HIV-Specific CD8+ T cell Responses in Infected Infants Enrolled on a Study of Early Highly Active Antiretroviral Treatment (HAART) and Supervised Treatment Interruption (STI)

Christina F. Thobakgale

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HIV Pathogenesis Programme
Doris Duke Medical Research Institute
Nelson R Mandela School of Medicine

UNIVERSITY OF KWAZULU-NATAL

HIV-Specific CD8+ T cell Responses in Infected Infants Enrolled on a Study of Early Higly Active Antiretroviral Treatment (HAART) and Supervised Treatment Interruption (STI)

By

Christina Fanesa Thobakgale

Submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy (Immunology) in the Department of Paediatrics and Child Health, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal 2011

DECLARATION

I, Christina Fanesa Thobakgale, declare that this is my original work except as acknowledged in the thesis and has not been submitted in any other form to another university. Most of the experiments were performed at the HIV Pathogenesis Programme (HPP) Laboratory, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal. Some of the experiments were carried out at the Goulder Laboratory, Peter Medawar Building for Pathogen Research, University of Oxford. The work was supervised by Dr Photini Kiepiela (University of KwaZulu-Natal), Prof Thumbi Ndung'u (University of KwaZulu-Natal) and Prof Philip Goulder (University of Oxford).

Christina Thobakgale

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Thumbi Ndung'u

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PLAGIARISM DECLARATION

I, Christina Fanesa Thobakgale declare that:

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PUBLICATIONS

First Author Publications

Thobakgale CF, Ramduth D, Reddy S, Mkhwanazi N, de Pierres C, Moodley E, Mphatswe W, Blanckenberg N, Cengimbo A, Prendergast A, Tudor-Williams G, Dong K, Jeena P, Kindra G, Bobat R, Coovadia H, Kiepiela P, Walker BD and Goulder PJ. HUMAN IMMUNODEFICIENCY VIRUS-SPECIFIC CD8+ T-CELL ACTIVITY IS DETECTABLE FROM BIRTH IN THE MAJORITY OF IN UTERO-INFECTED INFANTS. Journal of Virology. (2007); 81 (23): 12775-12784.

Thobakgale CF, Andrew Prendergast, Hayley Crawford, Nompumelelo Mkhwanazi, Danni Ramduth, Sharon Reddy, Claudia Molina, Zenele Mncube, Alasdair Leslie, Julia Prado, Fundi Chonco, Wendy Mphatshwe, Gareth Tudor-Williams, Prakash Jeena, Natasha Blanckenberg, Krista Dong, Photini Kiepiela, Hoosen Coovadia, Thumbi Ndung'u, Bruce D Walker, and Philip J R Goulder. IMPACT OF HLA IN MOTHER AND CHILD ON PAEDIATRIC HIV-1 DISEASE PROGRESSION. Journal of Virology (2009); 83 (19): 10234-44.

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CO-AUTHOR PUBLICATIONS

Mphatswe Wendy; Blanckenberg Natasha; Tudor-Williams Gareth; Prendergast Andrew; **Thobakgale Christina**; Mkhwanazi Nompumelelo; McCarthy Noel; Walker Bruce D; Kiepiela Photini; Goulder Philip. HIGH FREQUENCY OF RAPID IMMUNOLOGICAL PROGRESSION IN AFRICAN INFANTS IN THE ERA OF PERINATAL HIV PROPHYLAXIS. AIDS. (2007); 21(10):1253-61.

Prado JG, Prendergast A, **Thobakgale C**, Molina C, Tudor-Williams G, Ndung'u T, Walker BD, Goulder P. REPLICATIVE CAPACITY OF MOTHER-TO-CHILD TRANSMITTED VIRUS IS ASSOCIATED WITH PAEDIATRIC HIV-1 DISEASE PROGRESSION RATE. Journal of Virology. (2010); 84 (1): 492-502 .

Ramduth Danni; **Thobakgale Christina F**; Mkhwanazi Nompumelelo P; De Pierres Chantal; Reddy Sharon; van der Stok Mary; Mncube Zenele; Mphatswe Wendy; Blanckenberg Natasha; Cengimbo Ayanda; Prendergast Andrew; Tudor-Williams Gareth; Dong Krista; Jeena Prakash; Coovadia Hoosen M; Day Cheryl L; Kiepiela Photini; Goulder Philip J R; Walker Bruce D. DETECTION OF HIV TYPE-1 GAG-SPECIFIC CD4 (+) T CELL RESPONSE IN ACUTELY INFECTED INFANTS. AIDS Research and Human Retroviruses (2008); 24(2):265-70.

(See Chapter 7: APPENDICES section for author contributions to publications)

ORAL PRESENTATIONS

CD4+ and CD8+ T cell Responses in Acutely Infected Infants receiving Early HAART and STI **(2nd South African AIDS Conference, ICC, Durban, SA, 2005)**

HIV-Specific CD8+ T Cell Activity is Detectable from Birth in the Majority of *In-utero* Infected Infants **(3rd South African AIDS Conference, ICC, Durban, SA, 2007)**

Immunological Outcomes of Acute HIV-1 Infected Infants undergoing HAART and STI in Durban, South Africa **(2nd Ungandan AIDS Conference, Entebbe, Uganda, 2008)**

Impact of HLA in Mother and Child on Paediatric HIV-1 Disease Progression **(4th South African AIDS Conference, ICC, Durban 2009)**

POSTER PRESENTATIONS

CD4+ and CD8+ T cell Responses in Acutely Infected Infants receiving Early HAART and STI (12th Conference on Retroviruses and Opportunistic Infections (CROI), Massacchussets, USA, 2005)

HIV-Specific CD8+ T cell Activity in Acute Paediatric Infection **(Keystone Symposium, Colorado, USA, 2006)**

Impact of HLA in Mother and Child on Paediatric HIV-1 Disease Progression **(Keystone Symposium, Colorado, USA, 2009)**

T Cell Activation and Polyfunctionality Profiles in progressive and non-progressive Paediatric HIV-1 disease **(South African Immunology Society (SAIS) conference, Cape Town, 2009)**

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Last but not least, to God Almighty, through whom all this was made possible.

ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral therapy
AZT	Zidovudine
CTL	Cytotoxic T Lymphocytes
CTLA-4	Cytotoxic-T-Lymphocyte Antigen 4
DNA	Deoxyribonucleic acid
ELISPOT	Enzyme-linked immunosorbent spot
IFN-γ	Interferon gamma
IL-2	Interleukin 2
IL-10	Interleukin 10
IU	Intrauterine HIV infection
IP	Intrapartum HIV infection
HIV	Human immunodeficiency virus
HAART	Highly active anti-retroviral treatment
HLA	Humal leukocyte antigen
LTNP	Long-term-non-progressor
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein-1-beta
MTCT	Mother-to-child-transmission of HIV
NEF	Negative regulatory factor

PBMC	Peripheral mononuclear cells
PMTCT	Prevention of mother-to-child-transmission of HIV
PD-1	Programmed Death 1
REV	Regulator of gene expression
RNA	Ribonucleic acid
TAT	Trans-activator of transcription
TNF-α	Tumor necrosis factor alpha
TREG	Regulatory CD4+ T cells
SIV	Simian immunodeficiency virus
VIF	Viral infectivity factor
VPU	Viral protein U
VPR	Viral protein R
WHO	World Health Organization

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ABSTRACT

The manifestation of HIV-1 infection is different in children and adults. Most of the children who acquire HIV perinatally progress to disease within the first two years of life, while adults can remain asymptomatic for up to ten years. However, a small minority group of children can control the virus for years in the absence of antiretroviral therapy. We characterized CD8+ T cell responses critical for the containment of HIV infection in a cohort of infants HIV infected from birth using IFN-γ ELISPOT, multicolour flow cytometry and viral sequencing of the Gag protein. We investigated whether the age at the time of infection, specificity and functionality of the generated responses, genetic make up and the maternal immune responses to HIV, influenced disease progression in the child.

We found that the majority of in-utero infected infants mounted CD8+ T cell responses from the first days of life. In contrast to chronically infected children or adults, the specificity of the initial response in acutely infected infants was directed towards Env and Rev proteins and CD4+ T cell responses were minimal during the first 6 months of life. Slow progression to disease was associated with possession of one of the protective HLA-B alleles by either the mother or the child (P=0.007) and targeting of Gag epitopes presented by the protective HLA-B alleles. Mothers who expressed protective alleles but whose children did not possess these alleles, transmitted less fit viruses that benefited their children. Furthermore, slow progressor children had more polyfunctional CD8+ T cell responses in early infection when compared to rapid

progressors (P=0.05). The ability of infants to induce CD8+ T cell responses early in life is encouraging for vaccine interventions. The differences in the specificity of the initial responses between adults and children, insufficient priming of these responses as a result of minimal CD4+ T cell help during infancy and possession of non-protective HLA alleles shared between mother and child, may explain the rapid disease progression generally noted in most infants. However, slow progression to disease in the minority group of children may be attributed to functional capacity of the CD8+ T cells generated by the child, mediation by protective HLA alleles, acquisition of low fitness viruses from the mother or de novo attenuation of the virus by the child's own immune responses.

ETHICS

The study was granted ethical approval by the Ethics Committee of the University of KwaZulu-Natal, Ref H062/05.

The Postgraduate Education Committee at the Nelson R Mandela School of Medicine, Faculty of Health Sciences, granted approval towards studying for a PhD degree.

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1.1. HIV/AIDS: THE GLOBAL PERSPECTIVE

Globally, an estimated 33 million people were living with human immunodeficiency virus (HIV) in 2007, of which 2.7 million were newly infected (UNAIDS, 2008). The number of acquired immune deficiency syndrome (AIDS) related deaths since the discovery of HIV was 25 million at the end of 2007, and in the same year, 2 million people died of the disease. Sub-Saharan Africa still bears the most HIV burden accounting for 67% of all people living with the virus and 75% of AIDS deaths in 2007 (UNAIDS, 2008), Fig 1.1.

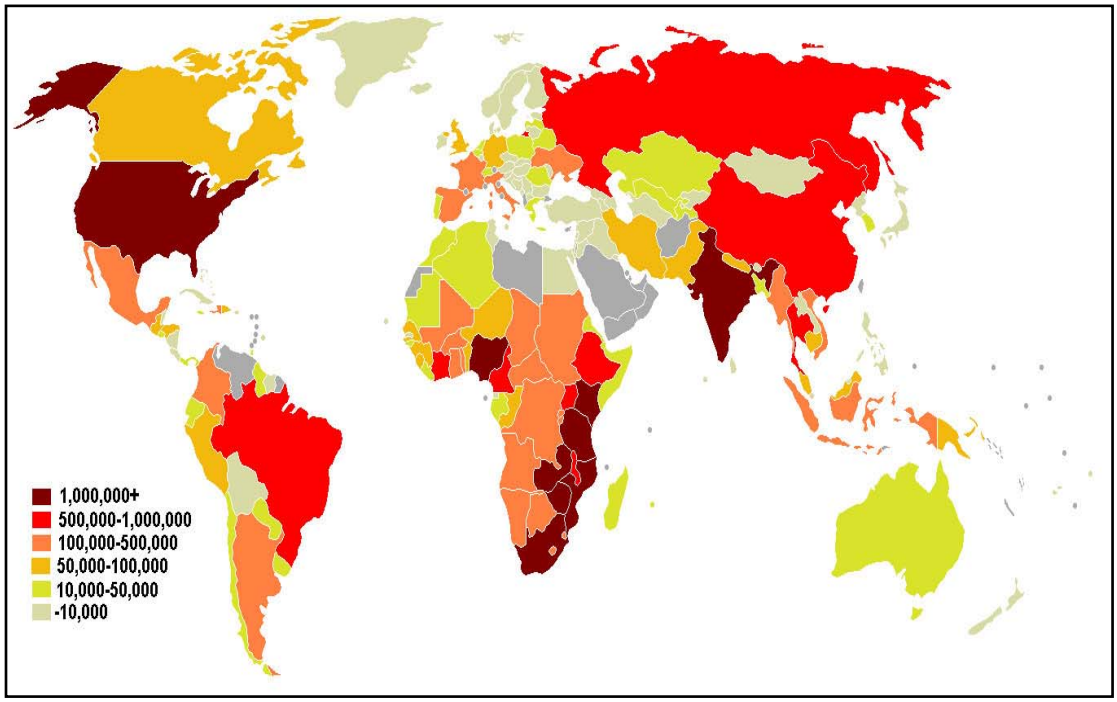

Figure 1.1. Global view of the estimated number of people living with HIV/AIDS in 2008 (UNAIDS, 2008).

HIV prevalence is highest in young women between the ages of 15 and 24, accounting for an estimated 45% of new infections worldwide. Children younger than 15 years accounted for 370,000 new infections and 2 million children were living with HIV in 2007 (UNAIDS, 2008).

1.2. HIV/AIDS EPIDEMIC IN SOUTH AFRICA

An estimated 5.2 million South Africans were living with HIV in 2008 and over 250,000 people died of AIDS in 2008 (South Africa Report, 2008, UNAIDS, 2008). The national prevalence is around 11% and varies by province, with the Western Cape (3.8%) and Northern Cape (5.9%) being the least affected, while Mpumalanga and KwaZulu-Natal are the most affected at 15.4% and 15.8% respectively. In children younger than 15 years, an estimated 280,000 were living with HIV in South Africa in 2007 (UNAIDS, 2008). In the absence of antiretroviral therapy (ART), HIV is the leading cause of death in children younger than 2 years in developing countries (Dabis et al., 2001, Obimbo et al., 2004). Mother-to-child-transmission accounts for the majority of infections in children while heterosexual transmission is predominant in young women of child bearing age; and HIV seroprevalence among antenatal women in South Africa was 29.3% in 2008, Fig 1.2, (South Africa Report, 2008).

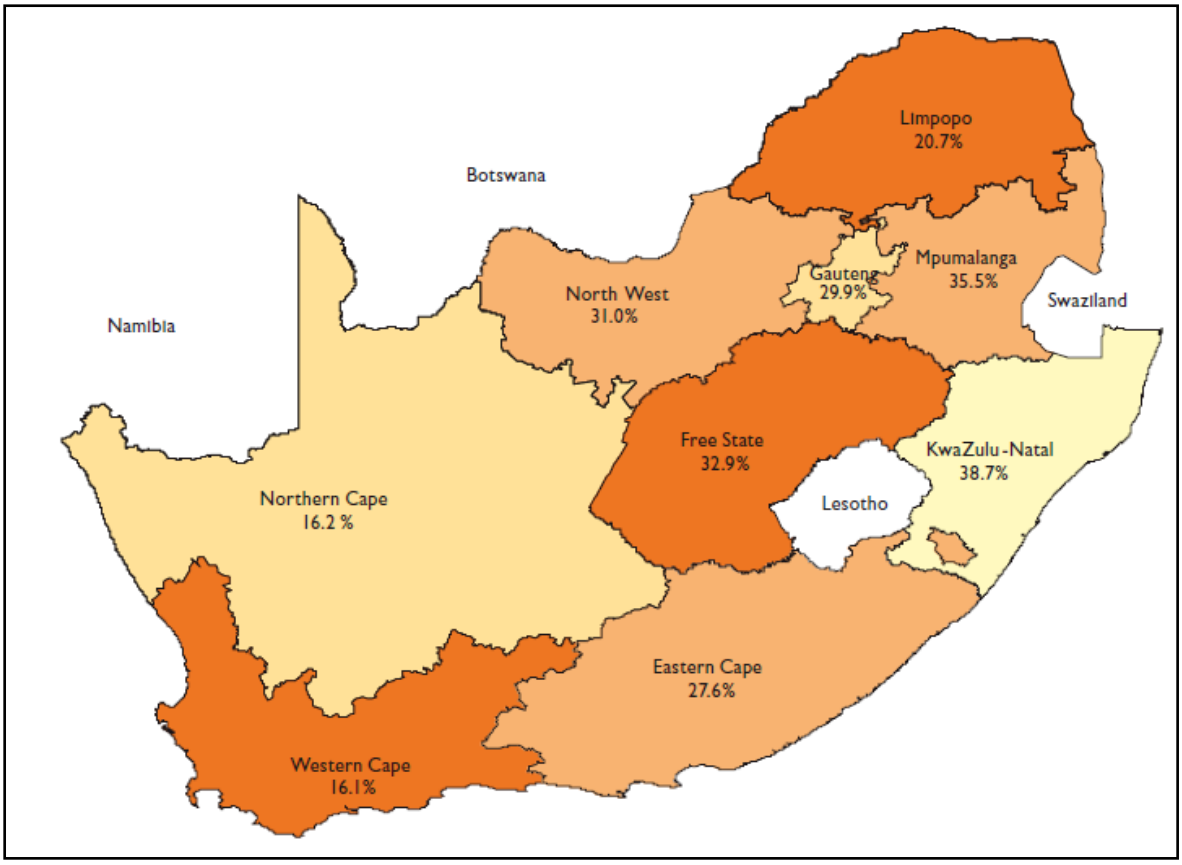

Figure 1.2. Distribution of HIV prevalence by province in antenatal clinic attendees, South Africa, 2008. (South Africa Report, 2008).

1.3 HIV-1 STRUCTURE AND REPLICATION

1.3.1 Structure

HIV is a retrovirus, an enveloped virus that replicates in a host cell by copying its RNA into a complementary DNA using reverse transcriptase encoded by the virus. The integration of the viral genome into the host genome is then mediated by integrase together with host cellular factors, allowing for many copies to be made in the host cells (Smith and Daniel, 2006). HIV encodes nine proteins; 3 major structural polyproteins, Gag, Pol and Env are required for the formation of new virus particles

and are common to all retroviruses, accessory proteins Vif, Vpr, Vpu and Nef found within the virion and the accessory proteins Tat and Rev are essential for regulatory functions and virion assembly (Frankel and Young, 1998).

The Gag gene encodes a polyprotein precursor Pr55Gag that is cleaved by viral protease to mature Gag proteins p17 matrix (MA), capsid (p24), p7 nucleocapsid (NC) and p6. In addition, 2 spacer peptides, p1 and p2 located between capsid and nucleocapsid and between nucleocapsid and p6 are also generated upon Pr55Gag processing (Fig 1.3), (Accola et al., 1998, Freed, 2001). The synthesis of Gag and Gag-Pol polyprotein Pr55Gag takes place in the cytosolic polysome before transportation to the plasma membrane (Murakami, 2008). The matrix, composed of viral protein p17, surrounds the capsid and ensures the integrity of the virion particle. The matrix is important for the incorporation of viral surface glycoproteins into virions (Dorfman et al., 1994, Yu et al., 1992), targeting of Gag to plasma membrane and early post entry events (Freed, 1998). The capsid forms the characteristic cone shaped shell of the mature virion that encloses the viral genomic RNA (Gelderblom et al., 1987) and plays a crucial role in virus assembly, maturation and post entry steps (Freed, 1998).

The nucleocapsid, found in the core tightly associated with the viral RNA (Meric et al., 1984), is required for the encapsidation and dimerization of the viral RNA, membrane binding and for stabilization of the preintegration complex and transcription. It also functions as a nucleic chaperone enabling refolding of nucleic acid molecules to more energetically favourable conformation (Accola et al., 1998, Freed, 2001).

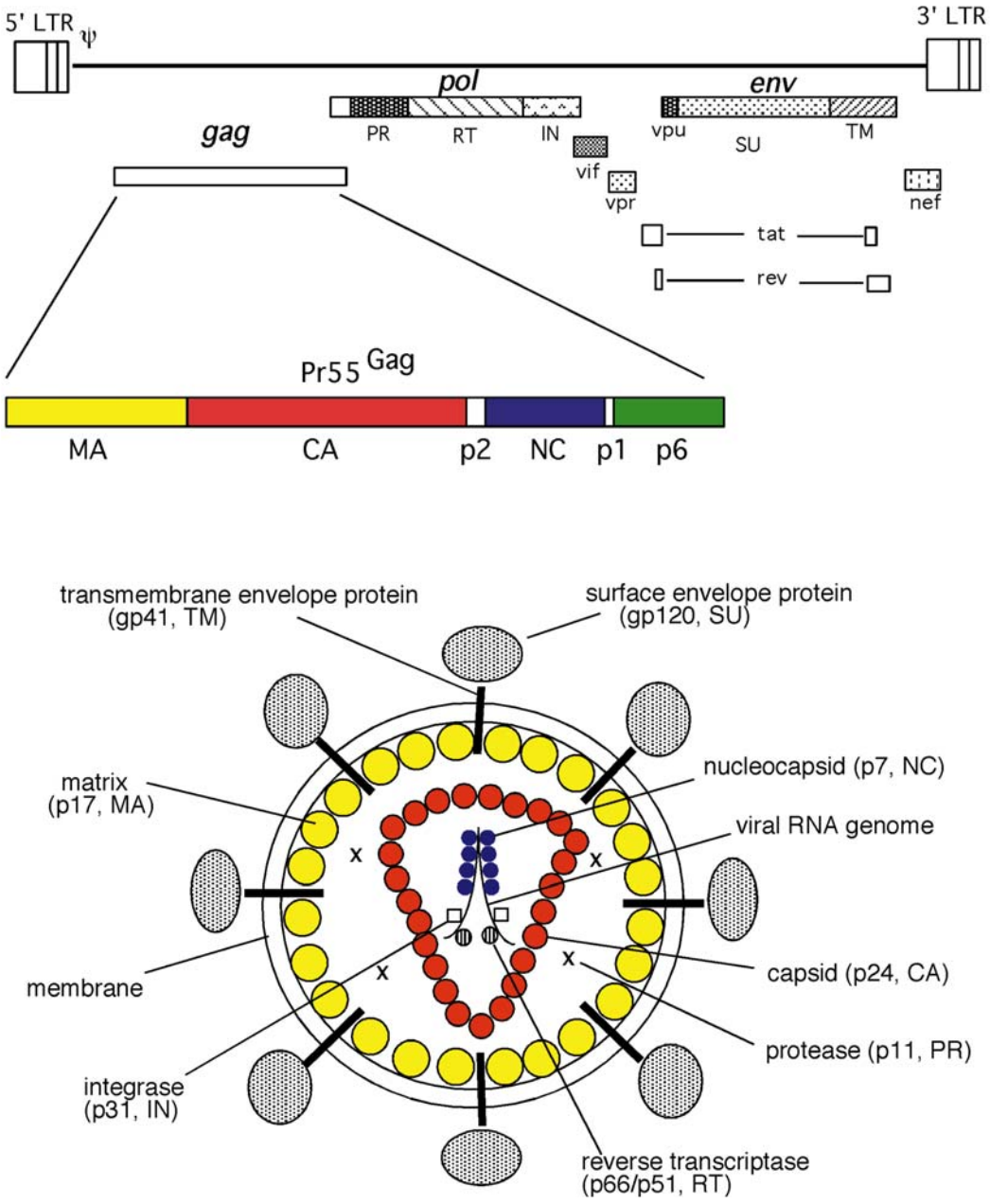

Figure 1.3. Schematic representation of the HIV-1 genome (top) and virion organization (bottom), indicating the location of the structural proteins and their encoded enzymes (Freed, 1998).

The proline-rich protein p6, located at the C-terminus of Gag is essential for the release of assembled virions from the cell surface (Gottlinger et al., 1991) and production of the Vpr protein (Kondo and Gottlinger, 1996, Paxton et al., 1993). The single stranded RNA is tightly bound to the nucleocapsid protein p7 and enzymes such as reverse transcriptase (RT), protease (PR), ribonuclease and integrase (IN) important for virion development.

The pol gene is synthesized as part of Gag-Pol fusion due to the lack of the initiation codon, resulting in overlap and shift of the pol gene in the minus 1 reading frame with gag. Pr160GagPol is cleaved by viral protease to produce viral enzymes reverse transcriptase, protease and integrase, (Fig 1.3), (Freed, 2001, Hill et al., 2005). Reverse transcriptase catalyses the conversion of single stranded RNA into double stranded DNA early post infection while integrase catalyzes the insertion of linear double stranded viral DNA into the host cell chromosome (Hill et al., 2005). Protease cleaves the Gag and Gag-Pol polyproteins and allows conformational changes within the particle to produce mature infectious viruses (Frankel and Young, 1998).

Synthesis of the Env glycoproteins gp160 takes place in the endoplasmic reticulum and these proteins are then transported via the secretory pathway to the plasma membrane (Murakami, 2008). The Envelope (Env) glycoproteins are produced as a polyprotein gp160, which is heavily glycosylated and cleaved by cellular protease into surface subunit (SU) gp120 and transmembrane subunit (TM) gp41 (Chan and Kim, 1998, Freed, 2001). gp120 determines interaction with the CD4 receptor and co-receptor

(either CXCR4 or CCR5) found on susceptible cells such as T lymphocytes and macrophages, this binding to target cell receptors activates gp41 to undergo a conformational change to a fusion-active state. gp41 anchors the gp120/gp41 complex to the membrane and facilitates membrane fusion between viral and host lipid bilayers during virus entry (Chan and Kim, 1998, Freed, 2001). In addition to the 3 structural proteins, the HIV-1 virus encodes 2 regulatory proteins Tat and Rev and accessory proteins Vpu, Vpr, Nef, Vif which are not essential in gene expression but important in viral infectivity, replication and contribute to the spread of the virus and disease onset. Transcriptional transactivator (Tat) is critical for activated transcription from HIV-1 long terminal repeat (LTR) and contributes to apoptosis through induction of mitochondrial permeabilization, while regulator of gene expression (Rev) mediates the transport of viral mRNAs from the nucleus to the cytoplasm (Seelamgari et al., 2004).

Negative regulatory factor (Nef) down regulates CD4 through rapid endocytosis from plasma membrane and MHC class I molecules from cell surface by protecting infected cells from cytotoxic T cell (CTL) recognition (Seelamgari et al., 2004). Viral protein R (Vpr) is essential for the transport of preintegration complex into the nucleus of infected cells and also induces cell cycle arrest in the G2 phase of the cell cycle. It is also involved in nuclear localization, apoptosis and has been shown to induce a proinflammatory cytokine tumour necrosis factor (TNF), known to activate HIV-1 expression and replication (Freed, 2001, Nakamura et al., 2002, Seelamgari et al., 2004). Recent data suggest that amino acid polymorphisms in specific residues in Vpr may affect epitopes significantly and contribute to the escape of the virus (Srinivasan et

al., 2008). Viral protein U (Vpu), is important in the late stages of infection in that it enhances the release of viral particles by counteracting host restriction imposed by tetherin (Neil et al., 2008, Nomaguchi et al., 2008). Vpu also promotes the degradation of CD4 and down regulates cell-surface expression of the CD4 receptor (Seelamgari et al., 2004) and also promotes the apoptosis of HIV-1 infected CD4+ T cells (Akari et al., 2001). Viral infectivity factor (Vif) is expressed late in infection and is localized in the cytoplasm (Goncalves et al., 1994, Karczewski and Strebel, 1996). Vif increases the stability and assembly of virion cores and reverse transcriptase complexes and may prevent premature degradation upon viral entry (Ohagen and Gabuzda, 2000). The critical role of Vif is to suppress the function of APOBEC3G and APOBEC3F, which are host proteins important to its anti-viral function (Malim, 2009, Swanson and Malim, 2008).

1.3.2. HIV-1 Replication

The HIV replication cycle can be summarized in six steps as illustrated in Fig 1.4; 1) binding and entry; 2) uncoating; 3) reverse transcription; 4) provirus integration; 5) virus protein synthesis and assembly and 6) budding (Fanales-Belasio et al., 2010). Entry of HIV-1 into target cells is mediated by the Env protein. The env gene encodes a polyprotein gp160 that is cleaved by cellular protease to yield the surface subunit gp120 and the transmembrane protein gp41 required for viral infectivity. Viral entry process begins when the viral surface envelope protein gp120 binds specifically to CD4; this interaction changes the conformation of gp120 which then increases the coreceptor affinity. A ternary complex composed of gp120, CD4 and coreceptor forms;

conformational changes in gp41 eventually trigger pore fusion formation between the virion lipid bilayer and the host cell plasma membrane releasing the viral core into the cytoplasm, (Freed, 2001, Gomez and Hope, 2005).

The fusion process is carried out by the "fusion peptide" located at the amino terminus of gp41 by insertion of gp41/CD4/receptor complex directly into the target membrane at an early step of fusion process. Following fusion, the uncoating process takes place, during which the viral RNA undergoes the reverse transcription process and a preintegration complex is formed.

During this process, the capsid is lost, while some matrix, nucleocapsid, pol-encoded enzymes reverse transcriptase and integrase and accessory protein Vpr remain associated as part of a high molecular weigh complex. In this process, the reverse transcriptase converts RNA genome into double stranded DNA before transportation of the preintegration complex to the cell nucleus (Freed, 1998, Freed, 2001), where the integration of viral DNA into the host cell chromosome is catalyzed by integrase.

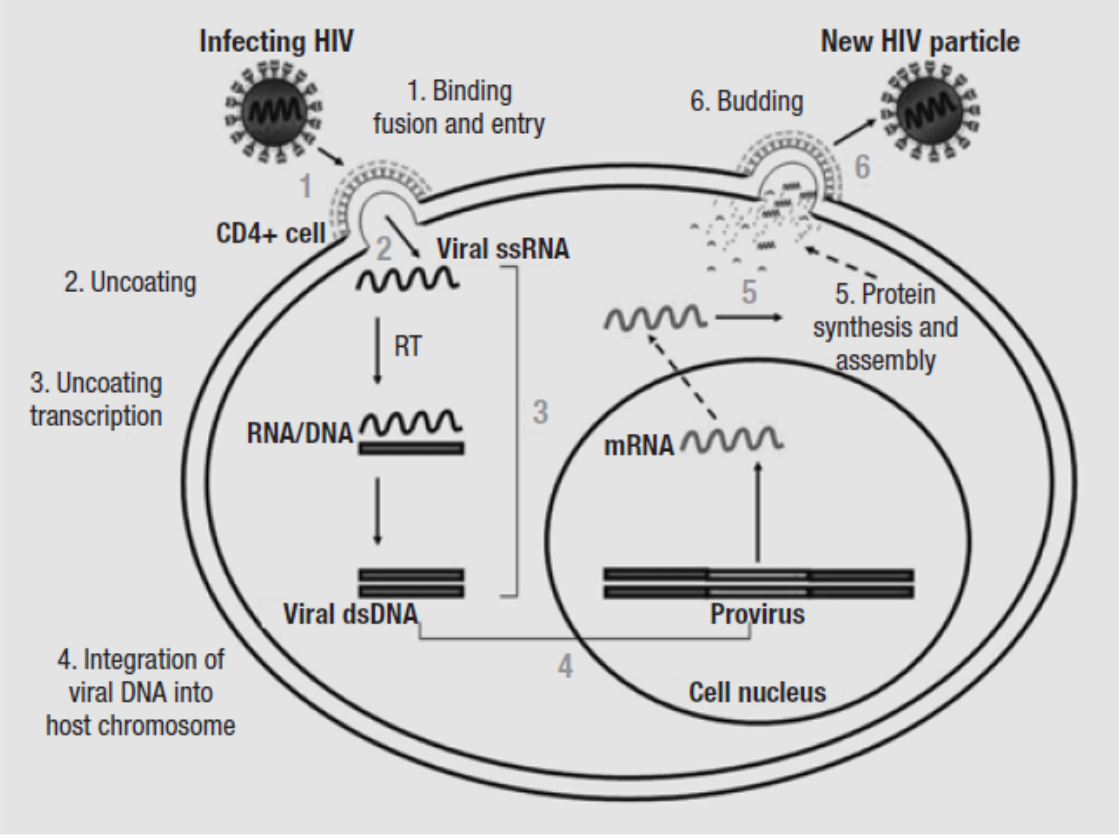

Figure 1.4. Schematic representation of the HIV-1 replication cycle (Fanales-Belasio et al., 2010).

The integrated provirus is then transcribed leading to the synthesis of viral RNAs which are transported to the cytoplasm. Translation of the mRNAs ultimately encodes the full complement of structural, regulatory and accessory proteins used to direct virus replication. The assembly process is mediated by the gag-precursor polyprotein Pr55Gag as it has determinants that target it to the plasma membrane, bind the membrane and encapsidate the viral genome. The Gag precursor also interacts with Gag-Pol precursor and the assembled Gag-protein complex induces membrane curvature leading to formation of a bud (Freed, 1998, Freed, 2001).

The budding process follows assembly and involves the incorporation of Env glycoproteins into new virus particles and is completed when the particles are released or pinched off from the plasma membrane. Maturation occurs during or shortly after the release of virions from the plasma membrane where the viral protease cleaves the Gag and Gag-Pol polyprotein precursors to generate mature Gag and Pol proteins. This leads to structural rearrangements that eventually generate mature infectious virions capable of initiating a new round of infection (Freed, 1998, Freed, 2001). The therapeutic approach of HIV/AIDS is through the use of different classes of antiretroviral drugs administered as a combination regimen that act at different stages of the HIV life cycle. Currently licensed and available classes of drugs include entry inhibitors, reverse transcriptase inhibitors, protease inhibitors and integrase inhibitors, named according to the phase of the HIV life cycle they inhibit.

1.4. THE NATURAL RESPONSE TO HIV-1 INFECTION

HIV-1 infection is characterized by an initial burst of high viral load, CD4+ T cell destruction and eventually stabilization to a viral set point indicative of the immune system's partially successful attempt to fight off the virus in the absence of antiretroviral treatment. The mechanisms underlying the establishment of viral set point few months after acute infection are poorly understood, but may involve the interplay between host immune responses and viral replication (Fig 1.5). The viral load levels generally rebound to the same set point following discontinuation of ART and the viral set point may be a good predictor of the rate of disease progression; the higher the set

point, the more rapid the immunologic progression to disease (Hatano et al., 2000, Lyles et al., 2000).

Studies from individuals with known dates of seroconversion have shown that it takes a median period of 10 years from the time of infection to onset of disease (Alcabes et al., 1993, Pantaleo et al., 1993). The outcome of disease may vary amongst individuals; some individuals may mount immune responses able to control viral replication and remain clinically asymptomatic with minimal CD4+ T cell loss and very low to undetectable viral loads even up to 12 years of infection, while some individuals may fail to control virus replication with a rapid CD4 count decline and may develop AIDS within months to a few years of acquiring HIV infection (Cao et al., 1995, Pantaleo et al., 1995, Paranjape, 2005). Several mechanisms including host genetic factors, age, virulence of the infecting strain, co-infection with other microbes, and immunological factors may influence HIV disease progression (Fauci, 1993, Pantaleo et al., 1993).

However, the adaptive immune response is thought to be the most critical component of the immune system in the control of HIV infection. Evidence of the importance of CD8+ T cells in viral control has been demonstrated in animal models, where CD8+ T cell depletion is associated with a dramatic increase in viral load in SIV infected macaques and rapid progression to AIDS (Schmitz et al., 1999).

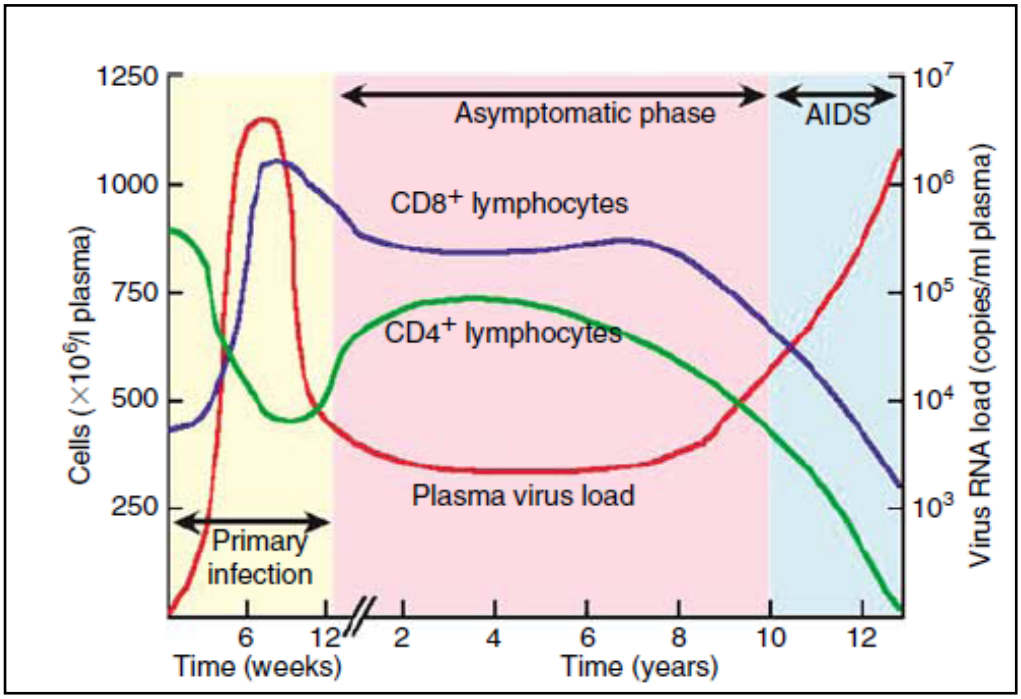

Figure 1.5. Representation of a typical course of HIV-1 infection showing CD8+ and CD4+ T cell dynamics and viral load changes over the time of infection (Munier and Kelleher, 2007).

In humans, the protective role of CD8+ T cell responses has been similarly demonstrated by the temporal association of the CD8+ T cell response with a decline in plasma viraemia following acute infection (Rosenberg et al., 2000), (Fig 1.5). Also, virus-specific CD4+ T cell responses are critical for the maintenance of effective CD8+ T cell function (Altfeld and Rosenberg, 2000, Kalams et al., 1999, Rosenberg et al., 1997). Thus immunologic control of viraemia may depend on a strong CD4+ T cell helper and CD8+ T cell responses.

1.4.1. The role of CD8+ T cell response in adult HIV-1 Infection

Cytotoxic T lymphocytes (CTLs) also referred to as CD8+ T cells, recognize HIV infected cells through HIV viral antigenic epitopes (8-11 amino acid in length), that have been processed intracellularly and are expressed on antigen presenting cells in conjunction with class I major histocompatibility complex (MHC) molecules and β2 microglobulin (Paranjape, 2005). The CD8+ T cell receptor recognizes the class I MHC, β2 microglobulin, viral peptide complex which triggers the CD8+ T cell to lyse the infected cell (Gandhi and Walker, 2002). Thus the CD8+ T cell response in an individual depends on HLA haplotype and the alleles expressed by the individual.

There is substantial evidence of the role HIV-specific CD8+ T cells play in combating viral replication during the acute infection phase. First, there is massive expansion of the CD8+ T cells in acute infection (Pantaleo et al., 1994) which coincides with the appearance of HIV-specific CD8+ T cells and a decline in plasma viraemia (Koup et al., 1994). Second, CD8+ T cells taken from patients with HIV-1 were able to inhibit viral replication in autologous CD4+ T cells in vitro (Yang et al., 1996). Third, depletion of CD8+ T cells from SIV infected animals using monoclonal antibodies results in failure to clear SIV viraemia after infection and rapid progression to disease (Schmitz et al., 1999).

Lastly, the immune selection pressure exerted by the CD8+ T cell response following early infection in animal and human infection suggest that the virus can rapidly mutate to escape CD8+ T cell recognition and control (Allen et al., 2000, Borrow et al., 1997).

The factors influencing the effectiveness of the CD8+ T cell response in controlling viral replication and in subsequently establishing the different viral set points in individuals remains poorly understood. It is suggested that the initial CD8+ T cell responses are low in magnitude and narrowly directed toward viral proteins such as Vpr, Nef, Tat and Env (Altfeld et al., 2001, Cao et al., 2003, Dalod et al., 1999). These responses are regarded as the most effective in controlling viral replication as they have the greatest antiviral activity (Cao et al., 2003); however, in the absence of treatment, the initial CD8+ T cell response continues to evolve and are less effective in further reducing viral load.

In contrast, the role of the CD8+ T cell response in chronic infection is still controversial as a large majority of patients lose viral control despite the presence of HIV-specific IFN-γ producing CD8+ T cells with high avidity or antigen specific CD8+ T cells that are able to recognize viral proteins such as Env, Gag and Pol (Addo et al., 2003, Draenert et al., 2004, Frahm et al., 2004, Gea-Banacloche et al., 2000). Furthermore, conflicting results have been reported between HIV-specific CD8+ T cell responses and viral load in chronic infection (Betts et al., 2001, Betts et al., 1999, Migueles and Connors, 2001, Ogg et al., 1998, Shankar et al., 2000). On the other hand, CD8+ T cell responses from therapy naïve long term non-progressors (LTNPs) have a greater capacity to proliferate coupled with perforin and IL-2 production and in addition display strong proliferative CD4+ T cell responses to Gag antigens, attributable to enhancement of the CD8+ T cell effector function (Migueles et al., 2002, Rosenberg et al., 1997, Zaunders et al., 2004). Also, recent data showed that LTNPs

displayed polyfunctional CD8+ T cells than progressors, suggesting that the quality of the CD8+ T cell response may be important for viral control (Betts et al., 2006). Thus, HIV-specific CD8+ T cells may become ineffective as the disease progresses and mere quantification with assays that measure IFN-γ production alone may not be adequate to understand mechanisms underlying this dysfunction in chronic infection.

Several mechanisms have been implicated in CD8+ T cell dysfunction. First, the depletion of essential CD4+ T helper cells by HIV may be an important cause for progressive loss of CD8+ T cell function since the CD4+ T helper cells are critical in providing effective CD8+ T cell function (Cardin et al., 1996, Gloster et al., 2004). Continuous viral replication and generalized immune activation may result in functional T cell defects, T cell exhaustion and activation-induced apoptosis of HIV-specific CD8+ T cells (Brenchley et al., 2003, Lichterfeld et al., 2004a, Streeck et al., 2008). The immature state of CD8+ T cells rather than end-stage effector cells may also affect the CD8+ T cell response (Chakraborty, 2005, Kostense et al., 2002). Finally, mutations of viral sequences recognized by CD8+ T cells can evade host recognition by CD8+ T cell responses resulting in uncontrolled viral replication (Geels et al., 2003, Goulder and Watkins, 2008).

1.4.2. CD4+ T cell responses in adult HIV-1 Infection

The hallmark of HIV-1 infection is the progressive decline in the number of CD4+ T cells. Activated CD4+ T helper cells are the main target of HIV and are killed by infection and HIV induced apoptosis (Cottrez et al., 1997, Groux et al., 1992, Levy,

1993), leading to a decrease in CD4 counts and defects in CD4+ T cell function (Musey et al., 1999). The CD4+ T cell recognize peptide antigens in association with class II MHC molecules after endocytosis of exogenous proteins by antigen presenting cells. The peptide-class II MHC complex is then recognized by the CD4+ T cell receptor and leads to activation of CD4+ T cells (Gandhi and Walker, 2002). Activated CD4+ T helper cells can mediate Th1 response by producing IL-2 and IFN-γ which are important for maintaining an effective CD8+ T cell response. The majority of anti-HIV-specific CD4+ T cell responses are detectable during primary infection in response to high viral load (Altfeld et al., 2001, Kalams et al., 1999, Rosenberg et al., 1997) and these responses may persist in chronic infection (Ramduth et al., 2005, Scriba et al., 2005). In the absence of ART, long term non-progressors maintain proliferating CD4+ T cell responses (Rosenberg et al., 1997, Schwartz et al., 1994). Strong T helper response were associated with strong CD8+ T cell responses implying that immunologic control in HIV infection may in part depend on both strong CTL and T helper responses.

With continuous viral replication, the CD4+ T cell response may produce IFN-γ but loose the ability to produce IL-2 or to proliferate (Iyasere et al., 2003, Younes et al., 2003), which is associated with lack of viral control. In addition, CD4+ T cells senescence has also been reported in HIV infection (Palmer et al., 2005). Thus, T cell control of HIV replication depends on the host immunological response and the ability of T cells to proliferate in response to viral antigen exposure, while maintaining low levels of immune activation (Emu et al., 2005).

1.5. MOTHER-TO-CHILD-TRANSMISSION

Around 70,000 babies are born HIV infected every year in South Africa, most of whom acquired the virus during pregnancy, birth or breastfeeding (WHO, 2009). Without any intervention, there is a 20-45% chance that a mother will transmit HIV to their new-borns (De Cock et al., 2000). The use of combination antiretroviral therapy given to the mother during pregnancy and to the child immediately after birth can reduce the risk of mother-to-child-transmission to less than 2%. Such interventions together with obstetric management and use of alternative infant feeding methods have made vertical transmission relatively rare in developed countries (EuropeanCollaborativeStudy, 2005). In developing countries, around 45% of HIV infected women received drugs to protect their children from infection during 2008 (WHO/UNAIDS/UNICEF, 2009, WHO, 2009), most women are not reached by prevention of mother-to-child-transmission (pMTCT) services due to lack of adequate health-care infrastructures and access to antiretroviral drugs or are unaware of their HIV status (De Cock et al., 2002, Menu et al., 1999).

Previously, single-dose nevirapine regimen to the mother at labour and to the child at birth according to the HIVNET-012 protocol has been widely used as a standard regimen for perinatal prevention due to its low cost, simple administration and high efficacy of the regimen (Abrams, 2004, Guay et al., 1999). However, the development of mutations associated with nevirapine resistance (Eshleman et al., 2001) and potential negative effect these mutations may have on the future use of the drug led to the current

use of long-course antiretroviral regimens that are more effective in reducing in utero and intrapartum transmissions (Scarlatti, 2004).

The recent South African guidelines require that mothers be given zidovudine (AZT) from 14 weeks of pregnancy until labour and single dose nevirapine during labour; to the infant single dose nevirapine from birth upto 6 weeks of life (National Department of Health, 2010). However, these guidelines fall short of the international standards as the World Health Organization (WHO) recommends that mothers take AZT and lamivudine (3TC) during and after birth to prevent transmission of the virus to the child and also as a cover tail strategy to reduce the risk of nevirapine resistance (WHO, 2006). Although suboptimal when compared to international standards, scaling up from nevirapine only using regimen to dual therapy is challenging in resource-limited settings but can be achieved to reduce transmission to rates as low as those seen in developed countries (Amornwichet et al., 2002, Geddes et al., 2008, Plipat et al., 2007, Tonwe-Gold et al., 2007).

1.5.1. Timing of infection and determinants of MTCT

MTCT can take place during pregnancy, at birth and through breastfeeding; however, the majority of infants acquire HIV infection despite repeated exposure to the virus even in the absence of antiretroviral drugs. In utero transmission accounts for an estimated 20-30% of vertical transmission (Abel, 2009) and is also associated with rapid disease progression in the infected infants (Dickover et al., 1998, Kuhn et al., 1999). Although transmission can occur throughout pregnancy, the majority of HIV-1

transmission occurs in the last trimester of gestation (Kourtis et al., 2001, Rouzioux et al., 1993). Generally, the placenta is an efficient barrier allowing for few in utero infections. However, MTCT in utero most likely occur through direct infection of the plancental or trophoblastic cells, although infection of the trophoblastic cells does not always result in infection of the fetus (Lee et al., 1997, Menu et al., 1999, Zachar et al., 1994).

Intrapartum transmission can occur during delivery by maternofoetal blood transfusions, amniotic fluid, vaginal secretions as a result of prolonged membrane ruptures or postnatal by breast milk. It is estimated that breastmilk transmissions bear the bulk of all MTCT cases ranging from 25-50% (Luzuriaga, 2007, Luzuriaga et al., 2006). It is hypothesized that the risk of breast milk transmission is the greatest during the first 6 weeks of life since viral load levels in human breast milk are the highest in the first few days after delivery (Rousseau et al., 2004), however, the risk of transmission remains high throughout the breastfeeding period (Nduati et al., 2000, Abrams, 2004, Coutsoudis et al., 2004).

Several risk factors influence MTCT transmission of HIV to the infant; high maternal plasma viral load, low CD4 counts or advanced maternal disease state, prolonged membrane rupture before delivery and increased duration of breastfeeding. In addition, child and maternal HLA mediated immune responses and viral characteristics also determine susceptibility to HIV infection (Abrams, 2004). The encouraging aspect is

that the use of antiretroviral drugs has been shown to minimize the risk of transmission, often alleviating the impact of these well-known risk factors.

1.5.2. Viral Kinetics in Paediatric HIV-1 Infection

Acute HIV-1 infection in adults is followed by the development of HIV-specific CD8+ T cell responses and temporal decline in viraemia from an average of 10 million copies to a median set-point of 30 000 copies/ml (Lyles et al., 1999, Rosenberg et al., 2000). In paediatric HIV-1 infection, there is no substantial decrease in viral load, instead viraemia remains over 100,000 to a million copies/ml in the first year of life and does not reach viral set point until after the first 2 or more years of life in surviving children, (Fig 1.6), (Abrams et al., 1998, Richardson et al., 2003, Shearer et al., 1997).

In the absence of antiretroviral therapy (ART) intervention, 45-59% of HIV-infected children in developed countries will die by the age of 2 years (Dabis et al., 2001, Obimbo et al., 2004, Spira et al., 1999), while the remaining children will progress slowly to AIDS by the age of 5-6 years (Barnhart et al., 1996, Blanche et al., 1990). It is still unclear whether the differences in viral kinetics following infection in paediatrics and adults contribute to rapid disease progression in children; however, it is possible as the reduction in viral load following ART is well established to result in clinical benefit.

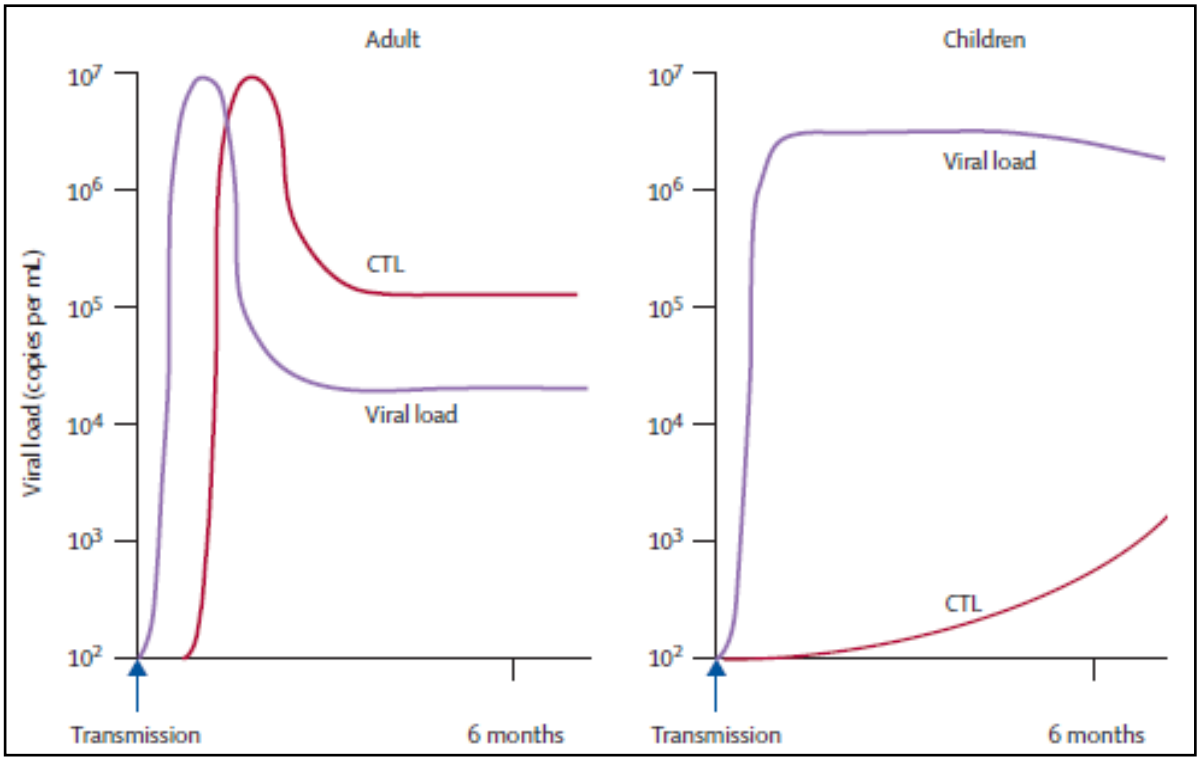

Figure 1.6. Schematic representation of viral load and levels of CD8+ T cell activity (CTL) in therapy naïve adult and paediatric HIV infection (Prendergast et al., 2007).

1.5.3. Immune Control in Paediatric HIV-1 Infection

In contrast to adults, several factors have been proposed to accelerate disease progression in children. First, the immature immune system at the time of infection may be incapable of generating immune responses essential for viral containment. Continued viral replication in infancy could be a result of the size of the pool of host cells permissive to viral replication, exposure of the thymus to HIV-1 mediated destruction at a time of active thymopoiesis (Chakraborty, 2005, Nahmias et al., 1998) or in some cases, destruction of the immune system happens before responses can be mounted (Kourtis et al., 2001).

Second, the genetic similarity between mother and child may be disadvantageous to the child as children acquire a virus with a history of encounter with maternal immune responses. Thus, the lack of immune control in the mother following adaptation of the virus to maternal HLA alleles shared with the child may result in failure to control the virus by the child (Tiemessen and Kuhn, 2006). The prognosis is different in children who acquire the virus through blood transfusions or who share protective alleles with their fathers (Frederick et al., 1994, Kuhn et al., 2004). Third, infected children are likely to inherit HLA alleles associated with poor control of HIV from their mothers than uninfected children since MTCT is associated with maternal viral load (Prendergast et al., 2007, Sperling et al., 1996). Fourth, although CD8+ T cell responses are detectable in infants, these responses are low in frequency and inconsistent during early life (Froebel et al., 1994, Luzuriaga et al., 1995, Buseyne et al., 1998) in comparison to older children and adults and do not offer immediate clinical benefit (Lohman et al., 2005, Riviere and Buseyne, 1998). Furthermore, CD4+ T helper responses, important for the maintenance of effective CD8+ T cell responses are of low magnitude during early infancy (Feeney et al., 2004, Thobakgale et al., 2007, Wasik et al., 2000, Huang et al., 2008), but are detectable in older children, although at lower frequencies than reported in adults (Pitcher et al., 1999). The lack of CD4+ T cell help may compromise CD8+ effector function in infants and contribute to the low frequency of polyfunctional CD8+ T cell responses in younger children (Huang et al., 2008).

There is evidence that CD8+ T cell responses may mediate control in paediatric HIV-1 infection. For example, HLA alleles such as B*2705 and B*5701 known to mediate control in adult infection have been shown to mediate non-progression in paediatric infection and the development of mutations in key epitopes presented by these alleles is followed by loss of control (Feeney et al., 2005, Feeney et al., 2004). However, there is also evidence of fitness cost to the virus if the mutations are within the conserved regions such as Gag, thus benefiting the child in the context of MTCT transmission (Leslie et al., 2004, Thobakgale et al., 2009). There are reports that CD8+ T cell responses exert selection pressure in known CD8+ T cell epitopes within the first few months of life suggesting that these responses may be effective (Pillay et al., 2005).

Taken together, these reports demonstrate that HLA class I alleles influence disease progression in paediatric infection through generation of CD8+ T cell responses or transmission of low-fitness viruses as has been shown in adult infection (Martinez-Picado et al., 2006). The sustained detection of CD4+ T helper activity in children with delayed disease progression highlights the importance of these cells in paediatric HIV-1 pathogenesis (Chakraborty et al., 2005, Wasik et al., 2000). HIV-1 may directly kill thymic cells required for normal thermocyte development (Stanley et al., 1993, Valentin et al., 1994). However, unlike in adults, the thymus is very active in children, the recovery of T cells is through the thymic production of new naïve T cells and generation of new T cells clones with various specificities capable of responding to pathogens (Correa and Munoz-Fernandez, 2002).

1.6. STUDY AIMS AND OBJECTIVES

The mechanisms underlying the lack of viral control following paediatric HIV-1 infection are poorly understood. The timing for the development of adaptive T cell responses remains controversial, and where detected, the effect of these responses in combating viral replication is not well established. The factors that mediate slow or long-term-non progression to disease in children are also not well studied in children. A cohort of 63 acutely HIV-1 infected infants was established and followed up over 3-4 years to address the following aims and objectives:

To determine the timing of virus-specific T cell development and the specificity of the CD8+ T cell response following acute HIV infection in infants

To determine whether or not the genetic background (HLA) of the mother or child will have a bearing on disease outcome in the child

To determine T cell functionality by longitudinally assessing T cell polyfunctionality, activation and senescence in a group of slow and rapid progressor children from birth up to 3-4 years of age.

1.7. OUTLINE OF THESIS

A detailed overview of the HIV epidemic and replication, determinants of transmission from mother-to child and the role played by T cell responses in the control of adult versus paediatric HIV-1 infection is covered in the first part of **Chapter 1**. The next chapters describe a series of investigations undertaken to understand the timing of the earliest detectable T cell responses in infants following acute infection and the factors that determine slow versus rapid progression to disease over time in these children. **Chapter 2** examined HIV-infected infants born to infected mothers and monitored the age of development and specificity of the HIV-specific CD8+ T cell responses using a panel of overlapping peptides spanning the entire HIV clade-C proteome. In addition, the CD4+ and CD8+ T cell responses were compared in infants with early infection versus older children with established HIV-1 infection.

Chapter 3 tested the hypothesis that HIV-infected children are likely to progress slowly to disease if they or the transmitting mother possess protective HLA alleles that are not shared between mother and child or if the mother transmits low-fitness virus to the child. The HLA-type, CD8+ T cell responses and viral sequences of mother-child pairs who were monitored from birth were investigated. **Chapter 4** further explored T cell functional characteristics that distinguished children with rapid versus slow disease progression within the same cohort of children after 3-4 years of longitudinal follow-up from time of birth. This was done by assessment of T cell quality by investigating T cell polyfunctionality, activation and senescence from early through to chronic infection in a group of identified rapid and slow progressor children. **Chapter 5**

summarizes the key findings of these studies with reference to previous literature and suggests future directions for paediatric research in T cell immunolgy. These studies demonstrate that like adults, children have the capacity to generate CD8+ T cell responses early after infection and mediate control through class I HLA alleles.

CHAPTER 2 : HIV-SPECIFIC CD8+ T CELL ACTIVITY IS DETECTABLE FROM BIRTH IN THE MAJORITY OF *IN UTERO* INFECTED INFANTS

(This chapter has been published in the Journal of Virology, 2007 Dec Issue.

See Appendices section)

2.1. INTRODUCTION

An association between HIV-specific CD8+ and CD4+ T cell responses and control of viral replication has been well documented in acute adult infection (Gloster et al., 2004, Goulder et al., 1997, Goulder and Watkins, 2004, Borrow et al., 1994, Allen et al., 2004) but it is less clearly established what role CD8+ T cells play in control of HIV in acute paediatric infection, which occurs both in utero and intrapartum. In adults, the appearance of HIV-specific CD8+ T cell immune responses in acute infection is temporally associated with a rapid decline in viremia from an average of 10 million copies/ml at peak to a median set-point of 30,000 copies/ml (Lyles et al., 1999, Rosenberg et al., 2000). In paediatric infection there is typically no such rapid decline in viremia following acute infection, viral loads remaining in an excess of 100,000 copies/ml over the first year of life, and only decreasing slowly over the next 2-3 years of life in survivors (Richardson et al., 2003, Shearer et al., 1997).

A potential explanation for the absence of a dramatic decline in viremia in early paediatric infection is either low-frequency HIV-specific CD8+ T-cell activity and/or ineffective CD8+ T-cell activity in infancy. Previous studies of limited numbers of HIV-infected infants have demonstrated that HIV-specific CD8+ T-cell responses can be detected at low frequency in some infants (Buseyne et al., 1998, Luzuriaga et al., 1995, Scott et al., 2001). Moreover, when detected in infancy, the CD8+ T cell responses generated had no immediate benefit in clinical outcome (Buseyne et al., 1998, Lohman et al., 2005, Riviere and Buseyne, 1998), and no studies have compared in utero, intrapartum and chronic paediatric infection. Recent reports that CD8+ T cell

responses in infants can exert selection pressure in vivo in known CD8+ T cell epitopes within the first few months of life (Feeney et al., 2005, Leslie et al., 2005, Pillay et al., 2005) suggest that in some instances, at least, these responses may be functional. In addition, slow progression to disease has been well described in children who express HLA-B*27 or HLA-B*57 (Feeney et al., 2005, Feeney et al., 2004), suggesting that CD8+ T cell responses can be important in paediatric infection as in adult infection.

The aim of these studies was to examine a large cohort of infants born to HIV infected mothers, to determine both the age at which HIV-specific CD8+ T cell responses are induced in infected infants, and the specificity of these responses using a panel of overlapping peptides spanning all the HIV proteins. These studies were undertaken in Durban, South Africa, a country in which its estimated that there are >100 newly infected infants born each day (http://www.avert.org/worldstats.htm).

2.2. MATERIALS AND METHODS

2.2.1. Study Subjects

Sixty-three HIV-1 infected infants born to HIV-positive mothers were enrolled from St Mary's and Prince Mshiyeni Hospitals in Durban, South Africa from 2003-2005 (Table 2.1). HIV-1 seropositive mothers were recruited during the last trimester of pregnancy and a single dose of Nevirapine was given to the mother during labour and to the infant within 48 hrs of birth, according to the HIVNET-012 Protocol (Guay et al., 1999, Jackson et al., 2003). A total of 719 mothers were enrolled and screened, 740 infants were born to the mothers; 623 infants were uninfected, 41 were untraceable and only 75

were infected. Sixty-three of the 75 infants met the clinical criteria for enrollment into the study. Exclusion criteria comprised prematurity, intrauterine growth restriction and congenital anomaly (Mphatswe et al., 2007).

Forty-five HIV-1-positive, antiretroviral therapy naïve children with chronic infection between ages of 2 and 12yrs were recruited from the Paediatric HIV outpatient clinic at King Edward VIII Hospital and McCord Hospital, both in Durban, South Africa. The median absolute CD4 count of this cohort of children with chronic HIV infection was 559, with a median CD4% of 17, and a median viral load of 110,000 copies/ml (Table 2.2). The mothers gave written informed consent for participation of their children both studies. These studies were approved by all the participating Institutional Review Boards.

2.2.2. Diagnosis of HIV-1 Infection in Infants

Infants were diagnosed as HIV-infected following detection of plasma HIV RNA by RNA-PCR (Roche Amplicor Assay). Blood was collected on day 1 and day 28 of life. A positive plasma viral load (>400 RNA copies/ml) on day 1 or day 28 was followed by a confirmation test before enrollment of the infant into the study. Infants with detectable virus on day 1 were defined as intra-uterine (IU) infected (n=44), and infants with undetectable virus on day 1 but with detectable virus on day 28 were defined as intrapartum (IP) infected (n=19). Since the majority of infants were breast-fed, it is possible that this 'IP' group includes some early breast-milk mother-to-child transmission.

2.2.3. Viral Load and CD4 Measurement

Plasma viral loads were measured using either the Roche Amplicor Monitor Assay detection limit of 400 HIV-1 RNA copies/ml plasma,) or the Roche Ultra sensitive assay detection limit of 50 RNA copies/ml plasma), according to the manufacturer's instructions. CD4 counts were determined from fresh whole blood using Tru-Count technology and analyzed on a four-color Flow cytometer (Becton Dickinson) according to the manufacturer's instructions.

2.2.4. Isolation of PBMCs

Blood was collected in EDTA tubes and processed within 6hrs of collection. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the Ficoll-Histopaque (Sigma, St Louis, Mo) density gradient centrifugation and used fresh in ELISPOT assays.

2.2.5. Synthetic HIV-1 Peptides

A panel of 410 overlapping peptides (18mers with a 10-amino acid overlap) spanning the entire HIV-1 clade C consensus sequence were synthesized on an automated peptide synthesizer (MBS 396, Advanced ChemTech) and used in a matrix system in screening assays.

2.2.6. ELISPOT Assays

Screening for T-cell responses was done ex vivo using the Interferon-γ ELISPOT assay as previously described (Kiepiela et al., 2004). The individually recognized peptides within the pools were determined by the use of a second Elispot assay.

Freshly isolated PBMCs were plated in 96-well polyvinylidene difluoride-backed plates (MAIP S45; Millipore) that had been previously coated with 100 µl of anti-human IFN-γ mAb 1-D1k (0.5 µg/ml; Mabtech) overnight at 4 ^{0}C. Peptides were added at a final concentration of 2µg/ml to a 96-well plate with 100 µl of R10 medium at 50 000 or 100 000 cells/well. Negative controls with cells and medium only were run in quadruplicate along with two positive controls containing phytohaemoagglutinin. The plate with contents was incubated overnight at 37 ^{0}C, 5% CO_2 and then processed. Following overnight incubation, the plate was washed with cold PBS and 0.5 ug/ml of IFN-γ mAb biotinylated secondary antibody (7-B6-1, Mabtech) was added for 90 minutes in the dark at room temperature. The plate was then washed with cold PBS, and 0.5µg/ml of streptavidin-alkaline phosphatase conjugate antibody (Mabtech) was added for 45 minutes in the dark at room temperature. IFN-γ producing cells were noted by direct visualization of the plate following development with alkaline phosphatase color reagents (Bio-Rad).

The IFN-γ secreting cells were quantified by counting the number of spots per well using an automated Elispot plate reader (AID ELISPOT reader system; Autoimmun Diagnostika GmbH, Strasburg, Germany). Results were expressed as number of spot forming cells (SFC) per million PBMC after subtractions of background wells. A

response was defined as positive, using previously adopted criteria (Addo et al., 2003, Kiepiela et al., 2004), if it was ≥100 SFC/million PBMCs and >3 standard deviations above the mean of 4 background wells containing PBMCs but no peptide. The mean background levels in all assays were always less than 120 SFC/10^6 cells, with a range of 0-120 SFC/10^6 cells.

Quantitation of CD8+ T cell responses towards each HIV protein was undertaken from the Elispot assays as follows: following subtraction of the background in each well, the number of SFC for each well containing peptides within a particular protein were summated; only positive wells with responses ≥100 SFCs were used to calculate responses to each protein. Wells with responses < 100 SFCs were treated as negative and were assigned a value of 90 SFCs for statistical analyses. To calculate the relative contribution of each protein to the total CD8+ HIV-specific response for each study subject, the total response to all 9 HIV proteins was summated, and the contribution of each individual protein derived by dividing the protein-specific response by the total response. Any protein-specific response that was <100 SFC/million PBMC represented 0% contribution to the total response.

2.2.7. Flow cytometric intracellular cytokine staining

Freshly isolated PBMCs (0.5 x 10^6) were incubated at 37^0C, 5% CO_2 for 90 min with peptide pools at a final concentration of 2 μg/ml per peptide following stimulation with anti-CD28 and anti-CD49 antibodies (Becton Dickinson). Brefeldin (Sigma) was added and cells were incubated for a further 4.5hrs at 37^0C, 5% CO_2. Cells were then stained with anti-human allophycocyanin (APC)-conjugated CD8 and anti-human phycoerythroerythrin (PE)-conjugated CD4 antibodies (Becton Dickinson), washed, fixed and permeabilized as previously described (Ramduth et al., 2005) before adding anti-IFN-γ FITC, IL-2 FITC or TNF-α FITC. Following 20 minutes incubation the cells were washed, resuspended in 200μl of PBS and acquired on a FACS-Calibur (Becton Dickinson). Duplicate negative controls with PBMCs alone together with a positive control containing PBMCs stimulated with phytohaemagglutin, were included in the assays. For the infant cohort, a minimum of 150 000 events were collected per subject, and a minimum of 100 000 events were collected for the chronic children cohort.

The total CD4+ and CD8+ T cell responses were obtained after subtracting the mean of two negative controls. Reference ranges were obtained from intracellular cytokine staining for Gag interferon-gamma in a total of 23 HIV-uninfected infants between the ages of 1 week to 17M. A response was considered positive if above 0.07% for CD8 and above 0.02% for CD4 responses. Gag-specific CD8+ responses ranged from 0.00-0.07% and CD4+ responses from 0.00-0.02% in these 23 uninfected controls.

2.2.8. HLA Typing

DNA for HLA typing was extracted using Puregene DNA isolation kit for blood (Gentra Systems, Minneapolis, Minn.) according to the manufacture's instructions. HLA Class I typing was done by DNA PCR using sequence specific primers as previously described (Kiepiela et al., 2004).

2.2.9. Statistical Analysis

Fisher's exact test was used to compare proportions of IU and IP infants with early detectable responses and also to compare the number of responders in acutely and chronically infected children. The Mann-Whitney test was used to compare differences in magnitude and contribution of each protein to the overall total response in the 9 HIV proteins targeted by both acute and chronic children. Mann-Whitney test was also used to evaluate CD4+ and CD8+ T cell responses measured by ICS in acute and chronic children.

2.3. RESULTS

2.3.1. HIV-specific CD8+ T cell responses are detectable from day-1 of life in infected infants

To determine whether in-utero HIV infection induces an adaptive immune response above the level of any response in exposed-uninfected infants, we enrolled 10 randomly selected HIV-infected mothers, and tested their newborn infants' HIV-specific CD8+ T cell responses on day 1 of life, blind to diagnosis. Three of 10 had detectable CD8+ T cell responses on day 1 of life, and all subsequently proved to be HIV-infected by plasma HIV RNA determination (Fig 2.1A). The remaining 7 subjects who all had undetectable CD8+ T cell responses (<100 SFC/million PBMC) proved to be HIV-uninfected.

In order to further define adaptive immune responses in acute infant infection, we studied an expanded cohort of infants who were HIV infected on day one of life (defined as in utero infected), and other infants who were HIV-negative on day-1 but became HIV-positive when retested on day 28 (defined as intrapartum infected infants), (Fig 2.1B).

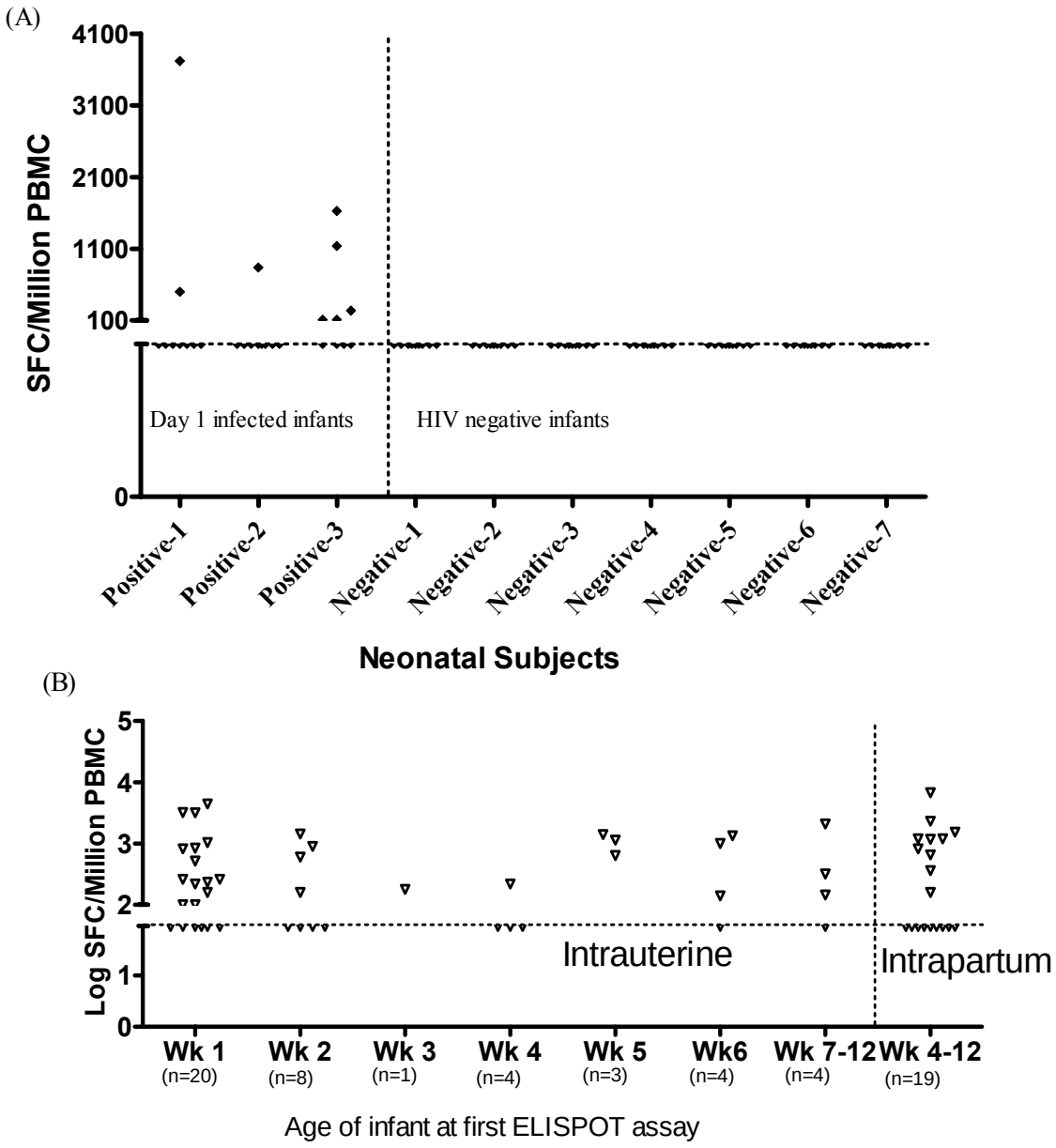

Figure 2.1. Early detection of CD8+ T cell responses in HIV-infected infants. (A). CD8+ T cell responses on day 1 of life in 10 infants born to HIV-positive mothers. (B). Detection of HIV-specific CTL responses in acutely infected infants. Infants were grouped by weeks depending on the earliest time the first assay could be done. For both plots, infants with CD8+ T cell responses above the horizontal dotted line (≥ 100 SFC/million PBMCs) were defined as to having positive responses, and those below, a negative response. Vertical dotted line divides intrauterine and intrapartum infected infants. Responses < 100 SFCs were assigned 90 SFCs and treated as negative.

A total of sixty-three infected infants were identified from 719 mothers enrolled in the study: 70% of these infants (44/63) were intra-uterine (IU) and 30% (19/63) intrapartum (IP) infected (Table 2.1). The initial IFN-γ Elispot assays were performed as soon after diagnosis as feasible, before antiretroviral therapy was initiated, at a median of 9 days of age (range 1-92 days) in IU-infected infants and at a median of 55 days of age (range 31-105 days) in IP-infected infants (Fig 2.1B). CD8+ T cell responses were detectable in 39/63 infants (29 of 44 in IU infected; 10 of 19 IP infected). In 3 of these infants, all of whom had detectable responses, peptides within Env and Vif were not included in the assay because of paucity of PBMCs. HIV-specific CD8+ T cell responses were detected in 14 of 20 in utero infected infants tested within one week of birth, and the majority of IU-infected infants tested after 4 weeks of life had detectable responses (Fig 2.1B and data not shown). Overall, a greater proportion (29/44; 65%) of IU infected infants had detectable cellular immune responses compared to IP-infected infants (10/19, 52%; not significant) with a median follow up of the intrapartum infected infants of 52 days (range 31-105 days). By comparison, all of 45 chronically infected children had detectable CD8+ T cell responses when initially tested at year 2 of life or greater (median 580 SFC/million PBMC).

2.3.2. Frequent targeting of Env in early paediatric infection and Nef in chronic infection

To identify the proteins principally targeted in early and chronic paediatric infection, the magnitude of the responses to each of the nine HIV proteins were documented from the first time point at which assays were performed for the 36 acutely infected infants (Fig 2.2A) and 45 chronically infected children (median age of 6 years; Table 2.2, Fig 2.2B), who had detectable responses upon comprehensive screening in ELISPOT assays.

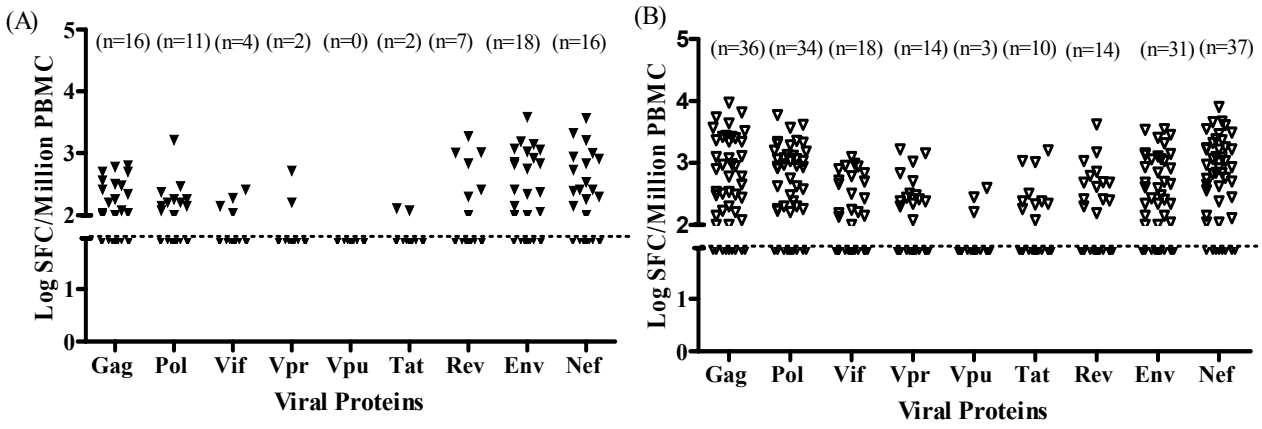

Figure 2.2. Hierarchy of responses to HIV proteins targeted by acute (A) and chronically (B) infected children. Analysis made from the 36 acutely infected infants (n=39, 3 excluded due to insufficient cells to carry out full matrix screen) and 45 chronically infected children all of whom made responses to at least one of the 9 HIV proteins on the first ELISPOT assay. The first ELISPOT assay was undertaken in infants at a mean of 31 days of age (range 1 day –105 days, IQR 7-46 days). The number of infants showing positive responses to each protein is shown in parentheses. Negative responses were assigned 90 SFC/million PBMC (dotted line): values ≥100 SFC/million were defined as positive responses and those <100 SFC/million PBMC negative (see Methods section 2.2.6).

Table 2.1. Characteristics of the cohort of acutely infected infants at the time of the first ELISPOT assay.

Subject	Age (Days)*a*	Transmission type *b*	HLA Class I Type	CD4 (%)	Viral Load (RNA Copies/ml)*c*
001-AC	Day 4	IU	A0101/3001, B4201/8101, Cw1701/1801	75	<400
021-AC	Day 1	IU	A0205/29, B4201/44 Cw0202/1701	18	23,900
046-AC	Day 36	IP	A3002/6801, B5802/5802, Cw0606/0602	36	18,100,00
081-AC	Day 92	IU	A29/68, B1516/44, Cw03/07	44	92
094-AC	Day 34	IU	A2301/6801, B0702/5802, Cw0202/0602	41	>100,000
097-AC	Day 44	IP	A0301/3601, B4501/5301, Cw04/1601	18	>750,000
102-AC	Day 15	IU	A2301/6602, B07/4201, Cw07/1701	47	>647,000
114-AC	Day 61	IP	A03/24, B07/08, Cw07/07	57	4,190
115-AC	Day 42	IU	A29/6801, B1503/5802, Cw04/0602	44	1,540
127-AC	Day 33	IU	A02/3402, B0801/1503, Cw02/04	46	436,000
133-AC	Day 31	IU	A68/7408, B5802/8101, Cw04/0602	54	241,000
135-AC	Day 69	IU	A01/3002, B08/8101, Cw07/18	13	>750,000
149-AC	Day 21	IU	A3001/8001, B1503/18, Cw0202/0202	51	948
188-AC	Day 32	IP	ND *d*	ND	2,330, 000
197-AC	Day 6	IU	A3001/3402, B44/5802, Cw04/0602	42	1,500
222-AC	Day 26	IU	A29/6802, B0702/1302,Cw0602/07	48	<400
227-AC	Day 5	IU	A0301/26, B1529/5201, Cw0202/07	ND	311
241-AC	Day 56	IP	A23/23, B44/81 Cw0202/04	54	4, 820
251-AC	Day 13	IU	A29/6802, B1510/1516, Cw03/04	56	12,400
268-AC	Day 27	IU	A3201/6802, B07/07, Cw0102/07	34	13,200
274-AC	Day 4	IU	A6601/8001, B18/4201, Cw0202/1701	45	40,400
275-AC	Day 55	IP	A23/6601, B1503/5802, Cw0202/0602	27	3, 890
284-AC	Day 36	IU	A3201/6801, B08/15, Cw04/07	38	3,030
298-AC	Day 42	IU	A29/6802, B13/14, Cw06/08	35	339,000
304-AC	Day 5	IU	A3402/6802, B1510/44, Cw03/07	44	14,200
312-AC	Day 84	IP	A0301/6802, B1510/5802, Cw03/0602	27	3,690
341-AC	Day 64	IP	A3002/3201, B1510/5802, Cw03/0602	13	1, 426, 000
344-AC	Day 52	IP	A29/6802, B44/5802, Cw0602/07	14	>750,000
349-AC	Day 4	IU	A2301/4301, B4501/5802, Cw0602/0602	ND	33,950
355-AC	Day 69	IP	A01/0301, B0801/5802, Cw0602/07	15	15, 800
360-AC	Day 47	IU	A02/3601, B3601/53, Cw0602/04	18	4,040,000
364-AC	Day 23	IU	A29/3002, B4102/44, Cw07/1701	34	87,200
380-AC	Day 9	IU	A2301/6801, B1510/1510, Cw03/08	56	16,100
385-AC	Day 105	IP	A3001/3402, B1503/1503, Cw0202/0202	37	2,760
413-AC	Day 4	IU	A01/3201, B07/8101, Cw07/1801	ND	5,650
423-AC	Day 31	IP	A2301/2301, B18/41, Cw04/1701	38	14,040,000
433-AC	Day 41	IU	A4301/6801, B1503/5801, Cw0602, 18	33	357,000
435-AC	Day 2	IU	A02/3004, B4201/44, Cw0202/1701	45	91,400
446-AC	Day 26	IU	A02/3001, B1503/4501, Cw0202/1601	20	3, 730,000
447-AC	Day 63	IP	A03/6802, B1401/4701, Cw0602/0802	34	8,860,000
458-AC	Day 41	IP	A4301/6801, B1503/1510, Cw03/18	37	2,340,000
464-AC	Day 44	IU	A29/6802, B15/18, Cw0202/03	28	5,720,000
468-AC	Day 3	IU	A0202/6801, B5703/5801, Cw06/07	51	5630
496-AC	Day 13	IU	A0301/6801, B1503/5802, Cw0602/06	32	882,000
517-AC	Day 31	IP	A3002/6802, B1510/4201, Cw03/1701	39	2,300,000
559-AC	Day 4	IU	A2301/6802, B0801/5801, Cw07/07	61	731
562-AC	Day 10	IU	A0205/3002, B1402/5801 Cw07/0802	36	2,580,000
568-AC	Day 33	IP	A3001/6802, B07/18, Cw07/07	45	>750, 000
576-AC	Day 4	IU	A29/6802, B1401/44, Cw07/0802	48	94,500
579-AC	Day 8	IU	A0202/3001, B4201/5703, Cw0701/1701	35	9,333
586-AC	Day 10	IU	A0101/6802, B1510/8101, Cw08/1801	45	997
590-AC	Day 8	IU	A02/68, B1503/5802, Cw0202/0602	33	>750,000
600-AC	Day 7	IU	A26/3002, B0801/8101, Cw04/07	28	8, 490
637-AC	Day 14	IU	A29/6601, B1302/5802, Cw0602/0602	34	2,200
639-AC	Day 7	IU	ND	31	31,600
641-AC	Day 35	IP	A2301/3002, B0801/4501, Cw07/1601	43	>750, 000
675-AC	Day 5	IU	A30/6601, B3910/5802, Cw0602/12	49	2,040
698-AC	Day 6	IU	A0205/24, B0702/1401, Cw07/08	52	1,150
720-AC	Day 1	IU	A3001/30, B0702/18, Cw02/07	47	66,300
729-AC	Day 103	IP	A29/6601, B1510/5802, Cw03/06	15	8,490
732-AC	Day 6	IU	A3002/6602, B4201/45, Cw1601/1701	33	21,800
737-AC	Day 69	IP	A0205/3001, B1510/4201, Cw08/1701	8	4, 140, 000
766-AC	Day 1	IU	A03/3402, B5802/5802, Cw0602/0602	33	2,780,000

[a] Time point of the initial Elispot assay in the Acute Infant Cohort. [b] Transmission type: IU (intrauterine), the viral load was detectable at >400 RNA copies/ml on the first day of life ; IP (intrapartum), the viral load was undetectable (<400) on day 1 of life but was detectable on day 28 of life. [c] Viral load measurement at the time-point of the initial ELISPOT assay, several days following intake of single-dose nevirapine (viral loads at birth for 001-AC, 081-AC, 221-AC and 227-AC were 1,370, 22,400, 142,000 and 7,940 RNA copies/ml, respectively). The initial viral load test for IU infants was done on a median of 1 day of life (range, day 0 to 7), and IP- infants were tested on a median 28 of life (range, 28 to 36). None of the infants were on treatment at the time of the first ELISPOT assay. [d] ND-not determined.

Table 2.2. Characteristics of the cohort of chronically infected children at the time of the first ELISPOT assay at baseline.

Subject	Age (yrs)	HLA Class I Type	CD4 (%)	Viral Load RNA Copies/ml
001-CC	5	A02/74, B1510/44, Cw0202/03	26	92,000
002-CC	3	A29/74, B15/35, Cw04/04	23	14,000
003-CC	2	A0301/2301, B1503/5802,Cw0202/0602	27	670,000
004-CC	5	A24/68, B0702/1510, 0304/08	24	58000
005-CC	2	A23/29, B08/44, Cw03/1403	23	35000
006-CC	4	A02/29, B44/44, Cw07/07	8	320000
007-CC	6	A23/29, B14/4201,Cw0802/1701	15	39000
009-CC	2	A4301/6802, B15/15, Cw03/1801	20	210000
010-CC	7	A29/3001, B4201/57, Cw07/1701	18	14000
011-CC	6	A0301/3001, B1503/5802, Cw0202/0602	22	4200
012-CC	3	A3001/6601, B4202/5802, Cw0602/1701	18	1000000
013-CC	11	A02/29, B1302/1401, Cw0602/08	10	83000
014-CC	6	A03/03, B4501/5802, Cw0602/06	12	12000
015-CC	8	A2301/4301, B1503/5802, Cw0202/0602	16	84000
016-CC	6	A01/3402, B1503/8101,Cw0202/1801	18	49000
017-CC	2	A23/3203, B1503/1503, Cw0202/0202	17	70000
018-CC	2	A02/2301, B1510/5801, Cw07/1601	24	170000
019-CC	4	A23/66, B1510/5802,Cw0602/1601	29	220000
020-CC	5	A0202/4301, B1503/5703, Cw07/18	13	120000
021-CC	3	A2301/6802, B1510/44, Cw03/03	12	150000
022-CC	7	A6802/7408, B14/41, Cw0802/1701	20	50000
023-CC	7	A3001/3201, B5703/5802, Cw1801/0602	17	270000
024-CC	5	A02/2301, B0801/1503, Cw02/04	26	230000
025-CC	5	A3402/6802, B1503/44, Cw04/04	20	6600
026-CC	5	A02/33, B1516/4201, Cw1601/1701	13	21000
027-CC	11	A2301/3201, B1503/1503, Cw0202/03	22	71900
028-CC	12	A23/6802, B0801/4201, Cw03/1701	17	4300
029-CC	8	A23/23, B0801/1503, Cw0202/03	12	24000
030-CC	5	A03/23, B0801/41, Cw07/1701	18	110000
064-CC	8	A29/6802, B1510/4403, Cw0701/0304	4	1670000
065-CC	8	A2301/74, B1503/5301, Cw0202/0401	8	1300000
082-CC	10	A29/66, B58/5802, Cw0602/06	5	454000
091-CC	4	A2301/4301, B1510/1503, Cw16/18	16	4060000
094-CC	9	A2301/24, B07/07, Cw07/07	17	100000
098-CC	8	A2301/3402, B0801/44, Cw0202/0701	23	123000
110-CC	4	A29/6801, B0702/5802, Cw0602/07	14	123000
118-CC	5	A02/33, B4501/53, Cw04/1601	15	647000
167-CC	7	A2301/3402, B4201/44, Cw04/1701	25	34400
184-CC	12	A0205/74, B5801/35, Cw04/07	6	233000
185-CC	9	A29/74, B1503/1510, Cw0202/16	15	100000
205-CC	6	A0301/29, B0801/44, Cw07/07	28	355000
261-CC	7	A24/3402, B0801/44, Cw04/07	25	4820
299-CC	10	A3002/4301, B08/1503, Cw0202/07	10	141000
314-CC	7	A0205/3001, B1510/4201, Cw08/1701	12	280000
419-CC	11	A2301/4301, B14/5802, Cw0602/0802	23	133000

There was a greater number of responders to Gag, Pol, Vif, Nef and Vpr (p=0.0011, 0.0001, 0.0051, 0.0008, 0.0051 respectively, Fisher's exact test) among chronically infected children compared to acutely infected infants. Further analysis confirmed a larger magnitude of responses to Gag, Pol, and Nef (p=0.0012, 0.0002, 0.0410 respectively, Mann-Whitney test) in chronically infected compared to acutely infected children. There were no significant differences between acute and chronically infected children in responses to Vpu, Tat, Env and Rev.

We next compared the proportional contribution of each protein to the overall total response per child in both acute and chronic paediatric infection (Fig 2.3A). The overall contribution of the Env and Rev response was greater in acute infection compared to that in chronically infected children ($p<0.0001$ and $p=0.0074$) and in chronically infected children the contribution of the Nef-specific response to the total response was greater than in acute infection (p=0.0027, Mann-Whitney test, Fig 2.3B). Of particular significance is the predominant targeting of Env in acute infection: in one-third (12/36) of infants Env was the dominant target, compared to 4% (2/45) of the chronically infected children (p=0.00083, Fisher's Exact test, Fig 2.3A).

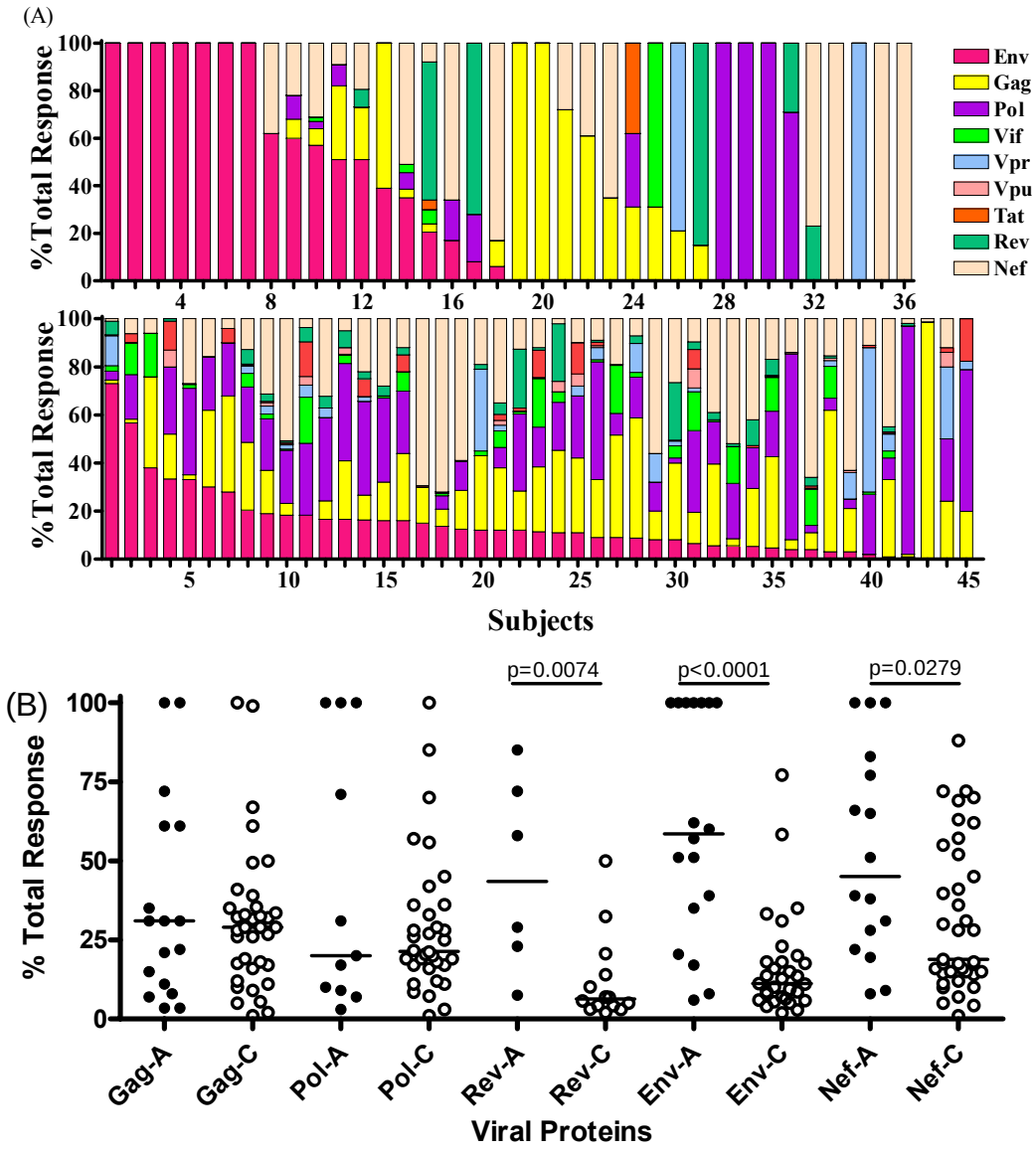

Figure 2.3.A. Relative contribution of protein response to overall response in acute and chronic infection. Reanalysis of the same data shown in Fig 2.2 is used to show the contribution of each protein to the total HIV-specific response in the acute infants (top panel) and chronic children (bottom panel) who had significant responses on the first ELISPOT assay. B. Comparison of the contribution of response to different proteins in acute (A) and chronic HIV-infected paediatric children (C) with detectable responses.

To determine whether the initial responses observed in children are maintained over time we tracked the responses longitudinally in four infected infants (Fig 2.4). These were the only four of 20 followed longitudinally from birth that did not meet the clinical and immunological WHO criteria to receive HAART within the first 12 months of age. Two of the infants, 349-AC and 447-AC, illustrate the initially high contribution to the total HIV-specific response made by Env-specific CD8+ T cells in early paediatric infection. 447-AC required HAART after 14 months of life. 349-AC initially had dominant Env responses which decreased over the first 12 months and were later replaced by Gag and Pol-specific responses, development of the latter coinciding with a significant decrease in viral load (186,000 RNA copies/ml at 14 months vs 2,280 RNA copies/ml at 24 months) and an increase in CD4 counts from 30% at 14 months to 41% at 24 months without HAART (Fig 2.4A and B).

133-AC who achieved successful control of viremia without the need for antiretroviral therapy (to 494 copies/ml and a CD4% of 29 at 31 months) showed a strongly dominant CD8+ Gag-specific response that persisted. Similarly, 517-AC initially had dominant Rev-specific responses that were soon replaced by dominant Gag-specific responses (Fig 2.4C and D). Although viremia has remained high in this child (>$1x10^6$ copies/ml), CD4% has also remained at high levels of >30%. However, these 4 anecdotal cases are insufficient to allow an analysis of specificity of the CD8+ response in infants and subsequent immunological and virological control of HIV infection.

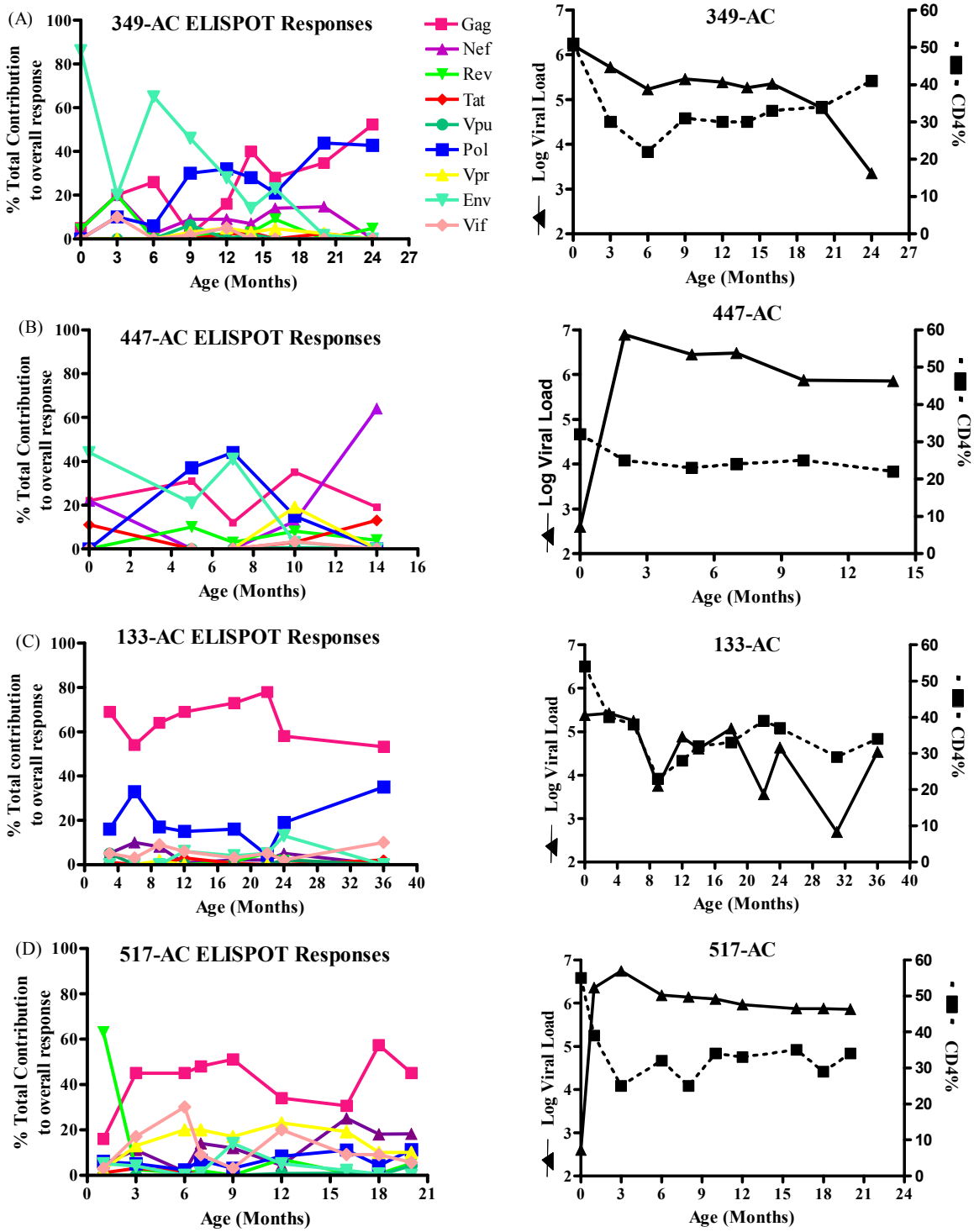

Figure 2.4. Longitudinal measurement of CD8+ T cell responses, CD4 and Viral load in therapy naïve subjects A-349 (A) and A-447 (B), A-133 (C) and A-517 (D). The CD4 counts and viral load measurements correspond to the time-points at which CD8+ T-cell responses were determined.

2.3.4. Weak $CD4^+$ Gag-specific T cell activity in early paediatric infection

Since CD8+ T cells require functional CD4+ T cell help to sustain their effector activity (Day and Walker, 2003) we examined Gag-specific CD8+ and CD4+ T cell activity in infants with early infection. We focused on Gag-specific responses since Gag is the dominant target for HIV-specific T-helper activity (Ramduth et al., 2005) and limitations on cell numbers precluded analysis of CD4+ T cell responses to non-Gag proteins. In addition, Gag-specific CD4+ T cell responses have been detected in acute HIV infection of adults (Zaunders et al., 2005). Gag-specific responses were measured at 2, 4 and 6 months of age only, as 50 and 75% of the infants required HAART by 6 and 12 months of age, respectively. The infants with acute infection had detectable CD8+ T cell responses at all 3 time-points (median Gag-specific CD8+ T cell responses were 0.095, 0.155, and 0.14% at 2, 4 and 6 months, respectively). These responses were lower but did not differ significantly compared to those measured in chronically infected children (median 0.31% Fig 2.5A). In contrast, infants with acute infection had significantly lower CD4+ T cell responses at all time points than the chronically infected children, median Gag-specific CD4+ T cell responses being 0.01, 0.01 and 0.02 CD4% at 2, 4 and 6 months, respectively, compared to 0.06% for chronically infected children ($p<0.0001$, <0.0001, <0.0008 respectively; Fig 2.5B).

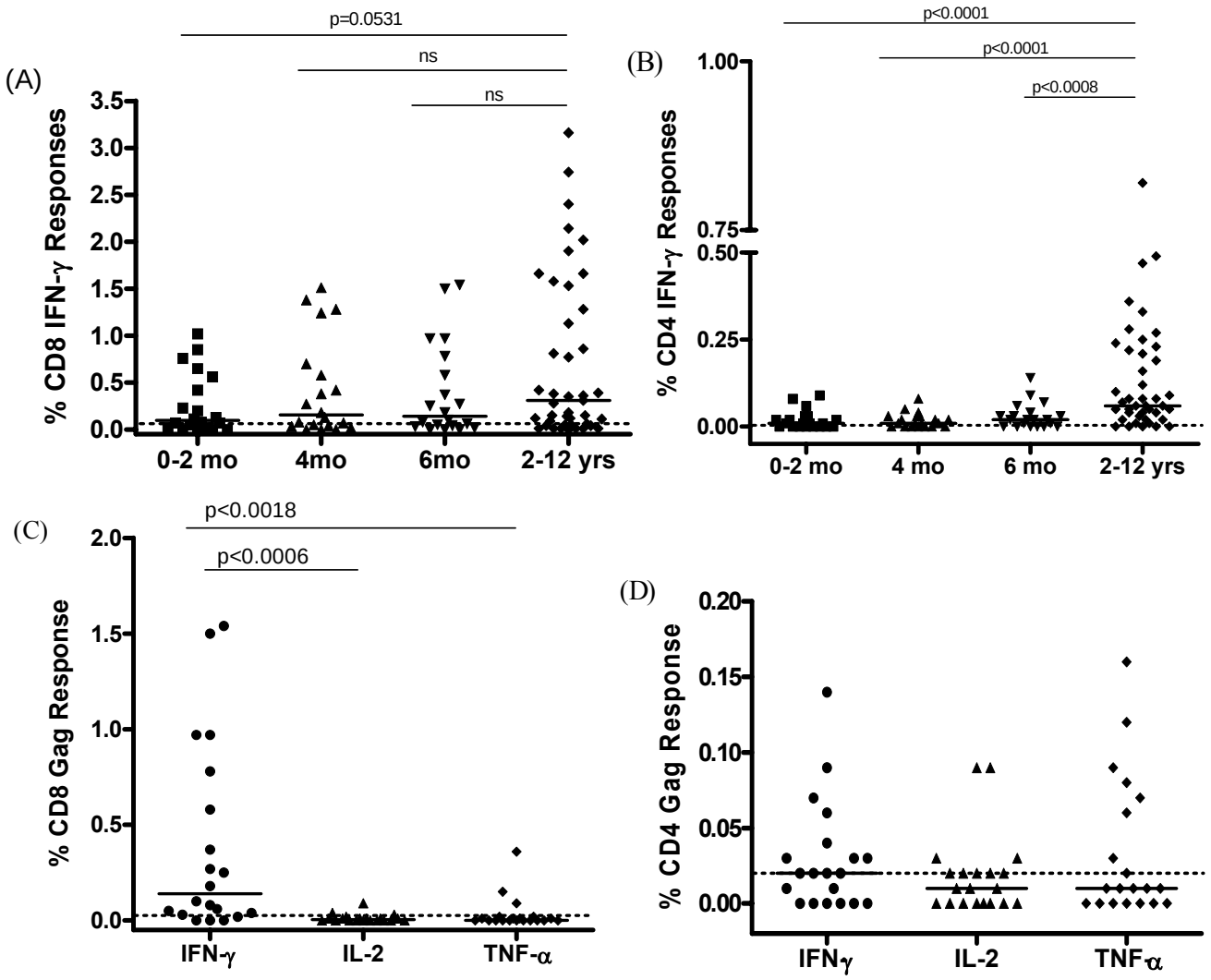

Figure 2.5. A, B. Gag-specific CD8+ and CD4+ T cell responses in acute and chronic children by intracellular cytokine staining. C, D. Gag specific CD8+ and CD4+ IFN-γ, IL-2 and TNF-α at 6 months of life in acute infected infants. All infants studied were antiretroviral therapy naïve at time of analysis. Dashed lines indicate the upper limit of responses detected from the same assays undertaken in 23 HIV-uninfected control infants (see Methods section 2.2.7). Horizontal bars indicate median responses in HIV-infected study subjects.

We also compared Gag-specific CD8+ (Fig. 2.5C) and CD4+ (Fig. 2.5D) IFN-γ, IL-2 and TNF-α production in the same of group of infants at 6 months of life. IL-2 and TNF-α responses were significantly less frequently detected than IFN-γ. In contrast, adults can generate substantial HIV-specific CD4+ IFN-γ and IL-2 responses during acute HIV infection (Zaunders et al., 2005), while chronic children on therapy show an increase in the frequency of IL-2 secreting CD4+ T cells (Correa et al., 2007).

2.4. DISCUSSION

These studies describe the early HIV-specific T-cell responses in paediatric infection. CD8+ T-cell responses are detectable from the first days of life in the majority (70%) of in utero infected infants. Responses were broadly directed, although Env specific CD8+ T-cell activity in particular contributed significantly more to the total HIV-specific response in acute infected infants compared to chronically infected children ($p<0.0001$). Gag-specific CD4+ T-cell responses were weak or undetectable in the first 6 months of life, contrasting with chronic paediatric or acute adult infection.

Previous studies using non-comprehensive screening assays and in very limited numbers of subjects have demonstrated weak HIV-specific CD8+ T cell responses in infancy (Luzuriaga et al., 1995, Luzuriaga et al., 1991). HIV-specific CTL have been detected in cord blood at birth previously (Luzuriaga et al., 1995) and HCMV-specific CTL in cord blood in utero (Marchant et al., 2003), indicating that immune responses to HIV can be detected very early in life. The studies described here demonstrate not only

that these early responses can arise, but that they are detectable in a high proportion (85%) of infants at the earliest time-point they were tested.

Studies evaluating the specificity of the initial CD8+ T-cell response in infants infected with HIV have not been systematically undertaken, and the finding that Env-specific CD8+ T-cell activity contributes substantially to this initial response is of interest in relation to recent studies in adults indicating that Env-specific CD8+ T cell responses are associated with higher viral loads, and Gag-specific CD8+ T cell responses with lower viral loads (Borrow et al., 1994, Mphatswe et al., 2007, Riviere and Buseyne, 1998). More studies will be needed to determine if this is related to the lack of a rapid reduction in viral load observed in early paediatric infection compared to early adult infection where Env makes a smaller contribution to the initial CD8+ T-cell responses (Lichterfeld et al., 2004b).

It was not possible here to relate the specificity of the early CD8+ T cell response and progression in this cohort as two-thirds of the study subjects were enrolled onto a study of early HAART in paediatric HIV infection. Of the 20 infants who did not receive early HAART, only 2 achieved viral loads of <100,000 in the first year. The finding of early CD8+ T cell responses combined with persistently high viral loads in the first year of life suggests that these CD8+ T cells are ineffective and additional functional studies will be needed to determine how this apparent dysfunction compares to that seen in adults (Borrow et al., 1994, Fauci et al., 1996). Comprehensive phenotypic analysis of CD8+ T cells in adults have demonstrated the association of polyfunctional

CD8+ T cells with control of viremia (Betts et al., 2006). These comprehensive analyses were not undertaken in these study infants, but the lack of IL-2 and TNF-α responses suggest that the majority of the CD8+ T cell responses detectable via the IFN-γ elispot assay may be monofunctional.

Limitations imposed by cell numbers allowed only Gag-specific CD4+ T-cell responses to be assessed. Gag was chosen since it is consistently the dominant target for HIV-specific CD4+ T-cell responses (Ramduth et al., 2005). The weak or undetectable Gag-specific CD4+ T cell activity in acutely infected infants seen in this study also contrasts with acute adult infection, where adults present with high levels of CD4+ T cell responses during acute infection (Zaunders et al., 2005). In addition, Gag-specific CD4+ T cell activity has been reported in chronic infection in children >5 years old, who have spontaneously controlled viraemia without antiretroviral therapy (Feeney et al., 2003). The marked absence of HIV-specific CD4+ T cell activity even to 6 months of age suggests a fundamental reason why CD8+ T cells in infected infants are ineffective (Gandhi and Walker, 2002, Lichterfeld et al., 2004a). These findings of a lack of HIV-specific CD4+ T cell activity are consistent with other studies (Luzuriaga et al., 1995), in one case showing minimal CD4+ T cell responses in HIV-infected children until 3-5 years of age (Sandberg et al., 2003). Furthermore, where detectable, HIV-specific CD4+ T cell responses have been reported as type 2 as opposed to type 1 (Wasik et al., 1997, Wasik et al., 2000) and therefore less likely to support the induction and maintenance of HIV-specific CTL activity. Of concern, and again in contrast to what is observed in acute adult infection (Rosenberg et al., 2000), early

treatment with antiretroviral therapy in infected infants did not result in increased HIV-specific CD4+ T cell responses (Ramduth et al., 2008). However, the extent to which these findings result from or cause the persistent high levels of viremia observed in early paediatric HIV infection is not known.

The encouraging aspect of these data is that in-utero infected infants mount CD8+ T cell responses from the first day of life, while those infected intrapartum have detectable responses a month after infection. Although 85% of infected infants (in this cohort) met current WHO criteria to initiate HAART within 12 months of infection, it is also clear that a small minority (2/20) of infected infants showed viral loads of <10,000 and CD4% of >30% by 24 months of age. Thus, spontaneous-control of HIV is possible in paediatric HIV infection. Identification of greater numbers of 'relative-controller' children will facilitate further definition of what constitutes an effective immune response in early paediatric HIV infection. Moreover, the fact that the neonatal immune system can generate adaptive immune responses to HIV provides important information for the development of vaccines to prevent peripartum infection.

CHAPTER 3 : IMPACT OF HLA IN MOTHER AND CHILD ON PAEDIATRIC HIV-1 DISEASE PROGRESSION

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See Appendices section)

3.1. INTRODUCTION

HIV-specific CD8+ T cells play a central role in controlling viral replication (Goulder and Watkins, 2008). It is the specificity of the CD8+ T cell response, particularly to Gag, that is associated with low viral loads in HIV infection (Geldmacher et al., 2007, Zuniga et al., 2006, Kiepiela et al., 2007). Although immune control is undermined by the selection of viral mutations that prevent recognition by the CD8+ T cells, evasion of Gag-specific responses mediated by protective class I HLA-B alleles typically brings a reduction in viral replicative capacity, facilitating subsequent immune control of HIV (Leslie et al., 2004, Martinez-Picado et al., 2006, Brockman et al., 2007). The same principle has been demonstrated in studies of SIV infection (Kobayashi et al., 2005, Matano et al., 2004).

Recent studies show that the class I HLA-B alleles that protect against disease progression present more Gag-specific CD8+ T-cell epitopes, and drive the selection of more Gag-specific escape mutations than those alleles that are associated with high viral loads (Matthews et al., 2008). These protective HLA-B alleles are not only beneficial to infected individuals expressing those alleles, but also benefit a recipient following transmission, since the transmitted virus carrying multiple Gag escape mutations may have substantially reduced fitness (Crawford et al., 2007, Chopera et al., 2008, Goepfert et al., 2008). However, there is no benefit to the recipient if he/she shares the same protective allele as the donor, because the transmitted virus carries escape mutations in the Gag epitopes that would otherwise be expected to mediate

successful immune control in the recipient (Goepfert et al., 2008, Goulder et al., 2001c).

The sharing of HLA alleles between donor and recipient occurs frequently in mother-to-child transmission (MTCT). The risk of MTCT is related to viral load in the mother, and high viral load is associated with non-protective alleles, such as HLA-B*18 and B*5802. This may contribute in two distinct ways to the more rapid progression observed in paediatric HIV infection (Prendergast et al., 2008, Prendergast et al., 2007, Mphatswe et al., 2007). First, because infected children share 50% or more HLA alleles with the transmitting mother, they are less likely to carry protective HLA alleles than adults (Kiepiela et al., 2004). Thus, infected children as a group carry fewer protective HLA alleles, and a greater number of non-protective HLA alleles. Second, even when the child has a protective allele, such as HLA-B*27, this allele does not offer protection if the maternally transmitted virus carries escape mutations within the key Gag epitopes that are presented by the protective allele (Goulder et al., 2001c, Kuhn et al., 2004).

However, it is clear that infected children who possess protective alleles, such as HLA-B*27, or HLA-B*57, can achieve durable immune control of HIV infection if the virus transmitted from the mother is not pre-adapted to those alleles (Goulder et al., 2001b, Feeney et al., 2004). HIV-specific CD8+ T-cell responses are detectable from birth in infected infants (Thobakgale et al., 2007). Furthermore, as in adult infection (Chopera et al., 2008, Goepfert et al., 2008), HIV-infected children have the potential to benefit from transmission of low fitness viruses in the situation where the mother possesses

protective HLA alleles, and the child does not share those protective alleles. Mother-to-child transmission of low fitness viruses encoding CD8+ T cell escape mutations has recently been documented (Prado et al., 2010, Sanchez-Merino et al., 2008).

In this study, undertaken in Durban, South Africa, we set out to test the hypothesis that HIV-infected children are less likely to progress rapidly to disease if either the infected child or the transmitting mother possesses a protective HLA allele that is not shared. The HLA alleles most strongly associated with low viral loads and high CD4 counts in a cohort of >1200 HIV-infected adults in Durban are HLA-B*57 (B*5702 and B*5703), HLA-B*5801 and HLA-B*8101 (Kiepiela et al., 2004, Leslie et al., 2010). These four alleles all present Gag-specific CD8+ T-cell epitopes, and in each case the escape mutations selected in these epitopes reduce viral replicative capacity (Brockman et al., 2007, Chopera et al., 2008, Crawford et al., 2007, Martinez-Picado et al., 2006, Goepfert et al., 2008, Matthews et al., 2008).

Analyzing a previously described cohort of 61 HIV-infected children in Durban (Mphatswe et al., 2007, Prendergast et al., 2008, Thobakgale et al., 2007), who were all followed from birth, we here address, first, the question of whether possession of any of these four alleles in either mother or child is associated with slower disease progression in the child; and, second, whether sharing of protective alleles by mother and child affects the ability of the child to make the Gag-specific CD8+ T cell responses restricted by the shared allele.

3.2. MATERIALS AND METHODS

3.2.1. Study Subjects

The paediatric study cohort has been previously described (Thobakgale et al., 2007, Prendergast et al., 2008). In brief, we enrolled 63 HIV-infected infants born to HIV-infected mothers at St Mary's and Prince Mshiyeni Hospitals in Durban, South Africa between 2003 and 2005. The HIV-1 seropositive mothers were recruited in the last trimester of pregnancy and received single dose nevirapine at the onset of labour and within 48 hours of birth to the child according to the HIVNET 012 protocol (Jackson et al., 2003, Guay et al., 1999). A total of 719 mothers were enrolled and screened and 740 infants were born to the mothers, 75 were infected. Sixty-three of the 75 infants met the criteria for enrolment into the study. Exclusion criteria comprised prematurity, intrauterine growth restriction and congenital anomaly.

Forty-three infants received short-term antiretroviral therapy (ART) immediately following diagnosis as part of a separate study investigating the effect of early, short-term ART on disease outcome in paediatric infection. In this group, ART was discontinued after 12 months of continuous therapy or 18 months of interrupted therapy. The remaining 20 infants did not receive ART immediately but were followed up longitudinally. All infants enrolled onto the study received ART once World Health Organization (WHO) clinical or immunological criteria were met (http://www.who.int/hiv/pub/guidelines/WHOpaediatric.pdf). Taking into account 85% of HIV-infected children progress to CD4<20% by 1 year (Mphatswe et al., 2007), we here define slow-progression as failure to progress to meet WHO criteria to start ART

within 12 months in the absence of ART; progressors were those children who required ART within 12 months of life or within 12 months of discontinuing ART.

HLA typing was obtained in 61 of the 63 enrolled mother-child pairs, two mother-child pairs withdrawing from the study shortly after enrolment. In addition HLA typing was undertaken antenatally on 236 HIV-infected mothers who did not subsequently transmit.

The mothers gave written informed consent for participation of their children into the study. The study was approved by all the participating Institutional Review Boards.

3.2.2. HLA Typing

DNA for HLA typing was extracted using Puregene DNA isolation kit for blood (Gentra Systems, Minneapolis, Minn.) according to the manufacture's instructions. HLA Class I typing was done by DNA PCR using sequence-specific primers as previously described (Kiepiela et al., 2004).

3.2.3. Synthetic HIV-1 Peptides

A panel of 410 overlapping peptides (18mers with a 10-amino acid overlap) spanning the entire HIV-1 clade C consensus sequence were synthesized on an automated peptide synthesizer (MBS 396, Advanced ChemTech) and used in a matrix system in

screening assays (Kiepiela et al., 2004). Previously defined optimal peptides were also similarly synthesized.

3.2.3. ELISPOT Assays

Ex vivo measurement of T-cells for interferon gamma production was undertaken as previously reported (Kiepiela et al., 2004, Thobakgale et al., 2007). As previously described, specific responses of >100 SFC/million PBMCs were defined as significant. In the analyses shown in Fig 3.2C, responses less than this level were treated as zero or non-responsive. In addition, defined optimal peptides were also tested according to the corresponding HLA type of the subject.

3.2.4. Viral Load and CD4 Measurement

Plasma viral loads were measured using the Roche Amplicor Monitor Assay (version 1.5) and CD4 counts were determined from fresh whole blood using Tru-Count technology as previously described (Thobakgale et al., 2007).

3.2.5. Viral Sequencing

Sequencing of HIV proviral DNA from mothers and children was undertaken as previously described (Leslie et al., 2004). Genomic DNA was extracted from peripheral blood mononuclear cell pellets; gag sequences were amplified by nested PCR, purified and directly sequenced.

3.2.6. Statistical Analysis

Fisher's Exact test was used to compare proportions defining progressor and slow progressor children, proportions of progressor and slow progressor children with protective and unshared HLA-B alleles. The same test was also used to determine the proportions of progressors and slow progressors with; ELISPOT responses to Gag epitopes, escape variants in epitopes presented by protective HLA-B alleles and proportions of escape variants in shared and unshared HLA-B alleles. The Odds ratio test was used to compare the number of sequence variations in children who made or did not make responses to the epitopes. The Mann-Whitney test was used to compare differences in the magnitude of Gag response in slow progressor and progressor children without protective HLA-B alleles.

3.3. RESULTS

3.3.1. Non-progression in infants is associated with unshared protective HLA B-alleles in the mother or child

Progression to disease is rapid in HIV-infected children, 85% of the children who did not receive ART immediately following diagnosis met the then WHO criteria to start ART (CD4 <20%) by 1 year (Mphatswe et al., 2007). For the purposes of this study, we defined slow-progression in these study subjects as failure to meet WHO treatment criteria within 12 months of birth, or within 12 months of cessation of ART in those who were treated with immediate, short-term ART after birth.

Of the 61 studied mother-child pairs, one or more of the protective HLA alleles B*5702, B*5703, B*5801 or B*8101, was present in either the mother or child in 16 pairs (Table 3.1). Ten of 61 transmitting mothers (16%) possessed one of the four protective HLA-B alleles, compared to 68 of 236 (29%) HLA-typed non-transmitting mothers (p=0.051, Fisher's Exact test, Fig 3.1A). Slow-progression, as defined above, was observed in 17 children (Table 3.1).

Table 3.1. Progressor and slow progressor mother-child pairs where child and/or mother express one or more of the protective HLA B-alleles (B*5801/B*8101/B*5701/B*5703).

SLOW-PROGRESSOR CHILDREN (protective allele in 8 of 17 pairs)

	CHILD	MOTHER
Protective allele in child only	133-C B*5802/**8101**	B*4201/5802
	559-C B*0801/**5801**	B*0801/4101
	586-C B*1510/**8101**	B*1510/5802
Protective allele in mother and child	001-C B*4201/**8101**	B*4403/**8101**
	468-C B***5703/5801**	B*4403/**5801**
Protective allele in mother only	114-C B*0702/0801	B*0801/**5702**
	349-C B*4501/5802	B*4501/**5702**
	568-C B*0702/1801	B*0702/**8101**

PROGRESSOR CHILDREN (protective allele in 8 of 44 pairs)

	CHILD	MOTHER
Protective allele in child only	433-C B*1503/**5801**	B*1503/1516
	562-C B*1402/**5801**	B*1402/1510
	600-C B*0801/**8101**	B*0801/5802
Protective allele in mother and child	135-C B*0801/**8101**	B*0702/**8101**
	241-C B*4403/**8101**	B*4701/**8101**
	413-C B*0701/**8101**	B*4403/**8101**
	579-C B*4201/**5703**	B*1510/**5703**
Protective allele in mother only	284-C B*0801/1501	B*0801/**5801**

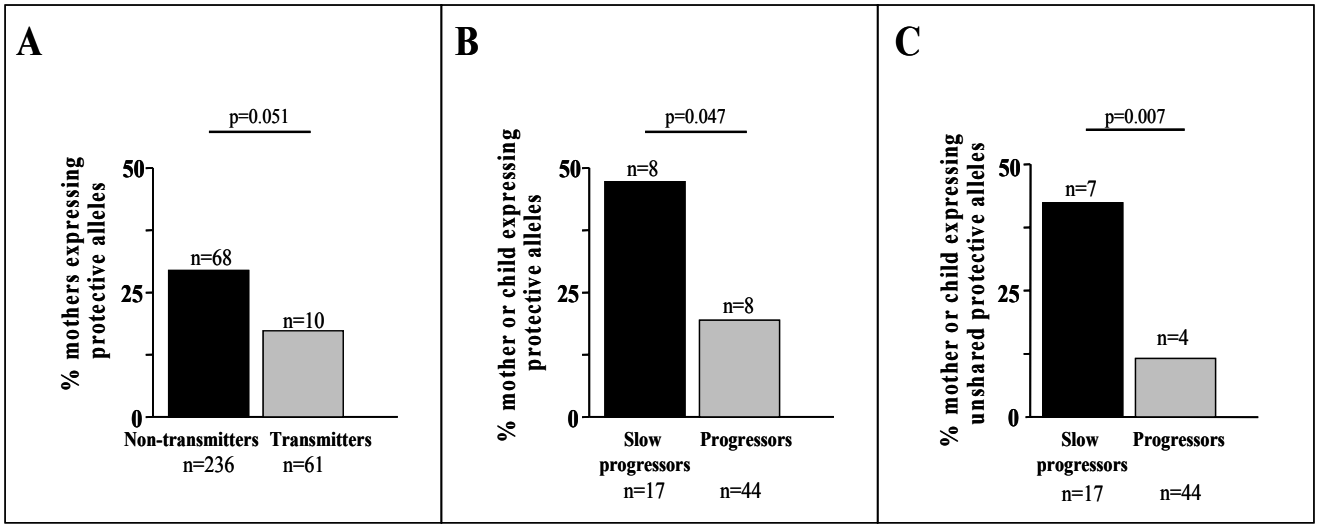


Figure 3.1. Relative contribution of protective HLA-B alleles in mothers and children. Comparison of protective alleles expressed in (A) transmitting versus non-transmitting mothers and (B) progressor versus slow-progressor children. (C) Comparison of protective HLA-B alleles unshared between mother and child.

In this slow-progressor group, a protective allele in either the mother or child was present in 8 of 17 pairs (47%), compared to 8 of the 44 progressor pairs (18%; p=0.047, Fisher's Exact test, Fig 3.1B). Furthermore, a significantly higher proportion of slow-progressor children had one of the protective HLA-B-alleles that were not shared by mother and child (7/17; 41%). In one pair, although the mother and child shared the protective B*5801 allele, the child (468-C) also carried B*5703 that was not shared with the mother. In contrast, in only four of the 44 (9%) progressor mother-child pairs was one of the protective HLA B-alleles unshared by mother and child (p=0.007, Fisher's Exact test, Fig 3.1C). These data therefore support the hypothesis that slow-progression of HIV in this cohort of children is influenced by protective HLA-B-alleles in either the mother or child, in particular where the protective alleles are not shared by mother and child.

3.3.2. HIV-specific CD8+ T cell responses in the slow-progressor infants tends to target Gag epitopes presented by protective alleles B*57/B*5801/B*8101

We next addressed the question of whether the slow-progressor infants possessing one of the 4 protective HLA-B alleles tended to generate detectable CD8+ T-cell responses to the Gag epitopes presented by these alleles. In the slow-progressor children expressing one of the protective HLA-B-alleles, 5/5 made IFN-γ ELISPOT responses to one or more of the Gag epitopes presented by these alleles compared to 3/7 progressor children (p=0.08, Fisher's Exact test; Fig 3.2A,B). Overall, the slow-progressor children targeted 6/8 Gag epitopes presented by these alleles, compared to the progressor children in this category who targeted 3/11 Gag epitopes presented by these HLA-B alleles (p=0.07, Fisher's Exact test; Fig 3.2A,B). We additionally analyzed Gag-specific CD8+ T-cell responses in slow progressor and progressor children without protective HLA-B alleles to optimal epitopes presented by their respective HLA alleles (Fig 3.2C). In the slow progressor children without protective alleles, 8/9 made IFN-γ ELISPOT responses to one or more Gag epitopes, compared to 20/36 progressor children (p=0.02, Fisher's Exact test). In addition, the magnitude of the Gag-specific response in the slow progressor

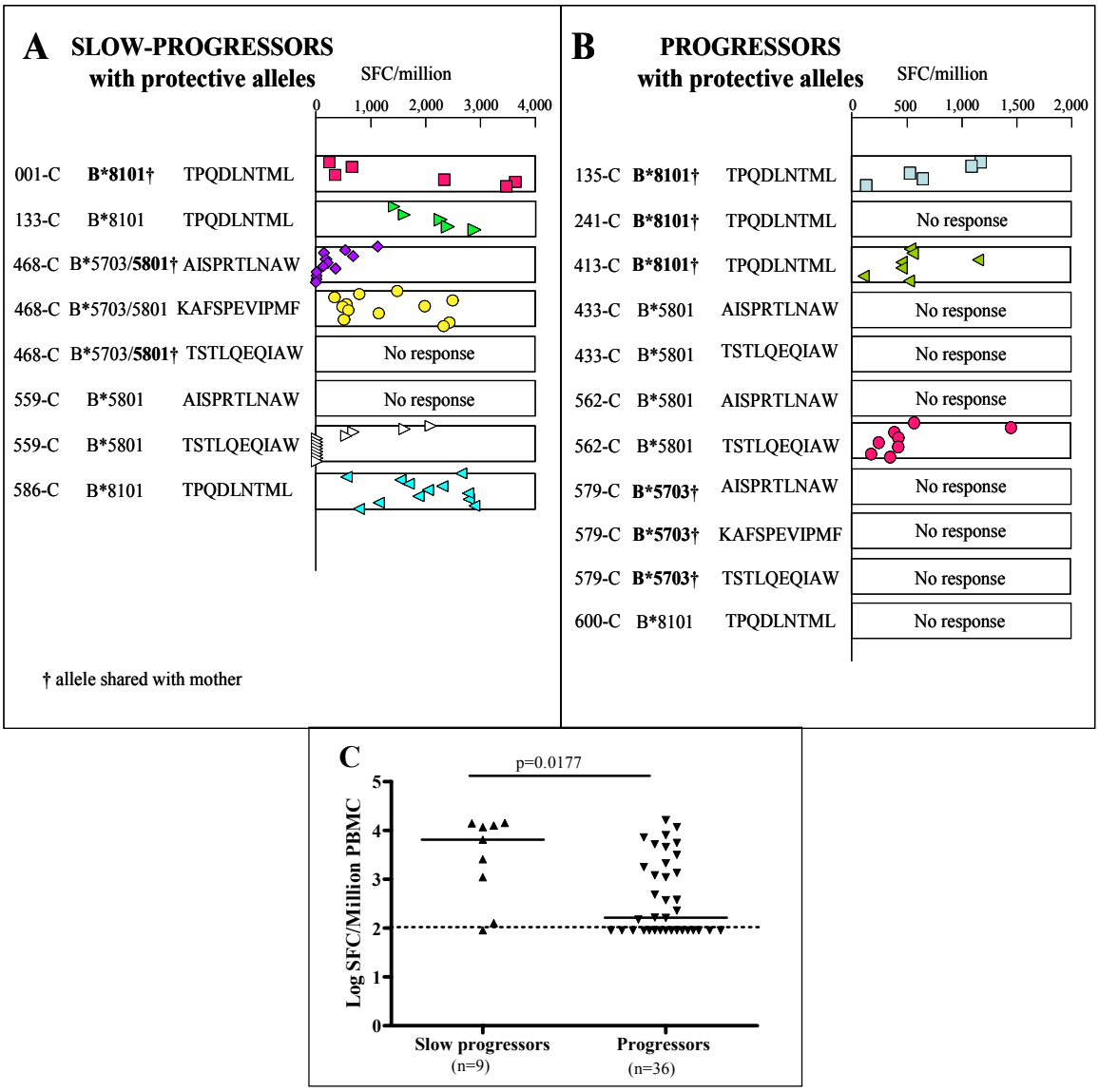


Figure 3.2. CD8+T cell responses to Gag epitopes in the first 12 months of life in children while not on HAART. Gag optimal epitopes presented by HLA-B*57/5801/8101 in (A) slow progressor and (B) progressor children with protective HLA-B alleles. (C) Gag optimal epitope responses in slow progressor and progressor children without protective HLA-B alleles. Symbols represent the magnitude of CD8+ T cell responses measured at different time-points by the ELISPOT assay in the first 12 months of life in children who deferred ART or in the first 12 months of cessation of ART in children who received immediate short-term ART. Magnitude of responses in panel C is shown by the median magnitude of the response for all time-points tested. A significant response was defined as a peptide-specific response of ≥100 SFC/million PBMCs (see Methods section 3.2.3).

children was higher (median, 6,539 SFC/million PBMC, range 0-14,350 SFC/million PBMC) than that of progressor children (164, range 0-16,428 SFC/million PBMC), ($p=0.0177$; Mann-Whitney test, Fig 3.2C). These data suggests that Gag-specific CD8+ T-cell responses tend to slow disease progression in HIV-infected infants, irrespective of the HLA clas I alleles presenting the Gag epitopes.

3.3.3. CD8+ T-cell responses and MTCT of escape variants

To investigate possible causes of the difference in responses to Gag epitopes presented by the protective HLA-B alleles that were observed in the progressor and slow-progressor groups, we next addressed the question of whether failure to generate detectable CD8+ T-cell responses to the protective epitopes was related to transmission of variants by the mother (Fig 3.3). Virus was sequenced from the mother and child at the time point closest to transmission that was available (Fig 3.4). Variants in child sequences were observed in 2/9 cases where there were responses to the epitopes detected, compared to 5/10 cases where there were no responses detected (odds ratio 2.25, p = ns; Fig 3.3A,B). Thus, whereas responses to epitopes are more likely to be observed if wild-type virus has been transmitted, in 50% of cases no response was detected even when wild-type was transmitted.

A SLOW-PROGRESSORS with protective alleles

			Child sequences		Maternal sequences	
001-C[a]	**B*8101†**	TPQDLNTML[c]	———————	447 days	———————	34d antenatal
133-C[a]	B*8101	TPQDLNTML[c]	———————	30 days	———————	17d antenatal
468-C[a]	B*5703/**5801†**	AISPRTLNAW[c]	———S——	1 day	———————	432d postnatal
468-C[a]	B*5703/5801	KAFSPEVIPMF[c]	———————	1 day	———————	432d postnatal
468-C[a]	B*5703/**5801†**	TSTLQEQIAW	—N——————	1 day	—N——————	432d postnatal
559-C[a]	B*5801	AISPRTLNAW	———————	42 days	———————	16d antenatal
559-C[a]	B*5801	TSTLQEQIAW[c]	———V——	42 days	———V——	16d antenatal
586-C[a]	B*8101	TPQDLNTML[c]	———————	13 days	———————	9d antenatal

B PROGRESSORS with protective alleles

			Child sequences		Maternal sequences	
135-C[a]	**B*8101†**	TPQDLNTML[c]	———————	74 days	———————	29d antenatal
241-C[b]	**B*8101†**	TPQDLNTML	—S—S——	33 days	—S—S——	36d antenatal
413-C[a]	**B*8101†**	TPQDLNTML[c]	—E————	93 days	—S—————	3d antenatal
433-C[a]	B*5801	AISPRTLNAW	———————	13 days	———————	55d antenatal
433-C[a]	B*5801	TSTLQEQIAW	———————	13 days	———————	55d antenatal
562-C[a]	B*5801	AISPRTLNAW	P———————	1 day	———————	30d antenatal
562-C[a]	B*5801	TSTLQEQIAW[c]	———————	1 day	———————	30d antenatal
579-C[a]	**B*5703†**	AISPRTLNAW	PL———————	44 days	PL———————	31d antenatal
579-C[a]	**B*5703†**	KAFSPEVIPMF	———————	44 days	———————	31d antenatal
579-C[a]	**B*5703†**	TSTLQEQIAW	—N——————	44 days	—N——————	31d antenatal
600-C[a]	B*8101	TPQDLNTML	———————	6 days	———————	7d postnatal

† allele shared with mother [a]intra-uterine infection [b]intra-partum infection [c]Detectable CD8+ T-cell response in child

Figure 3.3. Mother and child Gag epitope sequence variation in (A) slow progressor and (B) progressor children.

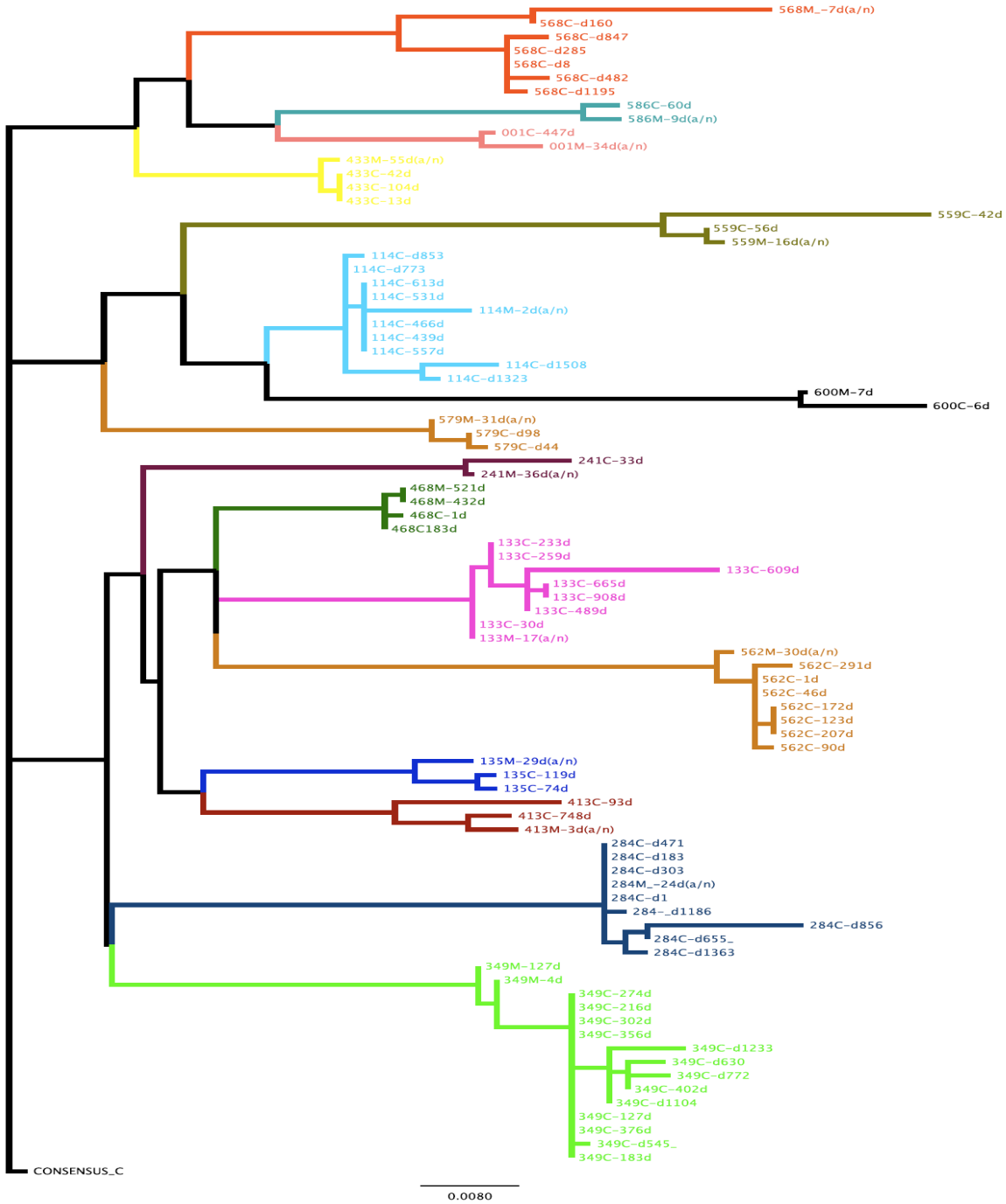


Figure 3.4 Phylogenetic tree of mother and child sequences. Gag p24 nucleotide sequences from M (mother) before (a/n-antenatal) or after delivery and C (child) at different days (d) after infection were edited and aligned with SeqAlv2.0. Maximum-likelihood trees were constructed with GARLI (Genetic Algorithm for Rapid Likelihood Inference) 0.96 program. Each colour represents a mother-child pair, and the scale represents substitutions per site.

3.3.4. Mother-child HLA sharing and MTCT of escape variants

To investigate further the impact of HLA sharing of protective alleles by the child and the mother, we determined the frequency of transmitted variants within the p24 Gag epitopes (ISW9, KF11, TL9 and TW10) presented by the protective alleles HLA-B*57/5801/8101. Sequences encoding the same epitopes, but in transmitting mothers not expressing the relevant alleles, served as controls. In sequences obtained from the mother as close to delivery as feasible (median 20 days prior to delivery; range 55 antenatally to 432 days postnatally) 12/16 (75%) epitopes presented by the B*57/5801/8101 alleles and expressed in the transmitting mother contained mutations, compared to 5/24 (21%) in epitopes presented by the B*57/5801/8101 alleles and not expressed in the transmitting mother (p=0.001, Fisher's Exact test, Fig 3.5A; Table 3.2). Very similar findings resulted from analysis of sequences obtained from the child as close to diagnosis as feasible (median 31 days of age; range 1-447 days of age), (p=0.0001, Fig 3.5B). Thus, sharing of maternal alleles by the child is likely to contribute to failure of children to make responses to epitopes presented by protective alleles, since in the majority of cases variants will be transmitted.

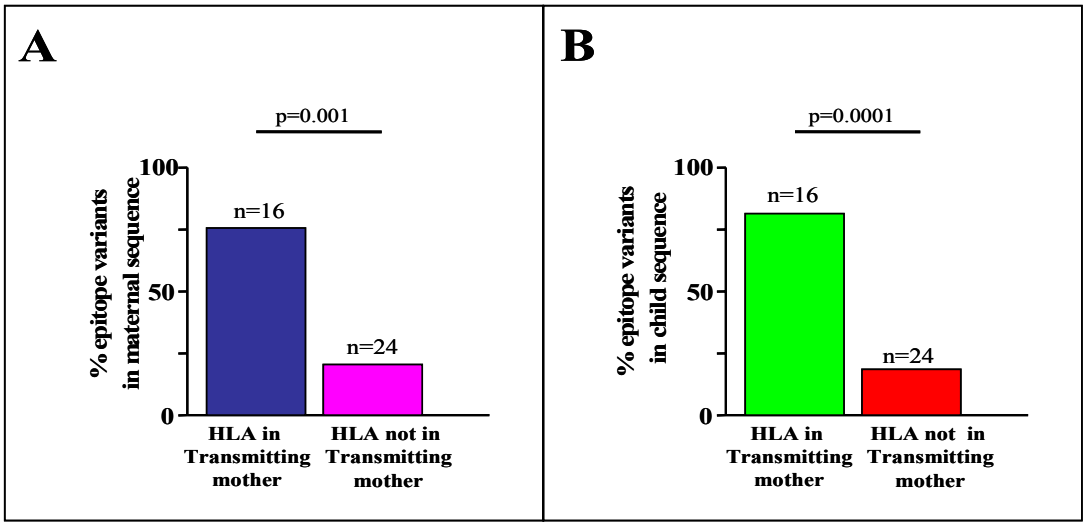


Figure 3.5. Gag epitope variants in mothers (A) and children (B) where the mother either expressed or did not express the protective HLA B-alleles.

Table 3.2. Deduced Gag epitope sequences in the 16 mother-child pairs where mother and/or child possess one or more of the protective HLA-B alleles.

	B*5702/5703/5801	B*5703	B*8101	B*5702/5703/5801		B*5702/5703/5801	B*5703	B*8101	B*5702/5703/5801
	AISPRTLNAW	KAFSPEVIPMF	TPQDLNTML	TSTLQEQIAW		AISPRTLNAW	KAFSPEVIPMF	TPQDLNTML	TSTLQEQIAW
Child	Gag 146-155	Gag 162-172	Gag 181-189	Gag 240-249	Mother	Gag 146-155	Gag 162-172	Gag 181-189	Gag 240-249
001-C	A146S	-	-	-	001-M	A146P	-	-	-
114-C	A146P/I147L	-	-	T242N	114-M	A146P/I147L	-	-	T242N/A248T
133-C	-	-	-	-	133-M	-	-	-	-
135-C	-	-	-	-	135-M	-	-	-	-
241-C	-	-	Q183S/T187S	-	241-M	-	-	Q183S/T187S	A248T
284-C	A146P	-	-	T242N	284-M	A146P	-	-	T242N
349-C	A146P/I147L/S148G	-	-	I247L/A248D	349-M	A146P/I147L/S148G	-	-	I247L/A248D
413-C	-	-	D184E	-	413-M	I147L	-	Q183S	-
433-C	-	-	-	-	433-M	-	-	-	-
468-C	N153S	-	-	T242N	468-M	-	-	-	T242N
559-C	-	-	-	I247V	559-M	-	-	T187M	I247V
562-C	A146P	-	-	-	562-M	-	-	-	-
568-C	A146S/I147M	-	L185M/T187S	-	568-M	A146S/I147M	A163G	Q183S/T187S	-
579-C	A146P/I147L	-	-	T242N	579-M	A146P/I147L	-	-	T242N
586-C	A146S	V168I	-	A248G	586-M	A146S	V168I	-	-
600-C	A146C/I147L	-	-	-	600-M	A146C/I147L	-	-	-

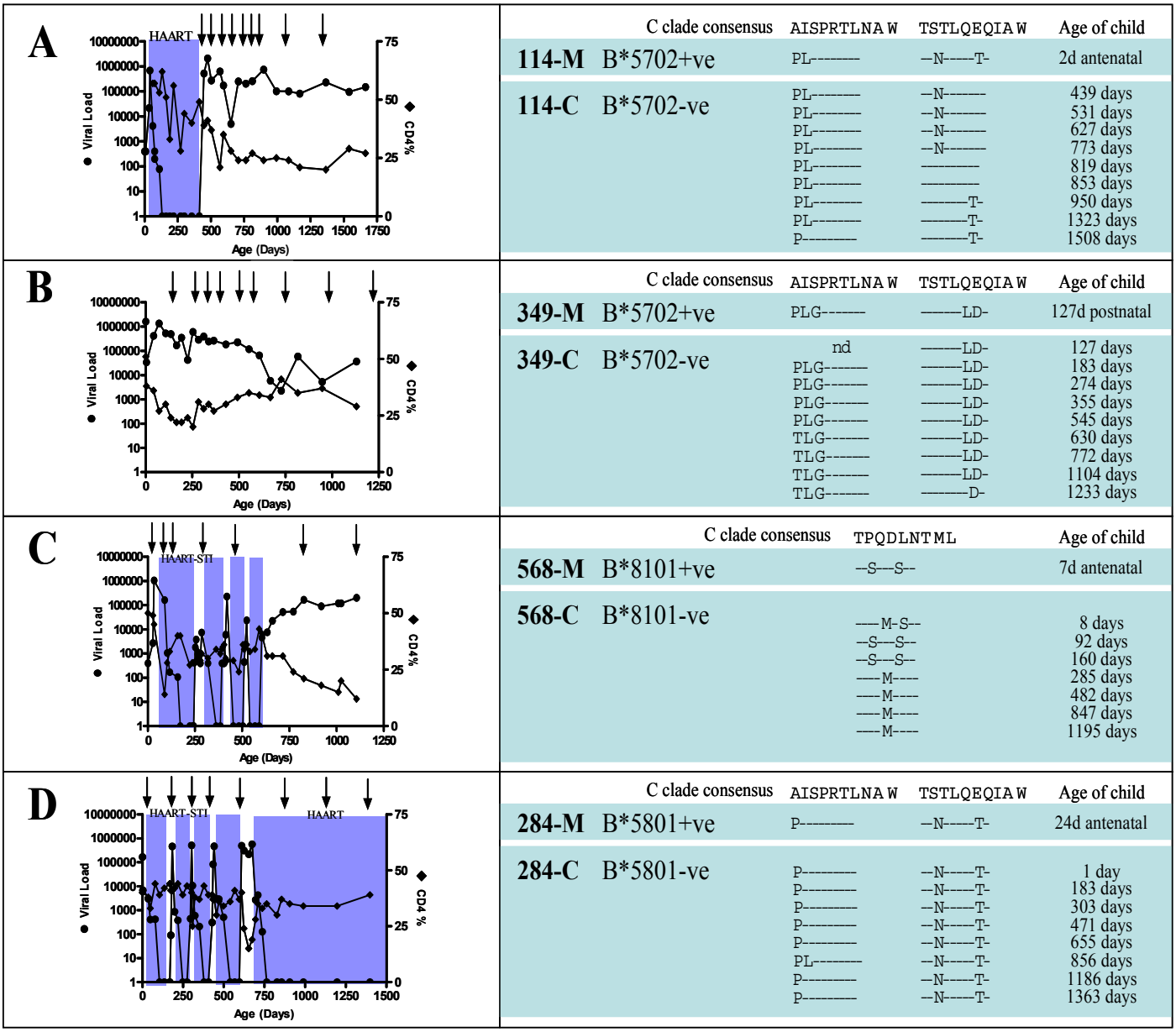
Mother or child possessing HLA-B allele presenting the epitope

Mother but not child possessing HLA-B allele presenting the epitope

3.3.5. Mothers carrying protective HLA alleles transmit Gag variants reducing viral fitness

Three of the 8 slow-progressor children and one of the eight progressor children had no protective HLA alleles themselves, but their mother possessed one. To address the question of whether the viruses transmitted by these mothers did in fact carry escape mutations in Gag that reduce viral fitness, virus from these mother-child pairs was sequenced from time-points as close as possible to transmission and tracked longitudinally to look for reversion of transmitted escape mutants in the HLA-mismatched child (Fig 3.6).

All the mothers transmitted escape variants to their children in each epitope presented by the protective maternal alleles. In all 3 cases where the child was defined as a slow-progressor, reverting escape mutations were observed in all the epitopes, indicating an in vivo fitness cost to T242N and I147L in B*5702-negative 114-C (Fig 3.6A), to A146P and I247L in B*5702-negative 349-C (Fig 3.6B), to Q182S and T186S in B*8101-negative 568-C (Fig 3.6C). In the B*5801-negative progressor, 284-C, even though low fitness mutants were transmitted, such as A146P and T242N, reversion did not occur before CD4 decline to the point where the criterion for starting ART was reached rapidly. Thus, from analysis of this small number of mother-child pairs, it is clear that some benefit to the child is likely to accrue from the transmission of Gag escape variants within epitopes presented in the mother by protective HLA alleles such as B*5702/5703/5801/8101.



		C clade consensus	AISPRTLNAW	TSTLQEQIAW	Age of child
114-M	B*5702+ve		PL--------	--N-----T-	2d antenatal
114-C	B*5702-ve		PL--------	--N-------	439 days
			PL--------	--N-------	531 days
			PL--------	--N-------	627 days
			PL--------	--N-------	773 days
			PL--------	----------	819 days
			PL--------	----------	853 days
			PL--------	-------T-	950 days
			PL--------	-------T-	1323 days
			P---------	-------T-	1508 days
		C clade consensus	AISPRTLNAW	TSTLQEQIAW	Age of child
349-M	B*5702+ve		PLG-------	------LD-	127d postnatal
349-C	B*5702-ve		nd	------LD-	127 days
			PLG-------	------LD-	183 days
			PLG-------	------LD-	274 days
			PLG-------	------LD-	355 days
			PLG-------	------LD-	545 days
			TLG-------	------LD-	630 days
			TLG-------	------LD-	772 days
			TLG-------	------LD-	1104 days
			TLG-------	-------D-	1233 days
		C clade consensus		TPQDLNTML	Age of child
568-M	B*8101+ve			--S---S--	7d antenatal
568-C	B*8101-ve			----M-S--	8 days
				--S---S--	92 days
				--S---S--	160 days
				----M----	285 days
				----M----	482 days
				----M----	847 days
				----M----	1195 days
		C clade consensus	AISPRTLNAW	TSTLQEQIAW	Age of child
284-M	B*5801+ve		P---------	--N-----T-	24d antenatal
284-C	B*5801-ve		P---------	--N-----T-	1 day
			P---------	--N-----T-	183 days
			P---------	--N-----T-	303 days
			P---------	--N-----T-	471 days
			P---------	--N-----T-	655 days
			PL--------	--N-----T-	856 days
			P---------	--N-----T-	1186 days
			P---------	--N-----T-	1363 days

Figure 3.6. Evolution of escape Gag variants in HLA-B*5702 and HLA-B*8101 negative children after transmission from HLA-B*5702 positive and HLA-B*8101 positive mothers. CD4%, viral loads and epitope sequence assessment in the four cases where mothers expressed protective HLA-B alleles and the child did not: (A) 114-C, (B) 349-C, (C) 568-C and (D) 284-C.

3.6. DISCUSSION

While most HIV-infected children progress rapidly to disease following MTCT, a minority of children control the virus for years without the need for ART. These analyses of 61 HIV-infected children followed from birth highlight some of the factors associated with slow and rapid progression in children. Slow progression was associated with possession of either one or more of the known protective HLA B-alleles, HLA-B*57, B*5801 or B*8101, particularly when these protective HLAs were not shared with their mothers ($p=0.007$). Slow progressors with or without protective HLA-B alleles were more likely to make HIV-specific CD8+ T-cell responses to Gag epitopes presented by the alleles, than progressors. Mothers expressing protective alleles were more likely to transmit escape variants within Gag epitopes presented by those alleles than mothers not expressing them ($p=0.001$). Reverting escape mutations were transmitted to, and subsequently reverted in all slow-progressing children whose mothers possessed protective HLA-B alleles. These data demonstrate the contribution of protective HLA-B alleles in either the mother or the child to slowing disease progression in paediatric infection.

These data suggest that HIV-infected children can benefit from protective HLA-B alleles in the same way as infected adults, via the targeting of Gag epitopes presented by these alleles (Kiepiela et al., 2007, Matthews et al., 2008), and also may benefit from the acquisition of transmitted escape mutations as described in adult infection (Goepfert et al., 2008, Chopera et al., 2008).

Although the presence of protective HLA-B alleles contributes to outcome in paediatric HIV infection, these studies illustrate that other factors are also operating to influence outcome. We have previously shown in studies from this same cohort that MTCT is related to maternal viral load (Mphatswe et al., 2007), and it would not be unexpected where factors other than the protective HLA-B alleles focused on in this study, play a role in determining maternal viral load. Nine of 17 slow-progressor children (53%) did not possess protective HLA-B alleles, and nor did their mother; and, of the progressor children, 8 of 44 (18%) carried a protective HLA-B allele or the mother did. Non-HLA-associated factors likely to play a role would include non-HLA genetic factors (Altfeld and Goulder, 2007, Shostakovich-Koretskaya et al., 2009), the presence of co-infections by pathogens such as HCV, CMV, tuberculosis, and the concomitants of extreme poverty (Mphatswe et al., 2007). The humoral response constitutes another arm that may be responsible for mediating HIV-1 control and disease progression in the slow progressor group without detectable T cell responses, however the function of neutralizing antibody responses in delaying HIV-1 infection in perinatal transmission remains poorly understood. Nevertheless neutralizing antibodies are frequently detected during mother-to-child-transmission. Several reports in subtype A, B and C demonstrate that transmitted viruses in children are less susceptible to neutralization by their maternal antibodies (Dickover et al., 2006, Kliks et al., 1994, Wu et al., 2006). The inability to neutralize transmitted viral isolates suggests that there is preferential transmission of escape variants in mother-to-child-transmission setting. Even when there was sensitivity to neutralization, no correlation with disease progression was noted implying that neutralizing antibodies did not protect against disease progression

in children (Zhang et al., 2010). Overall, these data suggest that neutralizing antibodies may protect against mother to child HIV transmission but do not appear to significantly impact on pediatric HIV control. However, further research is needed to evaluate factors that influence neutralization outcome of the viruses during perinatal transmission.

A further factor reducing the apparent impact of HLA-B on viral fitness may be that mutations reducing viral fitness are transmitted by as many as 21% of mothers not themselves carrying the protective alleles (Table 3.2). In some cases it is possible that the father may have transmitted the allele to the child, and the variant to the mother, as previously described (Pillay et al., 2005). For example, subject 559-C carried B*5801 that could be expected to select for mutations such as I247V in the epitope TW10, but the mother carried this mutant without having B*5801 herself. It is striking that a large proportion of the mother-child cohort carry HLA-B*57-associated mutations, but do not themselves possess B*57, for example 568-C/M. These data support the notion that these mutations, in spite of their having the capacity to revert in the absence of the restricting allele, nonetheless are seen at relatively high frequency even in HLA-mismatched subjects (Kawashima et al., 2009). A potential reason for the persistence of mutations reducing viral fitness is that in some cases they are 'neutralised' by compensatory mutations. For example, in the anecdotal case of child 284-C, the mutations I223N/M228I/A248T/S252N were observed, that have been shown to contribute to reducing the fitness cost of T242N (Brockman et al., 2007). In the child 114-C, whose mother similarly transmitted T242N but, unlike 284-C, in whom

reversion did occur subsequent to transmission, the virus consistently at all 4 time-points prior to reversion (days 439-773) showed wild type virus at all these positions. With such small numbers of study subjects in this category, these data are no more than suggestive explanations for lack of reversion over time seen in 284-C. A separate manuscript describing in detail the in vitro fitness of viruses from children in this cohort is has recently been published (Prado et al., 2010).

A potentially important observation is the relative inability of CD8+ T-cell responses to be made by infected children even when they carry the protective HLA allele and the unmutated epitope has been transmitted (Figs 3.2-3.3). The reasons for this failure are unclear, but maternal well-being has a strong influence on paediatric disease progression (Prendergast et al., 2007). For example, child 600-C, who had HLA-B*8101, and whose virus encoded the unmutated TPQDLTNML epitope, made no detectable response to it (or to any other epitope – data not shown); the mother 600-M had an absolute CD4 count of 4 cells/mm3 (CD4% of 2%).

An additional observation from the data presented in this study is the lack of precise correlation between transmission of escape mutants by the mother and either the ability of the child to make a response to the epitope or the disease outcome in the child. Previous studies (Goulder et al., 2001a) focusing on transmission of escape mutants within the HLA-B*27-restricted epitope, KK10 (KRWIILGLNK, Gag 263-272), suggested that escape variant transmission would result in no response being generated by the child, and more rapid progression in the child. Although the findings from this

present study are consistent with the B*27 study, the fact that some children can make a detectable response in spite of being infected by an epitope variant, and the fact that more rapid progression as a result of the MTCT of escape mutants within epitopes restricted by shared alleles is not always observed is, we would suggest, the result of two main factors.

First, the impact of transmitted escape mutants on the child's ability to make CD8+ T-cell responses would depend on the particular escape mutants transmitted. In the case of the HLA-B*27-restricted epitope, KK10 (KRWIILGLNK, Gag 263-272), and the escape mutants transmitted referred to above (Goulder et al., 2001a), were shown to be non-binding mutants, which therefore prevented any response being made by a B*27-positive child. However, where the mutant is still capable of binding to the HLA allele, such as T242N within the B*57/5801 TW10 epitope TSTLQEQIAW (Gag 240-249), a variant-specific response can be made by the child, as has been previously shown to be the case for the TW10 escape mutants (Feeney et al., 2005). A second possible explanation for the lack of a clearcut relationship between transmission of escape mutants and disease progression in the infant may relate to the impact on viral fitness of the escape mutants in question. Whereas the fitness costs of B*27-KK10 escape mutants at residue R264 are apparently fully compensated by compensatory mutations elsewhere in p24 Gag (Schneidewind et al., 2008, Schneidewind et al., 2009), this is not the case for the B*57/5801/8101 mutants focused on in this study, as is supported by the reversions in vivo shown in Fig 3.6. Thus, infants whose ability to generate a CD8+ T-cell response to a particular epitope may be hindered or abrogated by the

transmission of an escape mutant, may nonetheless benefit from the fact that some of these mutants reduce viral replicative capacity.

One further point to note in comparison of maternal viral sequences from samples taken as close to the time of transmission as possible (a median of 2-3 weeks prior to delivery) and child sequences, also taken as close to the time of transmission as possible (at a median of 31 days age), is the lack of identity between early child sequences and maternal sequences when variants are involved. Focusing on the 4 Gag epitopes highlighted in this study, and sequences in the 16 mother-child pairs where mother and/or child possesses one of the protective HLA-B alleles (Table 3.2) variants are seen in mother or child sequences in 24 of 64 epitopes. However, the variants differ between mother and child in as many as 9/24 instances. In some cases this may be due to the fact that sequences were not determined from viral RNA, but from proviral DNA. However, another possibility is that the most frequent variant present in the mother is not necessarily the variant most likely to be transmitted, a finding previously well described in relation to MTCT (Wolinsky et al., 1996). Finally, it is possible that very early escape may be selected in the infant, even in utero. In the case of 562-C/M, for example, the mother did not have an HLA allele driving variant selection at Gag-146, and only wild-type Gag sequences were observed. The child had HLA-B*5801, however, and at day 1 of life, the child has virus encoding the B*5801 Gag mutant A146P, and no response to the epitope ISPRTLNAW was detected. In contrast, a strong response was initially observed to the B*5801 epitope TSTLQEQIAW; the escape

mutant in this epitope, T242N, was only first noted at 10 months of age, coincident with a decline in the size of the CD8+ T-cell response (Fig 3.2).

Taken together, these data suggest that infected children have the potential to generate Gag-specific CD8+ T-cell responses and delay disease progression if they possess protective HLA-B alleles to which the transmitted maternal virus is not pre-adapted. As in adult infection, the protective HLA class I alleles that drive selection of Gag escape mutations are not only beneficial to the infected mother, but also to the infected child not expressing the same alleles. These studies and those reported before could help identify individuals who qualify for delayed treatment as a result of the advantage of acquisition of a less fit virus. This may be especially relevant to infected children, since the generally observed rapid progression observed in infancy currently dictates that all infants be put on immediate ART following diagnosis.

CHAPTER 4 : T CELL ACTIVATION AND POLYFUNCTIONALITY PROFILES IN PROGRESSIVE AND NON-PROGRESSIVE PAEDIATRIC HIV-1 INFECTION

(A sub-section of work described in this chapter has now been published in AIDS Research and Human Retroviruses, 2011, Sep Issue. See Appendices section)

4.1. INTRODUCTION

According to UNAIDS, an estimated 370,000 children became newly infected with HIV in 2007 (UNAIDS, 2008) and HIV-1-infection is the underlying reason for more than one-third of deaths in children younger than 5 years. The majority of these infections are through mother-to-child-transmission. In contrast to adults, the natural history of HIV-1 disease is very rapid in children, and without antiretroviral treatment (ART), more than 50% of HIV-1-infected children will die within the first 2 years of life (Brahmbhatt et al., 2006, Newell et al., 2004, Mphatswe et al., 2007). Several factors might account for this rapid disease progression in children, including the immature neonatal immune system, and the transmission of viruses that have adapted to the mother's HLA class I genotype and include viral escape variants within CTL epitopes restricted by those HLA class I alleles shared between mother and child (Goulder et al., 2001a, Mphatswe et al., 2007, Thobakgale et al., 2009). However, a small proportion of children are able to control the virus for years without the need of ART. The correlates of this delayed disease progression in children are not understood.

Superior control of HIV-1 replication in long-term non-progressors has been associated with CD8+ T cells that express multiple effector functions upon recognition of cognate antigen (Almeida et al., 2007, Betts et al., 2006, Seder et al., 2008). However, the generalised T cell activation that characterises HIV-1 infection has been suggested to lead to accelerated proliferation, expansion and death of CD4+ and CD8+ T cells (Grossman et al., 2002, Kovacs et al., 2001). Continuous stimulation of T cells in HIV-1 infection can also result in functional impairment, senescence and activation-induced

deletion of HIV-1-specific CD8+ T cell clones (Brenchley et al., 2003, Lichterfeld et al., 2004a, Palmer et al., 2005, Streeck et al., 2008). Furthermore, the level of HIV-1-induced immune activation has been strongly associated with the speed of HIV-1 disease progression in adults. In contrast, the consequences of HIV-1 infection on T cell functions are much less understood in paediatric HIV-1 infection, but several studies suggest an important role of virus-specific CD8+ T cells (Feeney et al., 2005, Feeney et al., 2004, Leslie et al., 2004, Thobakgale et al., 2009, Huang et al., 2008) and T cell activation (McCloskey et al., 2001, Paul et al., 2005, Sherman et al., 2002) in the control of paediatric infection.

Here, we analyzed T cell activation and function in HIV-1 infected children with rapid and slow disease progression over the first 3-4yrs of life, using samples from a previously described paediatric cohort (Thobakgale et al., 2009, Thobakgale et al., 2007). We observed that children with delayed disease progression had lower levels of T cell activation and senescence and more polyfunctional HIV-1-specific CD8+ T cells than children with rapid disease progression. These studies suggest that the quality of the antiviral CD8+ T cell response can make an important contribution to outcome in paediatric HIV-1 infection.

4.2. MATERIALS AND METHODS

4.2.1. Study Subjects

Fifteen perinatally HIV-1-infected infants were longitudinally studied from birth. This paediatric study cohort including exclusion and inclusion criteria has been previously described (Mphatswe et al., 2007, Prendergast et al., 2008, Thobakgale et al., 2009, Thobakgale et al., 2007). Briefly, we enrolled HIV-1-infected infants born to HIV-1-infected mothers at St Mary's and Prince Mshiyeni hospitals in Durban, South Africa between 2003 and 2005. The HIV-1 seropositive mothers were recruited in the last trimester of pregnancy. The study was approved by the Ethics Committee of University of KwaZulu-Natal. The mothers gave written informed consent for participation of their children in the study. The mothers and children received single-dose nevirapine at the onset of labour and within 48 hours of birth, respectively, according to the HIVNET 012 protocol (Guay et al., 1999, Jackson et al., 2003).

Study participants were subsequently enrolled into a clinical study, as described previously (Mphatswe et al., 2007) and were either followed longitudinally prior to initiation of deferred ART (arm A), or received one year of ART following diagnosis at birth (arm B). All infants enrolled onto the study received long-term ART once World Health Organization (WHO) clinical and/or immunological criteria for antiretroviral treatment were met (http://www.who.int/hiv/pub/guidelines/WHOpaediatric.pdf). Taking into account that 85% of HIV-1 infected children progressed to CD4 <20% by 1 year in the absence of ART (Mphatswe et al., 2007), we defined rapid progressors as children who met WHO criteria to receive ART within 12 months of birth or within 12

months of ART cessation, and slow progressors as children not meeting WHO criteria to receive ART for > 36 months. Since progression was rapid in the absence of ART in the majority of these children, we here analyzed the remaining 7 children who did not require ART after 36 months (slow progressors) and comparison was made with 8 children who progressed rapidly to disease by 12 months in the absence of ART (rapid progressors). The peripheral blood mononuclear cell (PBMC) samples from the fifteen individuals were assayed at 3 time points for T cell activation, senescence and polyfunctionality: early (median of 4 months, range 2-6 months), intermediate (median of 16 months, range 13-23 months) and late (median of 38 months, range 25-55 months). Clinical characteristics of the study participants are shown in Table 4.1.

4.2.2. Viral Load and CD4 Measurement

Plasma viral loads were measured using the Roche Amplicor Monitor assay (version 1.5) and CD4 counts were determined from fresh whole blood using Tru-Count technology as previously described (Thobakgale et al., 2007).

4.2.3. HLA Typing

DNA for HLA typing was done by DNA PCR using sequence-specific primers as previously described (Kiepiela et al., 2004).

4.2.4. IFN-γ ELISPOT Assays using Synthetic HIV-1 Peptides

Ex vivo measurement of T-cells for IFN-γ production was undertaken using an IFN-γ ELISPOT assay as previously reported (Kiepiela et al., 2004, Thobakgale et al., 2009, Thobakgale et al., 2007). A panel of 410 overlapping peptides (18mers with a 10 amino-acid overlap) spanning the entire HIV-1 clade C consensus sequence and defined optimal peptides matching CD8+ T cell epitopes described for the HLA of the patients were used in the ELISPOT assays.

4.2.5. Assessment of T cell activation by multicolor flow cytometry

Ex vivo measurement of T cells for expression of the activation marker CD38 and senescence marker CD57 were performed. In brief, freshly thawed cryopreserved PBMCs were resuspended to 1-2 x 10^6 cells/ml in R10 media (RPMI 1640 supplemented with 10% heat-inactivated FCS), 100 U/ml penicillin, 1.7mM sodium glutamate, 5.5ml HEPES buffer) and rested for 2 hours at 37^0C; 5% CO_2. Cells were then adjusted to 1x10^6 cells/ml, washed with PBS (2% PBS/FCS), stained for intracellular amine groups to differentiate between live/dead (violet viability dye (Invitrogen) and incubated for 30 min at 4^0C. Following incubation, cells were washed and stained with the following surface antibodies: anti-CD19 Pacific Blue (Caltag), anti-CD3-PE Cy5.5 (Caltag), CD4-Alexa 700, CD8-APC Cy7, CD57-FITC and CD38-PE Cy7 (all from BD Biosciences), and incubated for 20 min in the dark at room temperature. Cells were again washed and fixed in 1% parafomaldehyde (Fix Perm A, Caltag) for 20 min in the dark at room temperature. Following incubation, cells were

washed twice with PBS and resuspended in 200µl of PBS before acquisition on LSRII flow cytometer (BD Biosciences).

4.2.6. Assessment of T cell functionality by multicolor flow cytometry

PBMCs were stimulated with peptides corresponding to described optimal CD8+ T cell epitopes that represented immunodominant responses in the study subjects (see Table 4.2; Fig 4.4). T cell functionality was assessed by staining for 4 functions using the following antibody panel: anti-CD3-PE Cy5.5 (Caltag), anti-CD4-APC, anti-CD8-APC Cy7, anti-IFN-γ-PE CY7, anti-TNF α-Alexa 700, anti-MIP-1β-PE, anti-CD107a-PE Cy5 (all from BD Biosciences) as described previously (Streeck et al., 2008). Negative control tube with PBMCs alone and positive control containing PBMCs stimulated with phorbol myristate acetate (PMA) and ionomycin were included in the assays. The cells were then washed twice with PBS and resuspended in 200µl of PBS before acquisition on LSRII.

For both activation and polyfunctionality panels, between 500,000 and 1000,000 events were acquired per sample and data analysis was performed using FlowJo version 8.8.2 (TreeStar, Inc.). Initial gating was on CD3 versus time to eliminate random laser events, followed by removal of doublets by forward scatter height (FSC-H) versus area (FSC-A). Live/dead and B cells were discriminated by the use of viability dye and CD19 in the Pacific Blue channel respectively; subsequently lymphocyte population was gated on, followed by identification of CD8+ and CD4+ T cells. For functional

analysis, gates for each of the measured four CD8+ T cell functions (IFN-γ, TNF-α, MIP-1β, CD107a) were set based on the negative control (unstimulated, media only).

Boolean gating was used to create a full array of the 16 possible response patterns when testing four functions. Data reported here are after background subtraction and were further analysed using SPICE 5 and PESTLE software programmes (provided by Mario Roederer and Joshua Nozzi, NIAID, NIH). For the activation panel, gates were set as indicated above for polyfunctionality panel until CD4+ and CD8+ populations were identified. The respective gates for percentage of CD57+ cells and median fluorescence intensity (MFI) for CD38+ cells were set based on fluorescence minus one (FMO) control tubes for the respective markers.

4.2.7. Statistical Analysis

Statistical analyses and graphs were plotted on Graphpad prism (version 5). Mann-Whitney test was used to compare differences between the medians in the expression of CD38, CD57 and IFN-γ by T cells between slow and rapid progressor children. The same test was also used to compare differences between the medians in proportions of CD8+ T cells expressing multiple functions. The Spearman rank correlation test was used to determine correlation between T cell activation and senescence on viral load and duration of infection. SAS version 9.1.3 (SAS Institute Inc., Cary, NC) was also used for analysis. Multiple regression analysis was performed using linear mixed models, taking into account longitudinal measurements, for viral load, CD38 MFI,

CD57, age and effects of ART. In this analysis, log transformation was applied to viral load to ensure normality.

4.3. RESULTS

4.3.1. Longitudinal measurement of T cell activation and senescence in slow-progressor and rapid-progressor children

Nine of the 15 enrolled infants described here received short-term ART in the first 12 months of life as part of the primary clinical study; the remaining infants did not receive immediate ART upon diagnosis (Mphatswe et al., 2007). Children were assigned into 1 of 4 groups (Table 4.1), on the basis of whether they were randomized into early ART or not and whether or not they remained off ART for >36 months.

T cell activation has been described as a better predictor of disease progression than viral load in adult and paediatric infection (Deeks et al., 2004, Giorgi et al., 1993, Sherman et al., 2002, Paul et al., 2005). We investigated CD4+ and CD8+ T cell activation levels by measuring CD38 expression in slow and rapid progressor children in the first 3-4 years of life before and after ART. In the arm A group (deferred ART), rapid progressors showed higher levels of CD8+ T cell activation at the early time-point, which decreased at the intermediate and late time-points subsequent to ART (Fig 4.1A). Slow progressors at the early time point tended to have lower levels of CD8+ T

Table 4.1. Characteristics of the study participants.

Participants	HLA-Type	Early			Intermediate			Late		
		Age (mo)	VL [a]	CD4%	Age (mo)	VL	CD4%	Age (mo)	VL	CD4%
Arm A (n=6)										
Slow-Progressors										
A-517C	A3002/6802 B1510/4201 Cw03/1701	4mo	5,620,000	25	18mo	>750000	27	48mo	40,535	36
A-133C	A6801/7408 B5802/8101 Cw04/0602	6mo	180,000	38	16mo	40,600	32	55mo	18,300	32
Rapid-Progressors										
A-298C	A29/6802 B13/1401 Cw06/08	6mo	318,000	11	14mo	1,050	17	33mo	<50	23
A-562C	A0205/3002 B1402/5801 Cw07/0802	6mo	11,860	10	13mo	519	29	27mo	<50	27
A-458C	A4301/6802 B1503/1510 Cw03/18	3mo	583,000	17	20mo	<50	43	29mo	<50	48
A-447C	A03/6802 B1401/4701 Cw0602/0802	2mo	7,820,000	25	20mo	717	33	25mo	<50	34
Arm B (n=9)										
Slow-Progressors										
B-114C	A0301/24 B07/08 Cw07/07	N/A [b]	N/A	N/A	23mo	249,000	24	46mo	11,800	19
B-380C	A2301/6802 B1510/1510 Cw03/08	5mo	101	40	20mo	65,200	31	38mo	373,000	30
B-559C	A2301/6802 B0801/5801 Cw07/07	4mo	<50	53	21mo	973,000	25	38mo	356,000	21
B-586C	A0101/6802 B1510/8101 Cw08/1801	2mo	387	38	15mo	137,000	30	42mo	>750000	18
B-021C	A0205/29 B4201/44 Cw0202/1701	5mo	<50	33	16mo	>750000	33	38mo	99,895	24
Rapid-Progressors										
B-001C	A0101/3001 B4201/8101 Cw17/1801	4mo	<50	54	21mo	236,000	25	47mo	<50	29
B-222C	A29/6802 B0702/1302 Cw0602/07	N/A	N/A	N/A	13mo	56,900	11	N/A	N/A	NA
B-675C	A6601/6802 B1510/39 Cw03/12	4mo	1,290	47	16mo	714,000	14	37mo	<50	37
B-732C	A3002/6602 B4201/45 Cw1601/1701	3mo	2,000	22	15mo	654,000	17	N/A	N/A	NA

a-plasma HIV RNA measurement; dilutions for quantification of high VL >750000 was done for some and not all patients due to inadequate sample availability
b-N/A-sample not available for measurement
Shaded grey area indicates time on ART, applicable to Arm A rapid-progressor children at the intermediate and late time-points; all Arm B children at the early time-point and Arm B rapid-progressors at the late time-point

cell activation than rapid progressors, and these levels of activation declined but did not change significantly over time (Fig 4.1A). Similar trends were noted for CD4+ T cell activation, with the exception that slow progressors showed higher levels of activation than rapid progressors at the early time point. The immediate treatment group (arm B) had lower CD8+ T cell activation during early infection compared to untreated (arm A) infants. Activation increased in rapid progressors when treatment was interrupted and later decreased when treatment was resumed; in contrast, slow progressors maintained

stable CD8+ T cell activation levels throughout the measured time-points (Fig 4.1A, B). While rapid-progressors had higher levels of CD8+ T cell activation than slow-progressors within the two groups, which decreased with ART, the differences did not reach statistical significance (P=0.19, Mann-Whitney; Fig 4.1A,B).

Chronic stimulation with HIV-1 can result in proliferation-incompetent T cells which may lead to failure of the T cells to effectively suppress viral replication (Bestilny et al., 2000). Concurrent with activation measurement, we also monitored T cell senescence by measuring CD57 expression on CD8+ and CD4+ T cells at the 3 described time points (Table 4.1). In both the immediate and delayed treated groups, there was a trend towards more elevated CD57 expression levels in rapid-progressors than in slow progressors in both CD8+ and CD4+ T cells (Fig 4.1C, D); with a few exceptions (arm B slow-progressors had more CD57 CD8+ T cell expression). The expression of CD57 increased from early to late infection in slow-progressors in the absence of ART. Rapid progressors had higher levels of CD57 expression in the absence of ART; however, these decreased on CD4+T cells when ART was resumed (Fig 4.1C, D) and there was minimal change of CD57 expression on CD8+ T cells. These changes in CD57 expression over time were not significantly different between slow and rapid progressors. CD4+ T cells displayed much lower levels of CD57 than CD8+ T cells.

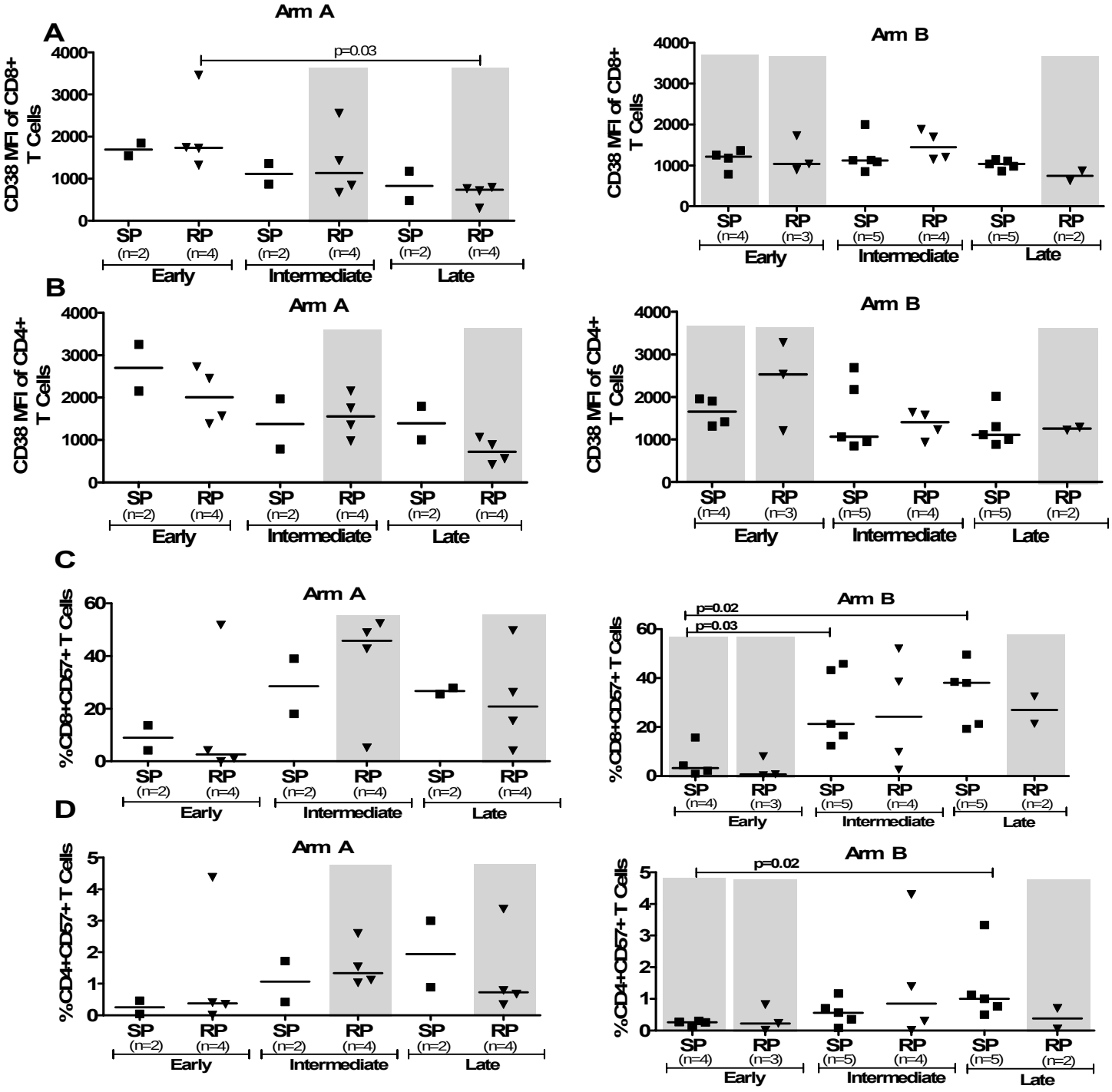


Figure 4.1. T cell activation profiles in slow-progressor (SP) and rapid-progressor (RP) children at different stages of HIV-1 infection. Assessment of CD38 (A, B) and CD57 (C, D) expression on CD8+ and CD4+ T cells in arm A (left panels) and arm B (right panels) children. Dot plots shows medians and shaded area in grey indicate time on ART.

In agreement with previous studies (Deeks et al., 2004, Sherman et al., 2002), the CD38 MFI significantly correlated positively with viral load on CD8+ T cells (P=0.036, Linear mixed model; Fig 4.2A) and on CD4+ T cells (P=0.027, linear mixed model; Fig 4.2B) in children through the course of infection. Furthermore, the expression of CD57 significantly correlated positively with age for CD8+ T cells (P=0.006, linear mixed model; Fig 4.2D) but not for CD4+ T cells (P=0.07, linear mixed model, Fig 4.2D). In addition, there was a significant negative correlation between viral load and the frequency of CD57 positive CD8+ (P=0.028, linear mixed model; data not shown) but did not reach significance on CD4+ T cells (P=0.17, linear mixed model; data not shown). Together these data show that high viral load levels induce T cell activation and senescence from early through to chronic infection, and that these T cell phenotypes are antigen driven as the expression of these markers is affected by ART.

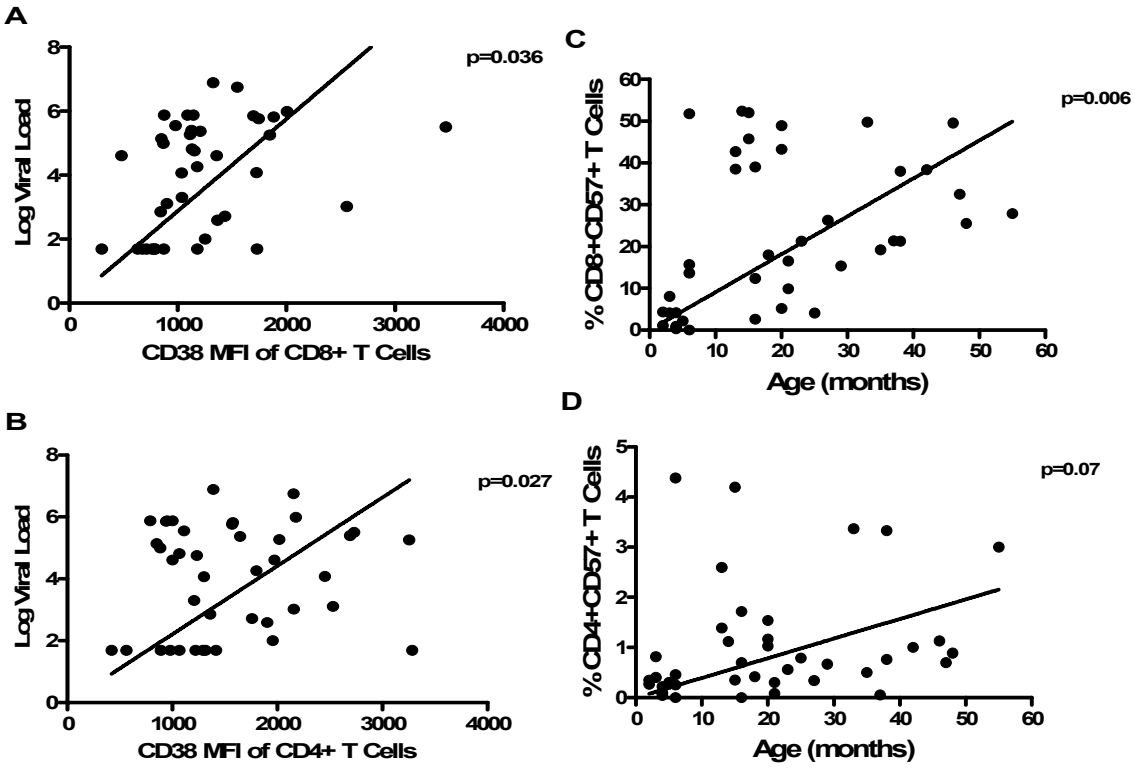


Figure 4.2. Correlation of CD38 and CD57 expression on T cells with viral load and period of infection in children. The graph show the correlations between Median Fluorescence Intensity (MFI) of CD38 on CD8+ (A) and CD4+ (B) T cells, respectively, and viral loads, and the correlations between the percentage of CD8+ (C) and CD4+ (D) T cells expressing CD57 and age (in months) in all participants plotted for all time-points studied (as detailed in Table 4.1).

4.3.2. Evaluation of dominant CD8+ epitopes for IFN-γ production by ELISPOT and ICS assays

We previously reported in this same cohort of children that the majority of in utero-infected infants mount CD8+ T cell responses in the first days of life (Thobakgale et al., 2007); however, disease progression is still rapid in most untreated HIV-1-infected children (Mphatswe et al., 2007). We first monitored the persistence of HIV-1-specific T cell responses over the first 3-4yrs post infection using the IFN-γ ELISPOT assay in

the fifteen slow-progressor and rapid-progressor children, and confirmed immunodominant responses by flow cytometry using ICS (Table 4.2). IFN-γ+ CD8+ T cell responses (gated as shown in Fig 4.3), increased over the course of infection in untreated children, and decreased following initiation of ART. However, no significant differences in the studied IFNγ+ CD8+ T cell responses were noted between slow-progressor and rapid-progressor children (Fig 4.4).

Untreated children (all arm A) had significantly higher IFN-γ producing CD8+ T cells than the children who received immediate ART upon diagnosis (all arm B) at the early time-point, (median 0.16% vs 0.00%; P=0.036; Mann-Whitney, data not shown); and there was a significant increase in the responses in arm B children when treatment was stopped at the intermediate time-point (median 0.0% vs 0.7%; P<0.0001, Mann-Whitney, data not shown), resulting in higher HIV-1-specific IFN-γ+ CD8+ T cell responses in children in arm B who discontinued ART compared to those in the untreated group (median 0.16% vs 0.7%; P=0.024, Mann-Whitney, data not shown). Taken together, these data demonstrate dynamic changes in the frequency of IFN-γ+ HIV-1-specific CD8+ T cell frequencies in response to HIV-1 replication, but the magnitude of these IFNγ+ responses did not allow differentiation between rapid and slow progressors.

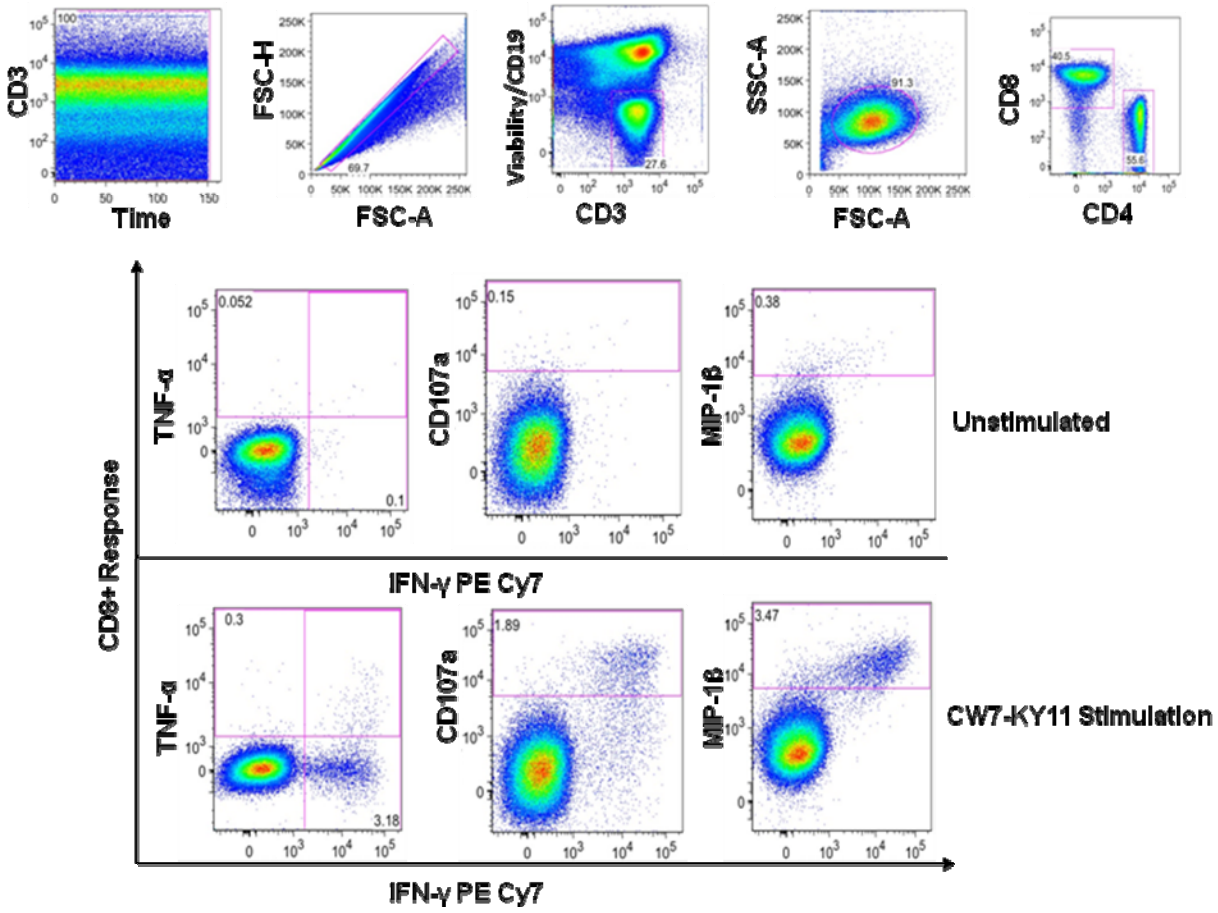


Figure 4.3. Measurement of HIV-1-specific CD8+ T cell functionality by multicolor staining in slow-progressor and rapid-progressor children Representative gating scheme for identification of polyfunctional HIV-1-specific CD8+ T cells following stimulation with HIV-1 peptide is shown for one individual. Initial gating was on the CD3 versus time to eliminate random laser events, followed by removal of doublets by forward scatter height (FSC-H) versus area (FSC-A). Live/dead and B cells were discriminated by the use of viability dye and anti-CD19 respectively versus CD3; subsequently lymphocyte population was gated on followed by identification of CD8+ and CD4+ T cells. Gates for each of the measured four CD8+ T cell functions were set based on the negative control (unstimulated, media only). Boolean gating was used to create a full array of the 16 possible response patterns when testing four functions.

Table 4.2. Intracellular IFN-γ measurement of CD8+ T cell in responses to dominant epitopes targeted by slow and rapid-progressor children over time of infection.

Participants	Optimal Epitope	Epitope Sequence	Early	Intermediate	Late
Arm A (n=6)				**% IFN-γ+ CD8+ T Cells**	
A-517C	B42-TL9 (Gag)	TPQDLNTML	0.00	0.33	0.11
	CW3-YL9 (Gag)	YVDRFFKTL	0.16	0.00	0.00
A-133C	B81-TL9 (Gag)	TPQDLNTML	0.08	0.37	1.05
A-298-C	A29-SY9 (Env)	SFDPIPIHY	0.45	0.00	0.04
	A29-RW10 (Gag)	RQANFLGKIW	0.02	0.00	0.12
A-562C	B58-TW10 (Gag)	TSTLQEQIAW	0.16	0.06	0.07
A-458C	CW3-YL9 (Gag)	YVDRFFKTL	0.36	0.11	0.00
A-447C	CW8-TL9 (Gag)	TPQDLNTML	0.86	0.11	0.00
Arm B (n=9)					
B-114C	CW7-KY11 (Nef)	KRQEILDLWVY	ND	0.16	2.35
	A23-RW8 (Nef)	RYPLTFGW	ND	0.32	0.26
B-380C	B1510-GL9 (Gag)	GHQAAMQML	0.00	0.46	1.70
	B1510-VL10 (Gag)	VHQAISPRTL	0.00	0.13	0.20
	A23-RW8 (Nef)	RYPLTFGW	0.00	0.22	0.96
B-559C	B58-TW10 (Gag)	TSTLQEQIAW	0.10	0.12	0.40
	CW7-KY11 (Nef)	KRQEILDLWVY	0.00	3.10	0.96
	B58-KW11 (Env)	KAYETEVHNVW	0.14	1.56	0.25
	B58-KAF9 (Nef)	KAAFDLSFF	0.42	0.81	ND
B-586C	B81-TL9 (Gag)	TPQDLNTML	0.05	0.64	0.52
B-021C	A2-YI9 (Pol)	YTAFTIPSI	0.06	2.18	0.08
	A29-SY9 (Env)	SFDPIPIHY	0.28	1.93	0.16
	B42-TL9 (Gag)	TPQDLNTML	0.00	0.39	0.74
B-001C	B81-TL9 (Gag)	TPQDLNTML	0.00	3.17	0.47
B-222C	B7-TL10 (Nef)	TPGPGVRYPL	ND	0.12	ND
B-675C	CW3-YL9 (Gag)	YVDRFFKTL	0.09	3.42	0.19
B-732C	B45-GV11 (Nef)	GEVGFPVRPQV	0.00	0.73	ND
	B7-RI10 (Env)	RPNNNTRKSI	0.00	1.19	ND

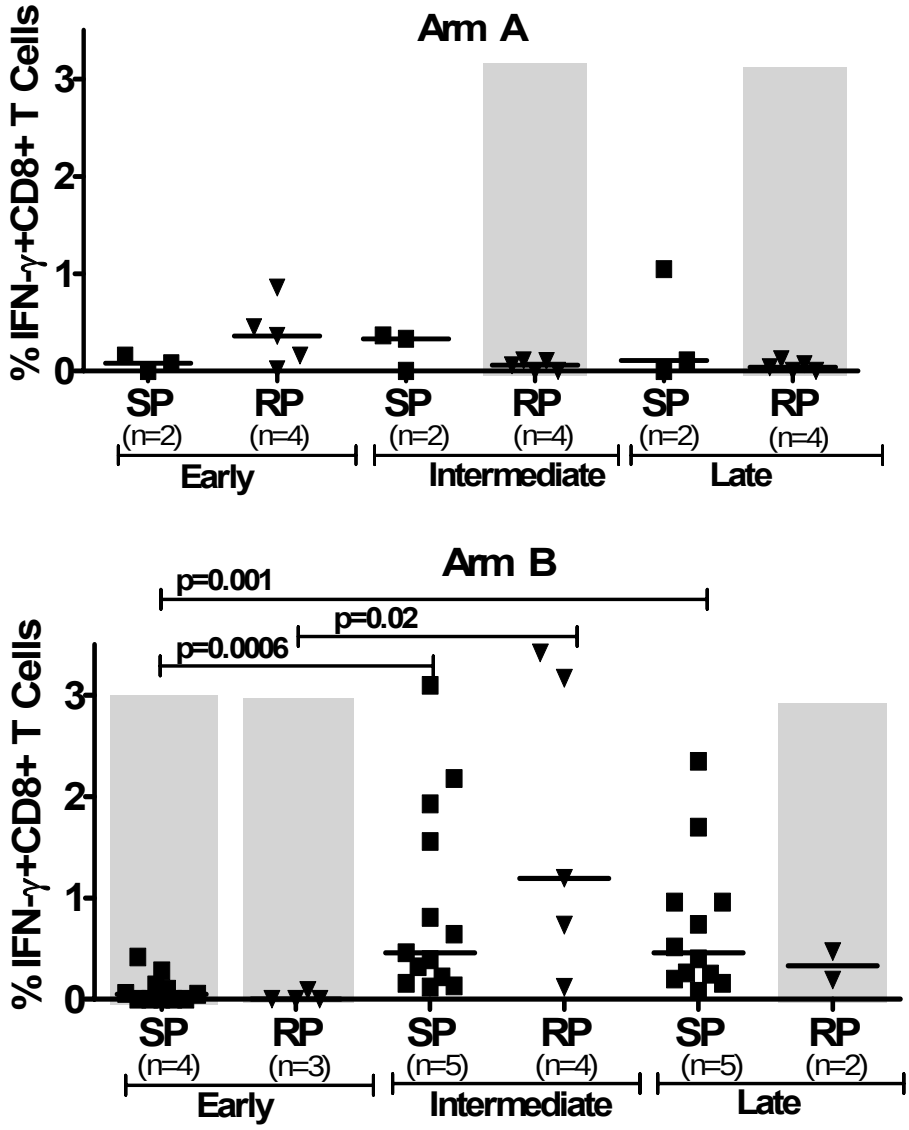
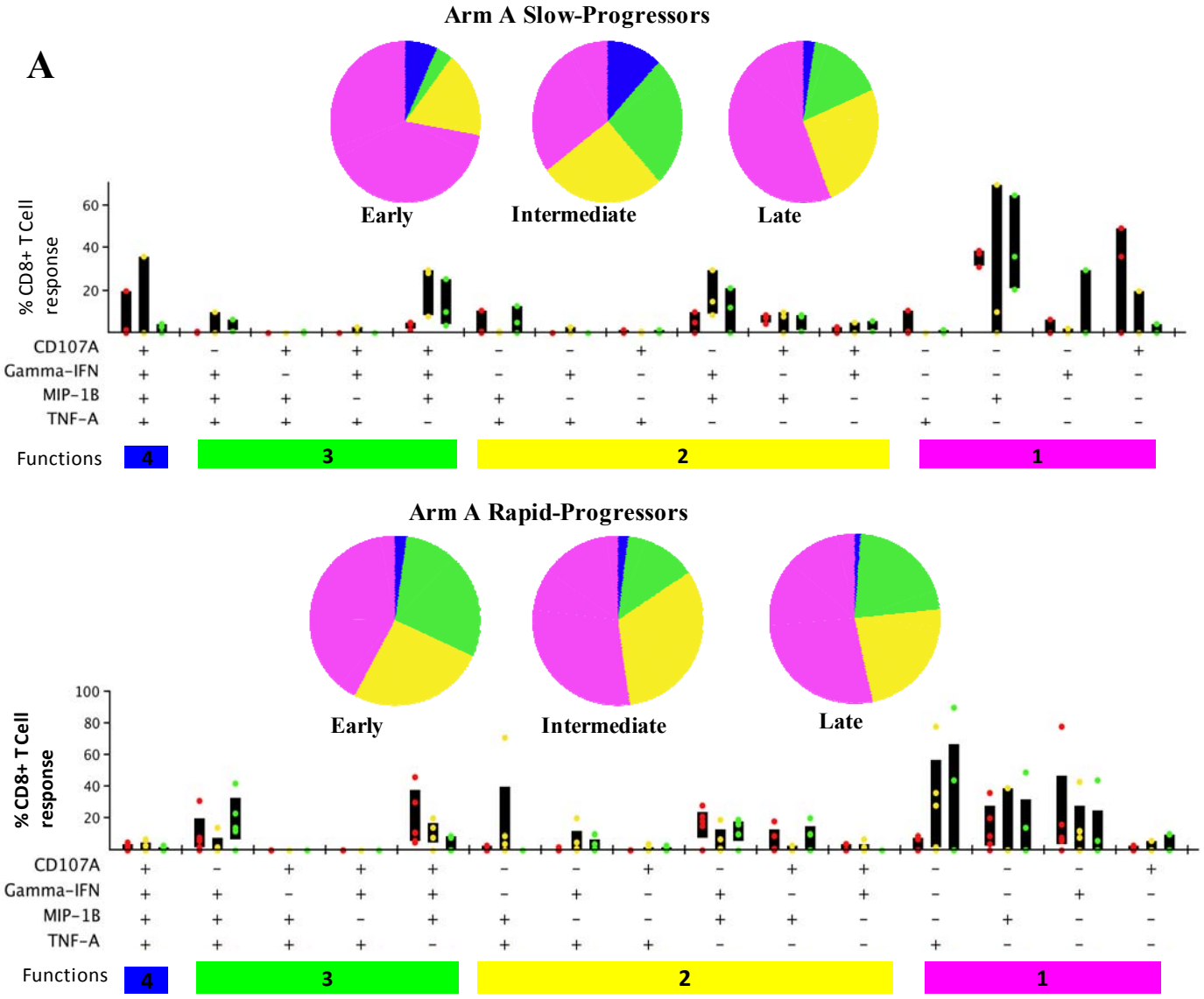


Figure 4.4. Longitudinal intracellular measurement of IFN-γ expression by CD8+ T cells. Measurement of CD8+ IFN-γ+ CD8+ T cells by ICS in arm A (top panel) and arm B (bottom panel) slow (SP) and rapid-progressor (RP) children throughout the first 3-4 years of life. Dot plots shows median values of the different epitopes (as shown in Table 4.2) targeted by different individuals in each group. Shaded area in grey shows time on ART.

4.3.3. Polyfunctionality of CD8+ T cell responses in slow and rapid progressor children over the course of infection

Since IFN-γ expression alone could not discriminate disease outcome in this cohort of HIV-1-infected children, we next evaluated T cell polyfunctionality (as represented in Fig 4.3); defined as the ability of HIV-1-specific-CD8+ T cells to produce multiple cytokines and chemokines in response to viral antigen. HIV-1-specific CD8+ T cell polyfunctionality has previously been associated with viral control in adults (Betts et al., 2006). Slow progressors in the delayed treatment group displayed more polyfunctional (4 functions) CD8+ T cells in the first 6 months of life than rapid progressors, which increased at the intermediate time point and decreased at the last time point. Rapid progressors in the same group had less polyfunctional HIV-1-specific CD8+ T cells than slow progressors, which decreased with disease progression and did not improve with ART at the intermediate and late time-points (Fig 4.5A).

Slow-progressors in the immediate treated group had more monofuntional to 3 function-expressing T cells at the early time-point while on ART, and only gained more functions (up to 4 functions) when viral load increased at the intermediate time point, subsequently decreasing with disease progression at the late time point. The proportion of ≤3 functions did not differ between slow and rapid progressors in the immediate treated group; however, CD8+ T cells expressing 4 functions were lower at the early and intermediate time point and increased at the late time-point when ART was resumed (Fig 4.5B).


A
Arm A Slow-Progressors
Early
Intermediate
Late
% CD8+ T Cell response
60
40
20
CD107A
Gamma-IFN
MIP-1B
TNF-A
Functions
4
3
2
1
Arm A Rapid-Progressors
Early
Intermediate
Late
% CD8+ T Cell response
100
80
60
40
20
CD107A
Gamma-IFN
MIP-1B
TNF-A
Functions
4
3
2
1

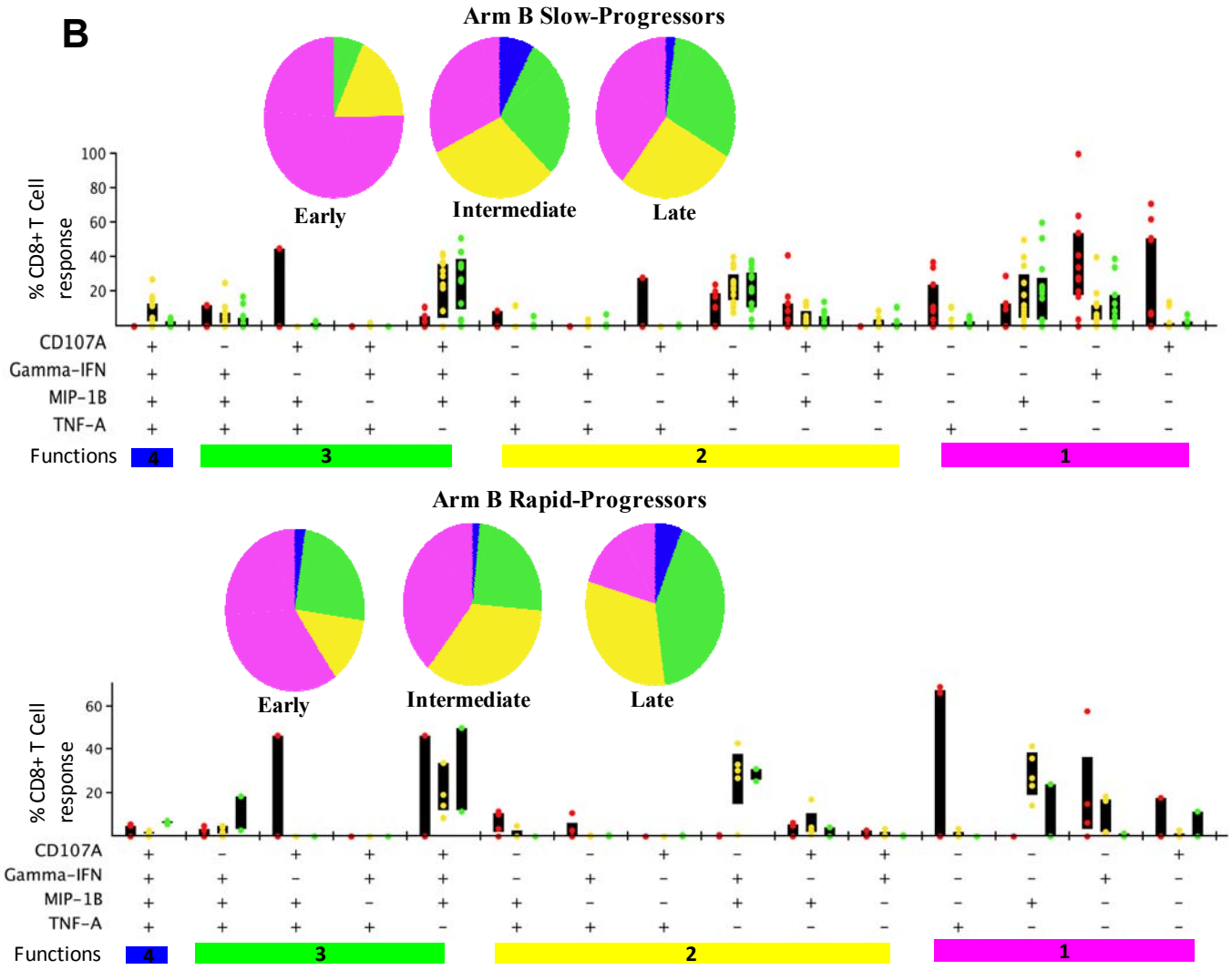


Figure 4.5.(A-B) Representative functional composition of CD8+ T cell responses in slow-progressor and rapid-progressor children. The functional flavour of the individual response patterns to IFN-γ, Mip-1β, CD107a, and TNF-α is denoted by coloured dots; red (early), yellow (intermediate), green (late time-points) for arm A (A) and arm B (B) children. The proportion contributed by the response pattern from slow-progressor (top panel) and rapid-progressor (bottom panel) individuals, and the inter-quartile ranges are shown. The response patterns for each individual and the epitopes studied (as depicted in Table 4.2) were grouped and colour-coded by the number of functions and summarized in pie chart form where each slice of the pie represents the fraction of the total epitope-specific response that consist of CD8+ T cells with the respective number of functions.

Next, we evaluated if the proportion of CD8+ T cells expressing all 4 functions could predict disease outcome in these children (Fig 4.6). Slow progressors were analyzed by combining arm A (early time point) and arm B (immediate time point) groups when off ART and determining the proportions of HIV-1-specific CD8+ T cells expressing different number of functions; a similar analysis was undertaken on rapid progressors. No significant differences were noted for 1, 2, and 3 functions (≤3 functions) between slow and rapid progressor children; however, there were more polyfunctional (4 functions) HIV-1-specific CD8+ T cells in slow progressors than in rapid progressors (P=0.05, Mann-Whitney; Fig 4.6). Taken together these data suggest that with continuous viral stimulation in the absence of ART, polyfunctionality of HIV-1-specific CD8+ T cells may predict slow disease progression in children.

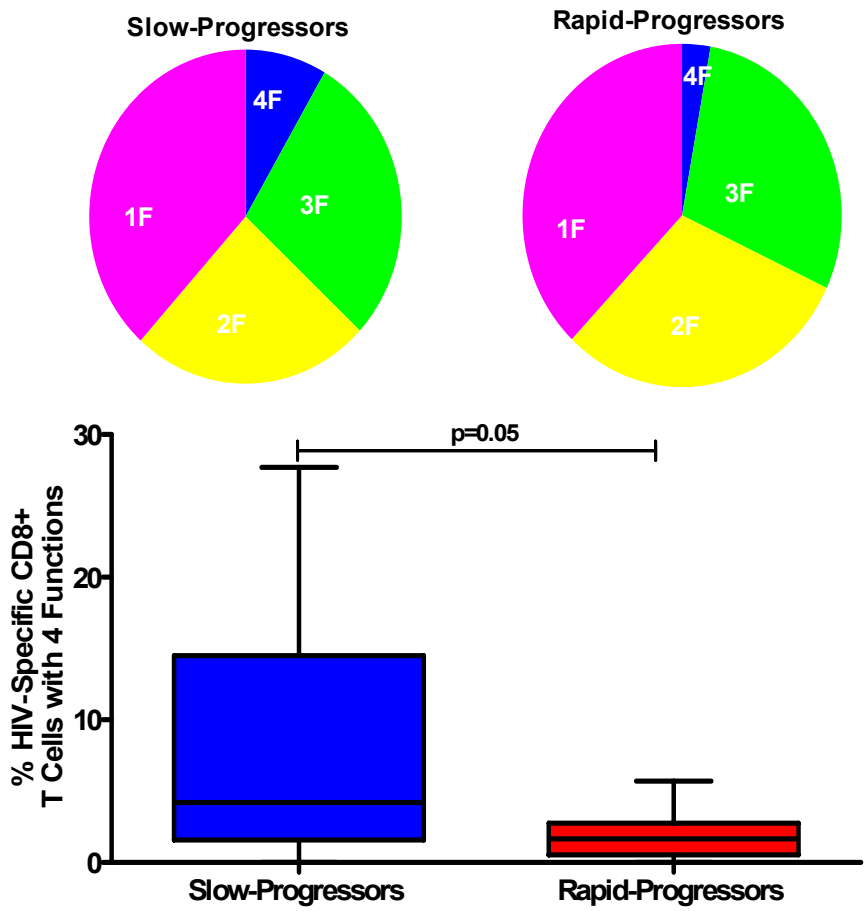


Figure 4.6. Summarized comparison of the overall proportions of polyfunctional CD8+ T cells in slow-progressor and rapid-progressor children. Top panel Polyfunctionality as depicted on the pie charts was obtained by grouping slow-progressors (left pie chart) and rapid-progressors (right pie chart) at comparable time-points while not on ART. For arm A this was the early time-point (slow progressors, n of individuals=2, n of epitopes=3; rapid progressors, n of individuals=4, n of epitopes=5) and for arm B this was the intermediate time-point (slow progressors, n of individuals=4, n of epitopes=13; rapid progressors, n of individuals=4, n of epitopes= 5). The pie charts represent the medians of the different functions for the two groups at the stipulated time points. Bottom panel The contribution of HIV-1-specific CD8+ T cells with 4 functions to the overall virus-specific CD8+ T cell response was compared between the combined slow (n of individuals=7, n of epitopes=16) and rapid (n of individuals= 8, n of epitopes=10) progressor children.

4.4. DISCUSSION

A small group of perinatally HIV-1 infected children maintain immunological control and display slow disease progression. Multiple factors may contribute to delayed HIV-1 disease progression in adults, however very little is known about these factors in paediatric infection. Here we assessed T cell activation and function in the slow progressor children compared to rapid progressors, and we observed variable levels of T cell activation and T cell senescence that did not differ significantly between slow and rapid progressors. We also noted low levels of polyfunctional CD8+ T cells in rapid compared to slow progressors. The expression of CD38 on CD8+ T cells correlated positively with viral load while CD57 expression levels on T cells increased with age and correlated negatively with viral load, supporting earlier findings that continued T cell activation leads to T cell dysfunction. These data show that differences in T cell phenotypes as a consequence of immune activation may exist in slow and rapid progressor children.

Previous studies in adults have found a strong relationship between the level of viraemia and the levels of both CD4+ and CD8+ T cell activation (Deeks et al., 2004). It has also been demonstrated that persistent viral stimulation gradually drives CD4+ and CD8+ T cells to lose their replicative capacity and become senescent; such loss of high quality T cells may play a role in the onset of disease progression (Appay et al., 2007). In line with this, our findings show a strong correlation in the level of CD8+ and CD4+ T cell activation and viraemia and also show a significant increase in CD8+ T cell exhaustion from acute to chronic paediatric infection but not on CD4+ T cells. The

CD38 expression levels were higher in rapid progressors than in slow progressors and as expected decreased with the introduction of ART, thus confirming that high expression of this parameter is associated with high viral load levels. It was unexpected to note a negative correlation between CD57 expression on CD8+T cells and viral load given that exhaustion of HIV-specific CD8+ T cells is driven by antigen stimulation. It is possible that continual viral stimulation results in generation of HIV-specific T cells that express high levels of CD57 that are prone to apoptosis as previously proposed (Brenchley et al., 2003), thus masking a positive correlation at a steady state.

A negative correlation between CD57 expression by CD8+ or CD4+ T cells and viral load as previously been noted by others (Lieberman et al., 1999) may be indicative of antiviral activity of cells bearing this phenotype (Landay et al., 1993, Roederer et al., 1997).

In our study, an additional factor reflecting differences between slow and rapid disease progression in children was the quality of the HIV-1-specific CD8+ T cell response. Expression of IFN-γ alone by virus-specific CD8+ T cells, as has been traditionally measured by ELISPOT and ICS assays, was not sufficient to discriminate between slow and rapid progressors. We noted that, individuals who received short-term ART (arm B) had higher frequencies of IFN-γ+CD8+ T cell responses when treatment was interrupted, in comparison to untreated children (arm A). Augmentation of T cell responses following interruption of ART has been well documented (Ortiz et al., 2001, Papasavvas et al., 2000). However, it cannot be ruled out that qualitative differences exist in the T cell responses generated by these children. This may in part account for

the rapid progression to disease observed in majority of infected children despite early detection of IFN-γ+ CD8+ T cell responses in the first months of life (Lohman et al., 2005, Thobakgale et al., 2007).

Indeed, differences were observed when more CD8+ T cell functions in response to antigen were measured by multiparameter flow assays; HIV-1-specific CD8+ T cell responses in slow progressors displayed a higher proportion of polyfunctional T cell responses than rapid progressors. Notably, these responses were different at the earliest time-point off ART when viral loads were not yet different between the two groups. This finding is consistent with previous observations in studies of adult long-term-non-progressors who displayed polyfunctional HIV-1-specific CD8+ T cells (Betts et al., 2006). An alternative explanation for this result may be that slow progressors had more antigen specific CD8+ T cells than rapid progressors as the number of epitopes targeted by individuals between the two groups was variable. Here, we detected polyfunctional HIV-1-specific CD8+ T cell responses in children younger than 6 months of age. In contrast, in a recent study, polyfunctional responses were less common in children younger than 24 months and hardly detected in children less than 12 months (Huang et al., 2008). A possible explanation for these differences could be viral subtype or host genetic differences, timing of infection as most infants in our cohort were infected in-utero, and also that peptides corresponding to optimal CD8+ T cell epitopes and not pools of overlapping HIV-1 peptides were used in our study.

The heterogeneity of the cohort studied here in terms of ART, and the limited number of patients did not allow us to establish a clear-cut relationship between polyfunctional HIV-1-specific CD8+ T cell responses and viral load, their maintenance over the time of infection, and changes in responses on ART. In addition, CD38 alone rather than CD38 and HLA-DR, was determined as a measure of T cell activation. A recent study examined both markers of activation on CD4+ and CD8+ T cells in Ugandan children with age ranges of 1-10 years, found a correlation with CD4 T cell counts but not with viral load (Ssewanyana et al., 2007). An additional limitation in our study is that the heterogeneity of slow and rapid progression noted here may have been confounded by antiretroviral treatment with some receiving deferred treatment (n=6) while the rest (n=9) were initiated on treatment at the time of diagnosis and then discontinued after one year. Therefore our observations need to be interpreted cautiously as they may not be generalized to the natural course of disease in the absence of antiretroviral therapy. In previous studies, the presence of 3 or more functions depicts polyfunctionality and is associated with slow disease progression. In our analysis, the effect of polyfunctionality as assessed by 3 functions did not differ between slow and rapid progressors but was only seen with regards to 4 functions. Follow-up studies to address these questions in a homogeneous cohort with large numbers of therapy-naïve children are warranted. However, these may be difficult to undertake in future since the high risk of rapid progression in children requires immediate ART intervention following diagnosis (Mphatswe et al., 2007, Violari et al., 2008) that is now provided to all HIV-1-infected children in South Africa.

To our knowledge, this is the first study to simultaneously assess T cell polyfunctionality, activation and senescence from early through to chronic HIV-1 infection in a cohort of slow and rapid progressor children. We conclude that rapid progression in paediatric infection is associated with early immune activation which can drive T cells to gradual functional exhaustion. In addition, we show that the quality of the HIV-1-specific CD8+ T cell response is associated with immunological outcome in paediatric HIV-1 infection as has previously been shown for adult infection. The introduction of early ART in infants following diagnosis as currently recommended, will overlook the few children likely to be able to spontaneously control replication of the virus. However, early initiation of ART will preserve T cell functionality in the majority of rapid progressor children and is clearly justified given the difficulty in discriminating between these two groups during routine clinical care. The potential to discontinue early ART following the initial period of rapid disease progression in children is currently under investigation (Violari et al., 2008). Studies of T cell function might help as a laboratory biomarker for guiding the management of HIV-1-infected children stopping ART.

CHAPTER 5 : GENERAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSION

5.1. GENERAL DISCUSSION

Paediatric HIV-1 infection is characterized by high viral loads that do not decline to viral set point until two or more years of life. This failure to control viremia is attributed to the delayed development of CD8+ and CD4+ T cell responses during early life and may thus contribute to the rapid disease progression noted in infants in the absence of antiretroviral therapy (ART). We examined a cohort of infants infected either during pregnancy (in-utero) or at birth during delivery (intrapartum) and we show that CD8+ T cell responses were detected in the first days of life in in-utero infected children and within the first months in infants infected intrapartum. Env and Rev were the most frequently targeted proteins during early infection compared to Gag, Pol and Nef that were dominantly targeted by chronically infected children (chapter 2).

The sharing of protective HLA alleles between mother and child influenced disease outcome in the child as a result of inability of the child to generate CD8+ T cell responses following adaptation of the transmitted virus in the mother and also brought benefit to the child as a consequence of acquisition of less fit viruses from the mother (chapter 3). The quality of the T cell response as assessed by simultaneous measurement of T cell polyfunctionality, activation and senescence determined slow and rapid progression to disease in children over time in the absence of ART (chapter 4).

These key findings are discussed in detail in the sections below, with particular reference to previous literature.

5.1.1. Timing, specificity and polyfunctionality of the CD8+ T cell response and disease progression in paediatric HIV-1 infection

The lack of viral load decline in children in the first years of life is a key distinguishing factor of paediatric when compared to adult HIV infection. In adults, acute infection is marked by high viral loads, the emergence of T cell responses and subsequent decline to viral set point within weeks of infection. The timing of T cell response development and its effect in slowing disease progression following vertical transmission remains controversial (Buseyne et al., 1998, Lohman et al., 2005, Luzuriaga et al., 1995, Luzuriaga et al., 1991, McFarland et al., 1994, Wasik et al., 1997). Prior studies in infants were limited by cell availability, small sample size and less comprehensive assays without defined timing of infection.

In this analysis of 63 HIV infected children, we set out to understand some of the factors that mediate pathogenesis in paediatric infection. We defined in-utero and intrapartum infections and systematically determined the age at which CD8+ T cell responses are generated and the specificity of the responses using peptides spanning the whole HIV genome. CD8+ T cell responses were detected as early as the first week of life in 70% of in-utero infected infants and after a month in intrapartum infected infants (Thobakgale et al., 2007). Our findings support previous suggestions by others that the capacity to generate CD8+ T cell responses may start from early foetal life (Luzuriaga et al., 1995, Marchant et al., 2003, Vermijlen et al., 2010), thus suggesting that

perinatal HIV vaccination of infants is feasible because they have the ability to mount immune responses in response to viral antigens.

The differences in the specificity of the response targeted towards Env and Rev in acute paediatric infection and towards Gag, Pol and Nef in chronic paediatric infection may have important vaccine implications following reports in adults that Gag responses are associated with low viral load and Env responses with high viral loads (Kiepiela et al., 2007). This may be the case for paediatrics as well since a recent study in infants found an inverse correlation between Gag responses and viral load in infants younger than 24 months (Huang et al., 2008), suggesting that Gag responses may be beneficial in neonates. The notable rapid progression to disease in the first year of life in 85% of the infants who delayed ART (Mphatswe et al., 2007) despite detection of T cell responses immediately after infection may suggest that these early responses are dysfunctional in infants. This is in contrast to acute adult infection where the development of CD8+ T cell responses coincides with a temporal viral load decline and subsequent viral control (Koup et al., 1994).

The long-term benefits of these responses could not be established between in-utero and intrapartum infected infants in our cohort as 75% of the infants started ART shortly after diagnosis. However, longitudinal follow-up over 12 months in a few infants suggested that these responses may have been beneficial in some infants (Thobakgale et al., 2007). An earlier study (Lohman et al., 2005), found no differences in peak viral load and magnitude of the early responses between in-utero and intrapartum infected

infants, indicating a lack of clinical benefit regardless of early detection of responses also in infants <12 months. In contrast to our study, many children failed to generate responses at the earliest time points measured, it is possible that defined optimal epitopes used in that study rather than the entire HIV proteome peptides may have accounted for the differences (Lohman et al., 2005).

Consistent with our findings, a recent study in clade C infection also detected CD8+ T cell responses at 6-10 weeks of age and at birth in in-utero infected infants. However, in contrast Nef was found to be dominantly targeted by most infants (Shalekoff et al., 2009). The differences in methodologies and the variability in the number of in-utero vs intrapartum infants studied between the two studies may explain the differences. Whole blood ICS rather than IFN-γ Elispot was used in the study by Shalekoff et al, and in addition, 18 infants (6 in-utero, 6 intrapartum and 6 with unknown timing of infection) were studied in comparison to 63 infants, (44 in-utero and 19 intrapartum) in our study. The majority of in-utero infections in our study were evaluated for T cell responses within the first 2 weeks of life, while Shalekoff et al measured T cell responses between 6-10 weeks in most infants. Thus it is likely possible that there is a temporal shift in the T cell specificity in neonatal infection. The long term benefits of these early T cell responses was also not demonstrated and it is likely that there was no benefit as no significant correlations were observed between the magnitude of the infants' T cell responses and viral loads (Shalekoff et al., 2009).

The studies done so far in infants may also highlight that IFN-γ alone as a marker is not sufficient for measurement of HIV-specific T cell immunity as it did not predict functionality of the CD8+ T cell responses. A possible explanation for the ineffectiveness of CD8+ T cell responses seen in infants may be attributed to the diminished CD4+ T cell help (Huang et al., 2008, Thobakgale et al., 2007) and the lack of CD4 proliferative capacity (Ramduth et al., 2008) during early life, which is in contrast to high levels observed in acute adult infection or chronic infected children older than 5 years (Feeney et al., 2003, Rosenberg et al., 1997, Zaunders et al., 2004). CD4+ T cell help is critical for the maintenance of CD8+ T cell memory, effective immunity to chronic viral infections through enhancement of CD8+ T cell effector function (Ridge et al., 1998, Zajac et al., 1998). The reasons for CD4+ T cell response deficiency during early childhood needs further investigation and it is proposed that it may be due to inefficient priming of CD4+ T cells by antigen presenting cells, reduced functional capacity or due to suppression by CD4 T regulatory cells (Adkins, 2000, Adkins, 2005, Delespesse et al., 1998, Hartigan-O'Connor et al., 2007, Wasik et al., 2000).

Host factors, timing of transmission and the maternal disease state at the time of delivery are some of the factors that can influence disease progression in children (Abrams et al., 2003, Dickover et al., 1994, Just et al., 1995). We assessed virological and immunological progression of infants infected either in-utero or intrapartum despite administration of single dose nevirapine to the mother during labour and to the child shortly after birth. As reported by others (Guay et al., 1999), we found that this

prevention of mother-to-child-transmission (pMTCT) strategy only managed to reduce intrapartum infections and did not have much effect on in-utero infections as 69% (44/63) of the infants were infected in-utero compared to 31% (19/63) of intrapartum infections.

As expected, the median viral load of the mothers of infected infants were higher than the mothers of uninfected infants and the median viral loads of the mothers of intrapartum infected infants were higher than those of in-utero infected infants. This finding further confirmed reports that the severity of the mother's disease state may have resulted in intrapartum transmission that would have otherwise been avoided by single dose nevirapine (Mphatswe et al., 2007). The finding that there was rapid progression in the majority of infants studied here, most of which were infected in-utero, support previous studies that in-utero infected infants progress rapidly to disease (Dickover et al., 1998, Kuhn et al., 1999, Mayaux et al., 1996).

Whereas this study enrolled infants between year 2003 and 2005 when single dose nevirapine was used for pMTCT and when infants were treated once CD4% declined to 20%, findings from this study highlighted two key issues in paediatric management. First, the urgent need for ART prophylaxis to reduce high viral loads from early pregnancy rather than only at labour to reduce both in-utero and intrapartum infections. Second, a need for immediate ART intervention in HIV positive infants due to the fast progression in the absence of ART. It is encouraging that the current South African treatment guidelines as of April 2010, recommends dual antiretroviral therapy in HIV

infected pregnant women from 14 weeks of gestation and to the child ART is given shortly after birth if confirmed HIV infected (National Department of Health, 2010)

Recent studies suggest that the quality rather than the quantity of the T cell response is an important immune correlate of effective control in HIV infection. Individuals who maintain low or undetectable viral loads in the absence of ART have proliferative T cells that also exhibit multiple effector functions and correlate negatively with viral load than individuals with high viral loads (Betts et al., 2006). Most of the studies undertaken in children only enumerated T cell responses; however there are limited data on the functionality of T cells on disease progression in children. We here demonstrated that polyfunctionality may also predict slow disease progression early in paediatric infection as shown by the ability of CD8+ T cells to degranulate (CD107a) and secrete IFN-γ, TNF-α and MIP-1β.

It is still unknown if polyfunctionality is a cause or effect of viral control. We noted differences in slow and rapid progressor children infected as early as the first 6 months of life in the absence of ART, when viral loads were not yet different between the two groups suggesting that polyfunctionality may lead to viral control (Thobakgale et al., 2011). However, the limitation of this analysis was the lack of longitudinal assessment of this finding due to the introduction of ART at later time points as a result of progression to disease in most children studied. While data so far suggest that CD8+ T cells may also control viral replication in children, it is possible that other non-CD8+ T

cell mechanisms are important in explaining control in children who neither generated Gag-mediated CD8+ T cell responses nor expressed protective class I HLA alleles.

5.1.2. HLA in the mother and child and viral replicative capacity on paediatric disease progression

We have shown that T cell responses are mounted shortly after birth and may contribute to the long term establishment of viral containment (Thobakgale et al., 2007). However, in the majority of the infants progression to disease still occured despite the presence of CD8+ T cell responses, thus other factors may operate to influence disease progression during early paediatric infection. The targeting of Gag epitopes by CD8+ T cells mediated by HLA-B alleles is associated with low viral loads in adults (Kiepiela et al., 2007), HLA-B alleles known to protect against disease progression also drive the selection of Gag-specific escape mutations (Matthews et al., 2008). However, the mechanisms underlying immune control by protective alleles are not well established.

Recent studies in adults have demonstrated that the accumulation of escape mutations may reduce fitness and result in lower viral loads benefiting not only the host, but also the recipient lacking the protective allele (Chopera et al., 2008, Goepfert et al., 2008). However, no benefit was noted in the recipient if the protective alleles are shared with the donor as the transmitted virus may have already adapted in the key Gag epitopes which are important for mediating control in the recipient (Goepfert et al., 2008, Goulder et al., 2001a). Thus the protective effect of the “good” alleles can be exerted in

two ways; directly through active CD8+ T cell mediation or through viral attenuation from mutations that reduce replication capacity of the virus.

The mother-to-child-transmission model afforded us an opportunity to explore this hypothesis in infants for two reasons. First, infants are able to mount Gag specific CD8+ T cell responses and induce escape mutations if they have HLA alleles to which no maternal adaptation occurred and second, infants also share over half of their HLA alleles with the transmitting mothers. We here demonstrate in a cohort of 61 mother-child pairs that like adults, the expression of protective HLA-B alleles (B*5701/B*5703/B*5801/B*81) in infants confers an advantage of slow disease progression through the targeting of Gag epitopes presented by these alleles and through acquisition of transmitted escape mutations that reduce the replicative capacity of the virus. Evidence that these mutations may have rendered the virus less fit was demonstrated by the reversion of the mutations in all slow progressor children without protective alleles but whose mothers expressed these protective alleles (Thobakgale et al., 2009).

The lack of benefit despite acquisition of escape mutations that incurred a cost to viral fitness such as the T242N substitution in the B57-restricted TW10 epitope, as was the case in one child with rapid disease progression in our study, was related to the development of upstream mutations in the cyclophilin A binding loop that restored the fitness defect. Indeed this was also noted in another paediatric study showing similar analysis that examined 13 mother-child pairs and showed that there was an advantage

of the HLA-B57 allele in clade B infected infants born to infected mothers and that there was exceptional control of viremia when mutations were within the B57-restricted epitopes (Schneidewind et al., 2009).

Consistent with our finding, Schleindenwind et al (2009), found that HLA-B*57 exerted its protective effect by driving and maintaining a fitness attenuation mutation in p24 Gag, interestingly, this benefit was regardless of the parental inheritance. In contrast to the latter, a previous study had found that transmission of escape mutations within HLA-B27 restricted epitope KK10 resulted in failure to control in children who shared these alleles with their mothers and benefited those who inherited the allele paternally (Goulder et al., 2001a) as they mounted high frequency responses to the KK10 epitope critical for B27 mediated control. Thus, this contrast in the heritability of HLA-associated control found in the study by Schleindenwind and others may suggest that there may be different mechanisms of protection by the HLA-B*57 and HLA-B*27 alleles as has been previously demonstrated by the effectiveness of these two alleles at different phases of infection (Gao et al., 2005).

Although a limitation to our study was that replication capacity could not be demonstrated, we later examined this in a separate study using the same cohort of children to further understand whether the biological property of the transmitted virus determines slow versus rapid progression in children. We noted differences in replicative capacity in slow and rapid progressors in plasma viral isolates obtained as early as 4 months of life (Prado et al., 2010). Other factors such as presence of Nef

polymorphisms, virus tropism and mutations within the B*57/5801 Gag CD8+ T cell epitopes known to influence replicative capacity were also investigated. No major differences were noted in viral tropism or presence of Nef polymorphisms between slow and rapid progressors (Prado et al., 2010). However, consistent with our study and others (Schneidewind et al., 2009, Thobakgale et al., 2009), mutations in Gag epitopes, particularly the accumulation of mutations in the B*57/5801 TW10 epitope in the very early virus isolates of slow progressors had a lower replicative capacity. The presence of upstream compensatory mutations in the cyclophilin A binding loop contributed to rapid progression as a result of high replicative capacity (Prado et al., 2010).

This model that the host immune response can have an impact on viral fitness has also been demonstrated in explaining non-progression to disease by 'elite controllers' versus individuals with progressive disease in adult infection (Blankson, 2010). In addition, a recent study has suggested mechanistic differences in the impact of escape mutations on viral fitness; mutations in Gag p24 were associated with a significant fitness cost while mutations in Env did not impact on viral fitness and contributed very little to viral control. The authors also showed that the rate of development of escape mutations may be associated with the impact on viral fitness; i.e. escape mutations that appear slowly may have a greater fitness cost than mutations that emerge rapidly in less conserved genes of the virus (Troyer et al., 2009). The latter may explain the accelerated disease progression in infants as the immunodominance of Env or Nef protein during early paediatric HIV infection could result in rapid emergence of escape mutations with little impact on fitness or viral control.

Taken together these series of studies have demonstrated the importance of Gag mediated control in children through active surveillance by CD8+ T cells. These responses then drive Gag escape mutations, resulting in virus attenuation through loss of replicative capacity, thereby facilitating prolonged viral control. The importance of measuring replicative capacity in therapy naïve individuals may be an additional indicator together with viral loads and CD4 counts in future monitoring of therapy naïve individuals following a recent report in a large adult cohort that suggested that measurement of replicative capacity may help predict out how fast or slow individuals will progress to disease (Goetz et al., 2010).

5.2. Future Directions

Efforts for the development of a prophylactic vaccine that prevents the establishment of chronic HIV infection have so far been unsuccessful or partially effective (Buchbinder et al., 2008, Rerks-Ngarm et al., 2009), as a result the focus has also shifted to developing a therapeutic vaccine that can boost immune responses and reduce viral load while prolonging survival of HIV-infected individuals. To better understand viral, immunologic and genetic factors that may provide models for effective HIV vaccination, over the past few years research has focused on "elite controllers" who are HIV-infected individuals who maintain viral loads <50 copies/ml without antiretroviral treatment (Blankson, 2010). Indeed, extensive work in these individuals has brought some understanding of the correlates of immune protection in adults; however these remain poorly studied in paediatrics.

Attenuation of HIV-specific T cell immunity by the programmed death-1 (PD-1) and CTLA-4 pathways has been described in humans and SIV models (Kaufmann and Walker, 2009). PD-1 expression levels are high in untreated subjects, correlates with viral load and disease progression and decreases on antiretroviral treatment (Day et al., 2006, D'Souza et al., 2007, Petrovas et al., 2006, Trautmann et al., 2006). Similarly, CTLA-4 directly correlates with viral load and indirectly with CD4+ T cell counts but unlike PD-1, CTLA-4 is overexpressed on CD4+ T cells and not highly expressed on CD8+ T cells (Kaufmann et al., 2007, Zaunders et al., 2006). Blockade of the PD-1 and CTLA-4 pathways enhances HIV-specific T cell activity and proliferation. Interleukin-10 (IL-10) has been identified as a determinant of viral persistence or acute clearance

following infection. Inhibition of this pathway by antibody blockade of IL-10 restored T cell function and also enhanced viral clearance during chronic lymphocytic choriomeningitis viral (LCMV) infection (Brooks et al., 2006, Ejrnaes et al., 2006). Simultaneous blockade of IL-10 and PD-1 pathways was found to be effective in restoring antiviral T cell responses and also eliminated persistent viral infection, suggesting that there may be an advantage of blocking both mechanistically distinct pathways (Brooks et al., 2008). These findings may have important therapeutic implications as IL-10 expression levels are high in many human chronic viral infections such as HIV, hepatitis C virus (HCV) and hepatitis B virus (HBV).

The role of these pathways needs to be elucidated in neonates and such data may help explain the CD4+ T cell defect during early childhood and failure of the CD8+ T cell responses to resolve viremia in the first few years of life. Limited data so far also suggest upregulation of PD-1 in untreated children in comparison to treated and uninfected children (Ssewanyana et al., 2009). Regulatory CD4+ T cells (Tregs) defined by the expression of the transcription factor FOXP3, maintain a balance in the immune system and may be beneficial in HIV infection by suppressing T cell activation but may also be detrimental as they might suppress the immune response to HIV (O'Connell et al., 2009, Seddiki and Kelleher, 2008). Depletion of Tregs in animal models have led to increased immune activation and high viremia (Cecchinato et al., 2008, Pandrea et al., 2008), illustrating the importance of these cells in containing infection. Treg maintenance in non-progressive disease as shown in various studies

could explain the enhanced HIV specific function in those individuals (Chase et al., 2008, Jiao et al., 2009, Seddiki and Kelleher, 2008).

In HIV infection, Treg studies are limited by the need to first identify markers that reliably predict function since to date the expression of the markers does not necessarily predict function (Ford et al., 2009). Although controversial in HIV, the importance of these cells in HIV control requires further investigation, particularly in children where their abundance from infancy may be the reason for impaired CD4+ T cell mediated control. A study done in infant macaques in SIV infection strongly suggested active suppression of the CD4+ T cell response by Tregs, depletion of which resulted in enhanced SIV T cell responses that were comparable to those observed in adult macaques (Hartigan-O'Connor et al., 2007). Thus it is possible that the high Treg cell activity in infants may account for the general lack of viral control to pathogens and low reactivity to vaccines in perinatal HIV infection. Alteration of Treg cell function through interventional studies might be important to improve infant T cell control and response to vaccinations (Hartigan-O'Connor et al., 2007). More studies are also needed to understand innate immune responses in children since this arm may be more important in children who don't have a well developed adaptive immune system.

While adaptive T cell responses are quick to develop, humoral responses on the other hand can take up to months to develop following infection, but once detectable are known to be well maintained. A successful HIV-1 vaccine would ideally elicit effective long lasting immune responses consisting of both neutralizing antibodies and CD8+ T

cell responses that recognize diverse strains of the virus and that can also protect at possible sites of infection (Fauci et al., 2008, Haynes and Shattock, 2008). Theoretically, neutralizing antibodies would have a prophylactic effect to prevent infection at mucosal sites, while CD8+ T cells would have a therapeutic effect at controlling viral load and slowing disease progression in cases where infection occurs (Munier et al., 2011). Recent advances in the vaccine field identified new monoclonal antibodies and demonstrated that broad neutralizing antibodies for multiple HIV-1 clades can be made (Walker et al., 2009, Wu et al., 2010). However, the challenge is that these antibodies are not easily induced in HIV-1 infection or by current vaccines (Haynes et al., 2010).

For example, the RV144 Thai trial that offered new hope that a preventative HIV-1 vaccine is possible did not induce neutralizing antibodies. The neutralizing antibodies induced by the vaccine regimen were of low titre and did not have broad specificity against heterologous strains (Rerks-Ngarm et al., 2009). Thus the correlate of protection for the modest level of protection is still unclear at this stage. There has been speculation that protection may have been mediated by some short-lived antibody response as considerable titres of binding antibodies to Env were induced. Antibodies that mediate antibody dependent cellular cytotoxicity (ADCC), antibodies that mediate other Fc receptor mediated antiviral activities such as induction of β-chemokines or IgM or IgA antibodies that inhibit virus movement across mucosal barriers and traditional neutralization antibodies are all being considered in the investigation to understand correlates of immunity of the vaccine regimen (Haynes et al., 2010, Munier

et al., 2011). Although our data suggests that immune-mediated viral attenuation, level of T-cell activation and T-cell functionality can impact on paediatric HIV disease progression; other mechanisms of immune control such as the ones proposed for the RV144 have not been comprehensively studied in children and should form the basis for future work.

Despite efforts to combat mother-to-child-transmission through administration of HAART, transmission is still inevitable as most women present late at antenatal clinics. Thus there is still a need for a vaccine that can prevent perinatal transmission. The ability to mount immune responses by the majority of intra-uterine infected infants in our cohort offers hope that most infants could benefit from a vaccine that prevents intra-uterine infections in those unfortunate cases. The differences observed in the specificity of the generated immune responses between infants and adults in our study setting may account for the lack of immune control in infants, thus our studies suggest that an ideal paediatric vaccine would need to be engineered to induce T cell responses of the right specificity that can mediate control from early life. However, our studies do not rule out that beyond T cell protein specificity there are other mechanisms mediating poor viral control by children. Further studies will be needed to address what specific immune defects they may have that may contribute to their inability to control HIV.

In an attempt to partially address these potential defects of CD4+ and CD8+ T cells in children, we simultaneously assessed T cell polyfunctionality, activation and

senescence in a small group of slow and rapid progressor children either undergoing ART or not as part of a pilot study. Our findings need to be interpreted with caution, as they might not represent the natural course of disease without ART and involved only small numbers of children. While the sharing of escape mutations between mother and child can be disadvantageous to the child, our data demonstrated that infants can benefit from acquisition of a less fit virus as has been demonstrated in adults. Larger studies in treatment naïve children are necessary to confirm our findings of T cell function and also further investigate viral and host factors that render the virus less replicative. Taken together, our studies of T cell function and HIV replicative fitness may help as laboratory markers in predicting disease outcome in children and in their clinical management in cases where ART is stopped or cannot be implemented. These data also suggest that immune-driven viral attenuation can impact on HIV clinical outcome in children, indicating that this would be a plausible rational strategy for a paediatric HIV vaccine.

5.3. Conclusion

Without antiretroviral treatment, the majority of children progress very rapidly to disease following HIV infection. However, a small subset of children can control virus replication for years without the need of ART. These series of studies examined the contribution of virus specific CD8+ T cells to relative control of viral replication and disease progression in paediatric HIV-1 infection. We illustrate for the first time using a cohort of infected children that CD8+ T cell responses are mounted in the first days of life in most in-utero infected infants and that the lack of viral containment in most children during infancy is not due to the inability to induce CD8+ T cell responses. Rather, we show that the specificity of the CD8+ T cell response is different in comparison to acute adult infection, and could thus explain the rapid progression noted in infants. Furthermore, we show that the polyfunctional effector phenotype of the CD8+ T cell response rather than IFN-γ measurement alone, together with reduced immune activation and reduced T cell senescence may determine slow progression in paediatrics. Finally, we demonstrate that as in adults, the targeting of Gag epitopes presented by protective HLA alleles in either the mother or child can impact on viral fitness and disease outcome in the child.

The failure of past vaccines may in part be explained by insufficient knowledge of correlates of immune protection and inadequate assays to evaluate such responses (Walker and Burton, 2008). Whereas a lot of effort has been made to define these mechanisms in adults, differences in disease manifestations between adults and children might require manipulation for success in vaccine interventions in children.

However, it is encouraging that there are some similar mechanisms in paediatrics and adults with regards to viral control. Further assessment of these mechanisms will benefit vaccine design.

CHAPTER 6 : REFERENCES

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CHAPTER 7 : APPENDICES

PUBLICATIONS AND AUTHORSHIP CONTRIBUTIONS