational change in Gp41 enabling the insertion of a fusion peptide into the host cell membrane. A fusion pore made from six helical bundles forms, bringing the phospholipid bilayers of the virion and host into proximity enabling membrane fusion (91). The viral capsid is subsequently released into the cytoplasm where un-coating takes place- a process aided through interaction with Cyclophilin A (92). Release of the content of the capsid results in the formation of the reverse transcription complex, which in addition to the 2 copies of ssRNA includes the viral RT enzyme, a tRNA^{Lys} primer, NC, Vpr and several host mediators that together associate with actin filaments (93). Reverse transcription takes place in the cytoplasm through the activity of RT. RT lacks the proofreading activity of other polymerases and therefore the cDNA molecule produced may contain point mutations and/or indels. In addition, RT may switch RNA templates, resulting in recombinant cDNA molecules (94). RNaseH activity degrades DNA-RNA hybrid molecules enabling the formation of double stranded cDNA. Following reverse transcription, the double stranded cDNA molecule associates with viral and host proteins to form the pre-integration complex (PIC). This structure undergoes compaction and traverses through nuclear pores into the nucleus. IN mediates the insertion of viral cDNA into the host genome through the formation of double stranded breaks in host genomic DNA. Integration of the provirus can take place in diverse chromosomal locations, though sites of active transcription are

favoured (95). Depending on the epigenetic environment of the integration locus, the provirus may be transcriptionally active or in a latent state.
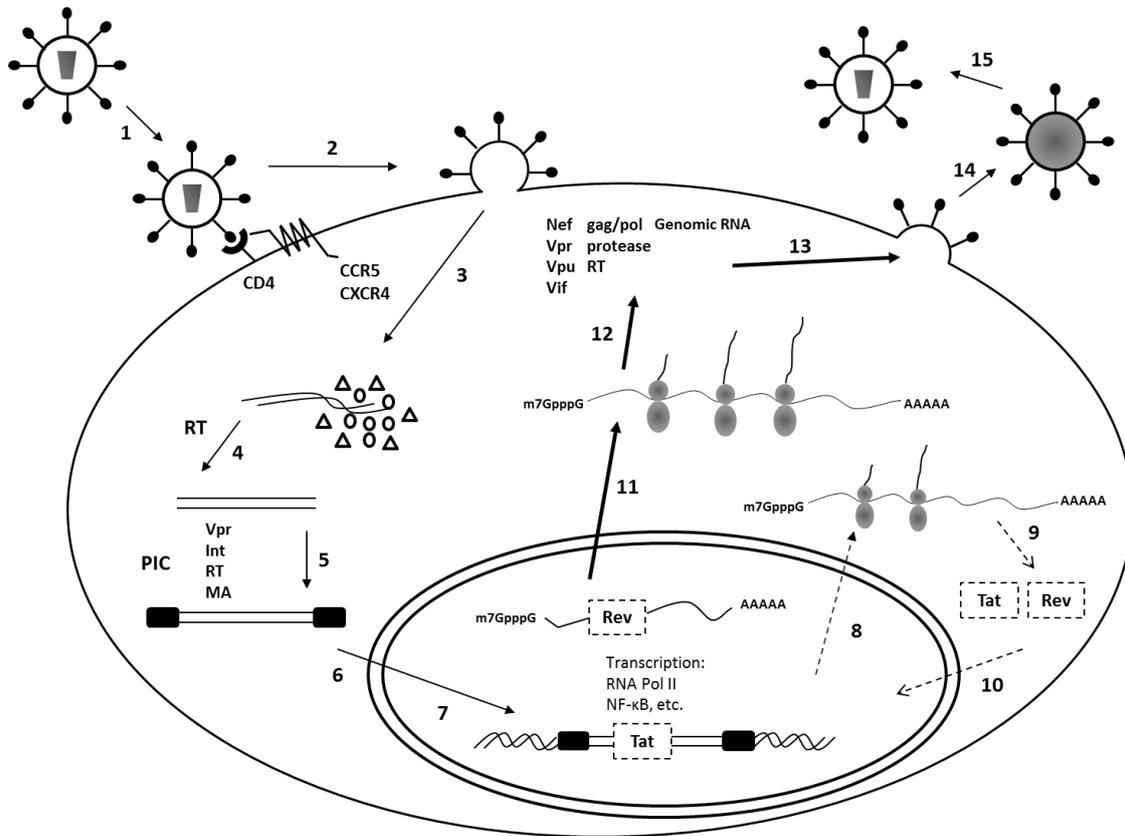
Figure 1.4. The HIV-1 replication cycle. 1) Engagement of Env glycoprotein with the CD4 receptor and either CCR5 or CXCR4 co-receptors; 2) fusion of viral and cellular membranes and entry of viral particle into the cell; 3) uncoating of viral genomic RNA; 4) reverse transcription; 5) pre-integration complex (PIC) formation; 6) nuclear import of the PIC; 7) integrase (Int)-mediated provirus integraction into the host genome; 8) viral transcripts are generated by RNA polymerase II (RNA Pol II) and host transcription factors such as NFκB; early multiply spliced transcripts are initially transcribed; 9) early regulatory proteins Tat and Rev are translated; 10) nuclear import of Tat and Rev; 11) unspliced mRNA transcripts bound to Rev are exported from the nucleus; 12) structural proteins translated; 13) viral genomic RNA is incorporated into

budding virions with protein components; 14) budding of nascent viral particles; 15) protease-mediated viral maturation. Thin arrows indicate steps from viral attachment to proviral integration; dashed arrows indicate the fate of early expressed viral genes; thick arrows indicate the fate of late expressed viral genes. Adapted from (96,97)

The 5'LTR of the provirus contains the transcriptional promoter and *cis* regulatory elements required for Pol II mediated gene expression and for the recruitment of host transcription factor such as NFκB. Initially, even in active genomic loci low levels of proviral transcription occurs. In addition, due to the block in nuclear export of incompletely spliced transcripts in host cells, only early HIV-1 proteins, Tat, Rev and Nef, derived from fully spliced transcripts are produced. Accumulation of Tat, a Pol II trans activator that binds the transactivation response element (TAR) located close to the 3' end of nascent mRNA, results in enhanced mRNA elongation and transcriptional efficiency. Enhanced expression of early proteins enables Rev-mediated nuclear export of unspliced and partially spliced transcripts that encode the 'late' proteins Gag, Pol, Env, Vpr, Vpu and Vif. This is achieved through binding of Rev to the rev response element (RRE), a secondary structure within the intron containing transcripts.

Gag and *pol* are transcribed as a single unspliced mRNA transcript. The Pr55 Gag protein precursor is produced from 90-95% of the transcripts (66). The remaining transcripts produce the Pr160$^{Gag-Pol}$ precursor through a -1 ribosomal frame shift at a poly T slippage site. Mirystolation of Gag targets the protein precursors to the plasma membrane where they accumulate in lipid rafts together with processed trimeric Env proteins (98). 2 copies of genomic RNA are packaged in each membrane coated budding virion. The cellular export machinery is usurped in the process of budding and the final release of the immature virion is mediated through p6 interactions with the

product of the tumor suppressor gene 10, a protein normally involved in endosomal processing (99).

1.2.5 HIV-1 epidemiology

HIV-1 was isolated for the first time in 1983(100) and recognised as the aetological agent of AIDS the year later (101,102). Early on in the epidemic it was recognised that in addition to horizontal sexual and parenteral infection, vertical transmission from mother to child was common. This discussion focuses on this important mode of transmission.

Epidemiology of MTCT of HIV-1

MTCT of HIV-1 is an important source of new infections that continues to drive the present-day epidemic. Of the 34 million people living with HIV-1 in 2011, 3.3 million were children under 15 years of age (103). 95% of these children were from Sub-Saharan Africa, the majority becoming infected following vertical exposure. In the absence of ART, 35-54% of HIV-1 infected infants from Sub-Saharan Africa progress rapidly to AIDS and die within the 2 years of life (104–106); the median time to AIDS in those surviving infancy has been reported to be 7 years (107).

A major driving force behind the infant epidemic is the disproportionate susceptibility of females to HIV-1 infection, in particular young women between 15 and 24 years of age (108). Heterosexual sex is the predominant mode of acquisition of HIV-1 in women from endemic countries, and the per-act transmission rate is higher in women than in men (109). Both biological and sociocultural risk factors contribute to this gender disparity, which often play a more prominent role in early adulthood. Genital micro-abrasions generated during sex occur more frequently in young women than in

men (110) and the large surface area of the female genital mucosa can remain exposed to semen for up to 3 days post coitus (109). In addition, immunological differences such as increased expression of the CCR5 co-receptor on CD4+ T cells of the female genital mucosa (110) and heightened systemic levels of cellular immune activation in females may enhance susceptibility to infection (111). These risk factors are compounded by an increased likelihood in females of having an STI at the time of sexual exposure to HIV-1 (108). Sociocultural factors influencing enhanced susceptibility to HIV-1 acquisition in females include gender disempowerment leading to lack of education and poverty, male resistance to condom use, age disparity in sexual partnerships, and intra-vaginal practices (110,112).

Our understating of the mechanisms of MTCT of HIV-1 and the development of potent ARVs over the past 10 years has led to progressive improvements in PMTCT interventions. Their implementation has virtually eliminated MTCT of HIV-1 in developed countries with universal access to care (113). In developing countries with high HIV-1 prevalence and constrained health care systems, public health approaches to PMTCT have had important epidemiological effects. **Figure 1.5** shows the impact of ARV prophylaxis on the number of new HIV-1 infections occurring through MTCT from 2000 to 2009 in the 25 countries with the largest number of HIV-1 positive pregnant women (114). These estimates show a 24% reduction in new child infections between 2000 and 2009 due to interventions with ARVs. Revised principles and recommendations for PMTCT and infant feeding practices were published by the WHO in 2009 (115,116). By scaling up the implementation of these recommendations through partnerships with the ministries of health of high burden countries, UNAIDS has set a goal of reducing MTCT of HIV-1 globally by 90% by 2015 with a reduction

in transmission rates to <5%. Meeting these targets at a national level has become health care priorities in countries with high HIV-1 incidence.
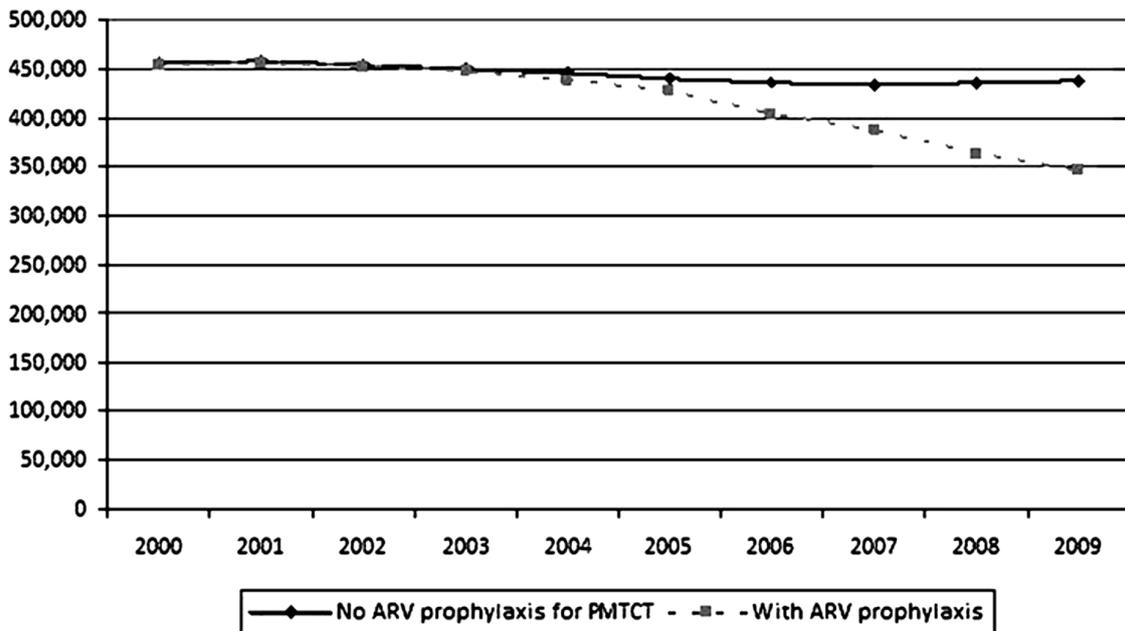
Figure 1.5. Estimates of the number of MTCT events from 2000 to 2009 due to the use of ARVs. The data indicates the number of new HIV transmissions estimated to have occurred in 25 countries with the highest number of HIV-1 positive women, in a scenario with no ARVs for PMTCT vs. that for the reported ARV coverage (adapted from Mahy et al. 2010)

In Kenya, the prevalence of HIV-1 infection in women of childbearing age is nearly twice that (8.0%) for age-matched males (4.3%) (117). Children make up 13% of the entire HIV-1 infected population and in 2011 12,894 children under 15 years of age were documented to have become infected with HIV-1 (117). A national PMTCT programme was formed in 2002 to oversee the formulation and updating of national

PMTCT guidelines. Although Kenya is among the countries with the highest number of children living with HIV-1 globally, significant advances have been recorded. 30% fewer children became infected in 2011 than in 2010 and prevalence among young women in Kenya has declined by 25% in the same period (118). 2008 figures indicate that Kenya has some of the highest rates of antenatal attendance in sub Saharan Africa with 88% women receiving 1 antenatal care (ANC) visit. However, only 52% of women attended the recommended 4 ANC visits and only 72% of HIV-1 infected expectant mother received ARV for PMTCT (118). Key targets for improvement of PMTCT provision have been set by national health authorities. The national rate of MTCT transmission in 2011 was 15%; the national target for 2013 is a reduction in the rate to 8% (117).

1.2.6 Transmission dynamics

MTCT of HIV-1 can occur at three stages: *in utero*, principally during the third trimester of pregnancy; *peripartum*, through exposure to maternal mucosal fluids during labour; and during breastfeeding. In the absence of ART and in breastfeeding populations these modes of transmission account for 10-25%, 35-40% and 35-40% of infections, respectively, with a cumulative risk of infection of between 40-45% (119). Determining the precise timing of infection, however, can be problematic. The most sensitive methods for early infant diagnosis rely on the detection of HIV-1 nucleic acid through PCR (120). Though it has been proposed that *in utero* transmission can be defined by the detection of HIV-1 nucleic acid within 48 hours of birth, this may, for instance, fail to distinguishing late *in utero* transmission from *peripartum* transmission (121).

Despite high levels of exposure more than 50% of infants remain uninfected in ART naïve populations. The question of whether this due to insufficient levels of exposure or to protective immunological mechanisms has been at the centre of efforts to define the key MTCT risk factors. Maternal plasma viral load is a correlate of transmission at all stages of infant exposure (122–125), however a cut off has not been established below which MTCT does not occur (123,126). The influence of physiological and virological factors at the localized environment at which exposure occurs is therefore of importance.

In utero *transmission*

In spite of a prolonged period of exposure *in utero* transmission is the least common mode of MTCT. Evidence for its occurrence includes the detection of HIV-1 in aborted foetuses (127) and in amniotic fluid (128). The main barrier separating infant and maternal circulation is the trophoblast, a compact layer of foetal-derived polar epithelial-like cells that mediate numerous maternal-foetal interactions. HIV-1 infection of trophoblast cells has been reported (129,130), though they lack CCR5, CXCR4 and likely CD4 expression (131,132) and are poor targets for HIV-1 replication. The foetal side of the placental barrier is populated by Hofbauer cells that have been shown to support HIV-1 infection *in vitro* and *in vivo* (127) and can adsorb HIV-1 through interactions with DC-SIGN (131). These cells likely sustain the initial stages of an *in utero* infection. Transient damage to the trophoblast (arising spontaneously, through placental infection, inflammation, chorioamnotitis or illicit drug use) may lead to passage of cell-free or cell associated virus or DC-SIGN adsorbed virus (120). In addition transcytosis of cell associated virus across the trophoblast layer is thought to play an important role in mediating placental transfer of HIV-1 (133) and may be

enhanced by inflammatory mediators such as TNF-α (134). A recent study identified high numbers of maternal immune cells in foetal lymph nodes which induced tolerization through FoxP3+ foetal T regs in response to the allogenic stimulation (135). This further highlights the complexity of maternal-foetal interactions and may have mechanistic implications for *in utero* transmission of HIV-1.

Peripartum *transmission*

Peripartum transmission accounts for approximately two thirds of transmissions in non-breastfeeding populations and is principally mediated through exposure to maternal blood and vaginal secretions during delivery (119). Caesarean sections can reduce transmission rates by up to 50%, presumably through reduced levels of exposure (136–138). During vaginal delivery, viral shedding from cervical and vaginal tissues is an important risk factor associated with *peripartum* transmission (139). Both parenteral and mucosal routes of infant acquisition likely mediate transmission: maternal-foetal micro-transfusions can occur during delivery due to breaks in the placental barrier (140) and HIV-1 nucleic acids have been identified in neonatal oropharyngeal (139,141) and gastrointestinal aspirates (141). Additional risk factors of *peripartum* transmission include prolonged rupture of membranes during labour (142) and maternal co-infections. Highly significant association between HSV-2 and transmission have been shown, with the presence of genital ulcers substantially increasing transmission (124).

Breast milk transmission

In the absence of ART, breast feeding infants are almost twice as likely to acquire HIV-1 infection as non-breastfeeding infants. Replacing breast milk with formula removes the risk of exposure; however, this can be problematic, particularly in low-income

countries. Early infant survival is highly dependent on the duration of breastfeeding in the developing world (143) and early weaning in HIV-1 exposed uninfected infants has been shown more than double the risk of death (144). In addition, issues of social stigma and HIV status disclosure play significant roles in infant feeding practices (145). Mixed feeding puts the infant at highest risk and has been shown to enhance the rate of transmission by 4 to 10-fold compared to exclusive breastfeeding (146,147). The precise mechanism mediating this effect is unknown, though bacterial infection leading to compromised integrity of the infant gastrointestinal tract and /or increased rates of clinical and subclinical mastitis due to milk stasis in the absence of suckling likely play a role (148). One of the key advances in the past 5 years has been the development of ART regimens that reduce systemic and breast milk viral load levels enabling exclusive breastfeeding by HIV-1 infected mothers (116).

Breast milk transmission is relatively inefficient, with estimates of the probability of transmission of 0.0005-0.00064 per litre of milk ingested (125,149). Viral loads in breast milk are typically 2-3 log10 lower than plasma (119) and high correlations between these parameters have been reported (150). Associations between transmission and breast milk viral RNA (149) and DNA (150) loads indicate that both cell-free and cell associated HIV-1 may be able to establish infection. In addition, the cumulative ingestion of cell free virus (a function of breast milk viral RNA concentration and volume of ingested breast milk) was associated with transmission, highlighting the increased risk posed by prolonged breast feeding (149). In a recent study, latently infected resting CD4 T cells were identified in breast milk with enhanced virion production potential compared to peripheral reservoirs (151) . These cells may be

refractory to ARV and are likely able to seed breast milk in women undergoing ART (152).

1.2.7 The natural history of infant infection

Vertical acquisition of HIV-1 in infancy significantly alters the dynamics of HIV-1 pathogenesis compared to acquisition in adulthood (153). Primary infection in adults is typically characterised by a sharp rise in viraemia that peaks at a median of between 5-6 log10 RNA copies/mL of plasma at 3-4 weeks after infection. A drop in plasma RNA associated with the onset of an adaptive immune responses leads to the establishment of the viral set point within approximately 6-9 months following infection. Typically 2-3 log10 lower than at peak, set point viral load is highly predictive of disease progression (154,155). In the absence of ART the progressive burden on the CD4+ T cell compartment and associated immunopathology leads to the onset of AIDS on average within 8-10 years in European populations and within 8 years in Sub-Saharan African populations (156,157). By contrast, vertically infected infants experience a greater rise in peak viraemia, with a median of 6 -7 log10 RNA copies/mL of plasma, with a gradual decline over the first 2 years of life indicative of a reduced immunological capacity to contain viral replication (158). In addition, disease progression proceeds rapidly with mortality rates in Kenyan infant cohorts of up to 52% within 2 years of life in the absence of ART (104). Plasma RNA levels and differences in the timing of infection have been shown to be significantly related to outcomes, with earlier infection being associated with increased peak and set point VL and increased mortality (153,159)

1.2.8   The T cell response to HIV-1 infection

Several lines of evidence indicate that HIV-specific T cell responses play critical roles in the control of viral replication in adults during acute and chronic stages of infection. Virus-specific CD8$^+$ T cells are detected before the appearance of HIV antibodies early in infection (160), CD8$^+$ T cell numbers correlate inversely with viral load during acute infection (161,162) and broad HIV-specific CD4$^+$ and CD8$^+$ T cell responses are associated with long term control of chronic infection (163,164). In the simian immunodeficiency virus model, depletion of rhesus macaque CD8$^+$ T cells results in uncontrolled viral replication which can be reversed upon the reintroduction of virus-specific CD8$^+$ T cells (165). In addition, specific escape mutations emerge in the viral genome as a response to the selective pressure exerted by cytotoxic T lymphocytes (CTLs) (166–169) . Most recently, a genome wide association study has implicated HLA class I alleles as the major host-genetic determinants of long-term control of HIV-1 infection (170).

The role of T cell responses during infant HIV-1 infection, particularly in infants from sub-Saharan Africa, is less clear. HIV-specific CD8 T cell IFN-γ responses have been detected from birth in African infants (171). Longitudinal data indicate that HIV-specific IFN-γ responses become stronger with age although the strength of these responses does not correlate with control of viral load (172). Additional reports have indicated that in terms of cytokine production and proliferation, CD8 T cell responses are compromised in infants compared to adults (173,174). By contrast, the emergence of *de novo* CTL-escape mutations in infant viral isolates is well documented(175–177) indicating the presence of functional CTL responses. In addition, studies on congenital cytomegalovirus infection demonstrate that infants are able to mount virus-specific

CD8 T cell responses with the same functional phenotype as those in adults (178). Such disparate outcomes suggest that infant HIV-specific CTL responses may undergo a process of functional maturation in early life. Alternatively, these effects could arise from HIV-mediated interference with CTL development, either through damage to the thymus or the dendritic cell compartment that may be more significant in infants than in adults. Furthermore, the failure of these emerging functional responses to control viral replication during acute infection may be accounted for by some of the unique features of MTCT of HIV-1. Firstly, the transmission pairs in MTCT will share a minimum of 50% of HLA alleles. As a consequence, escape mutations selected by maternal CTLs will be pre-adapted to infant CTL responses (107). Secondly, it has been shown in the rhesus macaque model that the pool of activated and proliferating CD4+ T cell targets is greatly increased in neonatal tissues compared to adults (10-fold in the gut), and that these cells are preferentially targeted and depleted by SIV(179). These factors may help explain the accelerated pathogenesis in acute infant HIV-1 infection in the face of the development of functional HIV-specific CTL responses.

1.2.9 HIV-1 evolution and diversity

Molecular evolution

A major factor linked to the lack of immune control of HIV-1 during infection that has also hampered vaccine development is the rate of viral evolution – one of the highest of any organism.(180). The high rate of evolution of HIV-1 primarily results from the very high mutation rate (mediated by the properties of RT discussed above) and high turnover rates associated with the viral replication cycle-the composite half-life of cell-associated and cell free virus has been shown to between 1 and 2 days(181). 10^{10} new virions are produced per day in an infected individual (182), enabling novel variants

to accumulate rapidly in a population. Viral evolution is also shaped by universal evolutionary forces such as genetic drift, and natural selection, which determine the rate at which novel variants may be become fixed in a population i.e. the rate of nucleotide substitution or evolutionary rate. The study of the evolutionary dynamics of HIV-1 or other organisms is frequently carried out within a phylogenetic framework (183). It is beyond the scope of this introduction to review the process of phylogenetic analysis. However, in brief, the inference of phylogenetic relationships relies on 1) the alignment of genetic sequences 2) selection of an evolutionary model that best fits the data set 3) phylogenetic inference though the implementation of a tree building algorithm and 4) statistical test of the phylogeny(180). This process enables evolutionary dynamics to be quantified, and studies of HIV-1 evolution frequently focus on measurements of divergence (the genetic distance from a reference point e.g. the transmitted sequence) diversity (the genetic variation within the viral quasispecies at a particular time point) and selection pressure (a specific environmental factor or set of factors, such as the host immune response, that influence molecular adaptation). The assessment of the ratio of synonymous (dS) and non-synonymous (dN) nucleotide substitutions has been shown to be a useful method for assessing molecular adaptation (184).

HIV-1 transmission in the majority of cases, either through MTCT (185–188) of through sexual contact (189,190), results in a single founder virion establishing an infection in the new host. This represents a large bottleneck in the genetic diversity of the viral population. However, during acute infection, rapid viral expansion and evolution leads to a large heterogeneous population of viral quasispecies. As discussed above the CTL response shapes early viral evolution through selection of CTL escape

variants. Escape variants harbour mutations that abrogate effective MHC-I presentation of viral peptides. These include mutations involving residues that contact the TCR but also ones that prevent HLA binding of the peptide or that impair antigen processing. Depending on the genomic region targeted by the CTL response, mutations may result in a fitness cost to the viral variant. Indeed evidence that fitness costs are incurred by escape variants comes from the observation of reversion mutations that occur upon transmission to a host lacking the specific restricting HLA allele (176,191). In addition, elite controllers (HIV-1 infected individuals who maintain viral loads <50 RNA copies/mL without ART) have been found to harbour viral escape variants with reduced replicative capacity (192). Based on these observations detailed models of how CTL responses drive viral evolution in acute adult infections have emerged in which early CTL responses have been shown to contribute to viral control. (193,194)

The effect of CTL selection pressure on viral evolution in early infant infection is less well understood. Relatively few studies have assessed the evolutionary dynamics of early infant infection and the few longitudinal analysis of evolutionary rates that have been carried out have been restricted to analysis of *env*, where antibody mediated selection pressure predominates (195–197). Cross sectional estimates of sequence divergence between mother and infants viral populations in *gag nef* and *pol* (predominant targets of the CTL response) have been estimated at 1.74% per year(198). There is evidence that some of this divergence is CTL driven as CTL escape has been documented in infants(177,199) and children(200), including within the well described B57-restricted TW10 epitope (an epitope in which escape mutation have been shown to significantly reduce viral fitness in adults with long-term viral control). Interestingly, and in contrast to adults, superior variant-specific CTL responses were detected in

children, indicating a greater plasticity of the CTL response to novel variants(200). Reversion has also been shown to play an important role in early infant infection, and as in adult infection(201), has been reported occur with a similar frequency as forward, putatively CTL driven, mutation(198).

Viral diversity

The evolutionary processes that give rise to intra-host genetic diversity have also shaped viral diversity in populations of infected humans. Based on genetic relatedness, HIV-1 viral isolates have been classified into 3 distinct groups: the M group, which stands for major, the O group which stands for outlier and the N group which stands for not-M, not-O group. A novel group, group P, standing for pending, has been recently described in a single patient of Cameroonian descent, and awaits assignment as a major group(202). The majority of viruses in the human population are from the M group; these have been further classified into subtypes (A-K, excluding letters E and I) sub-subtypes (A1-A4 and F1 and F2) and 55 circulating recombinant forms (CRFs). CRFs are recombinants of parental subtypes that are detected in 3 or more epidemiologically unlinked individuals; recombinant forms detected in isolated instances are termed unique recombinant forms (URFs).

The HIV-1 epidemics in different regions of the world are characterised by distribution of particular HIV-1 subtypes (**fig 1.6**). For instance the epidemics in Europe and North America are dominated by subtype B viruses and in South Africa the predominant viral subtype is C. The epidemic in Kenya is characterised by the circulation of a number of different subtypes and CRFs. The current distribution of viral sequences from Kenya submitted to the LANL database indicate that the predominant subtype in circulation is

subtype A (71.1%) followed by D (13.4%) and C (5.3%); subtypes G, A2, CRFs 01, 02, 10, 16 and 21, and URFs A1D and A1C have also been reported.
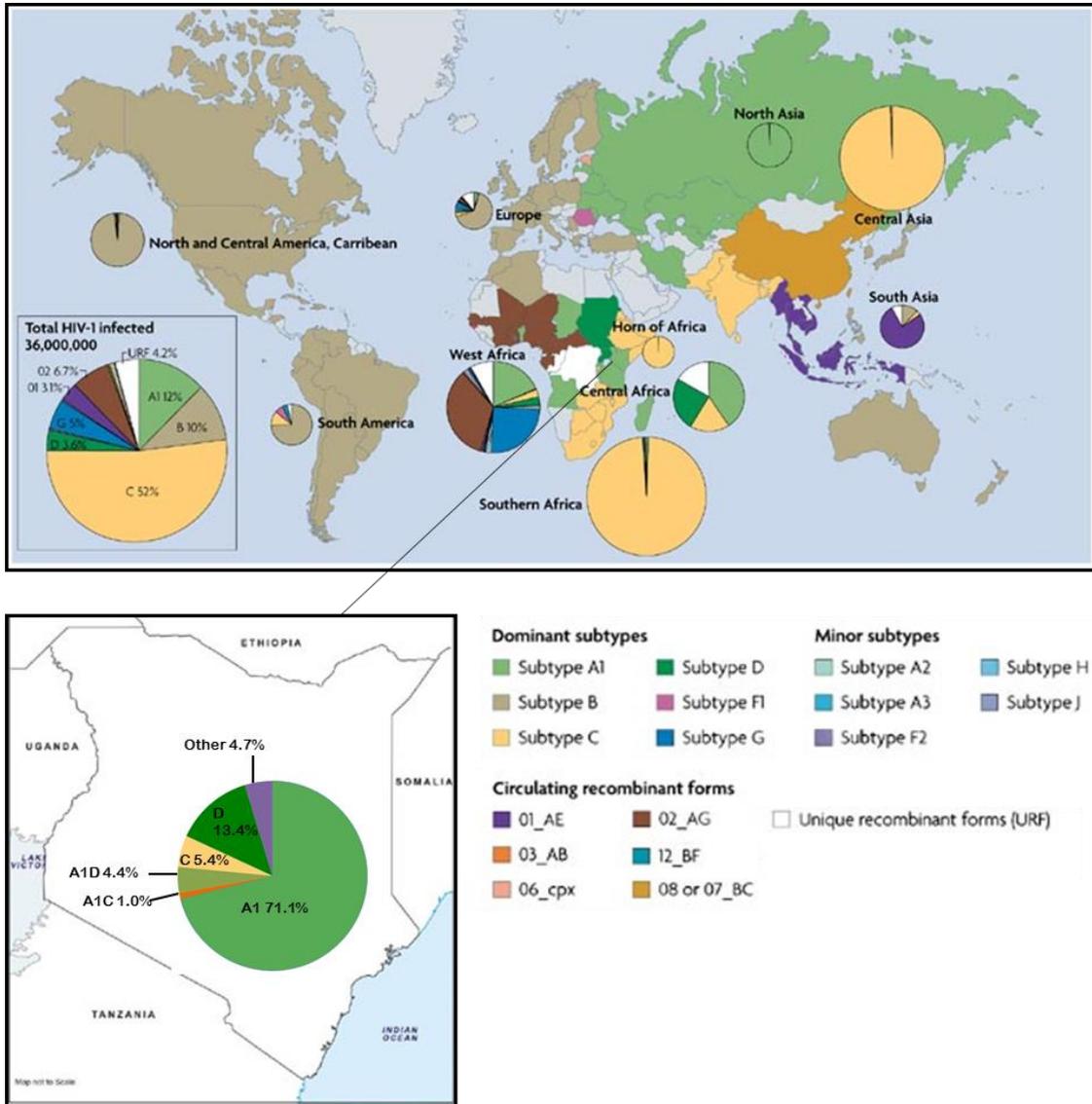
Figure 1.6. HIV-1 subtype distributions across the world and in Kenya. The world map indicates estimates of group M subtype frequencies in the total HIV-1 infected population and in different geographic regions. The countries coloured grey have a low level of HIV-1 prevalence or have limited data. The map of Kenya indicates the frequency of HIV-1 subtype viral sequences submitted to the LANLDB(203). Adapted from(204).

1.3   SECTION III: HIV-1 EXPOSED UNINFECTED INFANTS

As discussed in section II, the use of ART in pregnancy has enabled the elimination of vertical HIV transmission to become an achievable goal. In addition, recent advances in combination therapy have resulted in enhanced survival of the uninfected infant, particularly in low-income settings, by enabling HIV-infected mothers to safely breastfeed (205–207). These successes have resulted in increasing numbers of infants being born to HIV-1 infected mothers who are free from infection- HIV-exposed uninfected (HEU) infants. In countries with high HIV-1 prevalence, HEU infants constitute an important emerging population: in Southern Africa they account for >30% of all new born infants (208).

An accumulating body of evidence suggests that HEU infants face significantly higher rates of infectious disease-associated morbidity and mortality than infants born to HIV-1 uninfected mothers. A large study carried out in Zimbabwe in the pre-ART era that included over 14,000 infants showed that at 2 years of age, HEU infants had >3 fold higher mortality rate than unexposed control infants (209). Risk of death was highest between three and six months of age and was associated with lower respiratory tract infections. Similar increased mortality rates have been reported in Ugandan HEU infants (2-fold) (210), and in Malawian (>4 fold) (211) and Zambian (212) HEU infants following single-dose nevirapin prophylaxis. In the latter study, mortality (cumulatively at 13.6% in HEU infants within the first 2 years of life) was significantly associated with enteric infection and early weaning from breastmilk. Similar findings have been reported in a European setting (though the infants were of African descent) where the risk of invasive group B streptococcal infection was significantly higher among hospitalised HEU infants than aged matched controls (213).

Several factors unique to HEU infants may play a role in this heightened susceptibility to infection. HEU infants are born into an HIV affected household in which the capacity to provide care may be compromised. In addition, exposure to infectious diseases in this setting may be enhanced in early life. These factors are difficult to quantify though a case study has reported the apparent transmission of *Pneumocystis jiroveci* from an HIV-1 infected mother to her uninfected infant (214). HEU infants are also faced with a unique set of exposures during *in utero* development that can persist into early life: exposure to HIV-1 either as whole virus or soluble antigens; exposure to antiretroviral drugs; and exposure to a maternal immune system perturbed by HIV-1 infection. These factors are largely interdependent making it challenging to assess the individual contribution of each factor overall health status of HEU infant.

1.3.1 Exposure to HIV-1 virions and antigens

The transplacental passage of maternal cells into infant circulation is a well described phenomenon that serves as a mechanism for *in utero* HIV-1 MTCT. In the absence of transmission, there is well supported evidence that HEU infants are able to mount HIV-specific T cell responses. Both CD4 (215,216) and CD8 T cell responses (217–220) have been reported to a range of HIV-1 antigens. Furthermore, these responses have been shown to be subject to Treg-mediated immune regulation (216). The breadth and magnitude of HIV-specific T cell responses has been shown to be highest in the neonate (221), though persistent T cell responses have not been detected in 7 year-old HEU children (222). These data suggest significant levels of exposure to HIV-1 antigens in utero and/or in early life, that results in the priming of neonatal T cells. Reports that the magnitude of HIV-1 specific infant CD8 T cell responses are associated with protection (223) suggest that exposure to intact cell free or cell

associated virions may occur. On the other hand, HIV-1 specific T cell responses in HEU infants may also serve as a marker for exposure to soluble HIV-1 antigens and non-infectious particles. Moreover, exposure to viral antigens may have direct immunomodulatory effects. Soluble Nef has been shown to affect class switching on B-cells (224) and purified gp120 has been shown to have dose-dependent immunosuppressive effects on CD4 T cell proliferation and CTL cytotoxicity in response to EBV stimulation (225).

1.3.2   The immunological status of HEU infants

Numerous reports have indicated that significant immunological differences exist between HEU infants and unexposed infants. Perhaps the most consistent finding has been the reduced levels of *in utero* transfer of maternal IgG antibodies to infants. Neonatal levels of anti-tetanus antibodies have been shown to be reduced by 50% in HEU infants compared to controls(226) and anti-measles antibody titres have been shown to be significantly reduced in HEU infants (226,227), particularly infants born to mothers with high viral loads (228). HIV-1 associated immunopathology of the maternal B cell compartment likely mediates this outcome, although it remains unclear whether improved antenatal combination ART interventions will limit HIV-mediated B-cell immunopathology and restore maternal transfer of antibodies to normal levels. Furthermore, the specific effect of reduced maternal antibody transfer on HEU infant morbidity and mortality has not been addressed.

In terms of infant-derived immunity, there have been numerous reports of alterations that have been attributed to ARV exposure. ARVs, particularly NRTIs such as AZT, readily cross the placental barrier (229) and have been shown to induce varying levels of mitochondrial toxicity in HEU infants ranging from rare severe cases of neuropathy

(230,231) to subclinical yet sustained effects on mitochondrial function and DNA content (232–236). This broad set of metabolic alterations may underlie reported changes in haematological parameters in HEU infants exposed to various combinations of ARVs that have been consistently reported in large European and North American cohorts. Transient decreases in haemoglobin levels and more sustained decreases in platelets, total lymphocytes and CD4+ and CD8+ T cell subsets that persisted to at least 2 years of age were observed in French (237) and US cohorts (238). In addition, the European Collaborative Study found subclinical but sustained reductions in total lymphocyte and CD8+ T cell counts (239) and strikingly, reduced neutrophil counts over the first 8 years of life (240). Furthermore, exposure to more complex drug regimens (compared to mono-therapy) has been associated with greater depletion of haematological cell lineages (237,241). *In vitro*, AZT has been shown to reduce myeloid and erythroid CD34+ haematopoietic stem progenitor proliferation (242,243) . *In vivo,* low thymic CD4+ T cell counts and impaired CD34+ progenitor cell function were reported in a cohort of NRTI-exposed HEU infants (244).

Phenotypic and functional changes in lymphocyte populations have also been reported in HEU infants from various age groups. A number of these finding have been linked to maternal disease status and have been studied in cord blood. In comparison to HEU infants born to mothers with low viral load levels, cord blood mononuclear cells from HEU infants born to mothers with high viral loads have been shown to produce heightened pro-inflammatory cytokine responses (IFN-γ, TNF-α and IL-17) following polyclonal stimulation (245) and to show an increased proliferative capacity and a reduced IL-10 production capacity following stimulation with a mitogen (246). In addition, infant exposure to maternal immune activation and the associated cytokine

milieu *in utero* has been associated with increased spontaneous and activation-induced T cell apoptosis following anti-CD3 stimulation (247). These findings suggest a generalised heightened level of proinflammatory responsiveness in infant lymphocytes linked to uncontrolled maternal HIV replication and associated immune activation levels. This scenario represents a significant departure from the tolerogenic maternal immune environment present in the absence of HIV-1 infection, the implication of which has not been fully understood.

Studies assessing functional and phenotypic properties of HEU infant immune cell population in early life have largely found consistent findings to those from cord blood studies. In a cross-sectional analysis of HEU infants at 1 month of age and HEU children at 7 year of age, significantly expanded populations of activated (CD38+), memory (CD45RO+) and double negative (CD4- CD8-) T cells were found in HEU infants together with reduced levels of naïve T cells (222). In addition, significantly higher plasma IL-7 levels were found in both age groups compared to controls, indicating possible alterations in the homeostatic maintenance of T cells populations. Similarly, a study in HEU adolescents indicated that activated and terminally differentiated CD8 T cells together with CD19+ B cells were significantly higher than in age-matched controls, in contrast to Tem CD4 T cells and CD16+ NK cells which were reduced in frequency (248). Moreover, on *ex vivo* T cells, expression levels of CD40L (CD154), a co-stimulatory molecule expressed following cellular activation, was heightened in HEU infants at a median of 12 months of age compared to healthy controls (249). Effects on homeostatic regulation of T cell population in HEU infants have been further explored through assessments of thymic function. Reduced T-cell receptor excision circles as well as reduced CD34+ progenitor cell function was

described in cord blood samples from 19 neonates whose mothers had received ART during pregnancy (244). In addition, 15 month-old HEU infants have been reported have reduced thymic size (250), which in HIV-1 negative populations has been associated with increased susceptibility to infection and higher mortality rates (251,252). This same study assessed the frequency of regulatory T cell in circulation and found no difference between HEU and control infants, though this finding has not been consistent and other groups have shown dis-regulated Treg cell frequencies in peripheral circulation in HEU infants (216).

Relatively few studies have looked at the innate immune compartment in HEU infants. A report from a Colombian cohort of HEU infants indicated that frequencies of cord blood-derived mDCs were higher in HEU infants compared to controls and that following LPS stimulation, increased activatory co-stimulatory molecule expression (CD86 and CD80) but not inhibitory costimulatory molecule expression (PD-L1; B7-H1) was found on mDCs from HEU infants (253). In addition, IL-12 production was observed to be compromised in HEU infants following stimulation of cord blood-derived mDC with *Staphylococcus aureus* Cowan (254). These studies are difficult to reconcile, though they suggest that phenotypic and functional alterations may occur in HEU infant DC populations.

1.3.3 Vaccine responses in HEU infants

What are the *in vivo* implications of the alterations in the broad populations of immune cells described above? Immune responses to infant vaccinations provide a useful model to assess *in vivo* immune function following a controlled antigenic challenge. In addition, adequate vaccine-acquired immunity in infant populations is of critical public

health importance. A South African study of a large cross-sectional population of HEU infants showed robust antibody responses to EPI vaccinations that were similar or enhanced (in the case of pertussis and pneumococcus but not HBV) in comparison to unexposed controls (255). Consistent with previous reports, maternal antibody transfer to the infant was reduced and this was thought to account for the increased vaccination response in infants, through the reduced binding of maternal antibodies to vaccine antigens. A subsequent longitudinal study designed to compare antibody responses to EPI vaccination between HEU infants and control infants showed no significant differences between the two study populations (256). By contrast, following OPV vaccination, low poliovirus neutralizing antibody titres were associated with early weaning in a cohort of Zambian HEU infants (257). It remains to be determined what additional factors may contribute to the variations in vaccine-specific antibody responses in HEU infants and in particular whether memory B cell populations sustain long-term immunological memory.

The data on T-cell responses to vaccines in HEU children are less clear-cut. A number of studies have compared immune responses to the live attenuated Bacillus Calmette-Guerin (BCG) vaccine - given to infants at birth in tuberculosis-endemic areas - in HEU and unexposed controls, with some contrasting reports. Impairment of BCG immunogenicity was supported by an early study by Ota et al, which reported a significantly reduced occurrence of BCG scars after vaccination (258). More recently, altered proliferative responses in HEUs following *in vitro* BCG stimulation were reported at 10 weeks (259) and at a median of 7 months of age (260). A trend to lower IFN-γ production was seen in whole blood from HEU infants stimulated with purified protein derivative (PPD) at 6 weeks of age (64). By contrast, the same study, in which

the entire HEU cohort presented a BCG scar, found no demonstrable effect of maternal HIV-infection status on IFN-γ secretion after stimulation with BCG or ESAT-6. This finding is in accordance with others in which neither IFN-γ alone (259) nor IFN-γ, IL-13, IL-5 or IL-10 production (261) following *in vitro* stimulation with mycobacterial antigens was compromised in HEUs. Finally, the cytokine profiles of T cells responsive to mycobacterial antigens were explored in HEU infants longitudinally by flow cytometry over the first year of life (262) . A complex pattern of expression profiles dominated by CD4 T cells expressing one or more of the Th1 cytokines IFN-γ, IL-2 and TNF-α was shown that did not statistically differ between HEU infants and unexposed controls. However, HIV-1 infection was associated with significantly reduced BCG-specific T cell responses. Surprisingly, reduced BCG-specific T cell responses were not seen in a cohort of ART naive HIV-1 infected children with a median age of 3.5 years. This finding was thought to reflect a selection bias for infants with slow disease progression and preserved immune function (63). Collectively, these contrasting results reflect the wide variation in pro-inflammatory responses following neonatal BCG vaccination, though for the most part Th1 responses appear comparable between HEU and HIV-1 unexposed infants. However, it is of note that the correlates of protection of BCG vaccination are yet to be identified (263). By contrast to these studies, in a cohort of Ugandan HEU infants, T cell responses to tetanus toxoid following DPT vaccination were found to be significantly reduced compared to controls in terms of IFN-γ, IL-5 and IL-13 cytokine production following whole blood stimulation. In addition, to cytokine induction, it remains unclear whether the generation of vaccine-specific long-lasting memory T cell populations is affected in HEU infants following vaccination with BCG or other vaccines. The finding that

plasma IL-7 levels, a key cytokine in memory T cell homeostasis, are increased in HEU infants compared to controls (222) indicates possible effects on memory T cell induction or maintenance.

1.4   SUMMARY AND STUDY AIMS

The past 10 years has seen MTCT of HIV-1 shift from being one of the predominant forces in the global epidemic to a phenomenon that is largely preventable and on the path to elimination. This thesis pursues two main lines of enquiry with the aim to contribute to improved health outcomes of infants affected by HIV-1. The first seeks to further our understanding of the capacity of the infant cytotoxic T cell response to influence viral evolutionary dynamics in early infection. The second seeks to characterise the immunological status of an expanding population of HIV-1 exposed uninfected infants whom may benefit from targeted therapeutic interventions.

Chapter 3

Aims: To characterise infant HIV-1 evolutionary dynamics following MTCT

Specific aims

1) To determine the subtype distribution of HIV-1 in select mother-infant pairs

2) To estimate rates of evolution in *gag*, *pol* and *nef* within the first 2 years following infection

3) To assess the impact of CTL-driven selection on evolution in *gag*, *pol* and *nef*

Chapter 4

Aim: To carry out a cross-sectional characterisation of the frequency and phenotype of cellular immune parameters and antigen-specific immune responses to routine vaccinations in healthy Kenyan infants at 3 and 12 months of age

Specific aims:

1) To characterise complete blood cell count values

2) To characterise *ex vivo*:

   - Frequencies of T cells expressing activation and exhaustion markers

   - Frequencies of circulating T regulatory cells

   - Frequency of dendritic cell subsets and their expression of co-stimulatory molecules

   - Frequency of memory T cell subsets in circulation

3) To characterise, in healthy Kenyan infants, CD4 and CD8 T cell responses the mycobacterial antigen PPD, the vaccine antigen TT and the super antigen SEB

Chapter 5

Aim: To analyse the effect of HIV exposure on the immunological parameters and antigen-specific immune responses detailed in the aims of chapter 4

Specific aims:

To carry out cross-sectional comparisons between control and HEU infants at 3 and 12 months of age in order to assess the effect of HIV-1 exposure on:

   - Complete blood counts values

   - *Ex vivo* immune parameters

   - Antigen-specific immune responses

2   CHAPTER 2: MATERIALS AND METHODS

2.1   MATERIALS AND METHODS USED IN CHAPTER 3

2.1.1   Cohort characteristics

Samples were collected as part of the CTLs and the Prevention of HIV-1 Transmission Study (CTL cohort study) carried out in Nairobi, Kenya between the years 1999 and 2002. This was a prospective study designed to assess whether HIV-specific cytotoxic T cells were associated with protection from MTCT of HIV-1 and to identify correlates of disease progression in HIV-1 infected infants (223). The current study protocol was approved by the Kenyatta National Hospital Ethics Review Committee, the Institutional Review Board of the University of Washington and the Oxford Tropical Research Ethics Committee (OXTREC). To establish the CTL cohort, 31,731 pregnant women were screened for HIV-1 infection, 510 were enrolled in the study, 476 live singleton births were delivered, and 465 mother-infant pairs were followed for > 1 year postpartum. ART was not commonly available in the country at the time of the study so mothers were provided with Azidothymidine (AZT) during the final trimester to reduce the risk of HIV-1 transmission (264). Trimethoprim-sulphamethoxazole prophylaxis, routine vaccinations, CD4+ T cell counts and RNA viral loads were provided as part of the postnatal follow-up. Infant HIV-1 infection was determined by nested PCR amplification of *gag*; infection was defined as the detection of HIV-1 DNA or RNA at two separate visits. There were 90 transmission events with data on the timing of infection available for 87 infants. 30 infants were infected *in utero* (PCR positive within 48hrs of birth); 41 were infected *peripartum* (PCR negative at birth, PCR positive within one month of age); and 16 were infected through breast milk ingestion

(PCR positive after 1 month of age). Infant viral loads were determined from cryopreserved plasma specimens, using the Gen-Probe assay which has a limit of detection of 50 copies /ml. Infant and maternal HLA types were determined through an Amplification Refractory Mutation system PCR with sequence-specific primers optimized for African HLA alleles.

2.1.2 Blood sample collection, processing and storage

Cord blood was collected into EDTA-anticoagulated tubes (20mL) and peripheral blood from mothers (10mL) and infants (2-5mL) was collected at month 1, and then at 3-monthly intervals for up to a year from HIV-1 uninfected infants and for 2 years from HIV-1 infected infants. Ficoll hypaque density-gradient centrifugation was carried out to separate CBMC/PBMCs and plasma. Plasma was stored until use in the present study at -80˚C. Samples were transported to the collaborating groups involved in the CTL study, namely, the Lohman-Payne lab at the University of Nairobi, the Rowland-Jones lab at the University of Oxford and the John-Stewart lab at the University of Washington. A master inventory of the samples is maintained by the University of Washington (https://depts.washington.edu/ctlstudy/) and individual institutions maintain inventories of sample subsets. Databases are accessed through Microsoft Excel, Microsoft Access and SPSS.

2.1.3 Selection of sample subsets

Inclusion criteria for the sample selection were 1) infant HIV-1 infection at birth (*in utero* transmission) or by 1 month of age (early breast milk or peripartum infection) and 2) availability of 2 or more longitudinal infant plasma samples. PCR amplification was attempted on 32 HIV-1 infected infants and on a corresponding maternal sample

taken at the closest time point prior to transmission: 32 weeks of gestation in case of *in utero* transmission and during delivery in cases of *peri* or *postpartum* transmission.

2.1.4   Viral RNA extraction

The QIAamp Viral RNA extraction kit (Qiagen) was used to purify viral RNA from 140µl of peripheral blood plasma. Each sample was loaded onto separate columns and processed following the manufacturer's instructions. Purified RNA was eluted into 2x 40µl of elution buffer AVE and stored at -80˚C until use.

2.1.5   Primer design

Two pairs of PCR primers were designed per amplicon, using Primer3 version 0.4.0(67), to obtain whole *gag, pol and nef* by nested PCR (**table 2.2**). Briefly, a clade A source sequence (accession number [AN]: EU110092) spanning the gene of interest plus ~500bp flanking sequence was pasted in FASTA format into Primer3. Suggested primer pairs conformed to optimal primer characteristics (265) and were selected according to their location in conserved regions of the genome across HIV-1 clades A, C and D. To this end, a nucleotide sequence alignment consisting of 14 clade A (ANs: AM000055, AY521631, EU110092, AB287379, AY253314, AF004885, AB253429, AB253421, DQ396400, DQ676872, AY945736, AF286239, AF457051, AY371159) 10 clade C (ANs: AB254141, DQ369991, AY734556, AY713415, AY713416, AY713413, AF457054, EF469243, AY713417, AF443088) 7 clade D (ANs: EF633445, AF484499, AY253311, AJ488926, AY322189, AY371155 and K03454) and the HIV-1 reference clade B genome HXB2 (AN: K03455) was carried out using the ClustalW multiple sequence alignment application on the BioEdit Sequence Alignment Editor version 7.0.5.3(266) . Sequencing primers (**table 2.1**) were similarly

designed to sequence, with a minimum of 2-fold coverage, whole *gag, pol and nef*. All primers for PCR and sequencing were synthesised by MWG Operon.

2.1.6 Reverse transcription, nested PCR amplification of full length *gag, pol* and *nef*

HIV-1 *gag, pol* and *nef* sequences were amplified using a nested PCR protocol. The Titan One tube RT-PCR Kit (Roche Applied Science) was used for reverse transcription (RT) and first round PCR amplification of *gag, pol* and *nef* in a single reaction with primers, reaction components and thermocycling conditions listed in **table 2.2**. The Expand High Fidelity PCR System (Roche) was used to amplify the nested PCR products of *gag, pol* and *nef* with primers, reaction components and thermocycling conditions listed in **table 2.2**. Nested product were resolved on a 1% agarose gel (constituted in Tris-acetate- EDTA buffer [TAE]) ran with a 1kb DNA ladder (NEB) and the DNA visualized by ethidium brominde staining.

Table 2.1 Sequencing primers

Product	Primer	Sequence (5'-3')	Location relative to HXB2
Gag	CTLGagIF	AGCGGAGGCTAGAAGGAGAG	768-787
	CTLG01	ATCGTTCTAGCTCCCTGCTT	919-900
	CTLG00	GCATGGGTAAAAGTAGTAGAAGA	1249-1271
	CTLG03	ACTCTATCCCATTCTGCAGC	1433-1414
	CTLG02	TAGAAGAAATGATGACAGCATG	1817-1838
	CTLG05	TATGTGCCCTTCTTTGCCAC	1991-1973
	CTLG110	AGGCTAATTTTTTAGGGA	2078-2095
	CTLGagIR	AACCTCCAATTCCCCCTATC	2409-2390
Pol	CTLPolIF	GCTCTATTAGATACAGGAGCAGATG	2316-2340
	CTLGagOR	CCAATTATGTTGACAGGTGTAGG	2509-2487
	CTLP00	GCCTGAAAATCCATACAATACTCC	2702-2725
	CTLP01	AATATGCATCACCCACATC	2895-2877
	CTLP02	CAGTACAGCCTATAGTGCTGCCA	3268-3290
	CTLP03	GCCAATTCTAATTCTGCTTC	3460-3441
	CTLP04	AGTGGGAGTTTGTCAATACC	3787-3806
	CTLP05	ACTACAGTCTACTTGTCCATG	4400-4380
	CTLP06	CACAAAGGAATTGGAGGAAATG	4164-4185
	CTLP07	GAGCTTTGCTGGTCCTTTCC	4952-4933
	CTLP08	TAAGACAGCAGTACAAATGGCAG	4745-4767
	CTLPolIR	TAGTGGGATGTGTACTTCTGAACT	5217-5194
	AJB-1R	TATGGATTTTCAGGYCCAATTYTTG	2725-2702
	AJB-4F	ACACCAGAYAARAARCATCAGAAAG	3195-3219
	AJB-3R	TTCTGTATRTCATTGACAGTCCAGCT	3325-3300
	AJB-5R	GATTCCTAATGCATACTGTGAGTCTG	4064-4039
Nef	NefIF	CGAGGACTGTGGAACTTCTGG	8560-8680
	Nef00	ACACAAGGCTACTTCCCTGA	9557-9533
	Nef01	GTGTAATTCTGCCAATCAGGGA	9145-9164
	NefIR	GGTCTAACAAGAGAGACCCAGTACA	9179-9158

Table 2.2 Reverse transcription & Nested PCR primers, reaction components and thermocycling conditions

Product (bp)	Reaction	PCR primer, sequence (5'-3') and location relative to HXB2	Components of 1 reaction			Thermocycling conditions
Gag (1636)	RT-PCR	CTLGagOF: GTTCTCTCGACGCAGGACTC 680-699 CTLGagOR: CCAATTATGTTGACAGGTGTAGG 2509-2487	H$_2$0 5xBuffer dNTPs F primer R primer RNase inhibitor Enzyme Template	Vol. (µL) 10.5 5 0.5 1.25 1.25 1 0.5 5	Final conc. - 1x 200µM each 0.5mM 0.5mM 5 U - -	RT: 50˚C – 30 min. D: 94˚C – 2 min. D: 94˚C – 15 sec. A: 54˚C – 30 sec. 10x E: 68˚C – 2 min. D: 94˚C – 15 sec. A: 54˚C – 30 sec. 19x E: 68˚C – 2 min. Δt +5secs E: 68˚C – 7 min.
	Nested	CTLGagIF: AGCGGAGGCTAGAAGGAGAG 768-787 Gag IR: AACCTCCAATTCCCCCTATC 2409-2390	H$_2$0 10xBuffer dNTPs F primer R primer Enzyme Template	Vol. (µL) 39.25 5 1 1.5 1.5 0.75 1	Final conc. - 1x 200µM each 0.3mM 0.3mM -	Same as Gag RT PCR except RT step omitted
Pol (2902)	RT-PCR	CTLPolOF: TCCCTCAAATCACTCTTTGG 2251-2270 CTLPolOR: TGTATGCAGACCCCAATATGT 5262-5242	H$_2$0 5xBuffer dNTPs F primer R primer RNase inhibitor Enzyme Template	Vol. (µL) 10.5 5 0.5 0.5 0.5 1 0.5 5	Final conc. - 1x 200µM each 0.2mM 0.2mM 5 U - -	Same as Gag RT PCR except first two E steps proceeded for 3 minutes

Pol (2902)	Nested	CTLPolIF: GCTCTATTAGATACAGGAGCAGATG 2316-2340 CTLPolIR: TAGTGGGATGTGTACTTCTGAACT 5217-5194	Same as Gag nested PCR	Same as Pol RT PCR except RT step omitted
Nef (1014)	RT-PCR	CTLNefOF : TGTGCCTCTTCAGCTACCAC 8512-8531 CTLNefOR: CCCAGGCTCGATCTGGTC 9572-9554	Same as Gag RT PCR	RT: 50˚C – 30 min. D: 94˚C – 2 min. D: 94˚C – 15 sec. A: 54˚C – 30 sec.　　　40x E: 72˚C – 2 min. E: 72˚C – 7 min.
	Nested	CTLNefIF: CGAGGACTGTGGAACTTCTGG 8560-8580 CTLNef IR: GGTCTAACAAGAGAGACCCAGTACA 9557-9533	Same as Gag nested PCR	Same as Gag nested PCR except E temperatures at 72˚C and the first two E steps proceeded for 1 min

*RT: Reverse transcription D: denaturation A: annealing E:elongation

2.1.7 PCR product purification

PCR amplifications yielding a single sized band were directly purified using the PCR purification Kit (QIAGEN). PCR amplifications resulting in multiple sized bands were gel purified: 40μl of nested PCR product was mixed with 8μl of 6x loading dye and loaded, together with a 1Kb ladder (NEB), into a 1% agarose gel constituted in TAE buffer and electrophoresed for 30 minutes at 110V. The band corresponding to the desired product was cut out under a UV screen and either processed immediately or stored at -20˚C overnight and processed. The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen) by following the manufacturer's instructions taking care to carry out steps that increase sequencing efficiency. Purified DNA was eluted into 50μl of nuclease-free water and the DNA concentration was measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific) measuring absorbance at 260nm. Purified DNA was stored at -20˚C until used for sequencing.

2.1.8 DNA sequencing

gag, *pol* and *nef* products were sequenced using primers listed in **table 2.1** at a final concentration of 3.2 pmol/μl. Inner primers from the nested PCR reaction were used to sequence the 3' most and 5' most amplicon segments. DNA sequencing was carried out in a 48 capillary ABI-3730 DNA analyser using Applied Biosystems BigDye Terminator v3.1 chemistry.

2.1.9 Assembly of full length gag, pol and nef sequences

Overlapping sequence contigs were assembled and edited using the Geneious Software package (v 6.1.6, Biomatters Lmt.)(267). Sequence chromatograms were inspected for ambiguities. Ambiguous bases were defined using the Find Heterozygotes tool in the Geneious package v 6.1.6: peaks with quality scores >10 with a secondary peak height

above 25% of the maximal peak. Open reading frames were identified in Geneious and manual editing was carried out to correct premature stop codons following re-inspection of chromatograms.

2.1.10 HIV-1 subtyping and phylogenetic analysis of related HIV-1 sequences

Near full length HIV-1 *gag*, *pol* and *nef* sequences were analysed separately. Representative samples from the earliest infant sample following infection were used for subtype analysis. Pairwise alignments of all representative infant samples were made using MUSCLE(268) implemented in the Geneious package v6.1.6. Profile alignments were made in Clustal X2(269) of all infant sequences and the full LANLDB subtype reference alignment (2005). In a separate analysis, longitudinal infant sequences plus a maternal sequence, if available, were used to confirm appropriate phylogenetic clustering of related sequences. Pairwise alignments were carried out as above to make a final alignment of all mother-infant sequences. In all cases alignments were generated following manual inspection and editing in MEGA v5.2(270). The maximum likelihood method (ML) (271) was used to build phylogenetic trees. The online PhyML 3.0 interface(272) was used to construct ML phylogenetic trees under the General Time Reversible (GTR) model of nucleotide substitution (273), with estimated proportion of invariable sites and substitution rate heterogeneity. Branch support was estimated by the Approximate Likelihood Ratio Test (aLRT)-Shimodaira-Hasegawa-like (SH) procedure (274). Branches with SH values above 0.85 were considered as statistically well supported.

2.1.11 Recombinant subtype characterisation

Simplot and boostscan analysis was carried out to characterise recombinant isolates using Simplot v3.5.1 (275). A concatenated alignment (5009 base pairs) of near full

length *gag-pol-nef* sequences from infant isolates and the full HIV-1 LANLDB subtype reference alignment was made and gap stripped. A sliding window of 400bp was used with increments of 50bp. Bootscan analysis was carried out using the neighbour-joining method with the 2 parameter Kimura distance model, transition/transversion rate of 2.0 and 100 bootstrap replicated for each sliding window. Breakpoint coordinates relative to HXB2 were identified using the HIV Sequence Locator Tool(203) at the LANLDB. The Recombinant HIV-1 Drawing Tool(203) from the LANLDB was used to depict the breakpoint positions on the HIV-1 genome.

2.1.12 Bayesian inference of evolutionary rates

Time stamped longitudinal infant sequences were aligned using MUSCLE implemented in the Geneious package v6.1.6 and manually edited. Nucleotide substitution rates were estimated for each infant using a strict molecular clock model implemented in BEAST v.1.7.5 (276). The codon partitioning model SRD06 was chosen that implements the Hasegawa, Kishino and Yano (HKY) model of nucleotide substitution with discreet gamma-distribution rate variation among sites. A constant size coalescent model was implemented. This enabled evolutionary rates to be estimated separately at codon positions 1+2 and codon position 3 and to determine the rate ratio of codon position 1+2 to 3. Bayesian Markov chain Monte Carlo (MCMC) analysis was used to generate posterior distributions and the chains were run sufficiently long to ensure adequate mixing, determined upon inspection in Tracer v 1.5.

2.1.13 Evolution within an outside of CTL targeted epitopes.

Time-stamped longitudinal infant sequences were aligned using MUSCLE implemented in the Geneious package v6.1.6 and manually edited. Infant HLA types at

2-digit resolution and the current LANLBD annotation of CTL-restricted epitopes (http://www.hiv.lanl.gov/content/immunology/maps/maps.html) were used to partition sequences into regions restricted by infant CTL and those that were not. Evolution was then independently assessed in these regions through 2 approaches: 1) A maximum likelihood approach was implemented under the Tamura–Nei (TN93) substitution model assuming a strict molecular clock to estimate evolutionary rates; 2) Selective pressure was assessed under the codon based model Mause Gaut 94 (MG94)(184) and the non-synonymous (dN)/synonymous (dS) ratio were estimated. Both approaches were implemented in the HyPhy package (277) using *gag, pol* and *nef* evolution scripts(278)(279). Significance was assessed using the likelihood ratio test (LRT) with N-2 degrees of freedom.

2.1.14 Data analysis

Test statistics on evolutionary rates following Bayesian analysis were carried out using the Mann-Whitney test. Two-sided p values were calculated for analysis and values of $p<0.05$ were considered significant. Correlations between non-normally distributed data were computed using Spearman's rank correlation coefficient. Statistical analysis and graphical representations were made in GraphPad Prism version 6.0 (GraphPad Software inc.).

2.2   MATERIALS AND METHODS USED IN CHAPTERS 4 AND 5

2.2.1   Study participants

Participants were drawn from two cohorts that were recruited from localities within Kilifi District, Coastal Province, Kenya (**fig. 2.1**). The Infant Immunology Study (IIS) cohort is a prospective longitudinal cohort established to investigate immunological

health in HIV-1 exposed uninfected (HEU) infants. In addition a cross sectional cohort (CSC) was established to characterise age-normal values for the parameters under investigation in healthy infants from the community. The study protocol was reviewed and approved by the Oxford Tropical Research Ethics Committee (OxTREC; ref: 45-11) and the Kenyan Medical Research Institute Ethical Research Council (KEMRI-ERC; protocol no. SSC 2085).

2.2.2 The IIS cohort

The IIS cohort consists of HIV-1 exposed infants born to HIV-1 infected women who were recruited from the Comprehensive Care and Research Clinic (CCRC) located within Kilifi District Hospital, Coastal Province, Kenya. Mothers attending the CCRC with infants between 3 and 18 months of age were approached for recruitment and informed consent. Exclusion criteria were severe malnutrition based on mid-upper arm circumference and active acute or chronic infection. Participants received PMTCT care according to Kenyan national guidelines which are based on WHO 2009 updated recommendations (116). Infant HIV-1 status was determined by blood spot PCR at 6 weeks of age, by rapid antibody test at 9 months of age and by rapid antibody test at 18 months of age. Infants were followed longitudinally at 3 monthly-intervals from recruitment until exit from the study at 24 month of age. At each study visit, a blood sample (detailed below) was taken and routine clinical data, vaccination records, breast-feeding practices, starting or changing of ART, nutritional status, significant infections as well as CD4 counts were recorded for both the mother and infant. The present study was nested within the IIS cohort and designed as a descriptive cross-sectional study to characterise T cell responses to vaccinations delivered under the Expanded Programme on Immunisation (EPI) and to investigate immunological

parameters that may affect these responses. 20 3-month and 42 12-month old infants were included in the study.

2.2.3 The CSC cohort

The CSC cohort consists of infants recruited from 3 localities within Kilifi District: Ganze, Junju and Negerenya (**fig. 2.1**). Infants from Ganze were recruited from community clinics during routine vaccination visits. Infants from Junju and Ngerenya were participants of a community cross-sectional malaria surveillance cohort. Samples were obtained to establish cross-sectional age-normal values for the parameters under investigation and therefore a single sample was obtained per participant. Exclusion criteria were acute or chronic illness as established by clinical examination. Due to ethical considerations, infant HIV-1 status could not be determined in this cohort. Prevalence data for HIV infected infants in well-nourished infants at Kilifi District Hospital has been shown to be 2% (280) . The severity of neonatal HIV-1 pathology and the high level of antenatal screening for HIV-1 infection in these communities make it unlikely that HIV-1 positive or HEU infants were recruited in this cohort. 10 infants of between 2.5-4.2 months of age and 18 infants of between 9.7-13.9 months of age were recruited for the present study. Vaccination data was obtained from infant vaccination cards with records of the type and date of vaccination. Ethical approval was granted by OxTREC (ref: 45-11) and KEMRI-ERC (protocol no. SSC 2085).
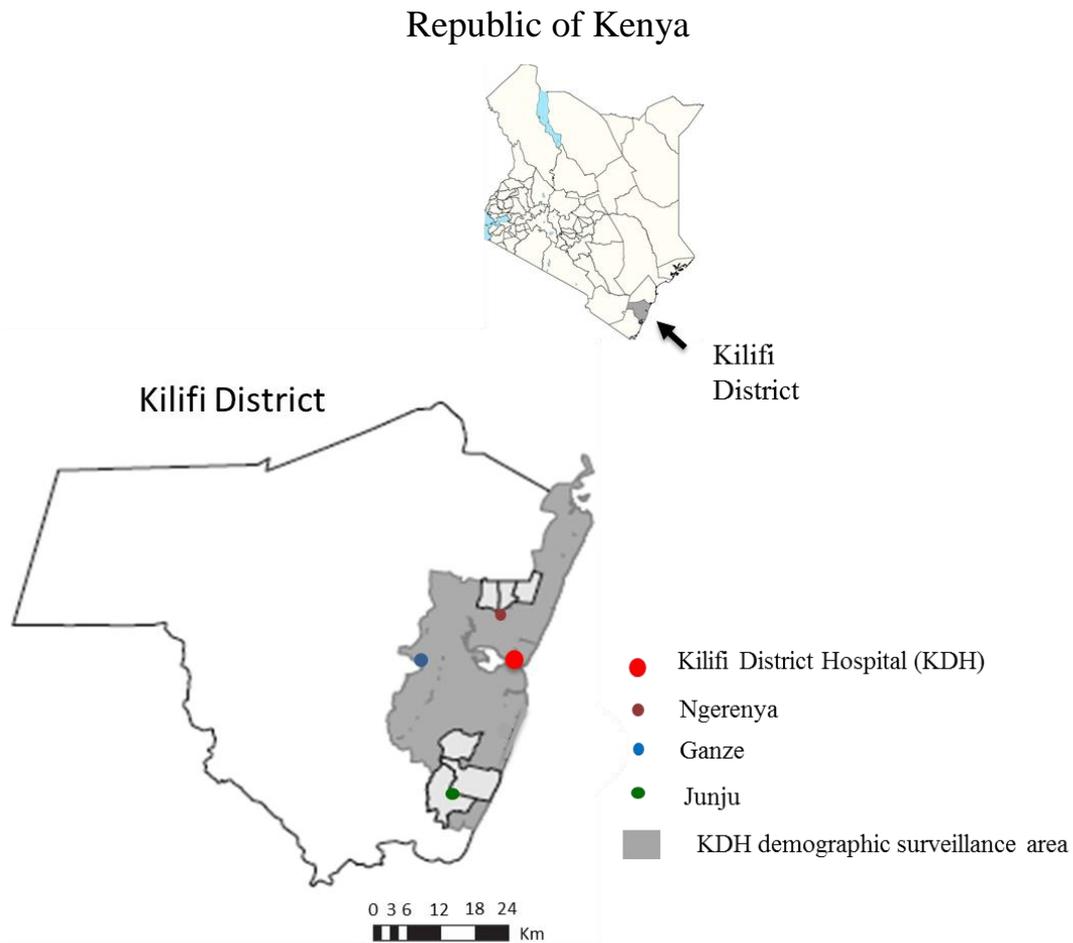
Figure 2.1. Study area indicating locations of patient cohorts. Participants residing in Kilifi District, Coastal Province, Republic of Kenya were recruited onto the study cohorts. The Comprehensive Care and Research Clinic (CCRC) is located within the Kilifi District Hospital (KDH; red). The CCRC cohort participants were mostly residents of Kilifi town and neighboring localities within the KDH demographic surveillance areas (grey). Control infants were recruited from geographically representative localities of Ngerenya (maroon), Junju (green) and Ganze (blue).

2.2.4 Blood collection and complete blood counts

At each study visit a maximum of 5mL of whole blood was collected by a study clinician, 3mL in heparin and 2mL in EDTA Vacutainer tubes (BD). Samples were

stored at room temperature until processing. EDTA blood was used to determine complete blood counts using a Coulter MDII-18 counter (Beckman-Coulter, Fullerton CA, USA)

2.2.5   IL-7 Enzyme Linked Immunosporbent Assay (ELISA)

IL-7 was measured in heparinised peripheral blood plasma using the human IL-7 Quantikine HS ELISA kit (R&D) according to the manufacturer's instructions. Plasma stored at -80°C was thawed at room temperature and assayed following the first thaw.

2.2.6   Titration of fluorescently-conjugated antibodies

Each antibody used in the study was initially titrated to estimate the optimal antibody concentration per test. Antibody titration was carried out by singly staining whole blood lymphocytes following the same staining protocol as used in the corresponding assay with the complete antibody panel (described below). Initially, 3 concentrations of a particular antibody were used, commonly 1, 3 and 5 μL/mL (the recommended volume stated by the manufacturer was used to guide the titration) and MFI of positive and negative events was plotted against concentration (**fig. 2.2**). The goal was to reach antibody saturation of the target molecules and not to add excess antibody which may result in non-specific staining. If saturation was not achieved at 3 concentrations, additional antibody concentrations were tested e.g. 1, 3, 5, 7, 10 μL/mL.
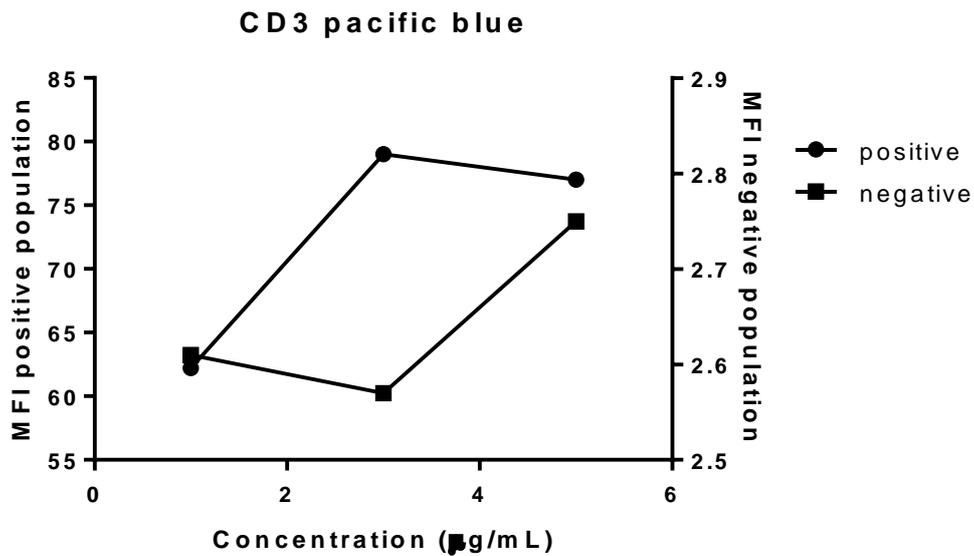
CD3 pacific blue

Figure 2.2. Titration of CD3-Pacific Blue (PB) antibody. Lymphocytes were gated on based on size and granularity and CD3-PB positive and negative populations were gated on. The MFI of these populations was used to set the cut off for saturation. Saturation is indicated by a lack of increase or a decrease in the MFI of the positive population with increasing antibody concentration; non-specific staining is indicated by an increase in the MFI of the negative population with increasing antibody concentration. The optimal concentration in this example was approximated to be 3μg/mL.

2.2.7   Optimisation of flow cytometry antibody panels

In order to assess cell phenotype and cytokine expression in specific immune cell populations the antibody panels shown in **table 2.4** were developed. Briefly, markers of interest were selected and these were grouped based on likely expression levels: high, intermediate or low. Fluorochromes were also grouped based on the brightness of the fluorescent signal emitted. Markers with high expression levels and/or that identify discreet cell populations (e.g. CD3 or CD4) were assigned to fluorochromes with the

65

dullest staining (e.g. PerCP). Those markers with unknown staining pattern, that required clear discrimination between positive and negative events (e.g. cytokines), that are weakly expressed or expressed on only a small fraction of cells were assigned to bright fluorochromes (e.g. PE) and/or fluorochromes with little spill over from other fluorescent channels (e.g. those exited by the 405nm violet laser). Fluorochrome/antibody combinations were also identified based on the degree of spectral overlap between fluorescent dyes. Minimization of spectral overlap was used to enhance the specificity of the detection of a particular maker following compensation (see below). The staining patterns were assessed following the step-wise addition of a particular conjugated antibody (281). High costs precluded the testing of all antibody combinations; a backbone of antigens with high expression levels conjugated to a limited number of fluorochromes was used and different fluorochromes (up to 3) were tested for markers with low levels of expression.

2.2.8 *Ex vivo* immunophenotyping

Remaining EDTA blood was spun by centrifugation at 1800 rpm and plasma was removed from packed cells, stored at -80°C and the volume of plasma replaced with RPMI supplemented with 10% inactivated new-born calf serum (NBBS) 0.01% β-mercaptoethanol, 1% penicillin/ streptomycin, 1% L-glutamate and 1% Hepes (R10). Packed cells were re-suspended in R10. Immunophenotyping of cell surface and intracellular markers was carried out to measure different parameters in a total volume of 100uL with 4 different antibody panels shown in **table 2.4**. At each independent experiment i.e. each separate day that cells were processed, and for each antibody panel, an isotype control staining was carried out using left-over infant cells. Isotype controls were used at the same concentration as their corresponding test antibodies and

the cells processed under the same conditions. Details of the antibodies used are shown in **table 2.3**. All stained cells were stored at 4°C until acquisition and acquired within 24hrs.

1. *T cell activation and exhaustion*

Whole blood cells were incubated with the following antibodies: anti-CD3 PB, anti-CD4 PerCP, anti-CD8 APCH7, anti-HLA-DR FITC, anti-CD38 PECy7, anti-PD-1 APC and anti-Tim-3 PE for 20 minutes at room temperature. RBC lysis was carried out using BD FACS lysing solution and cells were washed once in PBS before acquisition. Matching isotype controls conjugated to FITC, PE, PECy7 and APC were used in combination with all other surface stains to make the isotype control cocktail.

2. *T regulatory cells*

The human regulatory T cell whole blood staining kit (eBiosciences) was used according to the manufacturer's instructions. The importance of antibody clone and buffer use in detection of FoxP3 has been reported (282). Antibody clone PCH101 conjugated to FITC was used to detect FOXP3 expressing cells. Whole blood cells were initially surface stained with anti-CD3 APCH7, anti-CD4 PB and either anti-CD25 PE alone or anti-CD25 APC and anti-CD127 PE. Following RBC lysis, cells were washed once in FACS buffer (PBS + 10% NBBS + 1% penicillin/streptomycin) and incubated for 45 minutes in 1X fixation/ permeabilisation solution. Cells were pelleted and blocked with 2% rat serum for 15 minutes and incubated with anti-FOXP3 FITC for 45 minutes and washed in FACS buffer. A matching isotype control conjugated to FITC plus all other surface stains made up the isotype control cocktail

3. Dendritic cell and basophil frequencies

Whole blood was incubated with the following antibodies: anti-lineage FITC, anti-HLA-DR PerCP, anti-CD11c APC, anti-CD123 efluor 450, anti-CD86 PE and either anti-CD83 PECy7 or anti-CD274 (PD-L1) PECy7 for 20 minutes at room temperature. RBC lysis was carried out using BD FACS lysing solution and cells were washed once in PBS before acquisition. Matching isotype conjugated to PE and PECy7 were used in combination with all other surface stains to generate the isotype control cocktail

4. T cell memory subsets

Whole blood was incubated with anti-CD3 PB, anti-CD4 PerCP, anti-CD8 Qdot 605, anti-CD45RA PECy7, anti-CCR7 APC and anti-CD127 PE. To detect the intracellular marker Blc-2, surface stained cells were permeablised with 1X BD permeabilisation buffer and incubated with anti-Bcl-2 FITC for 20 minutes. Matching isotypes conjugated to PE, PECy7, APC and FITC were used in combination with all other surface stains to generate the isotype control cocktail.

Table 2.3 Antibodies used for immunophenotyping and ICS analysis

Marker	Fluorochrome	Clone	Manufacturer
CD3	APC H7	SK7	BD
CD3	Pacific Blue	SP34-2	BD
CD4	PerCP	RPA-T4	BioLegend
CD4	Pacific Blue	RPA-T4	BD
CD8	Qdot 605	3B5	Invitrogen
CD8	APC H7	SK1	BD
HLA-DR	FITC	LN3	BioLegend
HLA-DR	PerCP	L243	BD
CD38	PE Cy7	HIT2	Biolegend
CD25	APC	BC96	eBiosciences
CD25	PE	2A3	BD
CD279 (PD-1)	APC	eBioJ105	eBiosciences
Lineage cocktail (CD3/14/16/19/20/56)	FITC	Various	BD
Tim-3	PE	F38-2E1	Biolegend
FoxP3	FITC	PCH101	eBiosciences
CCR7	APC	150503	R&D Systems
CD45RA	PE Cy7	L48	BD
CD11c	APC	S-HCL-3	BD
CD123	eFluor 450	6H6	eBiosciences
CD86	PE	2331(Fun-1)	BD
CD83	PE Cy7	HB15e	BD
CD274 (PD-L1)	PE Cy7	MIH1	eBiosciences
CD127 (IL-7R)	PE	HIL-7R-M21	BD
Ki67	PE Cy7	B56	BD
Bcl-2	FITC	Bcl-2/100	BD
Bcl-2	Alexa Fluor 647	BCL/10C4	Biolegend
CD69	FITC	L78	BD
IL-10	PE	JES3-19F1	Biolegend
IFN-γ	APC Cy7	4S.B3	BioLegend
IL-2	FITC	MQ-17H12	BioLegend
IL-4	APC	8D4-8	BioLegend
IL-17A	PE Cy7	BL168	BioLegend
TNF-α	PE	MAb11	BD

2.2.9 Short-term whole blood stimulation with vaccine antigens

An optimised assay developed to quantitate cytokine responses by ICS from small whole blood volumes following short term stimulation was adapted (283). Soduim heparinised whole blood collected in Vacutainer tubes® (BD) was processed within 4 hours. Blood plasma was separated from packed cells by centrifugation, and the volume of plasma replaced with R10. 200uL of whole blood cells in R10 were cultured in standard polypropylene 2 mL screw cap tubes with the following: CD49d and CD28 costimulatory antibodies (eBiosciences) alone at a final concentration of 0.5ug/mL each, or in combination with either tetanus toxoid (Indian Serum Institute) at 10ug/mL final concentration, purified protein derivative of *Mycobacterium tuberculosis* (PPD; Statens Serum Institute) at a final concentration of 20ug/mL or staphylococcus enterotoxin B (SEB; Sigma-Aldrich) at a final concentration of 1ug/mL. Samples were cultured in a water bath at 37°C for 7 hours at which point brefeldin A (Sigma-Aldrich) was added to the cells at a final concentration of 10ug/mL. Cells were cultured for an additional 5 hours at 37°C at which point the water bath was programmed to switch off. After 10hrs, red blood cells were lysed in 2 mL of 1X BD FACS lysing solution and following centrifugation, the white blood cell pellet was re- suspended in NBBS with 10% DMSO, transferred to a CryoTube (Nunc), frozen in a step-wise manner and stored in liquid nitrogen.

2.2.10 Intracellular cytokine staining

In order to detect intracellular cytokines, stimulated whole blood cells were rapidly thawed at 37°C in a water bath, washed once in PBS and incubated for 10 minutes in 1X BD permeabilisation buffer. Following centrifugation, the supernatant was decanted and pelleted cells were re-suspended in 50 uL residual permeabilisation buffer. To the

cells, fluorochrome-conjugated antibody cocktails were added. 3 antibody panels were developed for the detection of intracellular cytokines detailed in **table 2.4**. These were composed of CD3 pacific blue (BD) CD4 PerCP, CCR7 APC, CD45RA PECy, IFN-γ APC-Cy7, IL-2-FITC, TNF-α PE, CD127 PE, Ki67 PECy7, Bcl-2 Alexa Fluor 647, IL-4 APC, IL-10 PE, IL-17 PECy7 and CD69 FITC. Cells were incubated at room temperature for 20 minutes and washed once in 1X permeabilisation solution before final re-suspension in PBS.

2.2.11 Flow cytometry

Flow cytometric acquisition was carried out on a Cyan ADP (Dako) configured with 3 lasers and 9 fluorescence detector channels, with Summit 4.3 software. Single stain compensation controls were set using anti-mouse kappa beads (BD) stained with the respective fluorochrome-conjugated antibody for all experiments. For the *ex vivo* immunophotyping of T cell populations, a minimum of 70,000 CD3+ events were collected. For DC and basophils immunophenotyping, a minimum of 200,000 cells within the leucocyte gates were acquired. For all ICS experiments, the maximum number possible of cellular events was acquired and the rate of acquisition was kept under 1000 events per second. In order to minimize doublet events, alterations to the event acquisition rate were kept to a minimum.

2.2.12 Data analysis

Compensation matrixes and analysis was carried out in Flow Jo version 7.6 for the PC (Treestar). Flow Jo template files were created to enable consistent gating strategies to be followed. Flow Jo tables were generated and exported into Excel (Microsoft). Statistical analysis and graphical representations were made in GraphPad Prism version 6 (GraphPad Software inc.).

Table 2.4 Antibody panels used for ex vivo phenotyping and intracellular cytokine staining

Channel	Fluorochrome	Immune activation	T reg	T reg	DCs & basophils	Ex vivo T cell memory	Antigen specific Panel 1	Antigen specific Panel 2	Antigen specific Panel 3
FL1	FITC	HLA-DR	FoxP3	FoxP3	Lin	Bcl-2	IL-2	IL-2	CD69
FL2	PE	Tim-3	CD25	CD127	CD86	CD127	TNFα	CD127	IL-10
FL3	Qdot 605	-	-	-	-	CD8	-	-	-
FL4	PerCP	CD4	-	-	HLA-DR	CD4	CD4	CD4	CD4
FL5	PECy7	CD38	-	-	CD83/PD-L1	CD45RA	CD45RA	Ki-67	IL-17
FL6	PB/eFluor450*	CD3	CD4	CD4	CD123*	CD3	CD3	CD3	CD3
FL8	APC/AF647*	PD-1	-	CD25	CD11c	CCR7	CCR7	Bcl-2*	IL-4
FL9	APCH7/Cy7*	CD8	CD3	CD3	-	-	IFN-γ*	IFN-γ*	-

3   CHAPTER 3:   EVOLUTION IN HIV-1 *GAG, POL* AND *NEF* IN EARLY INFANT INFECTION

3.1   INTRODUCTION

The rate of evolution in HIV-1 is one of the highest of any organism leading to a striking degree of diversity both within an infected host and amongst populations of infected humans(284). Numerous factors contribute to this rapid diversification, including the error prone nature of viral reverse transcription, the high rate of recombination, and a high replicative capacity. In addition, molecular plasticity enables rapid viral adaptation to host immune selection pressures. One of the principal immune selection pressures that drives evolution in early adult infection is the CTL response. However, the role of the CTL response in shaping HIV-1 evolution in early infant HIV-1 infection is poorly understood. Clear differences in the natural history of acute infection, in particular with respect to viral population dynamics, and in the nature of the CTL response exist between infants and adults. Vertically infected infants experience a greater rise in peak viraemia(153) and experience a gradual decline in viral load over the first 2 years of life(158). By contrast acute HIV-1 infection in adults is characterised by a decline in peak viral load over 12-20 weeks that reaches a set point(160). This viral load decline is associated with the onset of the CTL response and it is during this stage that the adult CTL response has been shown to select for multiple CTL-escape variants and drive molecular adaptation in the viral quasispecies(194,285). Numerous reports indicate that infants mount HIV-specific CTL responses early during infection (171–173). In addition, these responses have been shown to be able to select viral escape variants, indicating that at least in some cases, infant HIV-specific HIV-1

responses may be functional(177,199). However, it is unclear to what extent CTL-selection pressure drives HIV-1 evolution in early life. This study therefore aimed to 1) characterise rates of evolution in 3 distinct genetic regions of the HIV-1 genome that have been shown to be common targets of the CTL response, *gag, pol* and *nef* and 2) to assess the impact of CTL-driven selection on evolution in *gag*, *pol* and *nef* by comparing evolutionary rates within and outside of known CTL epitopes restricted by infant HLA alleles.

The evolutionary forces that drive intrahost viral diversification have also shaped the diversity of viral subtypes that constitute the global HIV-1 epidemic. Current data on the epidemic in Kenya indicate that A1 subtypes predominate (71.1% of sequences submitted to LANLDB(203)) followed by subtypes D (13.4%) and C (5.3%), with apparent low level prevalence of CRFs and other major subtypes. Accurate data on subtype distribution is important for epidemiological monitoring of the AIDS epidemic, and also plays important roles in viral diagnostics and vaccine design. This study therefore sought to characterise the subtype distribution of viral isolates from this cohort.

3.2 RESULTS

3.2.1 Cohort characteristics

Study participants were enrolled into the CTL cohort study between 1999 and 2002 at a time when ART for the prevention of MTCT was not widely available in Kenya. AZT monotherapy was provided for the women in the study in the last trimester of pregnancy as part of the trial. This was a prospective study designed to assess whether HIV-specific cytotoxic T cells were associated with protection from MTCT of HIV-1 and to identify correlates of disease progression in HIV-1 infected infants. Samples from a subset of 32 infants whom acquired HIV-1 through MTCT met the inclusion criteria for this study. 18 (56%) of the infants were male and had a median peak viral load of 2.9 x 10^7 viral RNA copies/mL of plasma (range $3.1x10^5$-$2.7x10^8$) and a mean CD4 count of 1599 cells/mL (range 165-3692); the majority of infections occurred *peripartum* or through early breast milk transmission (63%), the remainder *in utero* (**table 3.1**). Two analyses were carried out in this study. The first concerns the analysis of the HIV-1 subtypes that were transmitted to the infants. For this analysis all 32 infants were included. The second analysis concerns the characterisation of intra-patient evolution within the first two years of life following *in utero* or *peripartum* infection. This analysis was restricted to infants for whom 3 or more longitudinal sequences were obtained from *gag*, *pol* or *nef*: 17, 14 and 14 infants, respectively (**table 3.4**).

Table 3.1 Cohort characteristics and subtype distribution	
Number of infants	32
Male (%)	18 (56)
***Peripartum* transmission (%)**	20 (63)
Mean peak HIV-1 RNA copies/mL plasma (range)	2.87×10^7 (3.12×10^5 - 2.71×10^8)
Mean CD4 count in cells/mL (range)	1599 (165-3692)
A1 (%)	20 (62.5)
D (%)	4 (12.5)
Recombinants (%)	8 (25.0)

3.2.2   Viral subtyping

Viral subtypes were assigned to viral isolates obtained from the earliest time point available following transmission. *gag, pol* and *nef* genetic fragments were initially analysed individually. Alignments of infant isolates to the LANLDB subtype reference alignments (which contain reference sequences for each major HIV-1 subtype and CRF) were carried out. maximum likelihood phylogenetic trees were constructed as described in the methods section for *gag* (**fig. 3.1**) *pol* (**fig. 3.2**) and *nef* (**fig. 3.3**).

The majority of infant isolates (62.5%) clustered deep within the sub-subtype A1 cluster with high statistical branch support (SH >0.9) following analysis of all 3 genetic regions and are therefore likely pure A1 subtype isolates (**table 3.1**). This was consistent with the overall percentage of Kenyan A1 subtype sequences submitted to LANLDB(203) (71.1%). Similarly, 4 infant isolates (12.5% vs 13.4% from LANDB) clustered with reference D subtype references sequences with high statistical branch support (SH >0.9) in all 3 genetic regions analysed. This phylogenetic analysis also indicated that 8 isolates (25%) were likely recombinant forms of pure subtypes (shown in green in **figs. 3.1, 3.2 and 3.3**.). This was suggested by a) statistically supported clustering of an infant isolate with a known CRF (e.g. **fig. 3.2** isolate 454) b) statistically supported clustering with a particular subtype in one genetic region that

changed upon analysis of another genetic region (e.g**. Figs. 3.2 and 3.3** isolate 313) c)

indeterminate clustering across genetic regions (e.g. **Figs. 3.1 and 3.2** isolate 211) or d)

long branch lengths within a particular cluster (e.g. **Fig. 3.2** isolate 161).

Figure 3.1 Phylogenetic subtype classification of *gag* sequences from 32 mother-infant transmission pairs. The full Los Alamos (2005) subtype reference dataset was used to construct the tree rooted on subtype P. Viral isolates from the earliest infant sampling time point were used. Pure subtypes are shown in red and recombinant sequences in green. The maximum likelihood (ML) method (271) was used to build the phylogenetic tree under the General Time Reversible (GTR) model of nucleotide substitution (273), with proportion of invariable sites and substitution rate heterogeneity. Branch support was estimated by the Approximate Likelihood Ratio Test (aLRT)-Shimodaira-Hasegawa-like (SH) procedure (274); branches with support values >0.85 are shown in yellow and > 0.9 in red.
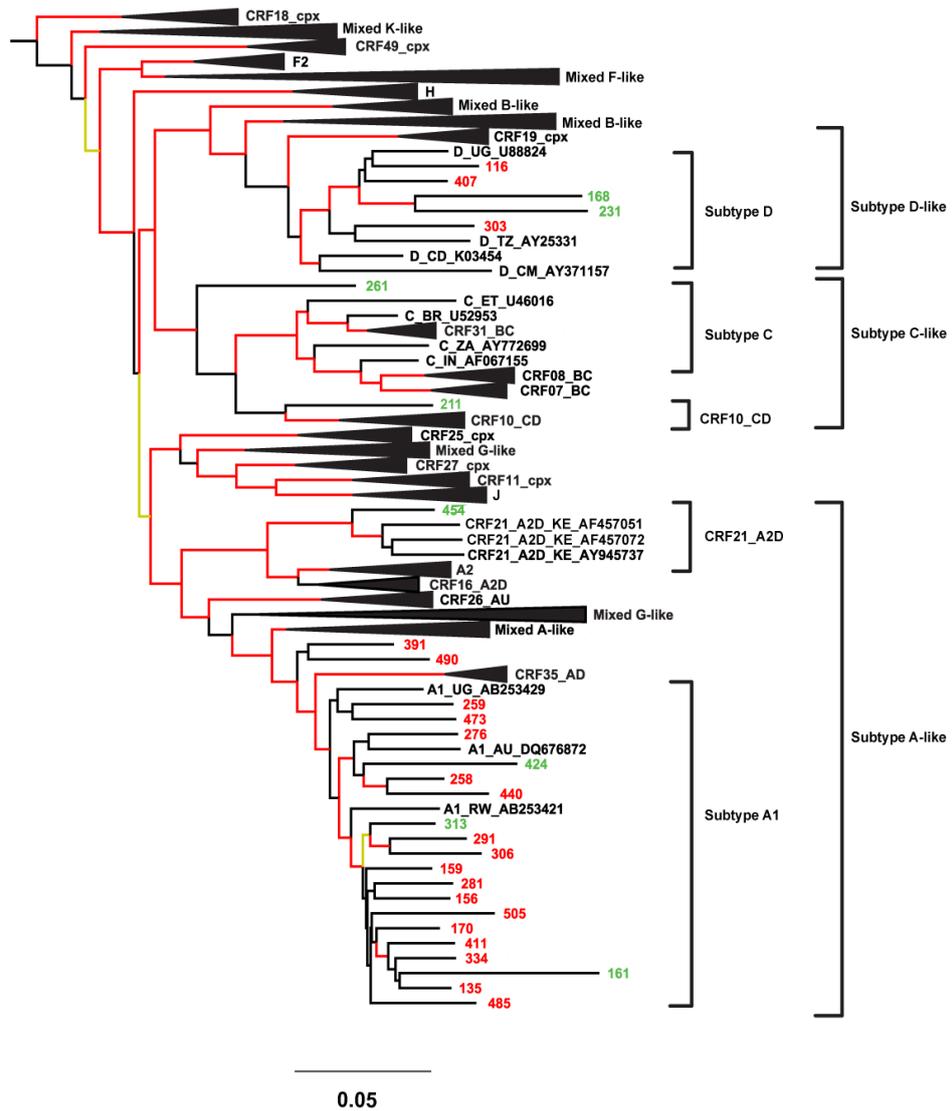
Figure 3.2 Phylogenetic subtype classification of *pol* sequences from 29 mother-infant transmission pairs. The full Los Alamos (2005) subtype reference dataset was used to construct the tree rooted on subtype P. Viral isolates from the earliest infant sampling time point were used. Pure subtypes are shown in red and recombinant sequences in green. The ML method (271) was used to build the phylogenetic tree under the GTR model of nucleotide substitution (273), with proportion of invariable sites and substitution rate heterogeneity. Branch support was estimated by the aLRT-SH procedure (274); branches with support values >0.85 are shown in yellow and > 0.9 in red.
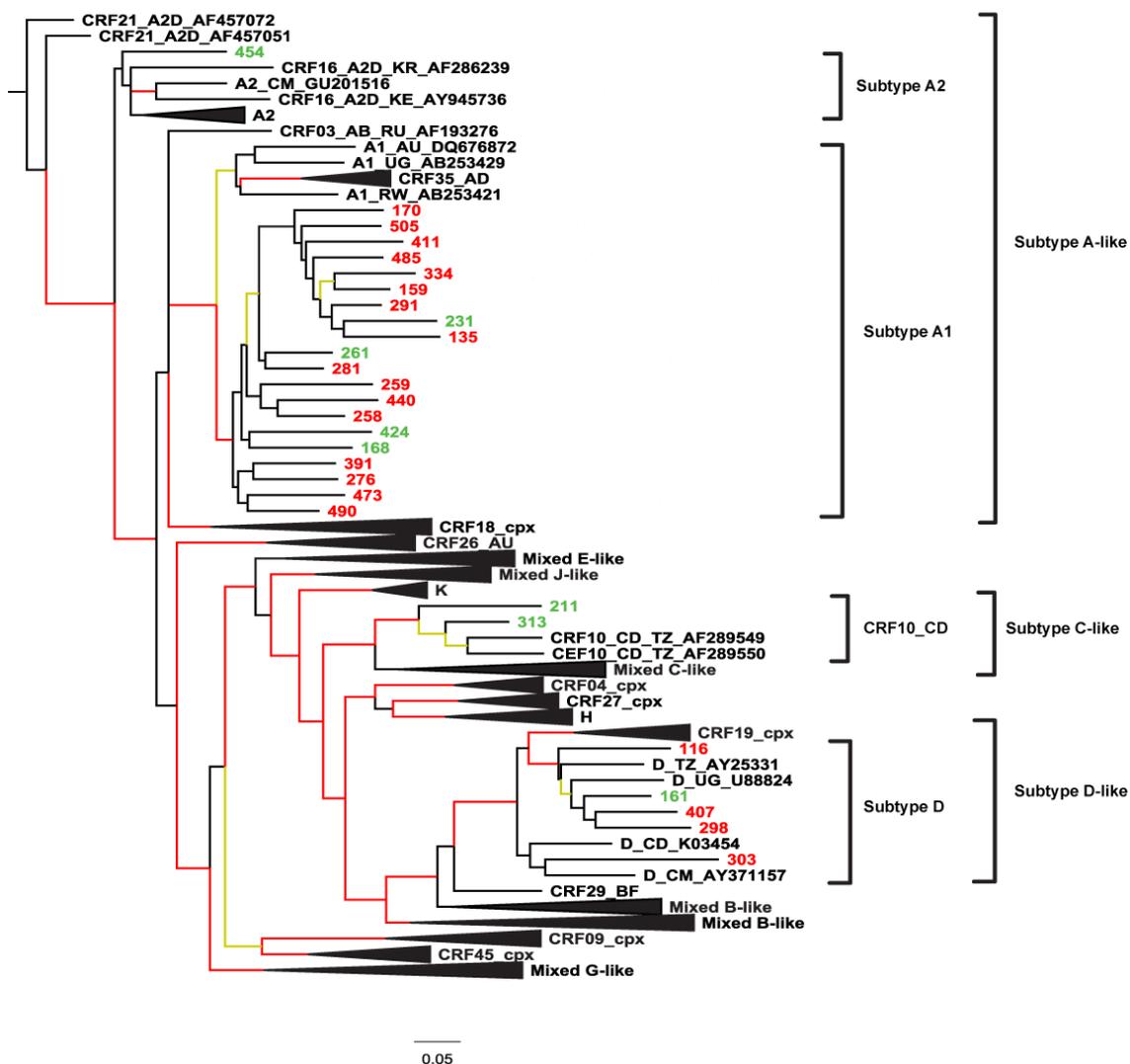
Figure 3.3 Phylogenetic subtype classification of *nef* sequences from 28 mother-infant transmission pairs. The full Los Alamos (2005) subtype reference dataset was used to construct the tree rooted on subtype P. Viral isolates from the earliest infant sampling time point were used. Pure subtypes are shown in red and recombinant sequences in green. The ML method (271) was used to build the phylogenetic tree under the GTR model of nucleotide substitution (273), with proportion of invariable sites and substitution rate heterogeneity. Branch support was estimated by the aLRT-SH procedure (274); branches with support values >0.85 are shown in yellow and > 0.9 in red.

In order to confirm that these isolates represent recombinant forms and to further characterise them, bootscan analysis was carried out in Simplot v3.5.1 (275), using a concatenated alignment of *gag-pol-nef* infant isolates and the LANLDB subtype reference alignment. **Figure 3.4** depicts a representative analysis from viral isolate 168 in which a double recombination event within *pol* produced an A1D recombinant; the A1 subtype corresponded to the 3' region of *pol* and this was also found for *nef*. **Table 3.2** summarised the results for all recombinant isolates identified, showing a high frequency of A1D recombinants, though none shared the same recombination breakpoints. The majority of breakpoints (71%) were found to occur in *pol*. No recombinant isolates correspond to previously identified CRFs classified in the LANLDB and are therefore considered to be unique recombinant forms (URFs).
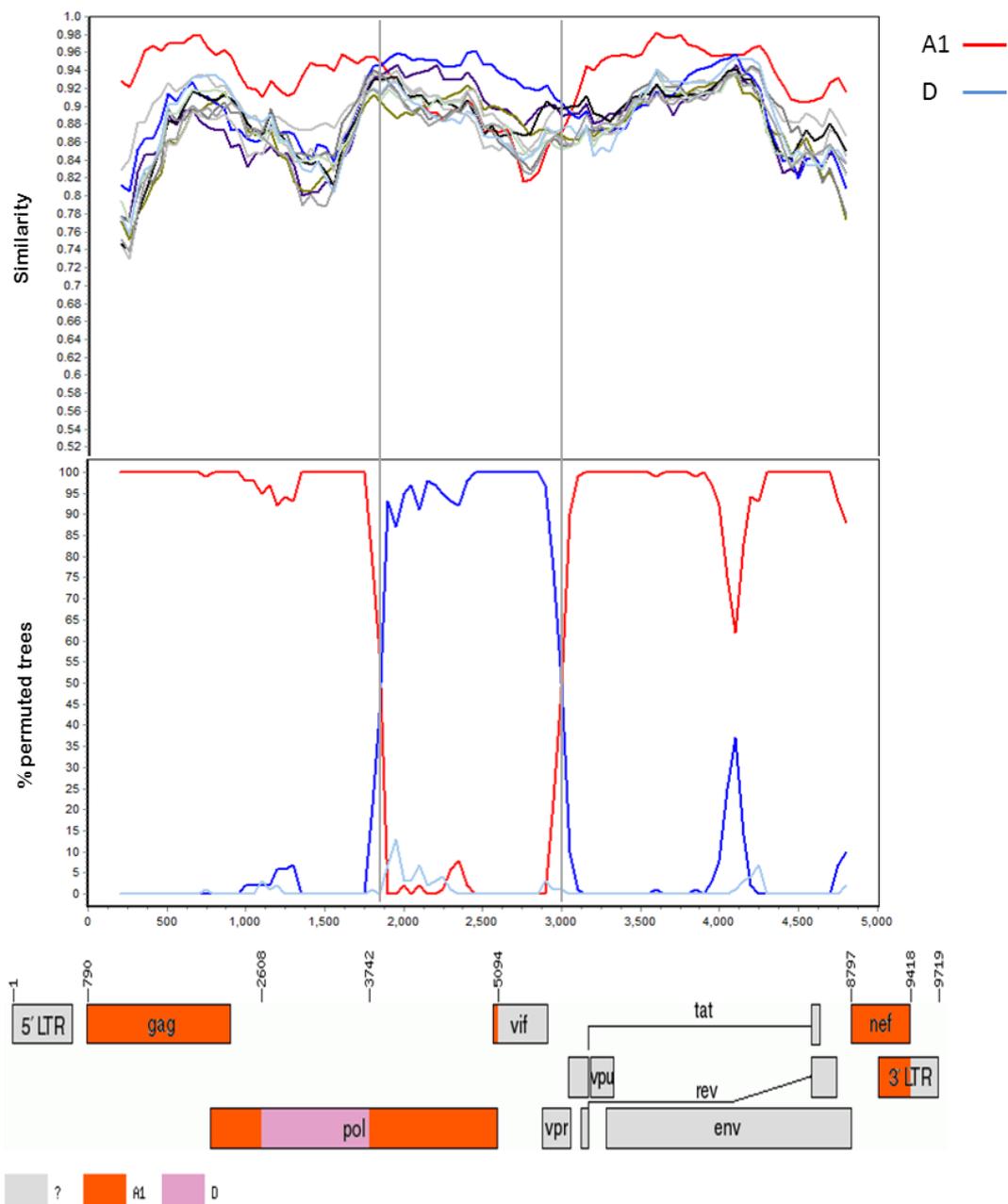
Figure 3.4 Recombination analysis of the viral isolate from infant 168. Simplot (upper plot) and bootscan (lower plot) analysis was carried on concatenated *gag*, *pol* and *nef* sequences of the viral isolate from infant 168 to assess subtype recombination. Recombination breakpoints are indicated with vertical grey lines and the positions were mapped onto the HIV-1 genome using the LANL database Recombinant HIV-1 Drawing Tool.

Table 3.2 Recombinant viral isolates

Patient	Parental subtypes	Breakpoints relative to start of HXB2 (bp)
161	A1, D	*2607*
168	A1, D	*1383*, 2720, 4362
211	CRF10, C	*1325*, 2920
231	A1, D	*1865*, 3494, 4124. 4814
261	A1, C	3361, 3776, 3869, 4887
313	A1, CRF10	4931
424	A1, A2, D	*1284*, *2173*, 2643, 4264, 4767
454	CRF21, A2	4984

Breakpoint within *gag* shown in italics, otherwise breakpoint in *pol*

3.2.3 Characteristics of individuals with sequences from 3 or more longitudinal time points

In order to assess the evolutionary dynamics of HIV-1 during early infant infection, a subset of infants with sequences from 3 or more longitudinal time points were selected. A total of 20 infants met this criterion for one or more genes, with 17, 14 and 14 infants meeting the criterion for *gag pol* and *nef* sequences, respectively (**table 3.3**). In total 13 (70%) were male and 13 (70%) became infected *peripartum*. The median peak viral load (range) was 1.14×10^7 viral RNA copies/mL of plasma (8.6×10^5-1.40×10^8); median (range) CD4 % and absolute CD4 T cell counts were 21% (7-34%) and 1500 cells/mL (576-3429 cells/mL) respectively.

3.2.4 Exclusion of sample mix-up and contamination

In order to exclude the possibility of mix-up or contamination of longitudinal samples during PCR/sequencing procedures a phylogenetic analysis was carried out of all infant samples and a maternal sample if available. **Figure 3.5** depicts ML phylogenetic trees in which monophyletic clusters were observed for all longitudinal infants sequences and corresponding maternal samples with robust statistical branch support (SH values>0.9) in all instances except 2 (*gag* 259 and *gag* 135).

Table 3.3 Characteristics of infants in which evolutionary dynamics were assessed

Patient	Sex	Mode of transmission	Peak VL$^{\delta}$ (HIV-1 RNA copies/mL plasma)	CD4 %	CD4 count (cells/mL)	Number of time points			HLA-A	HLA-B	HLA-C
						gag	*pol*	*nef*			
135*	M	IU	45033000	10	576	3	0	3	32, 6802	39, 44	04, 12
159	F	P	8690500	26	1194	6	0	4	29, 30	4501, 4501	06, 06
168*	M	P	140120000	22	2483	6	3	6	24, 29	15, 5802	02, 04
170	M	IU	862400	21	1500	4	4	3	30, 3402	4201/2, 57	07, 17
211	M	P	43129000	18	1279	6	4	6	30, 6802	4201/2, 4201/2	07, 17
231	F	P	1803300	22	2195	4	4	4	26, 34	35, 53	04, 04
258	M	IU	4775200	25	1124	4	4	2	03, 30	15, 7301	15, 17
259	M	P	17534000	34	3429	3	4	2	23, 74	58, 4501	07, 07
261	F	P	3700400	28	2180	5	3	5	2, 29	5802, 35	06, 07
281	M	P	3949100	25	1682	4	3	4	23, 74	15, 15	02, 02
291	M	P	17049000	14	902	6	4	6	29, 74	4201/2, 15	02, 17
303*	M	P	41542000	17	1639	0	3	0	30, 30	4501, 4501	1601, 1601
313	M	IU	28073500	21	976	4	0	3	33, 6802	15, 35	03, 04
334*	F	P	2907200	26	2372	4	0	4	29, 26/66	13, 15	02, 06
391	M	P	8345900	17	1643	0	3	0	74, 32	42, 58	06, 17
411	F	P	14114000	16	637	3	4	4	30, 30	15, 42	14, 17
424*	F	IU	28067000	7	669	3	0	3	02, 6802	51, 08	07, 1601
440	M	IU	7646400	19	760	4	0	2	02, 30	4501/03, 4902	06, 1601
454	M	P	43164500	29	2627	0	4	4	02, 31	08, 15	07, 08
485	M	IU	2462800	N/A	N/A	4	3	2	N/A	N/A	N/A

*Died <2 years of age. VL=viral load. $^{\delta}$ Maximum VL measurement within 6 months of infection $^{\Phi}$VL measurement at least 6 weeks after peak VL. IU= *in utero*; P= peripartum
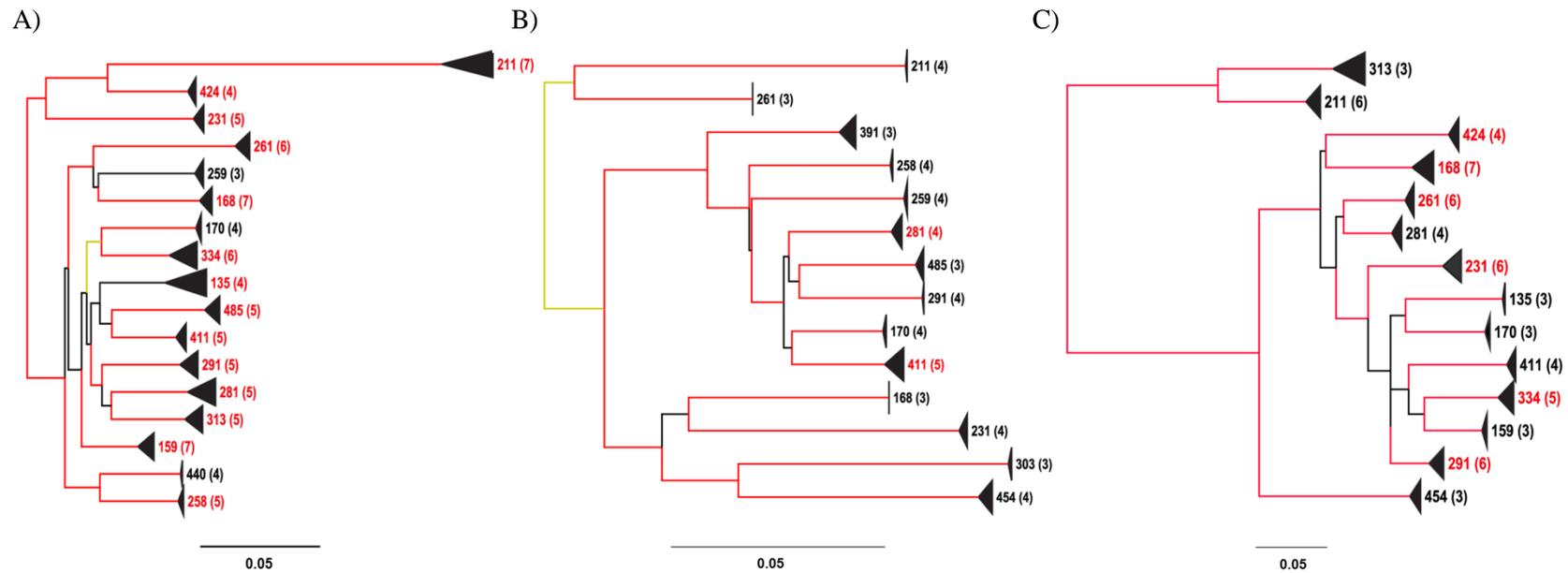
Figure 3.5 Phylogenetic analysis of gag (A), pol (B) and nef (C) sequences sampled longitudinally from mother-infant pairs. Infants from whom viral sequences were obtained at a minimum of 3 longitudinal time points were included for analysis (black tip labels) together with one maternal sequence if available (red tip labels). The numbers in brackets indicate the number of samples within the collapsed cluster. The ML method (271) was used to build the phylogenetic tree under the GTR model of nucleotide substitution (273), with proportion of invariable sites and substitution rate heterogeneity. Branch support was estimated by the aLRT-SH procedure (274); branches with support values >0.85 are shown in yellow and > 0.9 in red.

3.2.5 Evolutionary rates in infants across *gag, pol* and *nef*

The estimated median nucleotide substitution rates (inter quartile range [IQR]) for *gag, pol,* and *nef* were 3.39×10^{-3} (1.85×10^{-3}-5.58×10^{-3}), 4.19×10^{-3} (4.10×10^{-3}-4.37×10^{-3}) and 8.29×10^{-3} (5.73×10^{-3}-11.88×10^{-3}) nucleotide substitution per site per year, respectively. Median nucleotide substitution rates were significantly higher in *nef* compared to *gag* (p=0.001) and *pol* (p=0.0005) (**fig. 3.6B**); no significant difference was detected between median evolutionary rates in *gag* and *pol.* Noticeable variation was detected in the evolutionary rates across *gag* and *nef* though these parameters did not correlate (r=0.1, p=0.5). Evolutionary rates across *pol* were remarkably consistent between individuals (**fig. 3.6A**), indicating that this gene is under considerable evolutionary constraints.

Codon partitioning enabled relative evolutionary rate estimations to be made separately for codon positions 1+2 and position 3. The relative rate ratio at codon positions 1+2 and 3 (IQR) in *gag* was 1.12 (0.81-1.91) and 0.76 (0.62-1.37); in *pol* was 0.86 (0.40-1.21) and 1.27 (0.58-1.67); and in *nef* was 1.05 (0.81-1.23) and 0.90 (0.55-1.36). No statistically significant differences were observed between the relative rate ratios at codon positions 1+2 and position three in any of the genes analysed (p=0.4, p=0.2, p=0.9 for *gag, pol* and *nef,* respectively)

The rate ratio values at codon positions 1+2 and 3 were used to assess the ratio of the evolutionary rate occurring between positions 1+2 and 3 (**fig.3.6C**). No significant differences were detected in the median rate ratios of positions 1+2 to 3 in *gag, pol* or *nef.* However, in 12 of 17(71%) *gag*, 4 of 14 (29%) *pol* and 7 of 14 (50%) *nef* cases, the 1+2 codon position rate ratio was >1, indicating that positive selection may be acting predominantly in *gag* and to a to a large extent in *nef.* Conversely, codon position rate

ratios values in *pol* suggested that a number of sequences were likely under purifying selection.
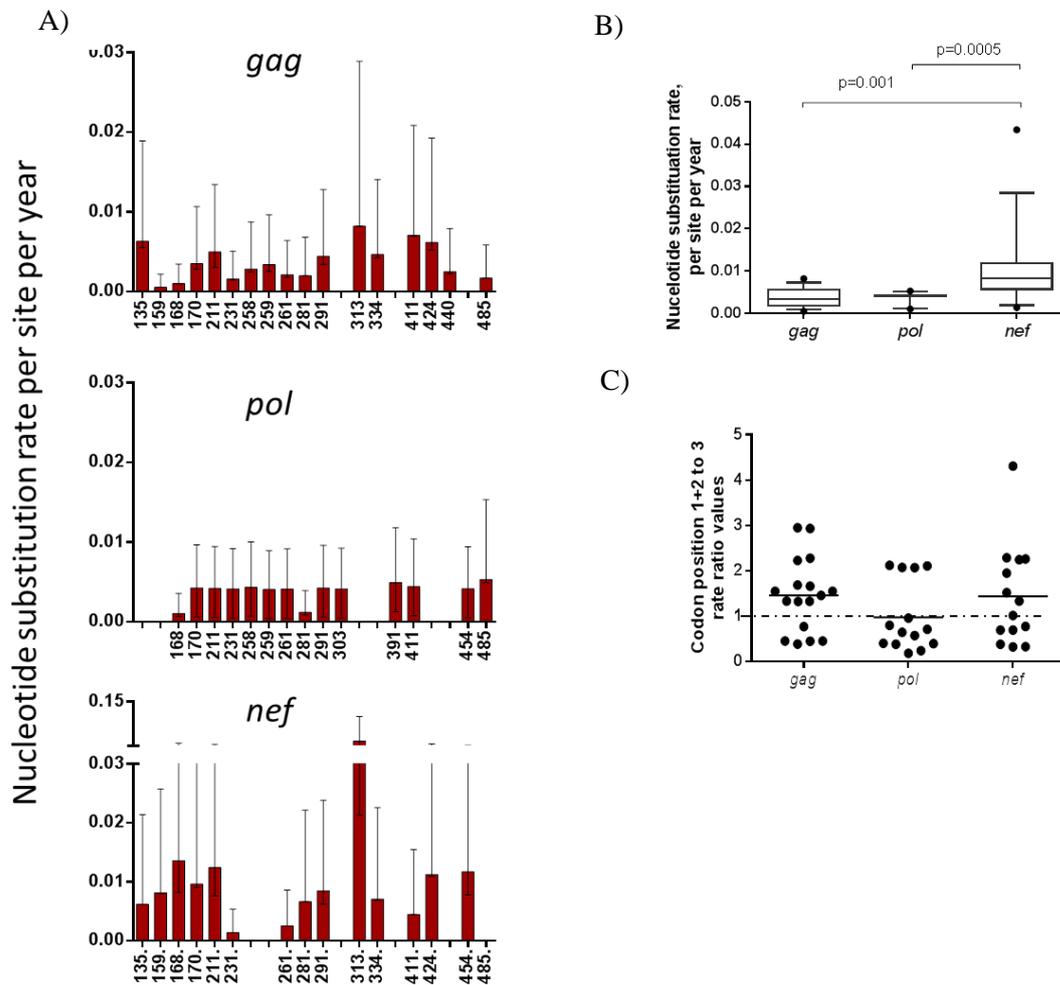
Figure 3.6 Nucleotide substitution rates across *gag, pol* and *nef genes* from infants sampled below 2 years of age. A) Comparative evolutionary rates in each individual showing the mean rate plus higher and lower highest priority density values. Blank spaces indicate a missing sample. (B) Summary median substitution rates across *gag, pol* and *nef* with 1st and 3rd quartiles (box) and the 10th and 90th percentile (whiskers). C) Codon rate ratio values of 1st+2nd to 3rd codon position. The horizontal line indicates the proportion of infants with values above and below 1. Mann-Whitney U test used to assess significant differences between median values. **p<0.01 ***p<0.001

3.2.6 Nucleotide substitution rates and infant clinical parameters

The association between evolutionary rate across *gag, pol* and *nef* and clinical markers of disease progression were assessed. Strong negative correlations were observed between the evolutionary rate in *gag* and both infant CD4 T cell absolute count (r=-0.57, p=0.02; **fig 3.7A**) and CD4 T cell % (r=-0,60, p=0.02; **fig 3.7B**) measured between 6 and 9 month of age. No statistically significant correlation was observed between peak viral load (defined as the highest viral load measurement within 6 months of infection) and evolutionary rate in *gag* (r=0.4, p=0.1). However, analysis of the evolutionary rate in *gag* in infants stratified into those with peak viral load above and below the median showed that a higher evolutionary rate was significantly associated with increased peak viral load (**fig. 3.7C**). No significant associations were observed between *nef* evolutionary rates and viral load (r= -0.4, p=0.4) or CD4 % (r=-0.1 p=0.7) or *pol* evolutionary rates and viral load (r= -0.3, p=0.3) or CD4 % (r= -0.2, p=0.5).

3.2.7 Evolution in *gag, pol* and *nef* in relation to CTL selection pressure

In order to assess the impact of CTL-mediated immune selection pressure on viral sequence evolution, we estimated evolutionary rates independently in regions of the genome known to encode CTL epitopes restricted by the infants HLA alleles and outside these regions. To this end the LANLDB annotation of current CTL-restricted epitopes was used, together with 2-digit resolution of infant HLA alleles, to partition genetic regions. A maximum likelihood approach was implemented as described in the methods section to estimate rates of nucleotide substitutions. In addition, the MG94 model of codon evolution was used to estimate dN/dS ratios for each individual across the distinct genetic regions.
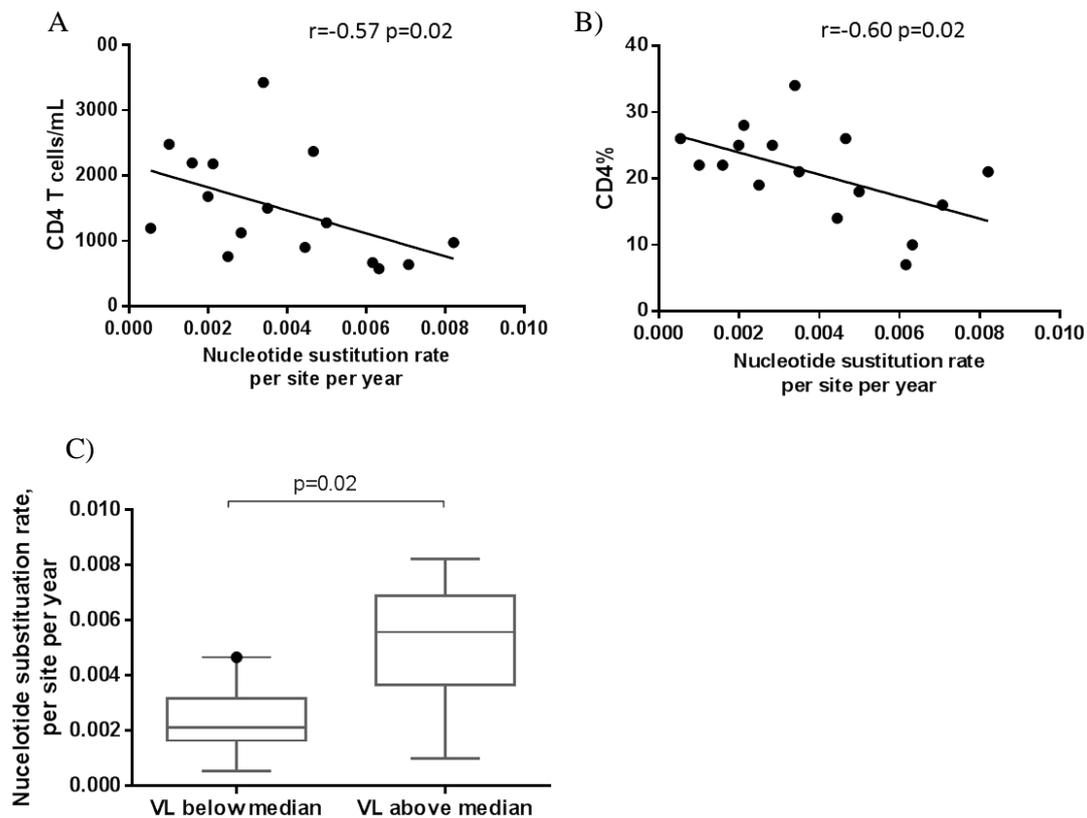
Figure 3.7 Nucleotide substitution rates in *gag* in relation to CD4 count and peak viral load. Spearman r was calculated between nucleotide substitution rate across *gag* and absolute CD4 T cell counts (A) or CD4 T cell % (B) measured between 6 and 9 months of age. C) Evolutionary rate across gag in infant with viral loads above and below the median peak viral load. Mann-Whitney U test used to assess significant differences between median values.

The analysis indicated that in *gag* the overall rate of evolution was significantly higher in genetic regions containing CTL epitopes restricted by infant HLA alleles (CTL+) than those that did not (CTL-) (0.0082 vs. 0.005 nucleotide substitutions per site, per year, p=0.008; **table 3.1**). Similarly in *nef,* evolution in CTL+ regions was significantly higher than in CTL- regions (0.0198 vs. 0.0103 nucleotide substitutions per site, per year, p= 0.001). By contrast the evolutionary rate in *pol* did not differ significantly

within and outside of CTL-epitopes restricted regions (0.0019 vs. 0.0014, p=0.454). These data indicate that *pol* evolves under distinct selective pressures to either *gag* or *nef*.

In order to further characterise the role of putative CTL selection pressure in driving viral evolution in the genes under study, a codon based model of evolutionary rates was used. This approach enabled dN/dS ratio to be estimated. The dN/dS ratio is a standard method used to assess selective pressure within protein coding genes. A dN/dS ratio >1 indicates that non-synonymous substitutions accumulate at a higher rate than synonymous substitutions over time and indicate that a genetic fragment is under positive or Darwinian selection. dN/dS ratios <1 indicate the converse and that the genetic fragment is under negative or purifying selection.

The result of this analysis are summarised in **table 3.1** and indicates that globally *gag, pol* and *nef* are under purifying selection. However within CTL+ regions dN/dS ratios in *gag* and *nef* were >1 and differed significantly from CTL- regions (p=0.001, p=0.003, respectively). This finding was in accordance with previous studies in adults that have reported marked differences in *gag* and *nef* evolution in CTL+ and CTL- regions (286,287). Reports of adult infection have also indicated that the dynamics of *pol* evolution are similarly CTL-associated (278,286), however the present findings indicate that codon evolution within *pol* CTL+ regions was associated with purifying selection and that the dN/dS ratio did not statistically differ from the that observed in CTL- regions.

Table 3.4 Evolution within and outside CTL epitopes

Gene	Substitutions per site, per year (95% CI)				dN/dS ratio (95% CI)			
	HLA targeted	Non-HLA targeted	P value	LRT	Global	HLA targeted	P¥ value	LRT
gag	0.0082 (0.006-0.011)	0.0050 (0.004-0.006)	0.008	7.10	0.57 (0.423-0.74)	1.26 (0.90-1.70)	0.001	10.16
pol	0.0019 (0.001-0.003)	0.0014 (0.001- 0.003)	0.454	0.56	0.30 (0.185-0.45)	0.18 (0.05-0.47)	0.398	0.71
nef	0.0198 (0.015-0.026)	0.0103 (0.008-0.014)	0.001	10.4	0.50 (0.34-0.70)	1.06 (0.75-1.44)	0.003	8.99

CI confidence intervals; LRT Likelihood ratio test; ¥P value of difference in dN/dS value between HLA-targeted and non HLA-targeted residu

3.3 DISCUSSION

In this study we initially sought to characterise the viral subtype distribution in the cohort. Molecular epidemiological studies have documented the presence of diverse subtypes within Kenya, although it is clear the country it is at the centre of the regional subtype A epidemic and to a lesser extent subtype D epidemic. The subtype distribution seen in the present cohort matched the overall distribution for subtypes A and D observed for Kenyan sequences submitted to the LANLDB. In addition, a substantial proportion (25%) of viral isolates from the cohort were URFs. AD recombinants dominated with cross over points occurring predominantly within pol (71%), though the structure of the recombinants was not the same between any two isolates. Interestingly, recombinants of CRF10 and 21, found to be circulating in Tanzania and Kenya respectively, were identified. The CRF10C isolate from patient 211 was the only isolate that did not have a subtype A component. Estimations that at least 25% of infection in western Kenya are represented by recombinant forms (288) are in accordance with the present data. The frequent emergence of recombinant forms suggests high rates of co-infection, which may have been common in Nairobi during the study period. Monitoring of subtype diversity is important for a number of reasons. Viral subtype may influence viral fitness and thus pathogenesis; natural polymorphisms may influence ARV drug resistance; viral diversity may influence accurate viral diagnostics and viral load assays; and identification of conserved immunogens for vaccine development relies on accurate data on sequence diversity. This data adds to the current understanding of viral diversity in Kenya and suggest that subtyping of limited genomic fragments may result in underestimations of the frequency of recombinant subtypes in circulation.

The principal aim of this study was to characterise the evolutionary dynamics of early HIV-1 infection in infants born before the era of effective interventions for the prevention of MTCT. In the absence of ART, a large proportion of infants experience an accelerated pathogenesis with distinct patterns of viral load dynamics from those seen in adult. In particular, infants frequently fail to establish a distinguishable set point viral load (104,173). During acute adult infection the onset of the HIV-specific CTL response is causally linked to the drop in peak viremia and the establishment of the set point viral load (194,285). This suggests that substantial differences may exist in terms of the functional capacity of the CTL response to limit viral replication between infants and adults. Indeed, despite detection of HIV-specific CTL response in infants, these have not been associated with improved outcomes during early life (223). However, the infant CTL response has been shown to be functional to the extent that *de novo* infant HIV-specific infant CTL responses have been shown to be able to drive immune selection of CTL escape variants(175,200). To what extent does CTL immune selection pressure drive HIV-1 evolution during early infant infection? This study sought to address this question by assessing evolutionary rates within a phylogenetic framework.

The analysis presented was carried out using two complementary approaches and assessed evolution within three principal gene targets of the CTL response, *gag, pol* and *nef.* In the first, a Bayesian phylogenetic framework was used to 1) estimate evolutionary rates 2) assess whether nucleotide substitutions rates differed between nucleotide positions within codons and 3) to correlate evolutionary rates with clinical parameters of disease. The results indicate that substantial inter-individual variation occurs in the evolutionary rates of *gag* and *nef* whereas in *pol* much less variation was observed. This suggests that considerable evolutionary constraints influence nucleotide

substitution within *pol*, as would be expected from gene encoding enzymes with critical function in the viral replication cycle. Viral substitution rates within *nef* were significantly higher than in either *pol* or *gag*. This in turn is likely a reflection of the molecular plasticity of *nef* and may similarly reflect the diverse functions of the accessory protein it encodes. The mean nucleotide substitution rates estimated in this analysis were $3.39x10^{-3}$, $4.19 x10^{-3}$ and $8.29 x10^{-3}$ nucleotide substitution per site per year, for *gag, pol,* and *nef* respectively. Previous estimates in adults of evolutionary rates in *gag* in adult subtype C infection(289) and *pol* subtype B(278) infection estimated the evolutionary rate at $5.22x10^{-3}$ and $7.2x10^{-4}$, nucleotide substitution per site per year, respectively. These estimates highlight the potential differences in evolutionary rates between infants and adults and the need to specifically characterise infant populations. The majority of evolutionary rate analyses focusing on infant infection have assessed evolution in *env*, and these reports have indicated a late onset of diversification linked to the induction of infant humoral response (195,197).

Analysis of evolutionary rate ratios at different codon positions indicated that although the mean rate ratio value did not statistically differ between genetic regions, a substantial proportion of infants had codon rate ratio values suggestive of positive selection within *gag* and *nef*. This was a further indication that non-synonymous nucleotide substitutions are restricted within *pol*, though it was apparent that in 4 individuals, positive selection was acting on this gene.

In order to assess the relevance of the evolutionary dynamics on clinical outcomes of HIV-1 infection, the correlation between peak viral load, CD4% or CD4 count and evolutionary rate was assessed. Whilst no apparent correlations were seen with respect to *pol* and *nef* evolution, the rate of evolution across *gag* correlated with markers of

disease severity. The indication was that higher rates of evolution are associated with worse clinical outcomes in terms of both reduced CD4 counts and percentage and increased viral replication. In addition, this finding indicates that a higher rate of viral replication during early infant infection results in higher mutation rate in *gag*.

We next assessed the role of the infant CTL response in shaping the evolutionary dynamics observed. The results indicate that the overall evolutionary rates in CTL+ regions across *gag* and *nef* were significantly faster than in CTL- regions. In addition, this localised high rate of evolution was driven by positive selection, indicated by significantly higher dN/dS ratios within CTL+ regions. These finding are in accordance with numerous reports in adults, showing CTL associated positive selection during early infection. The indication from the present results is that the infant CTL response may play a prominent role in driving early viral evolution. However, a significant limitation of the present analysis was that rates of reversion were not assessed. Lack of comprehensive maternal HLA data precluded this analysis. Reversion has been shown to account for a significant proportion (~42%) of non-synonymous changes in viral sequence in both infants(198) and adults(193). However, non-synonymous changes driven by reversion would likely be outside of infant restricted CTL epitopes. The data therefore suggest that the infant CTL response during early life infection represents a significant driving force of viral evolution within *gag* and *nef*. The indication however, that higher rates of *gag* evolution correlate with disease severity is difficult to reconcile with the notion that CTL escape occurs with an associated fitness cost to the virus. Further studies should address whether viral fitness is impaired following CTL escape in infants.

4 CHAPTER 4: CHARACTERISATION OF *EX VIVO* CELLULAR IMMUNE POPULATIONS AND VACCINE-SPECIFIC T CELL RESPONSES IN HEALTHY KENYAN INFANTS

4.1 INTRODUCTION

Infants and young children experience a heightened susceptibility to infection compared to adults. Age-related limitations in infant innate and adaptive immunity mediate these effects. One of the main goals of vaccination is to enhance immunological protection to infection during this period of vulnerability and this may be achieved by stimulating suppressed or maturing pro-inflammatory infant immune responses. It is therefore important to characterise age-specific immunological parameters during early life that may mediate vaccine outcomes and/or susceptibility to infection. To this end, this chapter focuses on the phenotypic characterisation of specific cellular subsets that play a role in mediating infant immunity and on the functional characterisation of T cell responses following routine vaccination. In addition this study is aimed at establishing baseline values that can be used to assess immunological alterations in HEU infants.

This study focuses on two vaccines known to elicit T cell responses, BCG and TT, and responses to the super antigen SEB. The peak BCG response has been shown to occur at 10 weeks of age following vaccination at birth (59,290). The response to TT vaccination in adults has been shown to peak 11 days following revaccination (291). The dynamics of IFN-γ responses to SEB stimulation have been shown to be subject to age-dependent functional limitations in CD4 T cell responses (292). 3 month old

infants were therefore recruited in order to assess near peak vaccine responses and early response to polyclonal stimulation- ethical and procedural considerations precluded blood collection from younger infants- and 12 month old infants were recruited to assess the development of the recall memory response to vaccination and responses to polyclonal stimulation at a later time point.

BCG is the only tuberculosis vaccine currently licenced for use in humans and is recommended for all infants in endemic countries to be administered at birth. BCG has been shown to confer ~80% protection against disseminated forms of childhood TB (293); however, wide variations, between 0 and 80% protective effect, have been reported against pulmonary TB (294). These variations have been attributed to numerous factors including, immunising strain (54), co-infections with helminths (295–297), age at vaccination (61,290) and exposure to environmental mycobacteria (298).

The immunological correlates of protection conferred by BCG vaccination have not been identified and although evidence from humans and mouse models implicate the Th1 cytokines IFN-γ (299,300) and TNF-α (301), these parameters alone are not sufficient to confer protection. In neonates, despite a bias towards to Th2 responses, BCG vaccination induces a Th1 response (37). Detailed characterisations of the T cell response to BCG vaccination has been carried out in South African (59,60) and Gambian (61) infants where a complex cytokine response dominated by CD4 T cells producing the Th1 cytokines IFN-γ, IL-2 and TNF-α has been found. Reponses from additional helper T cell subsets have been reported including those producing IL-17 (61,263) IL-10 (60,302), IL-4 (60) and IL-13 (61,261), highlighting the complexity of the T cell response and the capacity to induce what are considered to be suboptimal Th2 responses

The infant T cell response to tetanus toxoid vaccination is less well characterised. This may be in part a reflection of the known mechanism of immunity and the known antibody titre that confers protection (0.15 IU/mL) (303). However, maintenance of protective immunity requires booster immunisations and multivalent tetanus-containing vaccines are therefore provided at 3 time points in infancy and again in childhood to maintain protective titres. Characterisation of adult TT-specific T cell responses following revaccination have shown dominant CD4 T cells responses with mixed IFN-γ/IL-2 cytokine secreting populations during the peak responses and IL-2 dominated responses during the recall memory response (291,304).

A principal goal of vaccination is to achieve the induction of long-lived vaccine-specific memory responses with optimal functional properties. The ability of antigen-specific T cells to co-express more than one cytokine has been associated with protection following experimental vaccination in murine models of *Leishmania major* (305) and tuberculosis infection (306) and in improved outcomes during HIV-1 infection in humans (307,308). The quality of the T cell response is therefore an important functional component of the vaccine-specific T cell response.

Memory T cell populations can be distinguished through surface expression of CD45RA and CCR7 (51). Following the contraction phase of the primary T cell response, CCR7+ T cells that have encountered antigen (CD45RA-) can migrate along chemokine gradients into lymph nodes where they can reside as long-lived central memory T cells (Tcm) with rapid proliferative potential upon antigen reencounter. Lack of CCR7 expression on antigen experienced cells characterises effector memory T cells (Tem) that reside in the periphery and are able to migrate to inflamed tissues, where they carry out rapid effector functions upon cognate antigen recognition. Re-

expression of CD45RA on CCR7- T cells characterises populations of terminally differentiated effector cells (Temra). In addition to these well recognised markers of memory populations, the α chain of the IL-7 receptor (CD127) has been implicated in the maintenance of long-lived memory CD8 T cells in mice (309) and humans (310) through interactions with soluble IL-7. The literature indicates that these long-term memory T cells up-regulate anti-apoptotic markers such as Bcl-2 and down-regulate proliferation marker such as Ki67 (311).

Characterisation of BCG-specific CD4 T cells following neonatal vaccination has shown a dominant memory response from Tem and Tcm subsets (59,290). In adults, TT vaccination has been shown to induce a memory response dominated by Tem cells, and to a lesser extent Tcm cells; these cells up-regulate CD127 and Bcl-2 and down-regulate Ki67 (291). One of the principal aims of this study is to assess the dynamics of the generation of memory T cell populations in infants. To this end the distribution of memory T cells in bulk T cells *ex vivo* and in cytokine responsive cells following antigen stimulation were analysed using the markers discussed.

In addition to the functional characterisation of vaccine responses in early life, this study sought to characterise the frequencies and phenotype of *ex vivo* cellular populations that a) play a role in initiating, regulating and effecting T cell immune responses and b) have been described to be altered in HEU infants. To this end we characterised circulating populations of dendritic cells and their expression of co-stimulatory molecules; the frequencies of regulatory T cells; and levels of T cell immune activation and exhaustion marker expression. Dendritic cells play a key role in linking innate and adaptive immunity through their functions as professional antigen presenting cells with specific adaptations that enable priming of naïve T cells.

Regulatory T cells are important modulators of inflammatory responses and are thought to be induced following vaccination with BCG (294) . Finally, immune activation in infants may serve as a marker of pathogenic challenge in early life.

4.2 RESULTS

4.2.1 Study participants

We sought to characterise the frequency and phenotype of select immune cell populations and antigen-specific responses to BCG and TT vaccine antigens and to SEB stimulation cross-sectionally at 3 and 12 months of age in healthy infants. To this end, infants from 3 localities, Ganze, Ngerenya and Junju, all within Kilifi district (**fig 2.1**), were recruited as described in the Methods section. **Table 4.1** summarises the characteristics of the cohorts. Age in infants at the 3 (m3) and 12 (m12) month age groups ranged from 2.5-4.2 months (median of 3.5 months) and from 9.7 to 13.9 months (median 12.6 months) respectively. A well matched gender distribution was observed in both age groups (60% vs. 67% females at month 3 and 12 respectively). All infants reportedly received BCG and TT vaccinations; however these were often not administered according to guidelines. BCG vaccinations were administered >48hrs after birth in 15 out of 25 infants. Infants in the m3 group were excluded from analysis of BCG-specific vaccines responses if they received BCG vaccination >16 days after birth. All infants in the m3 group received at least 2 pentavelent vaccinations before the date of sampling and were all included in analysis of TT-specific immune responses. Analysis of immune responses to both vaccinations in the m12 group was carried out irrespective of the timing of vaccination.

Table 4.1 Summary of age, sex and vaccination dates from cross sectional cohorts

Infant	Locality	Sex	Age (months)	Age at BCG vaccination (days)	Age at 6 week pentavalent vaccination (weeks)	Age at 10 week pentavalent vaccination (weeks)	Age at 14 week pentavalent vaccination (weeks)
G016	Ganze	F	2.5	4.0	6.6	11.0	n/a
G020	Ganze	F	3.7	9.0	7.1	11.6	16.1
G021	Ganze	F	3.5	16.0	6.1	10.6	15.1
G024	Ganze	F	2.7	31.0	7.3	11.6	n/a
G032	Ganze	M	2.7	1.0	6.6	11.6	n/a
G035	Ganze	M	3.5	1.0	6.0	10.0	16.0
G036	Ganze	F	4.2	6.0	3.0	12.0	16.3
G037	Ganze	F	3.5	2.0	6.3	10.6	15.1
G038	Ganze	M	3.4	1.0	6.0	10.3	14.9
G039	Ganze	M	3.5	5.0	6.6	11.0	15.1
G009	Ganze	F	12.6	2.0	6.3	10.7	14.7
G012	Ganze	F	12.6	16.0	3.4	7.9	12.4
G018	Ganze	F	10.6	1.0	6.3	10.6	15.0
G025	Ganze	M	12.9	4.0	6.4	10.7	15.4
G028	Ganze	F	11.6	6.0	5.9	10.9	16.9
G029	Ganze	F	12.0	3.0	6.3	10.6	15.3
J871-4	Junju	F	13.7	4.0	6.3	11.6	16.1
J872-8	Junju	F	13.2	2.0	6.0	9.9	13.9
J873-3	Junju	F	13.9	1.0	6.3	10.4	14.4
J880-3	Junju	F	12.3	12.0	7.1	11.4	15.4
J883-4	Junju	F	12.4	1.0	6.1	10.6	14.9
J887-9	Junju	F	12.6	28.0	6.0	11.4	16.4
J890-4	Junju	M	9.7	2.0	15.1	19.7	24.1
N1220	Ngerenya	M	12.6	48.0	19.9	24.7	29.6
N1224	Ngerenya	M	12.1	35.0	6.9	15.9	20.7
N1242	Ngerenya	M	13.5	4.0	6.1	14.3	18.6
N1244	Ngerenya	M	12.6	75.0	6.3	11.3	15.4
N1247	Ngerenya	F	11.0	18.0	6.4	10.6	15.0

4.2.2   Whole blood count ranges at 3 and 12 months in healthy infants

Whole blood count ranges were determined as described in the Methods section and the values are summarised in **table 4.2**. A significant increase in the red blood cell count was observed in 12 month-old infants compared to the 3 month-old cohort (5.0 vs. 4.3 $x10^6$/μL respectively, p=0.003). No other age-dependent statistically significant changes were detected in this analysis. The median and 10th and 90th percentile values were all within the 95% reference intervals for previously published data from East African infants under 12 months of age (312,313) indicating a broadly healthy immunological status.

4.2.3   Characterisation of cellular immune activation and exhaustion markers at 3 and 12 months of age

The frequencies of *ex vivo* cellular subsets in peripheral circulation were quantified by flow cytometry. **Figure 4.1A-C** shows the gating strategy used to quantify the expression levels of the T cell activation markers HLA-DR and CD38 and the cellular exhaustion markers PD-1 and Tim-3 on CD4 and CD8 T cells. CD38 was found to be expressed by the majority of T cells (**fig. 4.1C**) in accordance with previous findings (314,315). Therefore immune activation was assessed by the combined expression of HLA-DR and CD38. In addition, the frequency of activated T cells co-expressing exhaustion markers was assessed and the data is summarised in **tables 4.3** (CD4 T cells) and **4.4** (CD8 T cells). The median expression level of all exhaustion and activation markers was significantly higher in CD8 T cells vs. CD4 T cells (data not shown).

Table 4.2 Median and reference intervals for whole blood cells count for healthy controls infants at 3 and 12 months of age.

Characteristic	3-months of age (n=10) Median (10th & 90th percentiles)	12-months age(n= 16) Median (10th & 90th percentiles)	P$^{¥}$ value	Median (95% reference intervals) Tanzania$^{±}$	Median (90% reference intervals) Uganda$^{±}$	95% reference intervals Europe/US$^{±}$
Red blood cells (10^6/μL)	4.3 (3.9-4.7)	5.0 (4.7-5.2)	0.003	-	4.2 (3.0–5.4)	-
Haemoglobin (g/dL)	10.2 (9.2-10.6)	10 (9.5-10.6)	*NS	10.7 (8.1–13.2)	10.0 (6.8–14.7)	9.4-13.0
Platelets (10^9/L)	446.6 (317.3-575.9)	452.4 (380.7-524.0)	NS	384 (25–708)	230 (123–487)	150–400
Neutrophils (10^9/L)	1.9 (1.1-2.7)	1.9 (1.5-2.3)	NS	1.7 (0.7–4.6)	2.1 (0.9–4.4)	0.7-8.0
Lymphocytes (10^9/L)	6.3 (4.9-7.6)	5.6 (4.7-6.5)	NS	6.2 (3.3–11.8)	5.1 (1.9–10.3)	3.3-11.5
Monocytes (10^9/L)	0.7 (0.3-1.0)	0.7 (0.5-0.9)	NS	0.7 (0.2–1.5)	0.7 (0.2–1.8)	0.2-1.3
Eosinophils (10^9/L)	0.3 (0.2-0.4)	0.4 (0.2-0.5)	NS	0.3 (0.1–0.8)	0.4 (0.1–1.9)	0.05-1.1
White blood cells (10^9/L)	9.3 (7.0-11.6)	8.7 (7.7-9.8)	NS	9.2 (5.0–17.3)	8.8 (4.1–15.8)	5.0-17.0

$^{¥}$ An unpaired T test was used to assess significant age related changes for all parameters (values normally distributed) except for monocytes and eosinophils were a Mann-Whitney U test was carried out (data not normally distributed).

*NS, not significant

$^{±}$Previously published values for healthy infants under 12 months of age from Tanzania (316) and Ugandan (313) and reference ranges for infants from Europe and the US are included for comparison (316)
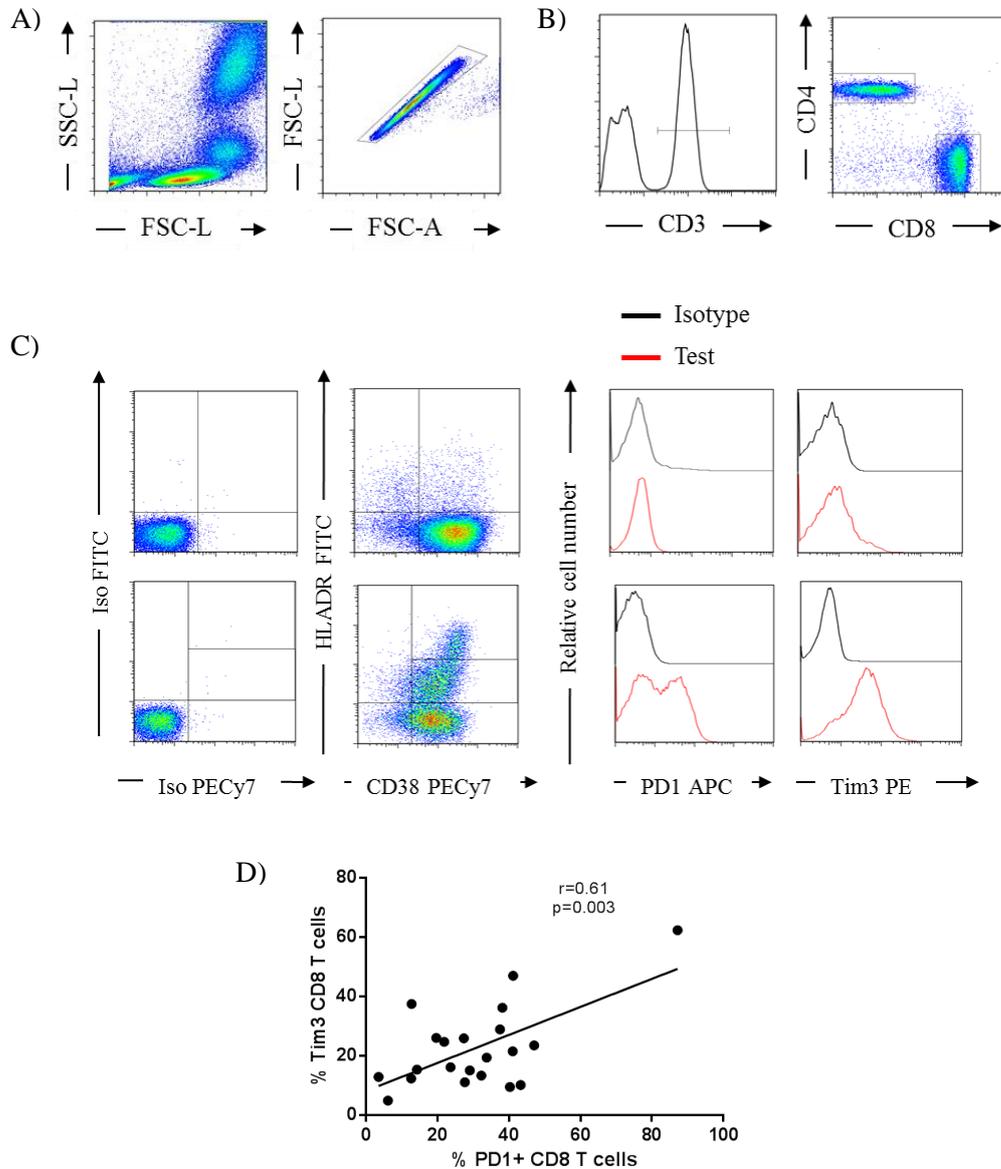
Figure 4.1 Gating strategy used to define populations of activated CD4 and CD8 T cells in peripheral blood. (A) Lymphocytes were identified using forward and side scatter profiles and cell doublet events were excluded. (B) CD3+ CD4 and CD8 T cells were identified. C) Isotype control stains were used to set gates in order to define the frequency of CD4 and CD8 T cell populations expressing the activation and exhaustion markers CD38, HLA-DR, PD-1 and Tim-3. D) Pearson correlation of the frequency of PD-1 and Tim-3 expressing CD8 T cells (r=0.61, p=0.003).

Table 4.3 Phenotype of CD4 T cells in peripheral blood of healthy community cross sectional cohorts at 3 and 12 months of age

Characteristic	3 months (n=10) Median % of CD4 T cells unless indicated (range)	12 months (n=16) Median % of CD4 T cells unless indicated (range)	¥P value
Activation & exhaustion			
CD38 HLA-DR	6.7 (1.7-12.1)	6.4 (2.48-14)	NS
CD4 PD-1	12.9 (8.7-22.9)	13.6 (7.22-25.1)	NS
CD4 Tim-3	3.0 (1.2-5.0)	1.8 (0.5-62.0)	NS
CD38 HLA-DR PD-1	3.6 (1.6-5.4)	3.1 (1.3-11.0)	NS
CD38 HLA-DR Tim-3	0.4 (0.2-1.2)	0.5 (0.2-3.9)	NS
CD38 HLA-DR PD-1 Tim-3	1.7 (0.2-10.8)	0.3 (0.1-30.6)	NS
PD1 Tim-3	1.0 (0.3-2.3)	0.7 (0.2-8.6)	NS
Immune regulation			
CD25hi FoxP3+	5.1 (1.4-7.0)	4.9 (1.8-8.0)	NS
Anti-apoptosis			
Bcl-2+	77.9 (59.0-93.6)	74.6 (22.2-92.2)	NS
Bcl-2-	23.2 (7.4-41.0)	26.0 (7.82-77.8)	NS
Memory			
CD127+	86.5 (74.6-95.0)	84.5 (69.8-93.4)	NS
CD127-	13.9 (5.0-25.4)	16.2 (6.6-30.6)	NS
CCR7+ CD45RA+ (Naïve)	74.8 (58.5-79.2)	66.2 (43.5-83.9)	NS
±**Naïve Bcl-2+**	85.6 (30.4-95.9)	72.9 (4.7-91.8)	NS
Naïve Bcl-2-	16.7 (4.8-74.2)	30.4 (9.3-96.3)	NS
Naïve CD127+	92.6 (89.0-99.7)	88.5 (62.3-99.9)	0.03
Naïve CD127-	7.9 (0.3-11.8)	12.2 (0.1-39.3)	0.03
CCR7+ CD45RA- (Tcm)	14.3 (9.5-33.5)	16.2 (8.5-42.8)	NS
Tcm Bcl-2+	58.3 (17.2-85.3)	54.3 (6.8-68.4)	NS
Tcm Bcl-2-	44.1 (16.5-84.9)	48.0 (33.7-94.1)	NS
Tcm CD127+	67.7(50.9-90.4)	68.7(38.5-93.1)	NS
Tcm CD127-	33.4 (10.1-50.3)	32.4 (7.4-62.6)	NS
CCR7- CD45RA+ (Temra)	3.2 (0.8-13.8)	5.6 (0.7-10.8)	NS
Temra Bcl-2+	78.6 (30.6-91.9)	63.3 (8.4-87.6)	NS
Temra Bcl-2-	24.5 (9.79-71.8)	39.7 (15.3-92.8)	NS
Temra CD127+	72.5 (45.8-97.0)	61.5 (21.3-98.7)	NS
Temra CD127-	28.7(3.9-55.6)	39.9(1.3-77.5)	NS
CCR7- CD45RA- (Tem)	7.2 (5.1-9.6)	10.2 (4.0-22.5)	0.03
Tem Bcl-2+	48.7 (16.1-85.9)	56.4 (15.0-76.9)	NS
Tem Bcl-2-	53.3 (15.7-85.2)	45.4 (24.8-86.5)	NS
Tem CD127+	58.8 (39.2-83.5)	62.6 (31.6-79.0)	NS
Tem CD127-	41.9 (17.1-61.6)	38.1 (21.6-69.0)	NS

¥ P values were calculated using a Mann-Whitney U test. ±Indented values indicate percentage of memory T cell subset

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Table 4.4 Phenotype of CD8 T cells in peripheral blood of healthy community cross-sectional cohorts at 3 and 12 months of age

Characteristic	3 months (n=10) Median % of CD8 T cells unless indicated (range)	12 months (n=16) Median % of CD8 T cells unless indicated (range)	¥P value
Activation & exhaustion			
CD38 HLA-DR	48.6 (2.5-65.2)	40.6 (14.8-75.5)	*NS
PD-1	28.3 (12.7-47.1)	32.3 (3.6- 87.2)	NS
Tim-3	18.4 (9.5-25.9)	19.4(4.8-62.4)	NS
CD38 HLA-DR PD-1	18.7(1.4-41.9)	20.0(1.4-39.6)	NS
CD38 HLA-DR Tim-3	7.2(0.5-16.7)	13.25(1.91-36.0)	NS
CD38 HLA-DR PD-1 Tim-3	3.79(0.3-11.7)	4.9(0.4-35.3)	NS
PD-1 Tim-3	6.41(3.0-13.4)	5.32(1.08-59.7)	NS
Anti-apoptosis			
Bcl-2+	55.8(27.5-96.8)	58.8(26.5-96.4)	NS
Bcl-2-	44.2(3.22-72.5)	41.25(3.62-73.5)	NS
Memory			
CD127+	58.6(25.8-96.2)	49.25(26.4-92.9)	NS
CD127-	41.5(3.8-74.1)	51.1(7.1-73.6)	NS
CCR7+ CD45RA+ (Naïve)	62.1(50.4-96.0)	49.1(25.6-86.8)	0.03
±Naïve Bcl-2+	67.9(48.6-98.7)	84.7(25.3-95.4)	NS
Naïve Bcl-2-	25.6(0.8-50.9)	9.0(4.0-72.4)	NS
Naïve CD127+	78.9(45.5-99.5)	80.0(20.1-97.5)	NS
Naïve CD127-	21.8(0.5-55.7)	21.1(0.3-79.3)	NS
CCR7+ CD45RA- (Tcm)	4.1(0.5-45.0)	2.9(0.7-9.53)	NS
Tcm Bcl-2+	26.4(0.7-71.6)	23.25(3.2-72.8)	NS
Tcm Bcl-2-	72.0(27.9-98.2)	75.4(20.4-95.2)	NS
Tcm CD127+	42.9(4.6-85.0)	31.4(5.8-68.4)	NS
Tcm CD127-	60.8(16.7-95.5)	70.1(30.0-93.3)	NS
CCR7- CD45RA+ (Temra)	9.9(0.4-37.4)	29.9(3.0-65.0)	0.006
Temra Bcl-2+	20.1(0-48.6)	29.1(0.8-71.3)	NS
Temra Bcl-2-	73.7(17.4-11.4)	49.6(8.9-78.5)	NS
Temra CD127+	31.7(3.6-91.8)	8.6(1.9-81.6)	0.04
Temra CD127-	57.1(8.5-90.6)	80.7(8.5-92.6)	NS
CCR7- CD45RA- (Tem)	7.4(0.4-26.4)	13.6(2.9-32.0)	NS
Tem Bcl-2+	10.5(0.7-52.5)	15.4(1.03-47.3)	NS
Tem Bcl-2-	78.0(28.6-98.7)	70.8(15.9-92.6)	NS
Tem CD127+	34.2(5.1-60.7)	13.2(2.4-35.4)	NS
Tem CD127-	68.4(39.3-95.1)	82.2(46.2-92.2)	NS

¥ P values were calculated using a Mann-Whitney U test.*NS, not significant. ±Indented values indicate percentage of naïve or memory T cell subset

CD8 T cells exhibited wide variation in expression levels, particularly in CD38/HLA-DR co-expression, with ranges of <5% to >70%, and in PD-1 expression, with ranges of<5% to >80%. A more restricted level of variation was observed in CD4 T cells in terms of CD38/HLA-DR co-expression and in PD-1 expression; negligible levels of Tim-3 expression on CD4 T cells was observed (median frequencies <4%). No correlations were observed between the expression levels of PD-1 and HLA-DR/CD38 co-expression or Tim-3 and HLA-DR/CD38 co-expression on CD4 and CD8 T cells (data not shown); however a positive correlation was detected between the expression levels of PD-1 and Tim-3 on CD8 T cells (**fig. 4.1D**) indicating that up regulation of exhaustion markers may occur through independent mechanisms than those involved in HLA-DR/CD38 co-expression. No statistically significant changes in median expression levels of these markers in either CD4 or CD8 T cells were observed between 3 and 12 month old infants.

4.2.4 Characterisation of T regulatory cell frequencies

We sought to quantify the frequency of FOXP3+ T regulatory cells in the circulation. Tregs were defined as CD25+ FOXP3+ CD4 T cells and identified according to the gating strategy shown in **figure 4.2A**. It has been reported that an inverse correlation exists between expression levels of FOXP3 and CD127 on CD4 T cells and that human Tregs can be identified as being CD25+ CD127low (317). It was not possible to include CD127 as a biomarker for the entire study. However, a strong positive correlation was found in a subset of 5 infants between the frequency of Tregs defined through CD25 and FOXP3 expression and those defined through CD25 and CD127 expression (**figs. 2B and C**). No statistically significant differences in the frequency of T regs among CD4 T cell were detected between 3 and 12 month old infants (**table 4.3**;

5.1 vs 4.9%, p>0.05) ; similar ranges in frequency were also observed at the two time

points (1.35- 7.03 at 3 and 1.81-7.96 % at 12 months of age).
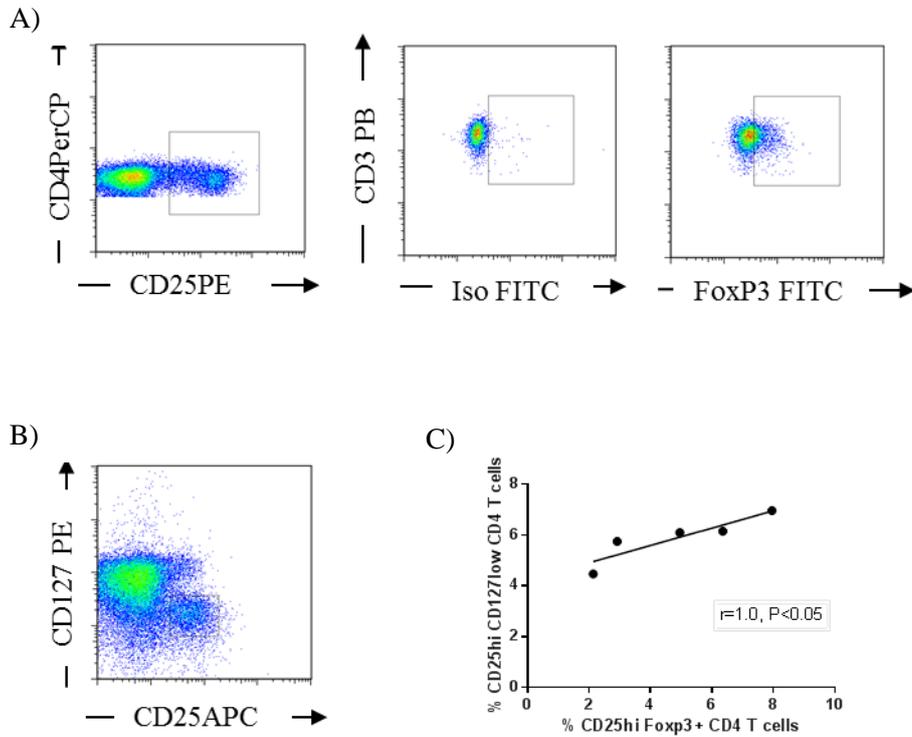
Figure 4.2 Gating strategy used to define FOXP3+ regulatory T cells. (A) CD3+ CD4+ CD25hi cells were gated on and an isotype control was used to define FOXP3+ events on a CD3 vs FOXP3 dot plot. B) Identification of T regulatory cell population on the basis of CD127low and CD25hi expression after gating on CD3+ and CD4+ T cells. C) Spearman correlation of the frequencies of Treg cells among CD4 T cells identified through CD25hi FoxP3+ expression and through CD25hi CD127 low expression(r=1.0,p<0.05).

4.2.5 Characterisation of circulating memory T cell populations

Figure 4.3 shows the gating strategy used to quantify the frequency of naïve and memory T cell populations based on CD45RA and CCR7 expression, and the expression levels of the IL-7R (CD127) and the intracellular marker Bcl-2. Use of CCR7 to distinguish antigen experienced memory T cell subsets is well described (51,318). CD127 expression plays a critical role in cell survival and homeostatic maintenance of resting naïve and memory T cell populations (319) and Bcl-2 is a marker that promotes cell survival through prevention of apoptosis (320) that may be maintained in long-lived cellular populations.

The data obtained from this analysis is summarised in **tables 4.3** and **4 4.** The majority of CD4 and CD8 T cells at 3 months of age had a naïve phenotype; a trend to a decreased proportion in CD4 T cells (74.8 vs. 66.2%, p>0.05) and a significant decrease in the CD8 T cells (62.1 vs. 49.1%, p=0.03) in 12 month old infants was observed. Among CD4 and CD8 T cells, the median frequencies of Tem and Temra memory subsets were higher in infants from the m12 group; this increase was statistically significant for CD8 Temra cells (9.9 vs 29.9%, p=0.006). Similar median frequencies of CD4 and CD8 Tcm cells were found in infants in m3 and m12 age groups (CD4: 14.3 vs. 16.2; CD8: 4.1 vs 2.9). Analysis of bulk T cell compartments showed that CD4 T cells were predominantly Bcl-2hi CD127hi indicating a population of anti-apoptotic cells many of which were maintained in a resting state; CD8 T cells showed more moderate expression levels of these markers with approximately 50% of cells exhibiting low expression levels for both markers, an indication of higher proportions of cells prone to apoptosis with low responsiveness to IL-7 stimulation. Analysis of the correlation between CD127 and Bcl-2 expression on bulk T cell subsets

showed a highly significant positive correlation on CD8 T cells (**fig 4.4A**; r=0.88, p=<0.0001) and a more modest significant correlation on CD4 T cells (4.4B; r=0.44, p=0.04).

In order to assess differences in memory phenotype distributions between CD4 and CD8 T cells, a pooled analysis was carried out with infants from both age groups. Significantly higher proportions of Tcm cells were observed in the CD4 compartment; in turn CD8 T cell had significantly increased proportions of cells with a Temra phenotyrpe (**fig. 4.4B**). The proportion of naïve and Tem cells were similar in the two T cell compartments.

In order to further characterise naïve and memory T cell subsets in terms of their expression levels of Bcl-2 and CD127, a similar pooled analysis was carried out in all healthy infants (**fig. 4.4C**). Naïve T cells in both CD4 and CD8 compartments were predominantly Bcl-2hi and CD127hi (p=<0.001 in both cases; **fig. 4.4C**). There were clear differences between CD4 and CD8 memory T cell subsets in the proportion of cells expressing either Bcl-2 or CD127. CD8 T cell memory subsets were all predominantly Bcl-2low CD127low (p=<0.001 in all cases). By contrast, the majority of all the cells within the CD4 memory subsets were CD127hi (p=<0.001 in all cases) and approximately 50% of cells within the Tcm and Tem subsets were Bcl-2hi.
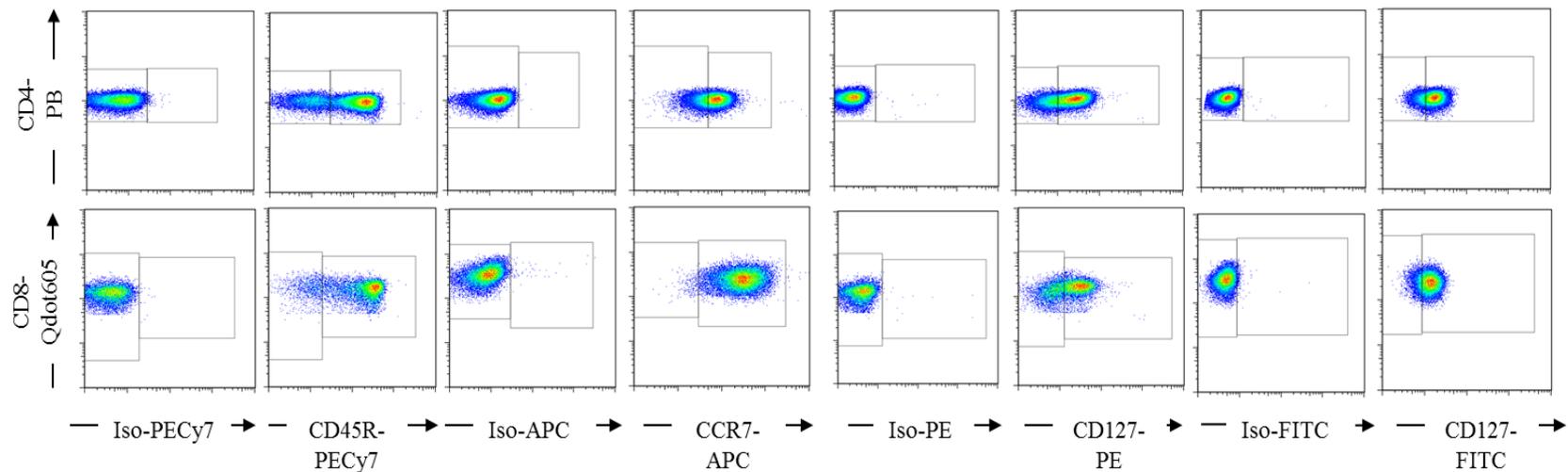
Figure 4.3 Gating strategy used to determine the frequency of memory CD4 and CD8 T cell populations in peripheral blood circulation. Cells gated on CD3 and CD4 or CD8 as shown in **figure 4.1B** were analysed for their expression of the memory marker CD45RA, CCR7, CD127 and Bcl-2. Isotype control antibodies (Iso) conjugated to the corresponding fluorochrome and used at the same concentration, were used to define positive and negative events.
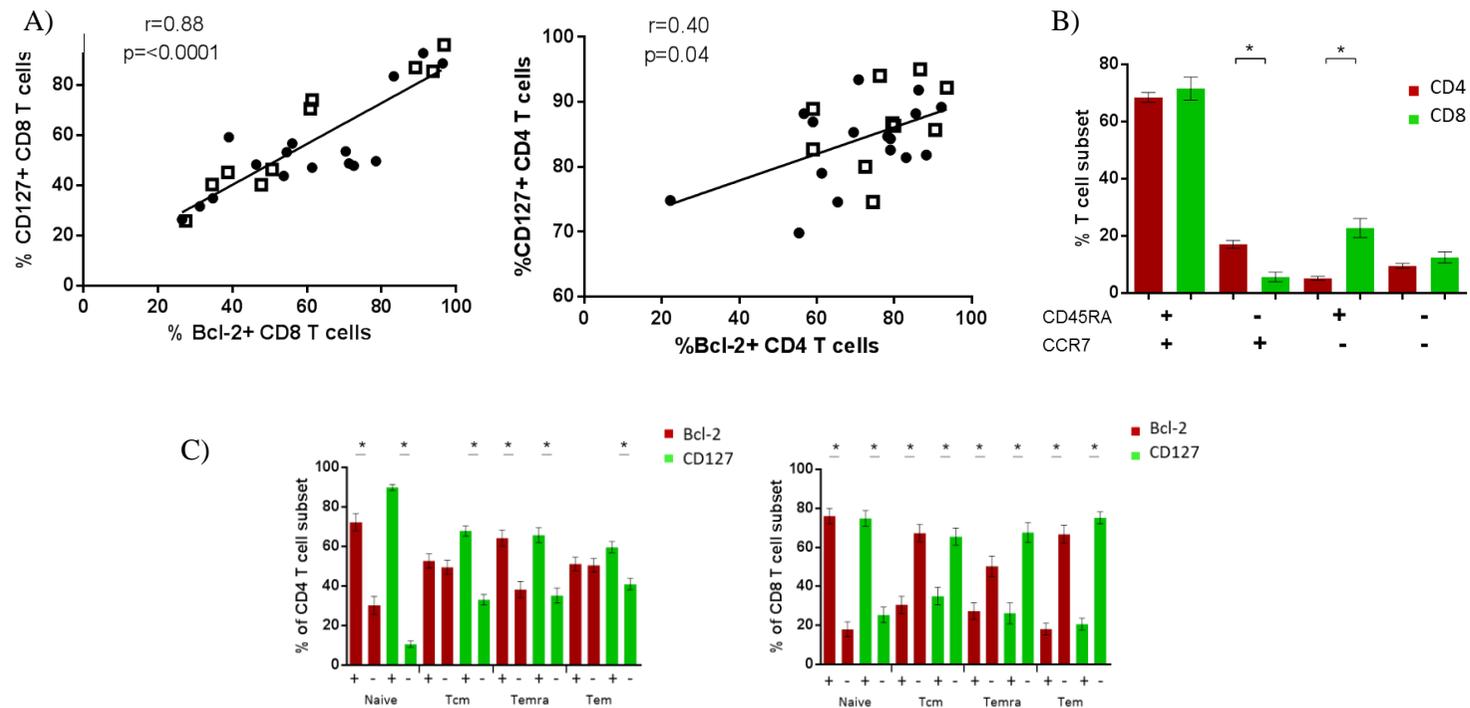
Figure 4.4 Memory T cell subset distributions and phenotype. A) Spearman correlations of CD127 expression and Bcl-2 expression on CD4 (right panel) and CD8 (left panel) T cells pooled from 3-month and 12-month infant age groups. Open boxes represent 3 month old infants and filled circles represent12 month old infants. B) Pooled analysis of memory T cell subset distribution in CD4 and CD8 T cells. C) Blc-2 and CD127 expression levels on naïve and memory T cell subsets from pooled m3 and m12 infant groups. Bar charts indicate the mean frequency ± the standard error of the mean. P values were calculated using a Mann-Whitney U test. *p<0.05

4.2.6 Characterisation of circulating dendritic cell frequencies and phenotype

This study next sought to characterise the frequency of dendritic cell subsets among leucocytes in the periphery and determine the expression patterns of the co-stimulatory markers CD86, CD83 and the co-inhibitory marker PD-L1 on these cells. The gating strategy used to define the populations analysed is shown in **figure 4.5**. **Table 4.5** summarises the results. mDC were found in higher proportions compared to pDCs in infants from both age groups as indicated by a median mDC/pDC ratio of 1.18 and 1.62 at 3 and 12 months respectively; however, instances of mDC/pDC ratios of <1 were observed. CD86 was found to be expressed constitutively on mDC (median expression levels >90%) but only in a subset of pDC. No significant changes were observed in CD86 expression levels on mDCs and pDCs between age groups. Negligible expression was observed in both dendritic cell subsets in terms of CD83 expression in 3 month old infants and PD-L1 expression in 12 month old infants. The staining protocol used to identify dendritic cells also enables the identification of basophils which form a distinct population as shown in **figure 4.5**. The proportion of basophils within the leucocyte gate was significantly lower in the m12 vs. the m3 group (0.23 vs. 0.17%, p=0.04). Considerable frequencies on basophils were observed to express CD83 in the 3 month old group (median 38.7%) and PD-L1 (median 26.4%) in the 12 month old group.
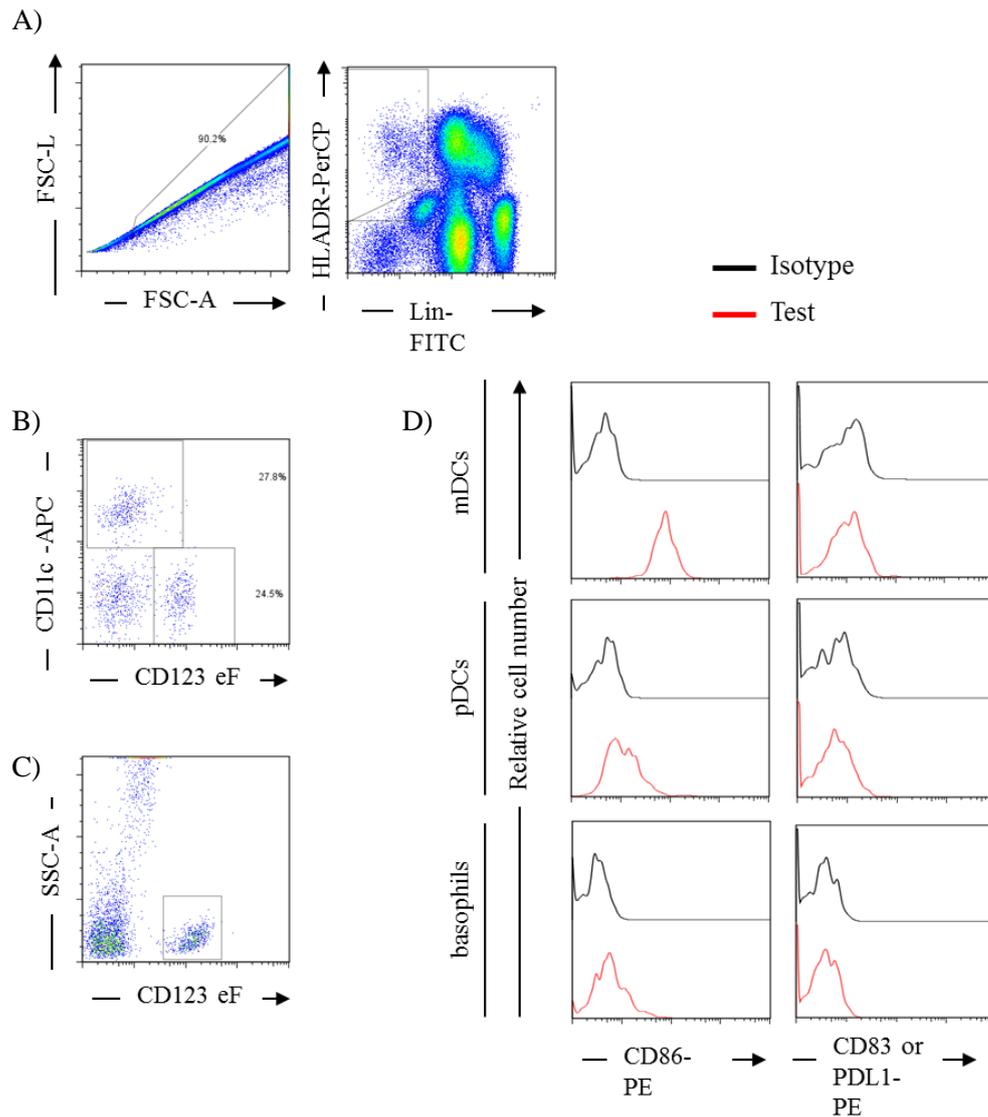
Figure 4.5 Gating strategy used to determine frequency and phenotype of DC cell subsets and basophils. A) Whole blood leucocytes were identified and singlet populations of cell events included. DC cells were defined as lineage marker negative (Lin) HLA-DR positive. Basophils were identified within the Lin- HLA-DR- population. B) CD11c and CD123 distinguish mDC and pDC respectively. C) Basophiles were identified by CD123 expression (eF= efluor450). D) CD86, CD83 amd PD-L1 positive events were defined through the use of isotype controls.

Table 4.5 Peripheral blood dendritic cell and basophil frequencies and phenotype in healthy community cross-sectional cohorts at 3 and 12 months of age

Characteristic	Median values (range)		
	Month 3 (n=10)	Month 12 (n=16)	¥P value
mDC (% of leucocytes)	0.23 (0.15-0.39)	0.26 (0.09-0.48)	±NS
CD86 (% of mDCs)	96.6 (92.0-98.9)	94.6 (66.10-99.1)	NS
CD86 MFI	117 (76-127)	84 (43-146)	NS
CD83 (% of mDCs)	0.06 (0.00-0.57)	-	*NA
CD83 MFI	199 (15-355)	-	NA
PD-L1 (% of mDCs)	-	0.10 (0.00-2.24)	NA
PD-L1 MFI	-	17 (10-212)	NA
HLA-DR MFI	841 (384-1218)	877 (371-1370)	NS
pDC (% of leucocytes)	0.15 (0.05-0.33)	0.17 (0.05-0.28)	NS
CD86 (% of pDCs)	26.0 (10.4-43.6)	27.7 (8.6-52.8)	NS
CD86 MFI	37 (25-146)	35 (18-124)	NS
CD83 (% of pDCs)	1.58 (0.73-4.24)	-	NA
CD83 MFI	75.95 (42.9-678)	-	NA
PD-L1(% of pDCs)	-	1.19 (0-3.45)	NA
PD-L1 MFI	-	48 (7-107)	NA
HLA-DR MFI	385.5 (172-546)	359.5 (179-715)	NS
mDC/pDC	1.18 (0.87-4.02)	1.61 (0.77-2.67)	NS
Basophils (% of leucocytes)	0.23 (0.13-0.59)	0.17 (0.01-0.33)	0.04
CD86 (% of basophils)	15.9 (4.3-26.3)	7.3 (2.3-25.5)	NS
CD83 (% of basophils)	38.7 (0.0-44.9)	-	NA
PD-L1 (% of basophils)	-	26.4 (0.00-45.7)	NA

¥ P values were calculated using a Mann-Whitney U test. ±NS=not significant. *NA= not applicable

4.2.7   Single cytokine responses to in vitro stimulation with PPD, TT and SEB

In order to characterise the response to the vaccine antigens PPD and TT and the superantigen SEB, short term whole blood stimulation was carried out as described the Methods section. Specifically, I was interested in characterising, at 3 and 12 months of age, the type of cytokine response induced by antigen stimulation, the magnitude of those responses, the quality of the cytokine response and finally the memory phenotype of cytokine responsive cells. To this end I stained cells with 3 ICS panels shown in **table 2.4**. **Figures 4.6A and 4.7B** show the gating strategy used to define the type of cytokine response induced following the different stimulation conditions. This analysis was carried out following ICS staining with antigen-specific antibody panels 1 and 3 . Due to the high background levels of IL-4 positive events in cultured cells, antigen specific IL-4 responses were defined through co-expression of the early activation marker CD69 (**fig. 4.7A**).

PPD stimulation resulted in a predominant Th1 response with negligible contribution from IL-17, IL-10, or IL-4 producing CD4 T cells (**figs. 4.8A & B**). The Th1 response was dominated by IL-2 and TNF-α production and to a lesser extent, IFN-γ. The magnitude of the IFN-γ, IL-2 and TNF-α response was consistently higher in infants from the m3 vs. the m12 groups. PPD stimulation also induced a response from CD3+ CD4- cells (**fig. 4.8C**), which will be referred to as CD8 T cells, though the magnitude of the response, compared to CD4 T cells, was significantly lower in both age groups (**fig. 4.8D**). There was no pattern of age related changes in the magnitude of the CD8 T cell response in any of the measured cytokines (**fig. 4.8C**).
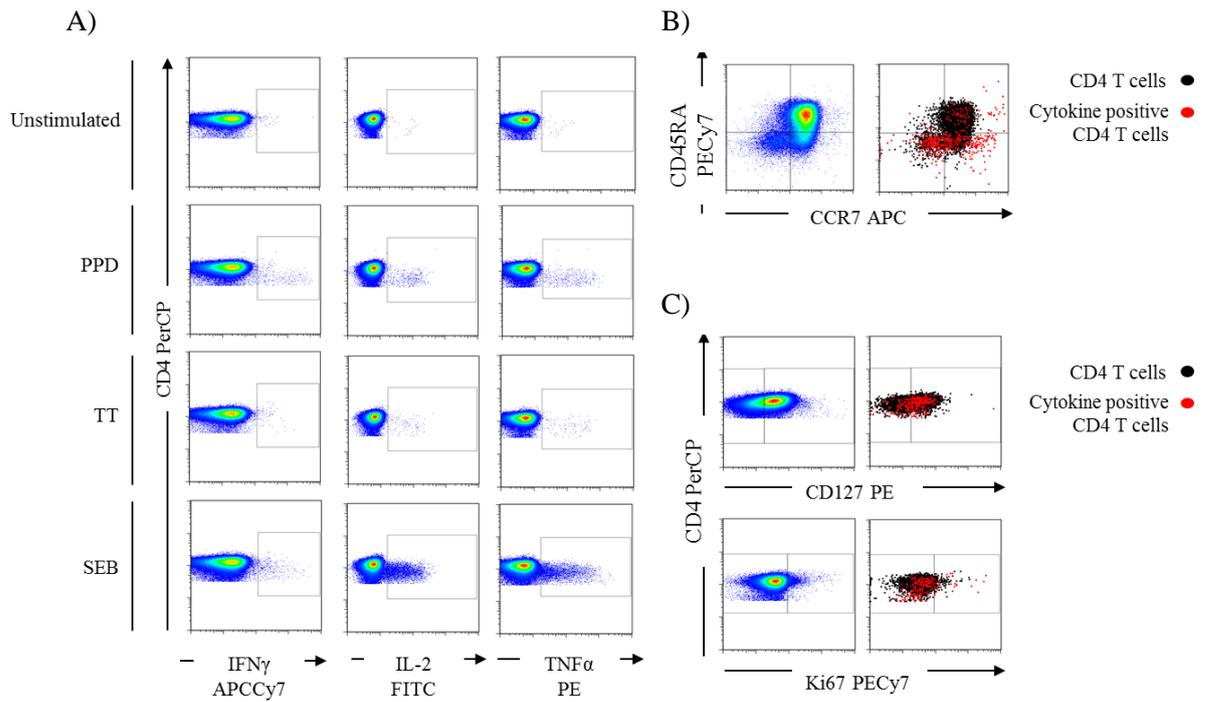
Figure 4.6 Gating strategy used to analyse CD4 and CD3+ CD4- T cell responses to antigenic stimulation by intracellular cytokine staining. (A) Antigen responsive T cells were stained with antigen-specific antibody panel 1. Shown is the gating on CD 4 T cell though an equivalent strategy was used to analyse CD3- CD4+ cytokine-responsive T cells. Unstimulated cells were used to set gates to define cytokine-positive events following PPD, TT or SEB stimulation. (B) Following staining with antigen-specific antibody panel 1, the memory phenotype of antigen responsive cells was defined according to CD45RA and CCR7 expression. C) Staining with antigen-specific antibody panel 2 was used to define the proportion of antigen-responsive cells that were CD127 (top panel) or Ki67 (lower panel) positive.
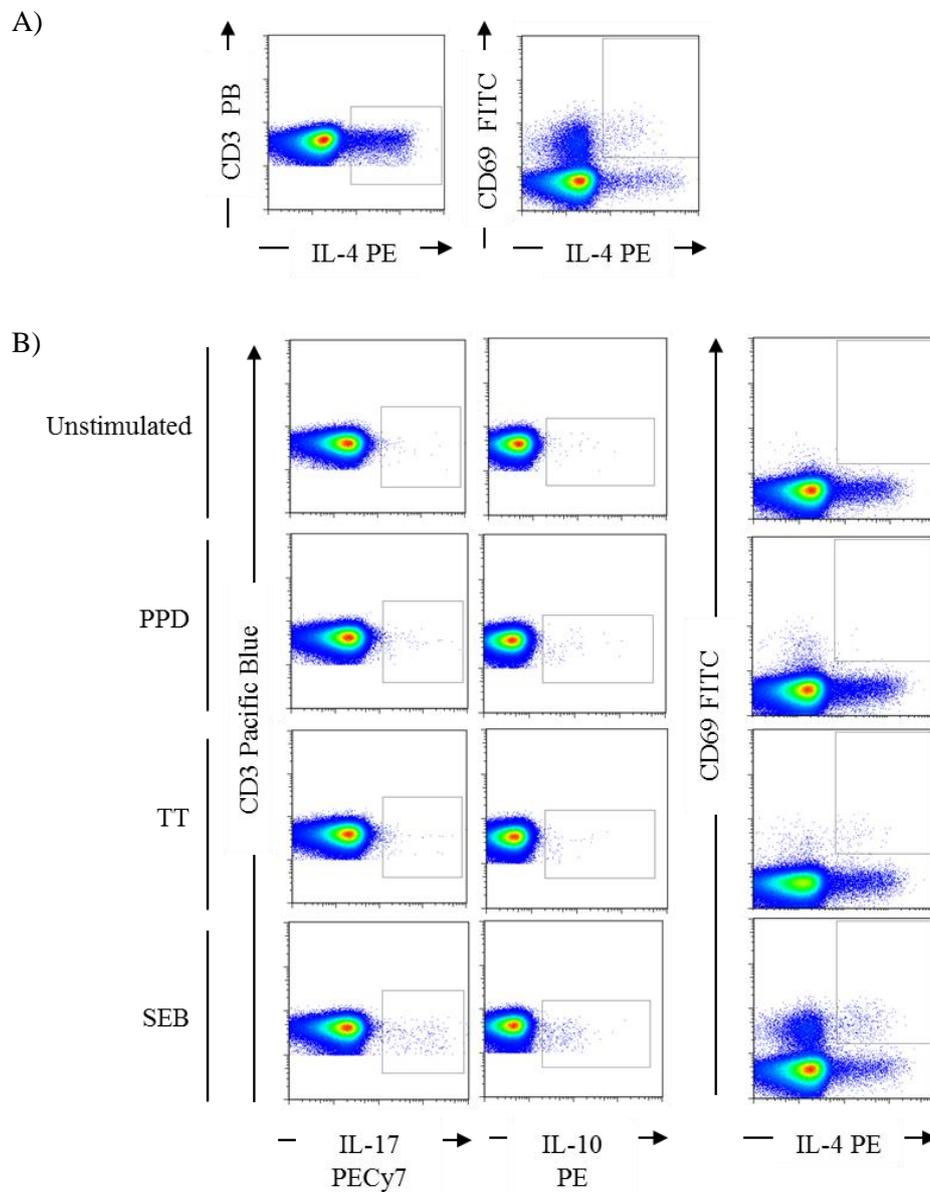
Figure 4.7 Gating strategy used to define populations of cytokine expressing cells stained with antigen-specific antibody panel 3. A) Antigen-specific IL-4 producing T cells were defined according to their co-expression of the early activation marker CD69 (right panel) due to high levels of non-specific staining of with the IL-4 antibody used (left panel). B) Positive cytokine events were defined by the gates set on unstimulated negative controls cells.

The response to TT stimulation was also dominated by a Th1 response (**figs. 4.9A & B**). In contrast to PPD stimulated cells however, the response to TT stimulation was almost exclusively driven by IL-2 and TNF-α secretion; negligible IFN-γ responses were observed. Mean TNF-α secretion in response to stimulation was higher in the 3 month age group and mean IL-2 secretion levels were equivalent between the age groups.

A direct comparison of the magnitude of the cytokine response to stimulation with PPD and TT in the 12 month age group showed that a similar magnitude of IL-2 and TNF-α expression levels were induced under both stimulation conditions (**fig. 4.10**). However, as noted, TT stimulated cells produced negligible levels of IFN-γ and a significant difference in IFN-γ expression level was therefore observed between stimulation conditions.

An assessment of the Th1 response following stimulation with the super antigen SEB was carried out. SEB stimulation induced expression of all 3 Th1 cytokine analysed in CD4 and CD8 T cells (**figs. 4.9C &D**). In CD4 T cells, the response was dominated by IL-2 and TNF-α and highly significant increases in the magnitude of the SEB response were observed in the month 12 group compared the month 3 group indicating heightened capacity to respond to antigenic stimulation at this age. This was particularly noted with respect to IFN-γ production, where a direct comparison between IFN-γ expression following PPD and SEB stimulations showed that in m3 infants similar frequencies of CD4 T cells were able to produce IFN-γ; however in m12 infants, the capacity to induce IFN-γ production was significantly increased following SEB stimulation (**fig. 4.10B**). A similar age-dependent increase in IFN-γ and TNF-α,

but not in IL-2 responses, was observed in CD8 T cells with the emergence of a dominant IFN-γ response to stimulation in the m12 group (fig. 4.9D).
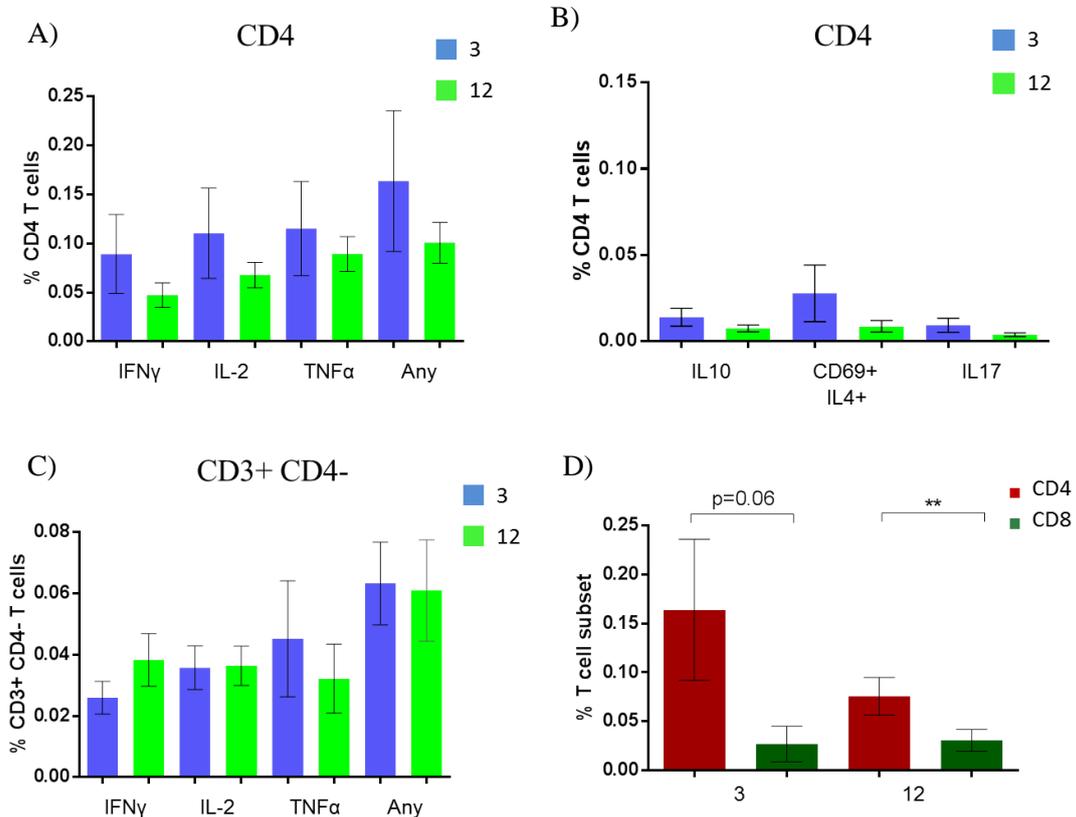
Figure 4.8 Cytokine responses in T cell subsets following short term stimulation with PPD in 3 and 12 month age groups. A) The percentage of PPD-responsive CD4 T cells expressing individual or any Th1 cytokines. B) The percentage of PPD-responsive CD4 T cells expressing IL-10, IL-17 or co-expressing IL-4 and CD69. C) The percentage of PPD-responsive CD3+ CD4- T cells expressing individual or any Th1 cytokines. D) A comparison between the cytokine expression levels of any Th1 cytokine expressed by CD4 and CD8 T cells following PPD stimulation of blood from infants in the m12 age group. Bar charts indicate the mean frequency ± the standard error of the mean. P values were calculated using a Mann-Whitney U test. **p<0.001.

1-way ANOVA analysis followed by Dunn's multiple comparisons test resulted in adjusted P value of **p=0.14.
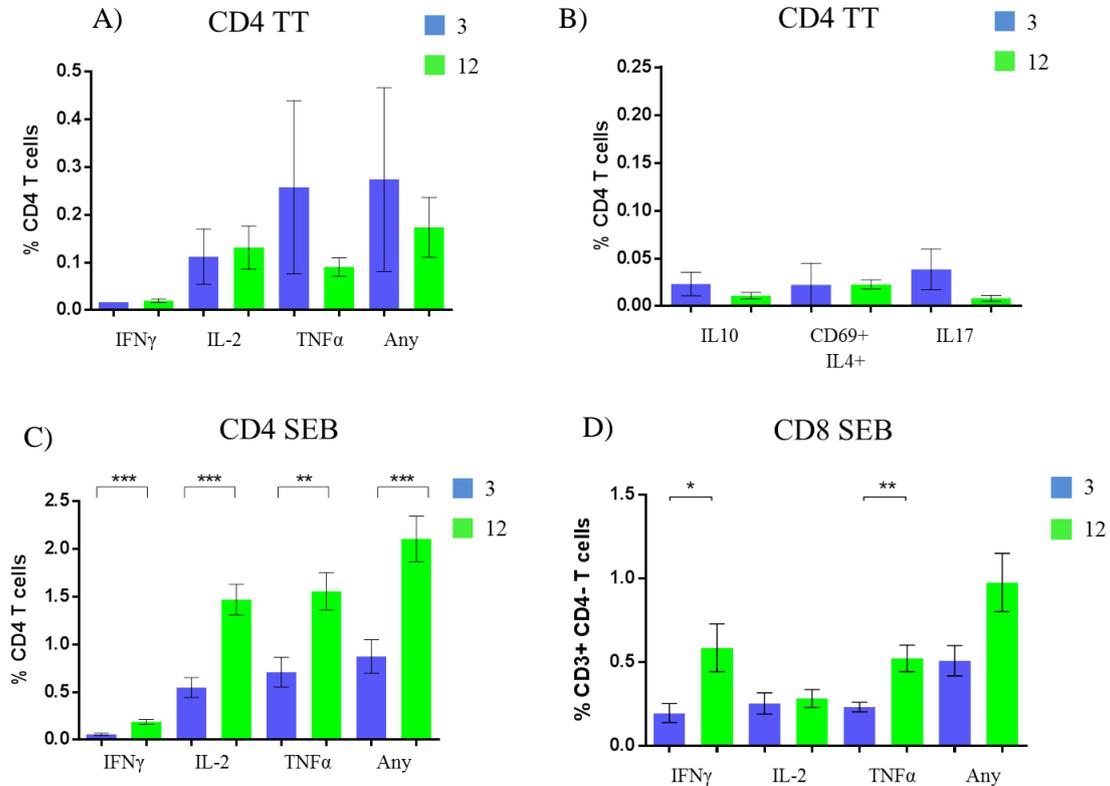
Figure 4.9 Cytokine responses in T cell subsets following short term stimulation with TT and SEB in 3 and 12-month age groups. A) The percentage of TT-responsive CD4 T cells expressing individual or any Th1 cytokines. B) The percentage of TT-responsive CD4 T cells expressing IL-10, IL-17 or co-expressing IL-4 and CD69. C) The percentage of SEB-responsive CD4 T cells expressing individual or any Th1 cytokines. D) The percentage of SEB-responsive CD8 T cells expressing individual or any Th1 cytokines. Bar charts indicate the mean frequency ± the standard error of the mean. P values were calculated using a Mann-Whitney U test. * $p<0.05$ **$p<0.001$, ***$p<0.0001$.
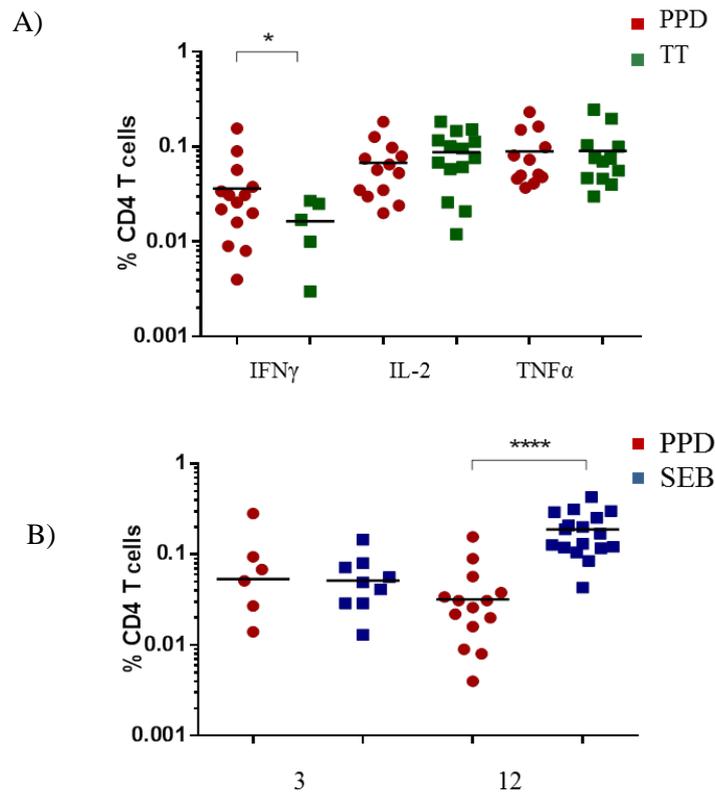
Figure 4.10 Comparisons of antigen-specific cytokine responses between stimulation conditions. A) A comparison of infants Th1 responses to PPD and TT stimulations in the m12 group. B) A comparison of IFN-γ responses to PPD and SEB stimulations in infants from m3 (3) and m12 (12) age groups. The black line indicates the mean. Mann Whitney U test was used to assess differences between groups.* p<0.05 ****p<0.0001

4.2.8   Polyfunctional cytokine responses following *in vitro* stimulation with PPD, TT and SEB

Several lines of evidence indicate that the quality of the T cell response as well as the magnitude is important in providing immunological protection against disease. An analysis was therefore carried out to characterise the quality of the CD4 antigen-specific and SEB responses in the two age groups. **Figure 4.11** summarises the

analysis. An almost equal distribution of single TNF-α, double IL-2/TNF-α co-expressers and triple expressers was observed in PPD-specific CD4 T cells, with the latter contributing to approximately 20% of the total response. No statistically significant differences were observed in these contributions between the two age groups. As mentioned previously, negligible IFN-γ responses were induced by TT stimulation and the response was dominated by a populations of IL-2/TNF-α co-expressing CD4 T cells in both age groups. Responses to SEB stimulation revealed a predominant dual IL-2/TNF-α response, with substantial contributions from single IL-2 or TNF-α producing cells and a significant age dependent increase in the proportions of cytokine combinations that included IFN-γ.

4.2.9 Memory phenotype of cytokine responsive cells following in vitro stimulation with PPD, TT and SEB

Lastly, the memory phenotype of antigens responsive cells was characterised under each stimulation condition in the two age groups. Analysis of CD45RA and CCR7 expression on CD4 T cells producing any cytokine (IFN-γ, IL-2 or TNF-α) showed that PPD-specific CD4 T cells were predominantly CD45RA- CCR7- (Tem) cells with a low proportion of CD45RA- CCR7+ (Tcm) and CD45RA+ CCR7+ (Naïve) responding cells and negligible responses from terminally differentiated CD45RA+ CCR7- (Temra) cells (**fig. 4.12A**). A trend to higher Tem responses was observed in the m3 group though this was difference was not statistically significant.
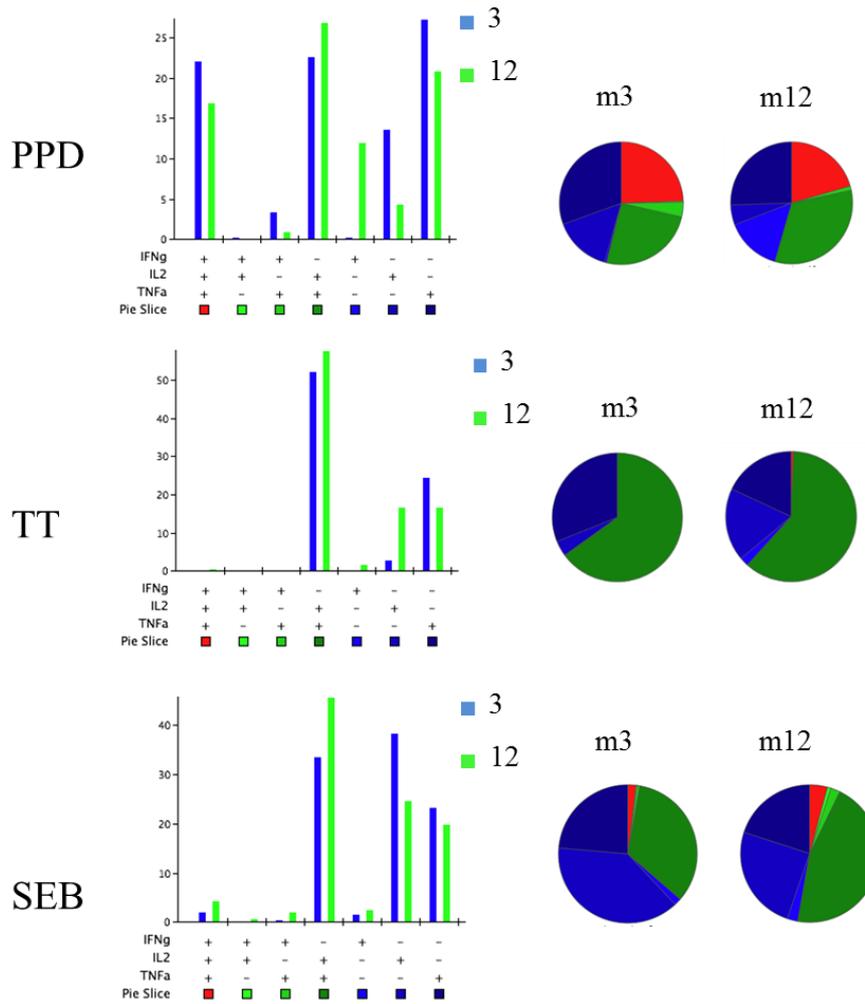
Figure 4.11 Analysis of polyfunctional cytokine responses in CD4 T cells following short term stimulation with PPD, TT and SEB in month 3 and 12 age groups. Boolean gating was used to assess the proportion of antigen-specific cells with single (blue), double (green) or triple (red) cytokine secreting capacity for IFN-γ, IL-2 and TNF-α. Normalized values are shown in the bar graphs, where the height of each bar represents the contribution of a particular cytokine combination relative to the overall response. The pie charts summarize the contributions to the total response with distinct colour shades corresponding to the indicated cytokine combination

Following TT stimulation, the majority of cytokine responsive cells were Tem cells, with low levels responses from Tcm and Temra memory subsets and from naïve T cells (**fig 4.12B**). A significant increase in the Tem response was observed in infants in the m12 group compared to infant in the m3 group.

Following SEB stimulation, cytokine responses were detected in all the memory subsets and in naïve cells (4.12C). This outcome likely results from the non-specific activation induced by SEB stimulation. The magnitude of cytokine responses increased significantly from 3 to 12 months of age in all memory subsets but not in naïve cells. This finding suggests that the age-specific increases in the magnitude of cytokine responses observed in **figure 4.9C** may be restricted to increased responses by memory CD4 T cells subsets and not by naïve T cells.

The expression of CD127, Ki67 and Bcl-2 was analysed following staining with antibody panel 2 (**table 2.4**). The Bcl-2 antibody used in this panel gave suboptimal staining outcomes which precluded analysis. Analysis of Ki67 expression on antigen responsive CD4 T and CD8 T cells showed >95% Ki67- phenotype following all stimulations conditions (data not shown) and time points and therefore no apparent variation. CD127 expression patterns on antigen-responsive cells are shown in figure 4. 13. PPD and TT-specific CD4 T cells were predominantly CD127+ at 3 and 12 month time points in similar proportions (**figs 4.13 A,B & D**). Following SEB stimulation, CD127- cytokine-responsive cells predominated, though a large proportion of CD127+ cells were also induced (**fig. 4.13C**).
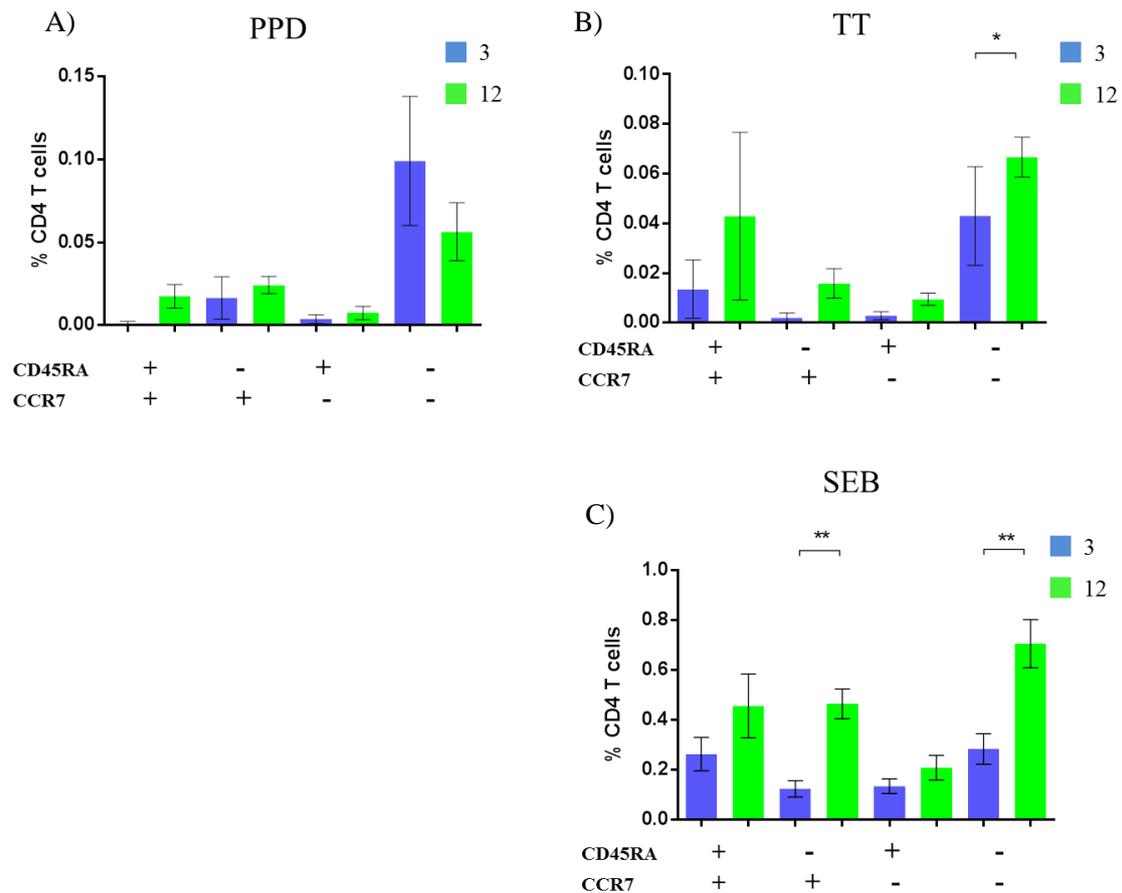
Figure 4.12 The memory phenotype of antigen-responsive CD4 T cells. Cross-sectional analysis of the frequency of memory CD4 T cell subsets secreting any cytokine measured in panel 1 following short-term stimulation with PPD (A), TT (B) and SEB (C). Comparisons were made between healthy control infants at month 3 (3) and month 12 (12). Bar charts indicate the mean frequency of CD4+ T cells expressing any cytokine ± the standard error of the mean. Mann Whitney U test was used to assess differences between groups.* $p<0.05$ **$p<0.01$
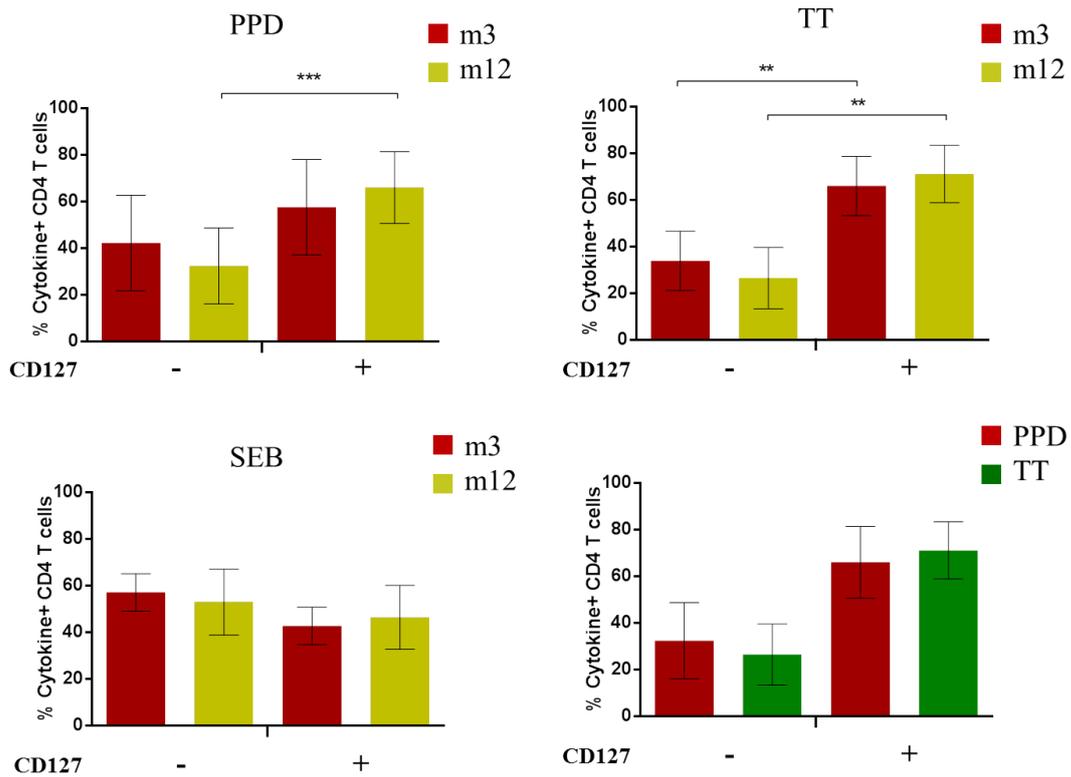
Figure 4.13 Percentage expression of CD127 on antigen responsive CD4 T cells following antigenic stimulation. The percentage of antigen-responsive CD4 T cells with positive or negative CD127 expression were measured following short-term stimulation with PPD (A), TT (B) and SEB (C) in 3 and 12 month old infants. D) A comparison between the percentages of antigen responsive CD4 T cells with positive or negative CD127 expression at month 12 following PPD or TT stimulation. Bar charts indicate the mean frequency ± the standard error of the mean. Mann Whitney U test was used to assess differences between groups.**p<0.001 ***p<0.0001

4.3 DISCUSSION

This study aimed to characterise the frequency and phenotype of cellular immune subsets that play a role in the induction of adaptive immune responses to immunological challenge. Infants are particularly susceptible to infection and undergo rapid developmental changes in phenotypic and functional immunological features. It is therefore important to characterise age-specific immunological parameters and describe any major alternations that may be associated with age. The data presented focus on two time points, 3 and 12 months of age.

The whole blood cell counts for the infants included in this study suggest that they have broadly healthy characteristics and may constitute a suitable control population to assess immunological alterations in HEU infants. Reference values from two East African countries were used as comparators as it has been shown that European reference ranges may not be appropriate for African populations (321). The median platelet value was the only parameter that was above the median East African reference values and this was consistent at both time points, though the upper 90th percentile value was within the reference interval. Significant changes to whole blood cell populations have been shown to occur in early life though no statistically significant changes in the parameters measured were apparent in this study at the two time points.

Ex vivo immune activation on T cells and expression levels of markers associated with immune exhaustion, PD-1 and Tim-3 were quantified. Immune activation in HIV-1 infection has been associated with poor outcome and is linked to the differentiation state of populations of T cells subject to chronic antigen stimulation (52,322). Concomitantly, chronically stimulated T cells have been shown to up regulate

exhaustion markers, such as PD-1 (52,323) and Tim-3 (48) that mediate a decline in functional capacity. In the context of this study, immune activation is likely a surrogate marker for early exposure to pathogen antigens either *in utero* or in early life. Co-expression of CD38 and HLA-DR is typically used to identify activated T cell populations. The constitutive expression of CD38 on CD4 and CD8 T cells in infants (314,315) was confirmed in this study and persisted up until 12 months of age. Little is known regarding the dynamics of exhaustion marker expression in infant T cells. It is surprising that PD-1 and Tim-3 expression were detected at all in healthy infant T cells; it is likely that their expression is indicative of immune activation on infant T cells. Data on the functional consequences of the expression of exhaustion markers on infant T cells is lacking. No correlation between PD-1 expression and CD38/HLA-DR co-expression was observed suggesting that the mechanism of induction of exhaustion marker expression and immune activation marker expression may not be the same. Overall, infant T cells showed striking levels of variation in immune activation and exhaustion marker expression with particularly wide levels of variation in CD8 T cells. Numerous factors may contribute to these variations including the effects of vaccinations, and concurrent infections. During recruitment, infants were excluded with clinical signs of infection. However, subclinical infections with pathogens such as CMV (324,325) and EBV (326) have been shown to drive the phenotypic redistribution of T cells. It was not possible to account for co-infections here though this is likely to be a major factor accounting for the observations presented. The influence of vaccination by contrast could be expected to be largely uniform on account of the standardised vaccination schedules (even if not rigidly adhered to), though genetic differences between individuals may mediate differential activation profiles in

response to vaccination. Lastly, CD8 T cells expressed higher frequencies of all markers assessed. This may be interpreted to indicate that CD4 T cell are less activated that CD8 T cells or that these markers may not adequately measure activation on CD4 T cells and other markers may be more appropriate.

The frequencies of FOXP3 positive T regulatory cells were found to be comparable at 3 and 12 months of age. Lack of standardised markers used to identify regulatory T cells in infants complicates cross-study age-matched comparisons, however the frequencies reported here were comparable to those reported in neonates using equivalent markers (40,327). An exploration of the use of CD127 as a marker for infant Tregs showed significant correlations with the use of FOXP3 albeit with an identification of higher frequencies of Tregs with the latter method. These results suggest that the method presented here to quantify Tregs offers a reliable readout of T cells with regulatory properties that can be used to assess deviations in these populations in HEU infants.

This study documented age-related changes in the *ex vivo* frequency and phenotype of memory T cell subsets. The data indicate the following general trends: an age-related decrease in naïve CD4 and CD8 T cells and increases in memory CD4 and CD8 T cell populations. Statistical significance was observed in relation to decreased naïve CD8 T cell frequencies and increased frequencies of CD4 Tem cells and CD8 Temra cells at month 12. These findings are in accordance with a detailed longitudinal report analysing the redistribution of memory subsets T cell subsets in HIV-infected South African infants (328). The indications from these data are that age-related changes in exposure to pathogens likely drive the expansion of memory T cell subsets resulting in the observed changes in subset frequency. Interestingly, differences were observed in the distribution of memory subsets within CD4 and CD8 T cells. CD4 T cells exhibited

significantly increased proportions of Tcm and significantly decreased Temra subsets compared to CD8 T cells. This is perhaps an indication of distinct differentiation dynamics among T cell subsets and may also reflect the natural history of the pathogenic challenges faced in the infants studied. A key outcome of this analysis relates to the relationship between expression of CD127 and Bcl-2 on T cells. The pooled analysis carried out revealed a highly significant positive correlation between the expression of these markers on CD8 T cells and also, though less striking, on CD4 T cells. This is in accordance with studies in mice where IL-7a receptor expression has been associated with long-lived memory CD8 T cells that express high levels of Bcl-2 (329). Limited data are available on the relationship between these markers in humans particularly in infants. The present results indicate that at least in infant CD8 T cells, the pathways that lead to IL-7Ra and Bcl-2 expression may be closely linked. The diversity of the CD4 T cell subsets may help explain the less robust correlation observed where for instance T regulatory cells that have been shown to express low levels of CD127 may nevertheless express anti-apoptotic markers such as Bcl-2.

The analysis presented here relating to the frequency and phenotype of circulating dendritic cell population revealed 1) a stable median frequencies of mDC and pDCs over the age time points; 2) an overall increased frequency of mDC compared to pDC in circulation; 3) constitutive expression of CD86 on mDCs and variable expression levels on pDCs; and 4) negligible levels of CD83 expression at month 3 and PD-L1 expression at month 12. There is limited knowledge regarding changes in DC cell frequencies and phenotype in infancy. In cord blood, pDC numbers have been reported to be higher than mDC and to decrease with age (21,22). The date presented here showed substantial variation in the mDC/pDC ratio, particularly in 3 month old infants,

perhaps due to on-going fluctuations in pDC cell subset frequencies. These changes may also be influenced by migration to peripheral lymphoid sites following activation. Phenotypically, the DC subtypes analysed were predominantly immature. Although mDC showed constitutive expression of CD86, lack of CD83 expression on both DC subsets indicates reduced functional capacity (330). This may indicate broad levels of DC immaturity or alternatively, a lack of detection of mature DC that relocate to peripheral lymphoid organs following activation. Expression of the negative costimulatory molecule PD-L1 has been detected on adult DCs *ex vivo*. Lack of the detection of PD-L1 expression in infant DC indicates that at this age negative co-stimulation of T cells through the PD-1 axis may not influence T cell function. Further characterisation of the role of negative co-stimulation infants would clarify this.

Functional characterisation of the cytokine responses in T cells following short term stimulation with PPD TT and SEB was carried out in this study. The results confirm that the PPD-specific T cell response is dominated by CD4 T cells producing the Th1 cytokines IFN-γ, IL-2 and TNF-α (**fig.4.8**)**.** Th2 responses following BCG-vaccination are considered sub-optimal and the IL-4 responses presented here were negligible (**fig. 4.8**). IL-10 secretion following BCG-vaccination has been hypothesised to result from the induction of BCG-specific T regulatory cells and IL-10 production has been detected following neonatal BCG vaccination (302). In addition, IL-17 responses have been described following BCG vaccination of neonates (61,263), and in adults they are thought to play a role in protection from TB infection (331). No significant IL-10 and IL-17 responses were found in the present study (**fig. 4.8**) indicating that BCG vaccination resulted in a narrow induction of T helper cell populations. PPD-specific CD8 T cells were also induced in accordance with previous reports (60) though due to

the low frequencies of these responses, subsequent analysis of poly-functionality and memory phenotype was not carried out.

Interestingly, at 3 months of age PPD-specific IFN-γ responses from CD4 T cells were of a similar magnitude to those achieved through polyclonal expansion of T cells stimulated with SEB (**fig. 4.10**). Specific impairments in infant production of IFN-γ have been described under diverse stimulation conditions that arise, particularly in CD4 T cells, as a result of epigenetic modifications of the IFN-γ gene promoter (332). Following SEB stimulation an age dependent increase in IFN-γ production has been shown to occur in infants, though adult levels are not reached even in late childhood (292). SEB activation of T cell bypasses the peptide-specific mechanism for antigen presentation and leads to an expansion of T cells expressing a T cell receptor with particular Vβ chains. The present data therefore suggests that events during the priming of BCG-specific populations lead to the development of clonal T cell populations with enhanced IFN-γ-producing capacities in comparison to other clonal populations of T cells. The quality of the innate response to BCG is likely to be implicated in mediating this outcome.

Little is known about the T cell response to TT in infants. The present study showed a dominant Th1 response to short term stimulation. This response was higher at the time point closer to vaccination and was mediated by IL-2 and TNF-α with negligible IFN-γ production. These finding are paralleled in a recent report from 6 month old infants who had received the full tetanus vaccination schedule (59). In adults revaccinated with TT, the IL-2 response was also found to be dominant over IFN-γ, though IFN-γ responses were readily detectable and persisted for a year post vaccination (291). The present data therefore suggest that the role played by CD4 T cells in eliciting protective

humoral responses following TT immunisation of infants is mediated through Th1 cells that lack IFN-γ production.

The capacity of PPD, TT and SEB to induce polyfunctional CD4 T cell responses was assessed. The combined expression of multiple cytokines has been associated with better outcome in HIV-1 infection (307) and with vaccine induced protection from intracellular pathogens such as *Leishmania major* (305) and *M. tuberculosis* (306) in mice. No age-related significant differences were observed between 3 and 12 month old infants under any of the stimulation conditions. These findings are largely in accordance with a previous longitudinal analysis (55) assessing polyfunctional responses following short-term BCG stimulation, though this report suggested a decrease in the proportion of cells producing 2 cytokines from 3 to 9 months of age. Polyfunctional responses to TT vaccination in infant have not been previously assessed and the results presented here indicate that TT-specific CD4 T cells with dominant dual (IL-2/TNF-α) cytokine producing capabilities are maintained up to 12 months of age. Previous reports in adults have not assessed TNF-α production following TT-stimulation, but have reported dominant IL-2 responses and low IFN-γ responses (291,304). The quality of the response following SEB stimulation, predominantly driven by combinations of IL-2 and TNF-α, underscores the observed limitations in IFN-γ production mentioned previously.

The final analysis presented in this chapter relates to an assessment of the memory CD4 T cell populations that contribute to the cytokine response under the different stimulation conditions. The main finding from this analysis is that both PPD and TT-specific CD4 T cells assumed a predominant effector memory phenotype at both time points analysed and are predominantly CD127+. Published reports from the Hanekom

lab, from which the stimulation protocol used in this analysis were adapted (283), have shown that following the contraction of the peak phase of the BCG response in infants, BCG-specific memory T cells principally assume effector memory (55,62) or central memory phenotypes (59). It is unclear what determines these distinct outcomes, though differences in anti CCR7 antibody clone have been suggested (59). TT-specific IL-2 CD4 T cells in adults have been shown to transition from Tem to Tcm following the peak response at 11 days after revaccination. A trend to an increase in TT-specific Tcm cells was observed at the 12 month time point though the data strongly suggest that infant TT vaccination predominantly induced responses from Tem CD4 cells. This is in contrast to what might be expected from a memory response to a non-persistent antigen response according to the model proposed during chronic viral infection (51). Further studies may help clarify if the developmental pathways of memory T cell subsets are distinct in infants and adults.

Staining with panel 2 presented some unexpected results. Firstly, the Bcl-2 antibody produced suboptimal staining and could not be included in the analysis. However, the finding that Bcl-2 and CD127 expression levels correlate *ex vivo* on CD4 T cells indicates that a substantial proportion of CD127+ antigen-specific cells assayed may well co-express Bcl-2 and represent cell populations that are not prone to apoptosis. Staining with Ki67 resulted in clear identification of bulk Ki67 expressing T cells (**fig 4.6**) however, analysis of antigen-specific cells that produced any cytokine indicated that >95% were Ki67 negative. Previous studies have detected Ki67 expression intracellularly following short-term stimulation (291), though there are other reports that expression levels are only detectable in antigen-specific cells after 6 days of

stimulation (333). The present data indicate modifications to the current protocol are required for adequate detection of antigen-specific Ki67 upregulation.

As mentioned in the text, numerous variables have been reported to influence the frequency and phenotype of *ex vivo* cell populations and vaccine outcomes. These include exposure to co-infections, gender, birth season and genetic background, among others. In order to minimise these confounding factors, the study participants were recruited from similar geographic locations, gender disparities were kept as limited as possible, and age was matched to with a close range. Nevertheless, it is important to interpret the findings presented here in the context of possible uncontrolled confounding factors. Therefore extrapolations of these findings to other human populations must be done with caution. Notwithstanding, the characterisation of the immunological parameters detailed at 2 time points enables age-specific comparisons to be made to infant populations within the same community.

5 CHAPTER 5: ANALYSIS OF ALTERNATIONS IN *EX VIVO* IMMUNE CELL POPULATIONS AND VACCINE-SPECIFIC T CELL RESPONSES IN HIV-EXPOSED UNINFECTED INFANTS

5.1 INTRODUCTION

One of the great success stories relating to the control of the HIV-1 epidemic has been the development of successful antiretroviral interventions that significantly reduce the likelihood of mother to child transmission (MTCT). This has led to virtual elimination of MTCT of HIV-1 in developed countries with universal access to health care. In many sub Saharan African countries, significant obstacles exist preventing universal access to health care including antenatal care; however over the past 10 years the number of babies born free from infection as a result of PMTCT interventions has increased significantly (**fig. 1.4**) (114).

Despite these gains, it has been repeatedly reported that infants born to HIV-1 infected mothers that are themselves free from infection, termed HIV-exposed uninfected infants, suffer from significantly higher rates of morbidity and mortality than infants born to HIV uninfected mothers (209,211,212,334). In addition, these reports suggest a heightened susceptibility to infectious disease (213,335–337). Currently in some areas of southern Africa, up to 30% of all new-borns are HEU (208). Therefore defining the underlying mechanism that mediate poor health in HEU infants observed in epidemiological studies is an increasing public health priority for countries with HIV-1 prevalence rates.

Obvious disadvantages exist from being born into an HIV-infected household in relation to care and heightened exposure to pathogens, and these influences are likely to contribute to the observed health outcome in HEU infants. In addition, a fundamental question is whether exposure to HIV-1 or antiretroviral drugs during the critical stages of foetal and/or neonatal development may have long lasting effects on infant immunity.

Numerous reports to date have described immune abnormalities in HEU infants. An unequivocal observation relates to the reduced capacity of trans-placental transfer of maternal antibodies to the foetus (226,338–340). Maternal antibodies are critical in mediating early infant protection from infection and their reduced levels likely contribute to poor health in HEU infants, though this has not been specifically addressed. Immunological alteration in the components of the infants own immune system have also been described. HEU infants receiving ARV-prophylaxis exhibit subclinical yet sustained reductions in various haematological cell lineages, including lymphocytes, neutrophils and platelets (237,238,341) that may persist into late childhood (240). Phenotypically, increased *ex vivo* populations of antigen-experienced T cells, identified through expression of memory and activation markers, have been reported as well as reductions in naïve T cells (222,249,342). These effects have been attributed to foetal exposure to HIV-1 following the trans-placental passage of HIV-1 antigens. The existence of this phenomenon is supported by the detection of HIV-1-specfic T cell responses in HEU neonates (217–220,343). Indeed, removal of regulatory T cells has been reported to reveal suppressed HIV-1 specific T cell responses in HEU infants (216). In addition, modulation of regulatory T cell frequencies has been observed in HEU cohorts (216), though this is not a consistent

finding (250). Alterations in dendritic cell populations have been assessed in a limited number of studies. Functional alteration in TLR-agonist induced IL-12 production was reported, though alternations in the frequency and basal expression of co-stimulatory molecules CD86 and CD80 were not detected (254). In addition, increased cord blood mDC frequencies were observed in a Colombian cohort of HEU infants (20).

The functional capacity of the adaptive immune response has been assessed in various studies. A common approach has been to measure responses to routine immunisation, as this presents a scenario of a controlled antigenic challenge. The protection elicited by the majority of infant vaccinations is antibody mediated. Conflicting reports have emerged with regards to induction of antibody responses following EPI vaccinations, with reports of increased (338), level (256) and reduced (257) antibody titres in HEU infants. Tetanus toxoid vaccination has been reported to induce protective antibody titres in HEU infants, though reduced geometric mean anti-tetanus titres were detected in comparison to healthy controls (344). In addition, the measurement of the secretion of T cell cytokines following *in vitro* stimulation of whole blood with TT resulted in reduced IFN-γ, IL-5 and IL-13 cytokine production from HEU infant cells (345).

The data on T-cell responses to BCG are also conflicting. BCG is the only EPI vaccine which is thought to mediate protection through T cell responses (though protective correlates have not been identified) and therefore suboptimal responses may have public health implications. Impairment of BCG immunogenicity was suggested in an early study in The Gambia, which reported significantly reduced occurrence of BCG scars after vaccination in HEU children (258). More recently, altered proliferative responses following *in vitro* BCG stimulation were reported in HEUs at 10 weeks(346) and at a median of 7 months of age(260). A trend to lower IFN-γ production was seen

in whole blood stimulated with PPD from 6 week-old HEU infants(64). By contrast, the same study, in which the entire HEU cohort presented a BCG scar, found no demonstrable effect of maternal HIV-infection status on IFN-γ secretion after stimulation with BCG or the *M. tuberculosis* antigen ESAT-6. This finding is in accordance with others in which neither IFN-γ secretion alone (346), nor IFN-γ, IL-13, IL-5 or IL-10 secretion (345), were compromised in HEU infants following *in vitro* stimulation with mycobacterial antigens. Finally, perhaps the most detailed study to date explored cytokine profiles of T cells responsive to mycobacterial antigens in HEU infants longitudinally over the first year of life by flow cytometry(347). The authors found no significant difference in the magnitude and polyfunctional capacity of BCG-specific T cells between HEU and control infants.

The present study was designed to assess immunological perturbations in a cohort of Kenyan HEU infants sampled at 3 and 12 months of age. The principal objective of this study is to assess whether there are significant alterations in memory T cell responses to vaccination with BCG and TT. I focused on several other features, discussed in this introduction, which have been found to be altered in HEU infants. Due to the significant differences in immunological characteristics between different human populations and between geographical locations, it is important to gain broad perspectives on immune health in infant populations. In addition, the incidence of HIV-1 in women of childbearing age is 8% Kenya and HEU infants represent significant proportions of new-borns. In Chapter 4, I characterised the immunological features under study here in a cohort of community age-matched healthy infants. In conjunction with these finding a cross sectional comparisons between age-matched HEU infants and control infants is presented

5.2 RESULTS

5.2.1 Study participants

We sought to assess whether HEU infants recruited into the IIS cohort presented significant alterations in 1) frequency and phenotype of select immune cell populations and 2) antigen-specific responses to BCG and TT vaccine antigens and to SEB stimulation. To achieve this, a cross-sectional analysis was carried out in 3 and 12 months old infants and comparisons were made to healthy infants described in Chapter 4. **Figure 5.1** illustrates the design of the study within the wider IIS cohort and the age matched infants from the CSC cohort. n=19 3 month old and n=39 12-month old HEU infants were initially recruited. By 9 months of age n=2 3 month-old infants from the IIS cohort seroconverted. These two infants remained in the study until 12 months of age, at which point an additional 2 infants were found to be HIV-1 infected. HIV-1 infected infants were excluded from the analysis resulting in n=17 infants available for analysis at month 3 and n=36 infants available for analysis at month 12. Analysis of antigen-specific responses were performed using this entire cohort. *Ex vivo* analyses were carried out on a reduced number of infants that are detailed in the text and in the results tables.
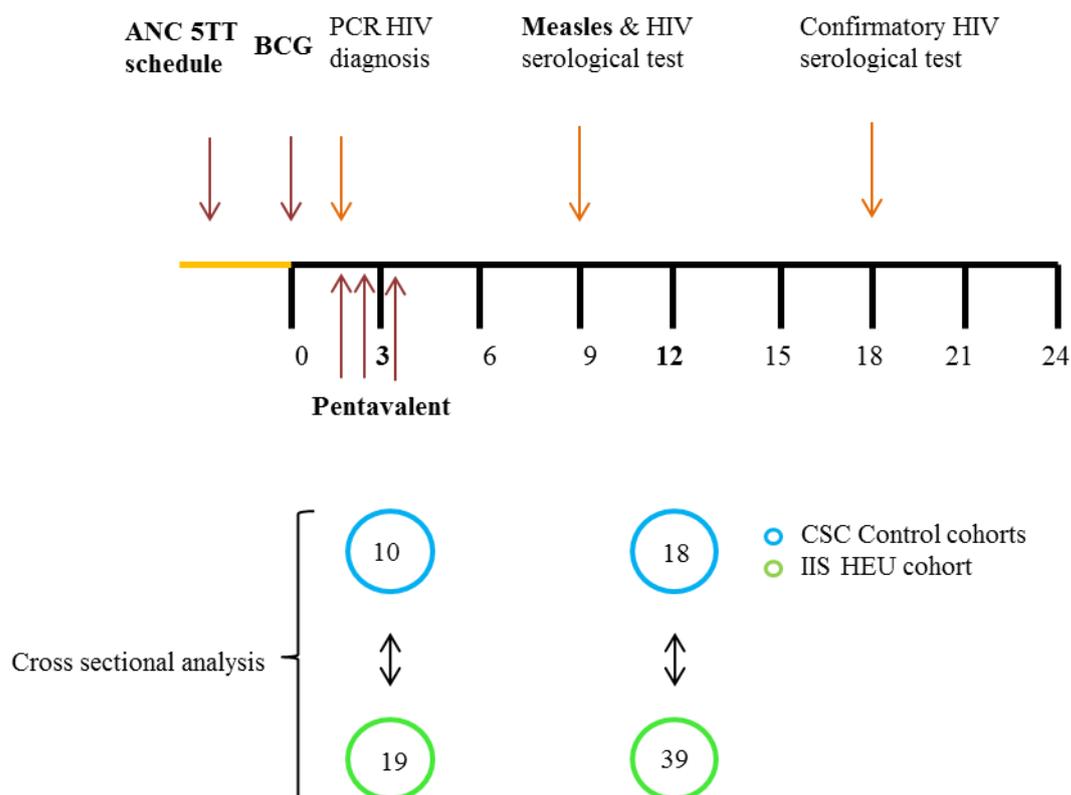
Figure 5.1 Diagrammatic representation of the study design. HEU infants aged 3 months and 12 months (bold) receiving care at the CCRC were recruited onto the IIS cohort as detailed in the Methods section. Kenyan EPI vaccination guidelines indicate vaccination with BCG and the pentavalent vaccine (**table** 1.1) at the time points shown. Infant HIV-1 diagnosis was carried at the time points indicated. A cross sectional study design was used to compare control infants cohorts and the HEU cohort at the two time points with the number of individuals shown inside the circles.

Age in healthy and HEU infant cohorts was well matched, with median age in months of 3.5 vs. 3.5 in the month 3 group and 12.3 vs. 12.0 in the month 12 group in control and HEU groups respectively (**table 5.1**). In addition, a well matched gender distribution was observed in both cohorts

All infants reportedly received full doses of the relevant vaccinations though as noted in Chapter 4, vaccinations were often administered off schedule. The median age of vaccination with BCG and with the 3 doses of pentavalent vaccine did not differ significantly between study groups (**table 5.1**). As in Chapter 4, analysis of cytokine responses to PPD, TT and SEB stimulation was carried on all HEU at 12 months of age. However, 3 month old HEU infants who received BCG vaccination >16 days after birth (n=3, all female) were excluded from analysis.

5.2.2 Whole blood count ranges at 3 and 12 months in healthy and HEU infant cohorts

Cross-sectional comparisons between control and HEU infant cohorts at 3 months of age showed no statistically significant differences in whole blood counts (**table 5.2**). Median values for all the parameters assessed were remarkably similar, though the 10th and 90th percentile ranges were wider in HEU infants. In the 12 month-old infant cohorts significant increases were detected in total lymphocyte counts (5.6 vs. 7.5 10^9/L, p=0.005) and in the number of white blood cells (8.7 vs. 10.5 10^9/L, p=0.003) in HEU infants. The upper 90th percentile value for white blood cell count in 12 month HEU infants also fell outside of the upper reference values (19.5 vs. 17.310^9/L) presented in **table 5.2** for East African infants, suggesting lymphocytosis. It is therefore likely that a number of infants in this cohort had concurrent infections during the time of sampling.

Table 5.1 Age, gender, age at vaccination and HIV-1 infection diagnosis in HEU and control infant cohorts

Characteristic	Month 3		P value	Month 12		P value
	Control (10)	HEU (19)		Control (18)	HEU (39)	
Median age in months at sampling (range)	3.5 (2.5-4.2)	3.5 (2.5-4.5)	¥NS	12.3 (9.6-13.9)	12.0 (10.7-14.0)	NS
Female sex (%)	6 (60)	8 (42)	-	12 (67)	20 (51)	-
Median age in days at BCG vaccination (range)	3.5 (1-31)	5 (0-117)	NS	4 (1-75)	8.5 (0-117)	NS
Median age in weeks at 6 week pentavalent vaccination (range)	6.5 (6.2-7.2)	6.1 (5.7-7.2)	NS	6.3 (3.4-19.9)	6.5 (5.7-16.3)	NS
Median age in weeks at 10 week pentavalent vaccination (range)	11 (10.3-12)	10.3 (9.7-16.7)	NS	10.8 (7.9-24.7)	11.0 (10-31.1)	NS
Median age in weeks at 14 week pentavalent vaccination (range)	15.2 (14.9-16.3)	14.6 (10.4-21.7)	NS	15.4 (13.8-30.0)	16.1 (11.1-35.0)	NS
HIV-1 RNA detectable at 6 weeks (n)	NA	0	NA	NA	2	NA
Anti-HIV-1 antibodies detectable at 9 months (n)	NA	2	NA	NA	4	NA

¥ An unpaired T test was used to assess significant changes for all parameters *NS, not significant

Table 5.2 Cross-sectional comparison of whole blood cells counts between healthy controls and HEU infants at 3 and 12 months of age.

Characteristic	3-months			12-months		
	Control (n=10) Median (10th & 90th percentiles)	HEU (n=16) Median (10th & 90th percentiles)	¥P value	Control (n= 16) Median (10th & 90th percentiles)	HEU (n= 24) Median (10th & 90th percentiles)	¥P value
Red blood cells (10^6)	4.3 (3.9-4.7)	4.24 (3.4-5.3)	NS	5.0 (4.7-5.2)	4.8 (3.8-5.6)	NS
Haemoglobin (g/dL)	10.2 (9.2-10.6)	10.5 (8.7-11.0)	NS	10.0 (9.5-10.6)	9.0 (7.6-11.0)	NS
Platelets (10^9/L)	446.6 (317.3-575.9)	441.0 (196.3-667.8)	NS	452.4 (380.7-524.0)	442.0 (163.8-687.0)	NS
Neutrophils (10^9/L)	1.9 (1.1-2.7)	1.9 (1.0-4.7)	NS	1.9 (1.5-2.3)	2 (1.4-4.4)	NS
Lymphocytes (10^9/L)	6.3 (4.9-7.6)	6.9 (3.7-9.9)	NS	5.6 (4.7-6.5)	7.5 (4.5-9.9)	0.005
Monocytes (10^9/L)	0.7 (0.3-1.0)	0.7 (0.3-1.4)	NS	0.7 (0.5-0.9)	0.6 (0.3 -1.3)	NS
Eosinophils (10^9/L)	0.3 (0.2-0.4)	0.3 (0.1-1.0)	NS	0.4 (0.2-0.5)	0.4 (0.1 -1.4)	NS
White blood cells (10^9/L)	9.3 (7.0-11.6)	9.2 (6.0-15.4)	NS	8.7 (7.7-9.8)	10.5 (7.6 -19.5)	0.003

¥ An unpaired T test was used to assess significant age related changes for all normally distributed parameters and a Man-Whitney U test was carried out where data were not normally distributed

5.2.3 Changes in cellular immune activation and exhaustion marker expression at 3 and 12 months of age in control and HEU infant cohorts

Ex vivo analysis was carried out to assess alterations in expression levels of markers of cellular immune activation (HLA-DR and CD38) and immune exhaustion (PD-1 and Tim-3) in HEU infants. Accounts in the literature have shown specific increases in populations of T cells with antigen experienced phenotypes in HEU infants that have been attributed to enhanced antigenic exposure in foetal or neonatal life (216,222,342). Characterisation of the markers used to assess immune activation in healthy infants in Chapter 4 showed that much higher levels of expression for all activation markers were observed on CD8 T cells compared to CD4 T cells at both 3 and 12 months of age (**tables 4.3 and 4.4**) The present data indicate that on CD8 T cells there were no significant differences in median expression levels of any of the markers investigated (**table 5.3**). In addition, wide ranges of HLA-DR+ CD38+ coexpression were observed in both groups at both time points. Surprisingly, immune activation marker expression and PD-1 expression on CD4 T cells was increased in control infants at 3 months of age with HLA-DR CD38 expression reaching statistical significance (6.7 vs. 3.2%, p=0.01) (**table 5.4**). These differences were not apparent at the 12 month time point. In both CD4 and CD8 T cells and across both time points, the median values for HLA-DR/CD38 co-expression and PD-1 expression tended to be higher in control infants. Only median levels of Tim-3 expression at the 12 month time point were higher in HEU infants.

5.2.4 Regulatory T cell frequencies in control and HEU infants

Tregs are key modulators of immune responses that function to limit inflammation and can thus influence levels of immune activation. It was hypothesised that alternations in

immune activation levels may also result in altered frequencies of Treg. The data presented here showed no significant variation in circulating Treg frequencies (CD25hi FOXP3+ CD4 T cells) between control and HEU infants at 3 months and 12 months of age [m3: control: 5.1% (range: 1.4-7.0%) vs. HEU: 4.6% (range: 1.7-6.8%); m12: control: 4.8% (range: 1.81-7.96%) vs. HEU: 3.9% (range: 1.2-7.7%)] although the median Treg frequency in control infants tended to be higher at both time points (**table 5.4**).

5.2.5 *Ex vivo* **memory T cell populations in control and HEU infants**

I assessed whether redistribution of memory T cell subsets occurs in bulk T cell populations in HEU infants. This analysis was restricted to infants at 12 months of age due to procedural limitations and is summarised in **tables 5.3** and **5.4**. Surprisingly, statistically significant increased proportions of naïve (control: 58.8% vs. HEU: 60.7%, p=0.03) and decreased proportions of Tem (control: 13.5% vs. HEU: 9.1%, p=0.006) CD8 T cells were found in HEU infants. Similarly, significantly higher frequencies of Tem CD4 T cells were observed in control infants (control: 10.2% vs. HEU 6.9%, p=0.03), with a trend to lower proportions of naïve CD4 T cells (control: 66.2% vs. HEU: 73.2%, p>0.05). Analysis of the expression of CD127 levels on naïve CD4 T cells showed that in HEU infants a significantly larger proportion of these cells expressed CD127 (control: 88.5% vs. HEU: 92.2%, p=0.03). By contrast, median CD127 and Bcl-2 expression levels on bulk CD4 and CD8 T cells were equivalent between control and HEU infants.

Table 5.3 Comparison of the phenotype of CD8 T cells in peripheral blood of healthy and HEU infants at 3 and 12 months of age

Characteristic	3 months Median % of CD8 T cells unless indicated (range)			12 months Median % of CD8 T cells unless indicated (range)		
	Controls (n=10)	HEU (n=19)	¥P value	Controls (n=16)	ΦHEU(n=16)	¥P value
Activation & exhaustion						
CD38 HLA-DR	48.6 (2.5-65.2)	37.8 (3.5-75.4)	NS[a]	40.6 (14.8-75.5)	36.0 (10.2-72.9)	NS
PD-1	28.3 (12.7-47.1)	22.7 (2.8-47.3)	NS	32.3 (3.58- 87.2)	24.6 (9.6-92.3)	NS
Tim-3	18.4 (9.5-25.9)	13.1 (2.6-78.1)	NS	19.4(4.82-62.4)	23.2(9.6-69.2)	NS
CD38 HLA-DR PD-1	21.4(1.4-41.9)	12.5 (1.0-36.3)	NS	20.0(1.43-39.6)	13.1(3.2-56.0)	NS
CD38 HLA-DR Tim-3	9.4(0.5-16.7)	5.8 (0.8-63.6)	NS	13.3(1.91-36.0)	7.6 (4.5-40.8)	NS
CD38 HLA-DR PD-1 Tim-3	4.5(0.3-11.7)	2.7 (0.2-24.1)	NS	4.3(0.4-35.3)	3.0(1.4-38.6)	NS
PD-1 Tim-3	6.41(3.0-13.4)	3.3 (0.5-31.4)	NS	5.32(1.08-59.7)	6.8 (2.3-59.3)	NS
Anti-apoptosis						
Bcl-2+	55.8(27.5-96.8)	-	NA*	58.8(26.5-96.4)	60.7(27.4-93.1)	NS
Bcl-2-	44.2(3.22-72.5)	-	NA	41.3(3.6-73.5)	29.4(6.4-72.6)	NS
Memory						
CD127+	58.6(25.8-96.2)	-	NA	49.3(26.4-92.9)	45.5(18.1-67.6)	NS
CD127-	41.5(3.8-74.1)	-	NA	51.1(7.12-73.6)	54.5(32.0-81.9)	NS
CCR7+ CD45RA+ (Naïve)	62.1(50.4-96.0)		NA	49.1(25.6-86.8)	60.7(21.0-97.1)	0.03
Naïve Bcl-2+±	67.9(48.6-98.7)	-	NA	84.7(25.3-95.4)	85.2(22.4-97.6)	NS
Naïve Bcl-2-	25.6(0.8-50.9)	-	NA	9.0(4.04-72.4)	13.8(2.4-73.0)	NS
Naïve CD127+	78.9(45.5-99.5)	-	NA	79.0(20.1-97.5)	70.9(21.0-93.2)	NS
Naïve CD127-	21.8(0.5-55.7)	-	NA	21.1(0.3-79.3)	29.9(7.3-78.6)	NS
CCR7+ CD45RA- (Tcm)	4.1(0.5-45.0)	-	NA	2.9(0.7-9.5)	2.0(0.3-9.9)	NS
Tcm Bcl-2+	26.4(0.7-71.6)	-	NA	23.3(3.2-72.8)	29.2(4.97-67.3)	NS
Tcm Bcl-2-	72.0(27.9-98.2)	-	NA	75.4(20.4-95.2)	72.3(35.1-94.5)	NS
Tcm CD127+	42.9(4.6-85.0)	-	NA	31.4(5.8-68.4)	22.6(12.1-67.6)	NS
Tcm CD127-	60.8(16.7-95.5)	-	NA	70.1(30.0-93.3)	77.6(35.2-88.3)	NS
	9.9(0.4-37.4)	-	NA	30.0(2.98-65.0)	27.1(0.7-63.3)	NS
CCR7- CD45RA+ (Temra)						
Temra Bcl-2+	20.1(0-48.6)	-	NA	29.1(0.8-71.3)	31.3(6.3-82.1)	NS
Temra Bcl-2-	73.7(17.4-11.4)	-	NA	49.6(8.18-78.5)	44.1(12.6-78.6)	NS
Temra CD127+	31.7(3.6-91.8)	-	NA	8.6(1.9-81.6)	10.2(3.9-34.6)	0.04

Temra CD127-	57.1(8.52-90.6)	-	NA	80.7(8.5-92.6)	78.1(66.1-90.4)	NS
CCR7- CD45RA- (Tem)	7.4(0.4-26.4)	-	NA	13.5(2.9-32.0)	9.1(0.2-46.0)	0.006
Tem Bcl-2+	10.5(0.7-52.5)	-	NA	15.4(1.0-47.3)	21.3(2.4-56.5)	NS
Tem Bcl-2-	78.0(28.6-98.7)	-	NA	70.8(15.9-92.6)	72.2(33.7-95.5)	NS
Tem CD127+	34.2(5.1-60.7)	-	NA	13.2(2.4-35.4)	19.7(7.63-50.0)	NS
Tem CD127-	68.4(39.3-95.1)	-	NA	82.2(46.2-92.2)	76.8(50.0-91.2)	NS

[Φ]CD127expression was measured on n=11 samples [¥] P values were calculated using a Mann-Whitney U test.

[±]Indented values indicate percentage of memory T cell subsets. [a] NS=not significant [*]NA= not applicable

Table 5.4 Comparison of the phenotype of CD4 T cells in peripheral blood of healthy and HEU infants at 3 and 12 months of age

Characteristic	3 months Median % of CD4 T cells unless indicated (range)			12 months Median % of CD4 T cells unless indicated (range)		
	Controls (n=10)	HEU (n=19)	P value	Controls (n=16)	$^{\Phi}$HEU (n=16)	$^{¥}$P value
Activation & exhaustion						
CD38 HLA-DR	6.7(1.7-12.1)	3.2 (2.0-12.8)	0.01	6.4(2.5-14.0)	3.4(1.4-11.9)	NS
CD4 PD-1	12.8(8.7-16.0)	8.7(1.1-16.8)	0.06	13.6(7.2-25.1)	12.5(8.8-26.1)	NS
CD4 Tim-3	3.0(1.2-5.0)	2.1(0.3-10.0)	NS	1.8 (0.5-62.0)	4.0(1.3-77.1)	NS
CD38 HLA-DR PD-1	3.6(1.6-5.4)	1.6(0.4-8.9)	0.01	3.1 (1.3-11.0)	2.025(0.8-9.1)	NS
CD38 HLA-DR Tim-3	0.5(0.2-5.6)	0.3(0.1-1.4)	NS	0.5(0.2-3.9)	0.6 (0.2-7.2)	NS
CD38 HLA-DR PD-1 Tim-3	2.0(0.2-10.8)	0.2(0.1-1.3)	0.001	0.3 (0.1-30.6)	0.6 (0.2-3.9)	NS
PD-1 Tim-3	1.0(0.3-2.3)	0.8(0.1-2.7)	NS	0.7(0.2-8.6)	1.2 (0.6-10.6)	NS
Immune regulation						
CD25hi FoxP3+	5.1(1.4-7.0)	4.6(1.7-6.8)	NS	4.8(1.81-7.96)	3.9(1.2-7.7)	NS
Anti-apoptosis						
Bcl-2+	77.9(59.0-93.6)	-		74.55(22.2-92.2)	72.1(47.9-89.6)	NS
Bcl-2-	23.2(7.42-41)	-		25.45(7.82-77.8)	30.6(10.4-55)	NS
Memory						
CD127+	86.5(74.6-95)	-	NA	84.5(69.8-93.4)	83.7(74.8-94.9)	NS
CD127-	13.85(5.01-25.4)	-	NA	16.2(6.59-30.6)	16.9(5.36-26.4)	NS
CCR7+ CD45RA+ (Naïve)	74.8(58.5-79.2)	-	NA	66.2(43.5-83.9)	73.2(19.2-83)	NS
Naïve Bcl-2+	85.6(30.4-95.9)	-	NA	72.85(4.7-91.8)	77.5(40.2-93.7)	NS
Naïve Bcl-2-	16.65(4.88-74.2)	-	NA	30.4(9.33-96.3)	25.5(7.3-63.8)	NS
Naïve CD127+	92.55(89-99.7)	-	NA	88.5(62.3-99.9)	92.2(85.1-98.3)	0.03
Naïve CD127-	7.925(0.291-11.8)	-	NA	12.2(0.063-39.3)	8.2(1.83-15.9)	0.03
CCR7+ CD45RA- (Tcm)	14.3(9.49-33.5)	-	NA	16.15(8.51-42.8)	14.9(4.52-20.5)	NS
Tcm Bcl-2+	58.3(17.2-85.3)	-	NA	54.3(6.81-68.4)	59.6(30.7-69)	NS
Tcm Bcl-2-	44.1(16.5-84.9)	-	NA	48(33.7-94.1)	43(33.2-71.5)	NS
Tcm CD127+	67.7(50.9-90.4)	-	NA	68.65(38.5-93.1)	70.2(57.7-78)	NS
Tcm CD127-	33.4(10.1-50.3)	-	NA	32.4(7.4-62.6)	30.8(22.9-43.6)	NS
CCR7- CD45RA+ (Temra)	3.215(0.825-13.8)	-	NA	5.56(0.732-10.8)	2.56(0.949-52.2)	NS
Temra Bcl-2+	78.6(30.6-91.9)	-	NA	63.3(8.35-87.6)	74.4(34.8-88.9)	NS
Temra Bcl-2-	24.5(9.79-71.8)	-	NA	39.7(15.3-92.8)	27.8(12.4-68.4)	NS
Temra CD127+	72.45(45.8-97)	-	NA	61.5(21.3-98.7)	57.9(30-89.9)	NS
Temra CD127-	28.7(3.87-55.6)	-	NA	39.9(1.3-77.5)	43.4(10.8-70.4)	NS

CCR7- CD45RA- (Tem)	7.2(5.1-9.6)	-	NA	10.2(4.0-22.5)	6.9(2.8-24.1)	0.03
Tem Bcl-2+	48.7(16.1-85.9)	-	NA	56.35(15-76.9)	64.6(29.6-73)	NS
Tem Bcl-2-	53.3(15.7-85.2)	-	NA	45.4(24.8-86.5)	37.4(28.6-72.7)	NS
Tem CD127+	58.8(39.2-83.5)	-	NA	62.6(31.6-79)	60.7(35.2-79.8)	NS
Tem CD127-	41.9(17.1-61.6)	-	NA	38.05(21.6-69)	40(20.9-65.6)	NS

[Φ]CD127 expression was measured on n=11 samples. [¥] P values were calculated using a Mann-Whitney U test. [±]Indented values indicate percentage of memory T cell subsets. [a] NS=not significant *NA= not applicable

5.2.6 Dendritic cell subset frequencies and phenotype in control and HEU infants

Dendritic cell frequencies were determined at 3 and 12 months of age in control and HEU infants in peripheral whole blood. The results are summarised in **table 5.5.** No significant differences were observed between control and HEU infants in median frequencies of mDC at 3 and 12 months of age (0.23 vs. 0.23, p>0.05 and 0.26 vs.0.25, p>0.05 respectively) and pDCs at 3 and 12 months of age (0.15 vs.0.22, p=0.1 and 0.17 vs. 0.20, p>0.05, respectively). A trend to higher pDC frequencies was observed in HEU infants at 3 months of age which resulted in a significant decrease in mDC/pDC ratio (1.18 vs. 0.90, p=0.03) in HEU infants at this time point. This difference between control and HEU infants resolved in the 12 month age group (1.61 vs. 1.61, p>0.05).

Phenotypically, mDC from HEU infants constitutively expressed CD86 as in control infants (>95% expression levels). At 3 months of age, CD83 expression was significantly higher in HEU infants (0.06 vs. 1.90, p=0.0001) though the median expression levels were low. By contrast the MFI of HLA-DR was significantly lower in HEU infants in mDC at this time point (841 vs. 576, p=0.05). At month 12, no phenotypic differences were observed between the two groups. On pDCs, a significant decrease in the MFI of CD86 was observed in HEU infants the 3 month age group (37 vs. 30, p= 0.02) though this was not reflected in the median percentage expression of CD86 (26.0% vs. 33.0%, p>0.05). In contrast to mDCs in month 3 infants, the percentage CD83 expression was significantly higher in pDC from control vs. HEU infants (1.58% vs. 0.72%, p=0.02). In addition, a trend to a higher MFI of HLA-DR was seen in control vs. HEU infants at this time point in pDC (385 vs. 241, p=0.07). At 12 months of age no significant differences were observed in pDC phenotype between

the two groups. Basal PD-L1 expression was negligible on DC from both groups at 12 months age group. In Chapter 4, I reported the unexpected detection of CD86, CD83 and PD-L1 expression on basophil populations, which can be identified thorough the staining protocol used to identify DCs (**fig. 4.5**). These results were confirmed in HEU infants, although at month 3 a significant reduction in median CD86 (15.9% vs 1.1%, p=0.003) expression and a trend to reduced CD83 expression (38.7 vs. 3.3, p=0.06) was observed in HEU infants. At month 12, the differences in CD86 expression between the two groups resolved, though a significant increase in basophil frequency was observed in HEU infants (0.17% vs. 0.24%, p=0.04)

Table 5.5 Comparison of dendritic cell and basophil frequencies and phenotype between control and HEU infants at 3 and 12 months of age

Characteristic	3 months Median values (range)			12 months Median values (range)		
	Controls (n=10)	HEU (n=19)	¥P value	Controls (n=16)	HEU(n=16)	¥P value
mDC (% of leucocytes)	0.23 (0.15-0.39)	0.23 (0.10-0.57)	±NS	0.26 (0.09-0.48)	0.25 (0.07-0.43)	NS
CD86 (% of mDC)	96.6 (92.0-98.9)	96.9 (85.3-98.6)	NS	94.6 (66.10-99.1)	95.4 (83.1-99.5)	NS
CD86 MFI	117 (76-127)	82.1 (68-158)	NS	84 (43-146)	99 (66-197)	NS
CD83 (% of mDC)	0.06 (0.00-0.57)	1.90 (0.00-13.30)	<0.0001	-	-	NA
CD83 MFI	89 (0-355)	123 (15-181)	NS	-	-	NA
PD-L1 (% of mDC)	-	-	*NA	0.10 (0.00-2.24)	0.20 (0.00-2.54)	NS
PD-L1 MFI	-	-	NA	17 (10-212)	112 (11-364)	NS
HLA-DR MFI	841(384-1218)	576(368-1372)	0.05	877 (371-1370)	777 (549-1194)	NS
pDC (% of leucocytes)	0.15 (0.05-0.33)	0.22 (0.09-0.64)	0.1	0.17 (0.05-0.28)	0.20 (0.08-0.50)	NS
CD86 (% of pDC)	26.0 (10.4-43.6)	33.0 (9.07-64.7)	NS	27.7 (8.6-52.8)	38.2 (7.7-67.9)	NS
CD86 MFI	37 (25-146)	30 (21-82)	0.02	35 (18-124)	37 (30-80)	NS
CD83 (% of pDC)	1.58 (0.73-4.24)	0.72 (0.00-9.18)	0.02	-	-	NA
CD83 MFI	75.95 (42.9-678)	75.15 (11.5-347)	NS	-	-	NA
PD-L1 (% of pDC)	-	-	NA	1.19 (0-3.45)	0.51 (0.00-1.99)	NS
PD-L1 MFI	-	-	NA	48 (7-107)	70 (9-181)	NS
HLA-DR MFI	385 (172-546)	241 (165-619)	0.07	359.5 (179-715)	362.5 (271-665)	NS
mDC/pDC	1.18 (0.87-4.02)	0.90 (0.47-2.50)	0.03	1.61 (0.77-2.67)	1.61 (0.55-3.00)	NS
Basophils (% of leucocytes)	0.23 (0.13-0.59)	0.18 (0.12-0.37)	NS	0.17 (0.01-0.33)	0.24 (0.02-0.72)	0.04
CD86 (% of basophils)	15.9 (4.3-26.3)	1.1 (0.0-22.6)	0.003	7.3 (2.3-25.5)	7.7 (0.1-51.4)	NS
CD83 (% of basophils)	38.7 (0.0-44.9)	3.3 (0.9-55.4)	0.06	-	-	NA
PD-L1 (% of basophils)	-	-	NA	26.35 (0.00-45.7)	18.35 (2.29-49)	NS

P values were calculated using a Mann-Whitney U test. ±NS=not significant. *NA= not applicable

5.2.7 Single cytokine responses to in vitro stimulation with PPD, TT and SEB in control and HEU infants

In Chapter 4, the characterisation of infant responses to vaccine antigens PPD and TT revealed a dominant Th1 response under both stimulation conditions and a robust polyclonal Th1 response following SEB stimulation. As a result this study sought to assess whether Th1 responses under these various stimulation conditions are impaired in HEU infants. The first analysis that was performed relates to the quantification of the magnitude of CD4 T cells producing Th1 cytokines. The data indicate that following PPD stimulation, there were no significant differences in the production of IFN-γ, IL-2, TNF-α or of any of these cytokines combined between control and HEU infants at 3 and 12 months of age (**fig. 5.2**). Infant Th1 responses to BCG have been shown to peak at 10 weeks of age following vaccination at birth. At 3 months, the time point closest to the peak response, a trend to increased IL-2 and TNFα responses were observed in HEU infants. At the 12 month time point, this trend was no longer apparent.

Following TT vaccination, no significant differences were observed in the production of IFN-γ, IL-2, TNF-α or of any of these cytokines combined between control and HEU infants at the 3 month time point, though a trend towards heightened TNF-α responses was observed (**fig. 5.2**). However, at month 12 a significant decrease in the frequency of IL-2-producing CD4 T cells was observed in HEU infants following stimulation (**fig. 5.2**). This difference was not observed in the production of any other cytokine.

Analysis of cytokine responses following stimulation with the super-antigen SEB indicated a pattern of age-dependent differences in cytokine production capacity between control and HEU infants (**fig. 5.2**). In the 3 month age group, statistically significant increased production of IFN-γ, IL-2 and TNF-α or of any of these cytokines

combined was observed in HEU infants compared to controls. At the month 12 time point the opposite observation was made, where, with the exception of IFN-γ, all parameters analysed were statistically increased in control vs. HEU infants.
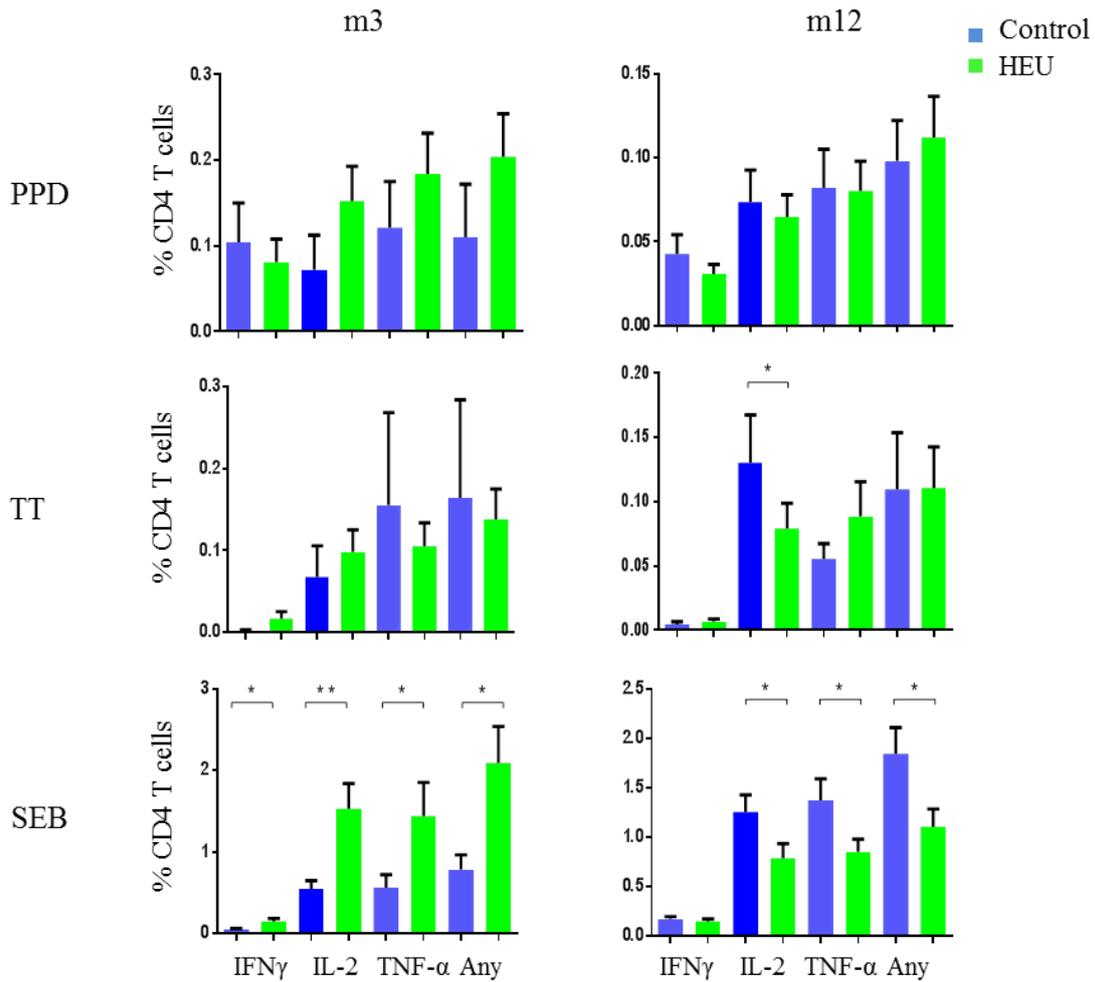
Figure 5.2 Single cytokine CD4 T cells responses. CD4 T cell cytokine responses following short term stimulation with PPD, TT and SEB in control and HEU infants in month 3 (m3) and month 12 (m12) age groups. Bar charts indicate the mean frequency of CD4+ T cells expressing the indicated cytokine ± the standard error of the mean.

Mann Whitney U test was used to assess differences between groups. * p<0.05 **p<0.001

5.2.8 Polyfunctional cytokine responses following in vitro stimulation with PPD, TT and SEB

The proportion of PPD, TT and SEB-induced CD4 T cells producing a combination of IFN-γ, IL-2 and TNF-α simultaneously was compared between control and HEU infants. The results are displayed in **figure 5.3**. No statistically significant differences were detected between control and HEU infants in either of the time points under any of the stimulation conditions. In the month 3 group there was a trend towards an increased proportion of mono-functional IL-2 secreting cells in HEU infants though this did not reach statistical significance (p=0.06).

5.2.9 Memory Th1 cytokine responses following in vitro stimulation with PPD, TT and SEB in control and HEU infants

One of the main study aims of the present Chapter is to assess whether the capacity of the immune system to induce appropriate memory T cell responses to vaccination is compromised in HEU infants. The data presented focus on the capacity of memory CD4 T cell subsets to respond to antigen stimulation through the secretion of any of the Th1 cytokine assayed. The memory phenotype of cytokine responsive cells was determined through surface expression of CD45RA and CCR7 as shown in **figure 4. 6**.
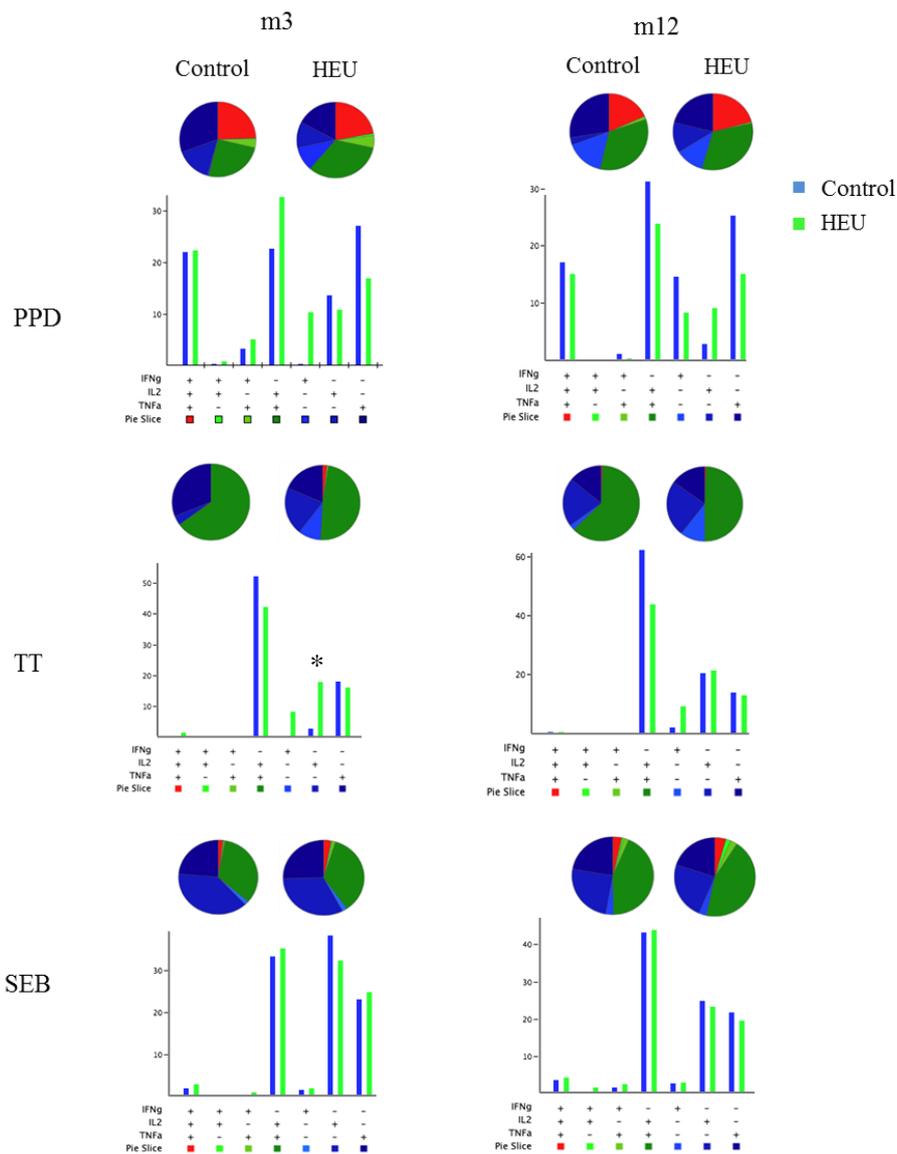
Figure 5.3 Polyfunctional antigen-specific responses in control and HEU infants. Comparison of the quality of the CD4 T cell cytokine response following short term stimulation with PPD, TT and SEB in month 3 (m3) and month 12 (m12) age groups. Boolean gating was used to assess the proportion of antigen-specific cells with single (blue), double (green) or triple (red) cytokine secreting capacity for IFNγ, IL-2 and TNF-α. Normalized values are shown in the bar graphs, where the height of each bar represents the contribution of a particular cytokine combination relative to the overall response. The pie charts summarize the contributions to the total response with distinct coloured pie slice corresponding to the indicated cytokine combination.*p=0.06

The characterisation of the memory response in control infants presented in Chapter 4 indicated that following PPD and TT stimulation, cytokine responses were mainly mediated by Tem cells at both time points. In HEU infants, a similar pattern of dominant Tem responses was observed in the 3 month age group (**fig. 5.4**). No statistically significant differences in the magnitude of the cytokine response in any CD4 T cell memory subtypes were observed between groups at this age, though the median Tem response was higher in HEU infants. However, in 12 month-old infants, a statistically significant reduction in the magnitude of the cytokine response by Tem cells was observed in HEU infants (**fig. 5.4**, p=0.05). Trends in increased responses by naïve cells were observed in HEU infants in the month 12 age group, though this was not statistically significant (p=0.3).

Following TT stimulation, a similar pattern of responses was observed to those following PPD stimulation: at month 3, HEU infants had a tendency to show increased cytokine response from Tem cells (not significant, p=0.2) and at month 12 a significantly reduced Tem response was observed (p=0.04) (**fig. 5.4**).

The contribution of memory subsets to the Th1 cytokine response following polyclonal stimulation with SEB mirrored some of the antigen specific-responses. In the 3 month age group, significantly more Tem cells produced Th1 cytokines following stimulation in HEU infants (p=0.03). In the month 12 age group, Tem and Tcm subsets in HEU infants showed significantly reduced cytokine responses (**fig. 5.4**).

In conjunction these data show enhanced Tem responses in 3 month-old HEU infants that are not sustained; at the 12 month time point HEU memory responses, particularly

those with a Tem phenotype experience a decreased cytokine production capacity relative to healthy infants.
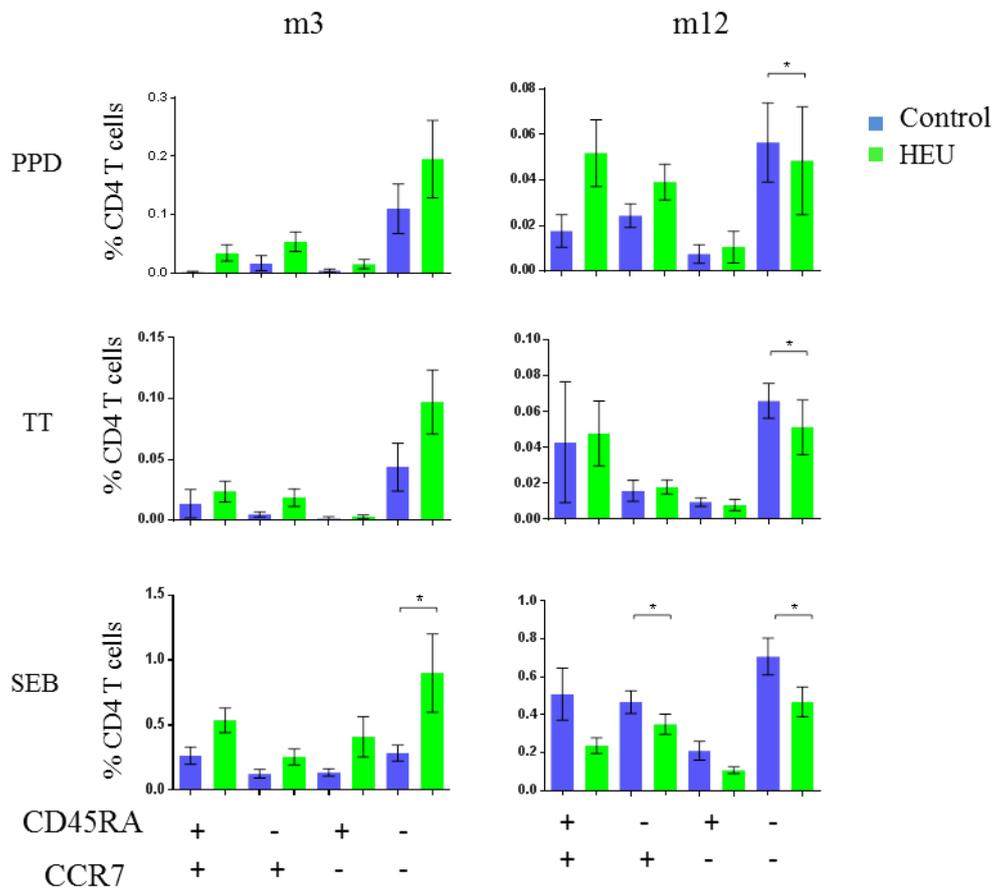
Figure 5.4 Memory phenotype of antigen-responsive cells. Cross-sectional analysis of the frequency of memory CD4 T cell subsets secreting any cytokine measured in panel 1 following short-term stimulation with PPD, TT and SEB. Comparisons were made between control and HEU groups at month 3 (m3) and month 12 (m12). Bar charts represent the mean frequency of CD4+ T cells expressing the indicated cytokine ± the standard error of the mean. Mann Whitney U test was used to assess differences between groups.* p<0.05

5.2.10 CD127 expression on cytokine responsive T cells following stimulation with PPD, TT and SEB in control and HEU infants

In order to further assess possible limitations in the induction of memory responses to antigenic stimulation in infants, the proportions of cytokine responsive cells expressing CD127 were compared in control and HEU infants. The results are summarised in **figure 5.5**. As detailed in Chapter 4, following antigen specific stimulation significantly higher proportions of antigen responsive cells express CD127 than ones that do not. Following PPD stimulation, the proportion of CD127 expressing PPD-specific CD4 T cells was similar between control and HEU infants in the 3 and 12 month age groups. Following TT stimulation, a trend was observed in which TT-specific cell from HEU infants showed reduced expression levels of CD127; this effect was not sustained in the 12 month age groups with equivalent CD127 distributions between control and HEU infants. Polyclonal stimulation similarly induced cytokine responsive CD4 T cell populations with similar CD127 expression distributions in control and HEU infants at 3 and 12 months of age.

5.2.11 Plasma IL-7 concentration in control and HEU infants at 3 months of age

To further assess potential differences in the induction of memory CD4 T cells, plasma IL-7 levels were measured by ELISA. Comparisons were made at the 3 month time point only. IL-7 is a key mediator of homeostatic maintenance of resting memory CD4 T cell populations. A trend towards increased concentrations of plasma IL-7 was found that did not reach statistical significance (**fig. 5.6**; p=0.08). A much wider range of concentrations was also observed in HEU infants, indicating possible modulation of IL-7 levels at 3 months of age in HEU infants under certain conditions.
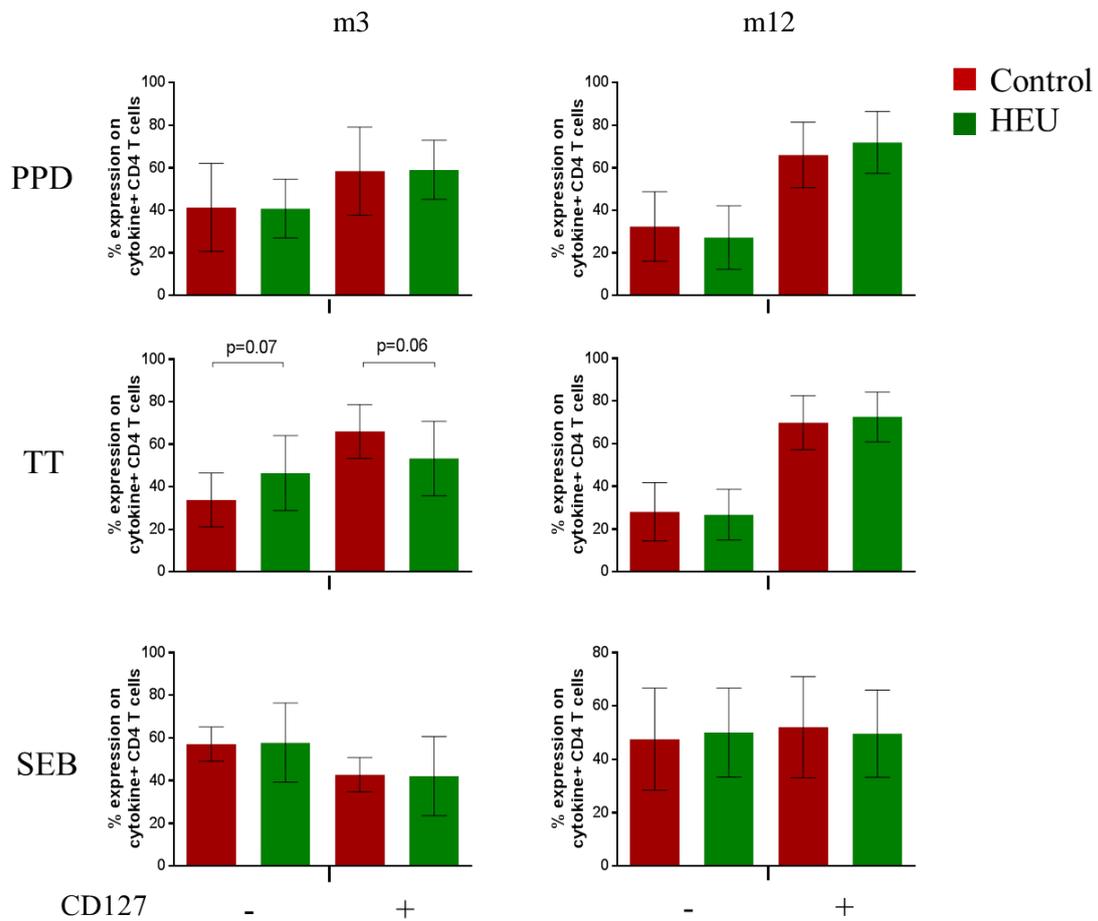
Figure 5.5 CD127 expression on antigen-responsive CD4 T cells. Analysis of the frequency of CD127 expression in CD4 T cell secreting any cytokine measured in panel 2 following short-term stimulation with PPD, TT and SEB. Comparisons were made between control and HEU groups at month 3 (m3) and month 12 (m12). Bar charts represent the mean frequency of CD4+ T cells expressing the indicated cytokine ± the standard error of the mean. Mann Whitney U test was used to assess differences between groups.

IL-7
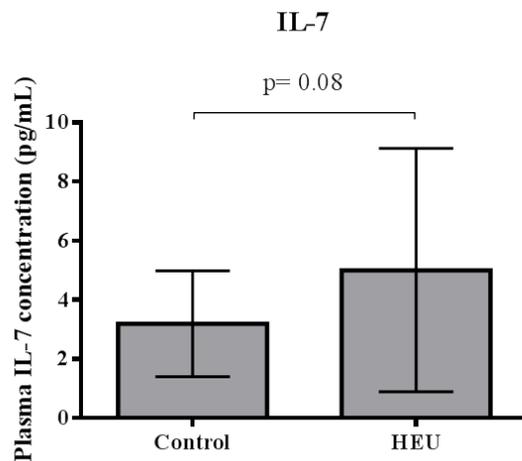
Figure 5.6. Plasma concentration of IL-7 in control and HEU infants at 3 months of age. Median IL-7 plasma concentration in pg/uL is indicated ± the standard deviation. Mann Whitney U test was used to assess differences between groups.

5.3 DISCUSSION

Analysis of *ex vivo* phenotypic parameters showed some unexpected results. Firstly, expression of activation markers on T cell subsets and Treg frequencies were well matched between groups. The exception was higher activation marker expression on CD4 T cells at 3 months of age in control infants, though expression levels were generally low and on its own this is difficult to interpret. Previous reports have indicated increased populations of CD38hi CD4 and CD8 T cells in HEU infants and interpreted this as increased immune activation (222,342). The present data indicate that constitutive expression of CD38 is maintained on HEU infant T cells as described in Chapter 3. Consequently, coexpression of CD38 and HLA-DR was considered a more unbiased approach to assessing cellular activation levels that is well described in the literature (46,47). In contrast to previous reports (222,342,346), the present analysis indicates that HEU infants resemble the control population in their activation profile. Reports of alterations in Treg frequencies have not been consistent (216,250). Changes

in Treg frequencies could indicate altered immune-regulatory potential in HEU infants and could be expected to be linked to levels of immune activation. The present data did not indicate that this was the case.

A second unexpected result was that HEU infants were found to have increased frequencies of naïve and decreased frequencies of Tem cells in circulation in the 12 month age group in both CD4 and CD8 T cells. This could indicate that levels of antigenic exposure were increased in the control population. This may be best explained through differential exposures to cotrimoxazole (CTM) prophylaxis. HEU infants are provided with CTM prophylaxis as part of routine care for infants at risk of HIV-1 acquisition until 18 months of age. This was not the case for the control population. This may have significantly reduced exposure to numerous bacterial infections in HEU infants compared to controls and had an impact on *ex vivo* activation parameters and bulk memory T cell differentiation. However, we observed increased total numbers of lymphocytes and white blood cells in HEU infants in the 12 month age group which was indicative of lymphocytosis and suggests on-going infection in some HEU infants. In this case reduced naïve T cell frequencies would be expected. Therefore these data are difficult to reconcile and contrast with published reports. Reduced frequencies of lymphocytes have been reported in HEU infants exposed to ARVs in numerous large studies (341,348). In addition, in cohorts of HAART exposed and unexposed HEU infants, naïve T cells frequencies have been shown to be reduced in HEU infants compared to healthy controls (222,342), and this has been interpreted as a mark of *in utero* or early life priming of naïve T cell through exposure to HIV-1 or other infectious agents. One of the limitations of the present analysis was the lack of assessment of maternal and infant co-infections which have been shown to drive

differentiation of memory populations and other phenotypic changes and may account for these findings.

Finally, *ex vivo* analysis of dendritic cell populations indicated that higher frequencies of pDC were present in HEU infants at 3 months of age, resulting in significantly altered mDC/pDC ratios. A previous study had indicated that mDC frequencies were increased in cord blood from HEU infants compared to healthy controls albeit transiently as analysis at 12 months of age indicted equivalent frequencies (253). The present data also support transient alterations in DC frequencies at 3 months of age driven by increased pDC in HEU infants. DC frequencies have been shown to fluctuate in early life and pDC decrease in number in the post natal period. The present effect therefore may indicate dysregulation of dendritic cell proliferation through exposure to HIV-1 antigens of ARV, or may be a reflection of normal fluctuations in DC cell frequencies in early life. Phenotypically, similar basal expression levels of CD86 were observed in mDC and pDC from both groups of infants. Basal expression levels of CD86 in infant DC have been shown to be similar to adults DC (253). The present data suggests that by 3 months of age mature CD86 expression levels are reached on pDC and mDC that are not significantly affected in HEU infants. By contrast HLA-DR expression was found to have a lower MFI on both pDC and mDC at 3 months of age indicating a transient less active phenotype. CD83 expression levels were found to be more variable, though in general the basal expression levels were of a very low frequency and the differences found may not have any functional significance. CD83 has been described to be expressed on mature dendritic cells following activation (330). The present analysis indicates that in this respect infant DCs are largely immature. Low/negligible basal expression levels of PD-L1 were also observed in both groups.

Basal PD-L1 has been detected on adult pDC (253). This may therefore constitute an important difference in the PD-1 exhaustion pathway between infants and adults. Further analyses would be beneficial to characterise the dynamics of CD83 and PD-L1 up-regulation following TLR stimulation as reports in the literature indicate that the functional properties of DC are altered in HEU infants (253,254). The analysis of basophils frequency and phenotype produced some unexpected findings: increased basophil frequencies with reduced expression of the co-stimulatory markers were found in HEU infants. This suggests that basophils may have antigen presenting properties, though this will have to be assessed in future studies.

In addition to the *ex vivo* analysis of immune parameters, the present study sought to assess differences in cytokine responses in CD4 T cells following short term stimulation with the vaccine antigens PPD and TT and the super antigen SEB. A consistent age-dependent trend was observed in the magnitude of cytokine production potential between HEU and controls infants under different stimulation conditions: in HEU infants at 3 months of age cytokine response were enhanced and at 12 months of age the magnitude of the response was reduced. This effect was least apparent following PPD stimulation with this trend only apparent in the 3 month age group. Following TT stimulation, this trend reached significance in the 12 month IL-2 response. However, most strikingly, this observation was statistically significant at both time points in relation to all cytokines following polyclonal stimulation with SEB. The consistency of this finding indicates an enhanced immunological reactivity in HEU infants at 3 months of age that becomes constrained through infancy leading to impaired cytokine responses at month 12 following both antigen-specific (except for PPD stimulation) and polyclonal stimulation. The infant IFN-γ response to SEB

stimulation in early life has been shown to mature throughout infancy and into childhood (292). In addition, the data presented in Chapter 4 indicates that the maturation of the response also occurs with respect to IL-2 and TNF-α production. The present results indicate that this maturation process is dysregulated in HEU infants. It seems reasonable to speculate that the early enhanced Th1 response is related to the contraction of the response at 12 months; however longitudinal analysis may best address this question. One interpretation of these finding is that a process of immuno-regulation or functional exhaustion may mediate the reduced late Th1 response. However, the *ex vivo* phenotypic data do not support this hypothesis. Additional regulatory or functionality mechanisms may influence these outcomes. The observation that these effects result following polyclonal and antigen-specific stimulation suggest that the effect may be independent of the effects of antigen-presentation and be intrinsic to Th1 cells. Finally, deregulated Th1 responses to SEB may have clinical relevance. Infants and children are particularly susceptible to staphylococcal infections and syndromes caused through toxin production (349,350) Further studies will be needed to assess altered patterns of susceptibility to these syndromes in HEU infants.

In addition to the magnitude of the cytokine response to stimulation, this study aimed to assess differences in the quality of the Th1 responses to vaccine antigens and SEB between HEU and control infants. As mentioned previously, the quality of the immune response (capacity to produce multiple cytokines) has been associated with protection from infection and is an important factor in assessment of the immunogenicity of novel T cell vaccines including those against tuberculosis (351,352). The data suggests that the quality of antigen-specific and polyclonal Th1 responses is similar between HEU and control infants. With respect to mycobacterial responses, this finding is in

accordance with a study carried out in South Africa(262). To my knowledge, this is the only study which has assessed poly-functional responses to TT and SEB stimulation in HEU infants.

One of the principal aims of this study was to assess differences in the induction of memory CD4 T cells between HEU and control infants. As shown in Chapter 4, PPD and TT stimulation induce Th1 responses with a predominant Tem phenotype. The main outcome of the present analysis is that at the 12 month time point significant reductions in Tem responses were observed in HEU infants. Conversely, at the 3 month time point, trends were observed towards increased Tem responses in HEU infants following antigen-specific stimulation. These observations mirror what was observed in the analysis of single cytokine expression and suggest that the increased reactivity at month 3 and reduced reactivity at month 12 of HEU infant Th1 cells to antigen-specific stimulation occurs predominantly in Tem cells. Similar outcomes were observed following SEB stimulation, though in addition to reduced Tem responses at month 12, responsive Th1 cells with a Tcm phenotype were also reduced. Studies assessing memory T cell induction following chronic and acute viral infection in adults have suggested that long term protective memory responses are mediated by Tcm cells (51,353). Reduced Tcm responses in HEU infants following polyclonal stimulation may therefore potentially influence long term protection from numerous infections, though its remains unclear whether cytokine responses following polyclonal stimulation accurately reflect broad antigen-specific responses.

Additional analysis of CD127 expression of cytokine specific T cells did not suggest that antigen specific memory populations bearing this marker were significantly altered, although a trend to a reduction in CD127 expression was observed in TT CD4

Th1 cells. Analysis of plasma IL-7 levels in 3 month old infants revealed a trend towards heightened IL-7 plasma levels in HEU infants. It may be possible that increased plasma IL-7 results in the haemostatic down-regulation of the IL-7R on CD4 T cells, though this hypothesis is not supported by *ex vivo* IL-7R expression levels or IL-7R expression levels on PPD and SEB responsive cells. Increased plasma IL-7 is however in accordance with previous reports in HEU infants (222). It is therefore conceivable that increased plasma IL-7 is a marker for dysregulated memory T cell homeostasis and related to the reduced memory Th1 responses discussed.

This data adds to the body of evidence that indicates specific defects in BCG responses in HEU infants. The consistent defects described so far are mainly with respect to antigen-specific proliferation (260,346), as reports of altered cytokine production havebeen conflicting. With respect to TT vaccination, a single study has assessed *in vitro* responses to vaccination in HEU infants in a large population of Ugandan infants and indicated a reduced capacity to produce Th1 and Th2 cytokines compared to control infants. The present data suggest that the induction of T cell memory responses is also hindered in HEU infants in response to antigen-specific and polyclonal stimulation.

6 CHAPTER 6: GENERAL DISCUSSION AND FUTURE PERSPECTIVES

CTL functionality during early infant HIV-1 infection

The analysis presented in Chapter 3 indicates that substantial variation exists in the evolutionary dynamics both between infants and between regions of the HIV-1 genome. The slow rates of evolution and limited inter-individual variation in evolutionary rates in *pol* were expected as this represents one of the most evolutionarily conserved regions of the HIV-1 genome. HIV-1 g*ag* and *nef* on the other hand showed much more wide levels of inter- individual variation. The lack of correlation between the evolutionary rates between *gag* and *nef* however, indicate that the selection pressure acting on these genes is not uniform. This is consistent with immune mediated selection which is able to target distinct antigenic epitopes depending on the genetic background of the host. Both antibody and cell mediated selection are known to drive HIV-1 evolution at different stages of the infection, though *gag* and *nef* encode numerous well described epitopes recognised by CTLs.

The data presented in chapter 3 indicate that infant CTL selection pressure is a strong evolutionary force that drives viral diversification in early infection. This finding implies that infant CTL responses are functional and act by limiting HIV-1 replication to the extent that viral populations with mutations that escape from CTL recognition are selected for. It therefore seems apparent that when considering the clear differences in viral load dynamics between infant and adults, lack of CTL functionality may not act a principal mediator in this outcome. This is in accordance with reports that describe adult-like CD8 T cell responses to CMV infection in neonatal cord blood(178). This

data therefore highlights the functional plasticity of the infant immune response under certain conditions, likely set during priming of the response. .

The data describing a positive correlation between evolutionary rate in gag and clinical outcome are difficult to reconcile in the context of a functional CTL response driving viral evolution. The data indicate that the fitness costs of some of the accumulated non-synonymous mutation in *gag* are limited. It also suggests that an expanding viral population undergoes a higher rate of diversification. It will be of interest to assess the outcome of infants containing known protective HLA alleles in terms of *gag* evolution and clinical outcome, though prospective studies of this nature are no longer ethically possible. Overall, the indication from this study is that additional factors other than CTL functionality may influence disease progression in infants. One possibility is the presence of large population of target CD4 T cell in early life that have been shown to be present in neonatal macaques(179). This large target cell pool may enable rampant viral replication in the face of an apparent t functional infant CTL response.

Immunity in the first year of life in healthy infants

Our understanding of infant immunity has undergone a paradigmatic shift over the last decade. Whereas infants were perceived to be largely immunodeficient, it has become clear that significant variability and plasticity exists in the capacity to induce protective immune responses during early life. Understanding the mechanisms that underpin the variability in infant immune responses is instrumental in the development of novel vaccination strategies and immunotherapeutics. The results presented in Chapter 4 indicate that much of the variation linked to immunological function and phenotype is, as would be expected, age dependent. The transition from a relatively sterile *in utero* immune environment to one of abundant antigenic exposure necessitates a carefully

regulated maturational process that balances the need for protective immunity yet limits potentially dangerous proinflammatory responses. It seems apparent from the literature that this balance in peripheral tolerance is achieved through a combination of active immune suppression of potentially mature immune responses (mediated by numerous pathways including Tregs, immunoregulatory cytokine production such as TGF-β, production of suppressive enzymes such as indole-amine 2,3-dioxigenase [IDO](354), among others) and specific developmental cues that delay the onset of potentially dangerous inflammatory responses. The high susceptibility to infection in infancy indicates that this balance is tipped towards immunosuppression and immaturity for a prolonged period.

Characterisation of the immune response in early life is particularly challenging due to numerous ethical and practical considerations. Therefore much of our current understanding of early infant immunity is derived from cord blood studies or studies in animal models. The dataset presented in Chapters 4 and 5 focused on two time points in early life: 3 months of age, which a period that is close to the peak vaccine responses under study, and 12 months of age, a period when the development of immunological memory during infancy can be assessed. The data in Chapter 4 indicated that age related immunological changes between 3 and 12 month of age were most apparent with regards to the *ex vivo* bulk memory phenotypes and functional potential of the T cell compartment.

The progressive age-related increase in memory T cell subsets differentiation is consistent with increased antigenic exposure, although a large proportion of T cells maintained a naïve phenotype even in 12 month old infants. Decreases in naïve and increased Tem cells at 12 months of age were particularly apparent in CD8 T cells. In

fact, a number of differences were observed between memory phenotypes in CD4 and CD8 T cells, particularly with respect to the induction of Temra cells. This population is maintained at very low levels in the CD4 T cells compartment. Temra cells are thought to represent terminally differentiated effector cells with limited functional potential(355). Perhaps differences in the maintenance of memory T cell subsets mediate the differences observed, particularly in subsets that lack the lymph node homing marker CCR7.

Age related differences in T cell responses to the vaccine antigen and polyclonal stimulation were clear in the analysis presented in Chapter 4. The data indicate that at the 3 month time point, the time point closest to the peak vaccination, the magnitude of the antigen specific Th1 response to both vaccines tended to be higher for their signature cytokine response. This is in accordance with previous characterisations of BCG vaccination in neonates and TT vaccinations in adults and indicates that Th1 cells from the expansion phase of the vaccine response mediate robust recall responses in the *in vitro* assay. Strong memory responses were also observed at 12 months, dominated by Tem cells, to both vaccines. This indicates that a stable memory CD4 T cell pool develops following vaccination with both BCG and TT that induces rapid, predominantly polyfunctional responses following antigen re-encounter. The memory and kinetics of the T cell response to TT vaccination have not been extensively studied in early life; the present data provide a reference for assessments of deviations from these responses during disease.

The Th1 response following polyclonal stimulation gave the clearest indication of age-related differences in immune response, particularly in the intrinsic capacity to produce signature Th1 cytokines, most strikingly IFN-γ. The IFN-γ response is a key pro-

inflammatory mediator that has been shown to be developmentally regulated, particularly in CD4 T cells. Differential methylation of the *ifn-γ* gene promoter has been shown to mediate this effect(332). As shown in Chapter 4, polyclonal stimulation of CD4 and CD8 T cell results in enhanced IFN-γ responses at 12 months of age compared to 3 months of age. This observation is in accordance with a detailed longitudinal assessment of the capacity of infants to produce IFN-γ following SEB stimulation that indicated that CD4 T cell responses comparable to those in adult are not seen until after the first 10 years of life (292). Th1 cell development is dependent on IL-12 production, and production of this cytokine has been shown to be similarly below levels reached in adults in the first decade of life (356). Interestingly, monocyte derived DC from neonates cultured with GM-CSF and IL-4 showed similar capacities to produce IL-12 as those derived from adults under the same culture conditions, indicating that under specific circumstances the nature of Th1 polarizing DC response can be equivalent to that seen in adults. Therefore it seems that reduced IFN-γ responses following SEB stimulation in infants is a consequence of the immunological environment in which CD4 T cells are primed. Indeed, this is supported by the observation in Chapter 4 that following BCG vaccination a strong IFN-γ response is induced at both time points and that by contrast, in the context of a rapidly cleared toxoid antigenic challenge, IFN-γ production is negligible.

Identifying factors that enable BCG to induce an IFN-γ response will be of importance and may help inform efforts to develop novel T cell vaccines. As is the case with numerous live-attenuated vaccines, the precise immunological pathways that are triggered following vaccination remain enigmatic. However, it seems likely that interactions between BCG and the innate immune system mediate the nature of the

ensuing T cell response. Recently, BCG vaccination has been shown to induce enhanced proinflammatory responses from monocytes that persisted months after vaccination (357). This enhanced innate inflammatory function was mediated by NOD2 receptor signalling and resulted in enhanced T and B cell independent protection from unrelated pathogens. These findings point to the adjuvant-like role of BCG vaccination that enable enhancement of secondary innate responses to infection- a form of innate immunological memory that has been termed trained immunity(358). Indeed, BCG vaccination (and the live-attenuated measles vaccination) has been shown to enhance survival in early life through non-specific protection from non-specific infections. The data presented in Chapter 4 indicates that the frequency of IFN-γ producing CD4 T cells following BCG stimulation and polyclonal stimulation were largely equivalent. This suggests that IFN-γ producing BCG-specific CD4 T cells account for a large proportion of the total CD4 T cell pool capable of producing IFN-γ following stimulation at 3 months of age. Therefore it may be reasonable to speculate that the non-specific effects of BCG that are IFN-γ mediated perhaps do not originate from the Th1 compartment.

A number of immunological parameters assessed in Chapter 4 remained at consistent levels between the 2 time points. These included frequencies of activated T cells, Tregs and DC. It may be beneficial to assess the activated Treg cell population that express HLA-DR, as these have been shown to identify highly suppressive T cells (359). In addition, it is not clear whether FOXP3 expression identifies all Treg cell. Nevertheless, the data indicate that a stable Treg pool is established in early infancy.

Analysis of immune activation marker expression revealed strikingly wide ranges in expression levels particularly with respect to CD8 T cells (<5%->70%) in infants at

both 3 and 12 months of age. It is likely that differences in intercurrent infection explain this variation, particularly viral infections such as CMV. It will be of interest to evaluate CMV infection retrospectively in this cohort to assess its effect on T cell activation. In addition it would be of benefit to investigate to what extent bystander activation of resting T cell populations may play a role. Bystander activation of T cells is a controversial phenomenon with poorly defined physiological consequences. Nevertheless, this phenomenon may help explain the striking levels of immune activation in some infants and would suggest that not all T cells bearing high levels of activation markers have been primed or represent T cells in the expansion phase of the immune response. A related observation was that all activation markers were expressed at higher levels in CD8 T cells compared to CD 4 T cells. This finding could arise from a number of possibilities. For instance a) due to enhanced levels of priming of CD8 T cells at the systemic level b) differences in localisation of highly activated CD4 T cell and CD8 T cells, with highly activated CD4 T cell relocating to lymphoid organs following activation. Alternatively, the activation markers used in this study may not be upregulated as readily on primed CD4 T cells as on CD8 T cells.

Significant levels of exhaustion marker expression were also observed on CD8 and CD4 T cells. This was not paralleled by the expression of PD-L1 expression in DC, indicating that baseline expression is not readily detectable in peripheral blood DC. Previous studies have shown robust PD-L1 up regulation on infant DCs following TLR stimulation, to a higher degree than in adults. It therefore does not seem likely that PD-L1 expression of DC is developmentally delayed. Rather activated PD-L1 (and possibly CD83) expressing DC may relocate to peripheral lymphoid to induce adaptive responses. Our understanding of the PD-1 pathway in the control of infant immune

responses in health and disease remains limited. For instance is the expression of PD-1 (and other coinhibitory molecules) simply a marker of immune activation or does the pathway play a role in active immunosuppression in early life? With the development of immunotherapies based on inhibitors of the PD-1 pathway to treat chronic viral infections, it seems important to further characterise the immunomodulatory roles of the PD-1 pathway in infants.

Assessing the extent of immune alterations in HEU infants

Despite being born free from HIV-1 infection, diverse populations of HEU infants have been shown to be significantly more susceptible to infectious diseases than their HIV-1 unexposed counterparts. This has had a measurable impact on mortality rates and in countries where increasing proportions of new-borns are HEU infants, it is critical to understand what mechanisms contribute to these outcomes. This endeavour, however, is complicated by the multiple factors that could potentially influence health outcomes in HEU infants. These include social/environmental factors, such as compromised care, that are particularly difficult to estimate and control but may play a substantial role. In addition, HEU infants are exposed to HIV-1, ARVs and a maternal immune system perturbed by HIV-1 infection. Numerous studies suggest that these factors affect the developing immune system. Identifying specific contributions of each of these factors to immune health is important to reduce their influence. In addition, this may enable specific mechanisms that lead to altered immune function to be identified. Randomised clinical trials, particularly in terms of ART regimens may be beneficial in addressing some of these questions- particularly in combination ART regimens for PMTCT that lack AZT- though precise estimates of maternal inflammation and exposure to HIV-1 antigens may be best addressed in animal models.

The aim of the present study was to further characterise possible immune abnormalities in HEU infants. In addition this study sought to confirm some of the observations in the literature in a population representative of HEU infants from the Kenyan Coastal Province. The principal focus was on alterations in the T cell compartment both *ex vivo* and in terms of their functional response following antigenic stimulation.

Numerous research groups have indicated that one of the principal immunological perturbations associated with HEU infants is an increased level of immune activation in T cell populations associated with an antigen experienced phenotype and reductions in naïve cell populations. The results presented in Chapter 5 indicate that in terms of *ex vivo* T cell activation and the distribution of memory cells populations, our observations contrasted with some of those in literature. The present data indicate that by and large immune activation levels were comparable between HEU infants and the control population, though there was an indication that immune activation may have been higher on CD4 T cell from control infants at 3 moth of age. In addition, the distribution of memory T cell populations at 12 months of age indicated that HEU infants in fact had higher frequencies of naïve T cells. This observation is perhaps best explained by the differential exposures to co-trimoxazole (CTM) prophylaxis. Reduced bacterial burden in HEU infants may result in reduced priming of naïve T cell and consequent lower levels of immune activation. CTM prophylaxis did not however seem to influence immune activation levels in all HEU infants. It remains possible therefore that immune activation may be a feature of HEU infants, though adequate prophylactic interventions may play a role in limiting this effect.

In contrast to the *ex vivo* phenotypic assessment discussed above, the functional assessment of Th1 responses following polyclonal and antigen-specific responses were

more consistent with the literature. The increased levels of immunological reactivity at 3 months of age in HEU infants, particularly following polyclonal stimulation, suggest a more sensitised T cell compartment, perhaps through previous antigenic exposures or through bystander mechanisms. This effect was however shown to be reversed at 12 months of age, indicating that a certain level of functional exhaustion or suppression exists at later time points. Indeed this effect is likely to have been central to one of the other main observations from this study, the reduced memory responses following polyclonal and antigen specific stimulation. Following BCG and TT stimulation, reduced memory responses were observed within the principal memory subset of Th1 responsive cells, Tem cells. In addition, apparent deficiencies in the Tcm cells were also observed following polyclonal stimulation. These observations indicate a broad alteration in Th1 cell reactivity in HEU infants with specific effects on developing memory T cell responses. The results in this study also suggest that these alterations may be related to increased levels of IL-7, which plays an essential role in memory T cell maintenance. Increased IL-7 levels may result from alterations in lymphopoiesis that has been described to result from exposure to numerous ARVs. Although no indication that the HEU infants in this study presented broad haematopoietic abnormalities, the size of the study cohorts may not have enabled significant alterations to be detected. Therefore, assuming that subtle alterations in lymphocyte numbers exist in HEU infants, this may result in compensatory levels of IL-7 production and consequent altered memory T cell reactivity. More detailed studies of the relationship between memory T cell maintenance and altered IL-7 levels will be required to address this hypothesis. In addition, further studies may be required to examine memory

responses from other components of the adaptive immune response, to assess whether

the perturbations in recall responses described here are broad in nature.

7 REFERENCES

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