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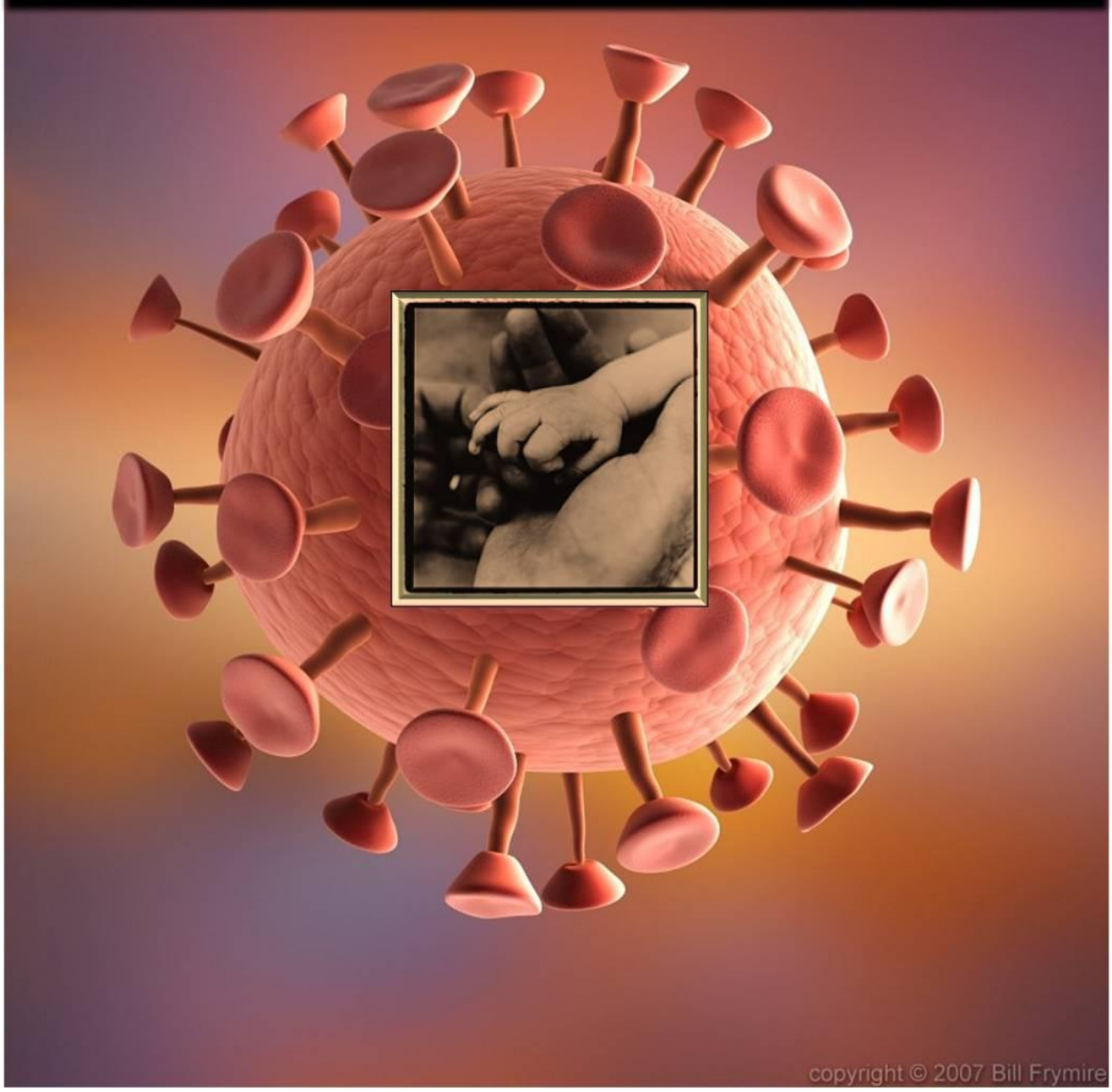
Eshia. S. Moodley-Govender

2011



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

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Cellular Immunity, immune activation and regulation in HIV-1 infected mother-child pairs: What are the determinants of protective immunity?

By

Eshia S. Moodley-Govender

Submitted in fulfilment of the requirement for the degree of
Doctor of Philosophy (Paediatrics and Child Health)
HIV Pathogenesis Programme (HPP),
Doris Duke Medical Research Institute,
Nelson R.Mandela School of Medicine,
University of KwaZulu-Natal.

2011

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Date: 14 February 2013

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Supervisor: Prof Thumbi Ndung'u

Date: 14 February 2013

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Publications and Presentations

Peer Reviewed Publications:

A. First Author:

1. Possession of HLA Class II Allele DRB1*1303 Is Associated with Reduced Viral Loads in 2 Independent Cohorts of Different Ethnicity and HIV Clades of Infection. JID. Jan 2011.

B. First Author-Currently been submitted:

1. Association of HIV-specific CD4+ T cell responses with biomarkers of disease progression in clade C treatment naïve chronically infected adults. Clinical Infectious Diseases.
2. Chapters 3, 4 and 5 have been written up as 3 stand-alone manuscripts: pending submission.

B. Co-author on the following publications:

1. The use of dried blood spots for the determination of genetic variation of IL-10, killer immunolglobulin-like receptor and HLA class 1 genes. November 2011.Accepted Tissue Antigens
2. Impact of select immunologic and virologic biomarkers on CD4 decline in chronic HIV-1 subtype C infection: Results from Sinikithemba longitudinal population-based cohort, Durban, South Africa. Clinical Infectious Diseases 2009; 49: 956-64.
3. Targeting of a CD8 T cell env epitope presented by HLA-B*5802 is associated with markers of HIV disease progression and lack of selection pressure. AIDS Res Hum Retroviruses. 2008 Jan; 24(1):72-82.
4. Human Immunodeficiency Virus-Specific CD8 T-Cell Activity Is Detectable from Birth in the Majority of In Utero-Infected Infants. Journal of virology. 2007, p. 12775–12784 Vol. 81, No. 23
5. Control of HIV-1 is associated With HLA-B*13 and targeting of multiple GAG-specific CD8+ T cell epitopes.J Virol. 2007 Jan 24;
6. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load.Nat Med. 2007 Jan;13(1):46-53.
7. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.Nature. 2006 Sep 21;443(7109):350-4.
8. Motif inference reveals optimal CTL epitopes presented by HLA class I alleles highly prevalent in southern Africa.J Immunol. 2006 Apr 15;176(8):4699-705.

International Conference Presentations

1. Co-Authored Poster, TIM-3 blockades, 2010, Harvard University, Boston, U.S.A.
2. HIV Vaccine Meeting, 2009, CTL mediated responses in children Paris, France.
3. Immunobiology Seminar, 2008. Greece.

Local Conference Presentations

1. 1st position in a call for African research oriented writing, Gauteng, SA
2. SA AIDS 2006 conference-*oral presentation*-Viral load and CD4 comparisons in Transmitter and Non Transmitter mums.

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I owe it all to my protector, creator and source of all wisdom the Lord Jesus "Philippians 4:13."

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I wish to dedicate this mere doctorate to the real success of my life, My Lord, My *bollywood* sweet-hearts Alea and Rea, My hottie, huge-hearted and ever so supportive husband Rodney (and his family).To my papa, Mr A Moodley, Thank you for always providing for us. You also make an awesome *Thatha*. Thank you for always going the extra mile. Your skills astound us. Your hard work has moulded the success of the 3 musketeers. My rock, my handsome brother Joshua, new found stunning sister Maneisha (and her family), my darling Godson AJ (love you so much) and to my future GOD-ordained nephews and nieces-you complement my life. My adorable angelic nephews Caleb and Chris-I can't get enough of you. To my loving, supportive, gorgeous sister and bro (Lilishia and Ryan)-you are so special. Savy-ma, you are one in a lifetime. Thank you Gloria for your love-you are an important part of my life. I am really proud of you.

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DEDICATED TO MY FAMILY; FRIENDS AND IRREPLACABLE LATE MUMMY-only the heavens can translate your beauty. Your faithful prayers and endless love-has extended and blessed us beyond our days. You may be missing in body but you are engraved in every heartbeat and facet of my life. You will forever live and breathe in me. My ways are your ways.

I am fuelled by HIS grace and the love and support of each of you.
"COUNT YOUR BLESSINGS. NAME THEM ONE BY ONE."

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ABBREVIATIONS

AIDS	Acquired Immune Deficiency syndrome
ANOVA	The Analysis of Variance
APC	Allophycocyanin
ART	Antiretroviral Therapy
ARV	Antiretroviral
BCIP	5-bromo-4-chloro-1H-indol-3-yl) Dihydrogen Phosphate
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DBS	Dried Blood Spot
DC	Dendritic cells
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
ELISPOT	The Enzyme-linked Immunosorbent Spot
ENV	Envelope Glycoprotein
FITC	Fluorescein Isothiocyanate
FMOs	Fluorescence Minus One
GAG	Group-specific Antigen
GFP	Green Fluorescent Protein
Gp	Glycoprotein

HAART	Highly Active Anti-Retroviral Therapy
HATP	HIV/AIDS Transmission to Tourism Prevention Programme
HIV	Human Immunodefiency Virus
HLA	Human Leukocyte Antigen
HLA-DR	Human Leukocyte Antigen Derived
HPP	HIV Pathogenesis Programme
IFN-γ	Interferon Gamma
IL-10	Interleukin 10
IQR	Interquartile Range
KZN	KwaZulu-Natal
LCMV	Lymphocytic Choriomeningitis
LTNP	Long Tem Non Progressor
mAB	Monoclonal Antibodies
MEC	Member of Executive Council
MHC	Major Histocompatibility Complex
MMX	Megamatrix
MOI	Multiplicity of infection
MTCT	Mother-to-child-transmission
NBT	Nitro Blue Tetrazolium
NEF	Negative Regulatory Factor
NHP	Normal Human Plasma
NK	Natural Killer
OLP	Overlapping Peptide
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cells

PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
PEHSS	Paediatric Early HAART STI
PERCP	Peridinin-Chlorophyll Proteins
PHA	Phytohaemoagglutinin
PMTC	Prevention of Mother to Child disease
PVDF	Polyvinylidene Diflouride-Backed
QS	Quantitation Standard
REV	Regulator of Virion Expression
RNA	Ribonucleic Acid
SA	South Africa
SIV	Simian Immunodeficiency Virus
SNP	Single-Nucleotide Polymorphism
STATSA	Statistics South Africa
TAT	Trans-Activator of Transcription
Tconv	Convential T cells
TCR	T cell receptor
TH	T Helper
TIM-3	T cell Immunoglobulin and Mucin domain 3
TNF	Tumor Necrosis Factor
TREGS	T Regulatory Cells
VIF	Virion Infectivity Factor
VPR	Viral Protein R
VTS	Vertically Transmission Cohort Study
VPU	Viral Protein U

UNAIDS United Nations Programme on HIV/AIDS

WHO World Health Organization

ABSTRACT

Background: Prevention of Mother-to-child transmission (PMTCT) of human immunodeficiency virus (HIV) remains a significant challenge in resource-poor settings despite the advances in antiretroviral (ARV) treatment. HIV-1 infected individuals are able to achieve viral control naturally, however the underlying mechanisms of immunological control in children remains poorly understood. This study was conducted from 2006 to 2010 to investigate correlates of immune control in HIV-1 clade C infected mother-child pairs in the absence of ARVs. Genotypic and phenotypic viral characteristics, cellular immune responses to HIV-1 and host genetics were characterized and correlated with clinical markers of disease progression.

Materials and Methods: To achieve the objectives of the study, three cohorts of mother-child pairs were investigated. The first cohort included 60 untreated mother-child pairs and a further ten uninfected children as controls. The second cohort comprised of ARV treated pairs (n=60). The third cohort consisted of 374 mothers and 374 children (infected, exposed uninfected, HIV negative). Plasma viral loads and absolute CD4+ T cell counts were routinely performed in all three cohorts. HIV-specific CD8+ T cell responses were analyzed by interferon gamma (IFN-γ) enzyme linked immunosorbent spot (ELISpot) assays. Viral replicative fitness was assessed using a green fluorescent protein reporter cell line (GFP). Multi-parameter flowcytometry allowed for the investigation of T cell regulation, exhaustion and activation using CD127/CD25, TIM-3/PD-1 and HLA-DR/CD38 markers respectively. IL-10 promoter single nucleotide polymorphisms (SNPs) at positions -592 and -1082 were determined by TaqMan allelic discrimination assays. Plasma IL-10 levels were measured using a luminex assay.

Results: To describe the CTL responses elicited to various regions of the HIV proteome in HIV-infected treatment naïve children. Sixty children under one year of age in the untreated

cohort were analyzed for CTL responses spanning the HIV genome, for which only 30 had detectable responses. There was no significant difference in viral load between responders and non-responders (p=0.2799). The responders predominantly targeted Nef (49%), Gag (17%) and Env (14%) regions. Markers of T cell exhaustion and regulation and their relationship to markers of disease progression, were next investigated as these parameters may explain the inability of T cells to effectively control HIV infection. T cell phenotyping compared treated, untreated and uninfected subgroups. In infected children, CD8+ T cells were significantly higher for both the inhibitory marker TIM-3 (p=0.001) and exhaustion marker PD-1 (p=0.0001) compared to uninfected children. Median expression of TIM-3 was higher on CD8+ T cells (46%) compared to CD4+ T cells (20%). TIM-3 and PD-1 expression on T cells were maintained at high levels over time. The frequency of absolute Tregs (p=0.0225) were found to be significantly higher in untreated compared to treated children. HLA-DR+CD38+ on CD8+ T cells were significantly up-regulated in untreated children compared to treated (p=0.002) and uninfected children (p=0.0177). HLA-DR+CD38+ was also significantly higher in children less than 6 months compared to older children on CD4+ (p=0.0437) and CD8+ T cells (p=0.00276). Interestingly, we observed a significant negative correlation between magnitude of CTL response and CD25+CD127- (p=0.0202; r=-0.7333) as well as HLA-DR+CD38+ (p=0.0408; r=-0.5516) on CD8+ T cells. IL-10 is an important immunoregulatory cytokine that has been shown to affect the outcome of chronic viral infections. IL-10 polymorphisms have previously been associated with IL-10 levels and HIV-1 outcomes in adults. Polymorphisms associated with different levels of IL-10 production and their relationship with transmission, markers of disease progression and immune responses were next investigated in this mother-child HIV transmission setting. Genetic analysis of IL-10 in cohort three revealed that HIV-1 acquisition was not associated with either IL10 -592 (AA/CA vs CC) or IL10 -1082 (AA/AG vs GG) single nucleotide

polymorphisms (SNPSs). There was a significant association between IL10 -1082 and HIV-1 transmission (p=0.0012). No correlation was observed between IL10 -592 (p=0.4279) or IL10 -1082 SNPs (p=0.6361) and mortality rates in children. IL10 -592C was associated with an elevated magnitude of IFN-γ CD8+ T cell response compared to IL10 -529A (p=0.0071). We found a significant positive correlation between IL-10 plasma levels and viral loads (p=0.0068; r=0.4759) and the ages of the children (p=0.0312; r=0.1737).

Conclusion: CD8+ T cell responses and viral fitness did not explain differences in disease progression in selected HIV-1 untreated clade C transmission pairs. T cell activation and regulatory markers influence CTL immune responses resulting in poor clinical outcome. IL10 -1082 polymorphisms may be used as a predictor of HIV-1 transmission. The association between increased IL-10 plasma levels and high viral loads suggest that IL-10 contributes to immune dysfunction in paediatric HIV-1 infection. This study has extended our understanding of immunological and genetic correlates of mother-to-child transmission and disease outcome in ARV naïve (naturally controlling) and HIV treated infected children.

ETHICAL APPROVAL

Full ethical approval was obtained from the Biomedical Research Ethics Committee (BREC), UKZN (E028/99). Informed consent was obtained from all participants in the study and good clinical practice guidelines were adhered.

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CHAPTER 1

Overview of the interplay between HIV-1, T cells and host genetics

1.1 INTRODUCTION: Why Human Immunodeficiency Virus (HIV)?

Our immune system is always at work, enabling us to survive the threat of infectious agents. The human immunodeficiency virus (HIV) is a virus that directly attacks the normal functioning of the human immune system. While many other viruses can be controlled, and infections can be cleared by the immune system, HIV targets and infects the very same critical immune defense cells. HIV preferentially infects activated CD4 cells. As the virus replicates the virions damage the immune system resulting in CD4 decline. Loss of CD4+ T cells and the progressive inability to defend the body ultimately result in Acquired Immune Deficiency Syndrome (AIDS). This inevitably leads to the weakening of the immune system (Sattentau, 1989). HIV destroys cell functionality which predisposes the host to opportunistic infections.

Evidence suggests that 40% of untreated HIV-infected infants die before they reach the first year of life and, if left unidentified and untreated, up to 50-60% die by age two (Newell et al., 2004). Possible eradication of paediatric HIV-1 infection lies in the development of an infant vaccine that can be administered at birth that would theoretically provide protection from infancy, through the adolescence period, and into adulthood. An overall understanding of the exact mechanism that drives HIV-1 disease pathogenesis is restricted due to the apparent multifactorial impact of the virus.

1.1.1 **Etiology and Epidemiology of HIV/AIDS: Global and local perspectives**

June 2011 marks 30 years since the discovery of HIV. Thirty years later, this devastating disease has claimed over 30 million lives. AIDS was first reported in 1981 as unusual infections and cancers such as Kaposi's Sarcoma (KS) and *Pneumocystis carinii* pneumonia (PCP) in young homosexual men in New York (Gottlieb et al., 1981, Levy, 2009). Two years later HIV was established as the causative agent of AIDS by the depletion of CD4+ T cells (Barre-Sinoussi et al., 1983). Today, 33.3 million people worldwide are still infected and living with HIV (Fig 1.1). In 2009, an estimated 2.6 million people were newly infected with HIV of which 370,000 infections were in children under the age of fifteen. In the same year, 1.8 million people died from AIDS of which 260,000 were under the age of fifteen. Since the beginning of the HIV epidemic, more than 60 million people have contracted HIV and more than 25 million have died of AIDS-related causes (UNAIDS).

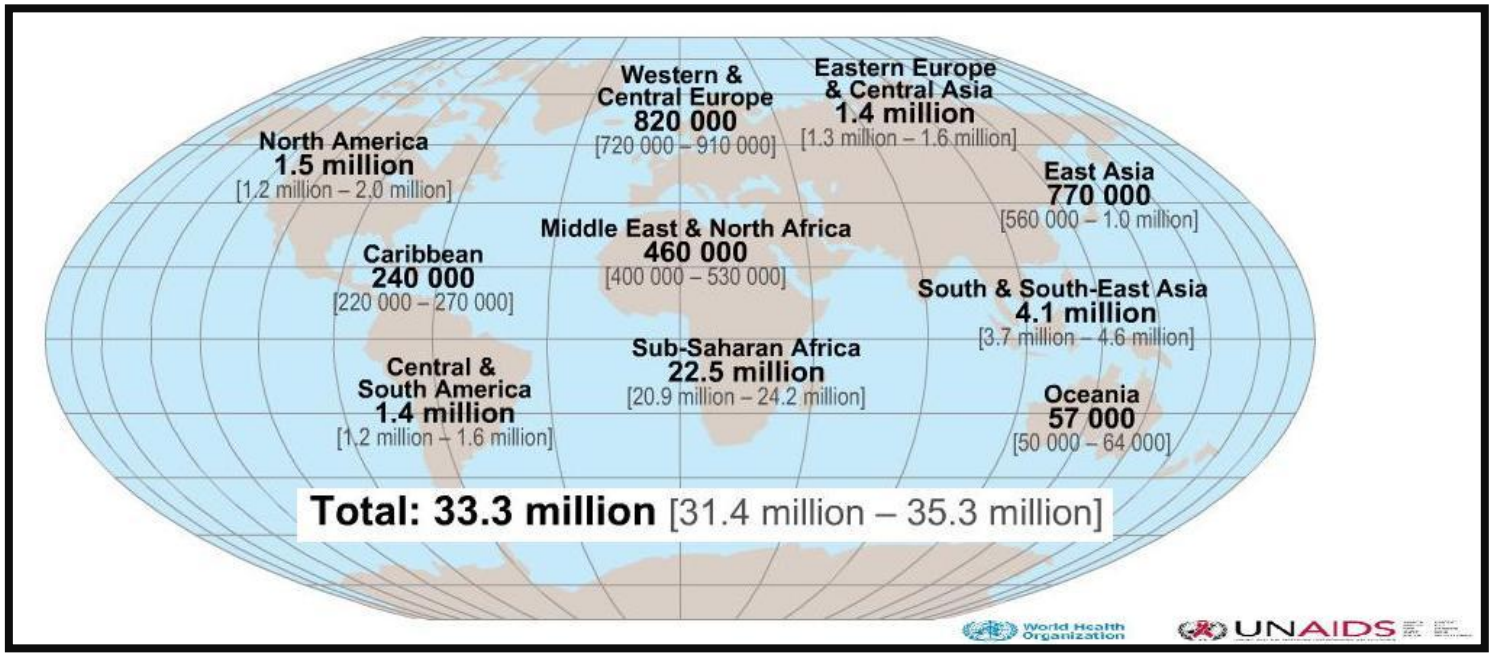

Figure 1.1 Estimated number of people living with HIV/AIDS in 2009 (UNAIDS & WHO report on global AIDS epidemic, 2010).

More than two-thirds, 22.5 million of all people living with HIV, live in sub-Saharan Africa-including 92 percent of the world's HIV-positive children. In 2009, an estimated 1.8 million people in the region became newly infected. South Africa has one of the highest infection rates in the world. Statistics South Africa's (StatsSA, 2011) reported the overall HIV-prevalence as 11 percent, one of the highest in sub-Saharan Africa and among the highest in the world. Approximately one-fifth of South African women in their reproductive ages are HIV positive. Life expectancy at birth had declined between 2001 and 2005, but had since increased partly due to the roll-out of antiretroviral therapy. More importantly, the report showed the elevation in the total number of people living with HIV from 2001 (4.10 million) to 2010 (5.24 million), represented in Table 1.1.

Table 1.1 HIV prevalence estimates of number of people living with HIV from 2001-2010, in South Africa *(adapted from statssa.gov.za).*

Year	Population % Women	15-49 years % Population	% of the total population	Total number of people living with HIV (in millions)
2001	18.7	15.4	9.4	4.10
2002	19.2	15.8	9.6	4.38
2003	19.4	16.1	9.8	4.53
2004	19.6	16.3	9.9	4.64
2005	19.7	16.5	10.0	4.74
2006	19.7	16.6	10.1	4.85
2007	19.7	16.7	10.2	4.93
2008	19.7	16.9	10.3	5.02
2009	19.6	17.0	10.3	5.11
2010	19.7	17.3	10.5	5.24

A related study stratified the HIV prevalence rate according to the different provinces in South Africa (Stats SA, 2011). KwaZulu-Natal (KZN), Mpumalanga and Free State had the highest HIV prevalence. The province of KZN showed an astounding prevalence of 15.8% averaged between 2002 and 2008. Adult mortality based on death notification data in South Africa: 1997-2004 revealed that the death rate among men aged 30-39 doubled, while that among women aged 25-34 more than quadrupled. In 2008, children aged between 0 and 9 years of age showed an increase in mortality to over 65,000 deaths when compared to older children (35,000). This elevation in child mortality number was largely attributed to HIV-1 Mother to Child transmission (MTCT). Prior to this report, another South African study stratified HIV incidence by children's age group revealing that children under the age of two had the highest number of new infections (Table 1.2). This high HIV incidence was attributed to increase in MTCT (Rehle et al., 2007).

Table 1.2 HIV incidence and number of new infections by age group, South Africa, 2005
Numbers rounded off to the nearest thousand (Rehle et al., 2007).

Age Group (years)	Weighted Sample (n)	HIV incidence per year %	Estimated number of new infections per year (n)
<2	44 513 000	1.4	571,000
2-14	13 253 000	0.5	69,000
5-9	4 820 000	1.1	49,000
15-24	9 616 000	2.2	192,000
15-49	24 572 000	2.4	500,000

1.1.2 HIV: Diversity, structure, replication and impact of antiretroviral drugs (ARVs)

Diversity

There are two types of HIV: HIV-1 and HIV-2. Both HIV-1 and HIV-2 are members of the retrovirus family which are characterized by viruses that are capable of copying RNA into DNA. Furthermore, HIV-1 and HIV-2 are lentiviruses. Lentiviruses are unique in comparison to other members of the retrovirus family in that, they a) deliver a large amount of genetic information into the DNA of the host cell, b) are able to replicate in non-dividing cells (one of the most efficient methods of transfer of genetic information) finally, lentivirus translates into the term 'slow virus' as these type of viruses slowly adversely affect the body.

HIV can be phylogenetically classified into four groups: group M (main); group O (outlier) and two new groups, N and P (Plantier et al., 2009). The classification of these groups is based on their discovery and likeness to simian immunodeficiency virus (SIV). Group O and Group P are rare HIV-1 strains and appear to be restricted to west-central Africa. The HIV-2 strain is very closely related to a strain of SIV found in sooty mangabees and African green monkeys (Hirsch et al., 1989, Li et al., 1989). The groups N and M are more infectious HIV-1 strains in comparison to HIV-2. More than 90% of HIV-1 infections belong to the HIV-1 group M, which can be divided into subtypes (Pieniazek et al., 2000).

To date, there are nine subtypes or clades. These clades have been assigned to letters A, B, C, D, F, G, H, J and K (Takebe, 2000). Certain clades are more prominent in some regions of the world in comparison to others. However, in regions where more than one clade is prevalent, clades can merge and viral recombination occurs. These mixtures of clades are referred to as circulating

recombinant forms (CRF's) (Gillies and Grossman, 1985). Figure 1.2 describes the distribution of the various clades worldwide.

- Clade A and CRF A/G predominate in West and Central Africa, with clade A causing much of the Russian HIV-1 epidemic (Gao et al., 1998).
- Historically, clade B was the most common clade/CRF in Europe however, other clades have emerged accounting for at least 25% of new HIV infections in Europe (Graf et al., 1998, Monaco et al., Steinrigl et al., Rangel et al., 2009, Lai et al.).
- Clade C is predominant in Southern and East Africa, India and Nepal. It has caused the world's worst HIV epidemics and is responsible for around half of all infections worldwide (Katzenstein, 2006, Khan et al., 2007, Jacobs et al., 2009).
- Clade D is generally limited to East and Central Africa. CRF A/E is prevalent in South-East Asia, but originated from Central Africa (Herbeck et al., 2007, Leitner et al., 1995).
- Clade F has been found in Central Africa, South America and Eastern Europe (Triques et al., 1999).
- Clade G and CRF A/G have been observed in West and East Africa and Central Europe (Ojesina et al., 2008, Ljungberg et al., 2002).
- Clade H has only been found in Central Africa; J only in Central America; and K only in the Democratic Republic of Congo and Cameroon (Vidal et al., 2000).

The HIV-1 and HIV-2 subtypes differ in transmission efficiency and pathogenicity (Levy, 2009). HIV-2 is also less virulent in comparison to HIV-1. HIV-1 clades differ by 20–50% (Gao et al., 1994) and are associated with varying disease progression (Ng et al., 2011, Kiwanuka et al., 2008, Cohen, 2000, Kanki et al., 1999). These clades may also differ in

immune responses elicited. Therefore, researchers worldwide are gathering data across different clades to develop an AIDS vaccine candidate that offers the broadest possible protection (Fig 1.2 (Kanki et al., 1999)).

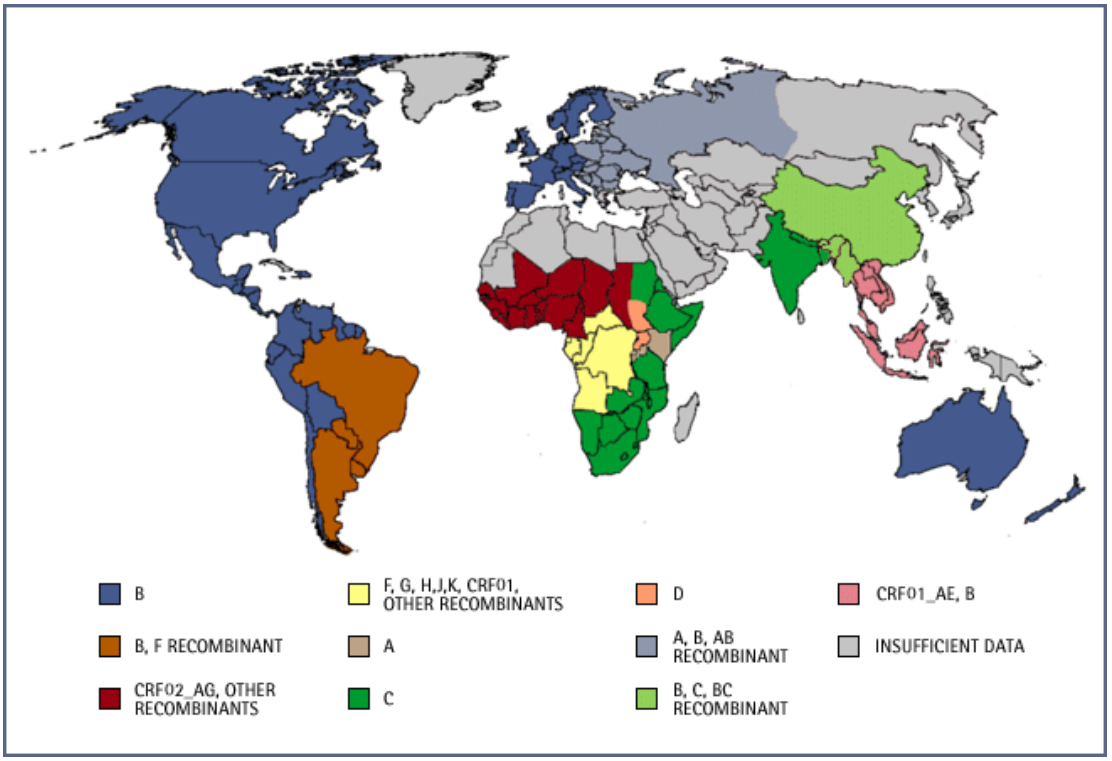

Figure 1.2 The distribution of the HIV clades and CRF's worldwide, Francine E. McCutchan, Henry M. Jackson Foundation (Rockville, Maryland).

Structure

HIV is different in structure from other retroviruses. Most virus sizes are defined by their internal structures however, as the HIV virions are variable in size, HIV is defined by its spherical morphology. The external surface structure of HIV virions (Fig 1.3) are formed when the capsid buds from the host cell, taking some of the host-cell membrane with it to form cone-shaped capsid particles. The external envelope membrane includes the two glycoproteins gp120 and

gp41. The genome encodes three structural proteins, three enzymes, and six accessory proteins (Turner and Summers, 1999, Andrea Rubbert, 2007).

HIV has two exact copies of single-stranded RNA (ribonucleic acid) as its basic genetic material which are located in the center of the organism. The RNA is tightly bound to the nucleocapsid proteins, p6 and p7, and enzymes such as reverse transcriptase and integrase. All of these are important for the development of the virion as shown by Figure 1.4 (Kuby, 2007). The virion particle also contains Vif, Vpr, Nef, p7 and viral Protease (Kuby, 2007).

HIV has nine genes that encode for its proteins as represented in Fig 1.3A (Hunt, Levy, 1993):

- Envelope: The Envelope is cleaved into proteins namely, gp120 which mediates viral binding to the cell, and gp41 which facilitates fusion of the viral and cellular membranes.
- Gag (Group-specific Antigen): Gag is an important core protein encoded by the HIV *gag* gene. Gag is the building block of the HIV particle core. HIV-seropositive individuals mount a significant immune response to Gag p24 supporting the use of Enzyme linked immunosorbant assay (ELISA) and Western blot assays for diagnosing HIV.
- Pol: Pol encodes several enzymes including reverse transcriptase and protease. Retroviral proteins are encoded by the *pol* gene. *Pol* and *Gag* are translated into large proteins which can be synthesized as protein precursors (polyproteins). These proteins are subsequently cleaved into final products that include reverse transcriptase, endonuclease/integrase and viral protease.

- Vif (Virion Infectivity Factor): The *vif* gene is essential in HIV infection. Vif is important during late stages of virus production and promotes stable reverse transcriptase complexes. Vif functions by suppressing innate anti-viral activities in T cells and macrophages. In the absence of Vif, HIV is not infectious in primary human T cells (De Cock et al., 2000).
- Tat (Trans-Activator of Transcription): The *tat* gene codes for the protein that enhances viral RNA transcription and promotes CD4+ T cell activation. Tat enables transcription as it binds to the HIV LTR region. It is a positive regulator of protein synthesis.
- Vpu (Viral Protein U): The Vpu protein promotes the proteolysis of the CD4 antigen of the host cell as virions are produced. The CD4 antigen and gp120 are synthesized in the endoplasmic reticulum of the same cell, making it more likely for them to bind one to another before reaching the plasma membrane. The Vpu protein prevents the degradation of this complex.
- Vpr (Viral Protein R): The Vpr protein promotes cell-cycle arrest and facilitates infection of macrophages, thereby contributing to the pathogenesis of HIV.
- Rev (Regulator of Virion expression): The Rev protein regulates viral RNA nuclear export and binds to a component found only in the mRNA for structural proteins (Gag/Pol/Env). Rev regulates the ratio of structural to non-structural protein (Tat/Rev) synthesis. Once Rev is expressed at elevated levels, structural protein synthesis increases.
- Nef (Negative Regulatory Factor): The Nef protein is synthesized early in infection. Nef promotes CD4 activation, enhances infectivity, prevents apoptosis and is associated with disease HIV progression. The translation of the Nef protein is a result of the virus' first influence on the internalization of the CD4 antigen from the surface of the cell.

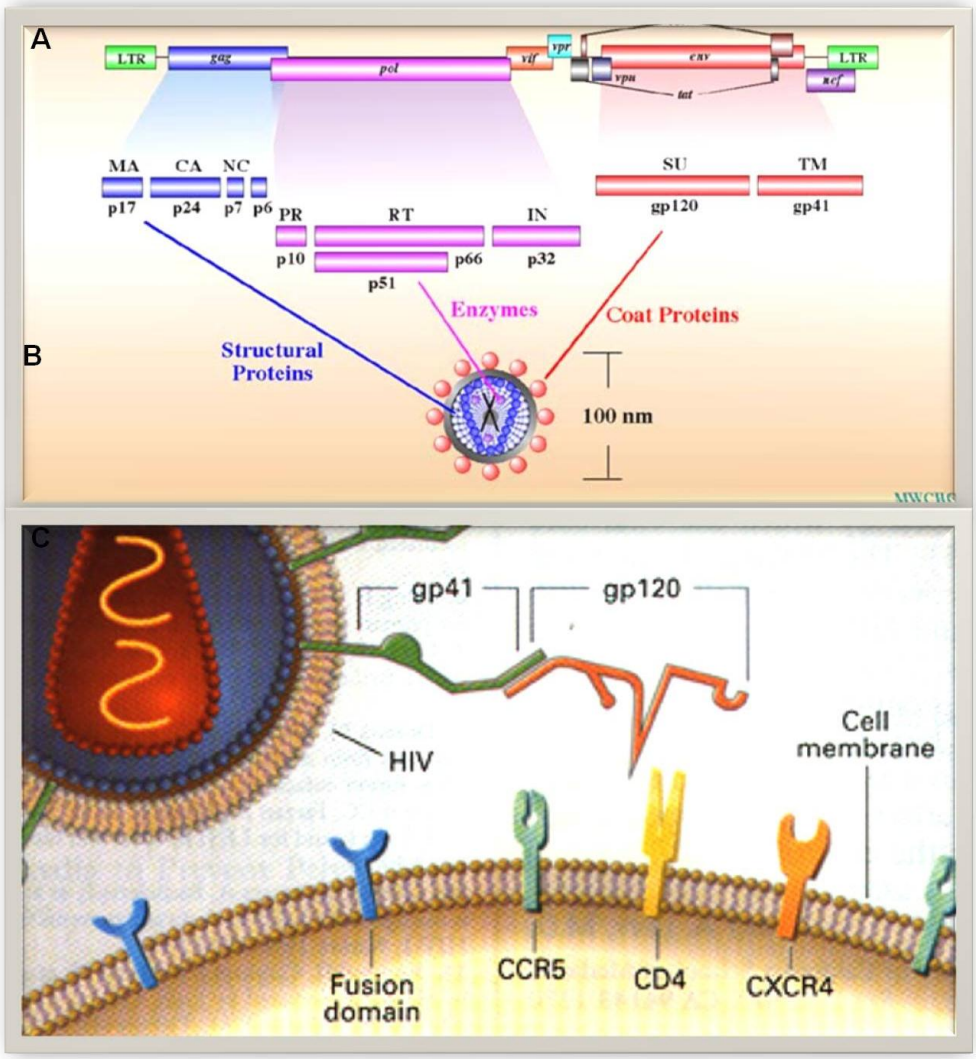

Figure 1.3 Schematic of A) HIV-1 genome, B) virion organization and C) viral attachment, indicating fusion receptors, the nine structural proteins, their encoding enzymes and coat proteins (adapted University of Chicago and Massachusetts medical society-http://hiv-aids-health.blogspot.com/2008_08_01_archive.html, 1996).

Replication

HIV begins its cycle by infecting susceptible host cells, preferentially white blood cells expressing the CD4 receptor. It binds to the CD4 receptor (Pudney and Song, 1994) which is present on the surface of T lymphocytes and macrophages. Both these types of white blood cells form a critical part of the immune system. During infection, HIV first attaches to the CD4 receptor and then to the chemokine co-receptors (CXCR4, CCR5) and other cell-surface proteins as indicated in Fig 1.3 B/C.

The gp120 protein on the HIV virion binds to CD4 and undergoes conformational change exposing the V3 loop, which facilitates binding to CCR5 (Rabehi et al., 1998). In individuals with the CCR5 delta 32 mutation (Martinson et al., 1997), the second step is impeded, therefore HIV cannot enter the macrophage after binding to CD4. However in normal individuals expressing CCR5, viral entry occurs unhindered. Upon entry, the genetic material of HIV (RNA) is released and undergoes reverse transcription into DNA (deoxyribonucleic acid) by the enzyme reverse transcriptase. The converted viral DNA enters the host cell nucleus. It is integrated into the genetic material of the cell by the presence of the enzyme integrase. Integration allows HIV to latently persist in the host cells for many years. However, activation of the host cells results in transcription of viral DNA into messenger RNA (mRNA). The new viral RNA forms the genetic material of the next generation of viruses. During translation the enzyme protease cleaves the viral RNA into useful viral proteins. The viral RNA and viral proteins assemble at the cell membrane and bud out of the cell forming a new fully functional virus. This cycle can be visualized in Figure 1.4 (Levy, 1998).

Impact of ARV on the lifecycle of HIV

The different phases of the HIV lifecycle can be interrupted through the use of antiretroviral therapy (ART). ART can be stratified into classes or groups based on the phase of the HIV lifecycle that they target. These groups are namely: entry inhibitors; nucleoside and nucleotide reverse transcriptase inhibitors; non-nucleoside reverse transcriptase inhibitors; protease inhibitors; integrase inhibitors and attachment inhibitors. Figure 1.4 demonstrates the inhibitors available, the phase in replication cycle that they inhibit and the sites of ART activity.

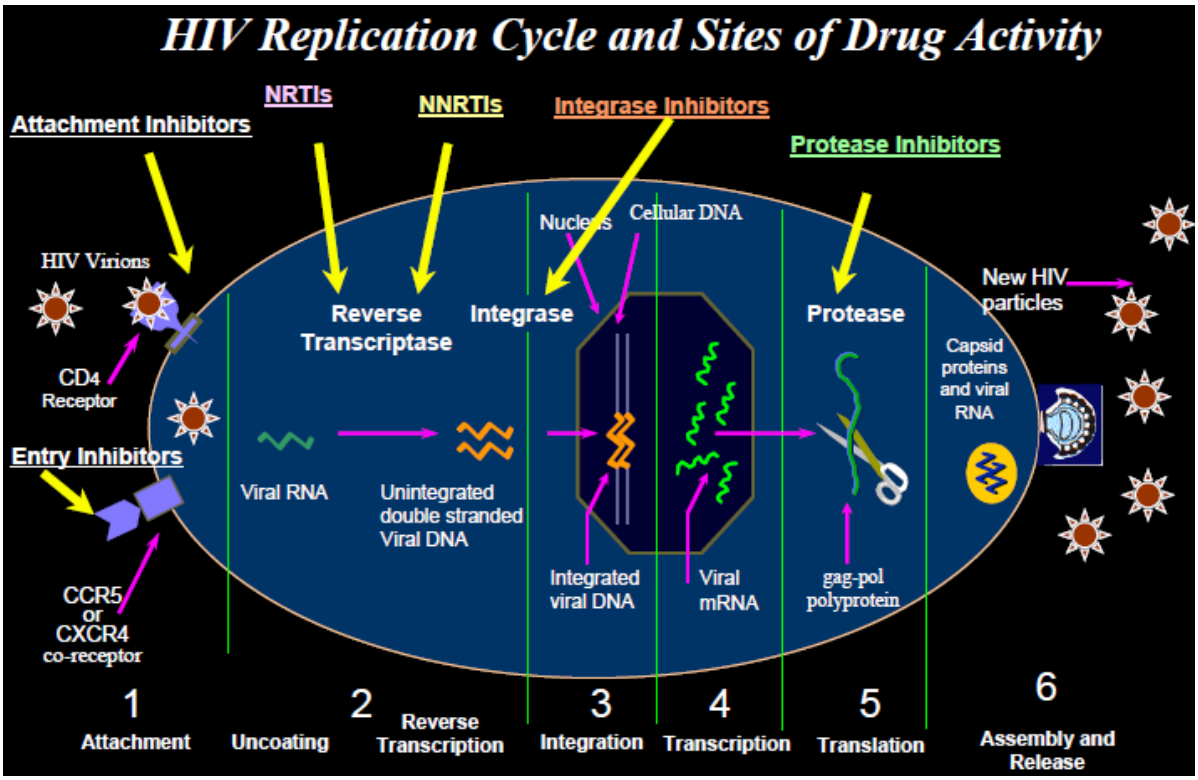

Figure 1.4 The HIV replication cycle (adapted (Levy, 1998); http://www.kaetc.org/slides/SEK-Dental-Study-Club-1-21-09.pdf).

1.2. Mother to child transmission (MTCT)

Transmission of HIV is dependent on various etiological factors, some of which are the concentration of HIV in bodily fluids and the nature of the host susceptibility, both at the cellular and immunological levels (Levy, 2009). HIV can be transmitted in many different ways. However, the most common ways that infections occur are through sexual intercourse, sharing of needles by drug users, blood transfusions and vertical transmission referred to as mother-to-child transmission (Jaffe et al., 1983).

Mother-to-child transmission of HIV occurs when an HIV infected pregnant woman transmits HIV to her unborn baby either before (intrauterine) or during birth (intrapartum/early postpartum), or after birth during breastfeeding (late postpartum). Although the mechanisms of *in utero* and intrapartum transmission are not well understood, it has been suggested that risk factors for *in utero* transmission probably include differences in cell mediated immunity and cells targeted. Additional, immunological or biological factors might protect the baby against infections in the uterus. Differences in maternal factors of viral load, CD4+ T-lymphocyte count may possibly play a role. Mechanisms underlying peripartum infection could be related to maternal viral load, by direct contact with blood or secretions.

1.2.1 MTCT epidemiology

The first cases of HIV/AIDS in children were described in 1982, where the link between infected mothers and their infants was established (CDC, 1982). Today, mother-to-child transmission of HIV still remains a global health problem. Acquisition of HIV is one of the main sources of

paediatric deaths, particularly in sub-Saharan Africa (Rehle et al., 2007). In 2009, WHO reported that around 400,000 children under 15 became infected with HIV, mainly through mother to child transmission, and most of these MTCT infections occurred in Africa. In the absence of ARV interventions, MTCT transmission rates range from 15-45% (WHO, 2011). This rate can be reduced to levels below 5% with effective treatment interventions (WHO, 2011). In 2010, the UNAIDS reported that MTCT infection rates had declined by 26% from 2001 to 2009 (UNAIDS, 2011). The South African (SA) AIDS conference recently held in Durban, KZN (International Conference Centre; 2011) reported a dramatic decrease in the rate of HIV mother-to-child transmission to 3.5% (HATP; 2011). Researchers propose the possible elimination of vertical HIV transmission by 2015 (Medical research council (SA), June 2011).

1.2.2 Intrauterine transmission

Of all the mother-infant infections in a non-breastfeeding population in the absence of ARV's approximately 30% occur as intrauterine and 70% intrapartum infections. Intrauterine HIV-1 infection is detected in fetal tissue by the presence of HIV on culture or PCR (polymerase chain reaction- a qualitative or quantitative laboratory method in which the genetic material, DNA or RNA, of a virus is detected and amplified). However, these types of tests are impractical. Intrauterine HIV-1 infection is more commonly defined as an infant with the first positive HIV-1 peripheral blood mononuclear cell culture and/or PCR at seven days of age or younger. Delivery of premature babies is a further risk of intrauterine transmission (Fawzi et al., 2001, Magder et al., 2005).

1.2.3 Intrapartum transmission

Intrapartum HIV transmission rate is as high as 60% in a non-breastfeeding, untreated infant population (Fleming et al., 2000, De Cock et al., 2000). Intrapartum infections occur when infants are diagnosed as HIV negative during the first seven days of birth and subsequently become HIV PCR positive within 28 days. Intrapartum infections occur when neonates are exposed to a large volume of HIV infected maternal blood during gestation and/or delivery. (Fawzi et al., 2001, Magder et al., 2005).

1.2.4 Transmission through breastfeeding

In the absence of ART, there is a 10-14% transmission rate during breastfeeding (Fleming et al., 2000, De Cock et al., 2000). Breastfeeding is an uncommon route of HIV transmission in developed countries, occurring at four to six weeks post-delivery. In resource poor settings however, the benefit of breast feeding outweighs the risk of HIV transmission (Coutsoudis, 2001, John-Stewart, 2007, Kakuma, 2002). The risk through breastfeeding is cumulative-the longer the HIV-infected mother breastfeeds, the greater the additional risk of transmission through breastfeeding. WHO and other research groups recommend exclusive breastfeeding for the first six months of life (Coutsoudis, 2001, John-Stewart, 2007, Kakuma, 2002). Flash heating and the use of donor breast milk has been shown to be feasible as a supply to infants in resource limited countries (Coutsoudis et al., 2011a, Coutsoudis et al., 2011b). The actual mechanism of HIV transmission by breast milk is not fully understood. However, as neonatal mucus membranes are not able to effectively prevent HIV infection and thus exposure to HIV in breast milk may result in viral infection directly through oral and gastric mucosa (Nduati et al., 2000). Cell-free and cell-associated virus transmissions may occur.

1.3 Clinical diagnostics and staging

The presence of the virus is evaluated by HIV antibody, PCR and p24 antigen tests. An antigen test detects the protein found on foreign particles that invade the body and trigger the production of antibodies in the body. When an individual immunity is compromised, their body produces specific antibodies to target the virus, which is detectable in an individual's bodily fluids. HIV antibody tests diagnose HIV infection by detecting such antibodies directed against HIV-1 antigens like Gag and Env. HIV antibody tests are accurate and reliable for routine diagnosis of HIV among adults. However, diagnosis by antibodies are challenging in infants, as these tests do not differentiate the maternally-derived antibodies from the infant-derived antibodies. Maternal antibodies can persist in the newborn's blood for up to 18 months. Therefore, rapid PCR DNA tests are used to diagnose HIV infection in the infant.

Once HIV infection is confirmed, the clinical course of HIV is monitored by absolute CD4+ T cell counts and viral load tests. The absolute CD4+ T cell count measures the number of disease fighting cells per milliliter of blood. It is an adequate indicator of the overall immune health of an individual and signifies the progression of HIV infection. The number of absolute CD4+ T cells determines whether an individual should initiate ART. In the developed countries WHO recommends initiation of ART as soon as an individual's absolute CD4+ T cell decline to below 350 cells/µl (EACS, 2009), and recently, similar guidelines have been adopted in SA (WHO, 2006). In adults, the absolute CD4+ T cell count indicates the degree of immune suppression. In children, the immune system is immature (Stewart et al., 1996, Ziegler et al., 1996) and the percentage of CD4+ T cells as a total of all lymphocytes is the best indicator of immune

suppression (WHO, 2007). The HIV viral load test measures the amount of the virus present in the bodily fluids of an individual and quantifies HIV RNA copies per mL. Both the viral load and the absolute CD4+ T cell count are used to assess HIV progression and suppression in individuals on ART (DOH, 2010).

The course of HIV infection is described as the duration from time of HIV acquisition to the development of opportunistic infections and ultimately (AIDS). HIV progression eventually leads to HIV pathogenesis (disease causing) (Levy, 2009). The stages of HIV infection differs clinically/symptomatically from one individual to another. The clinical course of HIV-1 infection generally includes three stages: primary infection, clinical latency and AIDS (Fig 1.5). HIV-infected individuals can remain asymptomatic during the primary infection phase (Hollander, 1988, McCutchan, 1990), but may show flu-like symptoms. Recently, many individuals have been found to exhibit symptoms in the primary stages of infection which manifests as rashes, fever and fatigue (Sudarshi et al., 2008). The clinical latency phase begins with a temporary decrease in viral load and an increase in absolute CD4+ T cells. However, due to the inability of the immune system to regenerate the absolute CD4+ T cells, a slow and constant decline in absolute CD4+ T cell count is observed. At this stage symptoms are moderately manifested. When the absolute CD4+ T cell count drops further the immune system is no longer capable of controlling the virus and other pathogens, resulting in AIDS-defining opportunistic infections.

The following guidelines are suggested for HIV management by The Department of Health in South Africa (DOH; 2011):

- Adults with an absolute CD4+ T cell count <350cells/mm^3 irrespective of their clinical stage.
- Nevirapine to be administered to HIV positive pregnant women at 28 weeks and mothers are placed in a PMTCT programme.
- Mothers who did not receive any ART before or during delivery to receive daily for at least six weeks, and continued for as long the child is under one year of age. Mothers are advised to exclusively breastfeed children for the first six months.
- Children aged between one and five with clinical staging three/four, or absolute CD4+ T cell percentage of 25 or below, or an absolute CD4+ T cell count < 750.
- Children aged 5-15 years with clinical staging three/four, or CD4+ T cell < 350.

The initiation of prophylaxis using ARVs can reduce morbidity and mortality in HIV infected individuals and may provide benefits in prevention of transmission.

Evidence suggests that 40% of untreated HIV-infected infants die before they reach the first year of life and, if left unidentified and untreated, up to 50-60% die by age two (Newell et al., 2004). HIV-related mortality in children can be attributed to the damage of the immune system, occurring in the first six months of HIV infection and/or HIV associated illnesses. In adults, HIV pathology is attributed to extensive damage of the immune system over time. However, other research has shown that the immune system can be impacted greatly during acute HIV and SIV infection (Grossman et al., 2006, Picker and Watkins, 2005).

1.4 The natural course and pathogenesis of HIV: Adult vs Paediatric

HIV is a rapidly spreading disease. HIV progression eventually leads to HIV pathogenesis (Levy, 2009). The stages of HIV infection differs clinically/symptomatically from one individual to another. Adults and children significantly differ in viral burden and therefore exhibit varying clinical outcomes (Holland et al., 2000, Resino et al., 2002b, Resino et al., 2002a). Vertically transmitted HIV progresses more rapidly in children, compared to HIV acquired in adults. (Ruiz Contreras, 1998, Walker, 1995, Cigielski, 1988). Unlike adults, in paediatric HIV-1 infection there is no rapid decline in viraemia after acquisition, and the viral burden in children remains elevated for the first year of life. This could be attributed to many reasons-the immune system of children is not fully developed; therefore they are particularly vulnerable to HIV and other common pediatric infections. HIV infected children cannot fight off infections as effectively as uninfected children or adults. Common infections in HIV-positive children include ear and sinus infections, sepsis, pneumonia, urinary tract infections, intestinal illness, skin disease and meningitis (Canosa, 1991, Scarlatti, 1996). In less developed countries, tuberculosis, diarrhea, and respiratory illnesses take precedence (Bobat et al., 1990, Jeena et al., 1998).

HIV-1 pathogenesis, chronic asymptomatic infection normally span from 3 to 20 years depending on the individual's rate of disease progression (Levy, 2009). About 10 to 15 % progress to AIDS within two to five years (rapid progressors), while long-term non-progressors (LTNP) , < 5% remain asymptomatic for at least 10 years (Curran et al., 1988). LTNP and elite controllers or slow progressors, gradually progress to AIDS. One of the main differences between a slow progressor and a rapid progressor is their ability to maintain a robust CTL response (Fig 1.5 (Goulder et al., 2001b, Altfeld et al., 2001, Hay and Rosenberg, 1998)).

Untreated HIV-1 infected children exhibit an elevated viral load within the first year of life. A slow decrease in viral replication is only visible over 2-3 years of age (Richardson et al., 2003, Shearer et al., 1997) which may be attributed to a mature strengthened, robust and more durable T cell response as demonstrated in Fig 1.6.

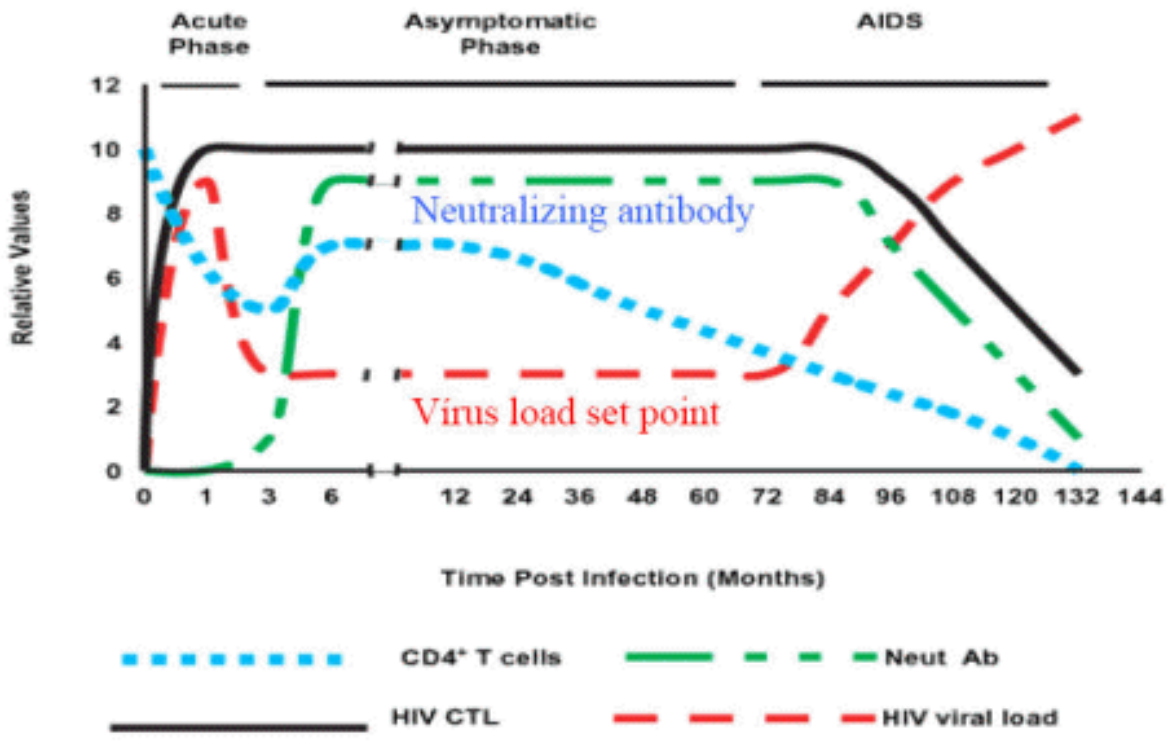

Figure 1.5 Schematic illustration of the course of HIV infection (Goulder et al., 2001b).

One of the main challenges in paediatric HIV-1 infection is the inability of the immune system to generate a sustainable immune response, particularly following vertical transmission. The CD8+ T cell response (CTL) has been documented to play a critical role in maintaining suppression of the virus (Borrow et al., 1994, Hay and Rosenberg, 1998, Kalams and Walker, 1994, Kaul et al., Papasavvas et al., 2003, Walker and Plata, 1990), particularly in primary infection (Fig 1.5/6). Accumulating evidence indicates that the immune system is potentially capable of effective

control of HIV infection and, events occurring in acute infection critically determine the final outcome (Paranjape, 2005, Borrow and Bhardwaj, 2008). The initial containment of HIV after acquisition impacts the progression of the patient to AIDS.

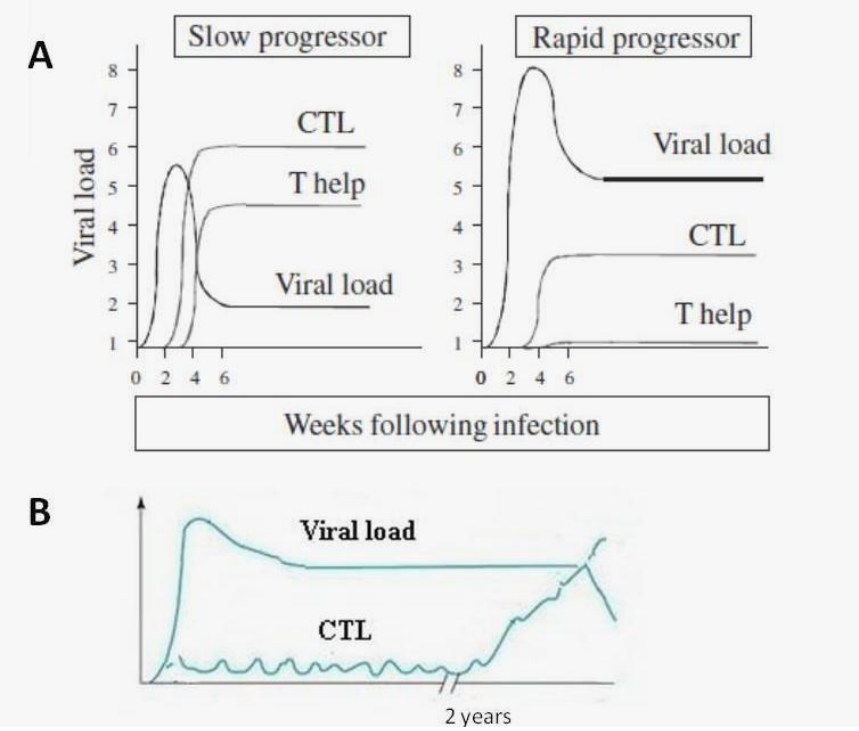

Figure 1.6 Schematic comparison of the kinetics of HIV disease progression in a) adults (adapted from (Paranjape, 2005, Borrow and Bhardwaj, 2008)) and b) children adapted (Goulder et al., 2001b).

Children fail at controlling HIV, firstly because of their immature immune system and inability to mount a significant immune response (Stewart et al., 1996, Ziegler et al., 1996). The next complication lies with the transmitted virus. As children share their parent's genetic make-up, the human leukocyte allele (HLA) inherited by a child may affect his/her capability to control the virus. The study of transmission pairs provides information of both donor and recipient virus.

Knowledge of the transmitted virus, sequence changes in the virus itself, innate genetic host factors, and host immune responses to HIV will direct our understanding of the correlates of protection and/or viral control.

Our review will cover the following in the sections to follow.

1) The virus and host interaction: host HLA and immune response, and virus escape/fitness,
2) The cells: immune activation (HLA-DR and CD38) and regulatory response: T cell immunoglobulin mucin protein (TIM-3); T regulatory cells (Tregs); Programmed death-1 (PD-1).
3) The host: immunogenetic regulatory factor (Interleukin-10).

1.4.1 HIV pathogenesis: The virus and host interaction

HIV-specific T cells play a crucial role in HIV control (Edwards et al., 2002, Kiepiela et al., 2007, Masemola et al., 2004, Novitsky et al., 2003). The negative correlation of strong HIV-1 specific CD4+ T helper (TH1) cell responses and viral load, indicates that CD4+ T helper cells play an important role in the immune response to HIV-1(Rosenberg et al., 1997). In particular, CD4+ T cells have repeatedly been described to impact viral control and disease progression, by secreting cytokines and other effector molecules that are essential in the orchestration of a potent antiviral CD8+ T cell mediated immune response (Rosenberg et al., 1997, Pitcher et al., 1999, Janssen et al., 2003, Shedlock and Shen, 2003, Snell, 1979).

HIV-specific cellular responses mediated by CD8+ T cells, are a major component of the host adaptive immunity and, have been implicated in the containment of viral load (Borrow et al.,

1994, Hay and Rosenberg, 1998, Kalams and Walker, 1994, Kaul et al., Papasavvas et al., 2003, Walker and Plata, 1990). CTL recognizes the infected cells through the signal produced by the processed portion of viral proteins on the infected cell surface. The T cell receptor (TCR) is somewhat promiscuous with respect to peptide binding as it is able to recognize and target multiple epitopes. CTLs with broad breadth can either be immunodominant or subdominant (Newman et al., 2002, Rolland et al., 2008) and if effective-should be considered in vaccine design.

The targeting of certain HLA restricted epitopes and viral protein regions successfully impacts viral set point (Kaul et al., Lyles et al., 2000, Mellors et al., 2007, Vidal et al., 1998), and have been associated with a dramatic decline in viremia in adults (Borrow et al., 1994, Altfeld et al., 2001, Goulder et al., 2001a, Streeck et al., 2009, Yang, 2004, Lindback et al., 2000). A similar outcome (Fig 1.6) was noted in paediatric studies (Feeney, 2004, Feeney et al., 2005, Goulder et al., 2001b, Goulder et al., 2001c, Thobakgale et al., 2009b, Thobakgale et al., 2007).

Recognition by CTL of epitopes derived from the HIV-specific protein coding genes, have been found to influence viral control. The conserved gene product, Gag has been associated with a decline in viremia in the primary and asymptomatic chronic period of infection. In adults, Gag-specific responses have been associated with low viremia, while Env-specific responses have been associated with higher viremia (Kiepiela et al., 2007). This suggests that in adult HIV-1 infection, immune responses to Gag play an important role in viral control while Env-directed responses may be associated with a detrimental outcome.

Very few studies have investigated cellular immune responses in paediatric infection or, the trends of protein recognition within mother-child pairs. Lohman *et al*. investigated HIV-1-specific IFN-γ responses over the first year of life. The infants demonstrated a diverse pattern of immune recognition and viral replication. Some infants completely lacked detectable responses while others demonstrated strengthening and broadening responses to HIV-1 over time (Lohman et al., 2005). In another study (Thobakgale et al., 2007), envelope specific responses were found to be frequently targeted in acutely infected infants, compared to chronically infected children. Gag-specific CD4+ T cell responses were undetectable during the earlier months of paediatric infection. The study revealed that some infants maintained suppression of the virus, partially attributable to CTL response (Thobakgale et al., 2007).

The HLA system is heterogeneous and is made up of a cluster of genes that influence immune functioning (McCusker and Singal, 1990, Scripcaru and Plesa, 1978). More than 300,000,000 genetically different individuals can be represented by HLA-A; -B and -C combinations (Bodmer, 1979, Bodmer and Bodmer, 1978). A distinct few HLA have been found to play a more influential role in clade C. HLA-B appears to have the strongest impact on viral load. More specifically, HLA-B*57; B*8101 and B*5801 had the strongest significant association with low viral load while HLA-B*5802 and HLA-B*1801 were documented to be associated with higher viral load (Fig 1.7 (Kiepiela et al., 2004)). CTL responses (epitopes) restricted by HLA-B alleles were further investigated in children. If the child or their mother possesses one of the protective HLA-B alleles, they progress slowly. Slow progressors tend to make more cellular T cell responses to Gag epitopes (Thobakgale et al., 2009a).

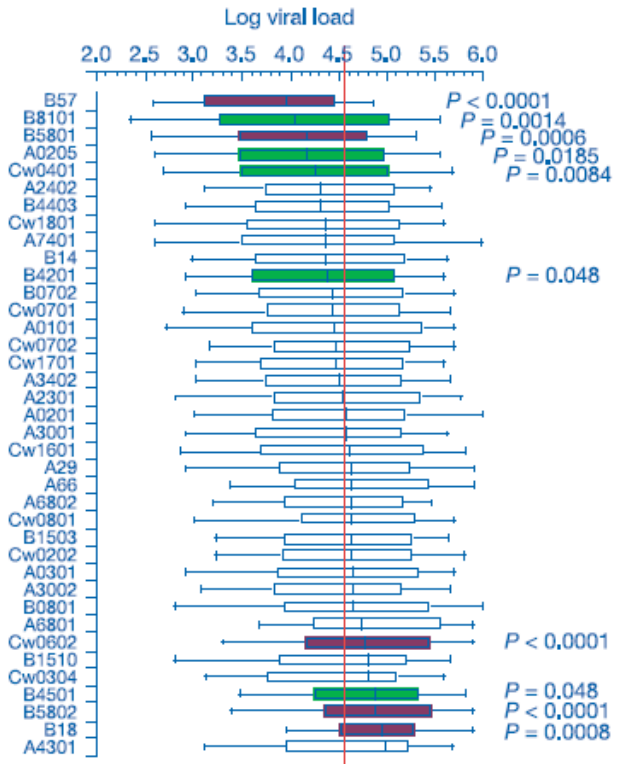

Figure 1.7 Impact of HLA-B on viral load (Kiepiela et al., 2004) Coloured bars denote epitopes previously found to significantly impact viral load.

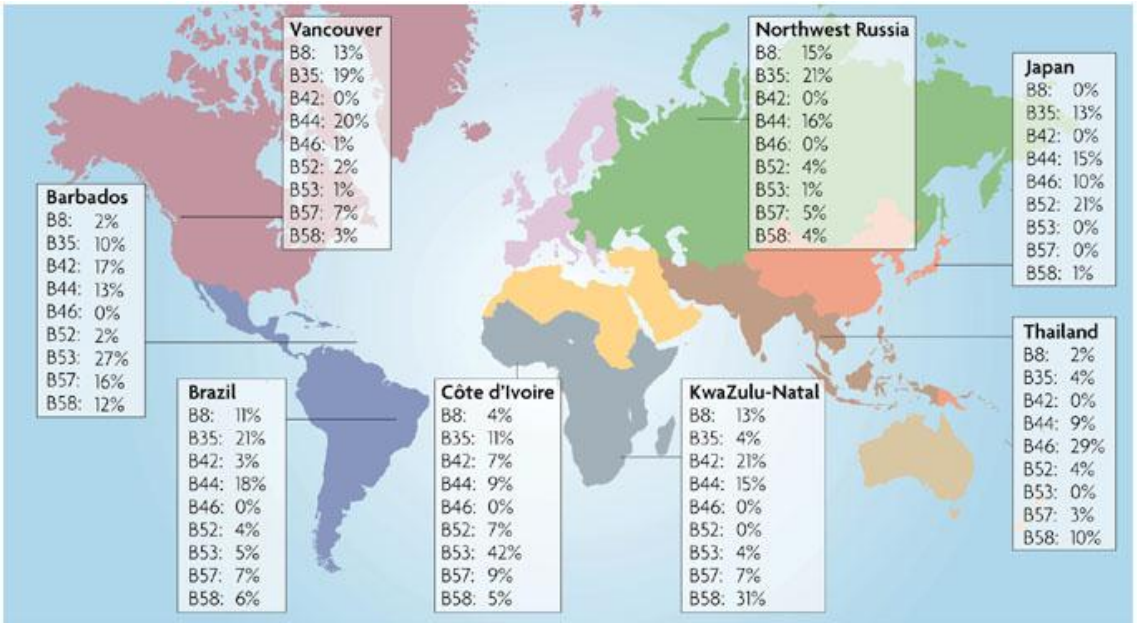

Figure 1.8 Global distribution of influential MHC class 1 HLA-B (Goulder and Watkins, 2008).

CTL studies provide information for the development of epitope-based vaccines. CTLs target different regions of the HIV genome across the different clades, posing variable targets for vaccine design (Newman et al., 2002, Cao et al., 1997, Cao et al., 2000). An effective global CTL-based vaccine is one likely to induce CTL responses that can suppress the virus across clades. HLA-B itself is similarly heterogeneous, varying in frequency across regions worldwide (Fig 1.8 (Goulder and Watkins, 2008)). Different HLA may be associated with strikingly different clinical outcomes. HLA-B*5801 has been shown to be associated with low viral loads while, HLA-B*5802 has been linked to high viral loads. (Ngumbela et al., 2008).

Transmission pairs provide information regarding donor and recipient virus. Knowledge of the sequence changes that subsequently occur during transmission, may direct us to protein sequences that play a role in viral containment. Mutational escape represents the major driving force for viral diversification. In adult HIV-1 transmission, transmitted escape variants may be associated with lower viral load in recipients. A significant association has been documented between the number of Gag escape mutations, and low viral loads in linked recipients. In HLA-B*5703 common Gag p24 mutations were found in epitopes IW-9; KF-11 and TW-10. Similarly, in HLA- B*5801, mutations were found in TW-10 and KF-9 (Goepfert et al., 2008). Accumulation of the three B*5703-associated Gag p24 escape mutations in succession, reduces viral replicative capacity in vitro (Crawford et al., 2009).

MTCT pairs represent an interesting cohort for investigation, as pairs share a partially matched genetic environment. The HLA inherited by a child will affect his/her capability to control the virus. In clade B investigations, HLA-B*27 has been strongly associated with low viral loads. In

adults, Gag specific CTL escape variants restricted by HLA-B*27, result in the loss of CTL recognition and disease progression. Accumulated CTL escape variants revert in the absence of the evolutionary pressure that originally selected the mutation (Leslie et al., 2004, Goepfert et al., 2008, Goulder et al., 2001b, Goulder et al., 2001c, Pillay et al., 2005).

HLA may drive the virus to a less fit state and thus influence clinical outcome. HIV-1 fitness is associated with, and contributes to, the rate of transmission and disease progression (Biesinger and Kimata, 2008, Chopera et al., 2008, Duda et al., 2009, Lal et al., 2005, Miura et al., 2009a, Miura et al., 2009b, Wright et al., Wright et al.). Viral fitness can be explored using a novel method that uses target cells that enable the entry of both CXCR4 and CCR5 tropic HIV-1 strains (Brockman et al., 2006, Wright et al., 2010, Wright et al.). This novel method measured replication capacity of the virus using an HIV-1 inducible green fluorescent protein reporter cell line (Brockman et al., 2006). The replication capacities of patient-derived Gag-protease recombinant viruses have been shown to positively correlate with viral load and negatively with absolute CD4+ T cell count in chronically infected individuals (Wright et al., 2010). However, the rate of decline demonstrated no influence over time. The protective allele HLA-B*81 seemed to play a significant role in driving sequence changes in the conserved regions of Gag, and may impact viral fitness (Wright et al.). These findings were associated to early HIV infection (Wright et al., 2011). Replication capacities did not correlate with viral load set points but were significantly lower in individuals with low viral load set points. Transmission or early Gag escape variants seem to result in reduced early CD8+ T cell responses and higher viral load set points. HIV disease progression is influenced by the ability to mount effective Gag CTL

responses, as well as the replication capacity of the transmitted virus (Wright et al., 2010, Wright et al., 2011).

Taken together, cellular immune responses provide at least partial success in mediating viral control, particularly when restricted by protective HLA. Certain HLA are associated with reduced viral replicative capacity. However, a better understanding of immune control in slow progressor survivors naturally containing viral load, may direct us to the underlying determinants of protective immunity. The broad scientific goal of our study was to explore HIV pathogenesis in genetically matched transmission pairs and/or children, in order to further contribute to understanding of the correlates of successful immune control in paediatric HIV-1 infection. The paediatric population is vulnerable to HIV owing to the immaturity of their immune system. In the absence of antiretroviral therapy, HIV-1 infected children die within the first two years of life (Shim et al., 2012, Bobat et al., 1999, Bobat et al., 1990, Goulder et al., 2001b, Newell et al., 2004, Ruiz Contreras, 1998, Prendergast et al., 2007, Mphatswe et al., 2007).

1.4.2 HIV pathogenesis: "The cells"

In neonates, components of the immune system are unable to function as maturely as the adult immune system. Understanding the difference in immunopathogenesis in children and adults, may provide further insights into the mechanisms leading to effective immune control. T cell immune response plays a role in immune control (Dyer et al., 2008, Kalams and Walker, 1994, Kaul et al., Liu et al., Paranjape, 2005, Rolland et al., 2008, Walker and Plata, 1990, Altfeld et al., 2002, Edwards et al., 2002, Huang et al., 2008, Prendergast et al., 2011, Ramduth et al., 2009, Wright et al., 2010, Wright et al., 2011) unless viral escape occurs (Autran et al., 1996,

Draenert et al., 2004). The sequence changes of escape variants make it hard for CTL to recognize and target them, often leading to the loss of immune control (Crawford et al., 2009, Thobakgale et al., 2009b). Many HIV infected infants seem to exhibit virus specific CD4+ and CD8+ T cell immune responses that appear to be functionally impaired and therefore, are unable to effectively control viral replication (Papasavvas et al., 2003). Previous adult cohort studies have shown that clinical outcome is further influenced by the functionality of T cells. In other words, the mere presence of T cells is not sufficient because, it is the level of functionality of these T cells that effectively mediate host immunity.

Functionality of T cell subsets can be investigated by the phenotypic characterization of lymphocyte subsets, and may contribute to the understanding of HIV disease pathogenesis and progression. There is variation in T cell frequency for T cells having phenotypes associated with T cell regulation and activation (Ssewanyana et al., 2009). T cell frequency differs during the course of infection, and may also differ between children and adults. The disparity between viral loads in infants compared to adults is considered to be a major contributing factor to observed clinical difference (Fig 1.6B). Unlike adults, the levels of plasma viremia in infected infants are persistently high, with declines seen only in the second year of life (Shearer et al., 1997). Approximately 25-40% of untreated infants will progress to AIDS within the first year of life (Newell et al., 2004). It is assumed that these neonates have ineffective T cell immunity, which may be attributed to T cell exhaustion, which can be measured by the increased expression of certain surface markers. However no studies have investigated this assumption in African children hence the need for our study.

Phenotypic evaluation of lymphocyte subpopulations can determine their maturational and functional capabilities. Activated specific CD8+ T cells present during virus replication frequently express activation markers CD38 and HLA-DR. CD38 is a surface marker of cell activation. In HIV negative individuals, CD38 is expressed in relatively greater numbers by naïve lymphocytes, while in HIV infected individuals, CD8+ memory T cells express more CD38 (Benito et al., 2005). HLA-DR is another cell surface receptor. The CD4+ HLA+DR CD38+ phenotype identifies activated lymphocytes and is increased in HIV-infected adults. However, the CD4+ T lymphocyte population may be different in children compared to adults. When lymphocyte activation in healthy infants are compared to that of HIV exposed uninfected infants, the proportions of activated CD4+ HLA-DR+ and CD38+ are increased in the exposed infants (Jennings et al., 1994).

CD4+ cells co-expressing CD38+ and HLA-DR+ have been found to be significantly higher in clade B HIV infected children compared to uninfected children(Plaeger-Marshall et al., 1994). When the CD4+ cells were compared between these uninfected and infected by age, the $CD4^+$ cells of the uninfected vs infected children exhibited a mean expression of 2 vs 6% for < 2 years of age, 3 vs 11% for 2-3 years, 2 vs 8% for > or = 4 years). There was a striking and significant increase in the proportion of CD8+ T cells co-expressing CD38 and HLA-DR with a mean of 5 vs. 25% for < 2 years, 10 vs 41% for 2-3 years, 6 vs 31% for > or = 4 years being exhibited for uninfected vs infected children, denoting the effect of immune activation on immune response (Gallagher et al., 1997, Plaeger-Marshall et al., 1994). Immune activation in children receiving ART may vary, depending on the viral load. The percentage of CD8+ CD38+HLA-DR+ T cells of children with VL ≥ 400 copies/ml was significantly higher than that of patients with VL < 400

copies/ml, suggesting that successful ART could significantly decrease immune activation in children (Jin et al., 2011).

T cell activation has also been found to be regulated by the suppressive ability of regulatory T cells (Treg). In humans, quantitative identification and viable enrichment of natural regulatory T cells are problematic. There exists a need to facilitate the analysis of these cells in disease states. Tregs are identified as being CD4+, with high levels of cell-surface expression of CD25, and can also be identified by their expression of the forkhead winged-helix transcription factor FOXP3 (forkhead box P3). Treg identification using these markers is not consistently accurate, as it is unable to uniquely define this specialized T cell subset in humans (Fehervari and Sakaguchi, 2004). Nevertheless, Tregs are critical regulators of immune tolerance. They prevent the generation of self-reactive T cells which are capable of generating autoimmune disease in the host.

Treg's suppressive ability may limit the magnitude of effector response, poorly contributing to viral control; but it may also suppress chronic immune activation, thus preventing disease progression. Immune activation during chronic HIV infection is a strong clinical predictor of death, and may mediate Treg depletion. Treg number is strongly correlated with both CD4+ and CD8+ T cell activation in adults (Eggena et al., 2004). In HIV-1 infected children, there has been an association between the number of activated CD8+CD38+ T cells and the viral load. In addition, the proportion of Tregs correlated positively with HIV-1 viral load and CD8+CD38+, but correlated inversely with CD4+ T cells. This suggests that the suppressive activity of Tregs may be sometimes unsuccessful in limiting immune activation (Freguja et al.).

CD4+ Tregs, mediated by the CTLA-4 pathway have been found to play a role in CD8+ T cell suppression *in vivo* (Sakaguchi et al., 2009). Stronger robust CD4+ and CD8+ effector T cell responses are important for control of HIV replication. After Tregs *in vitro* were sorted and removed, an increased HIV-specific effector T cell response was observed (Aandahl et al., 2004, Oswald-Richter et al., 2004, Weiss et al., 2004). Tregs may suppress the HIV-specific cytolytic antiviral response of CD8+ T cells (Kinter et al., 2007). Most of these studies examined the effect of Treg depletion on T cell proliferation and/or IFN- γ production, after stimulation with HIV antigens.

Another marker found to be associated with T cell regulation is the T cell immunoglobulin mucin (TIM) proteins. The TIM proteins are membrane surface glycoproteins that are expressed on T cells and exhibit common structural motifs. TIM-1 appears to be an activation molecule for all T cells. TIM-2 molecules function by negatively regulating T helper or TH2 immune responses, while TIM-3 is activated by galectin-9 and is specifically expressed on TH1 cells in both mice and humans. TIM-4 expression occurs in dendritic cells (DC) and promotes DC maturation, which plays an important role in the initiation of TH2 polarization.

Our investigation focused on the TIM-3, which to date has not been described in HIV-1 infected children and, of which no data is available in the context of HIV-1 clade C infection. TIM-3 has previously been associated with immune dysregulation of T helper cell responses (Hafler and Kuchroo, 2008, Koguchi et al., 2006, Kuchroo et al., 2006, Sakuishi et al., 2011, Sanchez-Fueyo et al., 2003, Zhu et al., 2011). TH1 cells represent the body's first line of defense against foreign microbes. When TH1 cells are activated, they help to initiate an attack and guard against

infection. However these TH1 cells can become counterproductively aggressive in their attack, leading to autoimmune diseases, and thus optimal TIM-3 function is needed (Anderson et al., 2007, Meyers et al., 2005, Sanchez-Fueyo et al., 2003). TIM-3 plays a role in controlling TH1 dependent immune responses by balancing aggressive responses, regulating immune tolerance (Hastings et al., 2009, Kuchroo et al., 2006, Sanchez-Fueyo et al., 2003). By increasing TIM-3 signal, the response of TH1 cells can be diminished. When the TIM-3 pathway is blocked and the TIM-3 signal decreased, the TH1 responses could be amplified, creating aggressive immune responses (Jones et al., 2008, Monney et al., 2002). In addition, TIM-3 signaling events exhibited differential expression on the innate naïve dendritic cells in comparison to adaptive immune cells (Anderson and Anderson, 2006).

TIM-3 fusion proteins could lead to hyper-proliferation and increased production on TH1 type cytokines, indicating that the function of TIM-3 may be to dampen effector responses (Sabatos et al., 2003). Conversely, a blockade of the TIM-3 pathway accelerated the onset of autoimmune disease in mice (Sanchez-Fueyo et al., 2003) The blockade of PD-1 together with TIM-3 pathway has been shown to rescue T cell immune responses in hepatitis (Golden-Mason et al., 2009, Callendret and Walker); leukemia (Zhou et al.) and HIV (Sakuishi et al.). These dually expressing T cells may also play a role in T cell exhaustion. No studies have investigated the role of TIM-3 in clade C HIV-1 infection, and even more so, in children, confirming the need for our investigation.

1.4.3 HIV pathogenesis-The host: Role of Interleukin-10

HIV pathogenesis and susceptibility is strongly reliant on host-pathogen interplay. How does genetic susceptibility translate into control or the lack thereof in children? Does genetic susceptibility in children affect immune responses as T cells and regulatory cells (Tregs) have been found to do? The immunoregulatory factor, interleukin-10 (IL-10), is a cytokine produced by lymphoid cells. IL-10 plays a key role in promoting viral persistence, possibly by inhibiting activation and effector functions of particular T cell and macrophage subsets (Brooks et al., 2008, Brooks et al., 2006). Thus immune activation appears to be a driving force in HIV pathogenesis, indicating a major role for IL-10 in this process.

Genetic polymorphisms within the IL-10 promoter region may influence the levels of IL-10 produced from various cells of the immune system (Ejrnaes et al., 2006, Lazarus et al., 2006, Turner et al., 2002). Our investigation focused on two single nucleotide polymorphisms (SNPs) found in the proximal promoter region at positions -1082 (A to G transition) and -592 (C to A transversion) of the 36-kDa IL-10 gene. The IL-10 gene has been mapped to human chromosome 1, between 1q31 and 1q32 and its promoter SNPs have been associated with different levels of IL-10 production (Kamali-Sarvestani et al., 2006, Lan et al., 2006) as shown in Fig 1.9.

IL-10 has also been shown to inhibit HIV-1 replication directly in macrophages and monocytes by blocking entry and/or post entry, and also suppressing and down regulating cyclin T1 and consequently Tat function (Wang and Rice, 2006). Lymphocytic choriomeningitis virus (LCMV) infection in mice is a model of chronic viral infections, and suggests that during the early phases of infection, IL-10 plays a critical role in the disruption of effector immune responses and viral

persistence (Brooks et al., 2006). The blockade of the IL-10 pathway allowed a DNA vaccine to further stimulate T cell responses and restore IFN-γ secretion, resulting in viral clearance. This suggests that a single immunoregulatory molecule can have a significant role in determining the outcome of persistent viral infection in mice (Brooks et al., 2006) There is a need for these studies to be extended to HIV-1, the most significant persistent viral infection in the human population. This will enable a better understanding of mechanisms that underlie immune dysfunction in HIV-1 infection.

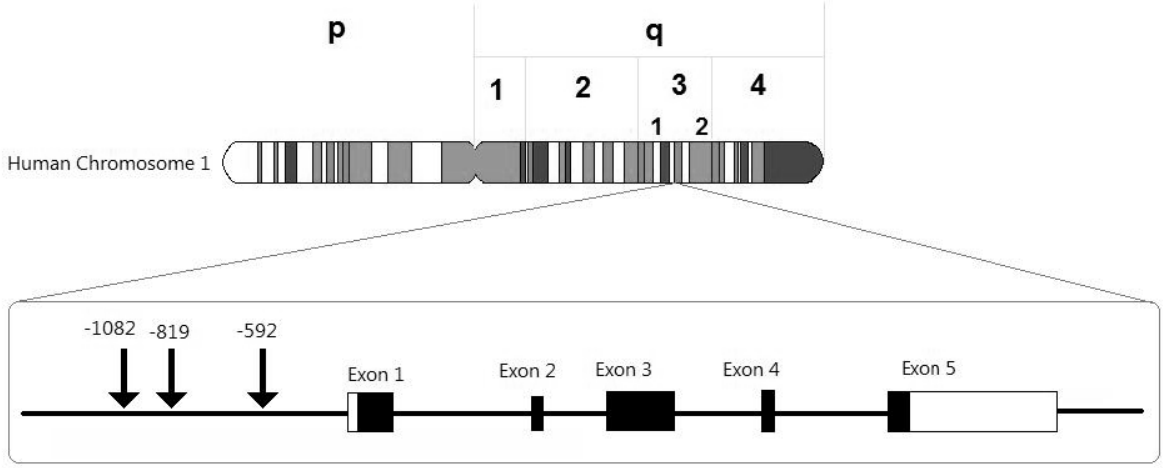

Figure 1.9 Location of -1082 and -592 SNPs on proximal promoter region of IL-10 gene on human chromosome 1 (adapted D. Naicker).

Polymorphic variants are likely to regulate susceptibility or resistance to HIV infection. Each of the genotypes of these SNPs is associated with different levels of IL-10 production (Kamali-Sarvestani et al., 2006, Lan et al., 2006). Polymorphisms associated with decreased IL-10 production have been associated with increased likelihood of HIV-1 acquisition, suggesting that high IL-10 production may reduce susceptibility to HIV-1 infection and protect against disease

progression. IL-10 genotypes do not appear to play a significant role in HIV pathogenesis. In HIV-1 CRF01_AE-infected northern Thais, IL10 promoter SNPs IL10-1082 (with the commonly expressed IL10-AA genotype and A allele) was not associated with CD4+ or CD8+ absolute T cell counts or viral load (Kingkeow et al.).

In African women at high risk for HIV-1 infection, a C to A transversion mutation at promoter position -592 (relative to the transcriptional start site) can be linked to increased likelihood of HIV-1 acquisition (Naicker D, 2009). On the other hand, A to G transition mutation at position -1082 showed a trend towards increased likelihood of HIV-1 acquisition. Thus genotypes linked to high IL-10 production may have a protective effect against HIV-1 infection. However, individuals with high IL-10 producer genotypes have exhibited significantly higher median plasma viral loads or lower absolute CD4+ T cell counts during the acute phase (≤3 months post infection). As HIV-1 infection progresses, the allele effect on median viral load or CD4+ T cell pattern reverses or may be lost (Naicker D, 2009). In carriers of IL-10 1082G, an allele linked to increased IL-10 production could be associated with decreased rates of mortality, and CD4+ T cells decrease may well be attenuated compared with non-carriers (Erikstrup et al., 2007). Taken together, these studies suggest that IL-10 promoter variants may influence susceptibility to HIV-1.

The IL-10 cytokine may have beneficial anti-HIV-1 effects. IL-10 promoter variants associated with different levels of IL-10 production may also affect markers of disease progression such as viral load and absolute CD4+ T cell counts, depending on the stage of infection. Pro-inflammatory cytokines have been found to induce transcription of latent HIV-1, and regulatory

cytokines play a role in suppressing type 1 cytokines. It is important to investigate functional levels of pro-inflammatory and anti-inflammatory cytokines; honing in on their levels of association with IL-10 promoter polymorphisms, clinical markers of disease progression, age and HIV-pathogenesis.

In acute HIV infection, up regulation of certain cytokines such as IFN-α, TNF-α (von Sydow et al., 1991) and IL-10 (Norris et al., 2006) have been reported. Furthermore, hepatitis studies revealed that a broad array of cytokine expression lead to eventual viral clearance in a majority of infected participants (Stacey et al., 2009). Recently, the association between plasma cytokine concentrations during primary acute infection, with clinical markers of HIV disease progression, such as absolute CD4+ T cell count and viral load measurements, has also been described (Roberts et al., 2010). Cytokines IL-12p40, IL-12p70, IFN-γ, IL-7 and IL-15 were able to predict 66% of the variation in viral load set-point 12 months post infection. Lower viral loads were significantly associated with IL-12p40, IL-12p70 and IFN-γ, whereas IL-7 and IL-15 were associated with a higher viral load. Moreover, IL-7 was associated with more rapid CD4 loss (Roberts et al., 2010). The investigators Jones *et al.*, 2005 are one of the very few investigators to have attempted to profile cytokines and their levels of production in HIV-1 infected children. Cytokine production, CD4+ T cell count and viral loads were monitored in HIV-1 infected children in Hong Kong (Jones, 2005) however, they did not find any conclusive correlations.

1.5 Study rationale: Hypothesis and specific aims

Our investigation was initiated in 2006 under previous ART guidelines when ART rollout was not widespread. The ART guidelines have since been revised (WHO/DOH; 2011) to ensure that

all pregnant mothers and HIV-1 infected infants are started on highly active antiretroviral therapy (HAART) to improve clinical outcomes, as was demonstrated by the CHER study (Violari et al., 2008). Unfortunately the limitation of resources in less developed countries still daunts HIV related MTCT. Possible eradication of paediatric HIV-1 infection lies in the development of an infant vaccine that can be administered at birth, providing protection from infancy, through the adolescence period, and into adulthood. Our investigation aimed to provide information that could aid and advance vaccine development.

The broad scientific goal of the study was to explore HIV pathogenesis in genetically matched transmission pairs, in order to further contribute to understanding of the correlates of successful immune control in paediatric HIV-1 infection.

1.5.1 CTL-Virus interplay

1.5.1.1 Hypothesis:

HIV-specific CTL responses in mothers or infants restricted by the protective HLA-B*27, B*5703, are associated with slow progression. We hypothesized that if a child carrying a protective allele is able to mount significant CTL responses, and/or if escape occurs-which lowers viral fitness-then the child is more likely to progress slowly. However, viral control may be lost if escape results in abrogation of immune responses, if the immune response was the actual underlying mechanism involved in viral containment. Alternatively, we hypothesize that a child will not mount an immune response because they inherit an escape variant, and therefore there will be no control, particularly if the child shares protective alleles with the mother. We tested these hypotheses in the following specific aims:

1.5.1.2 Specific Aims:

1. To describe the CTL responses elicited within various regions of the HIV proteome targeted by HIV-infected treatment naïve children using ELISpot assays.
2. To describe HLA restricted sequence changes and viral fitness of donor and transmitted virus within selected HLA matched cases.

1.5.2 T cell activation and regulation

1.5.2.1 Hypothesis:

Generalized T cell activation is the hallmark of adult HIV-1 infection, and is associated with CD4+ T cell loss and HIV disease progression. We hypothesize that T cell activation is elevated in HIV-1 infected infants, and contributes to T cell exhaustion and failure to control HIV viremia. We further hypothesize that regulatory T cell frequencies are elevated in HIV-1 infected infants, and can impact generalized immune activation, but also suppress HIV-1 specific immunity. We tested these hypotheses in the following two specific aims:

1.5.2.2 Specific Aims:

1. To investigate the expression of the markers of T cell exhaustion TIM-3 and PD-1 and their relationship to markers of disease progression such as absolute CD4+ T cell count and HIV-1 plasma viral load in a cohort of HIV-1 infected, exposed uninfected infants, ARV treated.
2. To assess the impact of regulatory T cell frequencies and T cell activation measured using multi-parameter flow cytometry on markers of disease progression such as absolute CD4+ T cell count and HIV-1 plasma viral load in a cohort of HIV-1

infected, exposed uninfected and ARV treated infants.

1.5.3 Host-pathogen interplay

1.5.3.1 Hypothesis:

Polymorphisms associated with increased IL-10 production are associated with a decreased risk of HIV-1 acquisition in infants-either after exposure, *in utero* or intrapartum. Furthermore, during paediatric HIV-1 infection, individuals possessing IL-10 polymorphisms associated with increased IL-10 production will display higher viral loads and lower absolute CD4+ T cell %. We tested these hypotheses using the following specific aims:

1.5.3.2 Specific Aims:

1. To assess the impact of genetic polymorphisms in the IL-10 promoter gene on the risk of HIV-1 acquisition among children and the risk of transmission among mothers.
2. To describe the associations between IL-10 promoter variants related to the breadth and/or magnitude of T cell immune responses.
3. To assess the impact of long term biomarkers of disease progression, such as absolute CD4+ T cell count and HIV-1 plasma viral load on cytokine levels, within groups of age-matched children.

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CHAPTER TWO

The influence of CD8+ T cell responses on HIV-1 pathogenesis in clade C infected treatment naive African mother-child transmission pairs.

ABSTRACT

Background: MTCT represents a significant proportion of HIV/AIDS burden in KZN, South Africa. The failure of the STEP vaccine trials and the partial success of the Thai trial have increased the urgency to extend HIV-1 vaccine development and design initiatives. An effective T cell vaccine against HIV-1 may need to elicit strong virus-specific T cell responses against functionally essential regions of viral proteins where escape mutations may result in significant fitness cost to the virus. HIV infected mother-child pairs have a partially matched genetic background and therefore offer a unique opportunity to study viral adaptation to host immune pressure. This study aimed to 1) characterize specific HIV-specific CTL responses in HIV-1 infected children 2) describe HIV-specific CTL responses in B*27 and B*57 and to characterize pathways of immune escape in mother-child transmission pairs.

Materials and Methods: One hundred and nineteen mother-child pairs were recruited from clinics in Durban, South Africa of which only 60 mother-child pairs met the inclusion criteria for the current sub-study. Ages of mothers ranged from 17-42 years while children ranged from 0.3-11 years. During follow-up, viral load measurement and absolute T cell CD4+ counts were performed at 3-6 month intervals. High resolution human leukocyte antigen (HLA) typing and comprehensive screening of HIV-1 specific CTL responses was performed by an interferon-gamma enzyme-linked immunosorbent spot (ELISpot) assay using pools of overlapping peptides (18mers) spanning the HIV genome. Subsequently, we studied HIV-1 clade C Gag from mother-child transmission pairs. A viral replication assay using an HIV-1-inducible green

fluorescent protein reporter cell line was used to investigate replication capacity of plasma-derived Gag-protease from the virus of selected transmission pairs.

Results: HIV-1 plasma viral load of mothers ranged from 1,170 to 750,000 copies/mL, while children ranged from 1,320 to 1,000,0000 copies/mL. Children exhibited a CD4+ T cell percentage ranging between 8-57% (median 24%). The viral regions targeted by children in order of frequency of expression were Nef (49%), Gag (17%), Env (14%) and Acc/Reg/Pol (6%). Mothers presented with a broad range of CTL responses which were predominantly different from children. CTL responses restricted by the possession of selected protective HLA alleles (HLA-B*2705 and B*5703), viral sequences and viral replicative capacities were described in 4 mother-child pairs. Magnitude of CTL responses was not associated with lower viral loads among mothers or children in this cohort.

Conclusion: HLA, CTL or viral fitness alone are not individual players as correlates of successful control.

Key words: CTL; viral proteins; CD8+ T cells; HLA; paediatric; children; HIV-1 and disease progression.

2.1 INTRODUCTION

The UNAIDS has reported remarkable progress in combating infection rates among children born to mothers living with HIV over the past decade (UNAIDS, 2010). Infection rates have declined by 26% from 2001 to 2009 (UNAIDS, 2011). However, despite HIV-1 PMTCT efforts in reducing new infections in children, there were an estimated 370,000 new paediatric HIV infections in 2009, with almost 65% HIV-1 exposed infants not receiving PMTCT drugs.

There is urgency for more to be done to prevent mothers and babies from morbidity and mortality incurred by HIV-1 infection. Adults and children differ significantly in viral burden and clinical outcome (Holland et al., 2000, Resino et al., 2002b, Resino et al., 2002a) as HIV-1 infection progresses rapidly in children compared to adults (Ruiz Contreras, 1998, Apostolopoulos et al., 1995, Takiguchi, 1994). To date, HIV immunopathogenesis in children remains poorly understood. Much of our understanding of paediatric infection and pathogenesis are drawn from adult studies and the underlying mechanisms may not always hold for paediatrics.

In **HIV-1 infection**, there appears to be a rapid decline in viraemia after HIV-1 acquisition. A slow decrease in viral replication is only visible over 2-3 years of age (Richardson et al., 2003, Soh et al., 2003). In the absence of antiretroviral treatment, **children** typically progress to AIDS or death within the first 2 years of life (Goulder et al., 2001b, Thobakgale et al., 2007). However, a minority group of children seem to progress relatively slowly to HIV disease, which is predicted to be attributed to a stronger and durable immune response. Several vaccine efforts are directed towards enhancing the induction of virus-specific T cell responses that are directed towards the early suppression of HIV. More so, the eradication of paediatric HIV-1 infection lies in the development of an infant vaccine that can be administered at birth that

would theoretically provide protection from infancy, through the adolescence period, and onto adulthood.

Virus-specific cytotoxic T lymphocyte (CTL) or CD8+ **T cell responses** play a significant role in viral control and the emergence of these responses in primary HIV-1 infection is associated with a decline in viremia (Rosenberg, 2000). Of the nine HIV proteins, Gag directed CTL responses have been largely associated with a beneficial role in HIV-1 containment (Zhou et al., 1993, Anderson et al., 1992, Geldmacher et al., 2007, Otto O. Yang, 2011, Kiepiela et al., 2007, Rolland et al., 2008). The CD8+ T cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in HIV seropositive participants (Anderson et al., 1992). The breadth of these Gag-specific responses is found to be associated with decreasing viremia (Kiepiela et al., 2007, Rolland et al., 2008). Gag-specific responses have been shown to be most associated with low viremia, the Env-specific responses have been associated with higher viremia in a clade C infected cohort in KwaZulu-Natal (Kiepiela et al., 2007) but this influence have been recently documented to not be intrinsically inferior to Gag responses investigated across several cohorts (Chen et al., 2011). Taken together these studies suggest that in adult HIV-1 infection, immune responses directed to Gag play an important role in viral control.

CTL recognize short peptide proteins presented by corresponding **HLA** (A, B and C), which are encoded by the major histocompatibility complex (MHC) (Townsend and Bodmer, 1989). Selected HLAs have been documented to be influential in viral containment more than others (Crawford et al., 2009, Goulder et al., 1997, Kiepiela et al., 2007, Kiepiela et al., 2004, Thobakgale et al., 2009b, Winchester et al., 2004). The heterogeneous HLA system contains a cluster of genes that influence immune functioning. More than 300, 000,000 genetically different individuals can be formed by HLA-A, B and C combinations (Bodmer, 1979, Bodmer

and Bodmer, 1978). HLA-B has the most influential impact on viral load (Leslie et al., 2010, Serwanga et al., 2009, Salgado et al., 2011, Kiepiela et al., 2007, Leslie et al., 2004). For example HLA B*57; B*8101 and B*5801 had the strongest association with low viral load while HLA B*5802 has been documented to be associated with higher viral load in clade C HIV infection (Kiepiela et al., 2004). Moreover, certain HLA alleles documented to be more associated with slower disease progression, tend to preferentially target the HIV Gag capsid (Borghans et al., 2007).

The presence of multiple subtypes of HIV-1 worldwide has created further challenges in the investigation of viral control of HIV-1 infection. HLA that may have been found be influential in certain clades may not be as influential in other clades. Investigation of HLA alleles associated with protection in both adults and children will benefit vaccine research. In clade B investigations HLA-B*27 has been strongly implicated in viral load associations. HLA B*27 has been shown to be associated with slow disease progression and accumulation of **Gag** escape variants result in loss of viral control in both adults and children in clade B infection (Leslie et al., 2004, Goepfert et al., 2008, Goulder et al., 2001b, Goulder et al., 2001c, Pillay et al., 2005). However the role of HLA-B*27 has not been assessed in clade C HIV-1 infection and hence we attempted to describe this role.

Adult studies suggest that protective HLA molecules associated with lower HIV-1 viral load and slow disease progression tend to present epitopes that predominantly target Gag. Very few studies have investigated cellular immune responses in **paediatric infection** and trends of protein recognition within mother-child pair context. These studies have been hampered by the difficulty of obtaining paediatric samples and by the early introduction of HAART (Highly Active Anti-Retroviral Therapy) to prevent mother-to-child transmission in developed

countries. Therapy naïve children demonstrate that HIV-specific CD8+ T cell immune responses are detectable during early life, however they may not offer immediate clinical benefit in the majority of infants (Lohman et al., 2005, Thobakgale et al., 2007). Furthermore, the role of CD4+ T cells in HIV-1 infection is controversial showing either lack of adequate responses or effective CD8+ T cell maintenance during early infancy (Feeney et al., 2003, Huang et al., 2008, Thobakgale et al., 2007, Wasik et al., 2000). More recently Gag-specific CD4+ T cell responses were detectable and correlated inversely with viral load in older children (Prendergast et al., 2011, Nqoko et al., 2011). It is encouraging to note that a small group of children could spontaneously control the virus without ART. Studies on rare controller cases are needed to further understand factors that mediate control in children.

Mother-child pairs represent a partially genetically matched environment and hence are a valuable cohort to study. These transmission pairs offer an opportunity to gather valuable information because unlike most studies, the source of the transmitted virus is known and can also be further studied. Knowledge of the sequence changes that subsequently occur during transmission will direct us to epitopes that may or may not impact viral control. The biggest obstacle to CTL based vaccine initiatives remains HLA associated polymorphisms called **CTL escape variants**. In the first years of life, many children are able to mount functional HLA driven immune responses that are able to influence immune escape (Feeney et al., 2005, Tang et al., 2010). The transmission of these HLA–B associated escape variants has been documented to be correlated to a lower viral load in recipients (Goepfert et al., 2008). Successive control seems to be significantly attributed to selective cellular responses within the protective HLA-B*27, HLA-B*57 and HLA-B*5801 whom have been implicated with slow progression to disease. Protective Gag driven HLA class I alleles select Gag escape mutations that benefit the mother/adult and child in clade C HIV-1 infection. Slow progression in children

have been significantly associated with the mother or a child possessing a protective allele (B*5702, B*5703, B*5801, or B*8101) (Thobakgale et al., 2009a). If the mother possesses a protective allele, she may transmit Gag variants that reduce viral fitness to children who lacked the HLA allele. Subsequently, the accumulation of certain mutations in a specific order seem to further reduce viral replicative capacity (Crawford et al., 2009); denoting the interplay between the cellular and viral components.

HLA may also drive the virus to a less fit state and thus influence clinical outcome. Studies have associated **HIV fitness** with the rate of transmission and disease progression (Duda et al., 2009, Biesinger and Kimata, 2008, Brockman et al., 2007). HIV evolves with its host in the attempt to avoid different immune responses, manipulating the fitness of the virus. The fitness of the virus similarly contributes to immune control or disease progression (Brockman et al., 2007). Viral fitness can be explored using a novel method that uses target cells that enable the entry through both CXCR4 and CCR5 tropic HIV-1 strains. The growth kinetics of a HIV-1-inducible green fluorescent protein reporter cell line GFP is measured to describe the replication capacity of the virus in clade B infection (Brockman et al., 2006). In chronically infected clade C participants, viral replication capacity (utilizing similar methodology) correlated positively with viral load and negatively with CD4+ absolute T cell count (Wright et al., 2010). However, no correlation was noted with viral loads in patients with early infection (Wright et al., 2011).

The precise location of the mutation in the viral genome sequence may influence the efficacy of certain CTL specificities to reduce viral fitness, hence relevant to vaccine design (Goulder and Watkins, 2004). The present study aims to try to identify the determinants of viral control so as to be able to provide information that could aid in therapeutic and ultimately protective

vaccine design. We aimed to describe HIV-specific CD8+ T cell responses, within the nine regions with the HIV genome, targeted by HIV-1 infected treatment naive children from a sub-group of a cohort of treatment mother-child pairs using ELISpot assays. We hypothesized that HIV-specific CTL responses restricted by the protective HLA- B*5703 and HLA-B*2705 will be associated with slow progression and/or a less fit virus in children. We also describe four case series of HLA-B*5703 and HLA-B*27 restricted sequence changes and viral fitness of donor and transmitted virus within selected HLA matched cases.

2.2 PARTICIPANTS, MATERIALS AND METHODS

2.2.1 Description of cohort and study sites

A cohort of treatment naive HIV-1 infected mother child transmission pairs was established in Durban, KwaZulu-Natal, to understand T cell immunity and HLA genetic associations with viral control. The study began recruitment in 2006 prior to the initiation of current PMTCT guidelines to prevent HIV-1 transmission from mothers to children. The guidelines at the time required that single dose of nevirapine be given to HIV-1 seropositive mothers during the last trimester of pregnancy and during labour. The infant received a single dose of nevirapine within 48 hrs of birth, according to the HIVNET-012 protocol, as previously described (Guay et al., 1999, Jackson et al., 2003).

A subgroup of 60 mother-child pair participants (Fig 2.1) from Zulu/Xhosa ethnic origin, with clade C HIV-1 infection were recruited at clinics in KwaZulu-Natal, Durban, South Africa. The recruitment clinic sites included King Edward Hospital; McCord Hospital; Saint Mary's Hospital and Prince Mshiyeni Hospital. At the study sites, transmission pairs were screened and mother-child pairs who met the inclusion criteria of HAART naïve mother-child treatment pairs and a child with PCR positive DNA results from birth were recruited. The inclusion criteria were further revised to include an age restriction of children aged three months of age having a confirmed positive DNA PCR result. Mothers and their children who were on ART were excluded from the study. Mothers provided written informed consent for their and their children participation into the study. For the case study, four transmission pairs in which either the mother or child carried HLA-B*27 or B*57 to study, and/or pairs which retrospectively sparked interest (i.e. family or siblings etc) as case studies. The research protocol was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, and the participating hospital review boards.

2.2.2 HIV Pathogenesis Programme (HPP)

All research (Fig 2.1) was conducted at the Doris Duke Medical Research Institute, Nelson Mandela School of Medicine (http://hpp.ukzn.ac.za) except for the Gag sequencing assay which was carried out at the Department of Pediatrics, Nuffield Department of Medicine, Oxford, UK. All assays except for the Gag sequencing assay, routine viral loads and CD4s were carried out by the candidate.

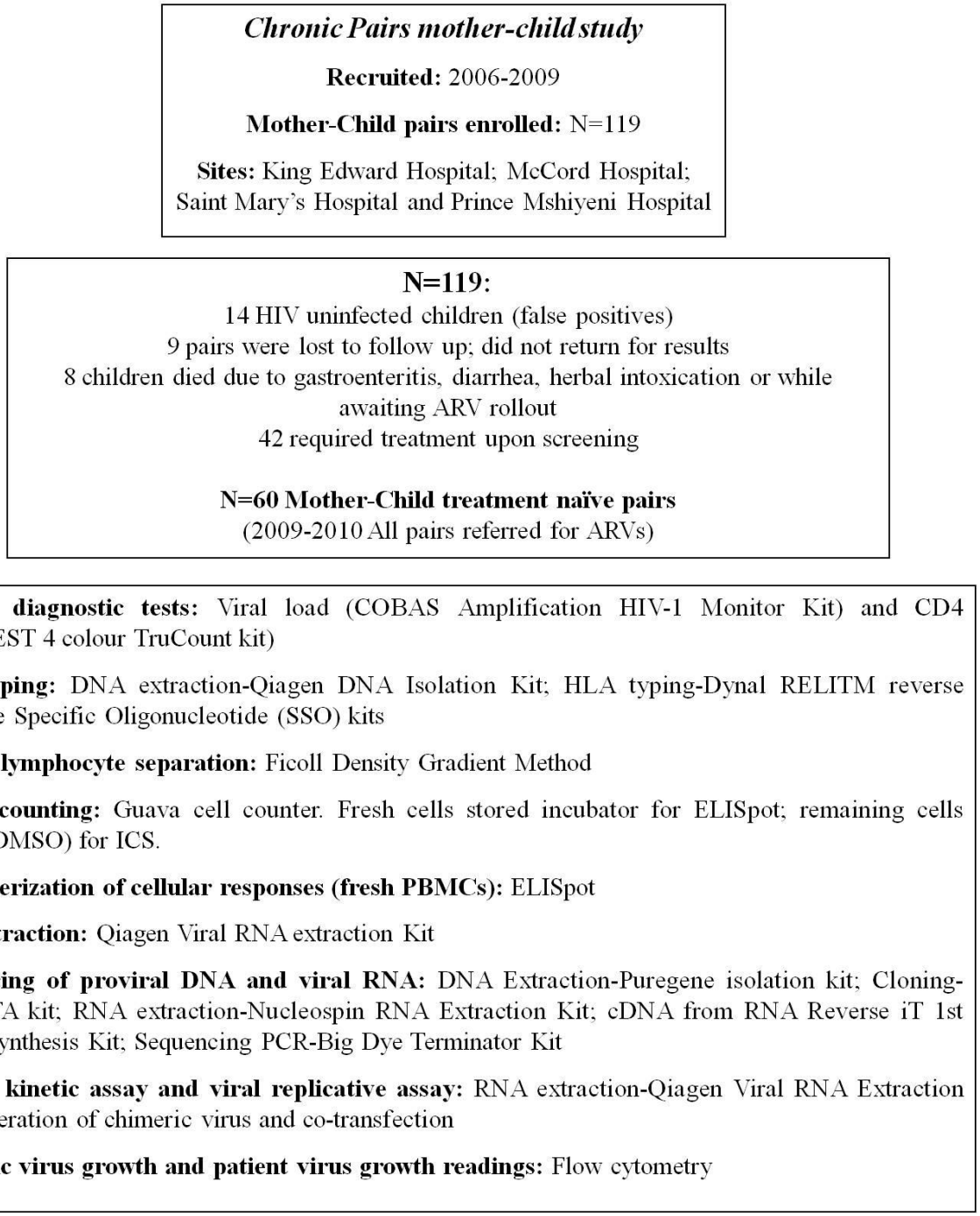


Figure 2.1 CP (Chronic Pairs) mother-child study design: Sites, participants and assays performed.

The blood samples were drawn at the clinic sites and transported at room temperature to the HPP lab. A single EDTA vial of 10 mL volume was drawn from children while three 10 mL EDTA vials were drawn from adults. Upon arrival at HPP, the blood sample were logged and dispensed for various immunological assays (Fig 2.2).

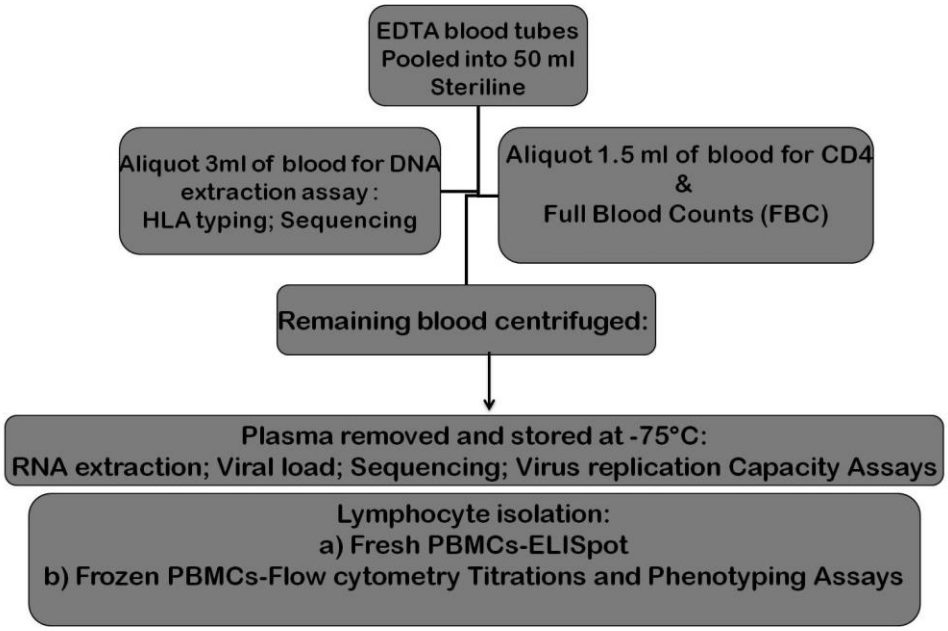


Figure 2.2 Summary of experimental procedures undertaken routinely at HPP.

2.2.3 Plasma viral load

HIV-1 plasma viral load was amplified and quantified using the Cobas Amplification HIV-1 Monitor Test, version 1.5 (Roche Diagnostics) and the Cobas Amplicor^{TM} Analyzer which can detect a limit of 50 HIV-1 RNA copies/mL plasma, according to manufacturer's instructions. The Amplicor HIV-1 Monitor Test Kit included three controls (Negative, Low positive and High positive control) at a specified threshold, and nine test samples. If controls failed the assay was repeated. Both test and control sample preparation were initiated by dispensing 100µl of HIV-1 Mn^{2+} (Manganese ion) solution to a vial of the HIV-1 matrix solution and mixed by inversion. 50 µl of this working stock was added to 12 A-tubes of an A-ring. The A-ring was stored at 4°C for a time maximum of 4 hours.

Subsequently, RNA was transcribed (reverse transcription) into cDNA using the following steps. The Lysis Buffer was first prepared by adding 100 µl of HIV-1 Monitor Quantitation Standard (HIV-1 QS) to the vial of HIV-1 Monitor Lysis Reagent. The Lysis Buffer was mixed well and 600µl was dispensed into test and control tubes. A volume of 200 µl of normal human plasma (NHP), together with 50 µl of patient plasma was added to control tubes while 200µl of patient's plasma was added to test samples. The sample tubes were vortexed and incubated for 10 minutes. 800 µl of 100% iso-propanol was added to each tube which was vortexed and centrifuged for 20 minutes. The supernatant was discarded and the pellet was washed with 1 mL of fresh 70% ethanol, vortexed and centrifuged for 5 minutes. The supernatant was discarded and the pellet was clearly visible. A volume of 400 µl of HIV-1 Monitor Specimen Diluent was added to each tube, followed by 50 µl of each processed controls and specimen into the MMX solution of the tubes of the A-ring, which was previously been stored at 4°C.

Next, the A-ring was transferred to the Roche Cobas Amplicor where the amplification, hybridization and detection steps were performed. HIV-1 viral RNA was validated using the HIV-1 Quantitation Standard which was added to the test specimen of a specific concentration. The results of the controls and test samples were exported in exponential log format.

2.2.4 Absolute CD4+ T cell measurement

Absolute CD4+ T cell counts and CD4+ T cells percentages were determined from fresh whole blood using Multitest four colour TruCount as previously described according to the manufacturer's instructions (Beckton Dickonson Technology). The technology that underlies the TruCount method uses specialized tubes which contain a pellet of a known quantity of fluorescent beads. A specific quantity of whole blood was added to the tubes, and the lymphocytes were stained with MultiTEST monoclonal antibodies (mAb). We assayed the absolute T cell counts of a full lymphocyte subset profile (CD3+, CD3+CD4+, CD3+CD8+,

CD3-CD19+, CD3-CD16/56+). The ratio of region events for each subset to bead event was calculated using the BD Biosciences MultiSET software.

2.2.5 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from whole blood

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the Ficoll-Histopaque (Sigma, St Louis, Mo) density gradient centrifugation, which allowed for the formation of a distinct opaque layer of mononuclear cells between the plasma/ histopaque stratum and Phosphate buffered saline (PBS)+R10 medium. Blood specimens were collected in EDTA tubes and processed within 6hrs of collection using the following steps.

The blood was diluted with PBS containing antibiotics in a 1:1 dilution and slowly layered over Histopaque 1077 using pipette aid (Drummond) in a second 50 mL Sterlin tube. The diluted blood was slowly pipetted at a 45 degree angle onto the Histopaque layer. The tube was centrifuged at 1,500 rpm for 30 minutes at room temperature. A Pasteur pipette was used to aspirate off the opaque interface containing the mononuclear cells. The mononuclear cells were transferred to a clean 50 mL Sterilin tube. The tube was topped with 40 mL of PBS containing antibiotics, and centrifuged to a pellet at 1,500 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet of cells were re-suspended, and washed with 40 mL of PBS containing antibiotics, at 1,500 rpm for 10 minutes at room temperature. The supernatant was discarded and the remaining pellet was gently re-suspended with R10 media (10mL). The PBMCs were incubated at $37^{o}C$ in a CO_2 incubator pending cell count and re-suspension to 1 million cells per mL.

2.2.6 Lymphocyte cell counting

To standardize cell quantification, the automated Guava cell counter laser technology **(**Guava Technologies, Guava PCA System) was used instead of the classical manual hemocytometer counting method. The Guava cell counter technology is based on the differentiation of permeability DNA-binding dyes; the nuclear dye binds DNA in all cells, whilst the viability dye exclusively binds DNA of dead cells. A Guava check kit containing calibration beads and diluents (room temperature) were used for routine calibration. The calibration beads were vortexed. Volumes of 25 µl calibration beads and 475 µl of diluent were pooled in a 1:20 dilution and ran on the machine. Test PMBCs were uniformly prepared using the Guava viacount assay by adding 20 µl cells to 180 µl Guava Counting Solution in a 1:10 dilution. The mixture was incubated for 8 minutes in the dark. PBMCs were then read on the Guava cell counter in a sterile microcentrifuge for cell counting to reveal final cell concentration. Samples were re-suspended for use in immunological assays.

PBMCs were used fresh in ELISpot assays and the remaining PBMCs were cryopreserved in 90% Fetal Bovine Serum (Sigma-Aldrich), 10% DMSO prior to storage in liquid nitrogen for subsequent Multiparameter Flow staining and immunophenotyping expression analyses (Chapter 3 and 4).

2.2.7 Cryo-preservation of cells

As any other cells PBMCs do not have a long life span and thus need to be frozen if they are not to be used immediately. These cells were centrifuged at 1,500 rpm for 10 minutes at 4 ^{o}C after which the supernatant was discarded. The pellet of cells was slowly re-suspended using a pasteur pipette tip and clumps were dissolved. The tube containing cells was transferred onto ice. A maximum concentration of 10 million cells per 1.8 mL volume was frozen in a cryovial.

A volume of 500 µl of fetal calf serum (FCS) was added to the resuspended cells and the mixture was gently mixed. A volume of 500 µl of FCS with 20% DMSO (dimethyl sulfoxide) was further added to the mixture dispensing a single drop at a time. The cryovial of cells were transferred to specialized freezing containers (Nalgene Mr. Frosty's). The freezing containers had iso-propanol at their base which allowed the temperature of the cryovial of cells to be lowered at a rate of 1 ^{o}C per minute when stored in the -80 ^{o}C freezer. Within 24hours, the cryovial of cells were transferred and logged into location in the long term liquid nitrogen storage freezer.

2.2.8 Deoxyribonucleic acid (DNA) extraction

DNA was isolated from whole blood using the Puregene^{TM} DNA Isolation Kit (Gentra Systems, Minneaplois, USA). The blood was first lysed by adding 9 mL of RBC lysis solution to approximately 3 mL of the blood. Steriline tubes were inverted and left to incubate for 10 minutes at room temperature. The mixture was centrifuged for 10 minutes at 2,000 rpm and the supernatant was discarded. This step was repeated after which cells lysis occurred. During the cell lysis step, excess supernatant was removed with a pasteur pipette. The DNA pellet was re-suspended with 3 mL of cell lysis solution and left to incubate for a minimum time of 24 hours. The proteins were precipitated by the addition of 1 mL protein precipitation solution to the cell lysate. This mixture was vortexed and centrifuged for 10 minutes at 2,000 rpm. Next, the supernatant containing DNA was transferred with a pasteur pipette into a sterile tube containing 3 mL of 100% iso-propanol. The steriline tube was inverted until strands of DNA formed. The tube was centrifuged for 3 minutes at 2,000 rpm. The supernatant was discarded and the pellet was washed with 70% ethanol solution. The supernatant was discarded once more and the DNA pellet was left to air dry. Finally, a volume of 50 µl of DNA hydration solution was added to the DNA pellet and the tube was stored at 4^{0}C.The following day the

hydrated solution was mixed and aliquoted into 1.5 mL eppendorf tubes which were stored in at -20^{0}C until assayed. DNA was quantified by pipetting 1 µl of DNA onto the sample port of a Nanodrop reader (ND-1000 Spectrophotometer - Inqababiotec™, Pretoria, South Africa). The sample was quantified at 260/280nm at a purity ratio of 1.8-2.

2.2.9 Human Leukocyte Antigen (HLA) typing

Human Leukocyte Antigen (HLA) typing is a DNA based genetic method that used the Dynal RELITM reverse Sequence Specific Oligonucleotide (SSO) kit to type HLA-A, HLA-B, and HLA-C loci (Dynal Biotech). HLA alleles were discriminated using the Dynal Biotech sequence-specific priming kits in conjunction with the previous SSO kit. Undetermined alleles were defined using sequence specific primers. The assay was performed at the transplant laboratory of the South African Blood Transfusion Services (Pinetown, Durban).

2.2.10 Synthetic HIV-1 Peptides

The HIV-specific peptides used for the ELISpot assays were either 410 overlapping individual peptides or a matrix of 408 peptides (18mers with a 10-amino acid overlap).These HIV peptides were synthesized from clade C consensus sequences using an automated peptide synthesizer (MBS 396, Advanced ChemTech).

2.2.11 Enzyme-linked immunosorbent spot (ELISpot) technique

CD8+ T cell responses were characterized using using HIV-specific individual peptides or a matrix of peptide pools as shown in Fig 2.3 and as previously described (Thobakgale et al., 2007) in an Interferon-γ Enzyme-linked immunosorbent spot (ELISpot) assay as per manufacturer's instructions (MAIP S45; Millipore).

Peptides representing each of the nine viral proteins regions were pooled into a matrix system consisting of Gag (66), Nef (27), Rev (13), Tat (12), Vpu (9), Pol (133), Vpr (11), Env (113), and Vif (24) were pooled together as previously described (Addo et al., 2003). Rows A, C and E contained respective proteins, which were then confirmed by matching wells to rows B, D and F. All positive pool responses were then traced to individual peptides by performing the secondary confirmatory ELISpot assay using individual peptides. Testing of individual peptides overlapping peptides (OLP) or HLA optimal epitopes were performed on a few participants depending on the paucity of cells.

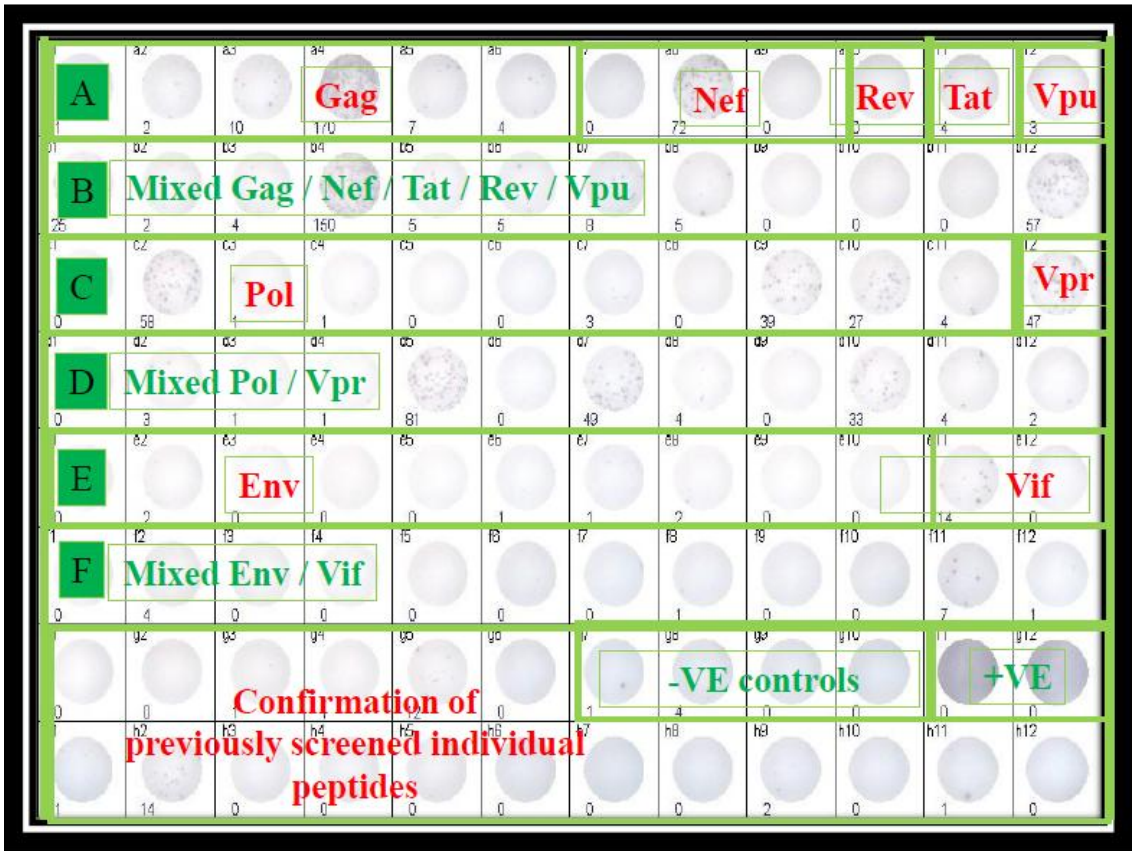


Figure 2.3 Representation of ELISpot matrix design of HIV-specific peptide pools testing all nine regions of the HIV genome. Rows A, C, E had individual peptides from each of the nine regions. Rows B, D, F were confirmatory with peptide pools (Adapted P.Kiepiela).

Briefly, a 96-well polyvinylidene diflouride-backed (PVDF) culture plate was coated with 100 µl of anti-human IFN-γ capture antibody (0.5µg/mL, 1-D1k, Mabtech) and stored for a minimum of 24 hours at 4°C. On the day of setup, the wells of the plate were washed five times with PBS. A volume of 50 µl of R10 medium was added prior to the addition of 10 µl of individual HIV-specific peptides (2 µg/mL) or 50µl of matrix of HIV-specific peptides (200 µg/mL) to all except control wells. The three negative controls contained R10 media, while the two positive controls contained 10 µl of a 200 µg/mL concentration of phytohaemoagglutinin (PHA) wells. Next, a volume of 100 µl of R10 medium containing either 50,000 or 100,000 PBMCs was added to each well of the ELISpot plate. The plate was incubated in an incubator at 37 ^{0}C, 5% CO_2 overnight. The next day the plate was processed by six washes with cold PBS, followed by the addition of PBS containing the secondary biotinylated IFN-γ monoclonal antibody (0.5 µg/mL, 7-B6-1, Mabtech) and 90 minute room temperature incubation. The plate was further washed with cold PBS, followed by addition of PBS containing the streptavidin-alkaline phosphatase conjugate antibody (0.5µg/mL, Mabtech) and incubated at 45 minutes at room temperature. Finally IFN- γ producing cells were stained with a substrate combination (Bio-Rad) of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt), which reacted with alkaline phosphatase to yield a black-purple colour, which allowed for visualization of IFN-γ T lymphocyte cells within 5 to 10 minutes of staining. The IFN-γ T lymphocyte cells were quantified by automated counting of spots per well using the Aid ELISpot plate reader (Autoimmun Diagnostika GmbH, Strasburg, Germany). The output was exported as the number of spots forming cells (SFC) per well using the ELISpot plate reader software. A response was defined as positive if it was above 100 SFC/million PBMC and above 3x standard deviations above the mean for the four background wells using previously adopted criteria (Addo et al., 2003, Kiepiela et al., 2004, Kiepiela et al., 2007).

2.2.12 RNA extraction

To investigate the HIV, RNA was extracted from patient plasma using the Qiagen QIAmp viral RNA extraction kit (Qiagen) as Fig 2.4

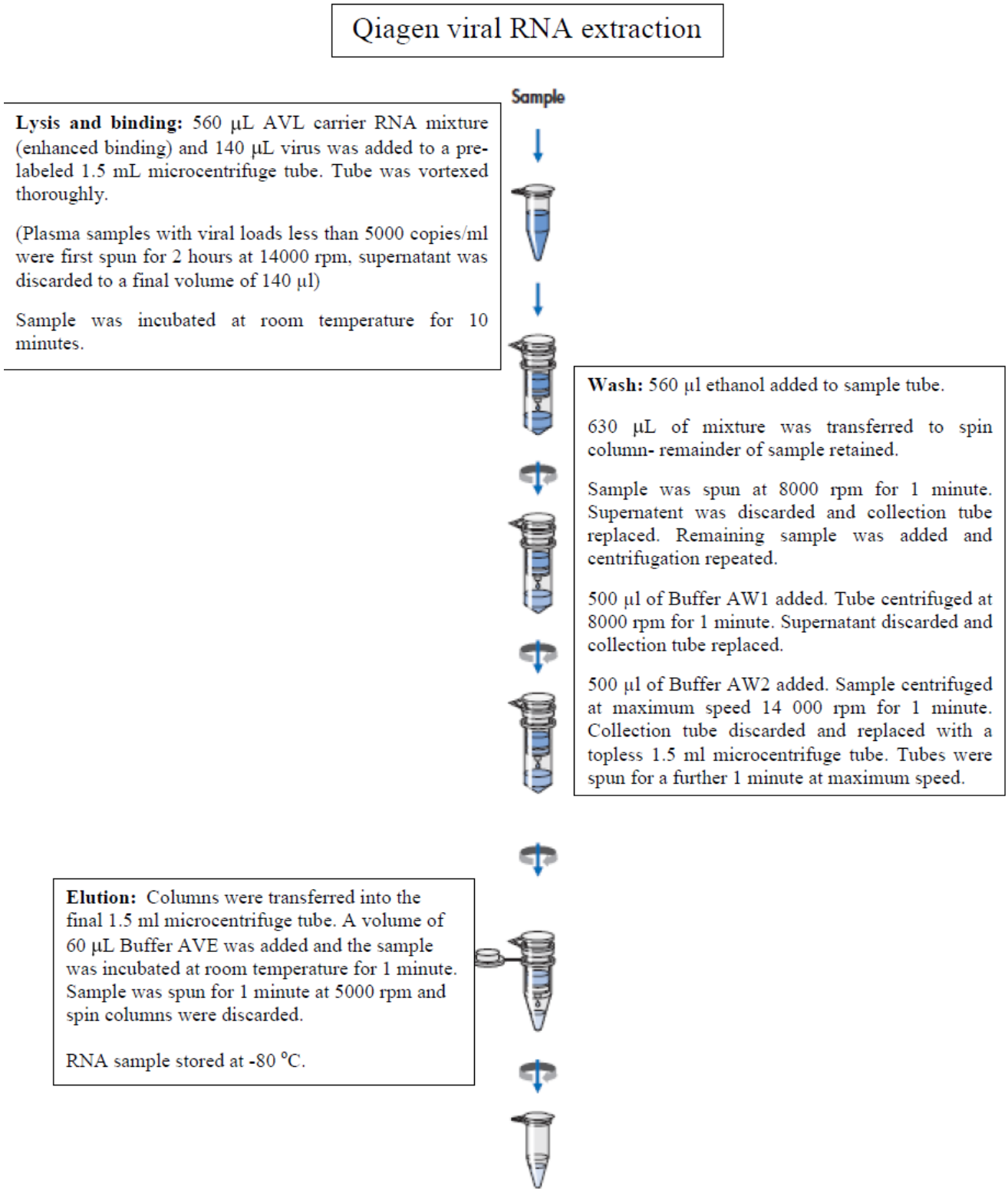


Figure 2.4 Viral RNA extraction protocol (adapted Qiagen protocols).

2.2.13 Sequencing of proviral DNA and viral RNA

Following genomic DNA extraction from PBMC, we set out to investigate patient Gag-specific sequences. The Gag-specific sequences were amplified and sequenced from DNA using a nested PCR as previously described (Leslie et al., 2004) at the Department of Pediatrics, Nuffield Department of Medicine, Oxford, UK. PCR primer for Gag specific amplification were 5`-CTAGCAGTGGCGCCCGAACA-3`and 5`-ACAGTCTTTCA TTTGGTGT CCTTC-3`(MWG Biotech, Germany) for first round PCR and 5`-TTCTCTCGACGCAGGACTC-3` and 5`-TTTTCCACATTTCCAACA GCC-3`(MWG Biotech, Germany) for second round PCR (Leslie et al., 2004). For amplification of Gag-specific sequences mastermixes (Bioline) were generated for the first and second round PCR amplification reaction (Appendix).

Following PCR amplification, 5 µl of the PCR product was loaded onto a 1% agarose gel [Sigma, St. Louis, MO] in TBE buffer (Tris-borate-EDTA, Promega). Ethidium bromide was added at 5µl/400mL gel. To determine the size of the amplified fragment, samples were run in parallel with Hyperladder I (Bioline) (Leslie et al., 2004). After running at 200V for 35 minutes, bands were visualized under UV light.

The PCR product was purified using ExoSAP-IT, containing Exonuclease I and Shrimp Alkaline Phosphatase, (GE Healthcare Life Sciences) as previously described as per manufacturer's instructions (Leslie et al., 2004).

The PCR product was directly sequenced using sequencing primers as per Table 2.1 and the BigDyeTerminator version v3.0 ready reaction mix (Applied Biosystems, Foster City, CA).

Table 2.1 Sequencing primers for HIV Gag regions p17 and p24) (Leslie et al., 2004).

Primer	Sequence
Frag1_5IP	TTCTCTCGACGCAGGACTC
Frag1_2	CTGCACTATAGGATAATTTTGAC
Frag1_3	GACACCAAGGAAGCCTTAG
Frag1_4	CTCCCACTGGAACAGGTG
Frag1_5	GGAACAAATAGCATGGATGAC
Frag1_3IP	TTTTCCACATTTCCAACAGCC

2.2.14 Viral replicative assay

The viral replication assay was performed to assess the replication capacities of the virus as previously described (Miura et al., 2009a). RNA was extracted from patient plasma. Gag-protease was amplified by a two round PCR reaction. Gag was selected based on its demonstrated role in viral containment, while protease was included due to its interaction with Gag by cleavage of the Gag polyprotein. The Gag protease was inserted into the plasmid NL4-3ΔGag-Protease, co-transfected, cultured, harvested and finally assessed viral fitness using an HIV-1-inducible GFP reporter cell line.

After RNA extraction reverse transcription-PCR (RT-PCR) was performed using a Superscript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) and the Gag-protease-specific primers: 5'-CACTGCTTAAGCCTCAATAAAGCTTGCC-3` (HXB2 nucleotides 512 to 539) and 5'-TTTAACCCTGCTGGGTGTGGTATYCCT -3` (nucleotides 2851 to 2825) (Miura et al., 2009a). A second round of PCR was performed with primers 5`GACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGA GTACGCCAAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAGATGGG-3` and 5`GGCCCAATTTTTGAAATTTTTCCTTCCTTTTCCATTTCTGTACAAATTTCTACTAAT GCTTTTATTTTTTCTTCTGTCAATGGCCATTGTTTAACTTTTG-3` (Wright et al., 2010,

Wright et al., 2011, Brockman et al., 2006, Miura et al., 2009a), which was exactly complementary to NL4-3 on either side of Gag-protease using the TaKaRa Ex *Taq* HS enzyme kit (Takara, Shiga, Japan). Two PCR reaction mixtures were prepared for each sample, comprising 37 µl diethyl pyrocarbonate (DEPC)-treated water, 5 µl, 10x Ex *Taq* buffer, 4 µl of deoxynucleoside triphosphates (dNTPs), 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.25 µl Ex *Taq*, and 2 µl of the first round RT-PCR product. (Miura et al., 2009a). Amplification conditions were as follows: 94°C for 2 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min; and 72°C for 7 min. PCR products from two reaction mixtures were pooled while 10 µl was reserved for sequencing (Wright et al., 2010, Wright et al., 2011).

The recombinant viruses Gag-protease deleted pNL4-3 plasmid was amplified from a stock of STBL3 cells that were previously heat shocked and co-transfected with plasmid (Brockman et al., 2006, Miura et al., 2009a, Wright et al., 2010, Wright et al., 2011). A volume of 200 mL LB (Luria Bertani or L-Broth) media was inoculated with 17.5 µl of STBL3 competent cells containing pNL4-3ΔGag-Protease. Following overnight culturing at 37^{o}C in a shaking incubator the DNA plasmids were harvested and prepared using the Qiagen plasmid DNA purification maxi kit. Plasmids were selected from the culture colony. The bacterial cells were harvested and centrifuged at 6,000 *g* for 15 min at 4°C. The bacterial cell pellet was resuspended in 4 mL of Buffer P1 (RNase A solution). A volume of 10 mL Buffer P2 (aid precipitation) was mixed and incubated at room temperature for 5 minutes. After adding Buffer P3 the solution was mixed and filtered using QIAfilters. The cleared lysate was washed with 30 mL Buffer QC. Solution was eluted with 15 mL Buffer QF. DNA was further precipitated by adding 10.5 mL isopropanol, Precipitated DNA was washed with 70% ethanol and centrifuged at 15,000 *g* for 10 minutes. The pellet was air dried and re-dissolved in TE (Tris and Ethylenediaminetetraacetic acid buffer).

Bacterial cultures were stored at -85°C using a 30% glycerol-LB solution. The glycerol was mixed with bacterial culture solution at 1:2 and stored at –80 ^{o}C. To confirm specificity of the maxiprepped product a Hind III digest was performed as Table 2.2.

Table 2.2 Confirmation of plasmid transfection using Hind III digest.

Reagent	**Volume (µl)**
Hind III	1
Buffer 2(New England BioLabs)	2.5
BSA (100X)	0.25
Water	16.25
Plasmid(1000 ng/µl)	3
Volume	400

The plasmid NL4-3ΔGag-Protease and the wild type pNL43 were digested for 1 hour at 37 ^{o}C after which they were visualized on a 1% agarose gel [Sigma, St. Louis, MO] in TBE buffer. Plasmid was represented by 4 gel bands while the wild type control by 5 gel bands. Prior to co-transfection we went on to prepare a digest reaction with circular pNL4-3ΔGag-Pro plasmid (Table 2.2) and we digested this for 2 hours at 60 ^{o}C in waterbath.

Table 2.3 Generation of chimeric pNL43 Gag-Protease viruses.

Reagent	Volume (µl)
Plasmid (amplified)	*10 µg per sample
10x Buffer (Promega BSTEII kit)	40 (1/10 reaction volume)
100X BSA	4 (1/100 reaction volume)
Enzyme (BSTEII from Promega)	2 U per µg plasmid
Water(to make up balance of final volume (400)	400µl-all above

*Plasmid concentration (c1v1=c2v2)

To generate the chimeric viruses (Table 2.3), we co-transfected the CEMGXR25 cells with digested pNL4-3ΔGag-Pro plasmid (Brockman et al., 2006, Miura et al., 2009a, Wright et al., 2010, Wright et al., 2011) and the second round PCR product while maintaining CEMGXR25 cells in culture every 2-3 days by feeding them with RPMI1640; containing 5mL 1M Hepes (Gibco); 5mL of 200mM L-glut (Sigma); 5mL of 5000U/mL Penstrep (Gibco) and 50mL 10% FBS (Gibco) (Brockman et al., 2006, Miura et al., 2009a, Wright et al., 2010, Wright et al., 2011). Concentrations of 2 million cells GXR cells were prepared per sample by spinning them at 1,500 rpm for 10 minutes at 20 ^{o}C. Supernatent was aspirated. A volume of 800 μL of cells, 80 μL of second round PCR product and a concentration of 10ug digested plasmid were combined in a cluster tube. After mixing, the solution was transferred to labelled electroporation cuvettes and they were electroporated at settings of 300 V; 500 μF; R= ∞ and 4mm BioRad GenePulsar II. Cuvettes were left to incubate for an hour to allow cell membranes to restructure themselves following co-transfection of product to plasmid. The solution was transferred to 25 mL flasks containing 4 mL media and incubated for 5 days. A volume of 5 mL R10 media was added to flasks and the flasks were further incubated. At day 12 samples were assessed using a flow cytometer to determine the percentage infected cells. The sample was run every second day until reaching a percentage of 30-40% infectivity and were then harvested and frozen.

Finally titration and viral replication capacities were (Brockman et al., 2006, Miura et al., 2009a, Wright et al., 2010, Wright et al., 2011) using a multiplicity of infection (MOI) of 0.0003. Viruses were thawed and based on their % infectivity they were added in different amounts to achieve 0.3% infection on day 2. The concentration of virus added (μL) = [0.3/ (% total cells fluorescing on day 2 of titre experiment)] * final volume (400 μL). This volume was subtracted from final volume of 400 μL to determine how much media to add. The solution and

contents were added to 100 μL of GXR cells containing 1 million cells which was transferred to a well of a 24 well culture plate and the plate was left to incubate overnight (Sigma). The following day 1 mL of media was added to the well. The day thereafter 500 μL sample was collected and acquired at 25,000 events on a flow cytometer. A volume of 500 μL of media was added to replace acquired sample. This process was repeated until day 6. Replication assays were performed at least in duplicate, and results were averaged.

2.2.15 Statistical analysis

Sequence data were analysed using Sequencher v.4.8 (Gene Codes Corporation). Nucleotides residues were matched and aligned against the HXB reference sequence manually in Se-Al v.2.0a11 (A. Rambaut, Department of Zoology, University of Oxford) or automatically in BioEdit Sequence Aligner Programme version 7.0.0(IBIS Biosciences, CA). Phylogenetic trees were derived from aligned nucleotide consensus sequences. Neighbour joining (NJ) trees were computed using ClustalX (Multiple sequence alignment tools). Bootstrap resampling (1000 replicates) was performed to validate individual nodes on a tree (Felsenstein, 1985). Flow data was analyzed with FlowJo software version 7.5 for PC. Microsoft Excel was utilized to transfer data and subtract FMO background values. Microsoft Excel was also used to calculate the mean slope of exponential growth for the viral replication assay from days 3 to 6 using the semi-log method [=ln(LOGEST({x1,x2,x3,{y1,y2,y3},0,0}))], which was divided by the slope of growth of the wild-type NL4-3 control that was included in each assay to generate a normalized measure of replication capacity. Nonparametric testing with the Mann Whitney U test was undertaken for a less than two group comparison and the Kruskal Wallis was performed for more than two group comparisons. Post test analysis was performed using Dunns Multiple Comparison Test. Normalization and subset discrimination and gating of data was performed using Flow Jo. The mean fluorescent intensity and % response per event was

calculated using the Wilcoxon-Mann-Whitney-U test. Significance levels were indicated using Mann Whitney U ($P<0.05$). Correlations were performed using Spearman rank tests (p and r values). This analysis was performed and graphically represented using Prism software (GraphPad, version 5).

2.3 RESULTS

2.3.1. Cohort Characteristics

A total of sixty HIV-1 infected mothers and children (mother-child pairs) were recruited for this study based on inclusion criteria (Appendix) as described in previous section.

2.3.2. Clinical characteristics (Absolute CD4+ T cell count and plasma viral load).

The investigators denoted CD4 counts as cells/mm^3 for mothers and % for babies as per WHO criteria which use these parameters as measures of immune deficiency in adults and children respectively. CD4% in children provides a more accurate indicator of a child's health compared to absolute CD4 counts.

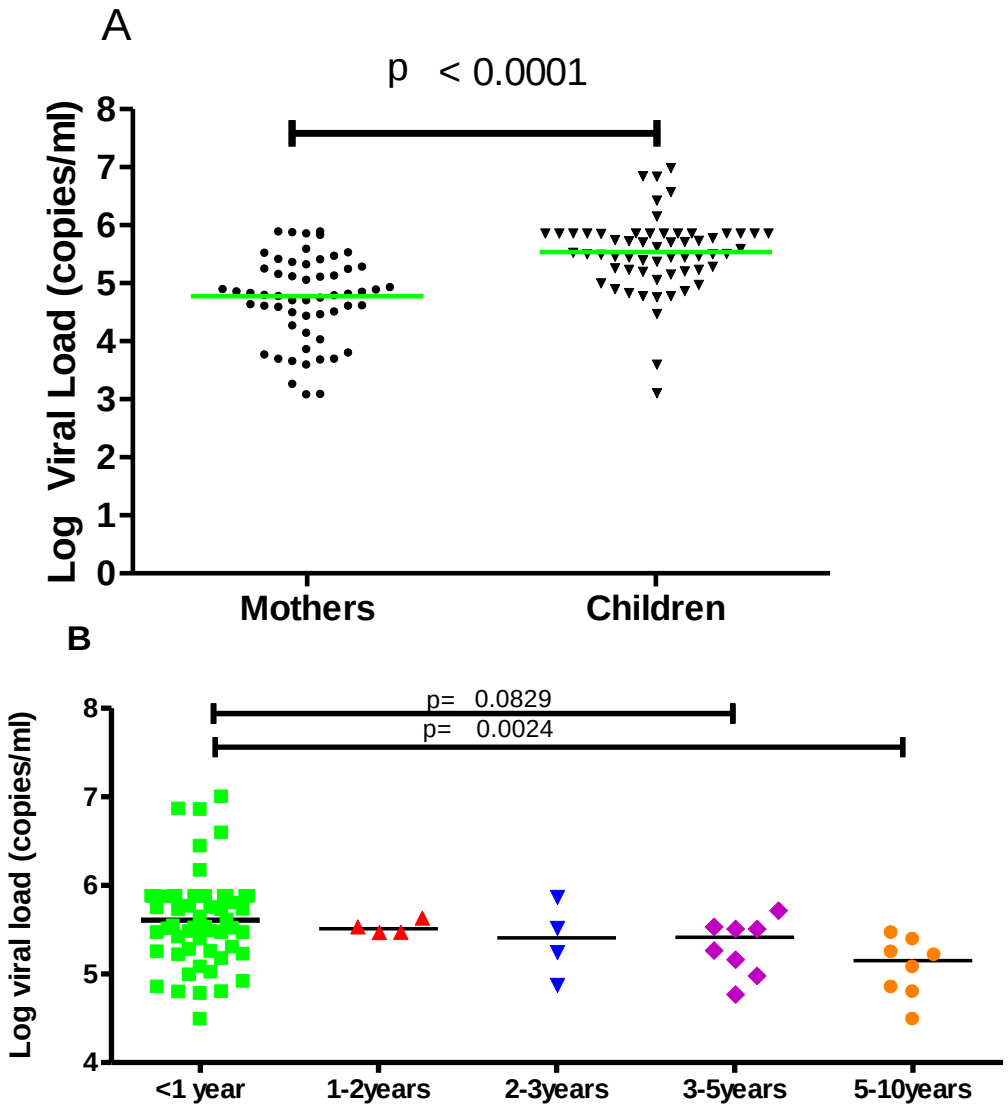
The median CD4 percentage in HIV infected children was 24% (range, 11-46%) while the median absolute CD4+T cell count in the mothers was 304 cells/mm^3 (range, 24-905 cells/mm^3), Table 2.9. The median age of the children studied was 12 months and ranged between 3 months-10 years, while the median age of mothers was 29 (17-34 years).

Table 2.4 Overall clinical characteristics of study cohort.

	Mothers	**Children**
N	60	60
Median Age in years (IQR)	29 (17-34)	1 (0.3-10)

Median Absolute CD4+ T cell count cells/mm3 (IQR)	304 (24-905)	-
Median CD4% (IQR)	-	24 (11-46)
Median Viral Load (copies/mL)	59 550	260,000
Viral Load IQR (copies/mL)	1170-750,000	1320-3,902,668

As expected, the median viral load in the children (260,000 copies/mL) was significantly higher than the mothers (59,550 copies/mL) ($p<0.0001$, Mann-Whitney U test) as represented in Figure 2.5 A.



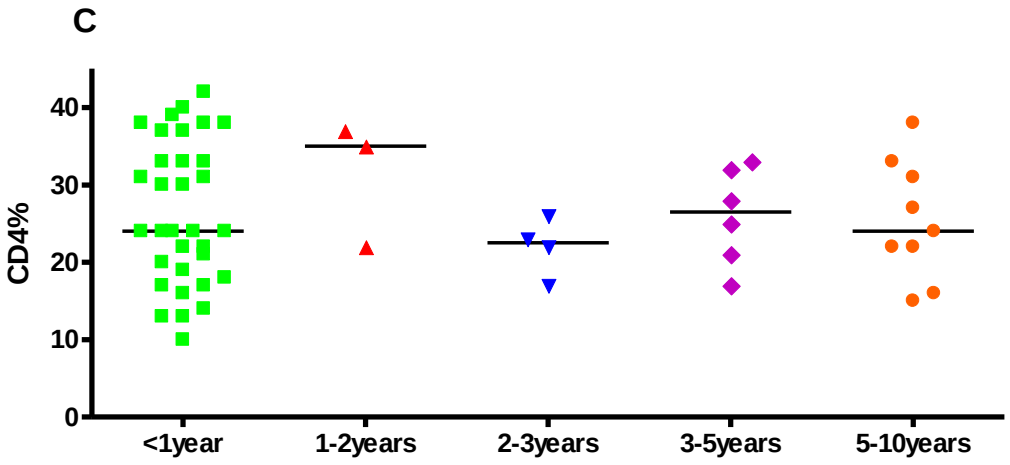


Figure 2.5 Comparison of A) Viral load (copies/mL) between mothers and children B) plasma viral load and) % CD4 T cell between children stratified by age.

As neonates have been shown to exhibit an elevated viral load in comparison to older children and CD4% has been documented to better depict HIV-1 infection in children clinically, we next sought to further describe HIV-1 infection by stratifying the children into groups based on their age (Fig 2.5). The younger group of children had a trend towards higher viral loads and lower CD4% in comparison to the older children.

2.3.3 CTL responses and viral loads in responder and non-responder children

We hypothesized that the presence of CTL responses in HIV-1 infected children would decrease on HIV-1 viral load and therefore HIV plasma Viral loads were compared between children who made CTL responses compared to non-responders. A total of 30 children made responses to at least one of the peptide pools and were thus responders, many children did not make any CTL responses (non-responders). Comparison of viral load between responders and non-responder showed no significant differences between the two groups. Although the non-responders had a higher median viral load (535,000 copies/ml) than the responders (375,000 copies/ml), the differences were not significant as per Mann-Whitney U (p=0.2799).

2.3.4 Specificity of the CTL response in children less than 12 months

Sixty children were investigated in this sub-study of which many infants did not make any detectable responses using the standard confirmatory ELISpot. The remaining 30 infants who did make detectable responses were older than 12 months and were not included in this investigation. As documented certain regions of the nine HIV proteins are targeted frequently at different stages of HIV-1 infection in comparison to other viral proteins. HIV-specific CD8+ T cell function in children have been documented to be critically influenced by age (Sandberg et al., 2003) and data on CTL responses in infants are scarce. Hence, the HIV regions targeted by children at different disease stages were assessed using ELISPOT. A previous study of infants in the same setting reported that the specificity of the response in the first month of life is directed to Env and accessory proteins RTVVV (Reg,Tat, Vif,Vpr, Vpu) (Thobakgale et al., 2007). Due to the paucity of samples in infants, we tested the ELISpot assay in a matrix pool approach (cut off 100 SFCs) in order to determine the HIV regions targeted by children between the ages of 3-12 months as previously described (Thobakgale et al., 2007) (Fig 2.6). This method did not confirm responses as the responses were low and not detected by day 2. Due to the low responses the magnitude of these responses could not be tested as this method is not comparable to the routine confirmatory ELISpots. All the infants under 12 months of age were tested using day 1 ELISpot pooled matrix results to ensure comparable results. There were a few infants that did show low level detectable responses in the original day 2 confirmatory ELISpot but for uniformity these infants were also analyzed using methodology described by (Thobakgale et al., 2007). Children aged less than 3 months of age dominantly targeted Nef. Gag was targeted next by children between 3 months to 9 months of age. Env was targeted less frequently in comparison to Nef and Gag in children between 3-12 months of age.

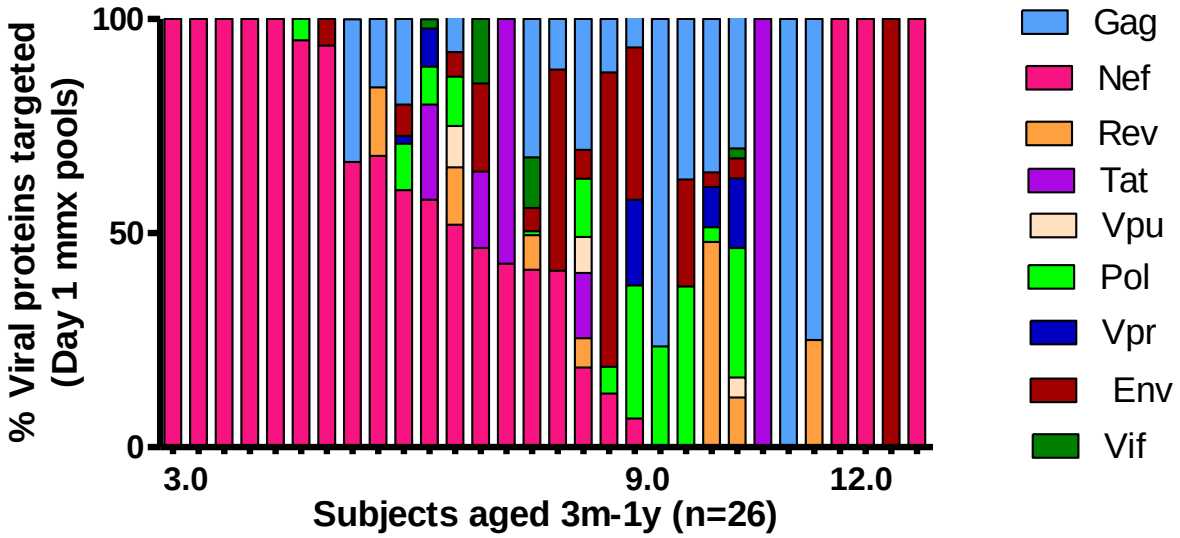


Figure 2.6 Contribution of HIV-specific protein response to overall response in 30 children using a matrix system (Thobakgale et al., 2007) in children aged 3-12 months. Nine proteins of the HIV proteome were tested with each colour representing the protein region described in the legend.

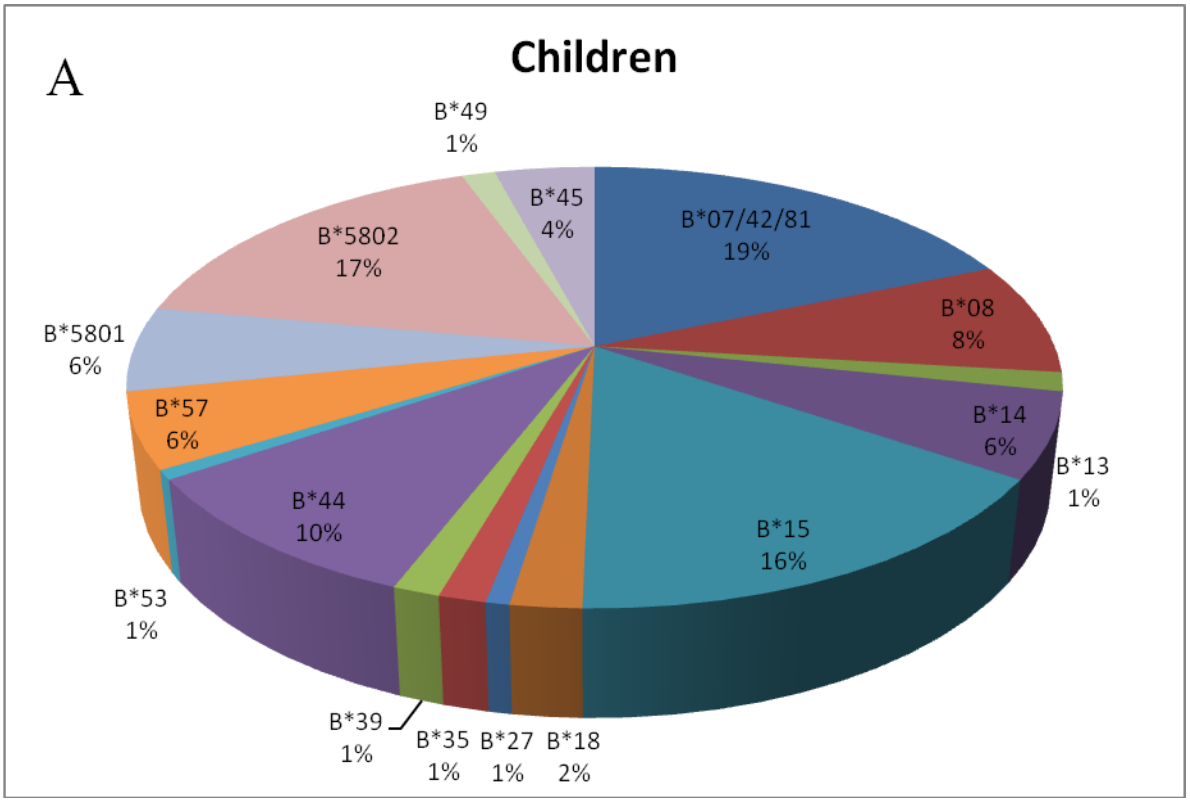
As observed in Table 2.5 and Fig 2.6 children less than a year of age targeted Nef, Gag and Env respectively. As children matured in age and stabilized in viral load-responders tend to target Gag more frequently than any other viral protein.

Table 2.5 Classification of viral proteins most frequently targeted in responder children over 12 months.

Age	**Region most frequently targeted**
1-2years (n=4)	Gag;Integrase Pol
2-3years (n=4)	Nef
3-5 years (n=8)	Gag;Nef
5-10 years (n=9)	Nef; RT Pol

2.3.5 Frequency of distribution of HLA-B and impact of HLA-B on disease in mother-child HIV-1 infected transmission pairs.

Due to the heterogeneity of HLA, genetic studies should ideally be performed in a large sample size to validate findings. As previously described in clade B, we next compared the distribution of HLA alleles in mother-child pairs (Kuhn et al., 2004). As T cell immune responses are restricted by different types of class I molecules designated HLA-A, HLA-B, and HLA-C, we next described the frequency of distribution of selected HLA-B restricted that have been found to be influential clade C HIV-1 infection. HLA-B*07/41/81, are similar alleles (epitopes target all 3 alleles) which were frequently distributed at 19% in children and 21% in mothers (Fig 2.7). Next HLA-B*5802 and B*15 were expressed in both mothers (17,17%) and children (17, 16%) respectively within this clade C cohort.



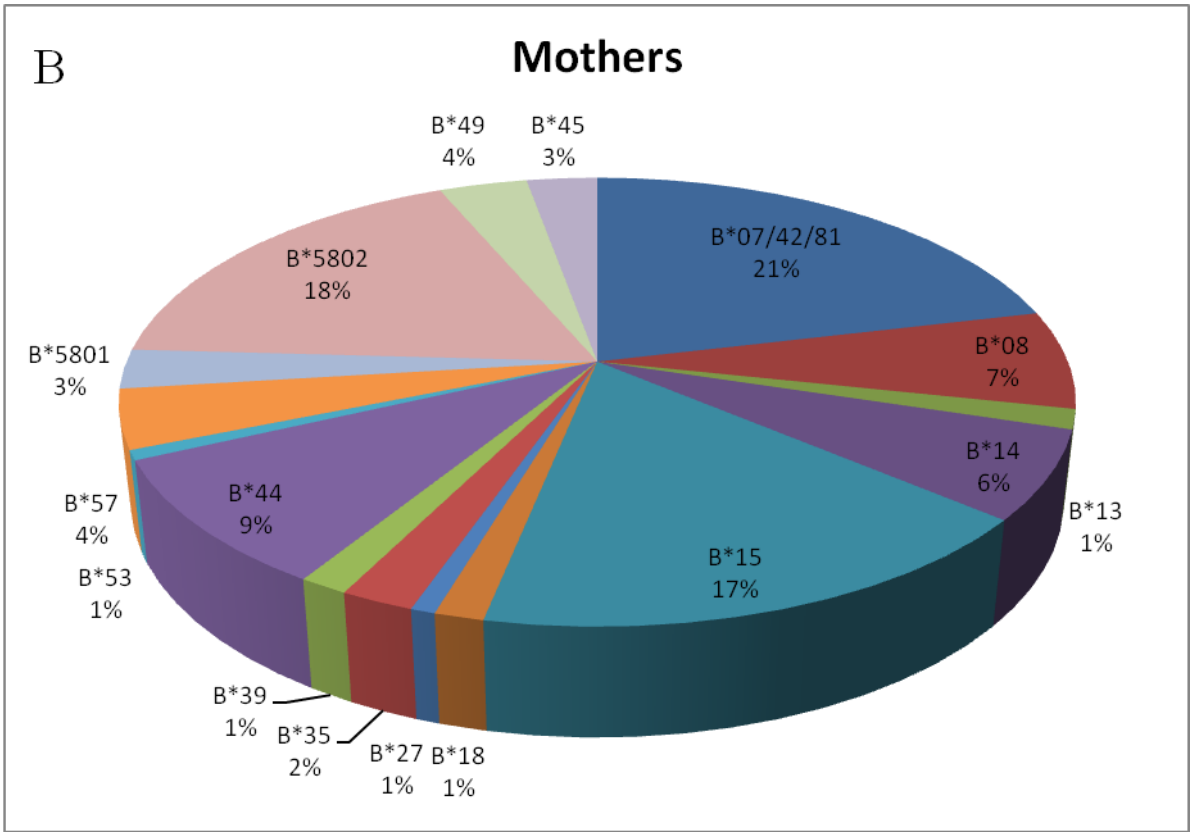


Figure 2.7 Frequency of distribution of HLA-B in transmission pairs A) children and B) mothers.

Based on the previous investigations that reported HLA-B alleles to be influential in viral control, we also investigated Gag-specific responses restricted by HLA-B alleles reported to be protective in children. Based on specific HLA-B allele findings related to the protective effect of HLA-B*27 and HLA*B57 in transmission pairs, we identified four transmission pairs in which either the mother or child carried HLA-B*27 or B*57 to study, and/or pairs which retrospectively sparked interest (i.e. family or siblings etc) as case studies. We investigated the mechanisms underlying viral control, viral escape a fitness in relation to HLA-B restricted Gag CD8+ T cell epitopes. The Gag epitopes in the positions highlighted in Figure 2.8 are the epitopes commonly targeted by HLA-B*27 and B*57. However, we focused our study on

B*27-IK9/KK10 in two HLA-B*2705 HLA transmission pairs and epitopes B*57-ISW9/KF11/TW10 in two HLA B*5703 transmission pairs.

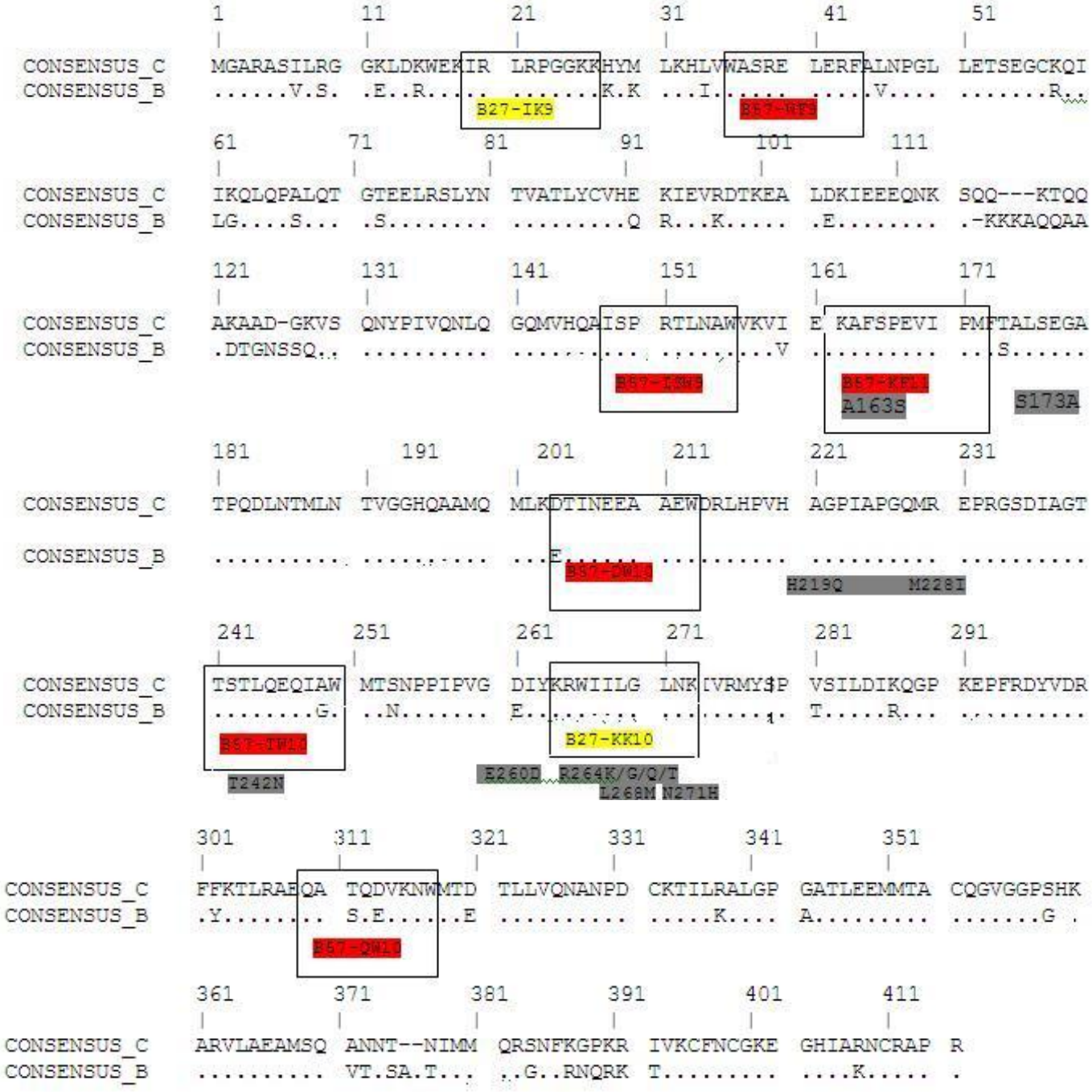


Figure 2.8 Illustration of epitope map (our study participant) of common HLA-B*27 and HLA-B*57 restricted Gag-specific CD8+ T cell epitopes and possible clade C related compensatory mutations**.**

2.3.6. A description of the protective HLA-B*27 in mother-child HIV-1 infected transmission pair

HLA-B*27 is a common allele in the Caucasian population in which clade B HIV-1 infection is the predominant subtype. HLA-B*2705, more specifically a Gag p24 KK10 response, has been previously associated with effective viral control in children (Schneidewind et al., 2007, Kelleher et al., 2001). However, the KK10 response is lost with the emergence of R264K mutant. If the transmitted virus carries an escape mutations in any key Gag epitopes HLA*27 tends to lose its protective effect (Kuhn et al., 2004). As documented by Goulder *et al.*(2001), infected children who carry B*2705 or B*5703 are able to effectively control the HIV infection particularly if the transmitted virus is not pre-adapted to either of these alleles hence if mothers or children do not share a protective allele they are likely to maintain viral control (Goulder et al., 2001a, Goulder et al., 2001b). We selected the only two HLA-B27 restricted transmission pairs in our cohort. In transmission pair CP3-005, only the mother carried HLA-B*2705 (Fig 2.9 A) while in transmission pair CP3-024, only the child carried HLA-B*2705 (Fig 2.9 A). In transmission pair CP3-005 the father volunteered his blood for testing so we were able to run tests on a single time point of the father as well.

As the mother carried HLA-B*27 we hypothesized that the mother would progress slowly, if she has effective CTL responses– but would progress rapidly following escape – unless escape lowers fitness and other responses can still control an attenuated virus. As the child did not carry HLA-B*27 we hypothesized that child would progress slowly if the virus in the child is less fit than the virus of the mother.

Clinically, the father required immediate treatment as had a viral load 510,918 copies/mL and an absolute CD4 T cell count of 129 cells/mm^3. The mother and child had stable viral loads and

relatively stable CD4 tests over 33 months of investigation (Fig 2.9 B). The mother exhibited an average absolute CD4 T cell count of 214 cells/mm^3 (Fig 2.9 C) however as time of infection was unknown it was difficult to identify if progression was slow or related to the possession of HLA-B*27. However, as the child was 8 months of age upon enrolment, we assumed a slower level of progression, as child consistently exhibited a CD4% ranging between 27-45% throughout investigation. A dip in CD4% was observed in children however we did not have clinical or co-infections data to further extrapolate. The data is presented as acquired. (Fig 2.9 C).

Based on the association of Gag-specific epitopes with slow progression, we next went on to investigate the role of HLA-B*27 restricted Gag-specific responses in transmission pair CP3-005 (Fig 2.9 D/E). CD8 T cell responses in this pair showed that the mother targeted Gag p17 (OLP 11) at low levels while no Gag-specific responses were detected in the child. A predominant B*27 restricted response to Nef (OLP 81) was detected at high levels in the child from the first till the last 15 month time point test. At the mother's 15 month timepoint it seems asif the assay may have been problematic as the positive control is not fully developed. We speculate the plate development stage may have not been fully completed. CTL data for this timepoint has been removed from analysis.

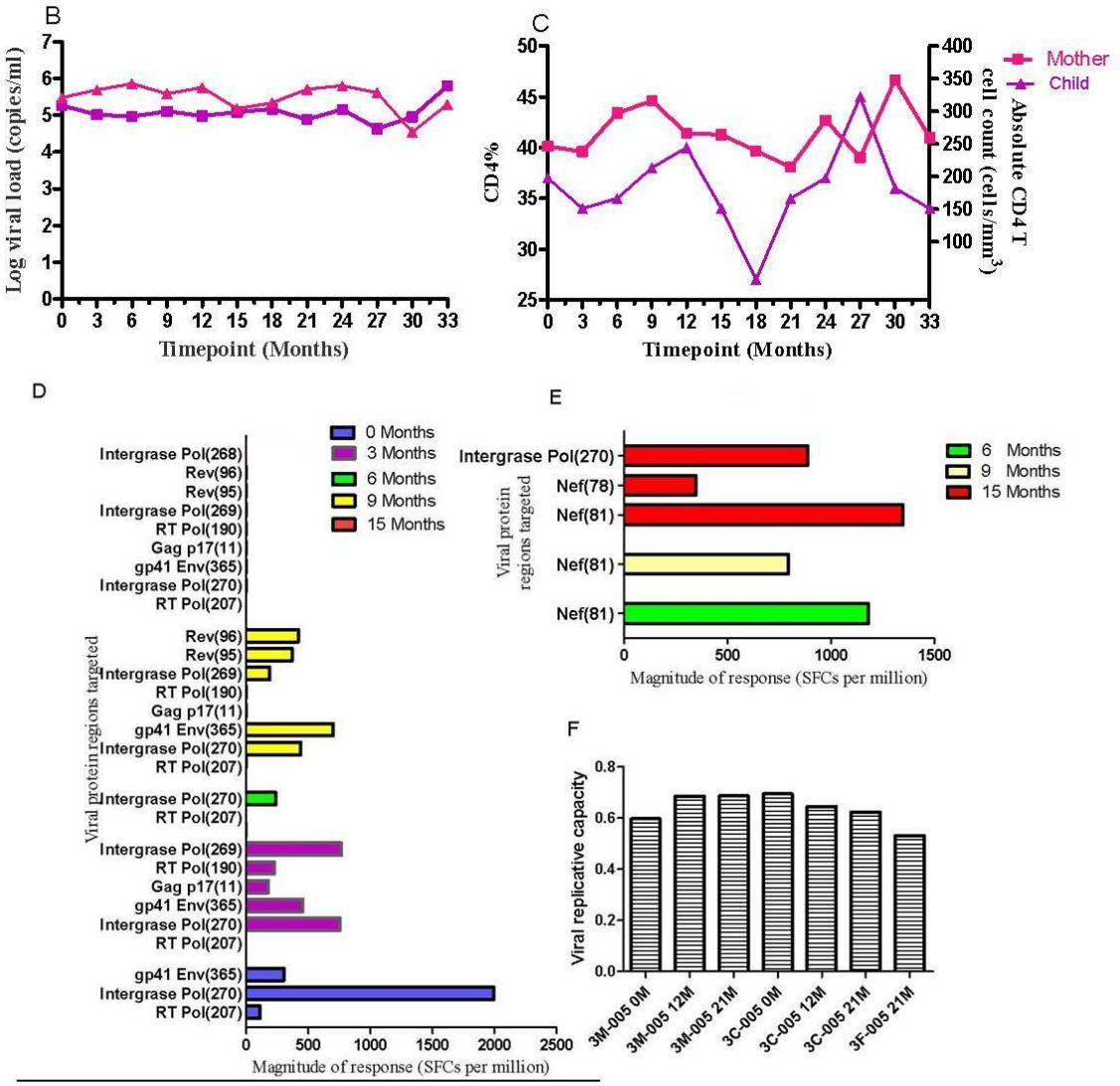
We next investigated whether the failure to generate effective Gag-specific responses was related to sequence changes by the transmission of variants from mother to child, based on the evidence of MTCT escape variants influencing CTL responses. We investigated the role of escape variant within the KK10 epitope as an effective KK10 response has been associated with slow progression in HLA-B *27 carriers and conversely late escape from KK10 as been linked to progression. In addition, as documented in clade B infection the R264K/R264G

mutation has been associated with the development of rare compensatory mutations, S173A and E260D. Our sequencing results showed that epitope B*27-IP9 was present as a wildtype in all family members, while epitope KK10 showed 3 sequence changes in position 264, 268 and 271 (Fig 2.9 G). In clade C infection we observed the appearance of a T173A variant. Interestingly as child and mum also possessed HLA-B*5802 we observed the presence of S165N/V168I in both B*58-KF11 and TW10 epitopes.

Based on the interplay between CTL response viral adaptation and viral fitness, we next compared the fitness of earliest timepoint to later timepoints and to the donor viruses within the family CP3-005. As CTL responses and viral adaption did not explain good clinical outcome, we expected viral fitness to play an influential role. However, we found no significant difference in viral replicative capacity between the virus of the mother, father or child (Fig 2.9 F). Furthermore, we found no difference in viral replicative capacity over a 21 month longitudinal investigation (Fig 2.9 F).

A HLA: CP3-005	A1	A2	B1	B2	Cw1	Cw2
Mother	3004	3004	2705	5802	0202	0602
Child (8months)	0301	3004	0801	5802	0602	0701

NOTE: Father (1 visit): HLA A*0301/0301 B*0801/0801 and C*0702/0702; Viral load 510918 copies/mL; Absolute CD4 T cell count 129cells/mm3.



G	*B27-IK9*	*B27-KK10*
CONSENSUS C	*(Gag19-27)*	*(Gag 264-273)*
	IRLRPGGKK	KRWIILGLNK
3M-005 0M	WT	KKWIIIGLHK
3M-005 6M	WT	KKWIIIGLHK
3M-005 21M	WT	KKWIIIGLHK
3M-005 24M	WT	KKWIIIGLHK
3C-005 12M	WT	KKWIIIGLHK
3C-005 21M	WT	KKWIIIGLHK
3C-005 24M	WT	KKWIIIGLHK
Note: F-005 24M	WT	KKWIIIGLHK

Figure 2.9 A) HLA, B) Viral load, C) CD4% and Absolute CD4 T cell count, D) CTL response of mother, E) CTL response of child, F) Viral replicative capacity and G) Sequence changes in epitope IK9 and KK10 of transmission family CP3-005 (C=child etc).

We next went on to investigate the role of B*27 in another transmission pair, CP3-024 in which the child carried HLA-B*2705 (Fig 2.10 A). As the child carried HLA-B*27 we hypothesized that the child would progress slowly, if he/she has and maintains an effective CTL response. As the mother did not carry HLA-B*27 we hypothesized that she would progress rapidly.

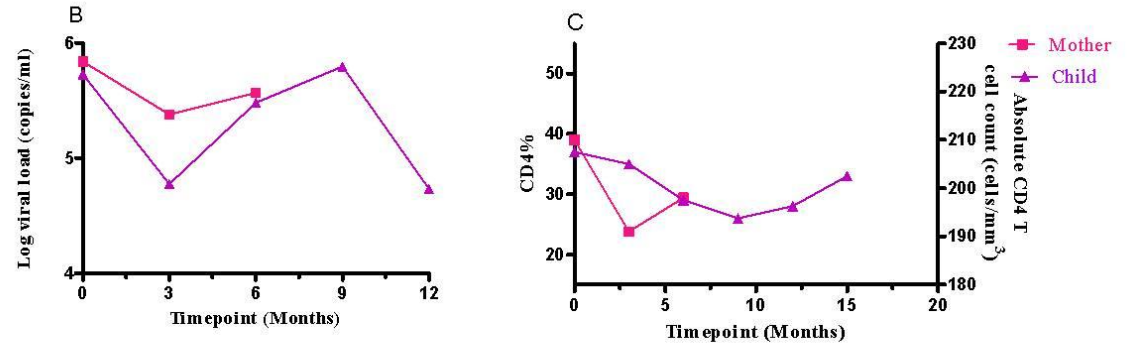
Clinically, the mother and child have stable viral loads and relatively stable CD4 tests months (Fig 2.10 B). The mother had an average absolute CD4 T cell count of 200 cells/mm^3 (Fig 2.10 C) warranting ART soon after recruitment. The child was 7 months of age upon enrolment and consistently exhibited a CD4% ranging between 26-37% throughout 15 months of investigation, assumed to be related to the possession of HLA-B*27 (Fig 2.10 C).

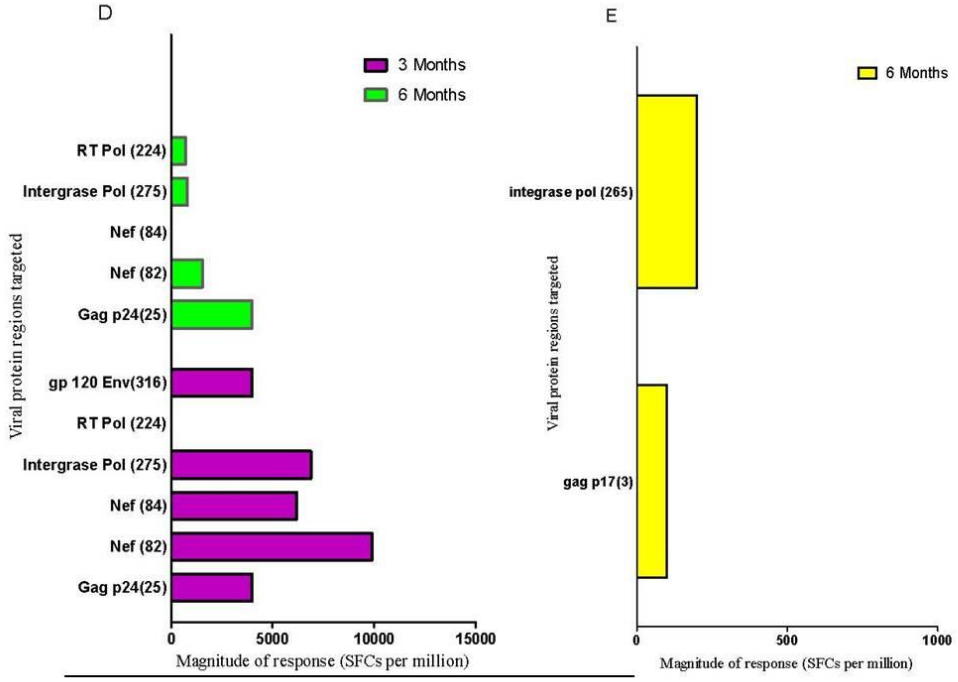
We assessed the association of HLA-B*27 restricted Gag-specific responses with slow progression in transmission pair CP3-024 (Fig 2.10 D/E). The mother targeted Gag p24 (25) at high levels at a three month interval time, while the child had no detectable responses at first ELISpot assay and a low level response to Gag p17 (OLP 3-IK9) and integrase (Pol).

As previously documented the transmission of escape variants within B*27-KK10 in children could results in lack of response, which in turn could be associated with rapid progression (Goulder et al., 2001a). We next investigated sequence changes in the transmission variants from mother to child focusing on HLA-B *27 restricted KK10 (OLP 36) and IK9 (OLP 3) epitopes compensatory mutations, S173A and E260D. Our sequencing results showed no changes in epitope B*27-IP9 in the mother but a variant change in position 2 of B*27-IP9 in the child. The epitope KK10 showed two sequence changes in position 264, and 268 (Fig 2.10 G). Similarly as in CP3-024, the variant T173A was present in contrast to S173A as in clade B

infection. We next compared the replicative capacity of the transmitted virus and found no significant difference in viral replicative capacity between the virus of the mother or child (Fig 2.10 F).

A HLA: CP3-024	A1	A2	B1	B2	Cw1	Cw2
Mother	2902	6601	4201	5802	0602	1701
Child (7months)	3004	6601	2705	5802	0202	0602





G	*B27-IK9*	*B27-KK10*
CONSENSUS C	***(Gag19-27)***	***(Gag 264-273)***
	IRLRPGGKK	**KRWIILGLNK**
3M-024 6M	WT	KKWIIMGLNK
3C-024 6M	IGLRPGGKK	KKWIIMGLNK

Figure 2.10 A)HLA, B)Viral load, C)CD4% and Absolute CD4 T cell count, D)CTL response of mother, E)CTL response of child, F) Viral replicative capacity and G)Sequence changes in epitope IK9 and KK10 of transmission pair CP3-024.

2.3.7. A description of HLA-B*5703 in mother-child HIV-1 infected transmission pairs

We next went on to investigate the impact of the HLA-B*5703 allele in HIV infection, as they have been reported to be most strongly associated with low viral loads and high CD4 counts in a clade C cohort (Kiepiela et al., 2004, Leslie et al., 2010). HLA-B*5703 also presents Gag-specific CD8+ T cell epitopes such as KF11, TW10 respectively and conversely escape mutations within these epitopes has been found to reduce the replicative capacity of HIV (Brockman et al., 2007, Crawford et al., 2009, Goepfert et al., 2008).

In order to describe the role of HLA-B*57 in clade C HIV-1 infection we investigated two transmission pairs. In transmission pair CP3-052 the mother carried HLA-B*5703 (Fig 2.11 A) while in transmission pairs CP1-002 two siblings carried HLA-B*5703 (Fig 2.11 A). The CP1-002 children were enrolled by the mother at different visits. The younger child was enrolled first and based on low CD4% was awaiting ARV treatment, while the older sibling joined the study a few months later but was still clinically stable.

We first investigated the mother who carried HLA-B*57 hypothesizing that the mother would progress slowly, if she has effective CTL responses and the correct succession of variants escaping. As the child did not carry HLA-B*57 we hypothesized that child would progress faster but that progression will also depend on the variants transmitted and/or the fitness of the transmitted virus.

Clinically, upon enrolment the mother and child were doing well but the child progressed rapidly after first visit and was disenrolled from our study and referred for treatment (Fig 2.11 C). The mother was doing well as she maintained an absolute CD4 T cell count of 583 cells/mm^3(Fig 2.11C). As the child was 5 months of age upon enrolment, the child seemed to be a rapid progressor requiring early treatment by second visit.

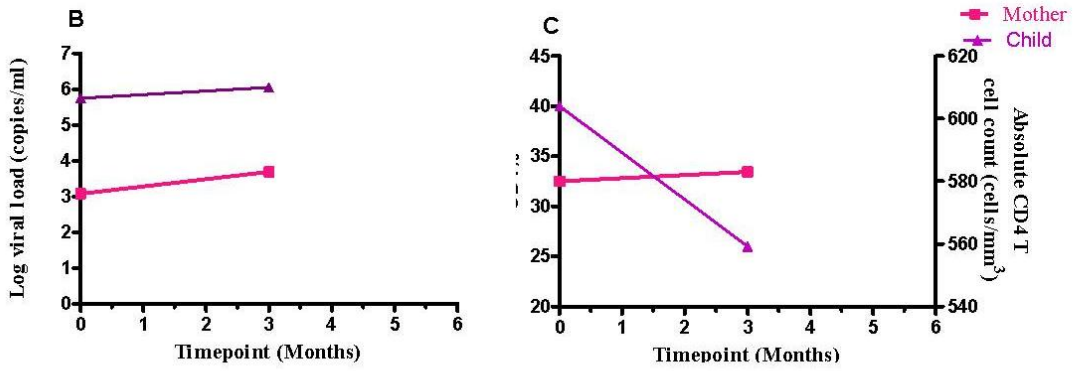
As the child rapidly progressed between 5 and 8 months of age from a CD4% of 40-25% we next investigated the child's HIV-specific responses (Fig 2.11 E). The child had one predominant B*7 restricted response to Nef (OLP 76). The mother also targeted Nef (OLP 76), together with Gag p17 (OLP 3-5) and Gag p24 (22) at higher levels.

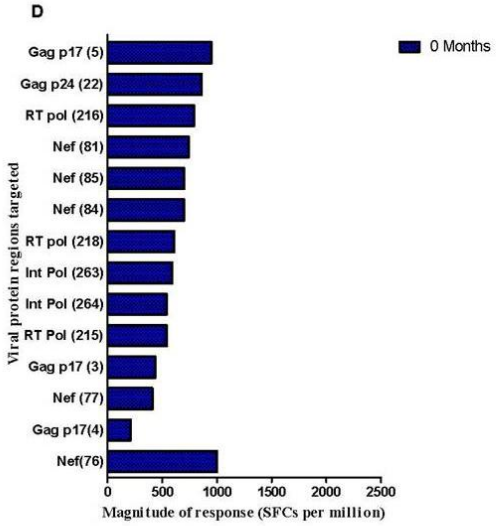
Based on the previous evidence of the association of B*57 MTCT escape variants on viral escape and viral fitness we next investigated whether rapid progression in the child was related to the child's inability to generate effective responses was related to sequence changes by the transmission of variants from mother to child. Do children benefit from transmission of escape variant within unmatched pairs and is its effect compensated by reversion (Thobakgale et al., 2009b)? We next investigated the role of escape variant Gag T242N escape mutation in Gag, which is known to decrease viral fitness early in the course of HIV infection, associated with TW10 epitope and the emergence of upstream mutations at residues H219, I223, and M228. We further investigated sequence changes in the ISW9 and KF11 epitopes. Our sequencing results showed that a sequence change in the first position (I147L) in ISW9 for the mother but the child still showed the presence of the wildtype. In addition, the mother had a sequence change in position 247 (I247M) in TW10 while the child still carried the wildtype. The child carried variant V168I in the KF11 sequence while the mum carried the wildtype.

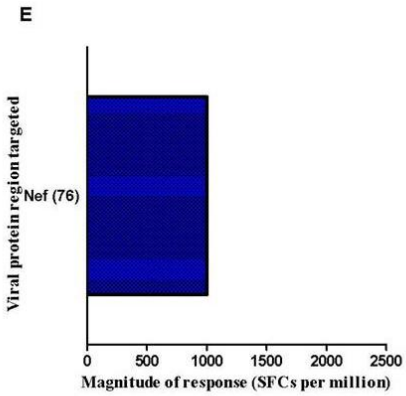
Interestingly we also observed the presence of possible compensatory mutations in A146P/A163G/ S165N and S252N.

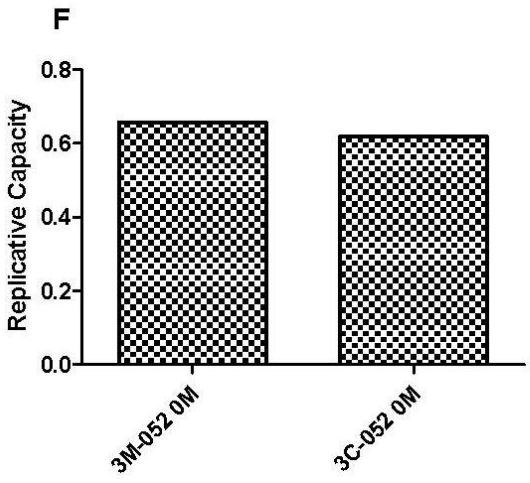
From the above observations it seemed possible that the child may have received a predominantly wildtype virus with ineffective compensatory mutations from the mother, hence we next compared the fitness of earliest transmitted virus of the child to the mother. We found no significant difference in viral replicative capacity between the virus of the mother, father or child (Fig 2.11 F).

A HLA: CP3-052	A1	A2	B1	B2	Cw1	Cw2
Mother	24	74	07	5703	07	07
Child 1 (5 months)	24	3402	07	44	04	07









G

	B5703-ISW9 (Gag147-155)	*B5703-KF11 Gag162-172)*	*B5703-TW10 (Gag240-249)*
CONSENSUS C	ISPRTLNAW	KAFSPEVIPMF	TSTLQEQIAW
3M-052	LSPRTLNAW	WT	TSTLQEQMAW
3C-052	WT	KAFSPEIIPMF	WT

Figure 2.11 A)HLA, B)Viral load, C)CD4% and Absolute CD4 T cell count, D)CTL response of mother, E)CTL response of child, F) Viral replicative capacity of pair at first timepoint and G) Sequence changes in epitopes ISW9, KF11 and TW10 of transmission pair CP3-052 (Child=C etc).

We next investigated the siblings who carried HLA-B*57 hypothesizing that these children would progress slowly depending on the transmitted variants and/or the fitness of the transmitted virus.

Clinically, upon enrolment the mother had a stable viral load and an average absolute CD4 T cell count of 275cells/mm^3 (Fig 2.12 B/C). At this visit, the mother enrolled her youngest child (3 years old). The child had a stable viral load and a low but relatively stable CD4% (Fig 2.12 B/C). However, only after 18 months of follow up the mother agreed for her child to be referred for treatment. After 9 months on the study, the mother also enrolled her older child (5 years). Interestingly, unlike the younger child, the older child was doing very well 5 years after transmission. The child had a low viral load averaging 1967copies/mL with a high CD4% of 43%. We next aimed to identify where the difference arose that enabled one child to progress and the other to control after both receiving the transmitted from the same donor but at different times.

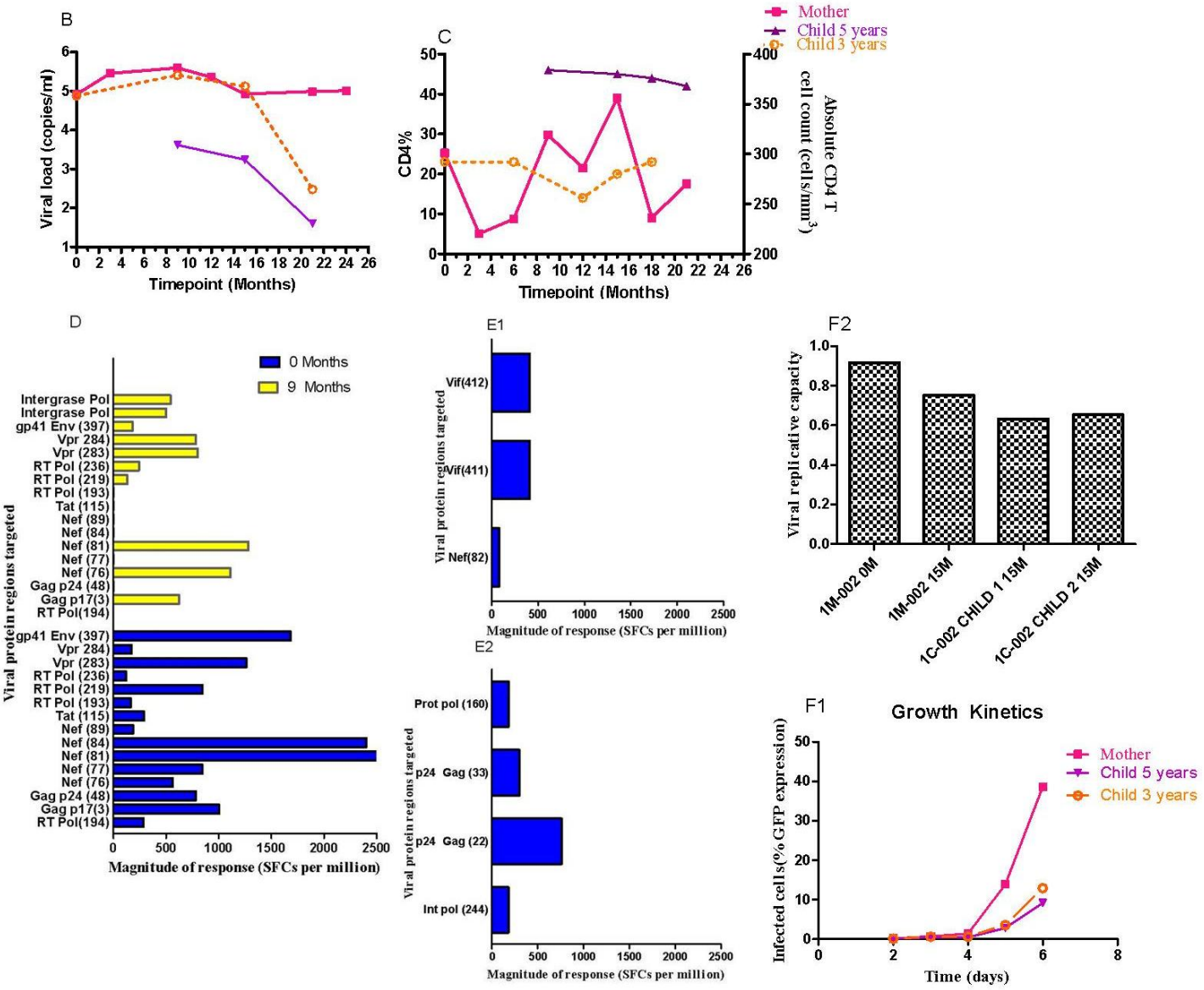
We next investigated if the children's HIV-specific responses played a role in their diverse clinical outcomes (Fig 2.12 E). The youngest child made a single dominant B*57 restricted response to Gag (OLP 22), which was an optimally defined KF11 response (Fig 2.12 E2). The older child targeted Vif and Nef at low levels but did not target Gag. We confirmed this finding using optimal epitopes. The mother exhibited the highest magnitude of response when she targeted Nef (OLPS 81and 84), together with low magnitude of responses to Gag p17 (OLP 3) and Gag p24 (48) when initially screened.

Based on the association of B*57 MTCT escape variants on viral escape and viral fitness we next investigated whether the difference in clinical outcome in siblings were related to

sequence changes in the transmitted virus. We next investigated the role of escape variant T242N escape mutation in Gag by investigating sequence changes in the TW10 epitope, and by further investigating sequence changes in the ISW9 and KF11 epitopes. Our sequencing results showed that no sequence changes in ISW9 for the mother and both children. Interestingly the mother carried the wildtype for all three epitopes while the children carried the same variant. The children carried a sequence change in position 242 (T242N) in TW10 and position 163 (A163N).

We concluded the investigation by comparing the growth kinetics and fitness of earliest transmitted virus from the mum to the children. When investigating growth kinetics, all viruses seem to grow similarly for first four days, after day 5 the growth of the donor virus increased exponentially (Fig 2.12 F1). The difference in growth filtered through when investigating viral replicative capacity. The donor virus seemed to show a higher replicative capacity than the children, whom unexpectedly did not differ from each other (Fig 2.12F2).

A HLA: CP1-002	A1	A2	B1	B2	Cw1	Cw2
Mother	3301	6802	0702	4102	0702	1701
Child 1 (5 years)	0202	6802	0702	5703	0701	0702
Child 2 (3 years)	0202	6802	0702	5703	0701	0702



G	*B5703-ISW9* *(Gag147-155)*	*B5703-KF11* *Gag162-172)*	*B5703-TW10* *(Gag240-249)*
Consensus C	**ISPRTLNAW**	**KAFSPEVIPMF**	**TSTLQEQIAW**
1M-002	WT	WT	WT
1C-002 Child 5 years	WT	KNFSPEVIPMF	TSNLQEQIAW
1C-002 Child 3years	WT	KNFSPEVIPMF	TSNLQEQIAW

Figure 2.12 A)HLA, B)Viral load, C)CD4% and Absolute CD4 T cell count, D)CTL response of mother, E1/E2)CTL responses of children, F1) Growth kinetics of virus F2) Viral replicative

capacity and G)Sequence changes in epitopes ISW9, KF11 and TW10 of transmission mother and siblings CP1-002.

2.3.8 Phylogenetic comparison of sequences

After sequence analysis it is imperative to identify if sequences correspond to clade C origin and are closely related to each other as expected with mother-child pairs. Our phylogenetic tree confirmed lineage which allowed and mother-child clusters which allowed investigators confidence to continue with successive implementation of viral replicative assays (Fig 2.13).

2.4 DISCUSSION

The majority of treatment naïve children typically progress to AIDS or death within the first 2 years of life. However, a minority of children progress slowly to AIDS. Effective immunity in these children could be related to genetics (eg. IL-10, HLA etc), transmitted virus, effective humoral immune response, and/or effective cytokine secretory response and can be related to virus to cell surface interactions (eg CCR5, CXCR4 etc). The humoral immune response consists of two main arms of immunity namely antibody defense (B cells) and CTL defense (T cells). Our investigation focused on the T cell arm of immunity predominantly in children, whom appear to be in immunologically impaired during the first few years of their life.

Our cross sectional evaluation of CTL responses in 60 treatment naive clade C infected children revealed that 50% of these children made low level responses that were not detectable when confirmed as individual pooled responses, while the remaining children had detectable responses. We found no difference in viral load between responders and non-responders ($p=0.2799$) in keeping with previous studies (Huang et al., 2008). Our analysis of responses

confirmed that viral regions targeted by responder children in order of frequency as Nef (49%), Gag (17%), Env (14%) and Acc/Reg/Pol (6%). Interestingly, Nef which has been previously associated with different clinical outcomes (Gomez-Icazbalceta and Larralde, 2009), was dominantly targeted from 3 months to 10 years of age confirmed previous findings (Thobakgale et al., 2007, Huang et al., 2008).

Knowledge of the known transmitted virus with an understanding of effective CTL responses may direct us to proteins that could impact viral control. Based on other studies that showed that HIV-1 infected adults (Goepfert et al., 2008, Matthews et al., 2009) and children (Feeney et al., 2005, Thobakgale et al., 2009b) benefit from exhibiting HLA-B restricted Gag-specific responses and from receiving transmitted escape mutations we next attempted to investigate the interplay between HLA, T cell and virus in a few MTCT transmission pairs. In the search for natural correlates of immune protection, our study was subsequently conducted in a rare untreated mother-child cohort. As protective HLA-B*27 (Goulder et al., 2001a, Schneidewind et al., 2007) and HLA-B*57 (Altfeld et al., 2003, Brockman et al., 2007, Crawford et al., 2009, Goulder et al., 2000, Leslie et al., 2004, Miura et al., 2009b, Feeney et al., 2005, Thobakgale et al., 2009b) restricted Gag-specific responses have been documented to play a role in slow disease progression within transmission pairs (Feeney et al., 2005, Thobakgale et al., 2009b, Goepfert et al., 2008), we focused on these HLA alleles.

We hypothesized that HIV-specific CTL responses restricted by the protective HLA- B*2705 and B*5703 would be associated with slow progression in children and/or sequence changes that may shape a less fit virus. However, our study was unable to relate specificity of any CTL targeting. Furthermore, we were also unable to associate Gag-specific CD8+ T cell response with slow progression in the selected children in our cohort, in keeping with previous studies in

children (Froebel et al., 1997, Huang et al., 2008). We were unable to show an obvious benefit in adults carrying a protective allele. The mothers carrying protective alleles B*2705 (CP3-024) and B*5703 (CP3-052) were doing well clinically but the study design was restricted in that we were unable to follow mothers or children from time of infection. In addition, mother CP3-052 defaulted once child was referred for treatment. Our study was restricted in that participants often defaulted after receiving clinical results, or once requiring ART as we were unable to provide treatment or aftercare based on funding constraints.

HIV-specific CTL responses are able to target many viral variants across clades represent possible targets (Newman et al., 2002, Cao et al., 1997, Cao et al., 2000). Certain HLA –B associated escape variants have been documented to be correlated to a lower viral load in recipients (Brockman et al., 2007, Feeney et al., 2005, Leslie et al., 2004, Miura et al., 2009b). We hypothesized that loss of viral control may be associated with sequence changes in Gag that facilitate viral escape, leading to the loss of effective immune responses influencing viral fitness. We were limited only able to investigate Gag sequence changes. The limited targeting of Gag (Huang et al., 2008)) could not explain why slow progressors, targeting Nef were still controlling HIV infection. As previously described, most chronically infected children frequently target Nef. We were unable to sequence Nef due to financial constraints.

We next went on to investigate replicative capacities of the viruses. The replicative capacities differ in slow and rapid progressor children (Prado et al., 2010). Furthermore, a reduced viral replicative capacity has previously been documented to be associated with the presence of certain mutation and/or compensatory mutations (Brockman et al., 2007). In B*57 restricted transmission pairs we observed the presence of mutations and compensatory mutations associated with TW10. The mother who carried B*57 was clinically stable even though follow

up period was restricted. She did not carry the T242N mutation, which has previously been associated with lower fitness cost (Brockman et al., 2007) but still carried the S252N compensatory mutation. However, both mother and child's virus had similar replicative capacities. The sibling carriers of B*5703 showed different clinical outcomes, which we presumed to be related to the changes in the virus that may have occurred between the time the mother transmitted to older and then younger child. However, the family was only recruited into our study many years after transmission, making it difficult to investigate the underlying mechanisms. Our investigation of sequences changes in this family revealed the presence of the T242N variants in both siblings at the time of investigation, however as we were unable to track sequence changes. We were thus unable to identify timing of sequence variants, and if changes were compensated in one child accounting for the difference in clinical outcome.

CTL based studies provide information for the development of T cell based vaccines. Overall, our study was unable to investigate transmission pairs as rigorously as required ie. time of transmission, larger HLA cohort, detailed sequencing etc. The overall study design was further affected due to the much welcomed changes in PMTC guidelines. Nevertheless, our investigation concluded that children make low level or no responses following transmission which could explain why a majority of children are unable to control HIV-1 infection. To complement this finding, children who make responses tend to target Nef-specific epitopes, which have been associated with differential outcomes. We were unable to link the interplay between HLA, CTL responses, transmission of escape variants with overall viral fitness in the selected transmission pairs which may be related to study design or show that HLA and CTL data alone is not sufficient in determining what enables control. There exists a need to describe the role of the other arm of immune defence such as neutralizing antibodies, and innate immunity such as natural killer cells which to date has yet to be described in children.

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CHAPTER THREE

TIM-3 and PD-1 contribute to T cell exhaustion in HIV-1 clade C infected African children

ABSTRACT

Background: Inhibitory receptors such as the T cell immunoglobulin and mucin-3 domain molecule (TIM-3), and Programmed Death-1 (PD-1) play a crucial role in regulating T cell function during chronic viral infections. A study in HIV-1 clade B infected adults suggested TIM-3 expression on T cells to be elevated in chronic HIV infection, and correlate with markers of HIV disease progression. TIM-3 expressing T cells exhibited an "exhausted" phenotype with impaired functionality; however blockade of TIM-3 resulted in restoration of T cell effector activity such as cytokine secretion and proliferation. To date, no studies have been performed to investigate the role of TIM-3 in HIV-1 infected children and no data is available in the context of HIV-1 clade C infection. In this study, we assessed T cell exhaustion profiles in HIV-1 clade C infected children, and investigated their association with markers of disease progression.

Materials and Methods: Our study investigated the expression profiles of the inhibitory molecules TIM-3 and PD-1 on CD4+ and CD8+ T cells in a cohort of 37 infected children, aged 24-942 days, and 9 HIV-1 seronegative children (controls) using multiparameter flow cytometry.

Results: T cells from HIV-1 infected children expressed significantly higher levels of both TIM-3 ($p=0.001$) and PD-1 ($p=0.0001$) on CD8+ T cells derived from HIV-1 infected children in comparison to uninfected children. Expression of TIM-3 was higher on CD8+ T cells compared to CD4+ T cells (46% vs 20%), in line with previous studies, and TIM-3 expression levels on CD4+ T cells and CD8+ T cells were positively correlated ($r=0.45$;

p=0.028). TIM-3 expression correlated with expression of PD-1 on CD4+ and CD8+ T cells. However, unlike adult HIV-1 infection, neither TIM-3 nor PD-1 correlated with HIV-1 viral load or absolute CD4+ T cell count. Longitudinal investigation revealed that TIM-3 and PD-1 expression levels on T cells were maintained at high levels over time.

Conclusion: We here demonstrate that the expression of the inhibitory molecules TIM-3 and PD-1 on T cells are elevated in paediatric HIV-1 infection. This elevated expression on T cells from vertically infected children suggests that both TIM-3 and PD-1 contribute to T cell exhaustion in children. Of note, the highest expression levels were observed in infants less than 6 months of age, where HIV-1 specific T cell immunity is of lowest magnitude and breadth. TIM-3 in concert with other inhibitory molecules such as PD-1 may therefore promote or result in the failure to control viral load and to accelerate disease progression in the paediatric HIV patient population.

Key words: TIM-3; PD-1; paediatric; children; HIV progression;

3.1 INTRODUCTION

Acquisition of HIV remains a major source of paediatric death particularly in sub-Saharan Africa (Stats SA, 2011). Despite the revision of treatment guidelines in favour of early initiation of ART, the impact of the HIV epidemic has had a devastating effect on the mortality rates of children (WHO, 2010). Recent estimates by Statistics South Africa- a government agency, revealed that 43% of deaths in South African children were AIDS-related (Stats SA, 2011). Clues obtained from studies of natural control of HIV on immune correlates may provide valuable information for vaccine design. Effector T cells such as CD8+ and CD4+ T cells have been implicated in successful viral control (Thobakgale et al., 2007, Honeyborne et al., 2007, Kiepiela et al., 2007, Yu et al., 2007, Gamberg et al., 1999, Ramduth et al., 2009). Functional effector T cells are initially generated during the early stages of infection. However, robust immune responses have been shown to gradually diminish over time. This loss of effector function, including the inability to produce cytokines and proliferate renders effector cells ineffective in viral control. Chronic infections are often characterized by varying degrees of functional impairment of virus-specific T cell responses, and this defect is the principal reason why the host is not able to eliminate the persisting pathogen. As these cells are not able to function at their optimum we refer to them as dysfunctional and in a state of "functional exhaustion". It has been demonstrated that by blocking the inhibitory pathways using antibodies against inhibitory molecules such as TIM- and PD-1, the CD8+ T cell effector function can be restored (Day et al., 2007, Jin et al., Jones et al., 2008b).

TIM-3 belong to the immunoglobulin super family, of which 4 molecules have been described in mice and humans (Kuchroo et al., 2003). TIM-1 appears to be an activation molecule for all T cells. TIM-2 molecules functions by negatively regulating TH2 immune

responses. TIM-4 expression in DCs promotes DC maturation, which plays an important role in the initiation of TH2 polarization. The present investigation focused on the TIM-3 molecule, which is a type I membrane surface glycoprotein expressed on T cells (Mariat et al., 2005). Interestingly, TIM-3 has been shown to engage with its ligand galectin-9 and thereby negatively regulates IFN-γ secretion (Zhu et al., 2005) and TH1 responses in general (Wang et al., 2009, Wang et al., 2008). TIM-3 has previously been associated with immune dysregulation of T helper cell responses in other disease settings (Koguchi et al., 2006, Zhang et al., 2011).

When TH1-helper cells are activated they help to initiate an attack thereby guarding against infection. However these T cells can also become overaggressive in their attack and thus their function needs to be kept in check. TIM-3 plays a role in controlling TH1 dependent immune responses by counterbalancing aggressive responses via interaction with its ligand galectin-9. Consequently, by increasing the TIM-3 signal the response of TH1 cells can be diminished, creating immune tolerance.

TIM-3 induces T cell tolerance in both mice and man. The effects of blocking the TIM-3 pathway were studied in mice who spontaneously develop insulin-dependent diabetes. A rapid onset of disease was observed in the mice treated with TIM-3-specific monoclonal antibodies. The study corroborated the role of TIM-3 in inhibiting autoimmune responses (Sanchez-Fueyo et al., 2003). The TIM-3 pathway blockade also played a role in other cells. TIM-3 was found to dampen the antigen-specific immunosuppressive function of CD4+CD25+ regulatory T cell populations (Anderson and Anderson, 2006, Sanchez-Fueyo et al., 2003, Wang et al., 2009). TIM-3 ligation has further been found to be associated with distinct signalling events exhibiting differential expression on the innate naïve dendritic cells

compared to adaptive immune cells (Anderson and Anderson, 2006, Kuchroo et al., 2008). Hence through differential expression on innate versus adaptive immune cells, TIM-3 can either promote or terminate TH1 immunity. Taken together, data supports a key role for TIM-3 in regulating immune responses in different disease settings.

When TIM-3 pathway was blocked and TIM-3 signal was decreased, the TH1 responses could be amplified, creating strong immune responses which could play a role in autoimmune diseases (Jones et al., 2008a, Monney et al., 2002). A study in HIV-1 clade B infected adults suggested that TIM-3 expression on T cells was increased in chronic HIV infection and correlated with markers of HIV disease progression. The frequency of TIM-3 expressed on CD8+ T cells correlated positively with HIV-1 viral load and CD38 expression as a marker of immune activation, and correlated inversely with absolute CD4+ T cell count in HIV-1 infected participants (Jones et al., 2008b). The highest TIM-3 expression was observed in HIV-1-specific CD8+ T cells. In addition, TIM-3 expressing T cells exhibited an exhausted phenotype (Sakuishi et al., 2011) with impaired functionality and blocking of TIM-3 signaling restored proliferation and increased cytokine production in HIV-1-specific T cells (Jones et al., 2008b).

Recent studies in the LCMV mouse model have documented CD8+ T cell exhaustion by co-expression of TIM-3 and PD-1 (Jin et al., 2010). Day *et al.* investigated the role of the inhibitory molecule PD-1 in a chronic human viral infection by examining PD-1 expression on HIV-specific CD8+ T cells in 71 treatment naïve HIV-1 clade-C infected participants (Day et al., 2006). The study demonstrated that PD-1 was upregulated on the surface of HIV-specific CD8+ T cells and that this expression was associated with increased T cell exhaustion and HIV disease progression (Day et al., 2006). The study reported that PD-1 was

significantly upregulated, and expression correlated with impaired HIV-specific CD8+ T cell function as well as predictors of disease progression: positively with plasma viral load and inversely with absolute CD4+ T cell count. In an additional blockade experiment of the PD-1/PD-L1 pathway, an enhancement of HIV-specific CD4+ and CD8+ T cell function, and the reversal of the exhausted T cell phenotype was observed (Day et al., 2006).

Even though there are a growing number of investigations on negative immuneregulators in adult study populations, the role of inhibitory molecules in paediatric HIV-1 infection has not been fully explored and no data exist on the expression of TIM-3 in children to date. A recent study demonstrated PD-1 to be a marked negative regulator of activated T cells in 93 HIV-1 infected untreated children aged (10.8 years) (Prendergast et al., 2011). PD-1 expression on CD8+ T cells, was found to be higher in HIV-infected children than HIV-uninfected children (Prendergast et al., 2011). However, unlike what has been reported for adult HIV-1 infection, the study found no correlation between PD-1 and HIV viral load (R=0.11, P=0.40). Interestingly, PD-1 expression on CD8+ T cells positively correlated with activation on CD8+ T cells which was measured by co-expression of CD38 and HLA-DR. The study finally demonstrated that both CD8 activation and PD-1 expression on CD8+ T cells were partially driven or driving the magnitude of the HIV-specific CD8+ T cell response (Prendergast et al., 2011).

Dual blockades of the TIM-3 and PD-1 pathway are able to rescue T cell immune responses in hepatitis C infection (Golden-Mason et al., 2009, Callendret and Walker), murine leukaemia (Zhou et al.), cancer (Sakuishi et al.), tuberculosis (Henao-Tamayo et al., 2011) and in LCMV infection (Jin et al., 2010). The LCMV study found that TIM-3 expression on CD8+ T cells was low after acute infection, but that TIM-3 was expressed at high levels

throughout chronic infection. Blockade of the TIM-3 pathway alone has also resulted in an improved T cell response. Interestingly as much as 80% of the LCMV-specific CD8+ T cells in lymphoid and non-lymphoid organs dually expressed TIM-3 and PD-1. The dual expression was associated with a severe exhaustion profile that was associated with the secretion of pro-inflammatory cytokines (Jin et al., 2010). In another study mice were infected with *Mycobacterium tuberculosis* (TB). The study documented that the exhaustion markers PD-1 and TIM-3, as well as the marker KLRG-1, were predominantly expressed on CD8+ T cells in 5 MTB infected mice. The expression levels of the exhaustion markers decreased upon treatment showing, changes as the bacterial load in the lungs dropped indicating a valuable marker to exploit therapeutically. The study further demonstrated the potential use of these markers for early detection of TB in comparison to conventional methods of screening TB (Henao-Tamayo et al., 2011).

To date, no studies have been performed to investigate the role of TIM-3 in HIV-1 infected children and no data is available in the context of HIV-1 infection. Given the recently published data on PD-1 in children and co-regulation of PD-1 and TIM-3 in LCMV, our study investigated the mechanism underlying the association between progressive T cell exhaustion and HIV replication in an African cohort of HIV-1 clade C infected children. We sought to answer the following research questions: (1) Do TIM-3 and PD-1 T cell expression profiles differ in HIV-1 infected children and uninfected children (2) Do TIM-3 and PD-1 expression correlate with markers of disease progression as observed within adult HIV-1 clade B populations? and (3) What is the interplay between TIM-3 and PD-1 expression in HIV-1 infected children?

3.2 PARTICIPANTS, MATERIALS AND METHODS

3.2.1 Cohort description

The study began recruitment in 2006 in South Africa, prior to the revision and initiation of the new ART guidelines. A cohort of untreated HIV-1 infected mother child pairs was established. The treatment guidelines at the time dictated that HIV-1 seropositive mothers during the last trimester of pregnancy received a single dose of Nevirapine during labour. The infant received a single dose of nevirapine within 48 hrs of birth, according to the HIVNET-012 Protocol, as previously described (Guay et al., 1999, Jackson et al., 2003).

The 46 study participants were recruited through clinics in KwaZulu-Natal, Durban, South Africa. The recruitment clinic sites included King Edward Hospital, McCord Hospital, Saint Mary's Hospital and Prince Mshiyeni Hospital. Transmission pairs were screened and mother-child pairs who met the inclusion criteria of mother-child treatment naïve pairs and positive HIV-DNA PCR results from birth hospitals were recruited. The participants were selected for a preliminary cross-sectional analysis based on sample availability. The exclusion criteria for the chronically infected cohort included treated pairs (mothers and/or children). The untreated cohorts were part of the chronically infected pair cohort and represented initially falsely diagnosed HIV negative participants. This study retrospectively investigated 23 HIV-1 infected, 9 HIV-1 exposed uninfected and 13 HIV-1 treated children (Thobakgale et al., 2009, Thobakgale et al., 2007, Mphatswe et al., 2007) from Zulu/Xhosa ethnic origin with HIV-1 infection. Treated children who had an undetectable viral load for at least one or more timepoints were included for analysis.

Written informed consent was obtained from all study participants and the research protocol for the study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa, and the Internal Review Board of Massachusetts General Hospital, Boston.

3.2.2 Plasma viral Load and absolute CD4+ T cell measurement

Plasma was isolated from whole blood after a single round of centrifugation. Plasma viral loads were assessed using the Cobas Amplicor Monitor Test version 1.5, detection limit of 50 HIV-1 RNA copies/mL plasma according to the manufacturer's instructions (Roche Diagnostics).

Absolute CD4+ T cell counts and percentages of CD4+ T cells were determined from fresh whole blood using Multitest four colour TruCount as previously described (Gratama et al., 2002) according to the manufacturer's instructions (Beckton Dickinson Technology) at the HIV Pathogenesis Programme, Durban, KZN, South Africa.

3.2.3 Isolation of PBMCs

Blood specimens were 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. PBMCs were used fresh in ELISPOT assays. The remaining PBMCs were cryo-preserved in 90% fetal bovine serum (Sigma-Aldrich), 10% DMSO prior to storage in liquid nitrogen for subsequent multiparameter flowstaining and immunophenotyping expression analyses.

3.2.4 Multiparameter flow cytometry staining for TIM-3 and PD-1 expression on bulk CD4+ and CD8+ T cells

3.2.4.1 Antibody titrations: Titrations were performed by staining ($1x10^5$) PBMCs at a range of 2 to 25µl antibody (depending on volume and concentrations specified by manufacturer). Antibody dilutions were halved covering the above range. The final optimal concentration was subsequently used for immunophenotyping. The optimal concentration was determined by choosing the dilution of the stain where the intensity plateau for the stained cells was most favourable.

3.2.4.2 Thawing: Cryo-preserved PBMCs were thawed using a warming method. R10 media, made up of RPMI 1640 + 10% FBS, was warmed to 37°C in a 15 or 50 mL Sterilin tube. Firstly, 20 µl nuclease was added to 2 mL 37 ^{o}C warm R10 in a 15 mL Sterilin tube. Frozen PBMC vials were left to sit at room temperature for approximately 5 minutes until nearing liquid state. The cryovial was opened and the rim was quickly swabbed with 70% ethanol before the icy cells were transferred into the 15 mL Sterilin tube containing nuclease and 2 mL of warm R10 media. An additional 8 mL of warm R10 was slowly added to the tubes and the mixture was spun down at 1,700rpm for 10 minutes. Supernatant was discarded removing all of the now toxic DMSO freezing solution and the cell pellet was re-suspended and cells counted using an automated cell counting Guava viacount assay and read on the Guava cell counter **(**Guava Technologies, Guava PCA System). Cells were re-suspended in R10 at a concentration of 1 million cells per mL.

3.2.4.3 Immunostaining: 1 million cells per mL were added to each experimental FACS tube and control Fluorescence Minus One (FMO) tubes. PBMC were first stained for viability in the dark, at room temperature, with 1µl of a 200 µl stock solution of

LIVE⁄DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen). After 15 minutes the dye was washed of using cold PBS (phosphate buffered saline) for 10 minutes at 1,500 rpm. PBMCs panels were stained (extracellular) as below (Table 3.1), at 4°C for 30 minutes. Due to the limitation of available fluorochromes for PD-1 and TIM-3 antibody staining at the time of investigation, single cell analysis could not be implemented.

Table 3.1 Experimental panels: antibodies and volumes.

PANEL1:			
Antibody	**Fluorochrome**	**Abbreviation (Company, Catalogue)**	**Volume(µl)**
CD14	Peridinin Chlorophyll Protein	PerCP (BD, 340660)	5
CD19	Pacific Blue	PacB (BD, 51-0199-73)	2
CD3	Phycoerythrin-Cyanine 7	PE-Cy7(BD,clone L3T4)	2
CD4	Allophycocyanin	APC (BD, 340443)	3
CD8	Alexa Fluor 700	Alexa Fluor 700 (BD, 557945)	2
TIM-3	Phycoerythrin	PE (R&D, FAB2365P)	8

PANEL2:			
Antibody	**Fluorochrome**	**Abbreviation (Company, Catalogue)**	**Volume(µl)**
CD14	Pacific Blue	PacB (EBioscience, 48-0149-42)	2
CD19	Pacific Blue	PacB (EBioscience, 51-0199-73)	2.5
CD3	Fluorescein Isothiocyanate	FITC(EBioscience, SK7, 11-0036-42)	3
CD4	Allophycocyanin	APC (BD, 340443)	3
CD8	Alexa Fluor 700	Alexa Fluor 700 (BD, 557945)	2
PD-1	Phycoerythrin	PE (EBioscience,clone MIH4, 558694)	10

After the above mentioned staining of PBMCs, they were washed twice using cold PBS. Supernatant was discarded and pellet was re-suspended in PBS. Compensation beads were

setup and read to standardize assay. Flow data were acquired on a LSR II flow cytometer (BD Biosciences). For the infant cohort, a minimum of 500,000 events were collected per participant, and a minimum of 250,000 events were collected for adult samples. Acquisition templates were drawn during acquisition to further confirm subset gating prior to analysis. The FMO control was used as the reference gate while the LIVE⁄DEAD® Fixable Violet Dead Cell Stain were used as an exclusion channel to eliminate dead PBMCs.

3.2.5 Statistical analysis

Flow data was analyzed with FlowJo software version 7.5 for PC. Microsoft Excel was used to transfer data and subtract fluorescence minus one (FMO) background values. Data was analyzed and graphically represented using Prism software (GraphPad; version 5). The Mann Whitney U test was performed when comparing less than two groups, Kruskal Wallis testing and ANOVA was performed when comparing more than two groups. Post-test analysis was performed using Dunns Multiple Comparison Test. Normalization and subset discrimination and gating of data including the mean fluorescent intensity and % response per event was performed using Flow Jo. Significance levels were indicated using Mann Whitney U ($P<0.05$).Correlations were performed using Spearman rank tests (p and r^2 values).

3.3 RESULTS

3.3.1 Cohort Characteristics

A total of forty six children were studied. As represented in Table 3.2 age range and clinical data shows that the HIV-1 uninfected children exhibit a median absolute CD4+ T cell count of 2,033 cells/mm^3 and CD4+ T cell % of 34%. The infected untreated children had a median viral load of 55,700 copies/mL with a median absolute CD4+ T cell count of 1,838 cells/mm^3 and CD4+ T cell% of 30% while the treated children had a median viral load of 292 copies/mL with an absolute CD4+ T cell count of 1,325 cells/mm^3 and CD4 T cell of 29% (Figure 3.1). The median ages of uninfected and infected untreated children were similar while the treated children were slightly older as represented in Table 3.2.

Table 3.2 Age and clinical characteristics of study cohort.

	HIV-1 uninfected	HIV-1 untreated	HIV-1 treated
N	9	23	14
Median Age in days (IQR)	61(62-945)	76(24-942)	1838(53-4025)
Median Absolute CD4+ T cell count (IQR)	2033 (194-3433)	1838(173-5561)	1325(605-4291)
Median CD4 T cell % (IQR)	34(24-61)	30(10-46)	29(16-54)
Median Viral Load (copies/mL)	-	55700	292
Viral Load IQR (copies/mL)	-	(10400-7281580)	(100-805)

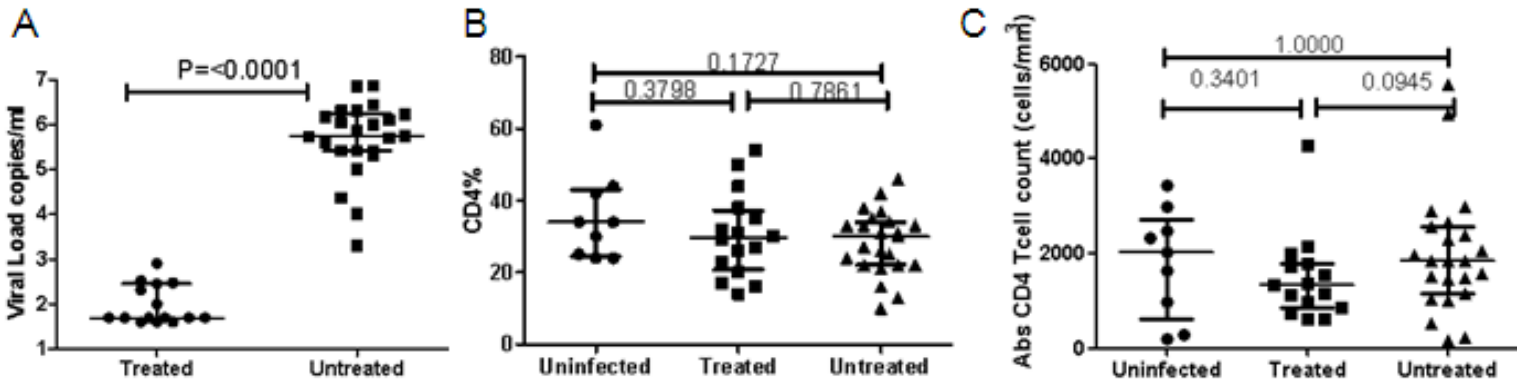

Figure 3.1 Graphical representation of the clinical characteristics of the cohort of children: A) Viral load (copies/mL), B) CD4+ T cell % and C) Absolute T cell count (cells/mm^3).

3.3.2 Representative gating scheme for the quantification of the expression of inhibitory molecules on CD4+ and CD8+ T cell subsets

Gating schemes for the PD-1 and TIM-3 experiments are represented in Fig.3.2 and Fig.3.3 In Fig 3.2/3A time represents a uniform flow rate. The live lymphocytes were then gated on removing doublets. Subsequently, gates were set to exclude dead cells (viability marker^{+}), monocytes (CD14+) and B cells (CD19+). Fig 3.2B represents the gating strategy for quantification of TIM-3 expression. The gating for viable cells was followed by the gating of viable lymphocytes (CD3+) and CD4+ or CD8+ T cells. The TIM-3 FMO and PD-1 FMO were used as controls to quantify TIM-3+ and PD-1+ expression as shown in Fig 3.2 and Fig.3.3

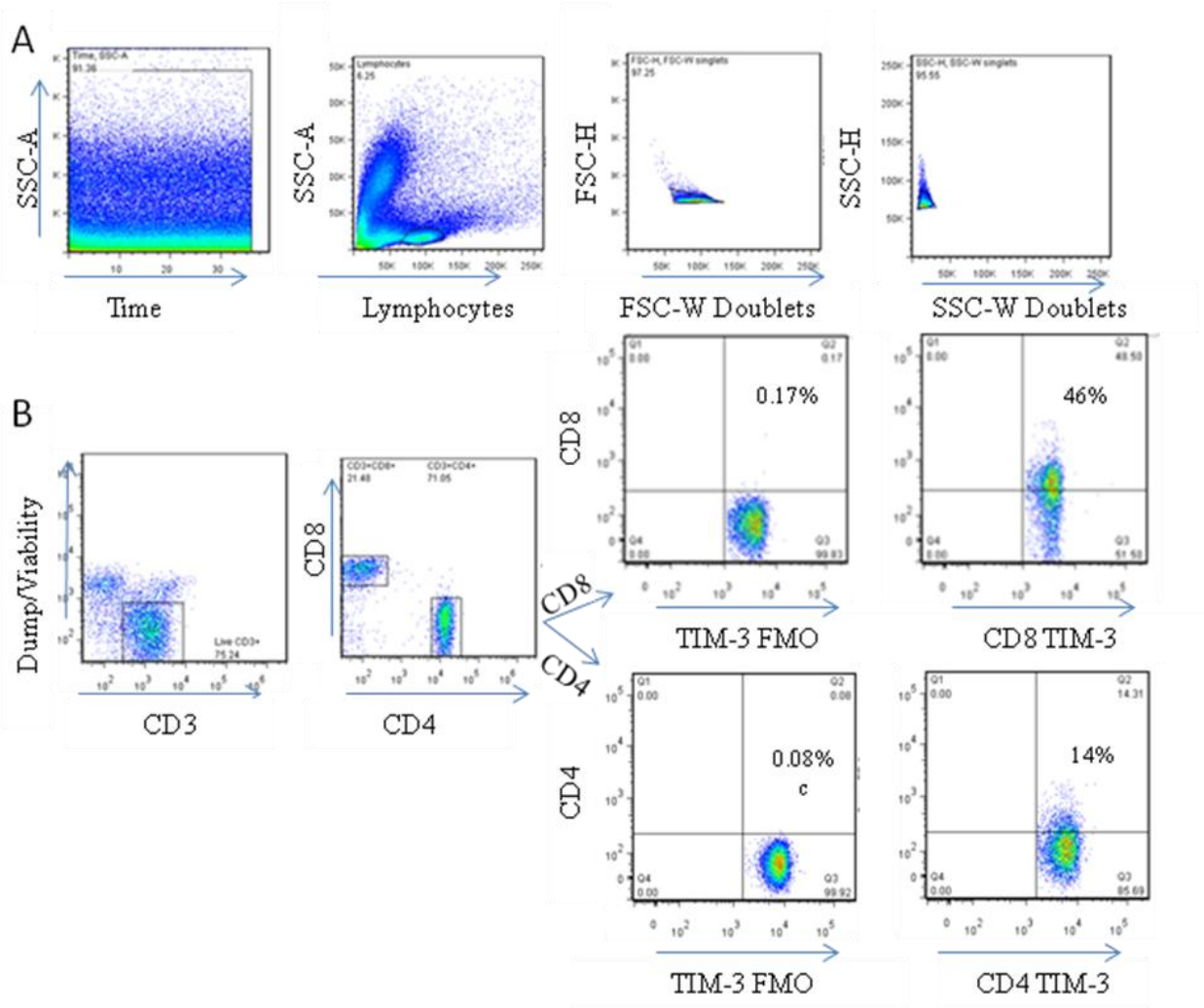

Figure 3.2 Representative flow cytometry gating scheme for the assessment of expression of TIM-3 for a single participant sample from the study cohort.

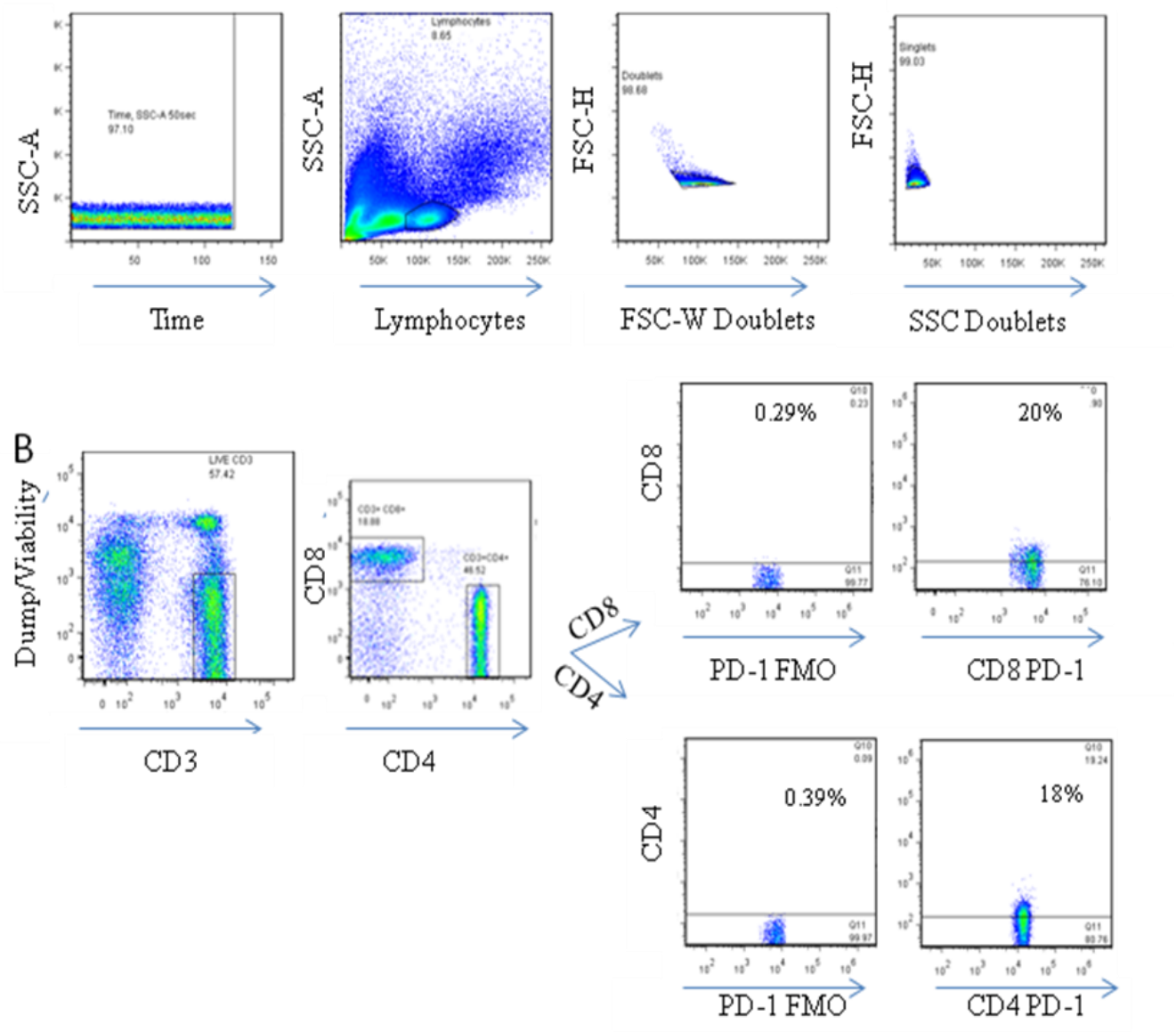

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Figure 3.3 Representative flow cytometry gating scheme for the assessment of expression of PD-1 for a single participant sample from the study cohort.

3.3.3 Elevated expression of TIM-3 on CD4+ and CD8+ T cells of the HIV-1 infected children.

We first evaluated TIM-3 expression levels on CD4+ T cells and CD8+ T cells as shown in Fig. 3.4 TIM-3 expression ranged from 8 to 87% for CD8+ T cells and 1 to 91% for CD4+ T cells. Median TIM-3 expression was higher on CD8+ T cells compared to CD4+ T cells. Moreover when comparing sub-groups median TIM-3 expression was higher on CD8+ T

cells from the group of treated children in comparison to uninfected children (p=0.0044). However untreated infected children had a broader expression range. The median TIM-3 expression in untreated uninfected children differed significantly from uninfected children (p=0.0134) but not from treated children (p=0.2647). The uninfected group was removed from CD4 TIM-3 analysis due problems related to antibody during sample processing.

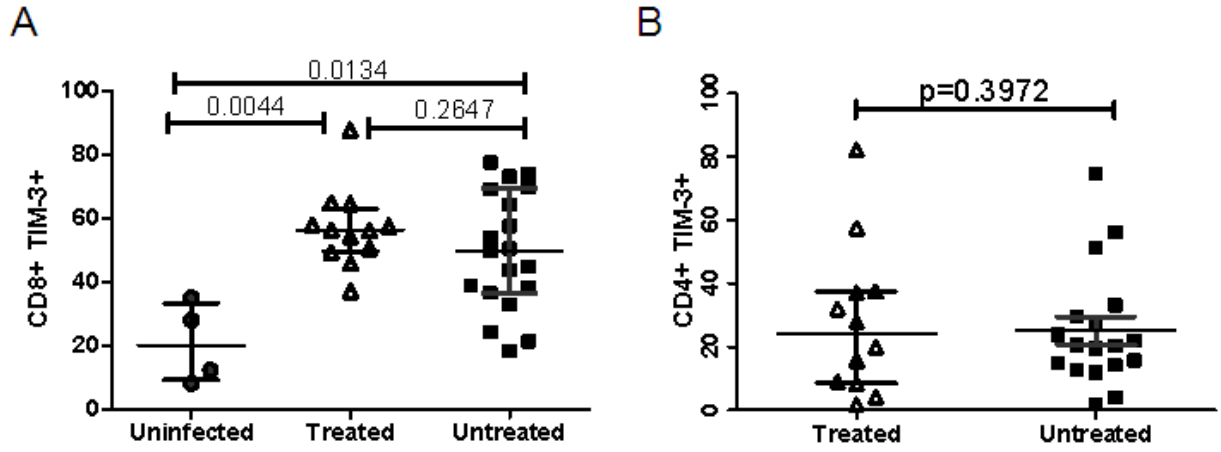

Figure 3.4 Comparison of CD8+ TIM-3+ and CD4+ TIM-3+expression levels within sub-groups: A) CD8+ TIM-3+ and B) CD4+ TIM-3+.

3.3.4 No association observed between clinical markers of disease progression and TIM-3+ expression

We subsequently examined the relationship between CD8+ TIM-3+ and viral load in untreated children. We decided not to examine this relationship in treated children as viral loads were undetectable and data may be conflicting due to age related differences. Noted, children on therapy may continue to express TIM-3 but have suppressed VL and relatively reconstituted CD4 counts, which could obscure correlations existing in children who were untreated. We found no relationship between CD8+ TIM-3+ and viral load. (p=0.6632, r=0.0763); absolute CD4+ T cell count (p=-0.3426, r=-0.1653) and CD4 T cell % (p=0.0842,

r=-0.2960) (Fig 3.5A/B/C.). The relationship between CD4+ TIM-3+ and viral load (p=0.4770, r=-0.1350); absolute CD4+ T cell count (p=0.7480, r=-0.0612 and CD4+ T cell % (p=0.2959, r=0.1973) showed insignificant associations (Fig 3.5D/E/F.)

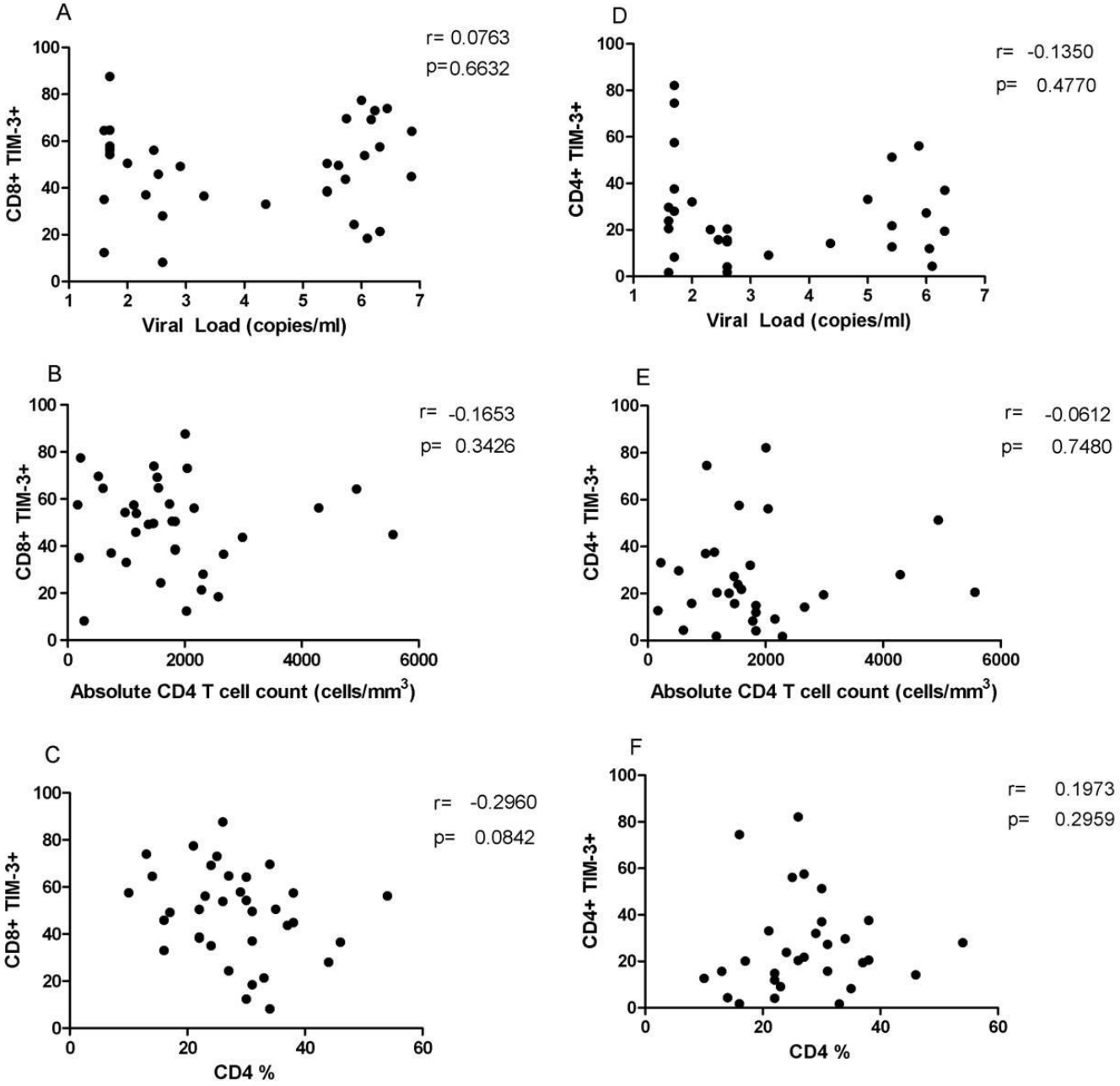

Figure 3.5 Correlation of clinical markers with expression of TIM-3 on CD8+ T cells: A) Viral load (copies/mL), B) Absolute T cell count (cells/mm^3) and C) CD4 T cell % and CD4+ T cells D) Viral load (copies/mL), E) Absolute T cell count (cells/mm^3) and F) CD4 T cell %..

3.3.5 Increased expression of PD-1 on T cells from HIV-1 infected children

We next investigated expression levels of PD-1 derived from HIV-1 infected children. A comparison of PD-1 expression on CD4+ T cells and CD8+ T cells reveals the following: PD-1 expression was elevated on CD8+ T cells in comparison to CD4+ T cells. When comparing the median of PD-1 expression across uninfected (5%), treated (19%) and uninfected (36%) sub-groups-PD-1 expression was elevated in the untreated children in comparison to treated children (p=0.02). In addition PD-1 expression was elevated in treated children in comparison to uninfected children (p=0.0348) and untreated children in comparison to uninfected children (p=0.0012) (Fig 3.6A). PD-1 expression on CD4+ T cells revealed that treated children exhibit a higher median of PD-1 expression in the untreated children (p=0.0074) and uninfected children (p=0.0002). PD-1 expression was significantly different between untreated and uninfected children (p=0.0058) on CD4+ T cells (Fig 3.6B). Additionally, PD-1 expression and TIM-3, were found to be expressed at higher levels in the children aged less than 6 months in comparison to older children although this differences were not statistically significant (p=0.1266; data not shown).

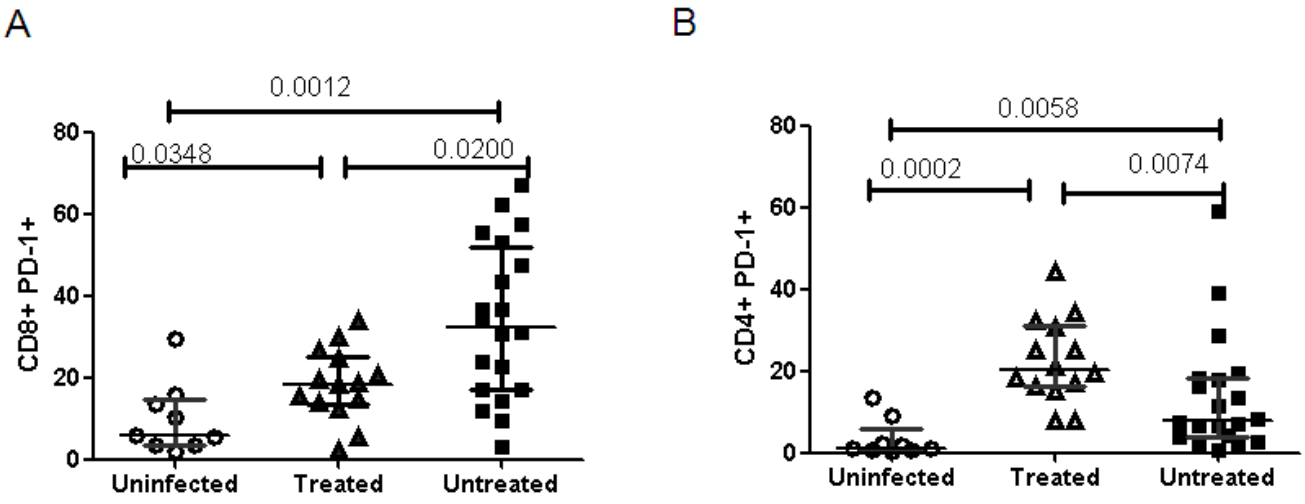

Figure 3.6 Comparison of expression levels of PD-1 on A) CD8+ T cells and B) CD4+ T cells between uninfected, treated and untreated children.

3.3.6 Correlations between clinical markers of expression and PD-1+ expression

Based on findings from adults where PD-1 expression correlated with viral load, we examined the relationship between CD8+ PD-1 and viral load and found a significant relationship between PD-1 and viral load ($p=0.0083$, $r=0.3974$) but not absolute CD4+ T cell count ($p=0.8347$, $r=0.00328$) and CD4+ T cell % ($p=0.1631$, $r=-0.2165$) (Fig 3.7A/B/C.). The relationship between CD4+ revealed the following associations: CD4+PD-1+ expression and plasma viral load ($p=0.1526$, $r=-0.2304$); absolute CD4+ T cell count ($p=0.1370$, $r=-0.2305$) and CD4 T cell % ($p=-0.2910$, $r=0.0583$) as represented in Fig 3.7D/E/F.

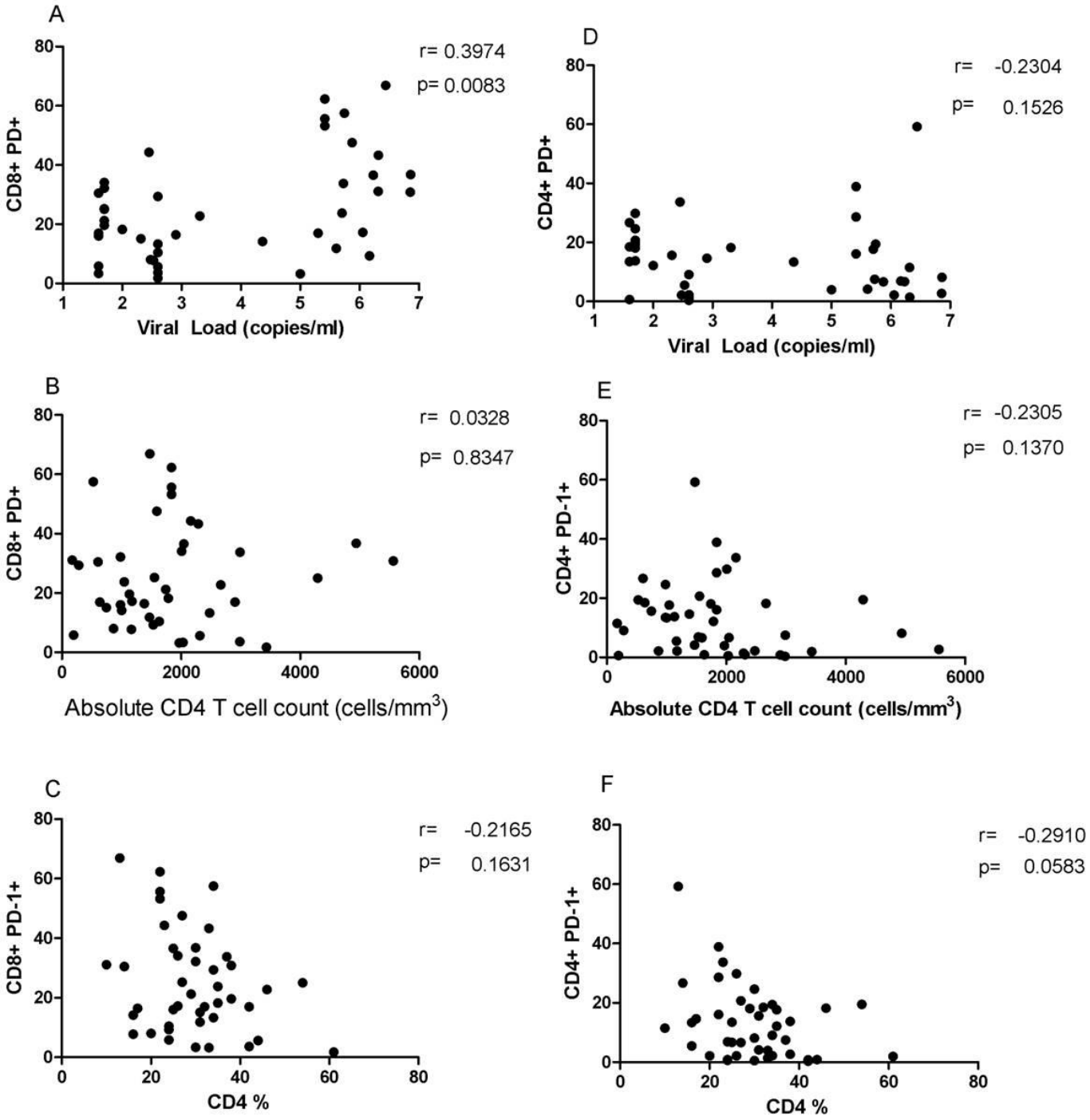

Figure 3.7 Correlation of clinical markers with expression of PD-1 on CD8+ T cells: A) Viral load (copies/mL), B) CD4 T cell % and C) Absolute T cell count (cells/mm^3) and CD4+ T cells D) Viral load (copies/mL), E) and CD4% F) Absolute T cell count (cells/mm^3).

3.3.7 Expression of PD-1 and TIM-3

A synergistic effect of PD-1 and TIM-3 blockades has been found to restore T cell exhaustion, we next investigated the expression of PD-1 and TIM-3on bulk cells. Based on the panel restrictions we were not able to correlate co-expression on identical cell. However, correlation analysis revealed positive correlation of CD4+ T cells and CD8+ T cells for PD-1 ($p<0.0001$; $r=0.6507$) which could be related to compensation controls, antibody staining or variation in subset expression. TIM-3 expression correlation analysis revealed positive correlation ($p=0.0131$; $r=0.4478$). There was a positive correlation of TIM-3 and PD-1 expression on CD8+ T cells ($p=0.0483$; $r=0.2941$) and no correlation of TIM-3 and PD-1 expression ($p=0.9737$; $r=-0.0006$) on CD4+ T cells.

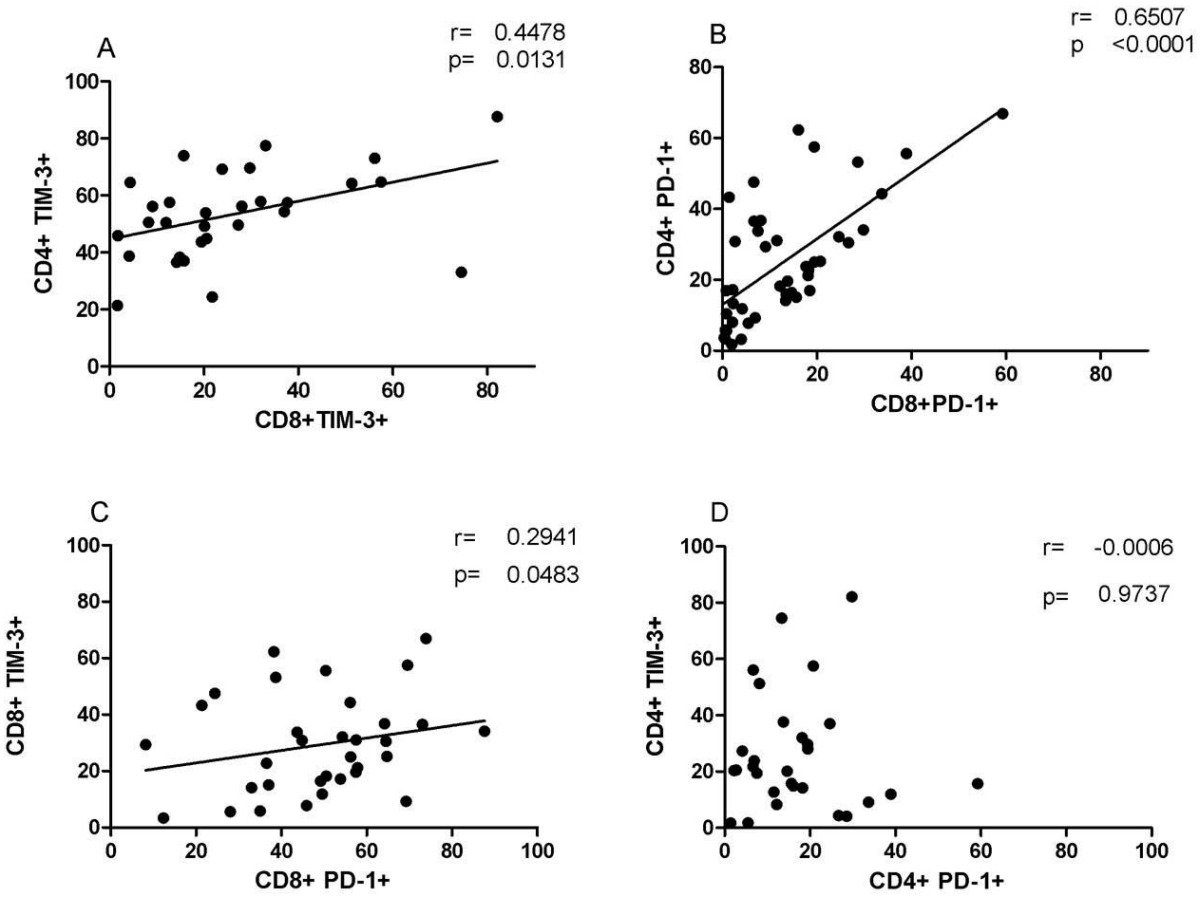

Figure 3.8 Correlation of expression on exhaustion markers A) CD4+ TIM-3+ and CD8+ TIM-3+ B) CD4+ PD-1+ and CD8+ PD-1+ C) CD8+ TIM-3+ and CD8+ PD-1+ and D) CD4+ TIM-3+ and CD4+ PD-1+.

3.3.8 Longitudinal follow-up: TIM-3 expression levels on T cells were maintained at high levels and tracked with HIV viral load.

In order to complement our cross-sectional data we next had the unique opportunity to investigate expression of PD-1 and TIM-3 longitudinally in an HIV-1 infected child over a 20 month period. PD-1 and TIM-3 expression on CD8+ T cells were maintained at high levels over time and in this participant appears to track positively with viral load and inversely with CD4 T cell %. Furthermore, expression of PD-1 and TIM-3 on CD4+ T cells track positively with viral load and inversely with CD4 T cell %.

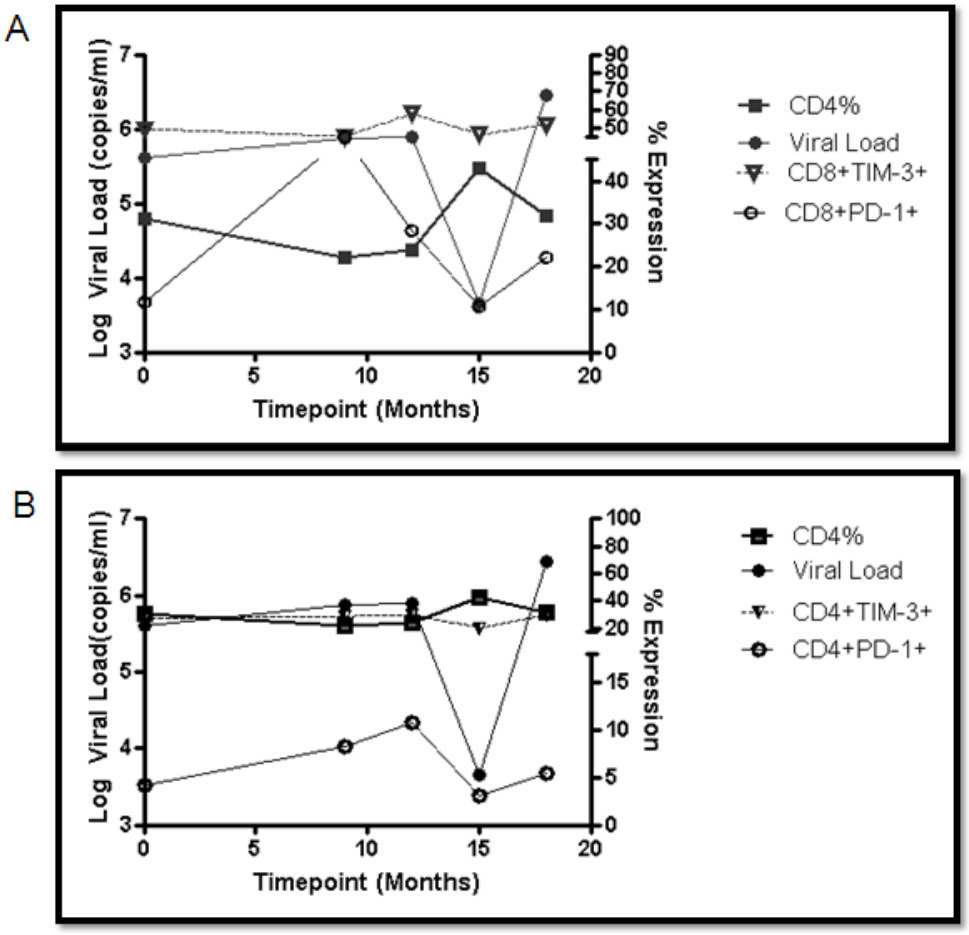

Figure 3.9 Longitudinal assessment of clinical markers and markers of exhaustion with A) CD8+ TIM-3+ and CD8+ PD-1+ and B) CD4+ TIM-3+ and CD4+ PD-1+.

3.4 DISCUSSION

Studies on paediatric HIV patient populations are limited but crucial to our goal of an HIV-free generation. There is an obvious difference in disease progression and immunology of children in reference to adults. Consequently, it is imperative not to extrapolate but rather to investigate the mechanisms underlying paediatric infection. To date no studies have been performed to investigate the role of TIM-3 in HIV-1 infected children and no data is available in the context of HIV-1 infection. The present study aimed to investigate the mechanism underlying the association between T cell progressive exhaustion and HIV replication in an African cohort of HIV-1 clade C infected children. Our results showed a significant difference in TIM-3 and PD-1 T cell expression profiles between HIV-1 infected children and uninfected children, with CD8+ T cells having an increased expression of TIM-3 in comparison to CD4+ T cells. Our findings demonstrated no association between TIM-3 and PD-1 expression markers with markers of disease progression in contradiction to observations in adults (Jones et al., 2008b, Day et al., 2006). Finally our study demonstrated a positive association between TIM-3 and PD-1 in an HIV-1 paediatric disease setting.

Paediatric infection is characterised by elevated viral loads (Mellors et al., 1997, Dickover et al., 1998, Shearer et al., 1997, Rouet et al., 2003). Higher viral loads results in persistent antigenic stimulation which lead to immune exhaustion rendering effector cells dysfunctional, as documented in HIV and HCV (Khaitan and Unutmaz, 2011, Kasprowicz et al., 2008). TIM-3 and PD-1 have previously been found to correlate with markers of disease progression such as viral load and inversely with absolute CD4+ T cell count (Wu et al., 2011, Vali et al., 2010, Jones et al., 2008b, Day et al., 2006). Our study found no association between clinical markers of disease and TIM-3+ expression, and a negative association between CD4 T cell % and PD-1+expression.

Children have an immature immune system with ineffective or suppressed effector function that contributes to the inability of cells to proliferate and effectively control HIV replication. It is still unclear whether CD8+ T cells in children are ineffective, and/or immature or completely suppressed? Our results, further confirm an elevated level of exhaustion on CD8+T cells compared to CD4+ T cells which could contribute to ineffective CD8+ T cell responses in children, or more simply could also mean that these markers better discriminate CD8+ T cells in comparison to CD4+ T cells. Hence, the difference between CD4 and CD8 cell could be attributed to CD8 or CD4 compensation controls, or variation in subset expression and may be a true reflection of expression of these markers on CD4 and CD8 cells in children. The analysis of an additional timepoint would have proven beneficial to confirm these results.

General T cell exhaustion was further corroborated by the next component that showed TIM-3 and PD-1 to be elevated in HIV-1 infected children in comparison to uninfected children. Other adult studies have confirmed elevation of TIM-3 on CD4+ T cells and CD8+ T cells in HIV infection (Jones et al., 2008b), chronic hepatitis C virus infection (Golden-Mason et al., 2009) and in pregnancy (Zhao et al., 2009). A recent study demonstrated PD-1 to be a marked negative regulator of activated T cells in children (Prendergast et al., 2011). PD-1 expression on CD8+ T cells was found to be higher in the HIV-infected group in comparison to the HIV-uninfected children. Our study confirmed these findings; we documented elevated expression levels on CD8+ T cells in comparison to CD4+ T cells for both TIM-3 and PD-1 inhibitory molecules. Levels of Tim-3 and PD-1 observed in this study of HIV-1 clade C infection were higher than those reported from previous studies in HIV-1 clade B infection.(Jones et al., 2008b).

Additionally, our study had the rare advantage of being able to document of expression of TIM-3 and PD-1 on T cells longitudinally over a twenty month period. The results revealed that TIM-3 and PD-1 expressing CD4+ and CD8+ T cells were maintained at high levels and tracked with HIV viral load. Due to limitation in sample size we were unable to describe immune responses over time which would have proven valuable as exhaustion markers have been implemented in the suppression of immune responses. Our investigation of longitudinal data also showed a change in CD4% (increase) and HIV viral load (sharp decrease). However, we did not have clinical data supporting concomitant infection, treatment or another clinical scenario explaining the drop in HIV viral load with concomitant mild increase in CD4 T cell count. The fact that CD4 count was higher as well would be consistent with a biological phenomenon; however a lab error in original sample acquisition cannot be entirely excluded. To rule out day to day assay variation all 5 timepoints were stained and processed for flowcytometric analysis on the same day, which makes a lab error at this stage less likely, given the consistent data for the other 4 time points.

In vitro experiments reveal the extent to which exhaustion markers suppress effector functions (Khaitan and Unutmaz, 2011, Day et al., 2006, Jin et al., 2010, Ju et al., 2009, Jin et al.). Blocking of the PD-1 and TIM-3 pathway alone restores immune responses which enable the immune system to mount a more vigorous attack against foreign infections. However, simultaneous blockade of TIM-3 and PD-1 pathways has been documented to have a synergistic effect in restoring antiviral immunity and viral control compared to the blockade of either pathway alone (Jin et al.). Reversal of T cell dysfunction may prove valuable in therapeutic vaccine development. Noteworthy, Phase I clinical trials are currently being carried out to evaluate the efficacy of the use of PD-1 blockade therapeutically in oncology

and infectious disease settings (Brahmer et al., 2010, Berger et al., 2008, Sakuishi et al., 2011).

As this was a retrospective design the investigators were restricted. The investigators were restricted in sample choice and availability. Given the dynamic nature of the developing immune system, age-related differences were noted but unavoidable and are acknowledged as a limitation in interpreting the data. The investigators also acknowledge that uninfected children from uninfected mothers would have been an additional relevant control cohort. Furthermore, our study was limited by the relatively small sample size for a phenotypic study and flow panel constraints that restricted our investigation of co-expression of PD-1 and TIM-3 on a single T cell. Noteworthy, functional studies would clearly have added an interesting and important component to the work. However, limited availability of cells from these paediatric samples restricted the investigators largely to the phenotypic studies described here (exception: functional Treg work in chapter 4). Furthermore, functional work best carried out on fresh samples. However, to our knowledge it represents the largest study addressing this topic matter in the literature to date. Our data is the first to report TIM-3 expression in HIV-1 infected children and suggests that expression of both PD-1 and TIM-3 exhaustion markers additively contribute to the failure to control viral load and to accelerate disease progression in this vulnerable paediatric population.

We conclude that Tim-3, in concert with other inhibitory molecules such as PD-1, were elevated in paediatric HIV-1 clade C infection. This elevated expression on T cells from perinatally infected children suggests that both TIM-3 and PD-1 contribute to T cell exhaustion in this paediatric patient population. This could explain why younger newly infected children are unable to control HIV infection and show low magnitude and breadth

suggesting limited HIV-1 specific T cell immunity. To reiterate, our study is the first to describe the contribution of TIM-3 to HIV pathogenesis in HIV-1 infected children and in HIV-1 subtype C infection no data is available in the context of HIV-1 infection. Exploring negative T cell regulation provides an obvious therapeutic advantage of reversing effects of the exhaustion markers Tim-3, in concert with other inhibitory molecules such as PD-1.

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CHAPTER FOUR

The role of regulatory T cells (Tregs) and immune activation in HIV-1 clade C infected African children

ABSTRACT

Background: Generalized immune activation has repeatedly been associated with poor outcome in HIV-1 disease progression. To date, limited studies have been performed to investigate the role of Tregs and activation in HIV-1 clade C infected children. We therefore aimed to study the impact of Tregs and activation expression profiles on HIV-1 disease progression, in clade C infected children.

Materials and Methods: Our study investigated the frequency of Tregs (CD4+CD127-CD25+) and T cell activation (HLA-DR+ CD38+) in 46 clade C infected children, using multi-parameter flow cytometric assays. Treg expansion with subsequent Treg suppression assay confirmed functional capacity of Tregs.

Results: We observed a statistically significant negative correlation between Treg frequencies and CD4+ T cell% (p=-0.0362, r=-0.3132). No significant correlations between Tregs and activation markers, or markers of disease progression such as absolute CD4+ T cell count and viral load were observed. The expression of HLA-DR+CD38+ on CD8+ T cells was found to be significantly higher on untreated compared to treated (p=0.002) and uninfected children (p=0.0177) Similarly HLA-DR+CD38+ expression on CD4+ T cells was significantly higher in untreated compared to treated (p=0.0495) and uninfected children (p=0.0312). The expression of activation markers on CD4+ and CD8+ T cells revealed a positive correlation (p<0.001, r=0.6376). Markers of activation were also found to be significantly higher in the children aged less than 6 months in comparison to older children

on CD4+ (p=0.0437) and CD8+ T cells (p=0.00276). In addition to our phenotypic studies we were able to expand Tregs from HIV-1 infected children and demonstrated their ability to suppress T cell proliferation.

Conclusion: We demonstrate that the expression of Treg and T cell activation markers are elevated in paediatric HIV-1 clade C infection. High Treg frequencies in perinatally infected children suggest that Tregs may contribute to T cell suppression in children, with highest frequency in the age group of < 6 months, when HIV-1 specific T cell immunity is of lowest magnitude and breadth.

Key words: Treg; Regulatory T cells; T cell Activation; paediatric; children; HIV-1

4.1 INTRODUCTION

Adults and children differ in disease progression denoting a variation in clinical outcome (Holland et al., 2000, Resino et al., 2002b, Resino et al., 2002a). In HIV-1 infected children, the levels of plasma viremia are persistently high, with declines only observed in the second year of life. In general, it is presumed that these young children respond ineffectively due to immature or defective cell functionality (Ashwood et al., 2011, Ly et al., 2009, Ochieng et al., 2006). T cell efficacy can be related to cell activation, the levels of cell expression and cell-cell interaction. Phenotypic markers facilitate the discrimination of cell populations, specific subsets and their functional capabilities. It is known that the pathogenesis of HIV-1 infection is characterized by CD4+ T cell loss in the context of generalized immune activation and dysregulation. (Papagno et al., 2004, Giorgi et al., 1999, Liu et al., 1998, Putnam et al., 2009). To date, limited research has been carried out that characterizes activation and regulatory T cell subsets between infected and uninfected African children.

HLA-DR and CD38 are surface markers of T cell activation. In HIV uninfected individuals CD38 is expressed in relatively greater numbers by naïve lymphocytes, while in HIV infected individuals, CD38 is expressed by CD8+ memory T cells (Benito et al., 2005). Immune activation is commonly characterized as either CD38+ or HLA-DR+ or dual expression of CD38 and HLA-DR, on T lymphocytes (Giorgi et al., 1994, Ho et al., 1993, Liu et al., 1997). Immune activation markers expressed on CD8+ T cells have been documented to be strong and independent predictors of disease progression (Liu et al., 1997). Elevated levels of CD38 in chronically infected individuals suggested that CD38 was the most predictive marker for the development of a clinical AIDS diagnosis and death. Activated T cells have also been shown to express HLA-DR at elevated levels (Saifuddin et al., 1998). Cross sectional and longitudinal studies also showed an increase of dual expression of CD38+ and HLA-DR+

over the course of infection. There is a progressive increase in expression towards later stages of HIV-1 infection (Kestens et al., 1992, Sindhu et al., 2003, Liu et al., 1996).

T cell subsets differ in **infants** compared to adults (Birle et al., 2003). When lymphocyte activation in healthy unexposed infants was compared to uninfected infants of HIV-infected mothers, the proportions of activated CD4+ cells were increased in uninfected infants of HIV-infected mothers compared to unexposed infants. (Jennings et al., 1994). Another study investigated co-expression levels of activation markers HLA-DR and CD38, in clade B age-matched children. The study demonstrated that CD4+ T cells co-expressing CD38 and HLA-DR were significantly increased in HIV infected children compared to uninfected children. The CD4+ cells of the uninfected and infected children exhibited a mean expression of 2 vs 6% for < 2 years of age, 3 vs 11% for 2-3 years, 2 vs 8% for ≥4 years respectively. There was a significant increase in the proportion of CD8+ cells co-expressing CD38 and HLA-DR with a mean of 5 vs 25% for < 2 years, 10 vs 41% for 2-3 years, 6 vs 31% for ≥ 4 years exhibited for uninfected vs infected children (Bhatia et al., 2010, Plaeger-Marshall et al., 1994) These findings confirm the possible effect of immune activation on immune responses.

A recent study investigated activation in 194 children receiving **HAART**. These children were divided into two groups according to the viral load: 59 patients with VL ≥ 400 copies/mL and 135 patients with VL < 400 copies/mL. The percentage of CD8+ CD38/HLA-DR+ T cells of patients with VL ≥ 400 copies/mL was significantly higher than that of patients with VL < 400 copies/mL, concluding that successful HAART could significantly decrease immune activation in children (Jin et al., 2011).

As immune activation appears to play a deleterious role in both progression and acquisition of HIV-1 infection, immune mechanisms with the ability to decrease inflammation may be beneficial, and play a significant role in maintaining the fine balance of the immunoregulatory equilibrium, in peripheral blood and at mucosal sites (Favre et al., 2009).

Regulatory T cells (Tregs) are a specialized subpopulation of T cells that are discriminated as 'naturally arising' CD25+CD4+ Tregs in which the frequency of the transcription factor forkhead box p3 (Fox3) occurs in the thymus (Curiel, 2007), or as 'induced' Tregs in which FoxP3 is induced in the periphery (Sakaguchi, 2004). Quantitative identification and viable enrichment of natural Tregs in humans are problematic, and warrants further investigation in order to understand these cells in disease. More commonly, Treg cells are defined based on the frequency of CD4+CD25+ and the transcription factor FoxP3+ however, alternate phenotypes CD4+CD25+CD127- have also been documented for use in Treg discrimination (Liu et al., 2006, Seddiki and Kelleher, 2008). Treg identification cannot uniquely define these specialized T cell subsets (Fehervari and Sakaguchi, 2004), however with methods such as sorting etc. the discrimination of Treg sub-populations have become more distinct. Studies have shown that IL-7R plays an important role in the proliferation and differentiation of mature T cells, and in vitro experiments show that the expression of CD127 is down-regulated following T cell activation. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ Treg cell (Liu et al., 2006). The phenotype CD3+CD4+CD25+CD127- has been used to successfully define and sort functional regulatory T cells in previous studies. Treg cells are defined based on the frequency of CD4+CD25+ and the transcription factor FoxP3+ however; alternate phenotypes such as CD4+CD25+CD127- have also been documented for use in Treg discrimination and isolation of functional Tregs for suppression assays .

Tregs are critical regulators of **immune tolerance**, averting the production of self reactive T cells which have the potential to generate autoimmune disease in the host. Tregs suppress activation of the immune cells and responses, and depletion of Tregs is associated with immune activation in HIV (Eggena et al., 2005). The suppressive activity may limit the magnitude of effector response, contributing to the lack of viral control. Concurrently, it suppresses chronic immune activation which indicates disease progression. Treg number is also strongly correlated with both CD4+ and $CD8^+$ T cell activation, and the relationship between Treg depletion and $CD4^+$ T cell activation has also been found to be constantly stronger than viral load and absolute CD4+ T cell count (Eggena et al., 2004).

Tregs have been **implicated** in many diseases such as lupus (Valencia et al., 2007), multiple sclerosis (Huan et al., 2005), HIV (Prendergast et al., 2011a, Hunt et al., 2011, Weiss et al., 2010, Sachdeva et al., 2010, Prendergast et al., 2010), HCV (Roe et al., 2009) and, have emerging function in renal transplant and tumour growth (Bestard et al., 2011, Jacob et al., 2009). A more recent study, one of the few carried out in the HIV-1 infected paediatric population, evaluated the correlation between viral load, immune activation and Tregs in HIV-1-infected children (Freguja et al., 2011). The study investigated Treg frequency in 89 HIV-1-infected children aged 6-14 years. The study found that the number of activated CD8+CD38+ T cells were increased in relation to viral load ($r=0{\cdot}403$; $P<0{\cdot}0001$). In addition, Tregs correlated positively with HIV-1 viral load ($r=0{\cdot}323$; $P=0{\cdot}002$) and CD8+CD38+ ($r=0{\cdot}305$; $P=0{\cdot}005$), but correlated inversely with CD4+ cells ($r=-0{\cdot}312$; $P=0{\cdot}004$). The study showed that the suppressive activity of Treg may be unsuccessful in limiting immune activation (Freguja et al., 2011).

Tregs limit the **immune response** by inhibiting proliferation and activity of CD4+ and CD8+ effector T lymphocyte cells. Strong robust CD4+ and CD8+ effector T cell responses are important for control of HIV replication. After Tregs *in vitro* were sorted and removed, an increased HIV-specific effector T cell response was observed (Aandahl et al., 2004, Oswald-Richter et al., 2004, Weiss et al., 2004). Tregs may suppress the HIV-specific cytolytic antiviral response of CD8+ T cells (Kinter et al., 2007). Most of these studies examined the effect of Treg depletion on T cell proliferation and/or IFN- γ production, after stimulation with HIV antigens.

The most notable evidence of the role of Tregs can be attributed to its use in human **clinical studies**. A cell expansion protocol focusing on the culture of pure Tregs, has been developed by the UCSF Diabetes Centre (Putnam et al., 2009). The expansion procedure enables enriched FpxP3 Treg cell cultures to be expanded to 1, 500 fold over a two-week period. Expanded Tregs can be infused back into patients to restore immune balance and possibly, even reverse type 1 diabetes. Successful Treg expansion may present a population of cells that can be expanded for use in autoimmune diseases (Putnam et al., 2009, McClymont et al., 2011, Bour-Jordan and Bluestone, 2007). Another study showed that the targeting and elimination of CD25+Tregs might be sufficient to eliminate smaller tumours. By depleting CD25 there may be an interference with the clonal expansion of tumor antigen specific T lymphocytes, if used in combination with other immunotherapeutic strategies.

High levels of **Tregs and activation** markers have been correlated with rapid disease progression (Shevach et al., 1998, Thornton and Shevach, 1998, Prendergast et al., 2011a, Macatangay and Rinaldo, 2010, Hunt et al., 2011, Weiss et al., 2010, Bernardes et al., 2010, Radziewicz et al., 2009, Cao et al., 2009a, Zhang et al., 2008, Langier et al., 2010, Rouse et

al., 2006, Sachdeva et al., 2010, Prendergast et al., 2011b). The activation of T cells is influenced by the suppressive activity of Tregs (Shevach et al., 1998, Thornton and Shevach, 1998, Prendergast et al., 2011a, Macatangay and Rinaldo, 2010, Hunt et al., 2011, Weiss et al., 2010, Bernardes et al., 2010, Radziewicz et al., 2009, Cao et al., 2009a, Zhang et al., 2008, Langier et al., 2010, Rouse et al., 2006, Sachdeva et al., 2010, Prendergast et al., 2011b). Tregs have been documented as the suppressor arm of the immune system due to ability to suppress immune responses (Langier et al., 2010, Rouse et al., 2006, Macatangay and Rinaldo, 2010, Sachdeva et al., 2010, Prendergast et al., 2011b). A recent study investigated activation, regulatory and exhaustion markers in HIV-1 infected clade B children (Prendergast et al., 2011a). The programmed death 1 exhaustion marker (PD-1), was positively correlated (R=0.41, p=0.002) to be a marked negative regulator of activated T cells. CD8+ T cell activation (HLA-DR+CD38+) was partially driven by the magnitude of the HIV-specific CD8+ T cell response (Prendergast et al., 2011b). The depletion in Tregs was associated with increased CD8 activation (R=-0.27, p=0.068), suggesting that the decline in Tregs may allow immune activation to increase. The study suggested an inverse role between Tregs and immune activation (Prendergast et al., 2011a). It will thus prove valuable to look at all these components of the immune system in unison.

Clade C infection has been reported to have one of the fastest rates of disease progression (Cohen et al., 2011, Spira et al., 2003). To date, no studies have been performed to investigate the role of Tregs and immune activation in children in the context of HIV-1 clade C infection. Given the recently published data on the interplay between exhaustion activation and regulatory markers, our study aimed to investigate Treg frequency association with generalized immune activation, and immune response in an African cohort of HIV-1 infected children.We aimed to answer the following research questions:

(1) Do Tregs and activation T cell profiles differ in HIV-1 infected children and uninfected children?

(2) Are Tregs and T cell activation associated with markers of disease progression?

(3) What is the relationship between the immunoregulatory cells, T cell activation and HIV-1 specific T cell responses in the setting of HIV-1 infection?

(4) Can functional Tregs be successfully expanded from HIV infected children?

4.2 PARTICIPANTS, MATERIALS AND METHODS

4.2.1 Cohort description

In South Africa, prior to the revision and initiation of the new ART guidelines, a cohort of untreated HIV-1 infected mother child pairs was established. The study began recruitment in 2006. The treatment guidelines dictated that HIV-1 seropositive mothers during the last trimester of pregnancy received a single dose of nevirapine during labour. The infant received a single dose of nevirapine within 48 hrs of birth, according to the HIVNET-012 Protocol, as previously described (Guay et al., 1999, Jackson et al., 2003). Treated children who had an undetectable viral load for at least one or more timepoints were included for analysis.

The 46 study participants were recruited through clinics in KwaZulu-Natal, Durban, South Africa. The recruitment clinic sites included King Edward Hospital, McCord Hospital, Saint Mary's Hospital and Prince Mshiyeni Hospital. Transmission pairs were screened and mother-child pairs who met the inclusion criteria of mother-child treatment naïve pairs and positive HIV-DNA PCR results from birth hospitals were recruited. The participants were selected for a preliminary cross-sectional analysis based on sample availability. The exclusion criteria for the chronically infected cohort included treated pairs (mothers and/or children). The untreated cohorts were part of the chronically infected pair cohort and

represented initially falsely diagnosed HIV negative participants. This study retrospectively investigated 23 HIV-1 infected, 9 HIV-1 exposed uninfected and 13 HIV-1 treated children (Thobakgale et al., 2009, Thobakgale et al., 2007, Mphatswe et al., 2007) from Zulu/Xhosa ethnic origin with HIV-1 infection. Treated children who had an undetectable viral load for at least one or more timepoints were included for analysis.

Written informed consent was obtained from all study participants and the research protocol for the study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa and, the Internal Review Board of Massachusetts General Hospital, Boston.

4.2.2 Plasma viral load and absolute CD4+ T cell measurement

Plasma was isolated from whole blood after a single round of centrifugation. Plasma viral loads were assessed using the Cobas Amplicor Monitor Test version 1.5: detection limit of 50 HIV-1 RNA copies per mL of plasma, according to manufacturer instructions (Roche Diagnostics).

Absolute CD4 T+ cell counts and percentages of CD4+ T cells were determined from fresh whole blood, using Multitest four colour TruCount as previously described (Gratama et al., 2002) according to the manufacturer's instructions, (Beckton Dickinson Technology) at the HIV Pathogenesis Programme, Durban, KZN, South Africa.

4.2.3 Isolation of PBMCs

Blood specimens were 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. PBMCs were used fresh in ELISPOT assays. The remaining PBMCs were cryo-preserved in 90% fetal bovine serum (Sigma-Aldrich), 10% DMSO prior to storage in liquid nitrogen for subsequent multiparameter flow staining and immunophenotyping analyses.

4.2.4 Multiparameter flow cytometry for Treg quantitation and investigation of T cell activation

4.2.4.1 Antibody titrations: Antibody titrations were performed by staining ($1x10^5$) PBMCs at a range of 2 to 25µl antibody (depending on volume and concentrations specified by manufacturer). Antibody dilutions were halved, covering the above range. The final optimal concentration was used subsequently in immunophenotyping. The optimal concentration was determined by choosing the dilution of the stain where the intensity plateau for the stained cells was most favourable.

4.2.4.2 Thawing: Frozen PBMCs were thawed using a warming method. The R10 media, made up of RPMI 1640 + 10% FBS, was warmed to 37°C in a 15 or 50 mL Sterilin tube. Firstly, 20 µl nuclease was added to 2 mL 37 ^{o}C R10 in a 15 mL Sterilin tube. Frozen PBMC vials were set aside at room temperature for approximately 5 minutes until sample was reaching liquid state. The cryovial was opened and the rim was swabbed with 70% ethanol before the cells were transferred into the 15 mL Sterilin tube containing nuclease and 2 mL of warm R10 media. An additional 8 mL of warm R10 was slowly added to the tubes and the mixture was spun down at 1,700rpm for 10 minutes. Supernatant was discarded, removing all of the toxic DMSO freezing solution, and the cell pellet was re-suspended. Cells were counted using an automated cell counting Guava viacount assay and read on the Guava cell

counter **(**Guava Technologies, Guava PCA System). Cells were finally re-suspended in R10 at a concentration of 1 million cells per mL.

4.2.4.3 Immunostaining: 1 million cells per mL were added to each experimental FACS tube and control Fluorescence Minus One (FMO) tubes. PBMCs were first stained for viability at room temperature in the dark, with 1µl of a 200 µl stock solution of LIVE⁄DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen). After 15 minutes the dye was washed off using cold PBS (phosphate buffered saline) for 10 minutes at 1,500 rpm. PBMCs panels were stained (extracellular) as below (Table 4.1), at 4°C for 30 minutes.

4.2.4.3.1 Extracellular immunostaining (Panel 1 and 2): PBMCs were stained with antibodies and volumes as per Panel 1(Table 4.1), at 4°C for 30 minutes or Panel 2 (Table 4.1). Subsequently, sample acquisition procedure was followed.

4.2.4.3.2 FoxP3 Intracellular immunostaining (Panel 3): Treg staining was performed per manufacturer's instruction (Ebioscience human regulatory T cell staining kit 2 catalogue number 88-8998-40). Briefly, PBMCs were stained (extracellular) with all antibodies as listed Panel 2 (Table 4.1) at stipulated volumes, for 30 minutes at 4°C. Excess antibodies were washed off with 2 mL PBS and a 10 minute spin at 1,500 rpm. Supernatant was discarded and cells were re-suspended with 2 mL fixation-permeabilization buffer (1:4 dilution). Experimental tubes were set aside to incubate for 30-60 minutes at 4°C. Thereafter, tubes were spun down at 1,500 rpm for 10 minutes. Supernatant was discarded and cells were re-suspended with 2 mL perm buffer (1:10 dilution with H2O). Once more, tubes were spun down at 1,500 rpm for 10 minutes. Supernatant was discarded and rat serum was added (2 uL rat serum per tube in 80 uL perm buffer). Tubes were left at 4°C for 15 minutes. 5uL FoxP3-

PE was added to sample tubes while no FoxP3 added to the FMOs. Tubes were left to stain for 30 minutes at 4°C. Subsequently, sample acquisition procedure was followed.

Table 4.1 Experimental setup: Antibodies and volumes.

PANEL1:	**Activation**		
Antibody	**Fluorochrome**	**Abbrev(Company; Catalogue)**	**Volume(µl)**
CD14	Pacific Blue	PacB (Ebioscience, 51-0199-73)	2
CD19	Pacific Blue	PacB (Ebioscience, 51-0199-73)	2.5
CD3	Fluorescein Isothiocyanate	FITC(Ebioscience, SK7, 11-0036-42)	3
CD4	Allophycocyanin	APC (BD, 340443)	3
CD8	Alexa Fluor 700	Alexa Fluor 700 (BD, 557945)	2
CD38	Phycoerythrin-Cyanine 7	PE-Cy7 (BD, 335790)	5
HLA-DR	Allophycocyanin-Cyanine 7	APC-Cy7(BD, 302618)	10

PANEL2:	**Tregs (CD25+CD127-)**		
Antibody	**Fluorochrome**	**Abbrev(Company; Catalogue)**	**Volume(µl)**
CD14	Pacific Blue	PacB (EBioscience, 48-0149-42)	2
CD19	Pacific Blue	PacB (Ebioscience 51-0199-73)	2
CD3	Phycoerythrin-Cyanine 5.5	PE-Cy5.5 (Ebioscience, SK7, MHCD0318)	2
CD4	Alexa Fluor 700	Alexa Fluor 700 (BD, 557922)	5
CD8	Peridinin Chlorophyll Protein Complex	PerCP(BD, 347314)	5
CD127	Fluorescein Isothiocyanate	FITC (Ebioscience,11-1278-73)	7.5
CD25	Allophycocyanin	APC(Ebioscience,17-0259-42)	5
FoxP3	Phycoerythrin	PE (Ebioscience,12-4476-42)	5
PANEL3:	**Tregs (FoxP3)**		
Antibody	**Fluorochrome**	**Abbrev(Company; Catalogue)**	**Volume(µl)**
FoxP3	Phycoerythrin	PE (Ebioscience,12-4476-42)	5

4.2.4.4 Acquisition: After staining of PBMCs, tubes were washed twice using cold PBS. Supernatant was discarded and pellet was re-suspended in PBS. Compensation beads were setup and read to standardize assay. Flow data were acquired on a LSR II flow cytometer (BD Biosciences). For the infant cohort, a minimum of 500 000 events were collected per subject, and a minimum of 250 000 events were collected for adults. Acquisition templates were drawn during acquisition, to further confirm subset gating, prior to analysis. The FMO control was used as the reference gate while the LIVE⁄DEAD® Fixable Violet Dead Cell Stain was used as an exclusion channel to eliminate dead PBMCs.

4.2.5 HIV-1 Peptides and ELISpot Assays

A matrix of 408 overlapping peptides (10-15mers overlapping by 10 amino acids) spanning the entire HIV-1 clade C consensus sequence were synthesized on an automated peptide synthesizer (MBS 396, Advanced ChemTech). Peptides were then pooled using a matrix screening system.

CD8+ T cell responses were determined using a matrix Interferon-γ ELISpot assay (Thobakgale et al., 2007) as described in chapter 2.

4.2.6 Flow-based cell sorting and expansion of functional Tregs

Human enriched CD4+ T cells were isolated using density centrifugation (RosetteSep, Stemcells technologies and Ficoll-Histopaque; Sigma-Aldrich). CD4+ T cell enriched PBMCs were labeled using a combination of surface markers: anti-CD3-Phycoerythrin-Cyanine 7 (PE-Cy7) (BD Pharmingen, clone SK7), CD4-Fluorescein Isothiocyanate (FITC) (eBioscience, clone RPA-T4), CD25- Allophycocyanin (APC) (eBioscience, clone BC96), and CD127-Phycoerythrin (PE) (BD Pharmingen, clone hIL-7R-M21). Next, the

CD3+CD4+CD25+CD127^{low} Tregs and the conventional T cells CD3+CD4+CD25-CD127+ subsets were sorted using a FACS Aria cell sorter (BD Biosciences) at 70 pounds per square inch with a 70-µm nozzle. For all populations, 250 000 cells were collected in X-VIVO 15 (BioWhittaker; Walkersville, Maryland) containing L-glutamine, gentamicin, and phenol red media (Lonza) supplemented with 10% human AB serum (Gemcell) and Penicillin/Streptomycin (50U/mL).The CD4+ Treg and conventional T (Tcon) cells were transferred in a flat-bottom 24-well culture plate, with 1mL of X-VIVO media 15 with human serum and anti-CD3/anti-CD28 coated microbeads (Invitrogen) at a 1:1 bead to cell ratio. At day 2, 1mL of media and exogenous IL-2 were added (300 U/mL final, NIH Aids Research & Reference Reagent Program). At day 5, cells were counted and transferred into a T25 cell culture flask at 250,000 cells/mL, with fresh X-VIVO 15 media supplemented with 10% human serum and IL2 (300 U/mL). At day 7, the expanded cells were assayed for their suppressive function and, the remaining cells were cryopreserved as observed in Fig 4.1B.

4.2.7 Assessment of Treg suppressive function using CFSE T cell proliferation assay

Suppressive Treg function was assessed using co-culture T cell proliferation assays, in which CD3+CD4+CD25-CD127+ T cells were used as responder cells and labeled with Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) at a concentration of 9 µM for 7 minutes at 37°C. 50x10^3 CFSE-labeled responder T cells were transferred to 96 round bottom well plates with RPMI 1640 medium (Sigma) containing penicillin/streptomycin (50U/mL), L-glutamine (2mM), HEPES buffer (10 mM), and 10% human serum (Cellgro). Responder T cells were co-cultured with the sorted Treg at different ratios, in the presence of anti-CD2/anti-CD3/anti-CD28 microbeads (Miltenyi Biotec) at a 1:1 bead to CD4+ T cell ratio. After 4 days of co-culture, cells were stained with anti-CD3-PeCy7 and anti-CD4-APC (BD

Pharmingen, clone SK3), acquired on a LSR II flowcytometer (BD Biosciences) and analyzed on FlowJo as observed in Fig 4.1A.

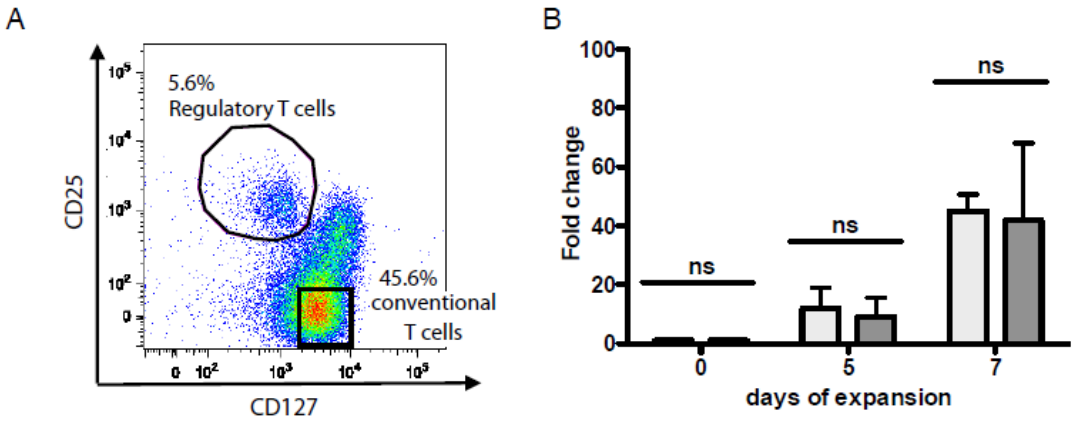


Figure 4.1 Graphical representation of expansion methodology A) Gating of regulatory T cells and conventional T cells and B) Observed fold change of Treg days of expansion from day 0 to day 7 (performed in collaboration with M. Angin-manuscript in preparation).

4.2.8 Statistical analysis

Flow data was analyzed with FlowJo software version 7.5 for PC. Microsoft Excel was used to transfer data and subtract fluorescence minus 1 (FMO) background values. Data was analyzed and graphically represented using Prism software (GraphPad; version 5). The Mann Whitney U test and the Kruskal Wallis test and ANOVA were used for group comparisons. Post test analysis was performed using Dunns Multiple Comparison Test. Normalization of subset discrimination and gating of data was performed using FlowJo. Correlations were performed using Spearman rank tests. P values of <0.05 were considered significant.

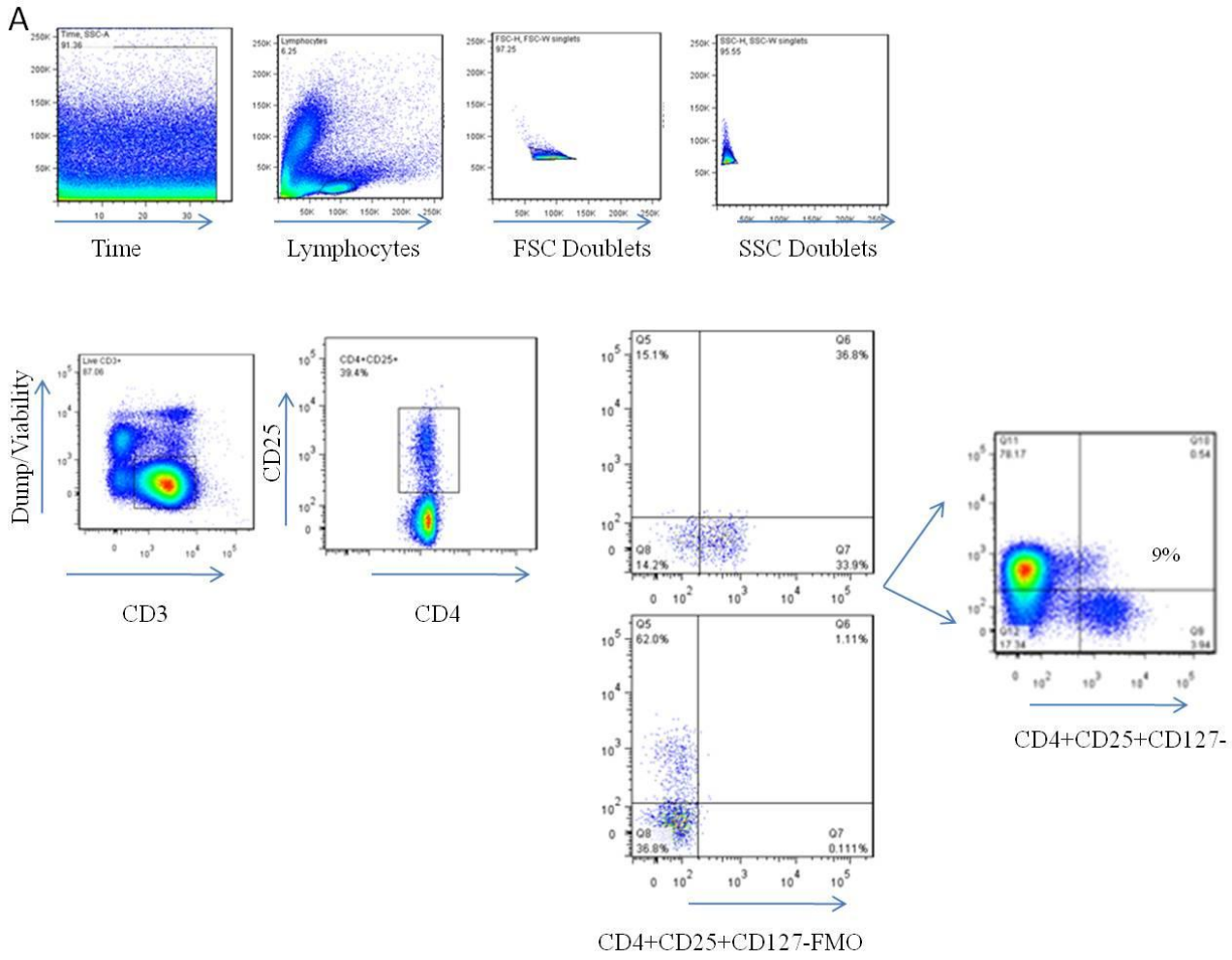
4.3 RESULTS

4.3.1 Cohort Characteristics

A total of forty six children and twelve adults were studied. As represented in Table 3.2, age range and clinical data show that the HIV-1 uninfected children exhibit a median absolute CD4+T cell count of 2,033 cells/mm^3 and a CD4 T cell% of 34%. The infected untreated children had a median viral load of 55,700 copies/mL with a median absolute CD4+T cell count of 1,838 cells/mm^3 and a CD4% of 30%, while the treated children had a median viral load of 292 copies/mL with an absolute CD4+ T cell count of 1,325 cells/mm^3 and CD4 T cell% of 29% (Figure 3.1). The median ages of the uninfected and infected untreated children are similar while the treated children are older in age as represented in Table 3.2. The adults exhibit a median viral load of 821,200 copies/mL, with a median absolute CD4+T cell count of 1582 cells/mm^3 and a CD4 T cell% of 34%.

4.3.2 Flow cytometry gating for the quantification of activation and regulation markers on CD4+ T and CD8+ T cell subsets

Gating schemes for the Tregs and activation experiments are represented in Fig.4.2. First we confirmed uniform flow rate (time plots). The live lymphocytes were then gated after doublets were gated out. Subsequently, gates were set to exclude dead cells (viability marker-), monocytes (CD14+) and B cells (CD19+). The gating for viable cells was then followed by the gating of viable lymphocytes (CD3+), and CD4+ or CD8+. The Tregs were described by gating of the Treg FMO, which were used as controls. Fig 4.2A represents Tregs defined as CD4+CD25+CD127- Fig 4.2B represents activation expression as HLA-DR+CD38+.



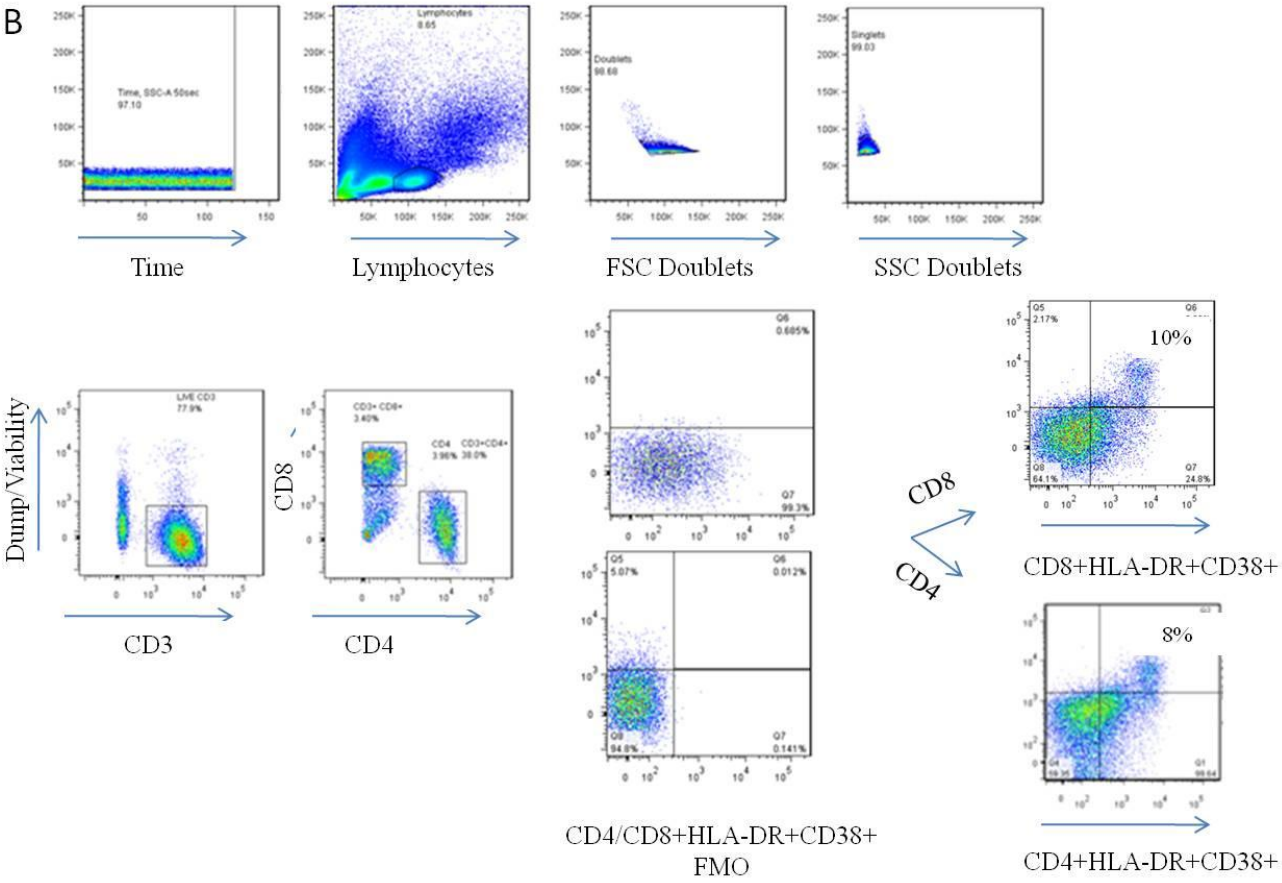


Figure 4.2 Representative gating for the assessment of A) Treg frequencies defined by CD4+CD25+CD127- and B) CD8+/CD4+CD38+HLA-DR+activation for a single participant from study cohort.

4.3.3 Elevated frequencies of Tregs in HIV-1 infected children

We first evaluated relative Treg frequencies between sub-groups, as it has been shown in adults that Tregs may differ in numbers based on stage of HIV infection (Schulze Zur Wiesch et al., 2011). CD4+CD25+CD127- Treg frequencies (% of total CD4+ T cells) ranged from 2 to 42% for CD4+ T cells as per Fig 4.3 When comparing the sub-groups, the median of absolute Tregs revealed a significantly higher frequency of Tregs in the group of untreated children in comparison to uninfected children (p=0.0225). Treg frequencies showed a trend toward elevated frequencies in HIV-1 infected children compared to their uninfected counterparts (p=0.056). We found no difference in the children aged less than 6 months in

comparison to older children (p=0.1275). We also observed Treg frequencies in 24 adults. We found absolute Tregs to show a trend of increased frequencies in 13 untreated participants in comparison to the 11 treated adult participants (p=0.0559).

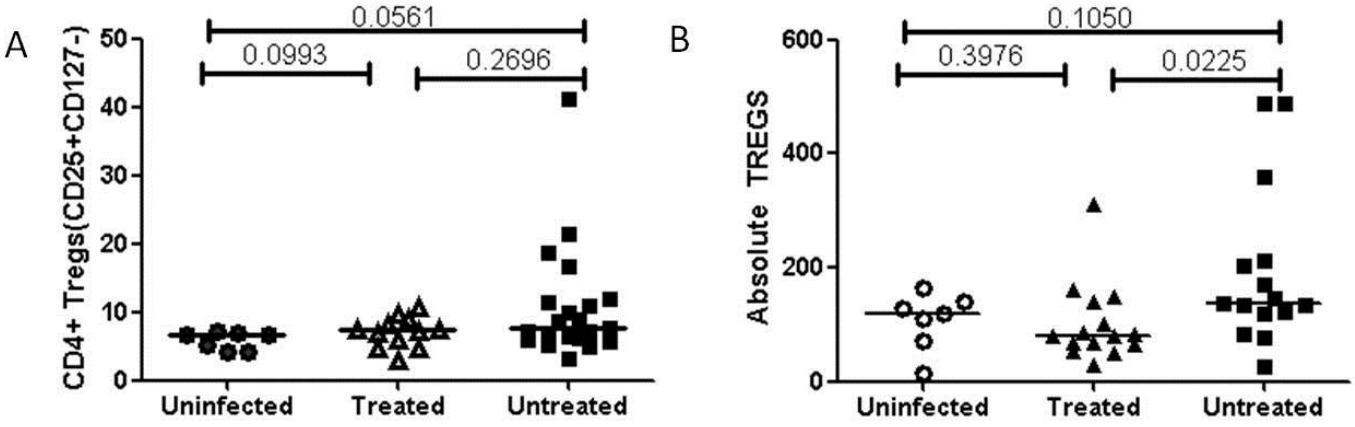


Figure 4.3 Comparison of Treg frequencies of A) Tregs and B) Absolute Tregs, between sub-groups of uninfected, treated and untreated children.

4.3.4 Association observed between clinical markers of expression and frequency of Tregs

We subsequently examined the relationship between frequency of Tregs and clinical markers of disease progression. We found a significant negative correlation between Treg frequency and CD4 T cell% (p=-0.0362, r=-0.3132). The relationship between Tregs and viral load (p=0.6295, r=-0.07866) and absolute CD4+ T cell count (p=0.4040, r=-0.1275) found no associations (Fig 4.4).

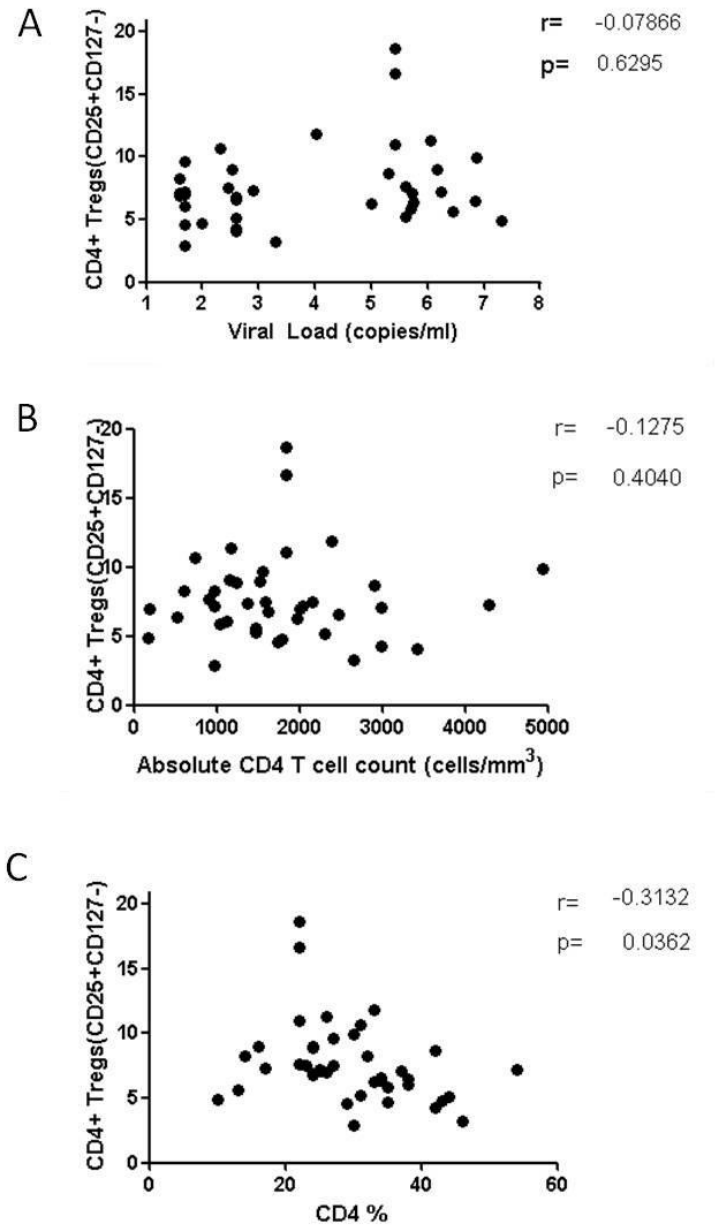


Figure 4.4 Correlation of clinical markers with frequency of Tregs: A) Viral load (copies/mL), B) Absolute T cell count (cells/mm^3) and C) CD4 T cell%.

4.3.5 Assessment of frequency of CD8+CD25+CD127-in HIV-1 infected children

The investigators included CD8+CD25+CD127- cells in the analysis as a potential surrogate for CD8+ Tregs. Recent data from the SIV model and in HIV suggest the presence of this cell type in infected subjects (Nigam et al., 2010, George et al., 2011). In SIV infected rhesus

macaques CD8+FoxP3+ Tregs were expanded in gut mucosa from infected monkeys and correlated negatively with immune activation. As information on CD8+ Tregs is limited, we next assessed the frequency of CD8+CD25+CD127- subset. The CD4+ Treg % was elevated in comparison to the percentage of CD8+CD25+CD127- (unsorted). The median of CD8+CD25+CD127- on T cells was significantly higher in the uninfected subgroup in comparison to the treated children (p=0.0094) but insignificant in the treated subgroup of children (p=0.2647), with an overall range of 0.1 to 2.2% frequencies on CD8+ T cells as Fig 4.5.

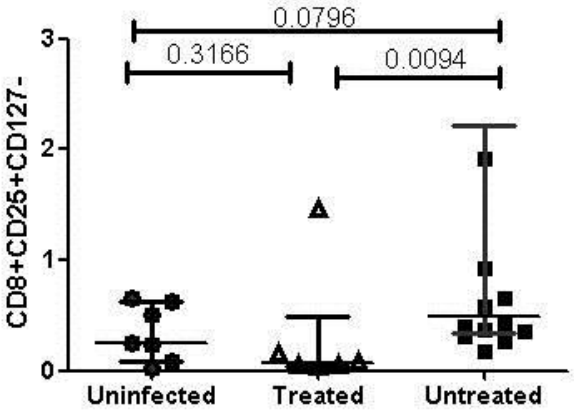


Figure 4.5 Comparison of CD8+ CD25+ CD127- frequencies within sub-groups.

4.3.6 Increased expression of HLA-DR+CD38+ on T cells from HIV-1 infected children

Based on a recent investigation in children that suggested that a decline in Tregs may allow immune activation to increase in HIV-1 infected in comparison to uninfected children, we first compared expression levels of HLA-DR+CD38+ expression between subgroups of children (Fig 4.6). A comparison of HLA-DR+CD38+ expression on CD4+ T cells and CD8+ T cells revealed the following: CD4 cells compared to CD8 T cells as previously described. HLA-DR+CD38+ expression was elevated on CD8+ T cells in comparison to

CD4+ T cells. When comparing the median of HLA-DR+CD38+ expression across sub-groups HLA-DR+CD38+ on CD8+ T cells, elevated expression was observed in the untreated children in comparison to treated children (p=0.0002). In addition, HLA-DR+CD38+ expression was significantly elevated in untreated children in comparison to uninfected children (p=0.0177), and no difference was observed between treated children compared to uninfected children (p=0.9151). HLA-DR+CD38+ expression on CD4+ T cells also revealed that untreated children exhibited a significantly higher median of HLA-DR+CD38+ expression then in the treated children (p=0.0495) and uninfected children (p=0.0312). HLA-DR+CD38+ expression did not differ significantly between treated and uninfected children (p=0.1550) on CD4+ T cells. However, in the children aged less than 6 months HLA-DR+CD38+ expression on CD4+ (p=0.0437) and CD8+ T cells (p=0.00276) was found to be significantly higher in comparison to children more than 6 months. When we went on to compare the median of HLA-DR+CD38+ expression to HLA-DR+CD38+ on CD8+ T cell in adults, elevated expression was observed in the untreated in comparison to the treated subgroup (p=0.0348).

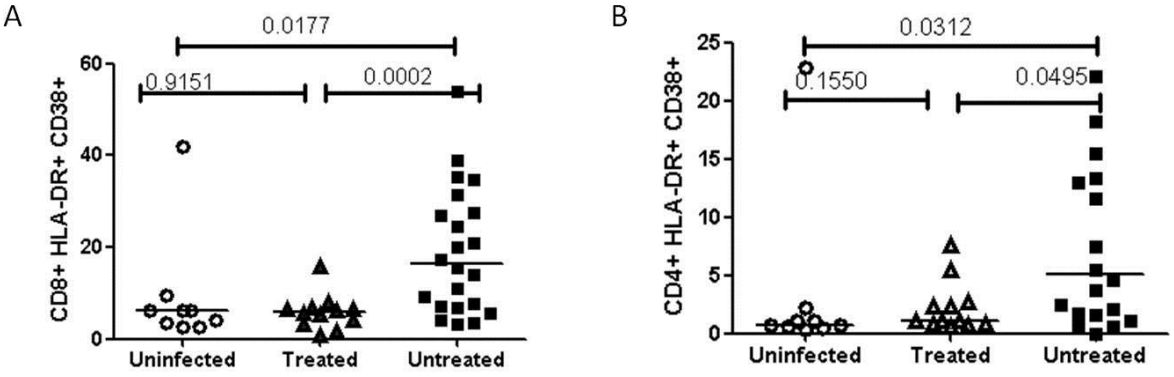


Figure 4.6 Comparison of expression levels of A) CD8+ HLA-DR+CD38+ and B) CD4+ HLA-DR+CD38+ within sub-groups.

4.3.7 No correlation observed between clinical markers of expression and HLA DR+CD38+ expression

Based on findings from adult studies where HLA-DR+CD38+ expression correlated with viral load, we subsequently examined the relationship between CD8+ HLA-DR+CD38+ and viral load (p=0.0946, r=0.2582); absolute CD4+ T cell count (p=-0.5888, r=-0.8000) and CD4+ T cell % (p=0.1259, r=-0.2240) (Fig 4.7A/B/C). Upon examination of CD4+ T cells the following associations were revealed: CD4+ HLA-DR+CD38+ expression and viral load (p=0.2597, r=0.1757); absolute $CD4^+$ T cell count (p=0.7127, r=-0.0546) and CD4+ T cell% (p=0.3529, r=-0.1371), as represented in Fig 4.7D/E/F. Additionally, our study confirmed the findings of other adult HIV-1 studies, showing a significant relationship between HLA-DR+CD38+ and viral load on CD8+ T cells, in our adult participants (p=0.0217;r=0.4761).

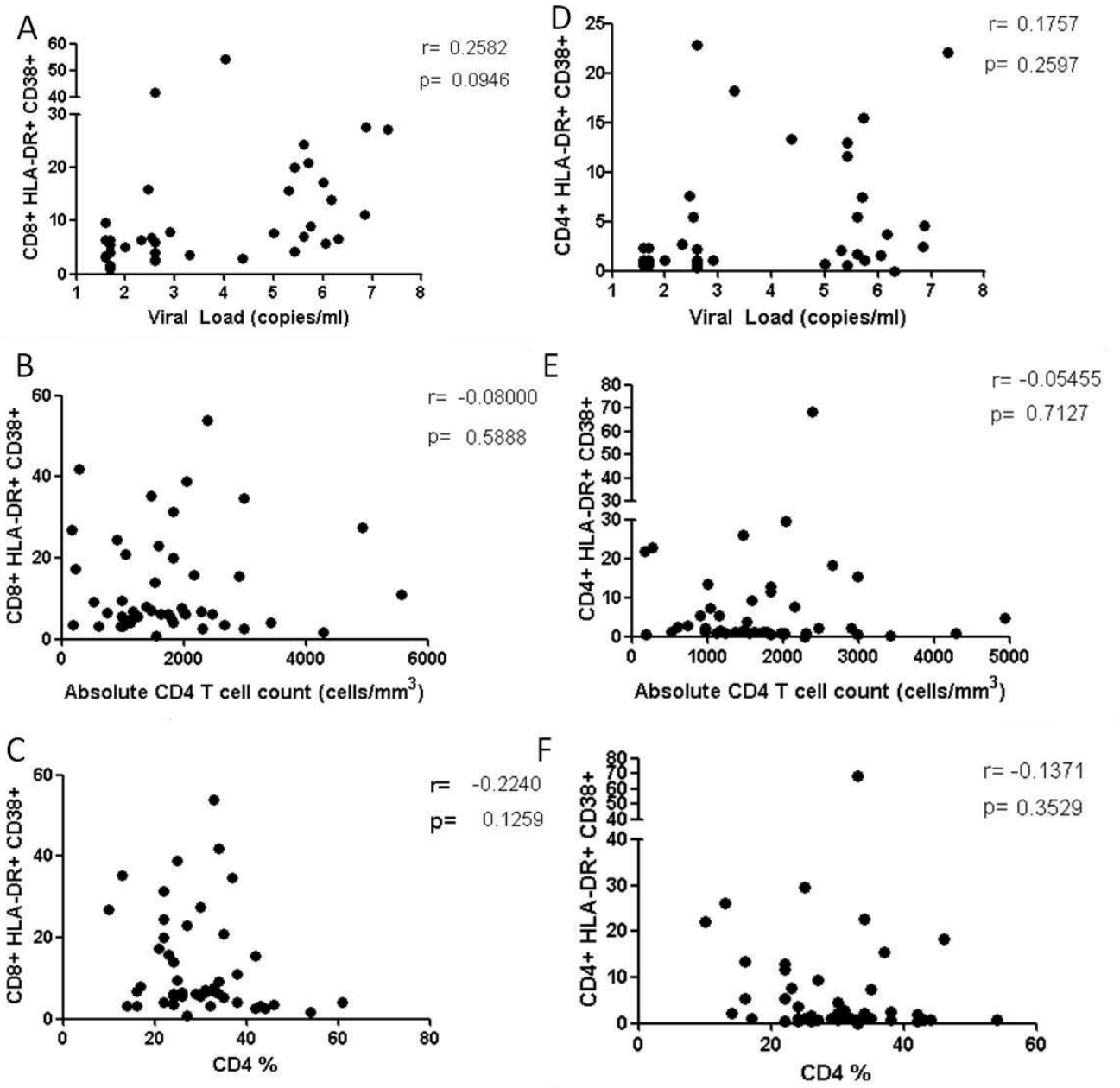


Figure 4.7 Correlation of clinical markers with expression of HLA-DR+CD38+ on CD8+ T cells: A) Viral load (copies/mL), B) Absolute T cell count (cells/mm^3) and C) CD4+ T cell% and D) Viral load (copies/mL), E) Absolute T cell count (cells/mm^3) and F) CD4%.

4.3.8 Correlation of HLA-DR+ CD38+ expression on CD4 and CD8 T cells

We next investigated the correlation of the expression of HLA-DR+CD38+ and Tregs respectively, between CD4+ and CD8+ T cells in children (Fig 4.8). There was a significant positive correlation of CD4+ T cells and CD8+ T cells for HLA-DR+CD38+ ($p<0.0001$;

r=0.6376). Based on the studies that reported an inverse relationship between Tregs and markers of activation, we next investigated the relationship between the expression of HLA-DR+CD38+ and the frequency of CD4+CD25+CD127- on bulk T cells. Taking into account diversity in frequency of expression, no correlation was observed between CD4+CD25+CD127- and CD4+HLA-DR+CD38+ (p=0.2097; r=0.2117), and CD8+HLA-DR+CD38+ (p=0.1994; r=0.1949).

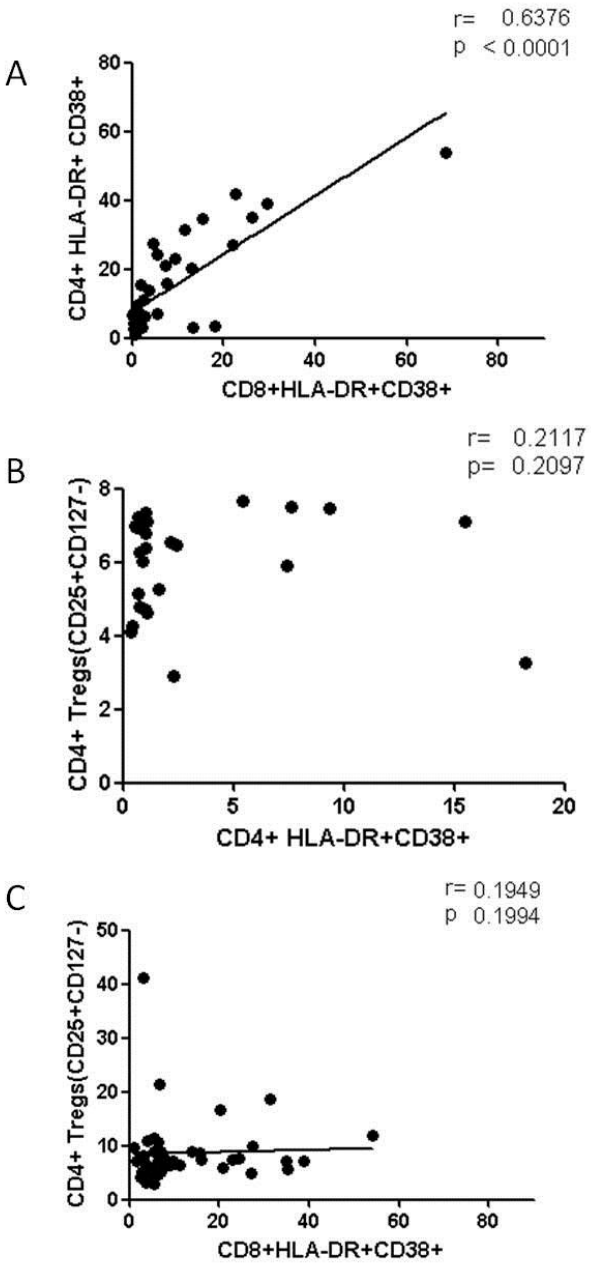


Figure 4.8 Correlation of expression of A) CD4+ HLA-DR+CD38+ and CD8+ HLA-DR+CD38+, B) CD4+ HLA-DR+CD38+ and CD4+CD25+CD127- and C) CD8+HLA-DR+CD38+ and CD4+CD25+CD127-.

4.3.9 Inverse relationship between Tregs and markers of activation and cellular immune response

Based on studies showing that Tregs are able to suppress immune activation and, other studies that have associated hyperactivation with both HIV susceptibility and disease progression, we investigated the relationship between expression markers and magnitude of CTL response (Fig 4.9) in a subset of individuals. As this was a retrospective analysis, CTL responses were available for a subset of individuals. Magnitude of response was assessed by the summation of all positive (>100SFCs) responses to pools of peptides spanning the entire HIV-1 genome. Of the 23 participants assessed using the ELISpot assay, ten participants made no responses. The remaining 13 children revealed a significant negative correlation between magnitude of CTL response, CD4+CD25+CD127- ($p=0.4569$; $r=-0.1716$), CD8+CD25+CD127- ($p=0.0202$; $r=-0.7333$) and CD8+HLA-DR+CD38+ ($p=0.0408$; $r=-0.5516$). There was no correlation with magnitude of response and age ($p=0.7503$; r=-00702).

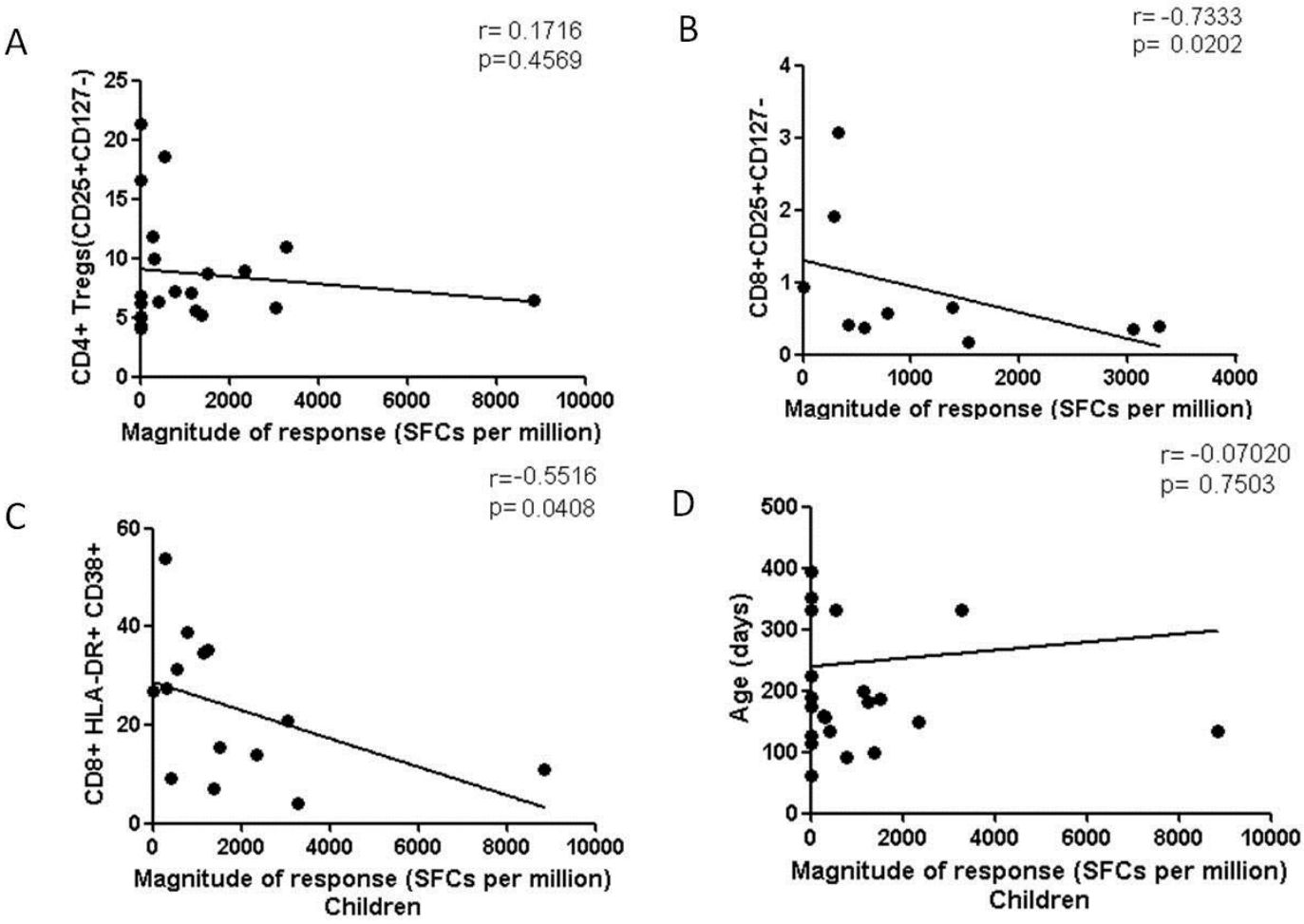


Figure 4.9 Correlation of magnitude of response with A) CD4+CD25+CD127-, B) CD8+CD25+CD127-, C) CD8+ HLA-DR+CD38+ and D) age in a subset of 10 uninfected children.

4.3.10 Longitudinal study: Treg frequencies, T cell activation expression levels and HIV viral load.

To complement our cross-sectional data, we investigated expression of HLA-DR+CD38+ and frequency of Tregs longitudinally over a 20 month period. HLA-DR+CD38+ and frequency of Tregs seemed to show an inverse relationship on CD8+ T cells, at certain time points earlier in the investigation (Fig 4.10). Both Treg frequency and activation markers seem to track positively with viral load and inversely with CD4+ T cell% in the participant studied.

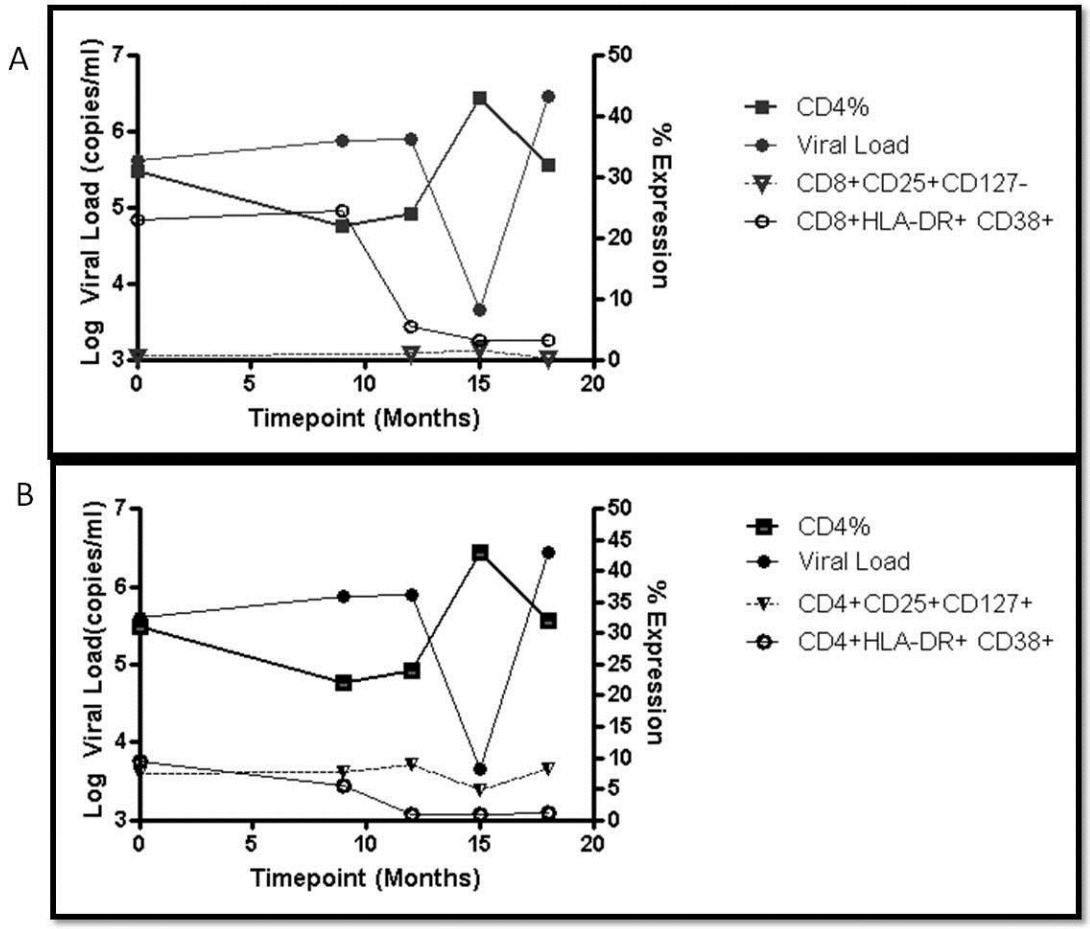


Figure 4.10 Longitudinal assessment of clinical markers and activation and regulatory markers of A) CD8+CD25+CD127- and HLA-DR+CD38+ and B) CD4+ CD25+CD127- and CD4+ HLA-DR+CD38+.

4.3.11 Lower frequency of Tregs when defined as $CD25^{high}FoxP3+$

Based on conventional phenotyping discrimination of Tregs, we next assessed the difference between CD25+CD127- and $CD25^{high}$FoxP3+. When assessing these markers in the same participant, we found lower frequency of $CD25^{high}$FoxP3+ in comparison to CD25+CD127- Tregs.

However the discrepancy appeared to be due to technical difficulties with the intracellular cytokine staining protocol for FoxP3 and no correlation was observed between CD25+CD127- and FoxP3.

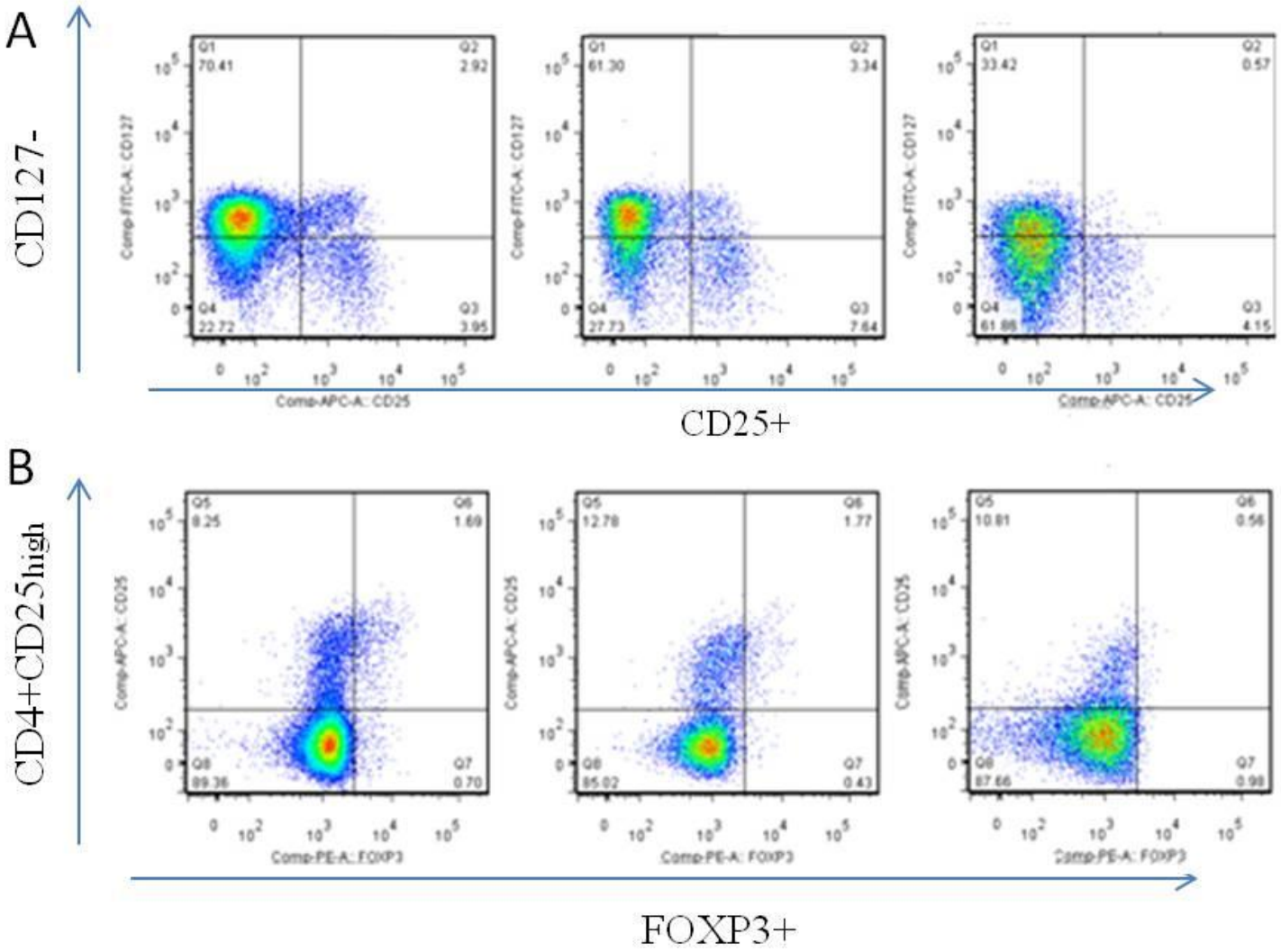


Figure 4.11 Comparison of Treg frequencies defined as A) CD4+CD25+CD127- and B) CD4+CD25^{high}FoxP3+.

4.3.12 Functional Tregs can be expanded from HIV-1 infected children

Building on the phenotypic investigations described above, we next sought to study Treg function in a subset of HIV-1 infected children. We next sought to study Treg function in a subset of HIV-1 infected children. These children's samples were selected based on cell

viability and saturated cell counts to investigate whether the proposed methodology could successful expand Tregs in children. One of the key issues in Treg studies lies in the ability of Tregs to suppress immune responses by expansion. We investigated this suppressive functionality in children. We first sorted cells to isolate Treg population as represented in Fig 4.12A. After expanding Tregs over 7 days using a CFSE proliferation assay, we found that Tregs were capable of suppressive activity in comparison to other CD4 T cells.

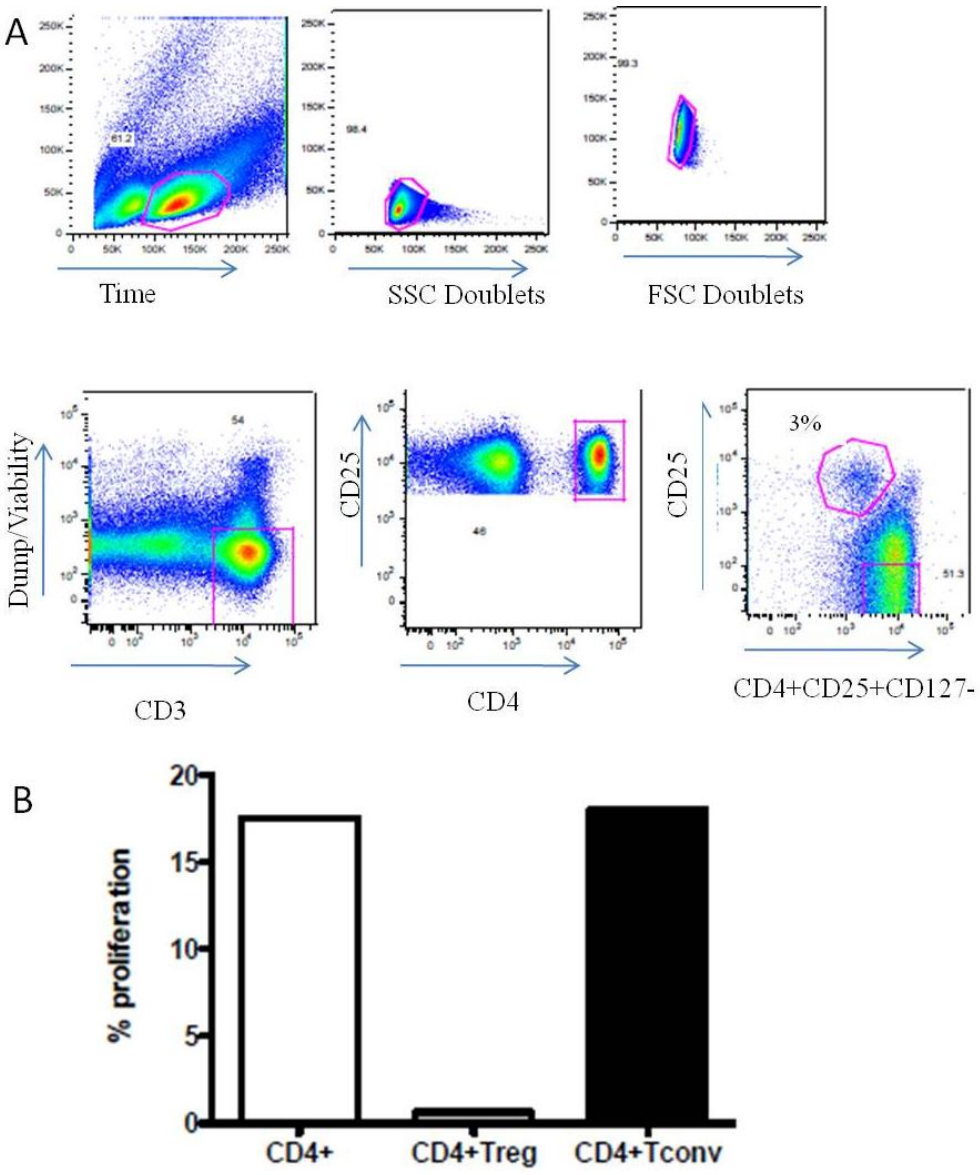


Figure 4.12 A) Gating strategy for identification of sorted CD4+CD25+CD127- Treg cells and B) Treg suppression assay showing CD4 proliferation in the absence of co-culture (white column, positive control), co-cultured with Treg (grey column) and co-cultured with

conventional T cells (black column) alone of successful suppressive capability of CD4+ Tregs, in relation to CD4 and conventional Treg cells (Mathieu Angin-manuscript in preparation (Investigator-co-author).

4.4 DISCUSSION

A multitude of viral, host and cellular factors may influence HIV pathogenesis. Nonetheless, persistent non-specific, generalized immune activation has been implicated as a major driver of HIV susceptibility and HIV disease progression (Paiardini et al., 2009, Hazenberg et al., 2003, Giorgi et al., 1999, Liu et al., 1998, Liu et al., 1997). Immune exhaustion has been found to occur, in parallel with immune activation and a decreased expression of Tregs in HIV-1 progression (Sachdeva et al., 2010). Moreover Tregs have been documented to play a role in activation of CD4+ and CD8+ cells (Eggena et al., 2005, Prendergast et al., 2011a). Taken together, the mechanism underlying T cell exhaustion, regulation and activation remain unclear.

Since their discovery in the early 1970s, the role of CD4+ CD25+ Tregs in both autoimmune and infectious diseases has continued to expand. Tregs actively engage in the maintenance of immunological self-tolerance and immune homeostasis (Sakaguchi, 2004). These controversial regulatory T cells (Coleman et al., 2007) have been found to have beneficial effects in preventing autoimmune diseases (2011, Meng et al., 2011, Mougiakakos et al., 2010) but conversely, they have harmful effects by limiting anti-tumour and anti-microbial immunity (Sakaguchi et al., 2010). There has also been increasing evidence for Treg involvement in allograft rejection (Dummer et al., 2011, Ge et al., 2010, Tao and Hancock, 2008, Nguyen et al., 2006, Cobbold et al., 2006, Battaglia and Roncarolo, 2006). The location of the Treg reservoir influences its role and function. Frequency of expression has been said

to differ in mucosal studies and peripheral studies (Ashwood and Wakefield, 2006). In addition there exists contradicting findings about the role of Tregs during HIV-1 infection, with some studies reporting increases (Xiao et al., 2011, Weiss et al., 2010, Kolte et al., 2009) and others associating depletion of Tregs with disease progression and immune activation in HIV in adults (Eggena et al., 2005, Sachdeva et al., 2010) and more recently, in children (Prendergast et al., 2011a).

More commonly, Treg cells are defined based on the frequency of CD4+CD25+ and the transcription factor FoxP3+ however; alternate phenotypes such as CD4+CD25+CD127- have been documented for use in Treg discrimination and isolation of functional Tregs for suppression assays. The phenotype CD3+CD4+CD25+CD127- has been used to successfully define and sort functional regulatory T cells in previous studies. We and others have shown a strong positive correlation between CD4+CD25+CD127low and CD4+CD25+FoxP3+ T cells. The investigators have data on FoxP3 expression by intracellular staining for subsets of study participants, however technical difficulties prohibited the availability of this date for the entire study cohort. FoxP3 staining would have been helpful to further demonstrate Treg phenotype for comparison with previous studies, We were restricted here and could not demonstrate the strong positive correlation that we and others have previously shown in this study cohort for technical reasons.

The investigators included CD8+CD25+CD127low cells in the analysis as a potential surrogate for CD8+ Tregs. Recent data from the SIV model and in HIV suggest the presence of this cell type in infected subjects (Nigam et al., 2010, George et al., 2011). In SIV infected rhesus macaques CD8+FoxP3+ tregs were expanded in gut mucosa from infected monkeys and correlated negatively with immune activation. In this study the CD25+ FoxP3+CD8+

were largely CD127 low and suppressive (Nigam et al., 2011). However we did not sort these cells in the present study and the extent to which these CD8+CD25+CD127low cells truly are of functional regulatory phenotype needs to be further explored in future studies.

As children represent a vulnerable population severely affected by limitations in immune defense, we too sought to investigate the role of suppressive Tregs and/or activation, as described in adults. As this was a retrospective design the investigators were restricted. Samples if treated children were selected based on earliest available timepoint. Age-related difference was noted but unavoidable. It could be attributed to population related differences. Nevertheless, our study described the frequency of Tregs and activation profiles to differ in uninfected children, treated and infected untreated children.

We observed an inverse correlation of Tregs with CD4+ T cell % which could be a naturally occurring relationship in HIV-1 infected children. Our data suggest that the lower a child's CD4 T cell count , the higher the percentage of Tregs. This finding is similar to observations made in adults, where Treg frequency (% Treg increases) correlates with loss of absolute CD4 T cell numbers. Biologically this may indicate that Tregs are either preferentially spared from cell death, are induced or expanded with more advanced HIV disease, possibly to counteract HIV associated immune activation (Shaw et al., 2011, Angin et al., 2012)

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Interestingly, in a subgroup of participants, we observed a significant inverse correlation between immune regulation and activation on CD8+ T cells. Our findings denoting increased expression of markers of activation on, untreated compared to treated, compared to uninfected, children were in keeping with previous investigations (Cao et al., 2009b, Prendergast et al., 2011a). Our investigation further confirmed a reduced frequency of Tregs in comparison to activation markers. A significant difference in frequency of Tregs and

absolute Tregs was observed on untreated in comparison to treated children, contradicting findings of previous study (Prendergast et al., 2011a). This could be related to the time of sampling of the treated group, age and population related differences. It is noteworthy, this together with our investigation was predominantly cross-sectional and this could influence long term changes at different stages of disease. Additionally, our study demonstrated changed in subsets in the context of f ARV treatment. Differential outcome may also be related to the selection of markers, which is controversial and differs from study to study.

The exact mechanisms of suppression of effector T cells by Tregs remain unclear, but there are in vitro and in vivo evidences showing that these cells are able to suppress antigen-specific responses via direct cell-to-cell contact, secrete anti-inflammatory cytokines such as TGF-beta and IL-10, and also inhibit the generation of memory T cells, among others (Dummer et al., 2011). Regulatory T cells have been documented to play a vital role in maintaining a balance between the induction of early virus specific immune responses and suppression of destructive hyperimmune activation; and have been shown to suppress the activity of both CD4+ and CD8+ T cells recruited to the same APC (Sakaguchi, 2009).

CD8+ T cell responses have been implicated in the effective containment of HIV in adults and children (Goulder, 2000). However, the direct impact of CTL seems to very controversial in children. Interestingly, when we investigated a group of participants tested for immune responses, we observed a significant inverse correlation between immune regulation and activation, which may provide additional evidence for rapid disease progression on children. Our findings are in line with another study, which described strong HIV-1-specific T cell responses after regulatory T cell removal in neonates (Legrand et al., 2006) and another finding in children that suggested CD8+ T cell activation to be partially driven by the

magnitude of the HIV-specific CD8+ T cell response (Prendergast et al., 2011a). We were restricted in that we were unable to test for CD4+ T cell responses and their association with Tregs and markers of activation. Additionally, our findings may be biased as we were only able to assess a single cytokine (IFN-γ) secreted by CD8+ and possibly CD4+ T cells as ELISpot is unable to differentiate CD4 from CD8 responses. These findings may be biased as we were only able to assess a single cytokine (IFN-γ) secreted by CD8+ and possibly CD4+ T cells as ELISpot on whole PBMC specimen is unable to differentiate CD4 from CD8 responses or less likely NK cell responses. Assays such as intracellular cytokine staining and tetramer staining could have differentiated between these possibilities but this was not possible in this study due to sample limitations. However, the ELISpot assay was optimized for CD8 rather than CD4 T cell responses (24h vs 48h incubation) and based on previous data from cohort, the majority of responses observed by ELISpot were CD8+. Assays such as intracellular cytokine staining and tetramer staining could have differentiated between these possibilities but this was not possible in this study due to sample limitations.

T cell expansion has been associated with suppression of immune responses and disease progression (Cao et al., 2009b). Tregs expansion experiments and murine modeling have shown that pharmacologic expansion of donor-derived, naturally occurring regulatory T cells can demonstrate a beneficial role in reducing graft-versus-host diseases (Duramad et al., 2011). Furthermore the suppressive capability of peripheral Tregs, have been documented to play a role in activation of CD4+ and CD8+ cells (Lim et al., 2007). Our study is the first to describe successful results in expansion of functional Tregs in HIV-1 infected children. We are aware that these findings are restricted by a small sample size and may be biased by the

phenotypic marker selected, particularly taking into account the controversy that surround Treg markers.

We conclude that an overall understanding of the exact mechanism which drive HIV disease pathogenesis is limited, due to the obvious multifactorial impact of the virus. Further functional studies that are able to dissect cause-and-effect during disease progression, will enhance our ability to manipulate Tregs and markers of activation in a clinically beneficial manner. Our study was the first to investigate the mechanism underlying Treg suppression on generalized immune activation and immune response in this African cohort of HIV-1 infected children.

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CHAPTER FIVE

The influence of Interleukin-10 (IL-10) Promoter Polymorphisms on HIV-1 Mother to Child Transmission (MTCT)

ABSTRACT

Background: Interleukin-10 (IL-10) is an immune regulatory cytokine that directly affects HIV-1 replication. The overall effect of IL-10 on susceptibility to HIV-1 infection and disease progression is not fully understood. IL-10 promoter polymorphisms influence susceptibility and have been reported to be associated with differential outcome depending on the clinical stage of HIV-1 infection. Data is lacking on the role of IL-10 and its genetic variants on mother-child transmission and paediatric HIV-1 acquisition. Our study investigated the association between two IL-10 promoter polymorphisms, with mother-to-child HIV transmission. Subsequently, we explored the role of IL-10 promoter variants on HIV-1 acquisition. Finally, we assessed the association of cytokine levels with biomarkers of disease progression such as absolute CD4+ T cell count and viral load.

Materials and Methods: We investigated 374 mother-child pairs from the previously described Mamanengane or Africa Centre Vertical Transmission (VTS) cohort and 60 mother-child pairs from the previously described PEHSS cohort. DNA was extracted using the Qiagen DNA isolation kit and the Qiagen QIAamp DNA Mini Kit for dried blood spots (DBS). Applied Biosystems (ABI) Taqman allelic discrimination platform facilitated the discrimination of IL-10 promoter single nucleotide polymorphisms (SNPs) at positions -592 and -1082, relative to the transcription start site. IFN-γ CD8+ T cell responses were screened using the IFN-γ ELISpot assay. Plasma IL-10 expression was measured using the Luminex

Millipore Multiplex MAP Human Cytokine high and regular sensitivity human high sensitivity assay.

Results: We demonstrated a significant association with IL10-1082 and HIV mother-to-child transmission (p=0.0012). Transmitter mothers had a lower proportion of IL10 -1082A, compared to women who did not transmit (79.5% vs 94.5%, p=0.0012), revealing an odds ratio of 0.23 (95% CI 0.09 – 0.58). No association with acquisition and IL10-592 (AA/CA vs CC) or IL10-1082 (AA/AG vs GG) was observed with p=0.6952 and p=0.3251. Absolute CD4+ T cell count and mortality revealed that for every 100 cells/µl increase children were 6% less likely to die (p=0.0703). We found no associated influence with IL10-592 (p=0.4279) or IL10-1082 (p=0.6361) on mortality rates of children in this cohort. Our investigation revealed a significant association between carriers of IL10-592 and magnitude of IFN-γ CD8+ T cell immune response. Carriers of IL10-592C were associated with an elevated magnitude of CD8+ T cell response in comparison to carriers of IL10-529A (p=0.0071). A positive correlation between viral loads and IL-10 plasma levels (p=0.0038; r=0.3872) as well as age (p=0.0199; r=0.4625) was observed. A significant negative correlation was observed with CD4 T cell% and IL-10 plasma levels (p=0.0007; r=-0.6190). No significant difference in IL-10 plasma levels according to IL10 -1082 (p=0.7148) and -592 (p=0.1374) allelic variants.

Conclusion: The data suggests a role for promoter IL-10 variants in HIV-1 mother to child transmission and disease progression. IL-10 plasma levels may play a role in immune dysfunction in HIV infected children.

Key words: IL-10; Mother to child transmission; cytokine; children; HIV transmission; HIV progression

5.1 INTRODUCTION

Interleukin-10 (IL-10) has been shown to play a significant role in promoting viral persistence by inhibiting activation and effector functions of T cells and macrophages (Brooks et al., 2006). IL-10 has been shown to inhibit HIV-1 replication directly in macrophages and monocytes (Wang and Rice, 2006, Ancuta et al., 2001) by blocking entry, and/or post entry through suppressing and down regulating cyclin T1 and consequently Tat function. Furthermore, the effects of IL-10 on HIV-1 replication may depend on the strain of the virus, the cell type targeted by HIV-1 and the stage of HIV-1 infection (Orenstein et al., 1997, Ancuta et al., 2001).

The lymphocytic choriomeningitis virus (LCMV) model of chronic viral infections in mice, demonstrate a critical role of IL-10 in the disruption of effector immune responses, in turn contributing to viral persistence particularly during the early phases of infection (Ejrnaes et al., 2006, Brooks et al., 2008). Furthermore, blockade of the IL-10 pathway allowed a DNA vaccine to further stimulate T cell responses and restore IFN-γ secretion, resulting in viral clearance. Hence, a single immunoregulatory molecule may be capable of playing a significant role in determining the outcome of a persistent viral infection in mice. These studies need to be extended to HIV-1, the most significant persistent viral infection in the human population. These studies may enable a better understanding of mechanisms that underlie immune dysfunction in HIV-1 infection.

Genetic polymorphisms in the IL-10 promoter region may influence levels of IL-10 produced from various cells of the immune system (Ejrnaes et al., 2006, Lazarus et al., 2006, Turner et al., 2002). Our investigation focused on two single nucleotide polymorphisms (SNPs) found in the promoter region at positions -1082 (A to G transition) and -592 (C to A transversion)

of the 36-kDa IL-10 gene. The IL-10 gene has been mapped to human chromosome 1, between 1q31 and 1q32 and its promoter. SNPs have been associated with different levels of IL-10 production (Kamali-Sarvestani et al., 2006, Lan et al., 2006). Allelic variants in the human genome can regulate susceptibility or resistance to infection. The allelic variants -1082A and -592A have been documented to be associated with decreased IL-10 production which in turn is further associated with an increased likelihood of HIV-1 acquisition and a rapid rate of CD4 T cell decline (Erikstrup et al., 2007, Shin et al., 2000, Shrestha et al., 2010, Vasilescu et al., 2003). This finding suggests that the recessive allele-1082G is associated with increased IL-10 production and may be associated with an AIDS delaying effect. Several studies have investigated the contribution of IL-10 promoter polymorphisms to HIV-1 susceptibility or disease pathogenesis (Erikstrup et al., 2007, Shin et al., 2000, Shrestha et al., 2010, Vasilescu et al., 2003). Studies suggest that allelic variants associated with lower IL-10 production predispose participants to higher likelihood of HIV-1 acquisition. Subsequently to being infected, the lower IL-10 producer alleles appear to be associated with rapid disease progression (Naicker et al., 2009, Shin et al., 2000). However, it should be noted that all these studies have been undertaken predominantly in adults. None of these studies have investigated the role of IL-10 promoter polymorphisms in HIV-1 mother to child transmission (MTCT) or HIV-1 acquisition in children.

The influence of IL-10 promoter polymorphisms within HIV-1 clade C was recently investigated in a cohort of high risk HIV-1 negative and acutely infected South African women (Naicker et al., 2009). The C to A mutation at promoter position -592 was associated with increased risk of HIV-1 acquisition. The IL10 -1082G allelic variant showed a trend towards decreased likelihood of HIV-1 acquisition. The study suggested that alleles associated with high IL-10 production such as IL10 -592C and IL10 -1082G may have a

protective effect against HIV-1 infection. However, these carriers exhibited significantly higher median plasma viral loads perhaps through IL-10 mediated suppression of effector antiviral immune mechanisms. Hence, IL-10 promoter polymorphisms may influence susceptibility to HIV-1 infection and may affect markers of disease progression such as viral load (Naicker et al., 2009). Our study described the association between genetic polymorphisms in the IL-10 promoter gene with the risk of HIV-1 acquisition among children; and the risk of transmission by mothers. Our study further described underlying mechanisms of IL-10 promoter polymorphisms and their interplay with cellular immune responses and cytokine production in an African HIV-1 clade C infected MTCT transmission cohort.

Cytokines have been implicated in disease progression. The immunosuppressive factor, IL-10, is a cytokine produced by lymphoid cells. Cytokine production, hyperactivation of antiviral T cells and the nature of the immunosuppressive environment play an influential role in HIV pathogenesis (Hedrich et al., 2010, Lubong Sabado et al., 2009, Song et al., 2011) and may provide valuable information to help understand the mechanism underlying viral control or the lack thereof.

Elevated IL-10 plasma levels have previously been documented to correlate with poor prognosis in many infections (Roberts et al., 2010). In acute HIV infection an upregulation of certain cytokines such as IFN-α, TNF-α (von Sydow et al., 1991) and IL-10(Norris et al., 2006) have been reported. Furthermore another study of acute Hepatitis B and C infections revealed a broad array of cytokine expression lead to eventual viral clearance in a majority of infected participants (Stacey et al., 2009). Chronic HIV-1 infection also revealed increased cytokine secretions due to persistent viral replication and antigenic stimulation. Both pro-

inflammatory and regulatory cytokines are produced during chronic immune stimulation and regulatory cytokines play a role in suppressing type 1 cytokines while anti inflammatory cytokine play a role in immune suppression (Oguariri et al., 2007).

Only few studies have profiled cytokines in children. The levels of cytokine produced in children have been found to be an important determinant of malnutrition (Jones et al., 2005). The study found an increased production of IL-4 and IL-10 cytokines in the CD4+ and CD8+ cells from malnourished children in comparison to the T cells from well-nourished children. The results suggest that malnutrition alters the capacity of T cells to produce the cytokine IL-10, thus explaining the inability if these children to develop a specific immune response and the predisposition to infection in these children (Rodriguez et al., 2005). Cytokine expression levels, absolute CD4+ T cell counts and viral load were also monitored in 12 HIV-1 infected children in Hong Kong. The study found alterations in cytokine profiles were not associated with adverse clinical events (Jones et al., 2005).

More recently, a study investigated the association between plasma cytokine concentrations during primary acute infection with clinical markers of HIV disease progression such as absolute CD4 T cell counts and viral load measurements (Roberts et al., 2010). The study showed cytokines IL-12p40, IL-12p70, IFN-γ, IL-7 and IL-15 to be able to predict 66% of the variation in viral load set-point 12 months post infection. The study found IL-12p40, IL-12p70 and IFN-γ to be significantly associated with lower viral load whereas IL-7 and IL-15 were associated with higher viral load. The study showed that IL-7 was associated with more rapid CD4 loss (Roberts et al., 2010). Our study aimed to correlate cytokine levels with long term biomarkers of disease progression such as absolute CD4+ T cell count and viral load. We assessed the median cytokine biomarker levels in age matched groups. The study also

described the levels of pro-inflammatory and anti-inflammatory cytokines and their influence on disease progression in an African clade C infected cohort of children.

5.2 PARTICIPANTS, MATERIALS AND METHODS

5.2.1 Cohort description

Participants were recruited from the previously described Mamanengane or Africa Centre Vertical Transmission (VTS) cohort (Bland et al., 2010, Coovadia et al., 2007). The mothers were enrolled during their antenatal care visits at the clinics in Umkhanyakude and the outlying areas of Durban, KwaZulu-Natal, South Africa. The eligibility criteria were woman aged 16 years of age or older, who were planning to stay in the study area for at least 3 months after delivery, and who were able to provide written consent. As illustrated in Fig 5.1, our study was a sub-study of a larger study. We selected Dried Blood Spots (DBS) samples collected from 374 South African Mother-Child transmission pairs. The transmitter and non-transmitter mothers were selected based on their matched viral load. The uninfected mothers were selected by matching the ages of the mothers to the ages of the infected mothers. Written informed consent was obtained from all study participants and the research protocol for these analyses were approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa, and the Internal Review Board of Massachusetts General Hospital.

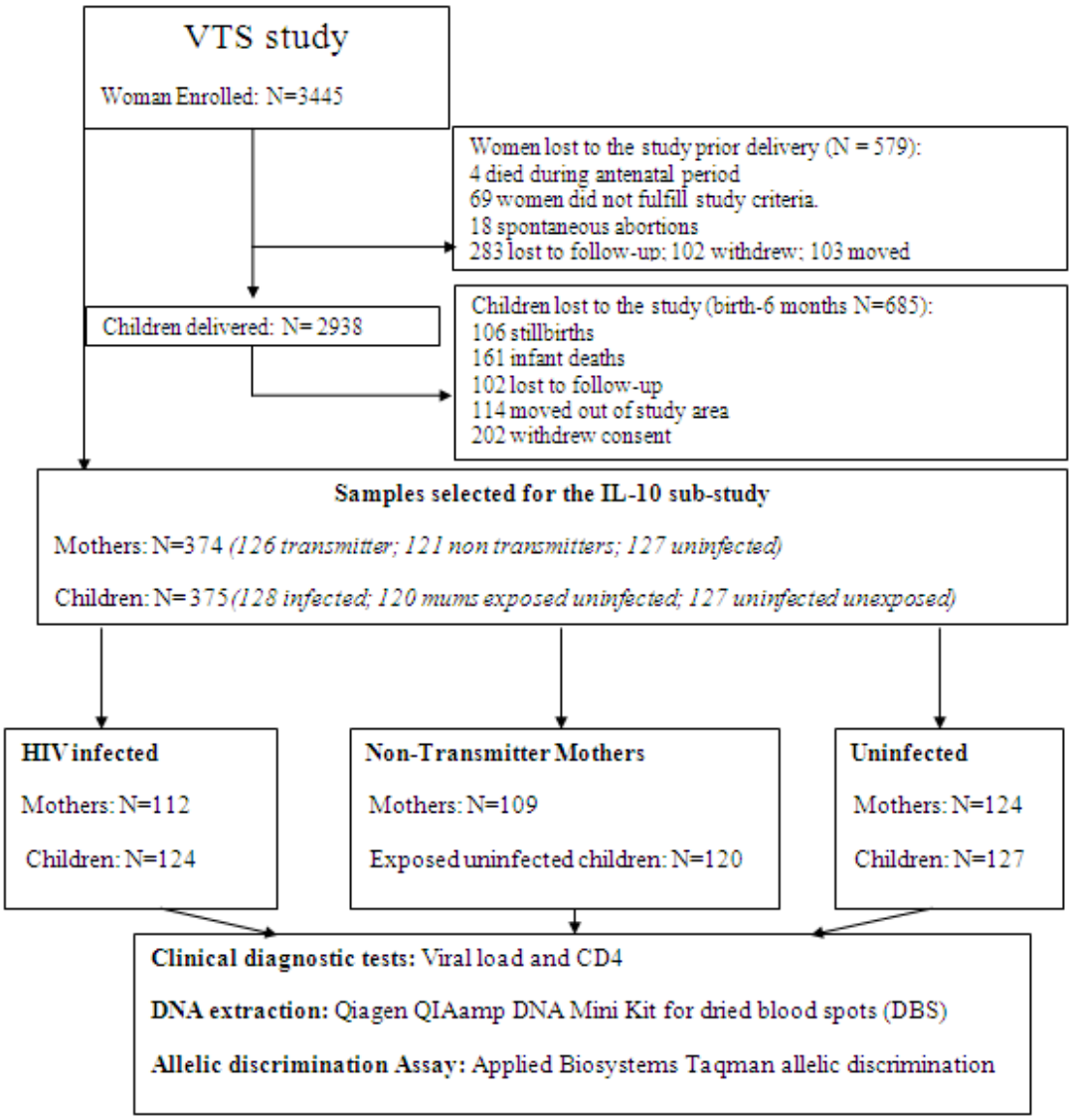


Figure 5.1 Flow diagram of participants selected from the VTS study for the current sub-study.

We also studied the previously described PHESS study cohort (Thobakgale et al., 2007, Mphatswe et al., 2007) in which we investigated immune response and cytokine production in association with IL-10 promoter polymorphisms. The study screened and recruited 719 HIV-1 infected mothers, 740 infants were born to the mothers; 623 infants were uninfected, 41 were untraceable and only 76 were infected. The study enrolled 60 infants whom met the study criteria. The 60 HIV-1 infected infants born to HIV-positive mothers were enrolled

from St Mary's and Prince Mshiyeni Hospitals in Durban, South Africa between the periods of 2003-2005 (Fig 5.2). The HIV-1 seropositive mothers were recruited during the last trimester of pregnancy and received a single dose of Nevirapine during labour. The infant received a single dose of Nevirapine within 48 hrs of birth, according to the HIVNET-012 Protocol, as previously described (Guay et al., 1999, Jackson et al., 2003).

The 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 viral load test (>400 RNA copies/ml) on day 1 or day 28 was followed by a confirmation test. Infants with detectable virus on day 1 were defined as intra-uterine (IU) infected, and infants with undetectable virus on day 1 but with detectable virus on day 28 were defined as intrapartum (IP) infected (Thobakgale et al., 2009, Thobakgale et al., 2007, Mphatswe et al., 2007). Written informed consent was obtained from all study participants and the research protocol for these analyses were approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa.

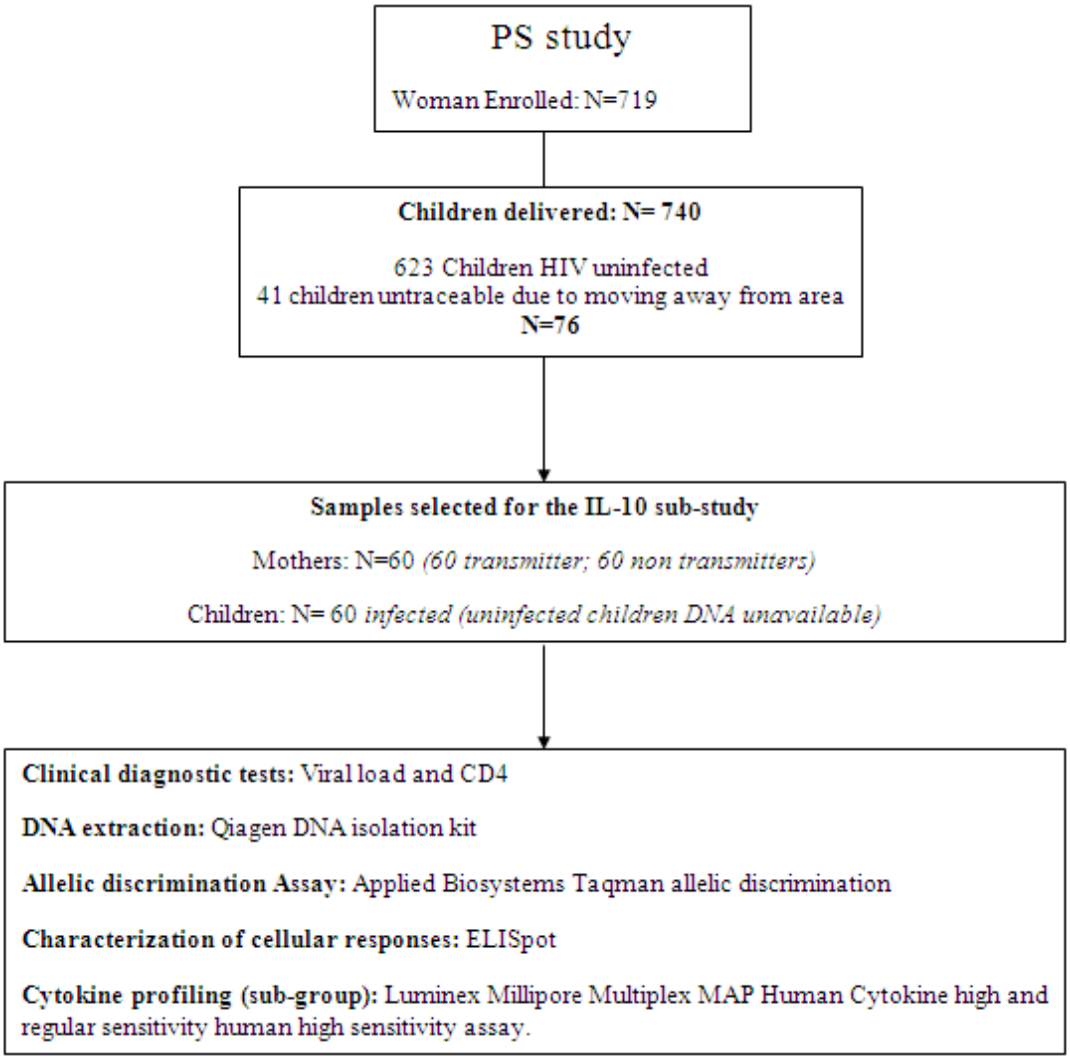


Figure 5.2 Flow diagram of participants selected from the PEHSS study for the current sub-study.

5.2.2 DNA extraction

For the VTS study the DNA was extracted from DBS blood cards (S&S 903 paper card, Whatman® Schleicher & Schuell, Sigma-Aldrich, St. Louis, USA). The DBS cards had been stored for approximately 8 years at varying temperatures, first at -80^{o} C for approximately 1 year, then at room temperature for 1 year and finally at -20^{o} C for 6 years. We isolated genomic DNA from a single punch from each DBS sample and followed manufacturers' instructions for extraction using the QIAamp DNA mini kit (Qiagen) as per Fig 5.3. This kit was selected based on results from a previous study that investigated the use of dried blood

spots for the determination of genetic variation as well as the quality of extraction of three commercially available kits as per Ndlovu et al, 2011; submitted). DNA was quantified on the Nanodrop 2000 spectrophotometer. Only DNA yield of 10-20ng of gDNA per 4.8mm punch of DBS card (Sjoholm et al., 2007, Singh and Spector, 2007, Wijnen et al., 2008) was used for genotypic analysis. DNA quality was validated by a 260/280 ratio and a purity of approximately 1.8.

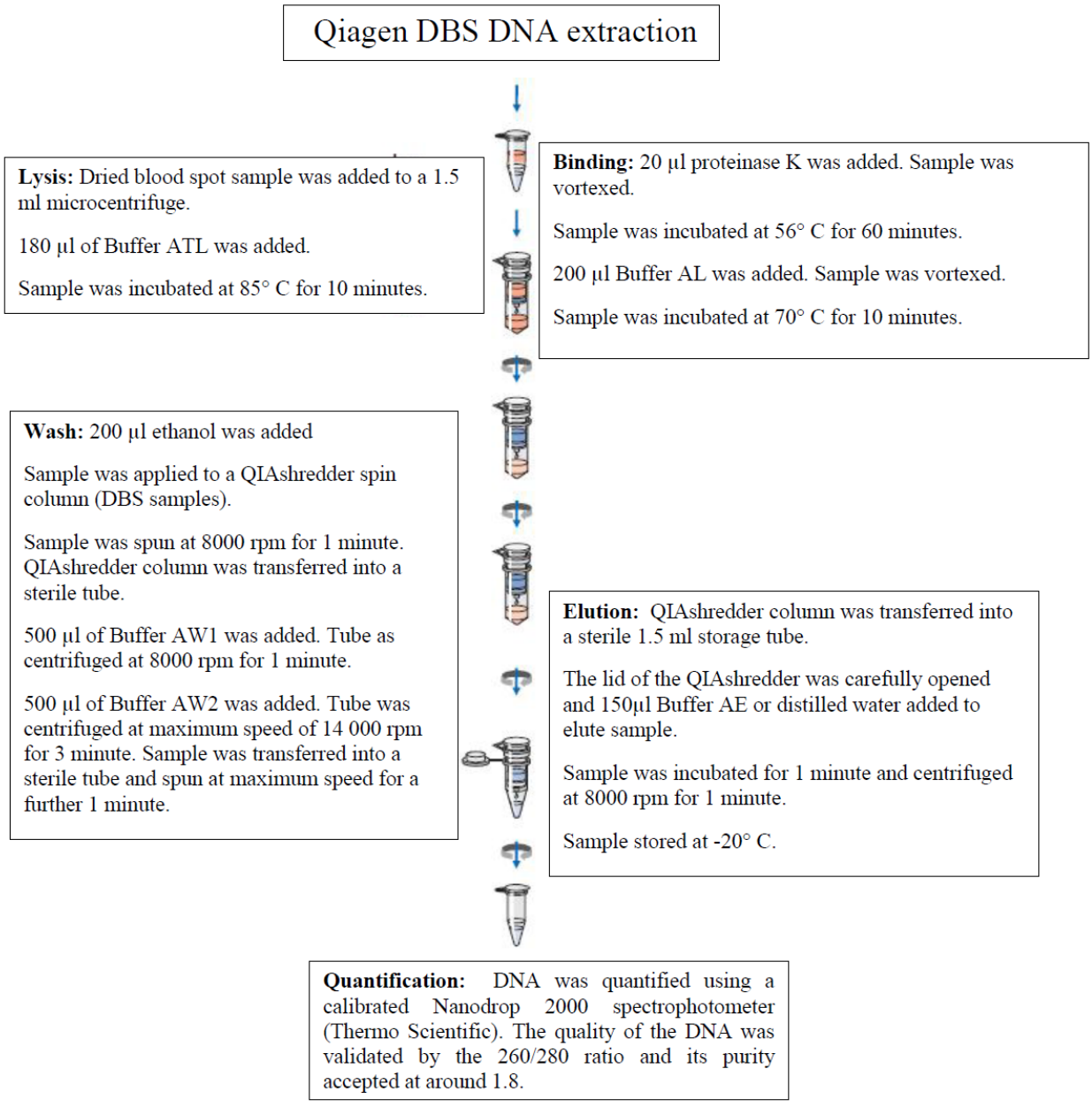


Figure 5.3 QIAamp DNA Mini Kit extraction protocol for dried blood spots (DBS) adapted.

5.2.3 Plasma viral load and CD4+ T cell measurement

In the VTS cohort the HIV viral load and CD4+ T cell counts were determined at the Africa Centre Virology laboratory in Durban. Total HIV RNA was isolated from plasma using guanidinium-silica methods (NucliSens EasyQ HIV-1 kit) and from plasma (RNA extraction was using Minimag extraction by Biomeriux). The NucliSens HIV-1 QT (bioMerieux, Inc., Durham, NC) assay has a quantitative range of 40 to >500,000 copies/mL of plasma. CD4+ T cell cell counts were measured on venous blood within 24 hours of sampling using an Epics XL cell counter (Beckman Coulter, Fullerton, CA) and a 4-color protocol.

In the PS cohort absolute CD4+ T cell counts were determined at the HIV Pathogenesis Programme, Durban, from fresh whole blood using Tru-Count technology on a four-color flow cytometer (Becton Dickinson) according to the manufacturer's instructions. Plasma viral loads were measured using 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.

5.2.4 Allelic discrimination Assay

The study assessed IL-10 genotyping using the TaqMan® SNP Genotyping Assay kit using MGB primers and probes for IL-10 at nucleotide positions -592 A/C (rs 1800872) and -1082 A/G (rs 1800896) as per manufacturer's instructions (ABI-Applied Biosystems) (Fig 5.3).

The Taqman SNP genotyping assay was setup in a 96 plate format utilizing the 5`to 3` nuclease assay principle where each of the taqman probes annealed to a specific complementary sequence between a forward (5`) and reverse primer (3`). The following components were combined to prepare a single sample reaction (Table 5.1):

Table 5.1 Optimized ABI Taqman SNP genotyping assay (per sample).

Reagents	Volume (µl)
PCR master mix	12.5
40x assay mix	0.625
Genomic DNA (10ng)	X
DNase free sterile H_20	(11.875-X)
Total	25

Note: PCR master mix (hot starter enzyme, optimized 50 mM MgCl2 (Magnesium chloride) buffer, AmpErase® Uracil N-glycosylase (UNG) and dUTPS (2´-Deoxyuridine, 5´-Triphosphate)-protects against contamination.

Assay mix-Minor grove binder (MGB) VIC™ or FAM™ labelled probes and sequences specific IL10 primer.s

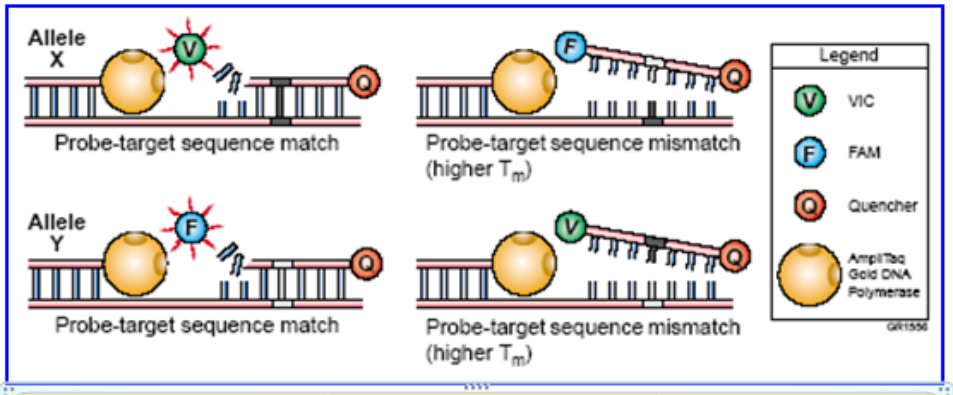
X=volume per sample of 10ng of final DNA concentration; purity 1.8-2.0(C1V1=C2V2).

For negative controls no template genomic DNA was added. For positive controls genomic DNA samples that we previously genotyped was used as internal standards for both IL10 SNPs -592 A/C and -1082 A/G. The standard thermocyling conditions were run on the Applied Biosystems Realtime PCR 7500 as follows (Table 5.2).

Table 5.4 PCR conditions for IL10 SNP genotyping assay (ABI Genotyping assay).

	AmpliTaq Gold Enzyme Activation	PCR	
	HOLD	CYCLE (40 cycles)	
		Denature	Anneal/ Extend
Time	10 min	15 sec	1 min
Temp	95 °C	92 °C	60 °C

The TaqMan Allelic Discrimination assay employs a probe technology that uses the nuclease activity of AmpliTaq Gold® DNA Polymerase to direct 5´-3´ reactions. Two TaqMan probes are used for allelic discrimination. The probe hybridizes to a target sequence within the PCR product. The AmpliTaq Gold enzyme cleaves the TaqMan probe with its 5´–3´ nuclease activity. The reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter. These reactions enable PCR product detection based on the detection of their fluorescent reporter. The reporter could either be VIC™ and FAM™ representing homozygosity of one allele or VIC™ and FAM™ representing heterozygous alleles as per Fig 5.4.



A substantial increase in:	Indicates:	Example:SNP 1082
VIC™ fluorescence only	Homozygosity for allele X	AA
FAM™ fluorescence only	Homozygosity for allele Y	GG
both fluorescent signals	Heterozygosity	AG

Figure 5.5 TaqMan Allelic probe discrimination assay.

We analyzed the results using the ABI Prism® Sequence Detection system 7000 software. The florescent probes were detected in a 96-well plate format and based on wavelength readout results; allelic variants were discriminated and graphically mapped as per Fig 5.5. The IL-10 allelic variants were analyzed as wildtype homozygous, mutation homozygous or heterozygous according to dominant/recessive model of mutants at each position.

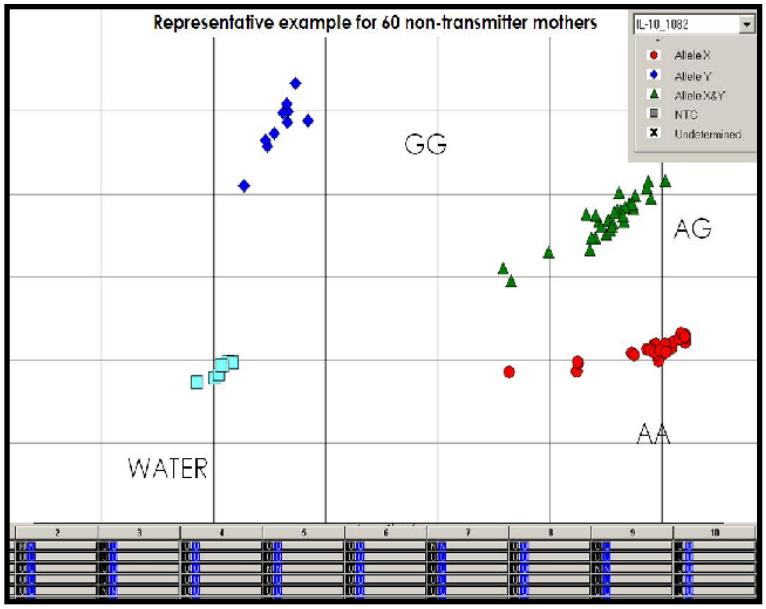


Figure 5.6 Schematic of allelic variant discrimination for 60 samples analyzed for IL-10 -1082 (ABI Prism® Sequence Detection system 7000).

5.2.5 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.

5.2.6 HIV-1 Peptides

A panel of 410 overlapping peptides (10-15mers overlapping by 10 amino acids) spanning the entire HIV-1 clade C consensus sequence were synthesized on an automated peptide synthesizer (MBS 396, Advanced ChemTech). Pepetides were used in a matrix system in screening assays.

5.2.7 ELISpot Assays

CD8+ T cell responses were determined using an interferon-γ ELISpot assay by Dr. C. Thobakgale (Thobakgale et al., 2007) as previously described in chapter 2.

5.2.8 Luminex Cytokine analysis

Plasma IL-10 cytokine expression levels were determined using the Luminex Multiplex MAP Human Cytokine high sensitivity and regular sensitivity immunoassay (Milliplex beads; Millipore) according to the manufacturer's instructions on a Bio-Plex 200 array system (Bio-Rad Laboratories). Multiplex assay enables a large quantity of data to be extracted, hence enabling multiple analytes to be measured speedily in parallel. Immunodetection of beads occur in a 96-well plate format. Different bead sets are combined as a kit or by selection to capture antibodies into one master mix. Plasma samples (50µl) were mixed and incubated overnight with the bead set mix in a microtiter plate. The assay was subsequently read using lasers for both-bead identification and analyte quantification. Each bead was individually discriminated based on capture bead and based on level of florescence produced as wavelength outputs which are compared to standards. Analytes are analyzed in a high sensitivity kit measuring GM-CSF, IFN-γ, IL-10, IL-12 (p70), IL-13, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, and TNF-α. The multiplex assay can be used for the quantification of analytes such as MIP-1B, EGF, Eotaxin, Fractalkine, G-CSF, IL-17, MCP-1, SD-40L, IP-10, TGF-A and VEGF on a regular sensitivity kit.

5.2.9 Statistical analysis

Data was analyzed and graphically represented using Prism software (GraphPad; version 5) and SAS version 9.2 (SAS Institute Inc., Cary) was used for further statistical analysis. Chi-square test compared allelic frequencies to verify that the analyzed SNPs were in Hardy-

Weinberg equilibrium in the study population. Fisher's exact test was used to analyze the association between HIV status and IL-10 promoter polymorphisms. Kruskal-Wallis test followed by Dunn's post test was used to compare differences between groups. Kaplan-Meier survival statistics and Cox proportional hazards models were used to assess mortality of infants. Odds ratio was used to determine the strength of association of transmission and allelic variants. A linear regression model was used to in order to determine any possible associations of IL-10 with age, clinical markers and CTL responses.

5.3 RESULTS

5.3.1 Cohort Characteristics

Three hundred and seventy four mothers and their children are selected as a subset of a larger study. After DNA extraction and TaqMan genotyping only three hundred and forty four mother and three hundred and sixty five children samples were evaluated. At the time of sampling both the mother and children received a single dose of NVP. The HIV-1 infected transmitter mothers were matched by viral load to HIV-1 non-transmitter mothers. The uninfected mothers are matched to the infected transmitter mothers by age. The study design was flawed in that the time of transmission was unknown.

Table 5.2 Characteristics of the children of the Vertical Transmission Study cohort analyzed in the current sub-study.

	Infected	**Exposed uninfected**	**Unexposed uninfected**
Children (n)	124	114	127
Median Age in days (IQR)	224 (1-1,627)	550 (1-1,657)	666 (1-1,351)
Median Absolute CD4+ T cell count (IQR)	1,404 (85-2,990)	1,920 (541-5,208)	2215 (1,049-9,250)
Median Viral Load (copies/ml)	176,000	-	-

IQR (copies/ml)	1,000-1,000,000

Table 5.3 Clinical characteristics of the mothers of the Vertical Transmission Study cohort analyzed in the current sub-study.

	Transmitter	**Non-transmitter**	**Uninfected**
Mothers (n)	112	108	124
Median Age in years (IQR)	24 (17-40)	24 (16-43)	25 (16-39)
Median Absolute CD4+ T cell count (IQR)	354 (15-1,020)	422 (35-1,419)	944 (301-1,928)
Median Viral Load (copies/ml)	45,000	35,500	-
IQR (copies/ml)	(230-17000,000)	(230-1,1000,000)	

5.3.2 Significant difference in absolute CD4+ T cell count (cells/mm^3) in children

As expected the uninfected sub-group of children exhibited the highest median absolute CD4+ T cell count of 2,215 with a range of 1,049-9,250 cells/mm^3 in comparison to the infected sub-group who exhibited a median CD4+ T cell count of 1,404 with a range of 85-2,990 cells/mm^3 ($p<0.0001$). The infected sub-group exhibited a median viral load of 176,000 with a range of 1,000-1,000,000 copies/ml. (Table 5.3&5.4; Fig 5.6).

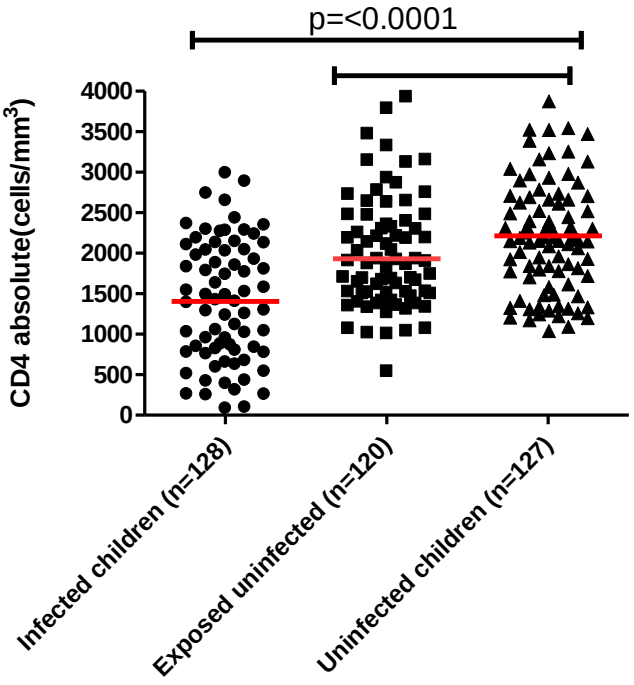


Figure 5.7 Comparison of Absolute CD4+ T cell count within children (cells/mm^3).

5.3.3 No relationship between distribution IL10 allele frequency and HIV acquisition in children

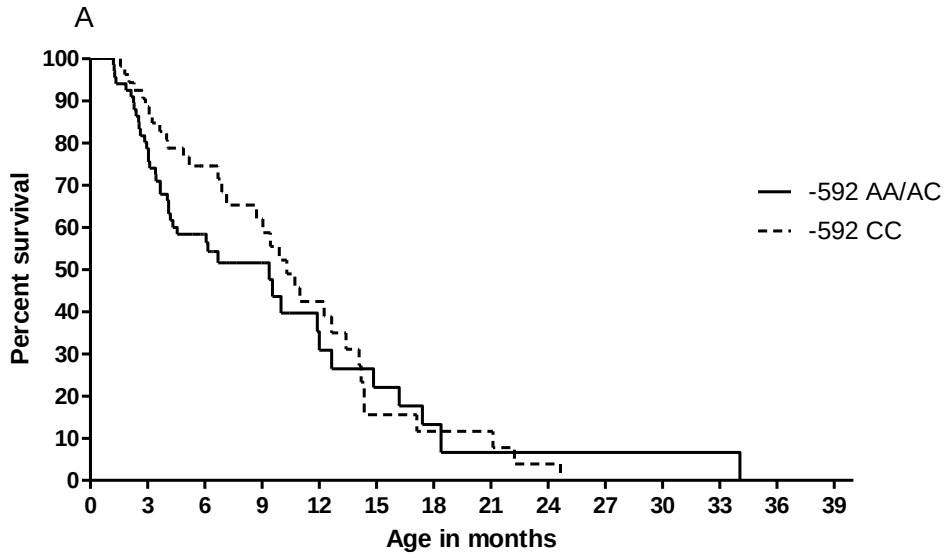
Based on a previous study (Shin et al., 2000) the -1082G mutant allele has been classified as the recessive allele and associated with an AIDS delaying effect while the -1082A (AA and AG) is associated with patterns of dominance. Similarly the -592 (AA and CA) has shown to have a dominant effect on carriers, which is further associated with AIDS susceptibility, while the -592C was classified as the recessive allele and associated with an AIDS delaying effect. These dominant and recessive patterns formed the basis of bulk of the analysis. As several studies have implicated IL-10 promoter polymorphisms to HIV-1 susceptibility or disease pathogenesis in adults (Erikstrup et al., 2007, Shin et al., 2000, Shrestha et al., 2010, Vasilescu et al., 2003)we wanted to assess the association of IL-10 promoter polymorphisms -592 and -1082 with HIV acquisition by comparing the distribution of the allelic variants between HIV-1 infected and uninfected babies of HIV-1 infected mothers. The allelic variants were distributed similarly for both IL10 -1082 and IL10 -592. There was no overall association (Table 5.5) between IL10 -592 (p=0.6952) and IL10 -1082 and HIV acquisition (p=0.3251).

Table 5.4 Comparison of distribution of genotype between HIV-1 infected and uninfected children.

IL-592 N (%)	**HIV infected (n=124)**	**HIV uninfected (n=114)**	**IL-1082 N (%)**	**HIV infected (n=124)**	**HIV uninfected (n=120)**
AA/CA	68 (54.8%)	66 (57.9%)	**AA/AG**	112 (90.3%)	103 (85.8%)
CC	56 (45.2%)	48 (42.1%)	**GG**	12 (9.7%)	17 (14.2%)
p-value	0.6952		**p-value**	0.3251	

5.3.4 No association between IL10-592 and IL10-1082and infant mortality

A previous study in a Zimbabwean adult cohort found that mortality was lower in carriers of the IL-10 -1082G allele which is associated with increased IL-10 production. We therefore sought to examine whether a similar trend would be found in children. These analysis was only preformed on the 124 HIV-1 infected infants of whom 78 (60.9%) died. The median age of death was 4.4 months (IQR 2.9 – 11.9). The association between allelic variants and clinical markers and their association with mortality was assessed using a Cox proportional hazards model. Firstly, distributions of allelic variants utilized for mortality assessment were as follows: AA/AC 60.0% (n=41) and CC 60.7% (n=34). In IL10 -1082 the distributions the different alleles were as follows: AA/AG 61.6% (n=69) and GG 58.3% (n=7). In adjusting for both alleles, dominant and recessive patterns, there was no significant difference in mortality rates between dominant and recessive genes (Fig 5.7) in either IL10 -592 or -1082 (p=0.4279 and p=0.6361, respectively).



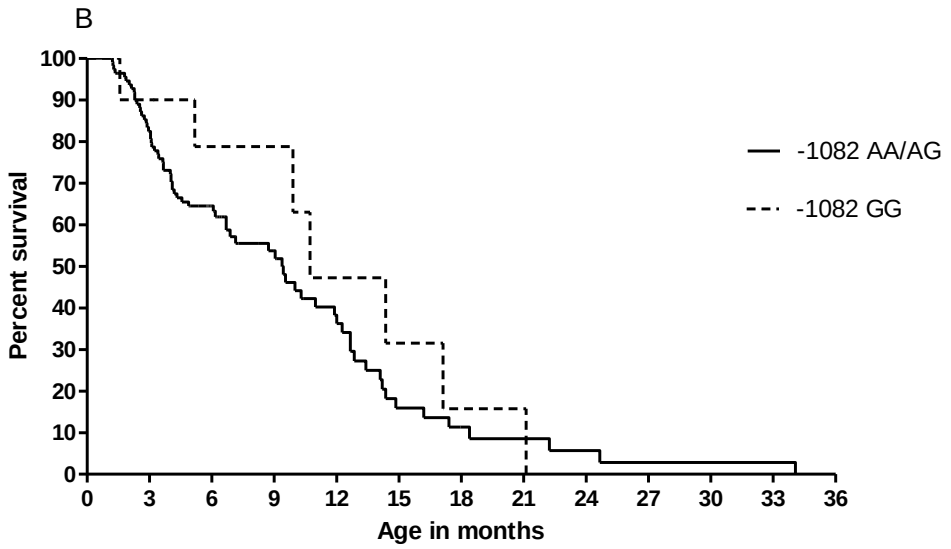


Figure 5.8 Mortality of children according to allelic variant: A) association with -592; B) association with -1082.

We next analyzed the degree of influence of clinical markers on infant mortality within the VTS cohort. The mean log viral load (closest to birth) for those children whom died and those children who did not die was 3.16 (SD=1.56) and 2.50 (SD=1.38) log copies/ml, respectively. The mean absolute CD4 T cell count for those infants who died and those who did not die was 190 (SD=20.30) and 188 (SD=27.37) cells/µl, respectively.

5.3.5 Proportional hazards modelling to determine the effect of IL-10 allelic variants and infant mortality

The first viral load for the infants who died and the infants who surivived, were taken at a median of 2.5 (IQR 1-8) and 2 (IQR 1-8) days after birth, respectively. The initial absolute CD4+ T cell count for deaths and survival were taken at a median of 185 (IQR 183-192) and 190 (IQR 180-195) days after birth, respectively. All infants had a first viral load measurement (n=128), but only 60 babies had a first absolute CD4 T cell count. A trend was observed demonstrating that for every 100 cell/µl absolute CD4+ T cell count increase 6%

children were less likely to die (95%CI). The multivariate analyses, adjusting for both IL-10 promoter polymorphisms as well as first viral load, revealed that there was no significant difference in infant mortality rates between carriers of dominant and recessive alleles for neither IL10 -592 nor -1082, p=0.4279 and p=0.6361 respectively.

5.3.6 Association of clinical markers viral load and absolute CD4+ T cell count on transmission

Several studies have suggested a contribution of IL-10 promoter polymorphisms to HIV-1 susceptibility or disease pathogenesis (Erikstrup et al., 2007, Shin et al., 2000, Shrestha et al., 2010, Vasilescu et al., 2003), we thus went on to investigate HIV-1 transmission in the mothers of the VTS cohort. The association between viral load and absolute CD4+ T cell count were first investigated 50 days prior to delivery in 60 women. Thirty four mothers transmitted HIV-1 to their children and 26 mothers did not transmit HIV-1 to their children. There was no association between viral load or absolute CD4+ T cell count and transmission with p=0.5584 and p=0.1497, respectively, adjusting for delivery.

Following transmission, the transmitter and non-transmitter sub-groups were matched to exclude variability associated with viral load. As expected there was no difference in viral load with transmitter and non-transmitter mums exhibiting median viral loads of 45,000 and 35,500copies/ml respectively (Fig 5.8). Absolute CD4+ T cell count was significantly different between the two groups. Transmitter mums exhibited a median absolute CD4+ T cell count of 354 cells/mm^3 in comparison to non transmitter (422 cells/mm^3) and uninfected mothers whom exhibit a median viral load of 944 cells/mm^3 ($p<0.0001$). No significant difference in viral load is observed between transmitter and non-transmitter variants within IL-10 promoter polymorphisms of both -592 and -1082.

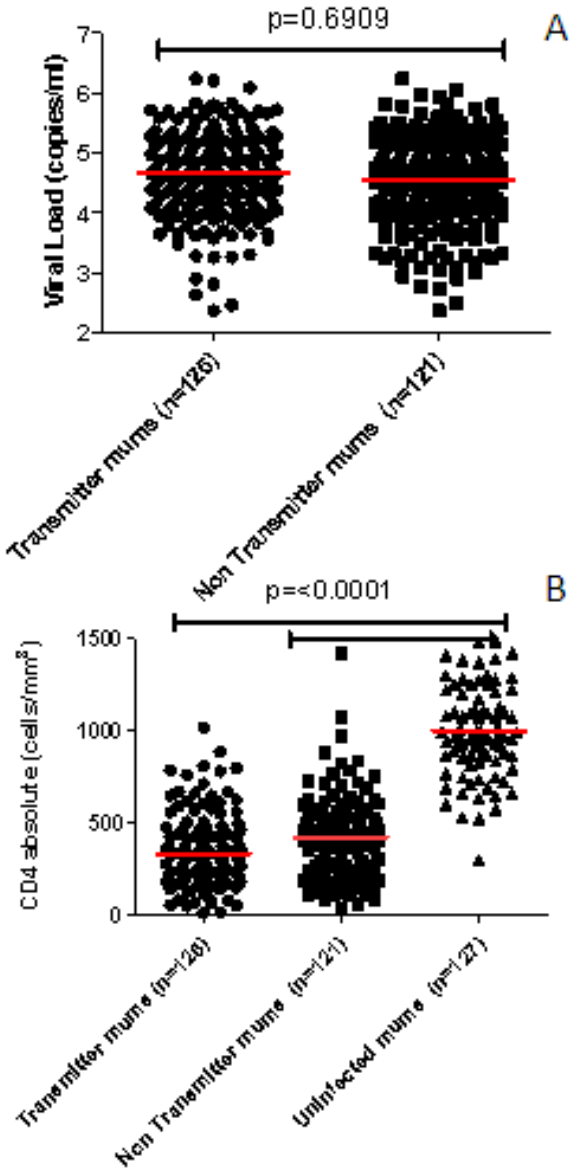


Figure 5.9 Comparison of a) viral load (copies/ml) between transmitter, non transmitter mothers, and b) Absolute CD4+ T cell count (cells/mm^3) between transmitter, non transmitter and uninfected mothers.

5.3.7 Significant association between distribution of IL-10 -1082 genotypic frequency and HIV transmission

To date, no studies have investigated the role of IL-10 promoter polymorphisms in HIV-1 clade C mother-to-child-transmission. Therefore we next investigated the association of IL-10 promoter polymorphisms and HIV transmission by comparing the distribution of allelic

variants of HIV-1 infected mothers who transmitted HIV to their babies, to those who did not transmit HIV to their babies. HIV-1 transmitter mums further exhibited a lower proportion of IL10-1082A in comparison to non-transmitter mums (79.5% vs. 94.5%, p=0.0012) (Table 5.6). This translates into carriers of 1082A having a 77% less odds of transmitting HIV to their infants (odds ratio 0.23 (95% CI 0.09 – 0.58)). There was no association between IL10 -592 and HIV transmission (p=0.4883). Noteworthy, distribution of allelic variations were unaffected by treatment as at the time of sampling all the infants were from a vertical transmission study cohort, where both the mother and children only received a single dose of NVP.

Table 5.6 Distribution of allelic variants between transmitter and non-transmitter mums.

IL-592 N (%)	**Transmitter mums (n=111)**	**Non-transmitter mums (n=108)**	**IL-1082 N (%)**	**Transmitter mums (n=112)**	**Non-transmitter mums (n=109)**
AA or CA	65 (58.6%)	69 (63.9%)	**AA or AG**	89 (79.5%)	103 (94.5%)
CC	46 (51.4%)	39 (36.1%)	**GG**	23 (20.5%)	6 (5.5%)
p-value		0.4883	**p-value**		0.0012

5.3.8 IL-10 promoter polymorphisms and CTL immune response have no effect on HIV-1 transmission

Based on findings from the LCMV model of chronic viral infections that suggested a critical role for IL-10 in the disruption of effector immune responses and viral persistence, we next investigated the effect of IL-10 on cellular responses. Due to retrospective study design we were compelled to investigate this role in another transmission cohort (n=60) (Mphatswe et al., 2007, Thobakgale et al., 2009, Thobakgale et al., 2007). As an overview, the transmitter

and non-transmitter sub-groups were matched to exclude viral load variability with the transmitter and non-transmitter mums exhibiting median viral loads of 99700 and 100000copies/ml respectively (p=0.9657). There was also no significance in absolute CD4+ T cell count. Transmitter mums exhibit a median absolute CD4+ T cell count of 299cells/mm^3 in comparison to non transmitter whom exhibit a median viral load of 388cells/mm^3 (p=0.1733). We found no significance in magnitude of immune response by looking at allelic variants previously found to be most associated with high IL-10 production and low IL-10 production in non-transmitter and transmitter mothers (Fig 5.9) for both IL10-592 (p=0.4281) and IL10-1082 (p=0.6132).

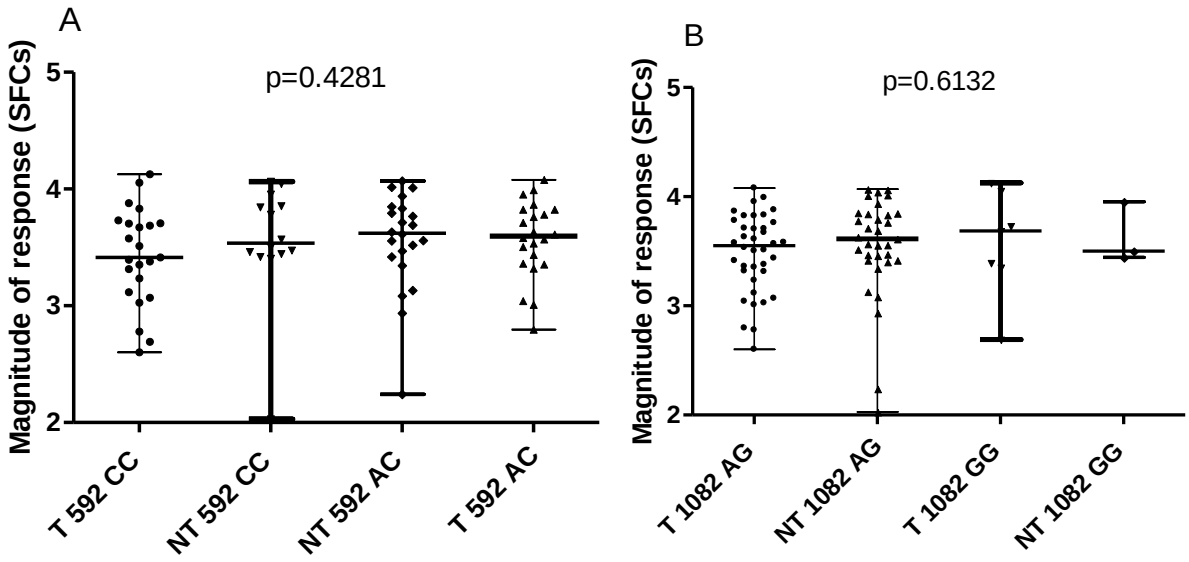


Figure 5.10 Association of magnitude of IFN-γ CD8+ T cell responses and IL-10 allelic variants A) 592 and B) 1082 between transmitters and non-transmitters.

5.3.9 Significant effect of IL-10 promoter polymorphisms on immune response and cytokine production in children

We subsequently went on to study the association of IL10 promoter polymorphisms with immune responses in a subset of the children of the PS cohort. Interestingly, a significant association was observed between magnitude of IFN-γ CD8+ T cell immune immune response within the HIV-1 infected children subgroup between children whom have the wildtype in comparison to children whom are mutant carriers IL10 -592 AA/AC (p=0.0071). The carriers of the mutant allele IL10 -592A was associated with a significantly lower magnitude of IFN-γ CD8+ T cell in comparison to the carriers of IL10 -592C in children. However as demonstrated in Fig 5.10 there was no significance for IL10 -1082 (p=0.3455).

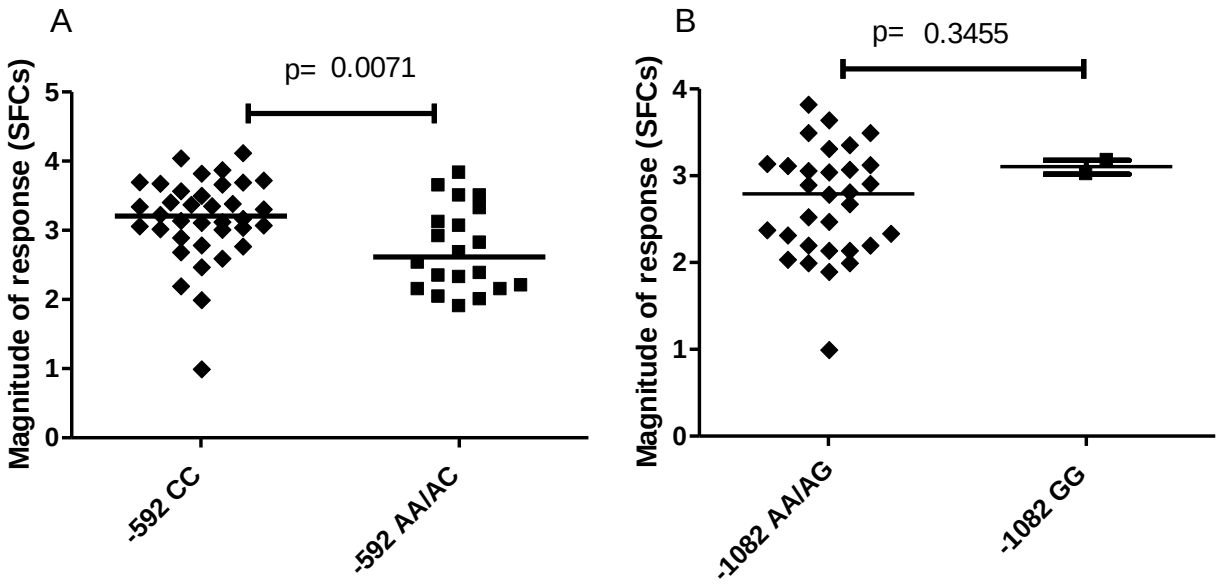


Figure 5.11 Association of magnitude of IFN-γ CD8+ T cell responses and IL-10 allelic variants A) 592 and B) 1082 in infected children.

Elevated IL-10 plasma levels have previously been documented to correlate with poor prognosis in many infections (Roberts et al., 2010, Lech-Maranda et al., 2006, Stanilova et al., 2005, Visco et al., 2004) including HIV (Norris et al., 2006, Brockman et al., 2009), based on these findings we next investigated IL10 cytokine levels in children.

Twenty seven of the HIV-1 clade C children from the PS cohort (Mphatswe et al., 2007, Thobakgale et al., 2009, Thobakgale et al., 2007) are investigated within the cytokine investigation. We observed the allelic variants to be distributed as 17.2% AA, 48.3% AC and 34.5% CC, while IL10-1082 was distributed as 55.2% (AA) and 44.8% (13) AG. We found no associations between allelic variants and levels of IL-10 (p=0.7932) and IFN-γ (p=0.6834) cytokines in-592 (Fig 5.11).

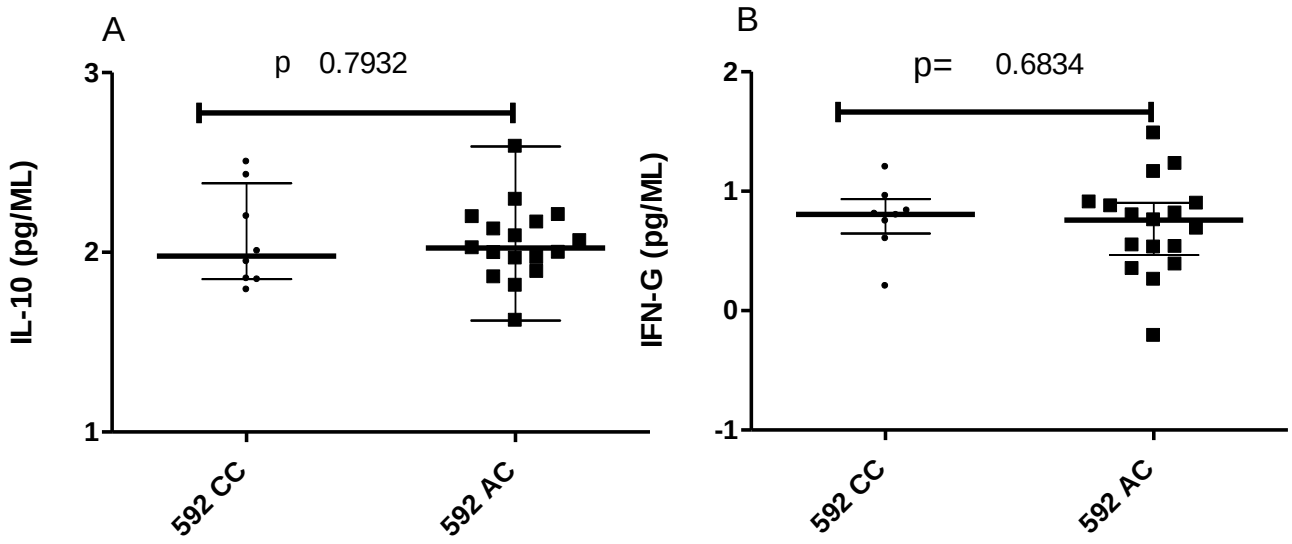


Figure 5.12 Comparison of plasma IL-10 and IFN-γ cytokine levels with allelic variants A) IL-10 -592 and B) -1082 infected children.

5.3.10 Correlation of clinical markers of disease progression with cytokine expression levels in children

Based on previous findings the allelic variants -1082A and -592A have been documented to be associated with decreased IL-10 production have thus associated with an increased susceptibility to HIV. A linear regression model was fitted to IL10 expression data in order to determine possible associations. In spite of the log viral load being significantly associated with IL10 expression in a univariate analysis (p=0.0113), when children were included age

seems to be a confounder. However, there was a significant negative association between viral load and age (p=0.0050), thus as age increases per month, viral load decreases at a rate of 0.02940 log copies/ml (SE=0.00962). At the same time age was also associated with IL10 expression (p=0.0014) with IL10 expression levels decreasing at a rate of 0.01028 log units per month increase of age. The younger aged children were associated with higher viral load, and lower age were associated with higher IL10 expression, thus this relationship makes it seem as if the higher viral load was associated with higher IL10 expression. Adjusting for age, using a multiple linear regression models, excluded viral load association to conclude that age was a confounder.

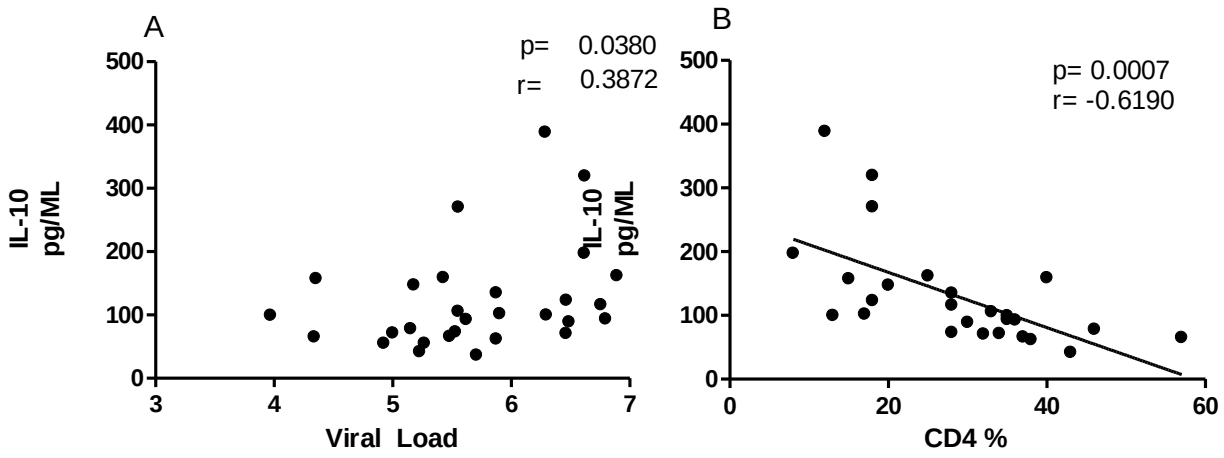


Figure 5.13 Correlation of IL-10 cytokine levels (pg/ML) with A) Viral Load B) CD4 T cell %.

Correlation of viral loads and cytokine levels revealed positive correlations for IL-10 (p=0.0380) and TNF-α (0.0065). As per Fig 5.12 correlation of absolute CD4 T cell % and cytokine levels revealed positive correlations for IL-10(0.0007); TNF-α (p=0.0188); IL-5 (p=0.0227); IP-10 (p=0.045) and MCP-1 (p=0.0482).All other cytokines showed no significant correlations.

5.3.11 Comparison of IL-10 cytokine levels within age matched babies

Grouping the babies by age reveals no significant changes in median levels of the IL-10 cytokine with age (Fig 5.13). An increase in viral load was inversely with absolute T cell count for children aged 1 to 3months of age. However a gradual stabilization in viral load coupled with an elevation in absolute CD4+ T cell count was observed 4 months of age and upwards.

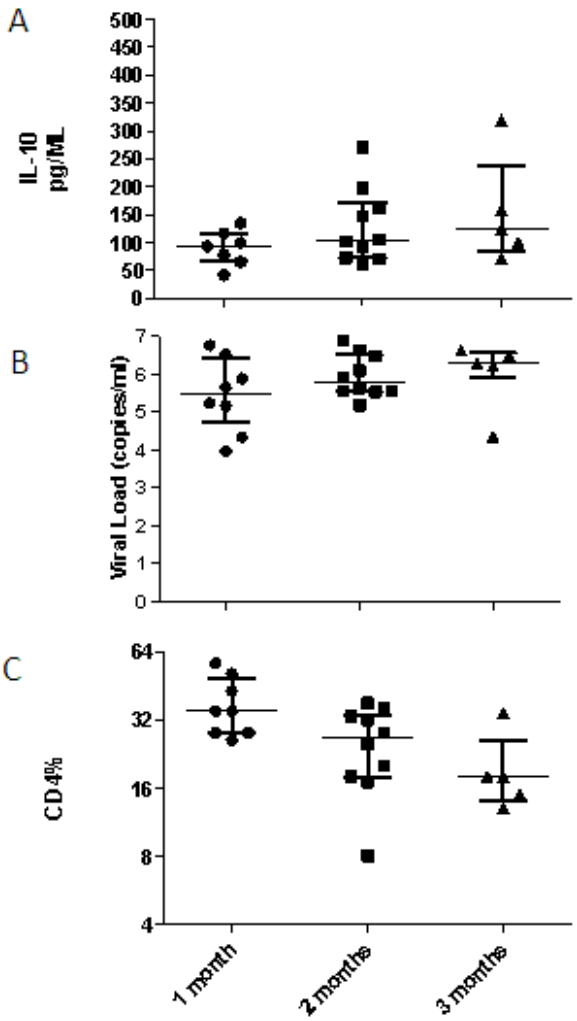


Figure 5.14 Stratification of: A) IL10 cytokine levels; B) Viral load and C) CD4 T cell % by age of children.

5.3.12 Comparison of cytokine levels cross sectionally by function and longitudinally over time

Expression levels are further investigated. Median levels of chemokines and cytokines were grouped by function. Pro-inflammatory cytokines are classified as IL-1β, IL-5, IL-6, TNF-α, MCP-1 and IL-12 (p70).

Growth factors are classified as EGF, TGF-A and VEGF; while haematopoietic classified as IL-7, GM-CSF and G-CSF. The anti-inflammatory function was only represented by cytokine IL-10. Chemokines are represented by IL-8, IP-10, MIP-1B, Eotaxin and Fractalkine. Adaptive function are classified as SD-40L, IL-2, IL-13, IL-4, SD, IL-17 and IFN-γ. As some cytokines are ubiquitously expressed without negative controls results proved difficult to interpret. It appears that chemokines are expressed at higher levels in comparison to other groups of cytokines within first month of life, which may or may not be related to age. Proinflammatory and adaptive function seems to be limited in comparison to the anti inflammatory cytokine IL-10 (Fig 5.14A).

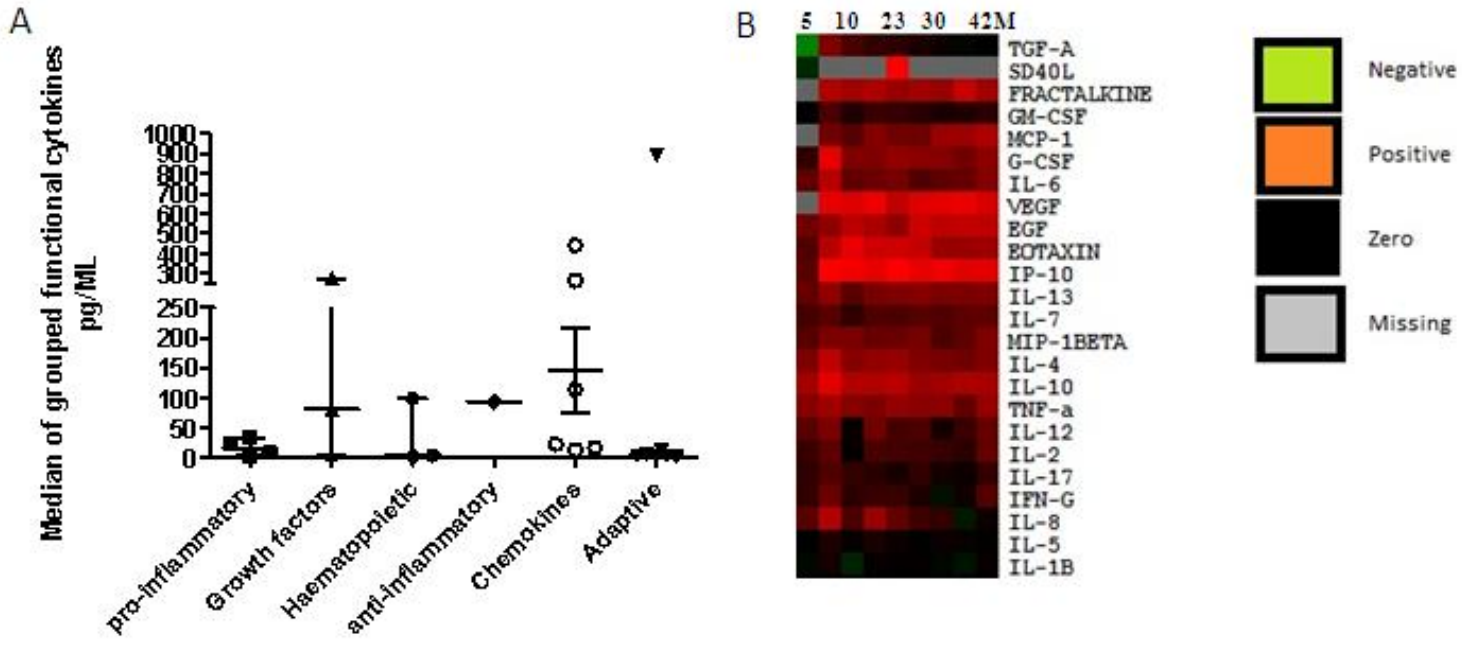


Figure 5.15 Expression levels of Cytokines/chemokines (pg/ML) A) Cross sectionally by function in children less than a month of age and B) longitudinally over time.

The study went on to investigate the individual cytokine and chemokine levels of expression over time from five months to forty two months of age. Chemokines (Eotaxin and IP-10) and

growth factors (EGF and VEGF) are expressed at highest levels throughout all 42 months of life. Anti-inflammatory cytokine IL-10 was expressed at increased levels to TNF-α. Interestingly chemokine IL-8 expression level decreased to undetectable levels over time. Expression levels of IL-7 and IL-13 remained stable over time. The study further investigated pro-inflammatory and anti-inflammatory function in one transmission pair over time. The mother and child are treatment naive and exhibited CD4 T cell % of 29-40% with a stable viral load. The child has a higher overall expression levels in comparison to the mother over time. The anti-inflammatory cytokine was expressed at a higher levels compared to pro-inflammatory cytokines in the child compared to the mother.

Within this cohort the children aged 1month or younger express lower IL-10 cytokine levels and exhibit lower viral loads that increased and then to be maintained at high levels with time. The children aged 1 month or younger exhibit a higher initial CD4+ T cell % which were found to decrease with age. The CD4+ T cell % seemed to stabilize after 3 months of age within this cohort.

5.4 DISCUSSION

The multifunctional role of IL-10 has sparked interest in understanding the mechanisms that underlie the function of IL-10 in signal transduction (Sarkar et al., 2011, Begue et al., 2011), cellular immune response (Tsilidis et al., 2009, Wang et al., 2009, Bialecka et al., 2008, Lazarus et al., 2006, Brooks et al., 2008), cellular activation (Hedrich et al., 2010, Sarkar et al., 2008, Hoiden and Moller, 1996), epigenetics (Saraiva et al., 2005, Chang et al., 2007) and cytokine production (Maurer et al., 2000, Hoiden and Moller, 1996, Mosmann, 1991). IL-10 has been shown to play an important role in the immune dysfunction and viral persistence in chronic viral infections (Brooks et al., 2008, Brooks et al., 2006). Immune dysfunction

associated with IL-10 appears to be reversible with IL-10 receptor blockade to restoring robust IFN-γ CD8+ T cell immune responses, promoting viral clearance and enhancing vaccine induced immune responses.

A study in an adult African cohort found that the carriers of alleles associated with high IL10 production in promoter polymorphisms were less likely to acquire HIV-1, however, this mechanism was reversed as the virus progressed suggesting a paradoxical role for IL-10 promoter polymorphisms in different stages of HIV-1 infection in adults (Eskdale et al., 1998, Naicker et al., 2009, Shrestha et al., 2010). This impelled us to investigate the role of IL-10 polymorphisms in HIV-1 mother-child transmission. Studies describing the role of IL-10 and its genetic variants on mother-child transmission and paediatric HIV-1 disease progression are limited. This study investigated association between two IL-10 promoter polymorphisms and mother to child HIV transmission and clinical outcomes. Our study explored some possible mechanisms underlying the role of IL-10 promoter variants on HIV pathogenesis. Furthermore, we assessed the association of cytokine levels with biomarkers of disease progression such as absolute CD4+ T cell count and viral load.

Absolute CD4+ T cell count is important clinical marker for disease progression in adults while the CD4+ T cell % is a more accurate indicator in children. As expected uninfected mothers and children exhibited higher absolute CD4+ T cell counts. Interestingly, in confirmation with other studies, we observed a significantly higher positive correlation of viral load with IL-10 levels, and an inversely a significant negative correlation with CD4+ T cell % in this particular cohort of children (Orsilles et al., 2006, Srikanth et al., 2000, Bebell et al., 2008, Imami et al., 1999). These results further confirm the strong role of IL-10 as a marker of disease progression. Despite the immature immune system and highly variable viral burden in these young children, IL-10 is able to predict disease as well the clinical

marker viral load and the marker CD4+ T cell %, which is used to dictate treatment guidelines in children.

Our study was the first to investigate IL-10 association in mother to child acquisition and transmission. We found no association between any allelic variants and HIV-1 acquisition in children. As the route of transmission and acquisition in children differ from that of adults, variations in findings were anticipated. Our results contradict studies in adults that found carriers of the IL10-592A to be more likely to acquire HIV-1 infection. Hence the allelic variants associated with low IL-10 production was significantly more likely to become HIV-1 infected. These results further contrast studies in adult South African and North American cohorts that found the carriers of the -592A allelic variant, linked to low IL-10 production, to be at a higher risk of HIV-acquisition (Naicker et al., 2009, Shin et al., 2000).

When Cox proportional hazards model was used to assess mortality or time to death there was no association between allelic variants and mortality in children which has been observed in studies in other infections (Liu et al., 2011). However, these results contradict observations in adults that linked -592A to increased risk of mortality (Erikstrup et al., 2007, Huebinger et al., 2010). When investigating time to death our study found 58% of participants to be carriers of the -1082G. The children whom carried the -1082G, an allele linked to increased IL10 production, were more likely to die within first 21months of life, in comparison to children with other allelic variants who survived as long as 12 additional months. These results are in contrast to other observations in adults which found carriers of -1082G to be associated with protection (Erikstrup et al., 2007). Survival rate in many carriers were approximately doubled (Erikstrup et al., 2007). The multivariate analyses, when

adjusted for both viral load and promoter polymorphism, showed no difference in clinical outcome irrespective of the allele being carried.

We next investigated the role of IL-10 promoter polymorphisms on HIV-1 mother to child transmission. This study is the first of its kind as IL-10 polymorphisms have not previously been documented within mother to child transmission. Many studies have indirectly implicated IL-10 expression levels, not allelic variants, in vertical transmission. It must be noted, that within this sub-study as the time of transmission was unknown mothers were matched by their viral loads and age of mothers. This may bias results if viral load is a confounding factor.

A study investigated IL-10 secretion levels in pregnant woman and found pregnant woman to express higher levels of IL-10 to help control HIV-1 replication and possibly reduce risk of transmission (Bento et al., 2009). Our study found significant differences in allelic frequency with the carriers of the IL10-1082G, the HIV-1 infected mothers who transmitted HIV to their babies (20.5%) in comparison to those mothers who did not transmit HIV to their babies (6%) in relation to IL10 promoter polymorphisms. There was no association between IL10 -592 and HIV transmission (p=0.6255).The non-transmitter mums were matched to control viral load for uniformity in our acquisition investigation of their children, but this may also bias the effect viral load has on the distribution of promoter polymorphisms during different stages of HIV-1 infection. Naicker et al. suggest a paradoxical role for IL-10 promoter polymorphisms on HIV-1 pathogenesis (Naicker et al., 2009). The study documented that IL-10 polymorphisms associated with higher IL-10 production were also associated with high viral load during the acute infection phase of HIV-1 infection. However, with the onset of

HIV-1 infection as the virus progressed towards the chronic stage of infection the association between viral load and allelic variants either disappeared or reversed (Naicker et al., 2009).

The LCMV model showed that IL-10 played an influential role in inhibiting adaptive T cell immune responses (Brooks et al., 2008, Ejrnaes et al., 2006). T cells have been shown to play an important role in viral control hence the lack of strong robust immune responses aggravate increased viral persistence (Brooks et al., 2006, Ejrnaes et al., 2006, Kiepiela et al., 2004, Kiepiela et al., 2007). In a vivo LCMV IL-10 blockade of the IL-10 receptor resulted in an enhancement of T cell immune response in persistently infected mice (Ejrnaes et al., 2006). Interestingly Brooks et al., document IL-10 to not just play a significant role in viral control but in complete viral clearance in a LCMV model after the genetic removal of IL-10. This removal of IL-10 enabled robust effector T cell response and subsequent elimination of virus (Brooks et al., 2008). Due to our study design we were not able to conduct these types of blockade experiments (Brockman et al., 2009). A deficit in IL-10 at birth translated into immune dysregulation has also been found to be associated with leukemia in children (Chang et al., 2011). However, these studies are currently underway in another cohort.

Polymorphisms in cytokine genes such as the anti-inflammatory cytokine IL-10 have further been implicated in clinical outcome perhaps through modulation of host adaptive response in humans via influence on cytokine levels (Vollmer-Conna et al., 2004). A study investigating woman with sexually transmitted disease detected higher levels of IL-10 in non-ulcerative STD and bacterial vaginosis in endocervical secretions suggested that these infections may alter susceptibility to HIV-1 infection in women (Cohen et al., 1999). When investigating the allelic variants of IL10 promoter polymorphisms the -592 A allelic variant is associated with lower IL-10 production which can be associated with strong anti-inflammatory function and

the presence of robust adaptive immune responses (van Exel et al., 2002, Naicker et al., 2009). Furthermore, previous studies have documented that carriers of the IL10-1082G allele to be linked to higher IL-10 production and hence a suppression of T cell immune responses (Naicker et al., 2009, Shin et al., 2000, Yanamandra et al., 2005).Our investigation confirmed that the carriers of -1082 G was associated with higher IL-10 production in comparison to the carriers of IL10 -1082A. A study of acute Hepatitis B and C infections showed that expressing an enormous cascade of cytokines lead to eventual viral clearance in a majority of infected individuals (Stacey et al., 2009).

We also investigated a broad array of cytokines and observed levels correlated positively with IFN-γ levels which represent immune responses. Proinflammatory and adaptive function seems to be less in comparison to the anti inflammatory cytokine IL-10 in children within this cohort. The carriers IL10-592A, which is generally associated low IL-10 production in adults, however who showed no association in our children cohort, was significantly associated with a lower magnitude of response. It has been documented that multiple cell types contribute to IL-10–mediated immune suppression in the presence of uncontrolled HIV viremia. The proliferative capacity of HIV-specific CD4 and CD8 T cells in individuals with ongoing viral replication differ which could attributed to why allelic polymorphisms did not correlate with plasma IL-10 levels (Brockman et al., 2009). Moreover, as the success of ART influences viral replication and thus cell proliferation it may also affect cytokine levels. Notably, due to the controversial role of IL-10 in maintenance of immune responses and the contradiction between our data in children in comparison to findings in adults, an understanding of the underlying mechanisms that regulate immune control is crucial.

This study did pose limitations worth highlighting. First, the sample size was relatively small for a genetic association study. The mother and children also received a single dose of NVP which could modify exposure outcomes. Furthermore, we matched viral load of transmitter and non-transmitter participants to allow for similar transmission setting, which could indirectly the effect of other factors that are driven by viral load that may influence IL-10. Moreover, in our investigation of cytokine levels and their association with IL-10 allelic variants and chronological measurements, we lacked a comparative uninfected control group which limits our data interpretation. Finally the cohort for the cytokine analysis was not a homogenous population due to varying age and hospital visits. Most importantly, we were unable to compare cytokine observations to a cohort of uninfected children to validate findings.

In conclusion the association of IL-10 polymorphisms vary in HIV-1 infection, and as we have described with age. We suggest that the mechanisms underlying the function of IL-10 may vary between adults and children. As our MTCT study was the first of its kind and our cohort represented a very rare untreated children's cohort. . A meta-analyses of all reported IL-10 promoter polymorphism studies within HIV-1 infection, as recently executed in cancer research (Zhu et al., 2011), will prove beneficial for a better understanding of the mechanism driving IL-10 regulation.

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CHAPTER SIX

6.1 GENERAL DISCUSSION

At the onset of the current millennium the international community established eight Millennium Development Goals (MDGs) to be achieved by 2015. The two goals at the heart of the memorandum was MDG 4, which seeks to reduce child mortality by 75% and MDG 5, which aims to improve maternal health resulting in reducing infant mortality (UNICEF, 2011). Many countries have made progress to reduce maternal, perinatal mortality and the mortality of children less than 5 years of age. However, it seems unlikely that South Africa would be able to adhere to this timeline. In particular, according to Stats SA (2011) and KwaZulu-Natal's (KZN) annual performance plan (http://www.kznhealth.gov.za/app2011-14.pdf), the province of KZN has reported high infant (55,8/1000 live births), child (87,5/1000) and maternal (210/100,000 live births) mortality rates. Furthermore, the government member of the executive council (MEC) of health has remarked on the damage the AIDS epidemic has inflicted on disease burden with unacceptable high morbidity and mortality rates in the province of KZN. (http://www.kznhealth.gov.za/app2011-14.pdf). The past decades of HIV research has brought substantial advances, but unfortunately mothers and children still remain vulnerable to the world's leading infectious health crisis

The paediatric population is vulnerable to HIV owing to the immaturity of their immune system. In the absence of antiretroviral therapy, HIV-1 infected children die within the first two years of life (Bobat et al., 1999, Bobat et al., 1990, Goulder et al., 2001b, Newell et al., 2004, Ruiz Contreras, 1998, Prendergast et al., 2007, Mphatswe et al., 2007). A better understanding of immune control in slow progressor survivors naturally containing viral load, may direct us to the underlying determinants of protective immunity. The broad scientific goal of our study was to explore HIV pathogenesis in genetically matched transmission pairs

and/or children, in order to further contribute to understanding of the correlates of successful immune control in paediatric HIV-1 infection. We carried out this investigation by comprehensively dissecting HIV-1 immunopathogenesis into the following investigational components: the transmitted virus, the cellular immune response to the virus, the functionality of elicited T cells, regulation of T cells, and finally host genetic determinants by investigating IL-10 gene variants within a mother-child transmission setting. We observed that CD8+ T cell responses and viral fitness did not explain differences in disease progression in the investigated transmission pairs within this cohort; activation and regulatory markers were shown to influence immune response or vice-versa. Our data showed a significant association between IL10 -1082 and HIV transmission and an association of high viral replication with increased IL-10 plasma levels which may contribute to immune dysfunction in paediatric HIV infection.

These findings are discussed in detail in the context of existing literature in the sections to below.

6.2 CD8+ T CELL RESPONSES, HLA AND VIRUS REPLICATIVE CAPACITY

Following the inability of the innate system to protect the host against some invaders, the host has evolved to include a second defense system. The adaptive arm of immunity includes dendritic cells, antibodies, CTLs, and T helper cells. The effect of both CD4+ and CD8+ T cell responses on disease progression in paediatric HIV infection has proven controversial (Feeney et al., 2003, Goulder et al., 2001b, Huang et al., 2008, Prendergast et al., 2011a, Salimi et al., 2000, Thobakgale et al., 2007, Lohman et al., 2005). Our study investigated CD8+ T cell antigen specificity using an IFN-γ ELISpot screening assay. Many children did not respond when we screened responses using peptide pooling methodology (Addo et al.,

2003, Kiepiela et al., 2004, Kiepiela et al., 2007, Luzuriaga et al., 1991). The inability to control viremia may be attributed to the lack, delay or ineffectiveness of T cell immune responses (Margolick et al., 1995). Subsequently, we stratified responses from our pooled peptide assay (cut off 100 SFCs) to discover that the non-responder children were ideally making low level responses. The viral regions targeted by these children in order of frequency of response were Nef (49%), Gag (17%), Env (14%) and Acc/Reg/Pol (6%). Children differ in that their immune system is less developed and their T cell responses may not be as detectable or effective in viral containment as observed in adults (Luzuriaga et al., 1991). These findings may be biased as we were only able to assess a single cytokine (IFN-γ) secreted by CD8+ and possibly CD4+ T cells as ELISpot on whole PBMC specimen is unable to differentiate CD4 from CD8 responses or less likely NK cell responses. Assays such as intracellular cytokine staining and tetramer staining could have differentiated between these possibilities but this was not possible in this study due to sample limitations. However, the ELISpot assay was optimized for CD8 rather than CD4 T cell responses (24h vs 48h incubation) and based on previous data from cohort, the majority of responses observed by ELISpot were CD8+. Furthermore, the study was limited in that the investigation was carried out cross-sectionally and hence the long term benefits of T cell specificity could not be assessed.

Our responder children confirmed findings of previous studies showing the viral region Nef to be dominantly targeted in children 3 months to 10 years of age (Thobakgale et al., 2007, Huang et al., 2008) while acutely infected children predominantly target Env (Thobakgale et al., 2007). In adults, Env-specific responses have been associated with higher viremia. Following vertical transmission, the viral loads in children do not decline and do not reach viral set point until a few years after HIV infection (Goulder et al., 2001b) unlike primary

infection in adults. The viral kinetics following infection is different to what is noted in adults (Holland et al., 2000, Resino et al., 2002b, Resino et al., 2002a); most children typically progress to AIDS or death within the first 2 years of life in the absence of antiretroviral treatment (Prendergast, 2007). We found no difference in viral load (Huang et al., 2008, Lohman et al., 2005). The functional assessment of T cell polyfunctionality suggested that it may be the quality of CD8+ T cell response rather than the quantity of responses that influences clinical outcome (Betts et al., 2006, Thobakgale et al., 2011).

We were unable to directly assess the effect of CD4+T helper cells as previously described in children (Huang et al., 2008, Thobakgale et al., 2007, Wasik et al., 2000). CD4+ T cells have also been shown to play a role in suppression of functional capacity in young children (Kolte et al., 2011) and young nonhuman primates (Hartigan-O'Connor et al., 2007). CD4+ Tregs, mediated by the CTLA-4 pathway have been found to play a role in CD8+ T cell suppression *in vivo* (Sakaguchi et al., 2009). Furthermore, a simultaneous blockade of TIM-3 and PD-1 pathways has been documented to have a synergistic effect in restoring T cell antiviral immunity (Jin et al., 2010).Interestingly in mice, blockade of the IL-10 pathway further stimulate T cell responses and restored IFN-γ secretion, resulting in eventual viral clearance. (Brooks et al., 2006). We were unable to test functional capabilities using blockade experiments. However taken together, regulating pathways, paediatric age, the peptides screened in relation to consensus sequence, cytokine used to measure CD8+ T cell responses contribute to our understanding of the mechanisms underlying T cell immunity in children.

Host and maternal factors are known to influence disease progression (Andiman, 1999, Bloland et al., 1995, Colognesi et al., 1997, Kuhn et al., 2004, Newell et al., 2004, Palomba et al., 1999). Our assessment of viral burden following vertical transmission revealed that

transmitter mothers had higher viral loads compared to non-transmitter mothers. Based on other studies that showed that HIV-1 infected adults (Goepfert et al., 2008, Matthews et al., 2009) and children (Feeney et al., 2005, Thobakgale et al., 2009) may benefit from exhibiting HLA-B restricted Gag-specific responses and from receiving transmitted escape mutations- we next investigated the interplay between HLA, CD8+ T cell and virus in MTCT transmission pairs. As HLA-B*27 (Goulder et al., 2001a, Schneidewind et al., 2007) and HLA-B*57 (Altfeld et al., 2003, Brockman et al., 2007, Crawford et al., 2009, Goulder et al., 2000, Leslie et al., 2004, Miura et al., 2009b, Feeney et al., 2005, Thobakgale et al., 2009) restricted Gag-specific responses have been documented to play a protective role in slow disease progression and within transmission pairs (Feeney et al., 2005, Thobakgale et al., 2009, Goepfert et al., 2008), we focused on these HLA alleles. We did not find any association between Gag-specific CD8+ T cell response and slow progression in the selected children in our cohort in line with other studies in children (Froebel et al., 1997, Huang et al., 2008). An alternative explanation for our findings could be sample size limitations as we examined a small number on children.

Studies have also associated HIV-1 fitness with the rate of transmission and disease progression (Biesinger and Kimata, 2008, Chopera et al., 2008, Duda et al., 2009, Miura et al., 2009a, Miura et al., 2009c, Plaeger-Marshall et al., 1994, Wright et al., 2010, Wright et al., 2011). We found no association with the absence of Gag responses and sequence variations in Gag. Even though many infants did respond to other viral regions, we were unable to sequence other regions of the virus due to funding restrictions. Furthermore, we were unable to associate CTL escape mutations with roles in attenuating the fitness of the virus, contrasting another study that demonstrated that replicative capacities differ in slow and rapid progressor children (Prado et al., 2010). Furthermore, the precise timing, occurrence and

location of escape mutations in the viral genome indicates the efficacy of certain CTL specificities and suggest a cost to the virus that may be important in vaccine design (Goulder and Watkins, 2004).

We were limited by the fact that we could not follow up all these transmission pairs in more detail over from birth. For example, the timing or the mode of transmission could not be determined in our study, contrasting to previous studies that demonstrated that in utero infected children rapidly progress to disease (Mphatswe et al., 2007). Details of other co-infections were also unknown. As the study was run through clinic sites many children were recruited as they were visiting sites due to symptoms related to ill-health. Genetically, we may have overlooked missing genetic links associated with the genetic environment of the father, as CD8+ T cell responses may be compromised both by the transmission of maternal escape variants and by MTCT of escape variants that originally arose in the father (Pillay et al., 2005). All in all, we did not find any association between HLA, CTL responses, transmission of escape variants with overall viral fitness in the selected transmission pairs. This may be related to study design or show that HLA and CTL data alone is not sufficient in determining what mediates control.

6.3 MARKERS OF EXHAUSTION: TIM-3 AND PD-1

HIV-1 pathogenesis is characterized by the rapid loss of CD4+ T cells leading to generalized immune dysfunction, including an exhausted CD8+ T cell phenotype. As previously mentioned- it may be the quality and not the quantity of response that influences the effectiveness of T cell immunity. An understanding of the underlying factors that drive the functional quality and effective antiviral T cell responses would inevitably improve therapeutic strategies to contain and possibly clear infection.

We investigated progressive cell exhaustion of TIM-3, in concert with PD-1. A synergistic effect of PD-1 and TIM-3 blockade has been found to revere T cell exhaustion. Based on the panel restrictions we were not able to correlate co-expression on identical cell. We found that these exhaustion markers were expressed at significantly higher levels in HIV-1 infected children in comparison to uninfected children in keeping with adult studies (Jones et al., 2008, Jin et al., Fourcade et al., Day et al., 2006). Our investigation revealed that PD-1 expression on CD8+ T cells was higher in the HIV-infected children in comparison to the HIV-uninfected children in keeping with recently described reports (Prendergast et al., 2011c). We documented elevated expression levels on CD8+ T cells in comparison to CD4+ T cells for both TIM-3 and PD-1 inhibitory molecules. Our findings demonstrate no association between TIM-3 and PD-1 expression markers with markers of disease progression in contradiction to adult HIV-1 studies (Jones et al., 2008, Day et al., 2006). Our study demonstrated a positive association between TIM-3 and PD-1 in an HIV-1 paediatric disease setting. PD-1 seemed to be a marked negative regulator of activated T cells in children (Prendergast et al., 2011c). Longitudinal investigation revealed that TIM-3 and PD-1 expression levels on T cells were maintained at high levels and tracked with viral load.

Taken together, our findings may explain why younger newly infected children are unable to control HIV infection and show low magnitude and breadth suggesting limited HIV-1 specific T cell immunity. Data on co-regulation of PD-1 and TIM-3 in a LCMV model showed that blocking of these pathways restored immune responses, suggesting that the immune system is able to mount a vigorous attack against foreign infections. Furthermore, a simultaneous blockade of TIM-3 and PD-1 pathways has been documented to have a synergistic effect in restoring antiviral immunity and viral control compared to the blockade of either pathway alone (Jin et al., 2010). Reversal of T cell dysfunction may prove valuable

in therapeutic vaccine development. It is noteworthy, Phase I clinical trials are currently being carried out to evaluate the efficacy of the use of PD-1 blockade therapeutically in oncology and infectious disease settings (Brahmer et al., 2010, Berger et al., 2008, Sakuishi et al., 2011).

Our study did pose limitations worth highlighting. We were limited by the relatively small sample size for a phenotypic study and flow panel constraints that restricted our investigation of co-expression of PD-1 and TIM-3 on a single T cell. We were further limited in that we were unable to describe these phenotypes in relation to T cell functionality. Nevertheless, to date no studies have been performed to investigate the role of TIM-3 in HIV-1 infected children, our data is the first undertake this investigations in clade C HIV-1 infection. It remains imperative not to extrapolate but rather to investigate the mechanisms underlying paediatric infection.

6.4 REGULATION AND ACTIVATION

Immune exhaustion seems to occur concomitantly with immune activation and decrease in regulatory T cells in viremic chronically HIV-1-infected adults (Sachdeva et al., 2010). Adults and children differ in disease progression denoting a variation in clinical outcome (Holland et al., 2000, Resino et al., 2002b, Resino et al., 2002a). In general, it is presumed that young children respond ineffectively due to immature or defective cell functionality associated with high viral burden (Ashwood et al., 2011, Ly et al., 2009, Ochieng et al., 2006). To date, no studies have been performed to investigate the role of Tregs and immune activation in children in the context of HIV-1 clade C infection. Our study was the first to investigate the mechanism underlying Treg suppression on generalized immune activation and immune response in this African cohort of HIV-1 clade C infected children.

Persistent non-specific, generalized immune activation has been implicated as a major driver of both susceptibility to HIV and HIV disease progression (Paiardini et al., 2009, Hazenberg et al., 2003, Giorgi et al., 1999, Liu et al., 1998, Liu et al., 1997). Tregs have been documented to play a role in activation of CD4+ and CD8+ cells during different stages of HIV-1 infection (Eggena et al., 2005, Prendergast et al., 2011b). Clade C infection has been reported to have one of the fastest rates of disease progression (Cohen et al., 2011, Spira et al., 2003). Our study showed that Treg frequency and activation expression profiles to differ between uninfected children and treated clade C infected children. We observed a significant inverse correlation of Tregs and CD4+ T cell%. Interestingly, in a group of participants tested for immune responses we observed a significant inverse correlation between immune regulatory and activation on CD8+ T cells.

Immune exhaustion seems to occur concomitantly with immune activation (Sachdeva et al., 2010)- we thus next investigated their role in paediatric HIV-1 infection. Our study investigated the frequency of Tregs using CD4+CD127-CD25+ and T cell activation using the markers HLA-DR+CD38+. It is noteworthy, that the selection of these markers is controversial and differs from study to study. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ Treg cell (Liu et al., 2006). The phenotype CD3+CD4+CD25+CD127- has been used to successfully define and sort functional regulatory T cells in previous studies. We and others have shown a strong positive correlation between CD4+CD25+CD127low and CD4+CD25+FoxP3+ T cells. We have data on FoxP3 expression by intracellular staining for a subsets of the study participants, however we encountered technical difficulties which prohibited having this data available for the entire study cohort. More commonly, Treg cells are defined based on the frequency of CD4+CD25+ and the transcription factor FoxP3+ however; alternate phenotypes such as

CD4+CD25+CD127- have also been documented for use in Treg discrimination and isolation of functional Tregs for suppression assays .

In contrast to adults (Sachdeva et al., 2010), we found no correlation between Tregs or activation markers and markers of disease progression such as absolute CD4+ T cell count and viral load. However, this may be attributed to the unstable high persistent viral loads children exhibit. The expression of HLA-DR+CD38+ was found to be significantly higher on untreated compared to treated and uninfected children on CD8+ T cells and similarly on untreated compared to treated and uninfected children on CD4+ T cells. The markers of activation on T cells were also found to be significantly higher in the children aged less than 6 months in comparison to older children, probably further related to the higher viral load observed on infants following transmission (Richardson et al., 2003).

Interestingly when we investigated the involvement of immune response we observed a significant negative correlation between magnitude of CTL response and both Treg and activation markers, in keeping with other studies (Prendergast et al., 2011c, Day et al., 2006). In addition to our phenotypic studies were able to expand functional Tregs from HIV-1 infected children, confirming the presence of functional Tregs, which could further explain CD8+ T cell suppression in children. As previously discussed, we were limited by sample size. In addition time and mode of transmission was unknown and we were thus unable to describe these subset frequencies in the context of MTCT. A larger scaled longitudinal investigation was not possible due to participants defaulting and retention of study participants. We were unable to test the effects of other cytokines apart from IFN-γ. Paediatric samples are limited and thus we were unable to run all experiments using fresh samples. However, a preliminary test indicated minor differences between samples tested.

In summary, we demonstrate that the frequency of Tregs and activation markers are elevated in paediatric HIV-1 clade C infection. High Treg frequencies, suggest that Tregs may contribute to T cell suppression in children, with highest frequency in the age group of < 6 months, where HIV-1 specific T cell immunity is of lowest magnitude and breadth. Further functional studies are needed to dissect the cause and effect during disease progression and will enhance our ability to manipulate Tregs and markers of activation in a clinically beneficial manner.

6.5 HOST GENETICS: IL-10

A multitude of viral, host and cellular factors may influence HIV pathogenesis. Polymorphisms in cytokine genes such as the anti-inflammatory cytokine IL-10 have been implicated in clinical outcome perhaps through modulation of host adaptive response in humans via influence on cytokine levels (Vollmer-Conna et al., 2004). The overall effect of IL-10 on susceptibility to HIV-1 infection and disease progression is not fully understood. IL-10 promoter polymorphisms have been shown to influence susceptibility and have been reported to be associated with differential outcome based on stage of HIV-1 infection investigated, however data is lacking on the role of IL-10 and IL-10 genetic variants on mother-child transmission and paediatric HIV-1 disease progression.

Our study explored possible mechanisms underlying the role of IL-10 promoter variants on HIV pathogenesis. Our investigation confirmed that the carriers of allelic variant-1082G were linked with higher IL-10 production in comparison to the carriers of IL10 -1082A and in turn associated with transmission. The stratification of investigated cytokines revealed that proinflammatory and adaptive function were limited, in comparison to the anti inflammatory cytokine IL-10 in children within this cohort. The carriers of IL10 -592A, which is generally

associated with low IL-10 production in adults, however who showed no association in our children cohort, which was significantly associated with a lower magnitude of response. Notably, due to the controversial role of IL-10 in maintenance of immune responses and the contradiction between our data in children in comparison to findings in adults, an understanding of the underlying mechanisms that regulate immune control is crucial.

The association of IL-10 polymorphisms vary in HIV-1 infection and with plasma IL-10 levels. This may vary with age, treatment and cell types. The population under investigation was a very heterogenous group which could account for variations. IL-10 polymorphisms alone cannot account for IL-10 levels. Other cells may further contribute to IL-10 production such as macrophages. Other cytokines may also affect IL-10 production. IL-10 levels may also be impacted by co-infections. Further work will need to be done to understand better the role of IL-10 and IL-10 genetic polymorphisms in HIV pathogenesis. A meta-analyses of all reported IL-10 promoter polymorphism studies within HIV-1 infection, as recently executed in cancer research (Zhu et al., 2011) and acute Hepatitis B and C infections (Stacey et al., 2009), will prove beneficial for a better understanding of the mechanism driving IL-10 regulation.

6.6 THE WAY FORWARD.....

We are constantly being exposed to infectious agents and yet, in most cases, we are able to resist infections. Unlike most infections, HIV infected individuals require ARV to prolong their survival, are unable to clear their infection, or develop a natural immunity to subsequent infection. The evaluation of protection against HIV is normally undertaken in exposed uninfected cohorts, elite, long term non-progressors and acute seroconvertors. The failure of the MERCK STEP vaccine trials (Bradac and Dieffenbach, 2009) and clues form the Thai

trial has increased the urgency to extend HIV-1 research to investigate viral, host, cellular and adaptive research concurrently.

As HIV preferentially targets CD4 cells and depletion of these cells characterizes HIV pathogenesis most researchers are on a quest to identify correlates of protection against HIV, which can be implemented into HIV vaccine design and efficacy trials. Only a few potential HIV vaccines have been clinically approved for testing, of which the recent T cell based RV144 vaccine has shown partial protection against HIV (Rerks-Ngarm et al., 2009). Further studies that focus their investigation on therapeutic vaccines that are able to boost quality immune response, provide protection and help in the containment of viral load will prove valuable. Efforts need to focus more towards CD4+ T cell responses which have more recently been implicated in viral containment in children (Prendergast et al., 2011a).

In macaques CD8+ T cell depletion have been associated with a rise in viremia (Jin et al., 1999, Metzner et al., 2000). T cells play a pivotal role in the host antiviral adaptive response to HIV infection. However, HIV does not only affect the cell mediated branch of the adaptive response but also influences the humoral arm of adaptive immunity. Ideally a successful vaccine should be able to induce effective cellular as well as humoral responses. Currently, researchers are trying to identify monoclonal antibodies that could elicit broad potent neutralizing antibodies that could prevent infection at sites of infection (Munier et al., 2011, Wu et al., 2011, Singh et al., 2011).

On the other hand, the host adaptive response is the body's second line of defence and requires time to react to an invading organism, whereas the innate immune system provides immediate defence and is ready to be mobilized upon infection. Second, the adaptive immune

system is antigen specific and reacts only with the organism that induced the response. In contrast, the innate system is not antigen specific and reacts equally well to a variety of organisms. Ideally both components of the immune system, have demonstrated central roles in antiviral immunity by shaping the quality of the adaptive immune response to viruses and by mediating direct antiviral activity(Koblin et al., 2011). Vaccine initiatives may also need to focus on natural killer T cells (NKT), which play an intermediary role in bridging the innate and acquired immune system enhancing both systems respective functions.

Pathways of exhaustion and regulation should also be further investigated. PD-1 and CTLA-4 have been found to be positively associated with viral load (Kaufmann et al., 2007, Day et al., 2006). PD-1 together with TIM-3, have been synergistically proven to improve CD8+ T cell responses and viral control in the LCMV model (Jin et al., 2010). Interestingly, CD8+ T cells expressing both inhibitory receptors produced the suppressive cytokine IL-10.The inhibition of the IL-10 pathway has also been shown to restore T cell function and result in eventual viral clearance (Brooks et al., 2008, Brooks et al., 2006, Ejrnaes et al., 2006). Tregs play a dual role and have been implicated in T cell suppression. Treg studies were limited by the need to identify markers that adequately predict frequency and function of Treg subsets. In addition, low frequencies of Tregs in HIV controllers have been found to contribute to an effective adaptive immune response, but may also contribute to generalized immune activation, potentially contributing to CD4 depletion (Eggena et al., 2005, Cao et al., 2009, Hunt et al., 2011, Prendergast et al., 2011b, Xiao et al., 2011). Our ability to expand functional Tregs from infected children contributes to the role of Tregs in HIV-1 infection.

Vertical Transmission

It remains unclear why some infants become infected while others do not, despite their considerable exposure to HIV-1 in utero, during delivery and while breastfeeding. The mechanisms underlying vertical transmission are thought to be elevated viral loads, genital secretions and breast milk. The immune responses, polyfunctionality of T cells, and the presence of selective escape variants impact viral load levels. Furthermore, neutralising antibodies have been found to successfully reduce infection rates. Both humoral and cellular responses are necessary to reduce vertical transmission. Taken together, reduction in transmission and suppression of HIV-1 may be possible and could be achieved by enhancement and manipulation of the potential pathways.

6.7 CONCLUSION

Our HIV-1 MTCT study was established prior to the revision of PMTCT guidelines and represents a rare cohort of untreated children. Due to the much welcomed revision of PMTC guidelines, our initially envisioned detailed study design changed, creating the need to broadly investigate paediatric HIV-1. However, there still exists a need to describe these roles in more detail in future investigations. Nevertheless these findings, much of which has not been assessed particularly role of Tregs, PD-1, TIM-3 and IL-10, contribute to our understanding and existing knowledge and understanding of paediatric HIV-1 infection.

Possible eradication of paediatric HIV-1 infection lies in the development of an infant vaccine that can be administered at birth that would theoretically provide protection from infancy, through the adolescence period, and into adulthood. An overall understanding of the exact mechanism that drives HIV-1 disease pathogenesis is restricted due to the apparent multifactorial impact of the virus.

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APPENDIX

A) CONSENT FORM

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

UNIVERSITY OF KWAZULU-NATAL

RESEARCH CONSENT FORM FOR UNINFECTED PARTICIPANTS (CHILDREN)

Protocol Title: MECHANISMS OF HLA-ASSOCIATED CONTROL AND LACK OF CONTROL OF HIV INFECTION.

BREC REFERENCE NUMBER **E028/99**

PHD SUBSTUDY CELLULAR IMMUNITY, IMMUNE ACTIVATION AND REGULATION IN HIV-1 INFECTED MOTHER-CHILD PAIRS: WHAT ARE THE DETERMINANTS OF PROTECTIVE IMMUNITY?

Principal Investigator: Professor Thumbi Ndung'u and Ms. Eshia Moodley

Study Clinician: Dr Fundisiwe Chonco and Dr Gurpreet Kindra

Description of Study Population: Children Uninfected with HIV

Information to Patients- English

This study will be conducted at 4 clinical sites as follows:

1) **Prince Mshiyeni Memorial Hospital.**
2) **St Mary's Memorial Hospital**
3) **McCords Hospital**
4) **King Edward Hospital**

(Information directed to the mothers of children >2 months <12 years as they are too young to make decisions)

Dear Parent and/or Caregiver,

You have been told that your child is HIV Negative. You have been informed about the study by the clinician and nursing staff of the HIV Pathogenesis Program (HPP).

If you agree to participate, you will be given a signed copy of this document. We would like to take some blood (5ml<2yrs, 10ml<12yrs) from your child to see the difference between immune responses made by children infected with HIV and those who are not infected. We will also study the genetic make-up of the donated blood cells. People with different genetic make-up respond differently to viral infections and our study will help us to understand the response to HIV. This is a research study that has no immediate benefits to the child. It is important as it will help us understand immune responses in young children and help us to develop a vaccine for HIV so that in future people will not be infected. The blood will be taken from your child every 3-6 months until the study is closed (estimated between 1 – 3 years).

USE AND STORAGE OF SAMPLES

We shall store your child's cells and they will be used to define his/her immune status and his/her genetic make-up. Your child's samples will be stored at laboratories that are specially designed to keep stored samples safely. Samples left-over from this study may be used in other approved studies in the future. These samples will not be used for studies not approved by the ethics committee. If we want to use the samples for a research purpose not described in this consent form, we will send our request to an Institutional Review Board or the UKZN Ethics Committee. The committee protects the rights and welfare of research subjects. They will determine if we need to contact you and ask your consent to do the research.

The samples will neither be used for any commercial interests, nor will they be sold or used in products that make money for the researchers. You will not receive payment or entitlements for any beneficial outcomes of these studies.

The blood may be analyzed in laboratories outside of South Africa. This is because we do not have the expertise to do all the testing here in South Africa. Only approved researchers working on this project will be able to access your samples. The people who work at these laboratories will have access to the samples when they store them and keep track of them, but they will not know the name of the child as they will be stored by number. There is no time limit on how long the samples may be stored. You will not forfeit your rights over these cells and you can request at any time that they be destroyed. The blood sample and personal information will be stored without the child's name or other information to identify him/her as a participant in this study to protect their privacy.

You will be given R75 on every scheduled clinic visit to cover transport costs. Participation is completely voluntary, and you may choose not to have your child participate. If you choose not to have your child participate, your care at the clinic will not be affected in any way. If you choose for your child to

participate, you may withdraw your consent at any time without any penalty if you so choose. Your child's participation will be treated with strict confidentiality.

Site-Investigator's Contact details: Professorf T Ndung'u on 0823587204 or Dr Fundisiwe Chonco on 0826871535.

Contact details of Research Ethics Committee administrator and Cha**ir**-for reporting of complaints/problems.

email: brec@ukzn.ac.za or phone: 031 260 4769.

Physical address: The Biomedical Research Ethics Committee, Room N40, Govan Mbeki Research Centre, Westville Campus, University of Kwazulu-Natal, Durban, South Africa.

Thank you for your help.

Doctor/Sister _____ of the Hospital Research team has explained to me that I am attending a clinic where HIV is being treated and studied. I have read the above document and I consent for my child to be involved in the study.

Date : _____

Client Name : _____ Client Signature : _____

Counsellor Name : _____ Counsellor Signature : _____

SAMPLE EXPORT

We ask your permission to send some of your blood to scientists outside of South Africa for investigations related to this study. We work closely with other scientists in the US and Europe who are able to do tests that we are not yet able to do here in South Africa. Your blood or your DNA (a product from your blood) will be sent without your name or other identifying information to protect your privacy. We will seek ethics approval and your consent before any new studies are done on the samples.

Date : _____

Client Name : _____ Client Signature : _____

Counsellor Name : _____ Counsellor Signature : _____

SAMPLE STORAGE

We ask your permission to store processed blood products for future testing. Your blood products will not be stored with any identifying information that can link your specimen to you as a participant in this study. We will seek ethics approval and your consent before any new studies are done on the samples.

Date : _____

Client Name : _____ Client Signature : _____

Counsellor Name : _____ Counsellor Signature : _____

RESEARCH CONSENT FORM

Isihloko: HIV Specific CD8+ T cell responses in chronically HIV infected children

Indawo: St Mary's Hospital, King Edward Hospital, Prince Mshiyeni Memorial Hospital, McCord Hospital

Umcwaningi omkhulu: Dr Philip Goulder

Abacwaningi abaseduze: Dr Fundisiwe Chonco; Srs Thandi Cele; Thandi Sikhakhane

Uhlobo lwabantu oludingekayo: Izingane ezitheleleke ngegciwane lengculazi isikhathi esingange minyaka emithathu

Ulwazi oluqondiswe kuziguli- IsiZulu

Ulwazi oluqondiswe kumama bezingane >2 izinyanga < 12 iminyaka njengoba bebancane ukuthi bangazithathela izinqumo

Mzali othandekayo

Ubusutsheliwe ukuthi umntwana wakho utheleleke ngegciwane lengculazi. Egazini lengane yakho kunezakhi zomzimba (T –cell) ezikwazi ukulwa nezinhlobo zezifo ezingamuhlasela, kubalwa negciwane lengculazi. Ucwaningo lukhombisa ukuthi uma lezizakhi zomzimba ziqinile futhi zisebenza kahle zenza ukuthi ingculazi ingasheshi ikugulise. Lokhu kuhambelana nohlobo lwegciwane lengculazi elihlasele umntwana wakho.

Singathanda ukuthatha igazi Kumntwana wakho elilingana nespuni (5ml<2,10ml<12) ukuze sihlole uhlobo lwegciwane nendlela igciwane elizibonakalisa ngayo kumntwana. Lolucwaningo alunanzuzo esheshayo kuwe. Lubalulekile ngoba wussizo ekwakheni umgomo wegciwane lengculazi kubantu bakusasa abangathelelekile. Igazi lomntwana wakho liyothathwa njalo emva kwezinyanga ezintathu kuya kweziyisithupha. Sizolilondoloza igazi lomntwana wakho, siyolisebenzisa kuphela uma sihlola isimo samasotsha omzimba kanye nohlobo lwezakhi zomzimba ezenza ufuzo (HLA) zakhe. (HLA- imele Leukocyte antigen yomuntu- okuyizinhlayiyana ezincane egazini okusiza umzimba ungangenwa yizifo.

Uhlobo lweHLA uluthola kubaba nomama wakho. Sikholwa ukuthi abantu abanezinhlobo ezahlukene ze HLA bangakwemukela ngezindlela ezahlukene ukumelana negciwane lengculazi. Kuyinhloso yethu ukuthi uma sesiqedile ngocwaningo emva kweminyaka emithathu kuya kwemihlanu (3-5 years) leli gazi eligciniwe sibulale izakhi ezikulo, ngeke asetshenziselwe ukuhweba noma olunye ucwaningo olwehlukile. Ngaphezu kwalokhu awulahlekelwa ngamalungelo akho ngaleligazi uma uthanda ungacela ukuthi abulawe.

Uyonikezwa imali yokugibela engamashumi amahlanu amarandi (R50) ngesikhathi uvakashele emtholampilo ukuyothathwa igazi. Ukubandakanya umntwana wakho kuyilungelo lakho awuphoqelekile, ungakhetha futhi ukungazibandakanyi. Uma ungazibandakanyanga lokho ngeke kuthikameze amalungelo akho njengesiguli emtholampilo. Ungakhetha ukuphuma kulolucwaningo uma ungasathandi. Ukuzibandakanya kwakho kuyogcinwa kuyimfihlo.

Siyabonga ngo sizo lwakho

UDokotela/uMhlengikazi omkhulu _____ wase St Mary's Hospital ungichazelile ukuthi ngivakashela emtholampilo lapho kwelashelwa khona isandulela ngculazi bese kwenziwa nocwaningo. Ngiwufundile lo mbhalo ongenhla futhi ngiyavuma ukuthi umntwana wami abandakanywe ocwaningeni.

Usuku : _____

Igama lesiguli: _____ Isishicilelo sesiguli: _____

Igama Likameluleki: _____ Isishicilelo sikameluleki: _____

Abacwaningi ongathintana nabo nezinombolo zabo zocingo: Srs Thandi Cele & Thandi Sikhakhane: 0836451759 noma u **Prof T Ndung'u pm 0823587204.** Ungaxhumana noSihlalo we Research Ethics Committee uma ufuna ukubika izikhalo zakho nezinkinga onazo. Ungakhona futhi ukuxhumana nosihlalo we Biomedical Research Ethics committe (Dr Jack Moodley) kanye noNkosikazi S. Buccas kulenombolo: (031) 260 4769; email address: buccas@ukzn.ac.za

I address Iakhona: The Biomedical Research Ethics Committee, University of KwaZulu-Natal, 719 Umbilo Road, Congella 4013, Durban

B) Sequencing PCR (Chapter 2)

Reagents for first round PCR.

Reagent	Volume (µl)
H_2O	34.0
X10 Opti Buffer	5.0
$MgCl_2$ (50nM)	3.0
5'-primer	1.0
3'-primer	1.0
dNTP	0.5
Bio*Taq* (5u/mL)*	0.5
DNA	5.0
Total	50.0

PCR amplification conditions for first round (Leslie et al., 2004) .

Step number	Temperature (°C)	Time
1	99	2 minutes
2	94	15 sec
3	62	30 sec
4	72	1 minute
5	*restart step 2,20 times*	

6	94	15 sec
7	59	30 sec
8	72	1 minute
9	*restart step 6, 20 times*	
10	72	7 minutes
11	4	for ever

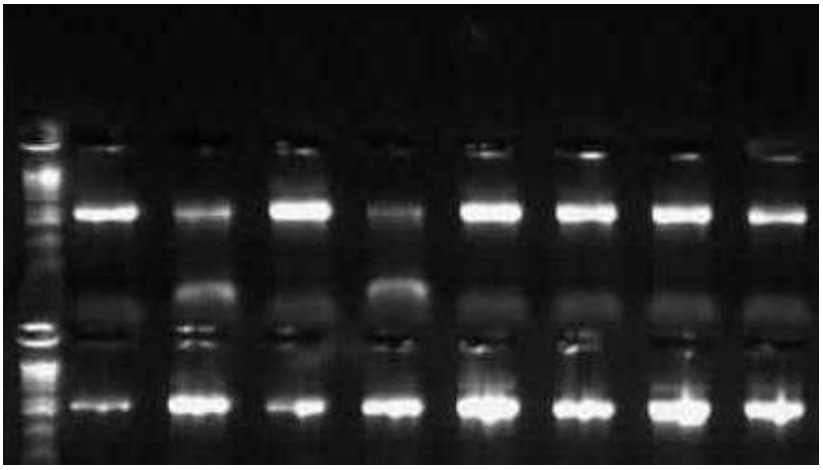
Reagents for second round PCR.

Reagent	Volume (ul)
H_2O	34.0
X10 Opti Buffer	5.0
$MgCl_2$ (50nM)	3.0
5'-primer	1.0
3'-primer	1.0
dNTP	0.5
Bio*Taq* (5u/mL)*	0.5
PCR product	5.0
Total	50.0

Primers as per Chapter 2.

PCR amplification conditions for second round.

Step number	Temperature (°C)	Time
1	99	2 minutes
2	94	15 sec
3	56	30 sec
4	72	1 minute
5	*Restart step 2, 20 times*	
6	94	15 sec
7	54	30 sec
8	72	1 minute
9	*Restart step 6, 20 times*	
10	72	7 minutes
11	4	for ever



Viral replicative assay MWM confirming Gag-Protease insert

C) HLA

Clinical characteristics of participants of ARV naïve study cohort **(Chapter 2)**

Mothers	**Age**	**CD4**	**VL**	***A***	**A**	**B**	**B**	**C**	**C**
CP1M- 001	35	347	13,400	02	3002/3010/3012	35	44	04	1601
CP1M- 002	30	301	82,600	33	68	07	41	07	17
CP2M- 001	22	173	173,000	24	3402/3404	08	1516	07	1402/1403
CP3M- 004	24	905	170,000	30	6602	39	45	1203	1601
CP3M- 005	28	246	185,000	3004/3006	3004/3006	27	5802	0202/0205	0602/0607/0610
CP3F- 005				03	03	08	08	07	07
CP3M- 006	33	259	40,100	nd					
CP3M- 007	17	418	376,000	68	68	07	1510	03	07
CP3M- 009	25	288	4,700	03	03	5802	5802	06	06
CP3M- 010	42	247	41,800	2301	6602	1402/1404	44	07	08
CP3M- 011	36	291	130,000	01	32	4207/4201	8101/8102	17	1801/1802
CP3M- 014	28	641	5,690	24	3002/3012	07	14	07	08
CP3M- 015	27	448	37,600	03	34	1503	44	02	04
CP3M- 016	23	225	223,000	29	3001/3018/3019/3014	5802	5802	06	06
CP3M- 018	36	287	69,600	30	68	4201	5802	06	17
CP3M- 019	25	281	127,000	03	74	1503	5802	02	06
CP3M- 020	29	200	286,000	0205/0208	68	1503	18	02	04
CP3M- 021	30	317	253,000	3	66	13	1510	NA	NA
CP3M- 023	29	211	31,200	02	68	07	5802	06	07
CP3M- 024	32	210	692,000	29	6601/6604	4201	5802	06	17
CP3M- 026	33	344	75,900	01	03	35	81	04	18
CP3M- 027	32	279	48,900	3001	6602	4201	44	07	17
CP3M- 028	40	397	30,200	30	32	1510	4201	08	17
CP3M- 033	22	201	111,000	02	3002/3010/3012	08	5802	06	07
CP3M- 034	32	176	1,170	23	66	5801	5802	06	07

CP3M- 036	36	217	7,080	30	30	1503	4201	1701	1801
CP3M- 039	28	526	4,790	3004/3006	74	1503	41	02	17
CP3M- 040	23	381	58,200	03	68	07	1510	03	07
CP3M- 041	30	113	726,000	23	3402/3404	08	08	07	07
CP3M- 042	29	450	4,790	23	3002/3007/3010/3012	4201	5802	06	17
CP3M- 043	34	176	60,900	0205/0208	33	4201	5802	06	17
CP3M- 044	30	647	1,790	2	03	08	5801	07	07
CP3M- 045	21	430	65, 500	1	68	44	44	04	07
CP3M- 046	25	450	10, 400	23	3402/3404	1503	4201	02	17
CP3M- 047	31	550	6,200	3002/3010/ 3012	3402/3404	08	44	04	07
CP3M- 049	22	428	63613	2	66	45	5802	06	16
CP3M- 050	28	821	4392	24	66	07	5802	06	07
CP3M- 051	21	269	3822	2	03	1510	45	03	1601/1607/1608
CP3M- 052	33	580	1202	24	74	07	5702/5703	07	07
CP3M-064	23	584	28,161	3	23	1510	5802	16	?
CP4M- 001	26	616	139,000	26	74	41	45	1601	17
CP4M-002	34	347	328,000	3402/3404	6802	07	44	04	07
CP4M-003	32	669	322,000	02	3002/3012	5802	5802	0602	602
CP4M-004	23	704	18,100	3001	3402/3404	4201	44	04	17
CP4M-005	34	147	39,800	24	6802	07	14	07	0802/0807
CP4M-006	32	244	75,300	2301	29	1510	15	03	1601
CP4M-007	37	345	54,200	3002/3012	74	1402	5703	07	08
CP4M-008	28	306	68,500						
CP4M-009	37	412	48,800	30	3402/3404	3910/3920	44	04	12
CP4M- 010	25	24	125,000	0205/0208	3004/3006	1401	5802	0602	08
CP4M-011	35	134	750,000	30	29	18	58	3	07
CP4M-012	31	253	249,000	29	6802	07	1510	0304/0305	07
CP4M-013	30	226	660000	4301	6802	1503	5802	0202/0210	0602/0607/0610
CP4M-014	26	143	750000	02	68	35	49	04	07

CP4M-016	29	nd	39,600	34	68	1503	49	2	4
CP4M-017	24	320	26,700	3	74	13	49	2	6
CP4M-018	24	515	205,000	3002	74	1503	49	0202/0210	0602/0607/0610
Children									
CP1C- 001	2.7	22	1,320	3402/3404	3002/3010/3012	35	44	04	1601
CP1C- 002 child 2	3	23	76,100	0205/0208	68	07	5703/5702	07	07
CP1C- 002 child 1	5	46	4,130	2	6802	07	5703/5702	07	07
CP2C-001	0.6	13	2765360	N/A					
CP3C-004	3	17	148,000	30	34	44	45	04	16
CP3C-005	0.8	37	303,000	03	30	08	5802	06	07
CP3C-006	0.5	20	750,000	03	68	0807/08	1509/1510	07	08
CP3C-007	0.3	39	750,000	68	68	07	5702	07	1801/1802
CP3C-009	0.4	17	750,000	03	3001/3018/3019	4201	5802	06	17
CP3C-010	0.3	38	750,000	2301	33	1402/1404	4201	08	17
CP3C-011	0.3	42	201050	2301	33	1402	4201	0802/0807	17
CP3C-014	0.7	21	328,000	24	68	07	58	07	07
CP3C-015	8	15	62,800	24	3402/3404	08	44	04	07
CP3C-016	0.5	30	7281580	0304/0305	3001/3018/3019	1510	5802	0304/0305	06
CP3C-018	2	22	178,000	3004/3006	68	07	5802	06	07
CP3C-019	0.4	24	750,000	3402/3404	74	1503	44	02	04
CP3C-020	0.5	14	322,000	02	02	18	45	02	16
CP3C-021	0.4	13	740000	03	30	13	39	06	17
CP3C-023	0.3	22	405,000	0202/0210	03	1503	5802	0202/0210	06
CP3C-024	0.6	37	537,000	30	66	27	5802	02	06
CP3C-026	0.3	16	750,000	01	30	42	81	17	18
CP3C-027	0.3	19	750,000	30	30	08	44	07	07
CP3C-028	0.5	18	292,000	23	30	1503	4201	02	17
CP3C-033	0.3	17	750,000	29	3002/3010/3012	44	5802	06	07

CP3C-034	1.8	12	579,000	23	68	1510	5801	07	08
CP3C-035	0.8	24	750,000	02	23	08	15	03	08
CP3C-036	0.4	24	1477305	30	30	08	4201/4208	07	17
CP3C-039	0.4	38	557,000	3004/3006	30	07	41	02	17
CP3C-040	0.6	31	750,000	6601/6604	68	07	5802	06	07
CP3C-041	1	31	750,000	23	3002/3007/3010/3012	08	39	07	12
CP3C-042	0.3	33	627,000	3002/3010/ 3012	3402/3404	1503	5802	02	06
CP3C-043	1.5	32	104,000	0205/0208	68	1510	5802	03	06
CP3C-044	0.9	22	260,000	0205/0208	02	1401/1407	5801	07	08
CP3C-045	1	24	530, 000	1	66	44	5802	06	07
CP3C-046	0.4	33	750, 000	34	34	1503	44	02	04
CP3C-047	0.4	38	7135185	3002/3010/ 3012	32	08	5801	07	07
CP3C-049	0.3	30	3,902,668	2	30	45	45	16	16
CP3C-050	0.4	33	82,227	23	24	07	08	07	07
CP3C-051	0.5	24	62,185	1	02	45	81	1601	18
CP3C-052	0.4	40	558,889	24	3402/3404	07	44	04	07
CP3C-064	0.5	30	1,000,000	23	30	1510	45	16	16
CP4C-001	3.3	28	59,900	0205/0208	26	1401	41	08	17
CP4C-002	3	34	529,000	24	3402/3404	07	44	04	07
CP4C-003	4.2	21	189,000	30	30	45	5802	06	1601/1602
CP4C-004	1.5	37	301,000	3001	6802	07	4201	07	17
CP4C-005	2.5	25	335,000	24	3001	1402	4201	0802/0807	17
CP4 C-006	3.1	25	246,000	2301	6801	1510	5801	03	06
CP4C-007	3	33	97,600	2301	74	1510	5703	07	1601
CP4C-008	2	26	348,000	02/0250	6802/6901	1401	5802	0602	08
CP4C-009	2.1	11	750,000	3402/3404	6601/6604	44	5802	04	06
CP4C-010	9	22	167,000	3004/3006	6802	1402	5802	0602	08
CP4C-011	2	11	439,000	30	30	13	18	6	7

CP4C-012	10	38	164,000	29	3402/3404	1510	44	0304/0305	04
CP4C-013	7	16	177,000	29	4301	18	5802	602	704
CP4C-014	4	32	331,000	3002/3010/ 3012	68	35	5802	04	06
CP4C-016	7	nd	71,300	3	34	1503	5802	2	6
CP4C-017	6	31	120,000	1	74	1503	45	2	16
CP4C-018	5	24	292,000	3002/3010/ 3012	6802/6818/6827	1510	5802	0304/0305	0602/0607/0610
CP4C-019	5	27	30,900	29	68	1503	5801	2	4

D) ELISpot-peptides routinely tested

HLA	Protein	Code	Clade-C Sequence	OLP#
B7	P24	B7-SV9(p24)	SPRTLNAWV	#20
	P24	B7/42/81-TL9(p24)	TPQDLNTML	#25
	P24	B7/42/81-HA9 (p24)	HPVHAGPIA	#29, #30
All these peptides should be tested for B7/42/81	P24	B7/42/81-GL9(p24)	GPSHKARVL	#48
	Nef	B7/42/81-FL9(Nef)	FPVRPQVPL	#76
	Nef	B7/42/81-RM9 (Nef)	RPQVPLRPM	#76
	nef	B7/42/81-RGF9(nef)	RPMTFKGAF	#77
	nef	B7/42/81-TL10(nef)	TPGPGVRYPL	#84
	RT	B7/42/81-SM9(RT)	SPAIFQSSM	#187
	vpr	B7/42/81-FL9(vpr)	FPRPWLHGL	#281, #282
	gp120	B7/42/81-RI10(gp120)	RPNNNTRKSI	#328
	gp41	B7/42/81-IA9(gp41)	IPRRQGFEA	#401
	vif	B7/42/81-HI10(vif)	HKRVSSEVHI	#407, #408
	Int	B7/42/81-LI9(Int)	LPPIVAKEI	#244
	Pro	B7/42/81-TL11(Pro)	TPVNIIGRNML	#163
	RT	B7/42/81-YL9(RT)	YPGIKVKQL	#202, #203
	REV	B7/42/81-RL10	RPAEPVPLQL	#102
	P17	B7/42/81-RLY10	RLRPGGKKHY	#3
	RT	B81/42/81-SL10	SPIETVPVKL	#166
	RT	B7/42/81-VM9	VPVKLKPGM	#166
	RT	B7/42/81-VL9	VASCDKCQL	#245/246
	RT	B7/42/81-RQL11	RPPLVKLWYQL	#221
	PROT	B7/42/81-TM10	TPVNIIGRNM	#163
	Vif	B7/42/81-KL9	KPKKIKPPL	#422
	RT	B7/42/81-TL11	TPGIRYQYNVL	#184

	RT	B7/42/81-GL9(RT)	GPKVKQWPL	#168
B27	P17	B27-IK9(p17)	IRLRPGGKK	#3
	P24	B27-KK10(p24)	KRWIILGLNK	#36
	nef	B27-KV10(nef)	KRQEILDLWV	#81
	gp41	B27-GR10(gp41)	GRSSLRGLQR	#393
	RT	B27-KY9	KRKGGIGGY	#265
B57	p17	B57-WF9(p17)	WASRELERF	#5
	P24	B57-ISW9(p24)	ISPRTLNAW	#20
	P24	B57-KF11(p24)	KAFSPEVIPMF	#22
Run B57 peptides	p24	B57-DW10(p24)	DTINEEAAEW	#28
for all B5801/5802/5803	P24	B57-TW10(p24)	TSTLQEQIAW	#33, #34
	P24	B57-QW9 (p24)	QATQDVKNW	#42
	nef	B57-HW9(nef)	HTQGYFPDW	#82
	Rev	B57-QY10(Rev)	QAVRIIKILY	#96
	RT	B57-IW9(RT)	IQLPEKDSW	#199
	RT	B57-IAW9(RT)	IAMESIVIW	#216
	Int	B57-SW10(Int)	SAAVKAACWW	#257
	Int	B57-KF9(Int)	KTAVQMAVF	#263, #264
	Vpr	B57-AW9(Vpr)	AVRHFPRPW	#281
	Env	B57-KW11(Env)	KAYETEVHNVW	#296
	Vif	B57-VF9(Vif)	VSRRANGWF	#405
	nef	B57-KAF9(Nef)	KAAFDLSFF	#78
	RT	B57-AF10	QATWIPEWEF	#220
	RT	B57-FF9	FSVPLDEDF	#181
	RT	B57-EY9	ETKIGKAGY	#226
	RT	B57-KI13	KAGYVTDRGRQKI	#226
	GP120	B5802-QL11	QTRVLAIERYL	#365
	Vif	B57-LW9	LGHGVSIEW	#411
	gp41	B57-TWS10	TTAVPWNSSW	#369
	RT	B57-LL9	LGIPHPAGL	#178
	RT	B57-FW11	FAIKKKDSTKW	#174
	POL	B57 KR10	KAGYVTDRGR	#226
	POL	B57 VI9	VTDRGRQKI	#226