MECHANISMS OF NON-PROGRESSING PAEDIATRIC HIV-1 INFECTION

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ABSTRACT

HIV-infected children progress more rapidly to AIDS than adults and, in sub-Saharan Africa, without antiretroviral therapy (ART), more than 50% will die before their second birthday. However, a minority of perinatally infected children will become paediatric non-progressors (PNP). The reasons for this rapid disease progression and the immunological correlates of protection against rapid progression are incompletely understood. This thesis focuses principally on paediatric HIV infection, and addresses the immunological and viral influences on disease progression.

This work shows that Human Leukocyte Antigen (HLA) class I variation does not significantly influence progression rates in paediatric HIV infection, in stark contrast to adult HIV infection where HLA class I allele variation substantially impacts disease progression. This study describes potent broadly neutralizing antibody responses along with strong virus-specific T-cell activity in HIV-infected children. These virus-specific immune responses, however, do not protect against progression. The primary driver of non-progression in HIV infected children is low levels of T cell immune activation despite persistently high viraemia. This suggests close similarities with mechanisms evolved over thousands of years of non-pathogenic SIV infection in non-human primates.

Further evaluation of the mechanisms underlying paediatric non-progression is relevant both to reaching a better understanding of HIV pathogenesis and to underpin future HIV cure strategies.

MANUSCRIPTS AND PUBLICATIONS

Adland E*, Muenchhoff M*, Laker L, Mori L, Csala A, Paioni P, et al. Reconstitution of functional HIV-specific CD4+ T-cell activity following ART in paediatric infection. *Manuscript in preparation*. **(*joint 1st author)**

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CHAPTER SPECIFIC CONTRIBUTIONS

The text, tables and figures in this thesis are my own work unless otherwise stated. Contributions from organizations and individual collaborators are listed below.

Chapter 1: Introduction

Sources of figures are acknowledged individually in the figure legends.

Chapter 3: Discordant impact of HLA alleles on viral replicative capacity and disease progression in paediatric and adult HIV infection

The Kimberley Hospital clinical team of doctors and nurses were instrumental to the data produced in this study. They undertook all clinical assessments and collected data and blood samples. The blood samples were then processed by lab technicians Luisa Mori and Leana Laker in Kimberley, and the NHLS undertook the viral load and CD4 count measurements.

HLA class I Typing was undertaken by Dr Steven Cate at the CLIA/ASHI accredited laboratory of William Hildebrand, PhD, D (ABHI) at the University of Oklahoma Health Sciences Center.

Proviral gag sequencing of the rapid progressing children shown in Figs 3.13 and 3.14 and Table 3.2 were performed by Christina Thobakgale and Julia Prado in 2008 during their time in the Peter Medawar Building, Oxford. ELISpot assays displayed

in figures 3.13 and 3.14 were performed at the HIV Pathogenesis Programme, Durban, South Africa by Christina Thobakgale in 2008.

The majority of this chapter was published in 2015 (Adland *et al*., PLoS Pathogens. 2015; 11:e1004954).

Chapter 4: Reconstitution of functional HIV-specific CD4+ T-cell activity following ART in paediatric infection.

The first stage of the whole blood ICS assay was carried out by Luisa Mori and Leana Laker in Kimberely, South Africa. In the course of one of several visits I have made to Kimberley, I provided the training they required to undertake this part of the assay. This assay is described in detail in section 2.10.3, but briefly; patient blood samples were antigen stimulated then cells cryopreserved and stored at -80°C in Kimberley. Cryopreserved cells were then shipped to Oxford where the intracellular cytokine staining and analysis was performed by myself.

HLA Class I Typing was undertaken by Dr Steven Cate at the CLIA/ASHI accredited laboratory of William Hildebrand, PhD, D (ABHI) at the University of Oklahoma Health Sciences Center.

Chapter 5: Sooty mangabey-like phenotype in non-progressing HIV-infected children.

The data in this chapter resulted from a multi-centre analysis, with data from 3 cohorts provided through collaborations with the following individuals:

Kimberley, South Africa: Pieter Jooste, Leana Laker, Luisa Mori

Great Ormond Street Hospital, London, UK: Margaret Clapson, Vas Novelli

Ithembalabantu cohort, Durban, South Africa: Max Muenchhoff, Thumbi Ndung'u

Cell surface staining of immune activation and exhaustion markers, and T cell memory subsets were performed by myself for the Kimberley Cohort and the Great Ormond Street Cohort; and by Max Muenchhoff for the Ithembalabantu cohort; and results were pooled.

Jacob Hurst performed the analysis of correlations between viral load and age.

Measurements of sCD14, iFAB and cell proliferation by CFSE were performed by Max Muenchhoff at the HIV Pathogenesis Programme, Durban, South Africa.

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ACRONYMS AND ABBREVIATIONS

°C	Degrees centigrade
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
AGM	African green monkey
AIDS	Acquired immunodeficiency syndrome
APC	Allophycocyanin
APC	Antigen presenting cell
ART	Antiretroviral therapy
BCL	B cell line
bNAb	Broadly neutralizing antibody
CD	Cluster of differentiation
CMV	Cytomegalovirus
CO2	Carbon dioxide
CRF	Circulating recombinant form
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme-linked immunospot assay
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein Isothiocyanate
GALT	Gut associated lymphoid tissue
GFP	Green fluorescent protein
glm	Generalized Linear Model
HAART	Highly-active antiretroviral therapy
HEU	HIV-exposed uninfected
HIV-1	Human immunodeficiency virus type I
HIV-2	Human immunodeficiency virus type II
HLA	Human leukocyte antigen
HTLV	Human T-lymphotropic virus
ICS	Intracellular cytokine staining
iFABP	Intestinal fatty acid binding protein
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin-
IP	Inner primer
IP	Intrapartum
IQR	Interquartile range
IU	Intrauterine
LASSO	Least absolute shrinkage and selection operator
LC	Langerhan cells

LPS	Lipopolysaccharide
LTNP	Long-term non-progressor
LTR	Long terminal repeat
M	Molar
Mab	Monoclonal antibody
mg	milligram
MHC	Major histocompatibility complex
MIP1β	Macrophage inflammatory protein 1β
mL	millilitre
mM	millimolar
MOI	Multiplicity of infection
MSM	Men who have sex with men
MTCT	Mother to child transmission
NAbs	Neutralising antibodies
n	Number
ng	Nanogram
NK	Natural killer
OLP	Overlapping peptide
OP	Outer primer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PEHSS	Paediatric Early HAART STI Study
PHA	Phytohaemagglutinin
pMTCT	prevention of mother-to-child-transmission
PNP	Paediatric non-progressor
PP	Paediatric progressor
RM	Rhesus macaque
RNA	Ribonucleic acid
rpm	revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Standard error of the mean
SFC	Spot forming cells
SHIV	Simian human immunodeficiency virus
SIV	Simian immunodeficiency virus
SM	Sooty mangabey
TBE	Tris-borate-EDTA
TCID50	50% tissue culture infective dose
TCR	T cell receptor
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
Tim-3	T cell immunoglobulin mucin
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Treg	Regulatory T cell
U	Units
V	Volts
VL	Viral load
VRC	Viral replicative capacity
WHO	World health organisation
WT	Wildtype
μg	microgram
μl	microlitre

TABLE OF CONTENTS

Chapter 1 Introduction **1**

1.1 Epidemiology of HIV-1 1

1.1.1 Historical context *1*

1.1.2 The current HIV epidemic *2*

1.1.3 The paediatric HIV epidemic *3*

1.1.4 HIV classification *4*

1.2 Structure and function of HIV-1 6

1.2.1 Structure of HIV-1 virion *6*

1.2.2 The HIV-1 genome *7*

1.2.3 Viral life cycle *8*

1.3 Pathogenesis of Adult HIV-1 infection 11

1.3.1 Clinical course of disease *11*

1.3.2 Transmission and viral dissemination *12*

1.3.3 Mother-to-child-transmission (MTCT) *14*

1.3.4 Immune activation in HIV-1 infection *14*

1.3.5 Lessons from primate models of disease *17*

1.4 Immunology of HIV-1 19

1.4.1 Innate immunity to HIV-1 *19*

1.4.2 Adaptive immunity to HIV-1 *21*

1.4.3 Role of antibodies *23*

1.4.4 Role of CD8+ T-cells *25*

1.4.5 Role of CD4+ T-cells *26*

1.4.6 Differences in immune control of paediatric versus adult HIV-1 infection *28*

1.5 CD8+ T cells and control of viraemia 32

1.5.1 Role of HLA class I molecules *32*

1.5.2 Role of HIV Gag as a CD8+ T-cell target *33*

1.5.3 Impact of HIV escape mutations *33*

1.5.4 Impact of HIV escape mutations upon transmission *34*

1.6 Towards a vaccine for HIV 35

1.6.1 The specific challenges of HIV-1 *35*

1.6.2 T-cell approaches to vaccine design *36*

1.6.3 Antibody approaches to vaccine design *38*

1.6.4 Non-human primate models *40*

1.7 Clinical aspects of HIV-1 infection 42

1.7.1 Natural history of HIV infection in adults *42*

1.7.2 Natural history of HIV infection in children *43*

1.7.3 Prevention of MTCT (pMTCT) *45*

1.7.4 Diagnosis and treatment of paediatric HIV infection *45*

1.8 Overview of this thesis 47

Chapter 2 Materials and Methods **48**

2.1 Patient cohorts 48

2.1.1 Kimberley cohort, South Africa *48*

2.1.2 Thames Valley cohort, UK *49*

2.1.3 Paediatric cohort, UK *50*

2.1.4 Durban cohort, South Africa *50*

2.2 Processing of blood samples 51

2.2.1 Blood collection *51*

2.2.2 Separation of plasma and PBMC *51*

2.2.3 Cryopreservation and thawing of PBMC *52*

2.2.4 DNA extraction *53*

2.2.5 Viral RNA extraction *53*

2.3 HLA class I typing 54

2.4 Molecular biology techniques 54

2.4.1 Quantification of DNA *54*

2.4.2 Agarose gel electrophoresis *54*
2.5 Plasmid preparation 55
2.5.1 Plasmid pNL4-3 *55*
2.5.2 Plasmid pNL4-3Δgag-protease *56*
2.5.3 Plasmid DNA extraction and purification *56*
2.5.4 Restriction enzyme digestion of plasmid *57*
2.6 Amplification of HIV genes by polymerase chain reaction (PCR) 59
2.6.1 Amplification from Amplification from proviral DNA *59*
2.6.2 Amplification from RNA *61*
2.6.3 Primers *61*
2.6.4 Visualisation of PCR product *63*
2.6.5 PCR purification *63*
2.6.6 Sequencing *63*
2.6.7 Caveats and quality control of PCR *65*
2.7 Cellular culture 67
2.7.1 Media and reagents *67*
2.7.2 Immortalised cell lines *67*
2.7.3 Cell counting *68*
2.8 Cellular assays 68
2.8.1 IFNγ ELISpot assay *68*
2.8.2 Screening for HIV-specific responses by megamatrix *69*
2.8.3 Whole blood ICS assay *70*
2.8.4 Cell proliferation assay *71*
2.8.5 Plasma levels of immune activation and inflammation *72*
2.8.6 Antibody neutralization assay *72*
2.9 Viral culture 73
2.9.1 Generation of HIV virus *73*
2.9.2 Generation of viral stocks *73*
2.9.3 Calculation of TCID50 of viral stocks *74*

2.9.4 Multiplicity of infection (moi) *74*

2.10 Viral replication assay 75

2.10.1 Linearisation of pNL4-3Δgag-pro plasmid *75*

2.10.2 Co-transfection of plasmid and gag-pro PCR by electroporation *75*

2.10.3 Culture transfected GXR cells *76*

2.10.4 Determine viral titres using GXR cells *76*

2.10.5 Viral propogation *76*

2.11 Fluorescent activated cell sorting (FACS) 77

2.11.1 Cell surface staining *77*

2.11.2 Intracellular staining *78*

2.11.3 Acquisition and analysis *79*

2.12 Statistical analysis and phylogenetics 79

2.12.1 Statistical tests used in Chapter 3 *79*

2.12.2 Statistical tests used in Chapter 4 *81*

2.12.3 Statistical tests used in Chapter 5 *81*

2.12.4 Phylogenetic analysis *82*

2.12.5 Corrgram *82*

Chapter 3 Discordant impact of HLA alleles on viral replicative capacity and disease progression in paediatric and adult HIV infection **83**

3.1 Introduction 83

3.2 Results 87

3.2.1 Lower VRC is associated with slow progression in children *87*

3.2.2 Impact of the maternally transmitted virus *93*

3.2.3 Differential impact of HLA class I expression on VRC in adult and paediatric infection *97*

3.2.4 Strong correlation between gag escape mutant number and VRC *99*

3.2.5 Low gag mutant number in adults with high VRC expressing *104*

disease-susceptible HLA alleles

3.2.6 Discordant impact of HLA alleles on progression in HIV-infected children and adults *107*

3.2.7 Lack of selection pressure mediated by protective HLA alleles in rapid progressor children *109*

3.3 Discussion 116

Chapter 4 Reconstitution of functional HIV-specific CD4+ T-cell activity following ART in paediatric infection **132**

4.1 Introduction 132

4.2 Results 135

4.2.1 Functional profile of CD4 and CD8 T-cell in ART-naïve HIV-infected children *135*

4.2.2 Viraemia is positively related to CD4 IFNγ, and inversely related to CD4 IL-2, TNFα and MIP1β responses to HIV gag stimulation *140*

4.2.3 Functionality of CMV-specific CD8+ and CD4+ T cells in HIV infection *142*

4.2.4 Influence of HLA type on functionality *144*

4.2.5 In ART-treated children, increased duration of viral suppression is associated with increased IL-2 and decreased IFNγ production in CD8+ and CD4+ T cells *146*

4.2.6 Enhanced T cell functionality is associated with low immune activation and low exhaustion *155*

4.3 Discussion 160

Chapter 5 Sooty Mangabey-like phenotype in non-progressing HIV-infected children **164**

5.1 Introduction 164

5.2 Results 168

5.2.1 Paediatric non-progressors maintain high CD4 counts despite persistently high viraemia *168*

5.2.2 Low levels of immune activation define PNP's *173*

5.2.3 Loss of IL-2 responses distinguishes paediatric progressors from non-progressors *179*

5.2.4 Chronic immune activation drives terminal memory differentiation in progressor children with a shift from IL-2 towards IFNγ dominated functional profiles *186*

5.2.5 Immune exhaustion is driven by immune activation and discriminates progressors from non-progressors *192*

5.2.6 CD4+ T-cell immune activation as the major driver of progression in paediatric HIV infection *200*

5.2.7 High titre, broadly neutralizing antibodies in PNP *205*

5.3 Discussion 210

Chapter 6 Discussion **219**

6.1 Introduction 219

6.2 The relative contribution of viral fitness on disease progression 220

6.3 The protective influence of HLA alleles in children and adults 221

6.4 Recovery of T cell function on ART 222

6.5 Central role of immune activation in HIV pathogenesis 224

6.6 Summary of results 227

References **228**

CHAPTER 1

INTRODUCTION

1.1 EPIDEMIOLOGY OF HIV-1

1.1.1 HISTORICAL CONTEXT

In 1981, the first reports were made of patients in Los Angeles and New York suffering from a novel immunodeficiency syndrome associated with a selective depletion in CD4+ T cells. Although initially restricted to homosexual men, reports started to emerge of a similar illness affecting injecting drug users (Brennan & Durack 1981). The clinical syndrome became known as Acquired Immunodeficiency Syndrome (AIDS). The first report of a retrovirus isolated from a patient with AIDS was published in 1983 (Barre-Sinoussi) and following initial classification as HTLV III was termed HIV-1 in 1986 (Coffin *et al.* 1986). Since the beginning of the epidemic, almost 78 million people have been infected with HIV-1 and current estimates report that 40 million people have died. Globally, 38 million people were living with HIV-1 at the end of 2013 (WHO 2014).

1.1.2 THE CURRENT HIV EPIDEMIC

Sub-Saharan Africa remains the region most severely affected by the global epidemic and accounts for 71% of the people living with HIV Worldwide (WHO 2014, Fig 1.1).

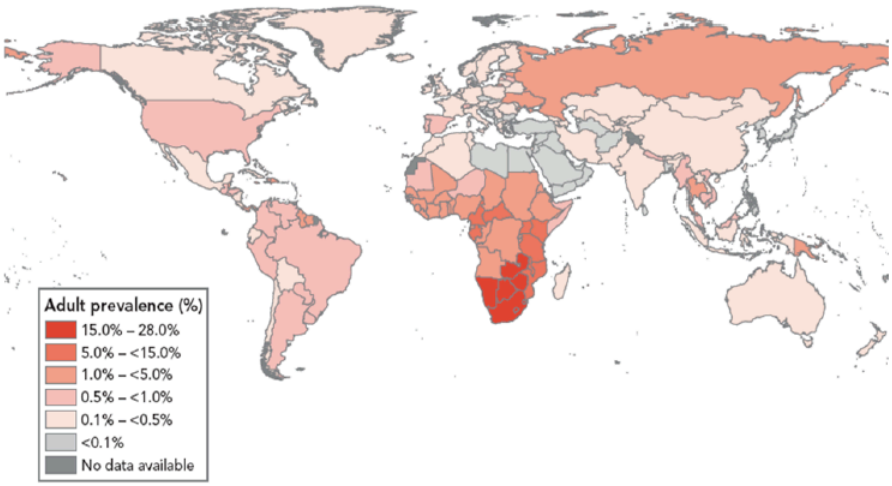


Fig 1.1. Prevalence of HIV globally. Adapted from www.unaidsrstesa.org showing the burden of disease in South Africa, Namibia, Botswana, Zimbabwe and Zambia.

Overall the global epidemic is stabilizing with a reduction in new HIV infections. However the total number of people living with HIV continues to increase because of improved access to ART (WHO 2014, Fig 1.2).

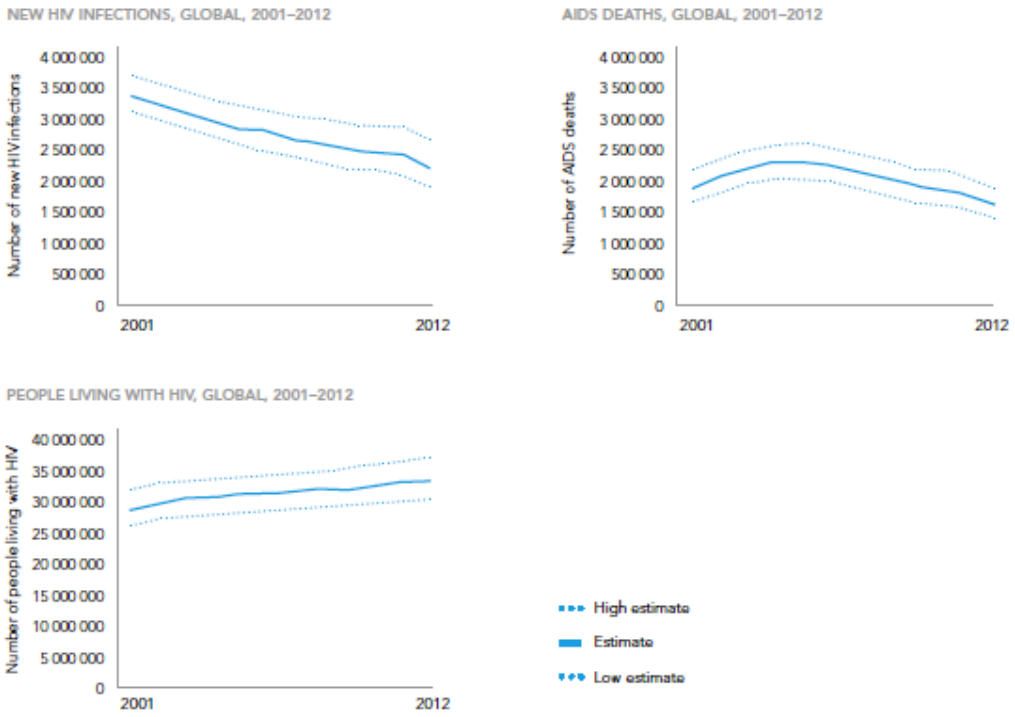


Figure 1.2. Numbers of people living with HIV, new HIV infections, and AIDS deaths, 2001-2012 globally. Source: UNAIDS 2012

1.1.3 THE PAEDIATRIC HIV EPIDEMIC

Paediatric HIV infection is a global health problem in its own right, which disproportionally affects children in the lowest income countries. Paediatric infections account for 10% of the total global epidemic, with an estimated 3.5 million children currently living with HIV-1 (UNAIDS 2014). Over 3 million children under 15 years of age were living with HIV-1 in sub-Saharan Africa in 2010, representing more than 90% of all children with HIV-1 in the World (WHO 2014). Sub-Saharan Africa is the region with the largest number of adult and paediatric infections worldwide, and consequently is one of the few places in the world where maternal and child mortality has increased since the 1990's. AIDS is the largest

cause of maternal mortality in South Africa and accounts for 35% of deaths in children under 5 (UNAIDS 2014).

The majority of paediatric infections are acquired during pregnancy, birth or breastfeeding. With the introduction of new strategies to reduce mother-to-child transmission (MTCT), the risk of transmission can be successfully reduced to <2% (WHO 2014) (Moodley *et al*. 2013). However, in countries such as South Africa, where the burden of HIV-1 is highest, and where >0.3m babies are born to HIV-positive mothers each year, even these interventions are not able to prevent MTCT altogether.

1.1.4 HIV CLASSIFICATION

HIV-1 belongs to the family Retroviridae, so named because the RNA genomes are reverse transcribed before integration with the host DNA; within this family it is further classified as a lentivirus on account of its long period of clinical latency. HIV-1 evolved from the chimpanzee virus SIVcpz which is likely to have been passed from the chimpanzee *Pan troglodytes troglodytes* to humans on at least 3 separate occasions, giving rise to the 3 main HIV groups: major (M), outlier (O), and non-major and non-outlier (N) (Gao *et al*. 1999; Korber *et al* 2000; Keele *et al.* 2006).

Group M is the predominant circulating form (Gao *et al.* 1999) and has evolved into 9 phylogenetically related clades (A-D, F-H, J-K) that vary genetically by 15-20% (Hemelaar *et al.* 2006; Buonaguro *et al.* 2007). Figure 1.3 shows the distributions of HIV-1 clades globally.

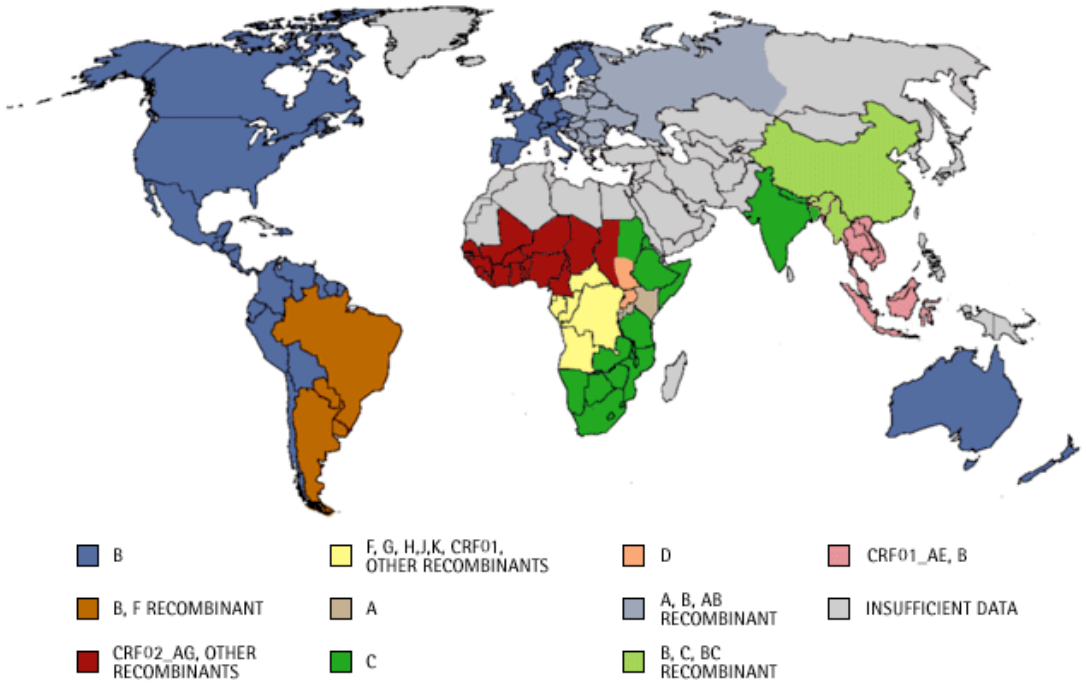


Figure 1.3 The current global distributions of HIV-1 strains. Adapted from *Hemelaar et al. AIDS, 2011*

Clade B is the predominant subtype in the USA and Europe and clade C predominates in sub-Saharan Africa. Recombination of viruses from different clades following co-infection or super-infection has resulted in circulating recombinant forms (CRF), which are now responsible for almost 20% of HIV-1 infections worldwide (Lau *et al.* 2013) and contribute to the enormous global viral diversity of HIV-1.

1.2 STRUCTURE AND FUNCTION OF HIV-1

1.2.1 STRUCTURE OF HIV-1 VIRION

The HIV-1 virion contains 2 copies of a 10kb single-stranded RNA genome of 9 genes. HIV-1 is approximately 80-120nm in diameter and enveloped by a host-derived plasma membrane. The trimeric gp120-gp41 complexes are embedded in the membrane. The cytoplasmic tail of gp41 interacts with the HIV-1 matrix protein p17. The capsid protein p24makes up the core, which contains the two positive-strand RNA copies of the genome that are surrounded by the nucleocapsid protein and summarized in Fig 1.4.

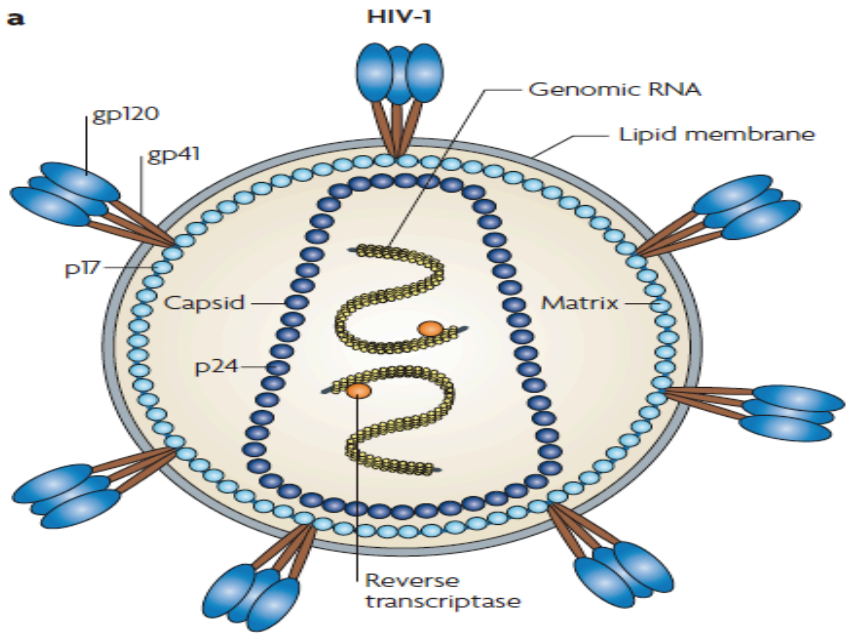


Fig 1.4 Schematic diagram of HIV-1. Adapted from Karlsson *et al.* 2008.

1.2.2 THE HIV-1 GENOME

The HIV-1 genome is approximately 9.7 kilobases, containing 9 genes and encoding 15 proteins (Fig 1.5). At either end of the genome are identical sequences, referred to as long terminal repeats (LTRs), which are involved in transcription and proviral DNA integration. As with all retroviruses HIV-1 possesses 3 principal genes: *gag, pol* and *env*. Of these the *gag* gene encodes the structural proteins p17, p24 and p7, which form the viral matrix, capsid and nucleocapsid respectively. *pol* encodes the viral enzymes protease (PR), reverse transcriptase (RT) and RNase H, and integrase (INT); and *env* encodes the surface glycoprotein's gp120 and gp41, which are involved in target cell binding and entry. In addition, HIV-1 possesses 6 accessory genes, *vif, vpr, vpu, tat, rev* and *nef* that regulate its replication cycle and interact with and modulate the host cell. The roles of the various proteins are summarized in Table 1.1.

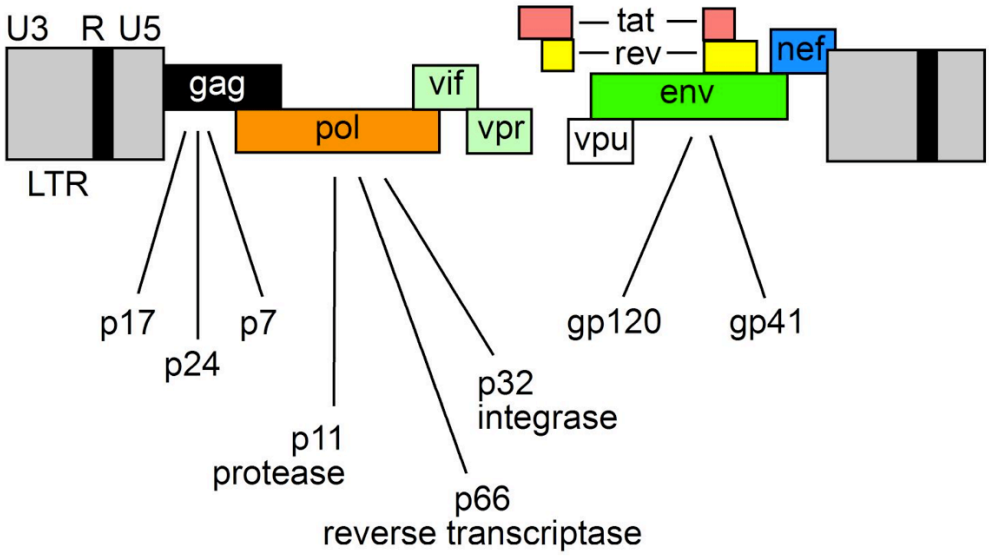


Fig 1.5 The HIV-1 Genome. Adapted from http://hivbook.com/tag/viral-genome/

1.2.3 VIRAL LIFE CYCLE

In intact mucosal surfaces, models suggest that free virions may penetrate gaps between epithelial cells or invade via microabrasions in epithelial surfaces to reach the deeper layers of the mucosa (Hladik *et al.* 2007).

The main cells targeted by HIV-1 are CD4+ T lymphocytes, though other cell types expressing the CD4 cell surface receptor are also susceptible to infection. These include macrophages and dendritic cells. Alongside CD4 a further co-receptor is required in order for HIV to attach to and gain entry to cells. These are the chemokine receptors CCR5 (R5 tropic), which is the co-receptor predominantly used, or CXCR4 (X4 tropic), use of which is associated with disease progression.

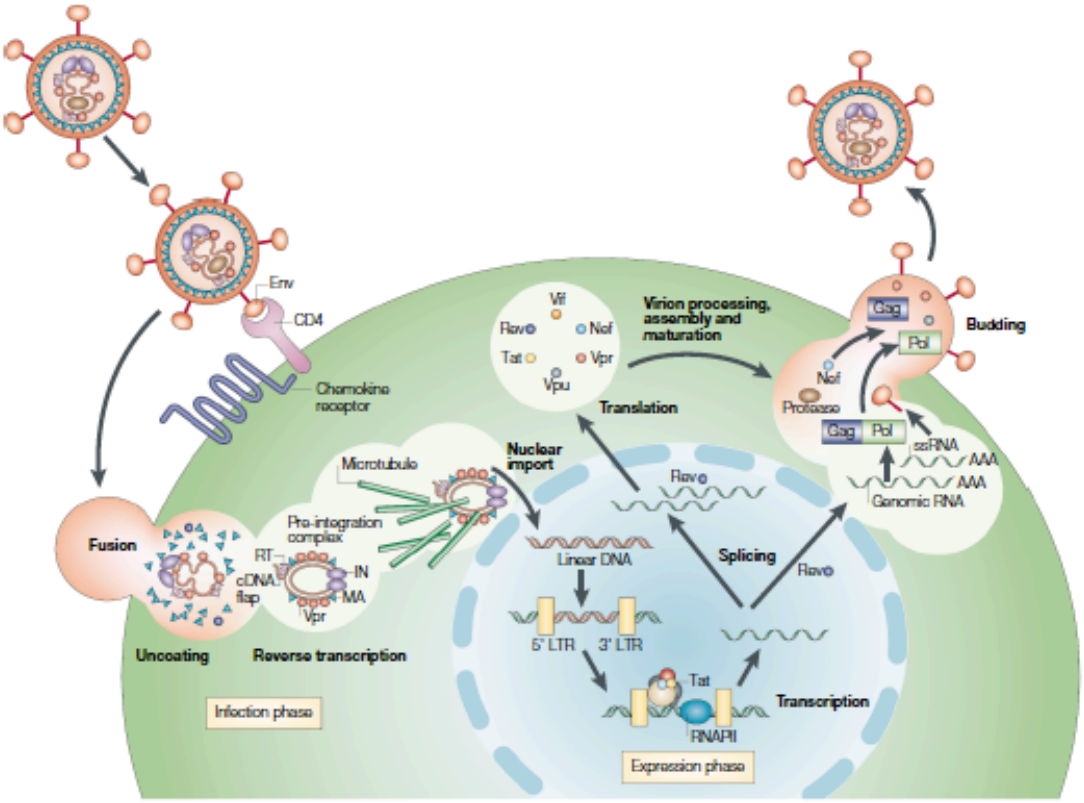


Figure 1.6 The lifecycle of HIV-1. Adapted from Peterlin and Trono 2003.

The lifecycle of HIV-1 is summarized in Figure 1.6. HIV entry into cells is facilitated by the env glycoprotein gp160, comprised of surface and transmembrane subunits, gp120 and gp41 respectively. Initially gp120 binds to its primary cell surface receptor CD4 and then, following conformational changes, to its coreceptor CCR5 or CXCR4 (Choe *et al.* 1996; Dragic *et al.* 1996; Feng *et al*. 1996). Binding of these 2 receptors exposes gp41, which then mediates cell fusion and internalization of the viral core into the host cell cytoplasm.

Once inside the cell, the RNA genome is converted to DNA by the viral RT enzyme. The double stranded DNA proviral genome generated by reverse transcription is then actively transported into the nucleus and integrates into the host genome. This process is facilitated by Vpr, Int and p17. Once integrated, the HIV provirus is either transcribed using host cell machinery to produce new viral proteins, or persists as a latent infection through mechanisms that, as yet, remain unclear (Dahabieh *et al*. 2015; Battistini and Sgarbanti 2014).

Transcription of the proviral genome is carried out by host cell RNA polymerases, and this transcript is spliced in various ways to form the viral proteins described above. The Tat, Rev and Nef proteins are produced first from completely spliced mRNA templates, followed by Gag, Pol, Env and accessory proteins Vpr, Vpu and Vif, which are encoded by unspliced mRNAs (Klotman *et al.* 1991). These viral proteins are transported to the cell membrane where they are assembled to form a viral particle, which then surrounds itself with a lipid bilayer membrane as it buds out from the host cell. Following budding, final processing of the Gag and Pol proteins by encapsidated viral proteases produce mature, infectious viral particles.

Gene	Protein(s)	Function
gag	p17 matrix (MA)	Targets Gag to the plasma membrane, has a role in the incorporation of envelope proteins to nascent particles
	p24 capsid (CA)	N-terminal domain is required for formation of mature virus core, binds CypA; C-terminal domain is important in both particle assembly and core formation
	p7 nucleocapsid (NC)	Essential for specific packaging of two copies of viral RNA into virion
	p6	Unique to HIV-1, variable in length and sequence, promotes detachment of assembled virions from cell and each other, binds Vpr
pol	Protease	GagPol cleavage, maturation of translated viral proteins
	Reverse Transcriptase	Reverse transcription of ssRNA into ds DNA
	Integrase	Integration of provirus into host cell genome
env	gp120	Protein on surface of virion that contains the binding site for CD4
	gp41	Mediates cell fusion
tat	Trans-activator of transcription	Activates viral gene expression
nef	Negative factor	Downregulates CD4 and MHC class I, increases viral infectivity
rev	Regulator of viral expression	Binds to the Rev-responsive element and promotes nuclear export and stabilization of viral mRNAs
vif	Viral infectivity factor	Promotes the infectivity of viral particles, prevents the action of host cell APOBEC-3G
vpu	Viral protein U	Degradation of CD4 in the ER, enhancement of virion release
vpr	Viral Protein R	Nuclear import of viral dsDNA, causes cell-cycle arrest

Table 1.1 HIV protein function. Adapted from www.hiv.lanl.gov

1.3 PATHOGENESIS OF ADULT HIV-1 INFECTION

1.3.1 CLINICAL COURSE OF DISEASE

The natural course of HIV-1 infection can be divided into three stages, acute infection, chronic infection and AIDS progression. In the first few weeks of acute HIV-1 infection, the level of viraemia typically rises to around 10^7 HIV RNA copies per ml of plasma. This subsequently declines during the following weeks by 10^2-10^3 copies per ml to a relatively stable viral set-point with a median of around 30,000 copies per ml, which is maintained during chronic infection (Fig 1.7). The particular set-point established in each individual is strongly predictive of the time it will take for that person to progress to AIDS; lower viral set-points predict slower progression to AIDS and higher viral set points predict more rapid progression (Mellors *et al*. 1996). HIV-1 preferentially targets and infects CD4+ T cells at mucosal sites and there is a dramatic depletion of CD4+ T cells in the gut during acute infection (Mattapalli *et al.* 2005). On average, the chronic stage of the disease spans 9 years, and is characterized by the progressive loss of systemic CD4+ T cells. When peripheral blood CD4+ T cell counts drop below 200 per ul of blood, HIV-infected subjects are prone to the opportunistic infections characteristic of AIDS.

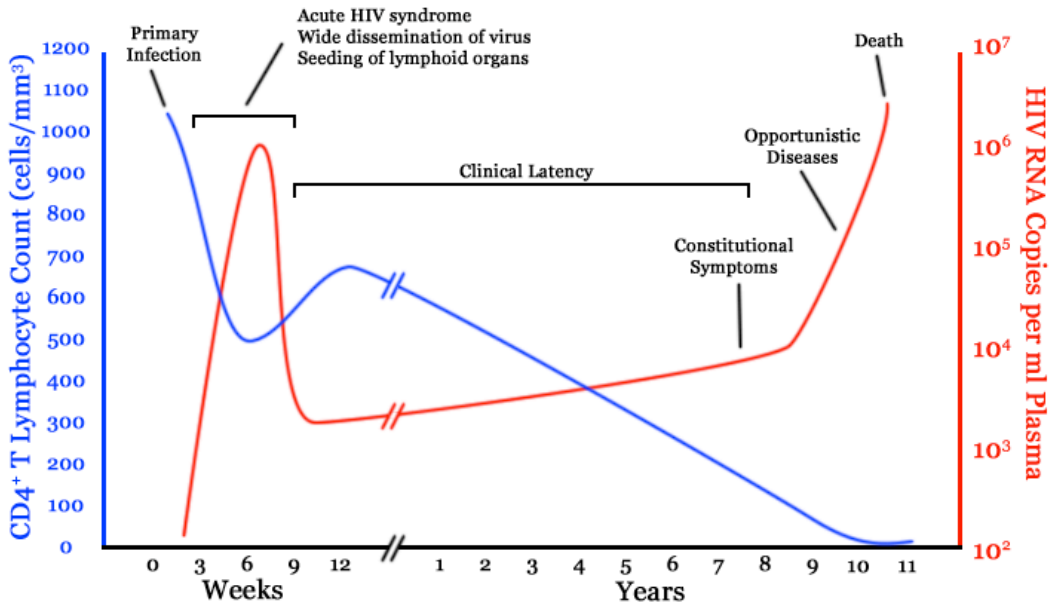


Figure 1.7. Typical relationship between HIV viral load and CD4 count over the average course of untreated HIV infection. Adapted from Rowland-Jones 2003.

1.3.2 TRANSMISSION AND VIRAL DISSEMINATION

The majority of HIV-1 infections worldwide are the result of transmission across mucosal membranes, including those found in the vagina, foreskin, rectum, and the lower genital tract (Shattock and Moore 2003), although the blood, placenta and gastrointestinal tract are also important sites of viral entry.

CD4+ T cells, macrophages and dendritic cells, such as those that reside in the vaginal mucosa, are early targets of HIV-1 infection and HIV-1 infects intraepithelial CD4+ T cells through CD4 and CCR5 receptor-mediated fusion. Dendritic cells (DCs) located in the vaginal mucosa, including vaginal Langerhans cells (LCs) and immature myeloid DCs take up viral particles by endocytosis (Hladik *et al*. 2007) but do not become productively infected. Immature DCs expressing C-type lectins, such

as DC-SIGN and langerin, capture HIV by binding the HIV envelope protein gp120 (Turville *et al*. 2002). These DCs can then migrate to lymphoid tissues and enhance viral dissemination through *trans*-infection of activated CD4+ T cells. *Trans*-infection of CD4+ T cells by DCs can occur via cell-cell contact also known as a virological synapse, (Arrighi *et al.* 2004) and may also occur by DC-derived exosomes (Wiley and Gummuluru 2006). Some DC subsets are susceptible to HIV infection, and they subsequently infect neighbouring CD4+ T cells through *cis*-infection, contributing further to viral dissemination.

Regardless of the initial route of infection, CD4 T cells in the gut associated lymphoid tissue (GALT) become particularly targeted within days of viral propagation. In the SIV-macaque model, up to 60% of resting memory CD4 cells in the GALT are infected during primary infection, and 80% of these are destroyed within 4 days, both by direct infection and bystander apoptosis (Mattapallil *et al.* 2005). This massive gut depletion is also seen in acute HIV infection with IL-17-producing mucosal CD4 cells (Th17 cells) being particularly targeted (Brenchley *et al.* 2008; Raffatellu *et al*. 2008; Bixler and Mattapallil 2013; Chege *et al.* 2011; Nigam *et al.* 2011; Klatt *et al*. 2012; Lu *et al*. 2015). The reason for preferential gut targeting is twofold: first, there is a high density of vulnerable CD4+CCR5+ cells in the GALT (Brenchley *et al.* 2004) and second, HIV is able to localize to the gut through interaction of its gp120 molecule with the gut-homing integrin $\alpha 4\beta 7$ (Arthos *et al.* 2008). Thus, very early in HIV infection there is profound CD4 T cell loss, damage to the gut mucosa, establishment of latent proviral reservoirs and widespread viral dissemination.

1.3.3 MOTHER TO CHILD TRANSMISSION (MTCT)

Without interventions to prevent MTCT transmission, 30-40% of infants with HIV-positive mothers acquire HIV, and this can occur in utero (5-10%), intrapartum (15%) or postnatally through breastfeeding (15%) (Prendergast *et al.* 2007). The timing of HIV acquisition has been shown to affect disease outcome, with an estimated 1-year mortality for perinatally infected children of 52%, compared with 26% for postpartum-infected children (Becquet *et al.* 2012). In spite of prevention strategies that have been developed, there were still 240,000 new infections in children in 2013, a result of suboptimal antenatal HIV testing, inadequate coverage of prevention strategies of MTCT, the occurrence of acute infections during pregnancy, and the overall benefits of prolonged breastfeeding (Prendergast et al. 2012). WHO guidelines instituted in 2008 recommended that all HIV-infected infants be placed on ART irrespective of CD4 count, and in 2013 recommended that all HIV-infected children 5 years old or younger receive ART. From 2016, recommendations now are that all HIV-infected children should receive ART. Currently the outlook for them is a life-time of ART. Aside from the long-term risks of drug toxicities, and the cost, ART adherence is also major issue especially in adolescence, raising the concern of a drug-resistant epidemic fuelled by HIV-infected adolescents.

1.3.4 IMMUNE ACTIVATION IN HIV-1 INFECTION.

Systemic chronic immune activation is considered a major driving force underlying CD4+ T cell depletion and AIDS. Levels of immune activation have been shown to predict CD4+ T cell decline better than, and independently of, viral load (Giorgi *et al.*

1994; Leng *et al*. 2001; Deeks *et al.* 2004; van Asten *et al.* 2004). Several mechanisms contribute towards the increased immune activation in HIV infection and are summarized in Fig 1.8.

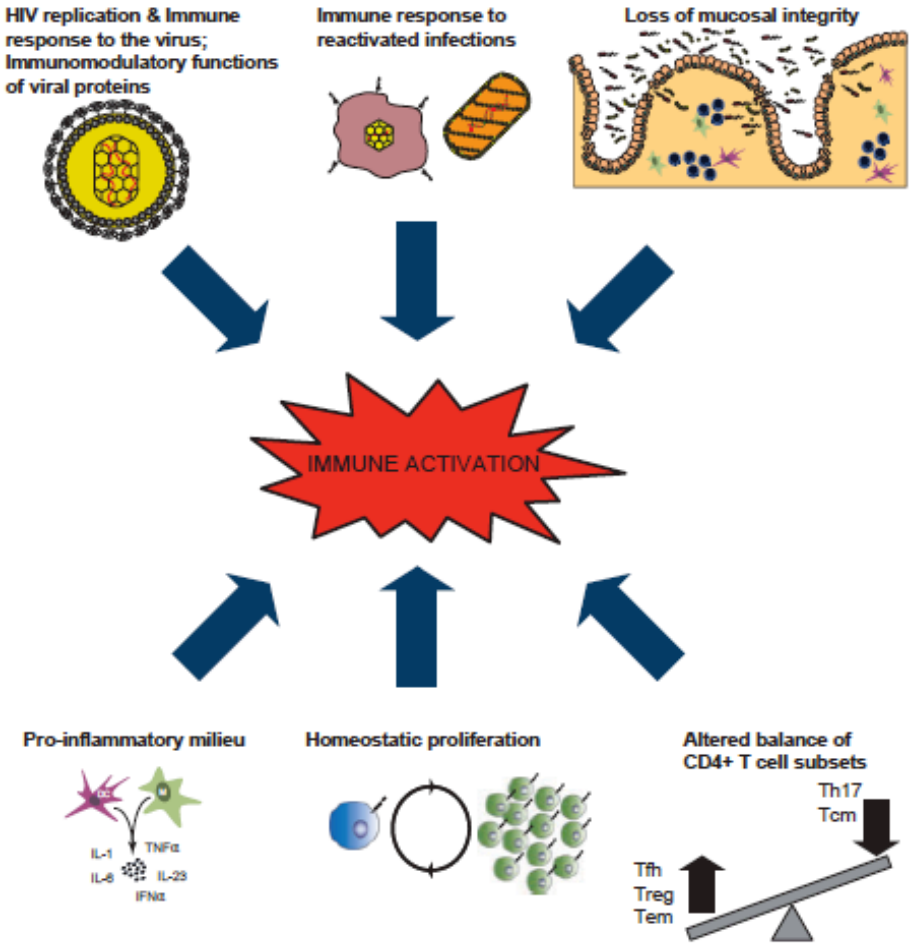


Fig 1.8. Potential contributors to HIV-associated chronic immune activation.

(Adapted from Paiardini, Muller-Trutwin. 2013).

The direct effect of the innate and adaptive immune responses against the virus are the most obvious cause of increased immune activation in HIV infection. Up to 20% of circulating CD8+ T cells in chronically infected individuals are HIV-specific and these cells are highly activated due to viral persistence (Appay *et al*. 2002). Components of HIV such as gp120 and Nef are capable of activating T cells directly

(Merrill *et al.* 1989; Wang *et al*. 2000; Simmons *et al*. 2001; Schindler *et al.* 2006). HIV components (such as ssRNA) also bind to pattern recognition receptors, such as the Toll-like receptors 7 and 9 (Beignon *et al.* 2005; Heil *et al.* 2004; Meier *et al.* 2007). In addition, during the process of entry and fusion, HIV may activate target cells by signaling through CD4 and the co-receptors CCR5 and CXCR4. Chronic herpesvirus infections, such as CMV and EBV, may reactivate during the course of HIV disease and virus-specific cells become activated in order to contain these co-infections (Doisne *et al.* 2004; Wittkop *et al*. 2013; Hunt *et al*. 2011). A key contributor to HIV-associated chronic immune activation is the damage caused to the GI tract. The massive depletion of CD4 cells observed in the GI tract in the first weeks of infection results in a loss of intestinal epithelial cells, disruption of tight junctions and compromised integrity of the mucosal intestinal barrier. Loss of mucosal integrity favours translocation of bacterial and fungal products – including peptidoglycan, lipoteichoic acid, LPS, flagellin, ribosomal DNA and unmethylated CpG-containing DNA, from the intestinal lumen to the systemic circulation, where they can activate lymphocytes and monocytes (Brenchley *et al.* 2004; Brenchley *et al.* 2006; Douek. 2007; Ancuta *et al.* 2008; Estes *et al*. 2010; Gordon *et al.* 2010; Wallet *et al*. 2010; Klatt *et al*. 2013). Through the stimulation of several TLRs, these microbial products may activate and induce production of pro-inflammatory cytokines such as TNFa, IL-6, IL1B and type I interferon's (Connolly *et al.* 2005; Alter *et al*. 2007; Appay *et al.* 2008; Iannello *et al.* 2009; Kwa *et al*. 2011). LPS levels are higher in chronically HIV-infected subjects compared with to HIV-uninfected individuals, and these LPS levels correlate with CD8 T cell activation (Wallet *et al.* 2010; Brenchley *et al.* 2006; Redd *et al*. 2009). Studies of a rare subset of HIV-infected individuals, termed adult viraemic non-progressors (AVNPs), who maintain

high and stable CD4+ T-cell counts for many years, despite levels of viral replication similar to those found in non-controllers, show a profile of very low immune activation (Klatt *et al*. 2014; Choudhary *et al*. 2007; Rotger *et al.* 2011).

1.3.5 LESSONS FROM PRIMATE MODELS OF DISEASE

The nonpathogenic nature of SIV infection in sooty mangabeys (SM) or African green monkeys (AGM), who maintain high CD4s in the setting of high viral loads, has been attributed to the low level of immune activation in these animals (Silvestri *et al.* 2003; Bosinger *et al.* 2009). This contrasts with the high levels of immune activation observed in pathogenic infection in HIV-infected humans and SIV-infected rhesus macaques (RM). Rhesus macaques are Asian primates, and, unlike sooty mangabeys or African green monkeys, are not naturally infected with SIV. Indian rhesus macaques experimentally infected with SIV represent the best-studied model of HIV. In acute infection in SM/AGM there is a robust, albeit transient, innate immune response (Bosinger *et al.* 2009; Harris *et al.* 2010), leaving unresolved high levels of viraemia (setpoint $\sim 10^5$ copies/ml). In contrast, resolution of high viraemia occurs in acute SIV infection in RM, which is dependent upon the activity of the CD8+ T-cell response (Schmitz *et al*. 1999). Blocking of the CD8+ T-cell response using monoclonal anti-CD8 antibodies in SM does not affect viral load. The lack of impact on viral setpoint of the CD8+ T-cell response in the SM/AGM compared to RM has been related to the early resolution of immune activation in SM/AGM, which is correlated with the early expression of PD-1 (Estes *et al.* 2008). In RM, immune activation is not more prominent in acute infection than in SM/AGMs but is maintained throughout the course of infection, and PD-1 expression is only

observed in chronic infection. Decreased immune activation enables SIV-infected SM or AGM to avoid the bystander damage observed in pathogenic infections and protects them from developing AIDS (Silvestri *et al*. 2003), this mechanism is summarized in Figure 1.9.

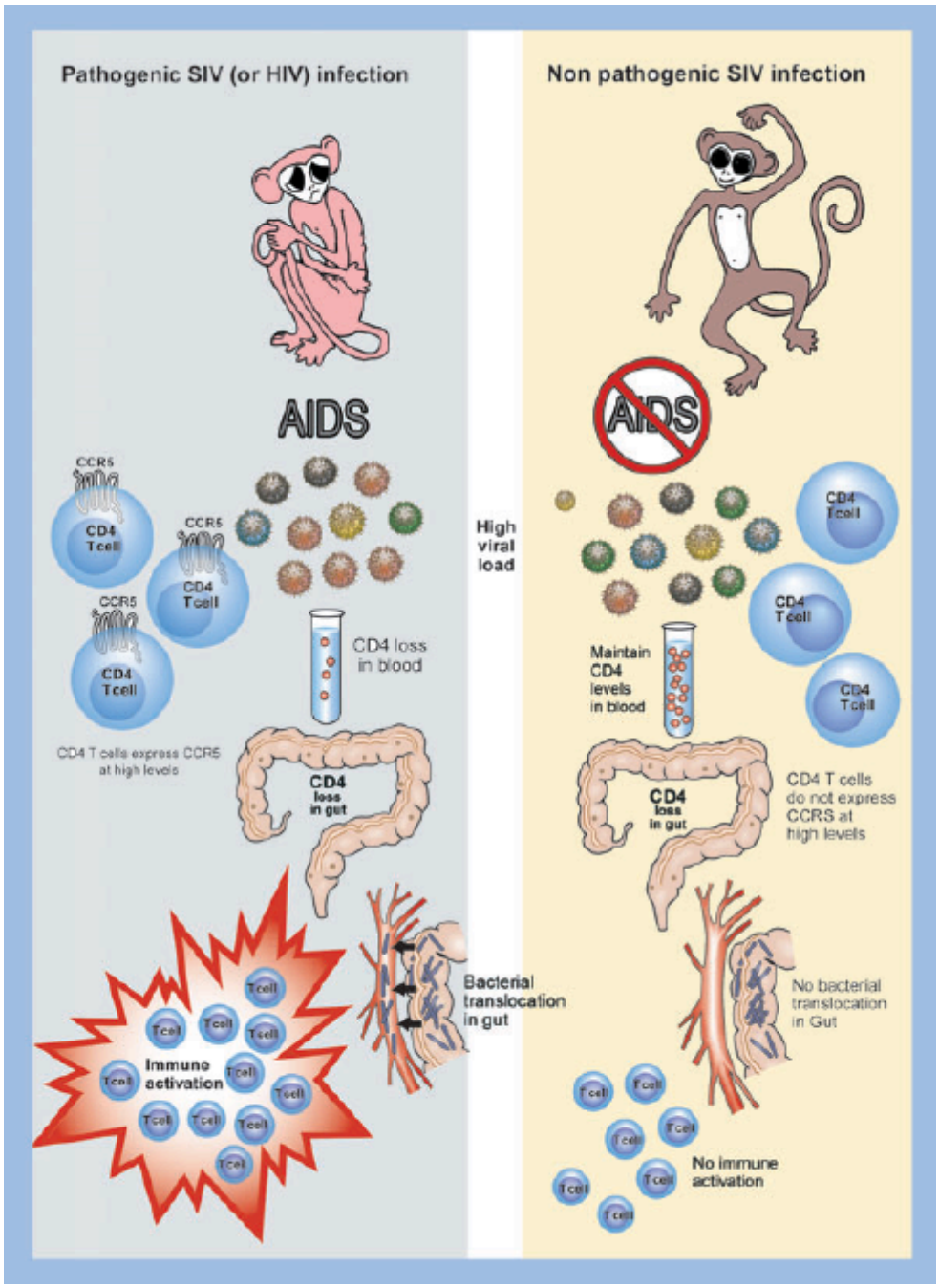


Fig 1.9 Comparison of pathogenic and non-pathogenic SIV infection. Adapted from Silvestri *et al.* 2008)

In the sooty mangabey, central memory CD4+ T-cells express low levels of CCR5, and in the African green monkey CD4 expression is low on CD4+ central memory cells. Thus, via distinct mechanisms, these natural hosts of SIV infection appear to ensure that viral replication arises principally within the effector cell population, a pool of cells that is already highly activated and programmed to die. In contrast, long-lived naïve and central memory cells are preserved (Pandrea *et al.* 2007; Paiardini *et al*. 2011).

1.4 IMMUNOLOGY OF HIV-1

1.4.1 INNATE IMMUNITY TO HIV-1

Mounting evidence suggests a role for innate immunity in the early control of HIV infection before the induction of adaptive immune responses. Certain innate immune defence mechanisms have been recently been identified in elite controllers of HIV-1.

NK cells can rapidly kill HIV-1 infected cells via direct cytolytic activity or through the secretion of cytotoxic cytokines (Altfeld *et al.* 2011). Inhibitory receptors interact with classical and non-classical class I MHC molecules, whilst activating receptors interact with surface-expressed proteins derived from pathogens or produced during cellular stress. By killing HIV-1 infected cells, NK cells can exert immune pressure on HIV-1 and influence viral sequence evolution (Alter *et al.* 2011). During HIV-1 infection, phenotypic and functional perturbations of NK cells

have been described, that are likely related to ongoing high-level viraemia and to the associated immune activation (Barker *et al.* 2007; Vieillard *et al.* 2010).

Plasmacytoid DCs (pDCs) possess pattern recognition receptors, called toll-like receptors (TLR), which recognize common components of microorganisms. Through binding to TLR 7 and 9, HIV induces secretion of type I interferon's (IFNα and β), which exert antiviral activities. Higher levels of IFNα secretion are seen in elite controllers compared to HIV-1 progressors (Barblu *et al.* 2012). The secretion of type I interferons causes cells to shut down viral production by limiting uptake of nutrients, degrading RNA and arresting cell cycle progression (Chang & Altfeld. 2009).

γδ T cells have also been shown to play a role in elite control. Typically the number of γδ T cells expressing the Vγ2Vδ2 TCR is decreased early in HIV-1 disease, however in elite controllers it has been shown that levels of fully functional Vγ2Vδ2 T cells are maintained in peripheral blood (Riedel *et al.* 2009).

Humans possess a number of additional innate defense mechanisms to protect against retroviral infection. Target cells possess intrinsic intracellular defence mechanisms termed 'viral restriction factors'. Three such factors that have clear antiviral effects in the HIV field are APOBEC3G, TRIM5α and tetherin. APOBEC3G induces G to A hypermutation by interfering with the reverse transcription step in the retrovirus life cycle. However, HIV-1 blocks the activity of APOBEC3 through the binding of the Vif protein (Kremer & Schnierle 2005). TRIM5α on the other hand potently blocks HIV-1 infectivity by binding to the capsid protein (Stremlau *et al.* 2004). Again HIV-1 circumvents this restriction factor by binding a second host-

derived protein, Cyclophilin A, which blocks the TRIM5α binding site (Towers *et al.* 2003). Tetherin prevents normally assembled HIV-1 particles from being released from the surface of the infected cell (Neil *et al.* 2006). Vpu is required to overcome this obstacle to HIV-1 particle release (Neil *et al.* 2008).

Protection from HIV infection and disease may be conferred by polymorphisms within the HIV coreceptors or their ligands. Homozygous expression of a 32-base pair deletion within the CCR5 coreceptor (CCR5Δ32) leads to a truncated protein, lack of CCR5 expression and protection from infection (Liu *et al*. 1996); heterozygous expression of CCR5Δ32 leads to a delayed progression to AIDS (Dean *et al.* 1996). The beta-chemokines MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) are the natural ligands of CCR5. Two additional variants named CCL3L1 and CCL4L1, encoded by genes arising from the duplication of CCL3 and CCL4, respectively have also been described (Menten *et al.* 2002). CCL3L1, also known as MIP-1αP, is the more potent CCR5 agonist and the strongest inhibitor of infection by R5 HIV-1 strains (Irving *et al.* 1990). CCL3L1 can influence susceptibility to HIV infection and disease progression (Gonzalez *et al.* 2005). Finally, polymorphisms in the non-coding region of SDF-1 (also known as CXCL12), a CXCR4-binding chemokine, are associated with delayed progression to AIDS (Winkler *et al.* 1998; Arya *et al*. 1999).

1.4.2 ADAPTIVE IMMUNITY TO HIV-1

The adaptive immune response consists of antibody and cell-mediated responses which are carried out by B lymphocytes and T lymphocytes, respectively. B cells are

the major cells involved in the secretion of antibodies which bind specifically to foreign antigens. Cell-mediated immunity involves the activation of macrophages, NK cells, antigen-specific cytotoxic T lymphocytes (CTL) and the release of various cytokines in response to antigen. The key feature of the adaptive immune response, and hence one distinction from innate immunity, is the specificity of the response and the development of immunological memory. However it takes several days for the adaptive immune response to be generated (Fig 1.10). Before it is presented to a T cell, antigen is first processed by an Antigen Processing Cell (APC). This involves the enzymatic degradation of the antigen into small peptides: typically peptides of 8-9 amino acids long become bound to HLA class I molecules, or peptides 12 amino acids or more in length to HLA class II. MHC molecules act as receptors for processed peptides. Class II MHC molecules process proteins that are taken up by the APC by phagocytosis or some other form of endocytosis and degraded within the lysosomal compartment. The delay in establishing an effective adaptive HIV-specific response contributes to the rapid seeding of latent reservoirs that occur before antiviral T cells are generated. Establishment of latent reservoirs is one of the major challenges to HIV eradication (Alexaki *et al.* 2008; Groot *et al*. 2008; Sattentau *et al*. 2008; Richman *et al*. 2009; Lewin & Rouzioux 2011).

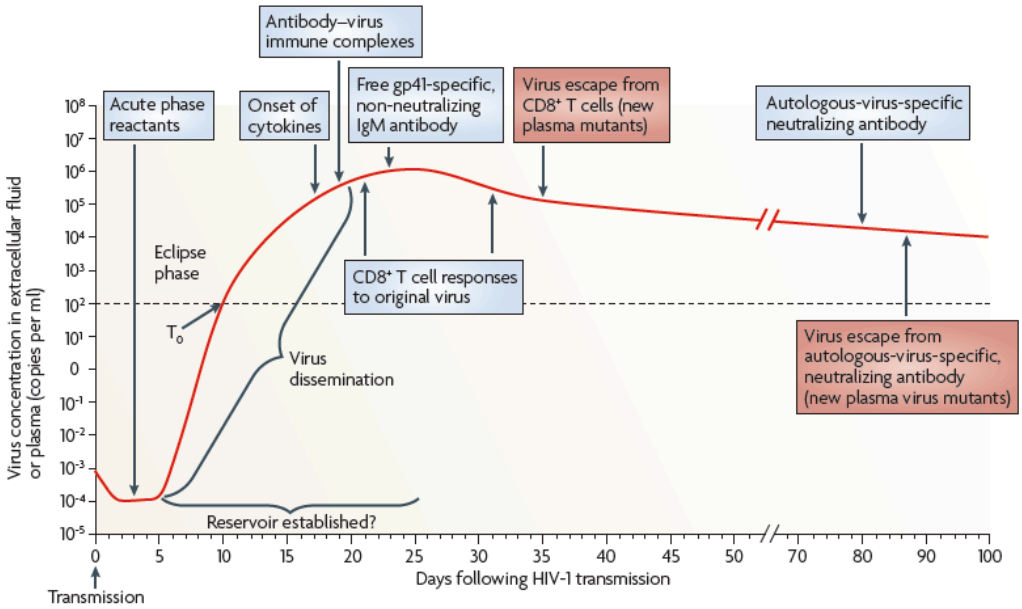


Fig 1.10. Earliest innate and adaptive immune responses to HIV-1 (adapted from McMichael *et al.* 2010).

1.4.3 ROLE OF ANTIBODIES

HIV-1 infection elicits antibody responses specific for a range of proteins including Env, Gag, Tat, Nef, Int and RT and appear in a predictable order following infection. The first antibody response develops within one week of detectable viraemia and consists of an anti-gp41 IgM which undergoes class switching to IgG and IgA (Tomaras *et al.* 2008). This is followed by non-neutralising anti-gp120 antibodies, which primarily target the V3 loop. Neutralising antibodies (NAbs) appear several weeks later (Wei *et al*. 2003; Richman *et al*. 2003; Gray *et al.* 2007; Mikell *et al*. 2011; Tomaras *et al*. 2011) and can be detected 3-8 weeks later in C clade infected subjects and up to 13 weeks post infection in B clade infected subjects (Davis *et al.* 2009).

Antibodies have the potential to block cell-cell HIV spread by various mechanisms. NAbs can bind and neutralise cell-free virus, HIV-1 specific antibodies can form a complex between the IgG Fab portion of antibody bound to envelope protein on the cell surface and the Fc portion to the Fc receptors on effector cells thereby leading to antibody-dependent cellular cytotoxicity (ADCC) (Overbaugh *et al.* 2012). Antibodies are also able to exert immune pressure on the virus that leads to escape.

Antibodies that effectively neutralize HIV-1 target the HIV-1 viral spike – a trimeric heterodimer composed of gp120 and gp41 (Wyatt & Sodroski. 1998; Pantophlet & Burton 2006; Moore *et al.* 2009, Rong *et al.* 2009).

The V1V2 region and sites at the base of the V3 loop are major targets for autologous NAbs in HIV-1 subtype B infection (Pinter *et al.* 2004; Rong *et al.* 2007; Tang *et al*. 2011). However in subtype C infection, the C3 region, specifically the α2-helix are the major target of Nabs, this is due to the amphipathic nature of the subtype C α2-helix in combination with a shorter V4 loop (Gnanakaran *et al.* 2007; Rong *et al.* 2009).

NAbs tend to increase in potency over time and broadly cross neutralizing responses capable of recognizing heterologous HIV-1 variants develop only in approximately 20% of individuals after primary infection (Binley *et al*. 2004; Li *et al*. 2007; Sather *et al*. 2009; Stamatatos *et al*. 2009; Doria-Rose *et al*. 2010; Walker *et al*. 2010; Gray *et al*. 2011). The reason why some individuals develop BCN antibodies is unclear but is related to the duration of infection and viral levels suggesting that years of persistent viral stimulation are necessary for their generation (Piantadosi 2009, Sather *et al*. 2009; Euler *et al.* 2010; Gray *et al.* 2011).

1.4.4 ROLE OF CD8+ T CELLS

CD8+ T cells (also known as Cytotoxic T Lymphocytes; CTL) are an arm of the adaptive immune response that recognize and kill virally infected cells through recognition of viral antigen in complex with HLA Class I molecules on the surface of infected cells. The specificity of this recognition is due to the highly specific T cell receptor (TCR) of the CD8+ T cell, which engages with the HLA allele/peptide complex.

There are several strands of evidence indicating the importance of CD8+ T cells in the control of HIV viraemia. *In vivo* studies demonstrate that, following acute infection, the decline in peak viraemia is temporally associated with the appearance of HIV-specific CD8+ cytotoxic T lymphocyte populations, suggesting that CTL may be responsible for reducing viral load at this stage of infection (Borrow *et al.* 1994; Koup *et al.* 1994). Subsequent studies in the SIV-macaque model have confirmed the effect of CTL by administering CD8-specific monoclonal antibodies to block the CD8+ T cell response. In acute infection, viraemia remains high and sustained, and in chronic infection viral load rebounds, suggesting uncontrolled viral replication (Jin *et al.* 1999; Schmitz *et al.* 1999).

The critical role of HLA class I in shaping disease outcome (Goulder and Walker 2012) also points to the importance of the CD8+ T-cell response in control of HIV replication. Amongst a multitude of possible mechanisms, a key one would appear to be the ability of protective HLA class I molecules such as HLA-B*57 to present a

broad array of Gag-specific epitopes (Kiepiela *et al*. 2007). This is discussed further below.

In addition to their specificity, the functionality of CD8+ T cells has been also associated with viraemic control. Their ability to exert a selection pressure on HIV (Kiepiela *et al*. 2004; Leslie *et al*. 2004; Fellay *et al.* 2009; Leslie *et al*. 2010; Pereyra *et al*. 2010; Kloverpris *et al.* 2012; Carlson *et al*. 2012; Matthews *et al*. 2008), proliferative capability (Migueles *et al.* 2002; Horton *et al*. 2006; Day *et al*. 2007; Appay *et al* 2008; Streeck *et al* 2008), the capacity to produce multiple cytokines (Appay *et al*. 2000; Akinsiku *et al*. 2011, Betts *et al* 2006; Harari *et al*. 2007; Almeida *et al*. 2007; Jagannathan *et al*. 2009), perforin expression (Miguelles *et al*. 2002), and lytic granule loading (Harari *et al*. 2009) are all important for viral clearance.

1.4.5 ROLE OF CD4+ T CELLS

CD4+ T cells recognize antigenic peptides in the context of MHC class II molecules. CD4+ T cell activity is required for both effective CD8+ T cell responses against HIV (Shedlock *et al*. 2003; Sun *et al*. 2003), and for the induction of NAbs by B cells.

Preliminary evidence for the role of these cells comes from observations that the preservation of HIV-specific CD4+ T cell populations and sustained IL-2 production are correlated with improved immune control (Rosenberg *et al.* 1997) and that certain MHC Class II molecules in Rhesus macaques are associated with enhanced protection (Giraldo-Vela *et al*. 2008). CD4+ T cell responses directed towards Gag and Nef specific macrophages have been demonstrated in CD8-depleted macaques,

suggesting their primary role may be in suppressing infection in the macrophage population (Sacha *et al.* 2009).

CD4+ T cells can be divided into subpopulations based on functional attributes. Th1 cells are defined by their ability to produce cytokines such as IFNγ, TNFα and IL-2 in response to viral infections and play a critical role in anti-tumor immunity (Mossmann *et al*. 1986; Kennedy *et al.* 2008). Th1 cells have also been shown to have a direct cytotoxic ability against HIV (Orentas *et al*. 1990; Sacha *et al.* 2009). Th2 cells, in contrast, are activated in response to bacterial, helminth or parasitic pathogens (Zhu & Paul 2008) and provide help for B cells to produce IgM, IgA, IgE and IgG isotype antibodies which induce the humoral immune response (McHeyzer-Williams & McHeyzer-Williams 2005). Th17 cells are IL-17 secreting CD4+ T cells and are important for resistance to extracellular bacteria and fungi (Harrington *et al.* 2005; Park *et al*. 2005). A further subpopulation of CD4+ T cells are the follicular helper T cells (Tfh). Upon antigenic stimulation the Tfh cells produce IL-21 and home to B cell follicles where they play an essential role in the differentiation of B cells into germinal center B cells and antibody secreting plasma cells (Breitfeld *et al*. 2000; Crotty *et al*. 2011). Finally there is an additional subpopulation of CD4+ T cells called regulatory T cells (Tregs) that negatively regulate the immune system and serve to prevent autoimmunity and immunopathology (Wing & Sakaguchi. 2010). Tregs at the sites of infection are associated with dysfunctional CD8+ T cells and can inhibit both HIV-specific CD4+ and CD8+ T cell responses (Andersson *et al.* 2005; Kinter *et al*. 2004; Aandahl *et al*. 2004). In HIV patients, Treg numbers are reduced in peripheral blood (Kinter *et al.* 2004). This is associated with an increase in Tregs in lymphatic tissues, suggesting either redistribution upon infection and/or active

proliferation in infected tissue (Nilsson *et al*. 2006; Andersson *et al*. 2005). In HIV controllers a lower frequency of Treg responses has been reported (Hunt *et al*. 2011) suggesting that Tregs indeed contribute to effector T cell dysfunction and inability to clear infection.

Although, like HIV-specific CD8+ T-cell responses, the magnitude of the CD4+ T cell response is not related to viral control (Ranasinghe *et al*. 2012), a broad Gag-specific CD4 T cell response is associated with viraemic control (Ranasinghe *et al*. 2012). However since activated CD4+ T cells are attracted to the site of infection and preferentially infected by HIV they are rapidly depleted, resulting in an impaired immune system; the degree of activation on CD4+ T-cells indeed predicts progression to AIDS (Roos *et al*. 1995).

1.4.6 DIFFERENCES IN IMMUNE CONTROL OF PAEDIATRIC VERSUS ADULT HIV-1 INFECTION

Fetal and neonatal immune adaptations facilitate intrauterine survival and provide early postnatal protection against extracellular pathogens, but they leave infants susceptible to intracellular pathogens, such as HIV, that are acquired perinatally. HIV-infected infants progress to disease more rapidly than adults (Prendergast *et al*. 2012). The median time to AIDS and death in adults is 10 and 11 years respectively (Collaborative Group on AIDS Incubation and HIV Survival, including the CASCADE EU Concerted Action. 2000) whereas in children mortality exceeds 50% by 2 years of age (Newell *et al*. 2004). In infected children viral loads are high, with a median of 10^5-10^7 copies/ml plasma in the first year or two of life (Mphatswe *et al*. 2007;

Obimbo *et al.* 2009) and these levels do not typically decline to reach adult levels (approx 10^4 copies/ml) until 3-5 years of age (Mcintosh *et al.* 1996), Fig 1.11. The CD4 count declines rapidly from birth, leading to severe infections and delayed growth and neurological development.

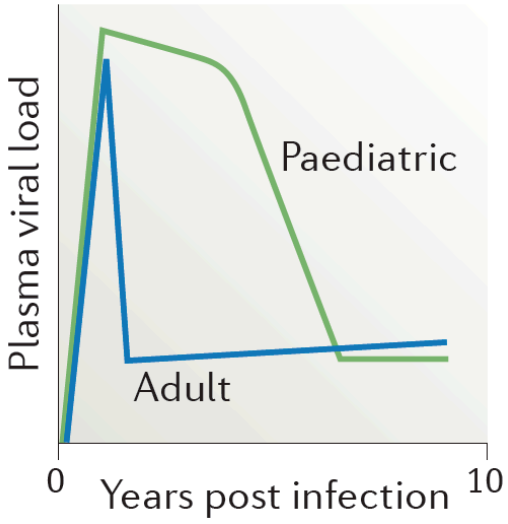


Fig 1.11. Differential outcome of HIV infection in paediatric and adult life

(adapted from Prendergast *et al* 2012).

The fetal immune system is skewed towards T helper 2 (Th2) cell (Prescott *et al*. 1998) and Treg cell populations (Mold *et al*. 2008) to avoid pro-inflammatory Th1 type responses that could be damaging in utero (Levy *et al.* 2007). During pregnancy both the fetal and maternal immune system are adapted to a state of 'immune tolerance' to prevent allogeneic T cells from damaging the fetus (Aluvihare *et al.* 2004). Regulation of Treg populations is one mechanism by which immune

tolerance is achieved. The maternal Treg pool is expanded during pregnancy and the fetus generates Treg cells that persist postnatally (Mold *et al.* 2008). Treg cells constitute up to 15% of the CD4+ T cell pool during fetal life, and are maintained above adult levels throughout childhood (van Gent *et al* 2009).

Neonatal innate antiviral immunity differs from that in adults in that it is skewed towards protection against extracellular bacterial pathogens rather than intracellular bacterial pathogens and viruses, thereby providing protection from otherwise fatal bacterial infections in early life. Antimicrobial peptides line the surfaces of epithelial cells in adults which contribute to antiviral defences but levels of these antimicrobial peptides are lower in the cord blood of newborns (Strunk *et al*. 2009).

Neonatal pDC's are less polyfunctional than those of adults (Kollmann *et al.* 2009) and in response to TLR activation produce lower amounts of type I IFN's, important for the prevention of viral spread and the induction of the adaptive immune response (Stetson *et al*. 2006). After the neonatal period type I IFN production increases and results in an increasingly effective immune response to intracellular pathogens.

Neonatal immune cells (such as monocytes, macrophages and DCs) produce lower levels of IFNγ and IL-12 than adults, these being the cytokines that support Th1 cell differentiation, and higher levels of IL-6, IL-23 and IL-1B which support Th17 cell differentiation (Kollmann *et al.* 2009, Belderbos *et al.* 2009) as summarized in Fig 1.12. Thus the immune responses of innate cells in the neonate are not 'smaller' than in adults, but qualitatively distinct, in a manner that reflects optimal adaptation to

the environment in utero and in early life. In general this immune strategy that has evolved in early life is not successful against HIV, however.

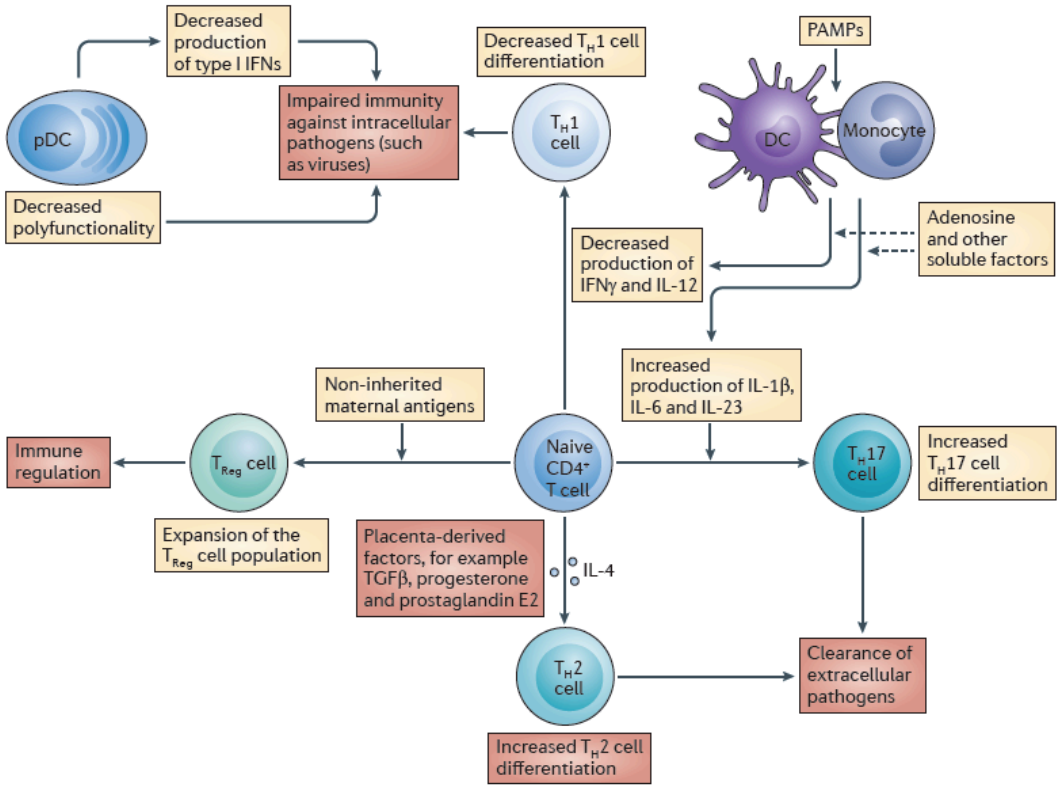


Fig 1.12 Differences in innate immunity in early and later life (adapted from Prendergast *et al*. 2012).

The adaptive immune system in infants also differs to that in adults. Although CD8+ T cell activity is detectable from birth in HIV-infected infants (Shalekoff *et al.* 2009, Thobakgale *et al*. 2007; Goulder *et al.* 2001) these responses are not sufficient to reduce viral load. Neither the breadth nor magnitude of CD8+ T cell responses predict mortality within the first year of life (Lohman *et al*. 2005). HIV-specific D8+ T-cell breadth and magnitude increase with age, however, (Shalekoff *et al*. 2009; Thobakgale *et al.* 2007; Lohman *et al*. 2005; Huang *et al.* 2008; Prendergast *et al*. 2011), the main targets of CD8+ T cells during infancy being t Nef and Env (Shalekoff *et al*. 2009; Thobakgale *et al.* 2007; Huang *et al*. 2008; Prendergast *et al*. 2011).

The most striking difference between adult and paediatric HIV infection are the undetectable or very low levels of CD4+ T cell responses that are observed in children (Thobakgale *et al*. 2007; Huang *et al*. 2008), resulting in a lack of help that is required to generate effective antiviral CD8+ T cells.

1.5 CD8+ T CELLS AND CONTROL OF VIRAEMIA

1.5.1 ROLE OF HLA CLASS I MOLECULES

The central role of HLA class I molecules and CD8+ T cell responses in control of adult HIV infection is underlined by studies that have shown characteristic rates of disease progression, depending upon the particular HLA molecules expressed (Pereyra *et al.* 2010; Fellay *et al*. 2009; Fellay *et al.* 2007; Goulder & Walker 2012). HLA-B*57, B*5801 and B*8101 are the class I molecules most consistently associated with slow progression to HIV disease, from hereon in termed 'protective HLA alleles', whilst HLA-B*1801, B*5802 and B*4501 are examples of HLA alleles associated with high viral load and therefore rapid disease progression in Sub-Saharan African populations, and are therefore termed 'disease-susceptible HLA alleles' (Pereyra *et al*. 2010; Costello *et al*. 1999; Kiepiela *et al.* 2004; Leslie *et al*. 2010; Tang *et al.* 2011; Lazaryan *et al*. 2011; Carlson *et al*. 2012). It is notable that in HIV infection, the HLA alleles that have the greatest impact on outcome are HLA-B alleles (Kiepiela *et al*. 2004).

Genome-wide association studies also pointed to the MHC locus as being predictive of disease control (Fellay *et al.* 2007, Fellay *et al*. 2009; Pereyra *et al*. 2009),

confirming previous data that had picked out protective and disease-susceptible HLA alleles, respectively. However a striking finding of the GWAS studies was the virtual exclusivity of the SNPs

1.5.2 ROLE OF HIV GAG AS A CD8+ T CELL TARGET

The mechanism by which particular HLA-B alleles mediate viral control of HIV provides a crucial clue to defining which CD8+ T cell responses need to be induced by an effective HIV vaccine. Recent studies have suggested that a critical factor linking these protective HLA-B alleles is the fact that they all present epitopes from within the HIV Gag protein (Matthews *et al.* 2008). Studies of immune control of SIV in several different macaque models also suggest a key role for Gag as an immune target (Goulder & Watkins 2008). CD8+ T cell targeting of Gag might be particularly important in immune control of HIV infection as Gag p24 is a particularly conserved region and sequence changes within this region result in significant fitness costs to the virus (Leslie *et al*. 2004; Martinez-Picado *et al*. 2006; Schneidewind *et al.* 2007; Matthews *et al*. 2008; Crawford *et al*. 2009; Miura *et al*. 2009; Wright *et al*. 2012; Boutwell *et al*. 2013), Gag is highly immunogenic (Kiepiela *et al*. 2007) and presentation of gag epitopes occurs very early in as little as 2 hours post infection (Sacha *et al*. 2007).

1.5.3 IMPACT OF HIV ESCAPE MUTATIONS

Recent analysis suggests that there is a significant impact of Gag escape on viral fitness (Leslie *et al*. 2004; Martinez-Picado *et al*. 2006; Schneidewind *et al.* 2007;

Matthews *et al*. 2008; Crawford *et al*. 2009; Miura *et al*. 2009; Wright *et al*. 2012; Boutwell *et al*. 2013).

Indeed the majority of escape mutations do not significantly affect viraemia (Kiepiela *et al*. 2007, Matthews *et al*. 2008). However the greater the number of escape mutations in Gag associated with each HLA-B allele, the better the immune control linked to that HLA-B allele (Matthews *et al.* 2008). This association was shown to be stronger for reverting mutations, supporting the notion that CD8+ T cell targeting of Gag epitopes is beneficial due to selection of mutations that affect viral fitness.

1.5.4 IMPACT OF HIV ESCAPE MUTATIONS UPON TRANSMISSION

The transmission of viral escape mutations that have been selected in response to the donors HLA alleles have been shown to have two different consequences when transmitted to a new host. For recipients who are HLA allele matched with the donor, such as MTCT, the transmission of viruses encoding escape mutations in epitopes, commonly presented by the matched allele, has been shown to be disadvantageous (Goulder 2001, Feeney 2004). MTCT represents a special case where donor and recipient share at least half of their HLA alleles. Adaption of transmitted virus to the mothers alleles and therefore pre-adaption to at least some of the child's HLA alleles, may be one possible factor contributing to the more rapid progression observed in infected children compared to adults. Studies of horizontal

transmission pairs have shown that transmission of escape variants may in fact be advantageous due to a reduced replicative capacity of transmitted virus (Chopera *et al*. 2008; Goepfert *et al*. 2008; Hollingsworth *et al*. 2010; Hecht *et al.* 2010; Prince *et al*. 2012). Indeed, escape mutations that incur a fitness cost to the virus may be of long-term benefit to an HLA allele mismatched recipient, despite subsequent reversion, by reducing viraemia during acute infection. Whilst these studies have highlighted the potential clinical benefit of transmission of viruses with HLA allele selected escape mutations, it is important to emphasise that, to date, only Gag-specific escape mutations have been shown to mediate this effect (Kiepiela *et al.* 2007; Goepfert *et al.* 2008; Ngumbela *et al*. 2008; Troyer *et al*. 2009).

1.6 TOWARDS A VACCINE FOR HIV

1.6.1 THE SPECIFIC CHALLENGES OF HIV-1

The development of an effective HIV vaccine is complicated by several factors. The first is HIV latency. HIV-1 can establish a state of latent infection, primarily residing in memory T cells where the virus is able to persist indefinitely even in patients on ART. This latent reservoir provides a major barrier to effectively eradicating the HIV-1 virus. HIV-1 has a high error rate of RT and recombination, which results in an ever-evolving quasispecies that can escape vaccine-induced responses. There is a large amount of genetic variability between clades of HIV-1 infection, this means that targeted epitopes may not be conserved between subtypes making a cross reactive vaccine very difficult to design. HIV-1 infects via multiple routes so a robust

immunity at all sites of potential infection would be necessary to prevent infection. HIV-1 has evolved many immune evasion strategies, for instance HIV-1 Env, which is the target of HIV-1 directed neutralizing antibodies (Wyatt *et al*. 1998; Pantophlet *et al*. 2006; Overbaugh *et al*. 2012), is protected by a shield of glycans, variable immunodominant loops and confirmational masking of key viral epitopes (Pantophlet *et al*. 2006; Kwong *et al.* 2002; Wei *et al*. 2003; Burton *et al*. 2005). One of the major barriers to effective vaccine design is that the correlates of protection against HIV-1 infection are still relatively unknown.

In the absence of natural protection against HIV-1 in human populations, research interest has focused on studies of acute HIV-1 seroconverters (Koup *et al.* 1994; McMichael *et al.* 2010), subjects with lack of progression in HIV-2 infection (Leligdowicz *et al.* 2008), exposed but uninfected individuals (HEU) (Fowke *et al*. 1996), with the most emphasis on studies of long-term non-progressors (LTNPs) and elite controllers (Baker *et al.* 2009).

Long-term non-progressors (LTNPs) provide hope that durable control of viraemia is achievable. These individuals have stable CD4 counts, low viral loads and remain free from clinical signs of AIDS for >10 years. LTNPs represent a heterogeneous group of HIV-infected individuals; numerous studies have linked HLA types, CCR5d32 polymorphism and limited lymphocyte activation to their lack of progression (Samson *et al.* 1996; Kiepiela *et al.* 2004; Choudhary *et al*. 2007; Matthews *et al*. 2008; Leslie *et al*. 2010; Rotger *et al*. 2011; Klatt *et al* 2014).

1.6.2 T-CELL APPROACHES TO VACCINE DESIGN

A successful protective T cell vaccine should be capable of stimulating both CD4+ T cells (Rosenberg *et al*. 1997) and CD8+ T cells (Edwards *et al*. 2002; Ogg *et al.* 1998). These T cells should have central (Burgers *et al*. 2009) or effector (Addo *et al*. 2007; Hess *et al*. 2004) memory phenotype, and be capable of cell proliferation (Day *et al.* 2007). The T cells stimulated should be able to directly kill infected targets (Hersperger *et al*. 2010; Migueles *et al*. 2008; Yang *et al.* 1996) and/or be able to inhibit viral replication (Blackbourn *et al*. 1996; Spentzou *et al*. 2010; Yang *et al*. 1997). T cells should also be polyfunctional - able to secrete several cytokines (Betts *et al*. 2006) - but should not express markers of exhaustion (Day *et al*. 2006; Petrovas *et al*. 2006; Trautmann *et al.* 2006). The elicited T cell response would need to preferentially target HIV epitopes in Gag but not epitopes within Envelope (Kiepiela *et al*. 2007; Rolland *et al*. 2008; Zuniga *et al.* 2006). The Gag epitopes should ideally be conserved across strains (Goulder *et al*. 1996; Turnbull *et al.* 2006), similar to those restricted by protective HLA class I alleles (Gao *et al*. 2001; Hendel *et al.* 1999; Kaslow *et al.* 1996; Migueles *et al.* 2000), in which mutation is detrimental to viral fitness (Leslie *et al*. 2004; Martinez-Picado *et al*. 2006; Schneidewind *et al.* 2007; Matthews *et al*. 2008; Crawford *et al*. 2009; Miura *et al*. 2009; Wright *et al*. 2012; Boutwell *et al*. 2013).

To date, only a handful of potential HIV-1 vaccines have undergone efficacy testing in humans (Buchbinder *et al.* 2008; Flynn *et al.* 2005; Pitisuttithum *et al.* 2006) with only one showing any partial protection against infection, the RV144 vaccine (Rerks-Ngarm *et al*. 2009). The vaccine tested by Merck & Co in the STEP and Phambili trials was based on the hypothesis that CTL alone would provide adequate protection from HIV acquisition, preclinical testing of this vaccine in SIV infected

non-human primates supporting this hypothesis (Shiver *et al*. 2002). The vaccine expressed HIV-1 Gag, Pol and Nef, with exclusion of Env, and therefore Env-specific antibody responses were absent (Buchbinder *et al*. 2008; McElrath *et al*. 2008). Although the vaccine induced some CTL responses, these were not of great magnitude or breadth, and the vaccine both failed to protect volunteers from acquisition of infection and also to reduce viral loads after infection (Buchbinder *et al*. 2008; McElrath *et al.* 2008). Table 1.2 summarises efficacy trials for HIV-1 vaccines to date.

Vaccine Trial	Schedule (months)					Population	Immunity	Efficacy
	0	1	2	3	6			
Vax003	Env protein	Env protein			Env protein	MSM & IVDU	Antibody & CD4+ T cells	0%
STEP	Adenoviral vector	Adenoviral vector			Adenoviral vector	MSM	CD8+ & CD4+ T cells	-31.50% (N/S)
RV144	ALVAC vector	ALVAC vector		ALVAC vector and Env protein	ALVAC vector and Env protein	General population	Antibody, CD8+ & CD4+ T cells	31.20% p=0.4
HVTN 505	Plasmid DNA	Plasmid DNA	Plasmid DNA		Adenoviral vector	MSM	Antibody, CD8+ & CD4+ T cells	-25% (N/S)

Table 1.2 HIV-1 vaccine efficacy trials to date (adapted from Koup *et al.* 2011)

1.6.3 ANTIBODY APPROACHES TO VACCINE DESIGN

Several human antibodies that can effectively neutralize diverse strains of HIV-1 have been identified, and these are summarized in Table 1.3.

Antibody	Neutralising Breadth	References
CD4-binding site		
b12	33%	Burton *et al*. 1994; Zhou *et al*. 2007
HJ16	30%	Corti *et al*. 2010
VRC01	87%	Wu *et al*. 2010; Zhou *et al*. 2010; Scheid *et al*. 2011; Wu *et al*. 2011, Zhou *et al.* 2013
8ANC131	57%	Scheid *et al*. 2011
CH103	34%	Liao *et al.* 2013
V1/V2		
PG9	70%	Walker *et al.* 2009; Pejchal *et al*. 2010; McLellan *et al*. 2011; Pancera *et al.* 2013; Julien *et al*. 2013
PGT145	60%	Walker *et al*. 2011
Glycan V3		
2G12	18%	Trkola *et al*. 1995; Calarese *et al.* 2003
PGT121	53%	Walker *et al*. 2011; Mouquet *et al*. 2012; Julien *et al*. 2013
PGT128	56%	Walker *et al*. 2011; Pejchal *et al.* 2011
PGT135	<30%	Walker *et al*. 2011; Kong *et al*. 2013
MPER		
2F5	48%	Muster *et al*. 1993; Trkola *et al*. 1995; Ofek *et al*. 2004
4E 10	88%	Muster *et al*. 1993; Trkola *et al*. 1995; Cardoso *et al.* 2005
10E 8	97%	Huang *et al.* 2012
Z13	>20%	Zwick *et al.* 2001

Table 1.3. Broadly neutralizing HIV-1 antibodies adapted from Kwong *et al*. 2013.

These antibodies are generated in individuals who have had at least two years of HIV-1 infection and have shown broad neutralization of the virus. Although immunization with Env proteins have been shown to induce high levels of HIV-1 specific antibodies, they have been unable to effectively neutralize most circulating primary HIV-1 isolates (Mascola *et al*. 2010; McElrath *et al.* 2010; Pantophlet *et al*. 2006; Overbaugh *et al*. 2012), and as HIV-1 evolves to evade these NAbs it has been proposed there may be minimal clinical benefit to the individuals in whom these antibodies do eventually develop (Sather *et al*. 2009; Gray *et al*. 2011; McMichael *et al.* 2002; Kwong *et al.* 2013). However, in animal models in has been shown that passive delivery of such antibodies can completely prevent infection (Shibata *et al.* 1999; Mascola *et al*. 2000; Mascola *et al.* 2003; Hessell *et al*. 2009; Hessell *et al*. 2009).

There have been various vaccine strategies used to elicit effective HIV-1 neutralising antibodies. This included an approach that involved viral spike mimics of the Env spike of HIV-1 (Tong *et al*. 2012; Julien *et al.* 2013). A second approach was to use epitope immunogens which focus on a particular epitope or site of overlapping

epitopes on the Env spike (Ofek *et al.* 2010; Azoitei *et al*. 2011; Zhou *et al*. 2007; Joyce *et al* 2013). A third approach is to use class immunogens to expand B cell populations that specifically produce bNAbs (Kwong *et al.* 2013). A recent study described generation of a fusion protein, termed eCD4-Ig which contains an immunoadhesin of CD4 with a small CCR5-mimetic sulfo-peptide. This construct has been shown to neutralize a very broad and diverse panel of HIV-1 isolates and protects from multiple SHIV challenges in rhesus macaques (Gardner *et al*. 2015).

1.6.4 NON-HUMAN PRIMATE MODELS

A further challenge in HIV vaccine development is identification of a suitable animal model. Small animal models that are readily available for research purposes are not easily infected by HIV so research efforts have turned to the use of a similar virus to HIV-1, the simian immunodeficiency virus (SIV) in non-human primates. However this model is not ideal when comparing mucosal transmission of HIV, which is relatively inefficient in humans where dozens of encounters of virus are typically required for mucosal transmission to occur even during peak viraemia in the infected partner (Gray *et al.* 2001; Wawer *et al*. 2005) and usually results in the establishment of a single clone of replicating virus from the numerous viruses present in the inoculum (Salazar-Gonzalez *et al*. 2009). In the SIV model, repeated low-dose mucosal viral challenges are required to transmit a single SIV variant across the mucosal surface (Keele *et al*. 2009). Due to the limited number of SIV

isolates in circulation it is difficult to test protection across genetically diverse strains, which is a major obstacle for development of an HIV-1 vaccine (Koup *et al.* 2011).

A further limitation of the SIV model is that SIV Env differs substantially from HIV Env. Since it is clear that broadly neutralizing antibodies play a key role in the prevention of HIV infection, an SHIV recombinant virus has been constructed that consists of an SIV backbone with an HIV Env protein. This in itself however is an unreliable predictor of vaccine efficacy in humans, as differences exist in how SHIV and HIV-1 are transmitted, which CD4+ T cells they infect and the mechanisms of disease that they induce (Morgan *et al.* 2008; Nishimura *et al.* 2004; Shedlock *et al*. 2009).

To date, T cell vaccines that have been tested in non-human primate models have shown an effect on viral load rather than viral transmission. However, in the human STEP vaccine trial (Merck & Co) there was a significant lack of impact on viral load observed in human participants (Buchbinder *et al*. 2008; McElrath *et al.* 2008; Shiver *et al*. 2002), once again questioning the SIV model as a predictor of vaccine efficacy in humans.

1.7 CLINICAL ASPECTS OF HIV-1 INFECTION

1.7.1 NATURAL HISTORY OF HIV-1 INFECTION IN ADULTS

Following acute infection in adults, there is a rapid peak in viraemia to ~10^7 RNA copies/ml plasma, which may be accompanied by 'seroconversion illness' characterized by influenza-like symptoms, rash and lymphadenopathy (Daar *et al.* 1991; Clark and Shaw 1993). This is followed by an asymptomatic period, median 10 years (Mellors *et al* 1996) during which viral load in plasma reaches a 'set-point' plateau, median 30-40,000 HIV RNA copies/ml (Kiepiela *et al*. 2004). This set-point is one of the most reliable clinical predictors of the time to AIDS (Mellors *et al*. 1996).

Following loss of viraemic control, CD4+ T cell count declines, rendering the host susceptible to AIDS at an estimated rate of 2.8-4.2% per annum (Tindall *et al*. 1988). The timing of these events in different individuals is likely to reflect the balance between host immune responses and the fitness of the circulating viral quasispecies. In the absence of HAART, the associated opportunistic infections and malignancies that occur when CD4 counts are <250 cells/mm^3 render AIDS almost universally fatal.

Progressive decline in CD4+ T cell count is brought about, in part, by direct lysis of infected cells by viral infection. However, given the relatively small proportion of total CD4+ T cell pool that is productively infected, this mechanism of cell death does not exclusively account for the overall depletion (Hazenberg *et al.* 2000; Brenchley *et al.* 2004). The expression of PD-1 (Programmed-Death-1), a marker of

an exhausted phenotype, is increased on the surface of CD4+ T cells and HIV-specific CD8+ T cells from chronically infected individuals and correlates with low CD4 counts (Day *et al*. 2006). Exhaustion of CD4+ T cells may occur as a direct consequence of increased cell turnover.

In the context of chronic HIV infection, immune activation indirectly leads to a loss of CD4+ T cells, as well as B cells, NK cells and CD8+ T cells (Hazenberg *et al.* 2000; Moir & Fauci 2009). This 'bystander effect' due to immune activation may arise as a consequence of bacterial translocation (Paiardini, Muller-Trutwin. 2013), as described above. HIV also leads to chronic dysregulation of homeostatic mechanisms that operate between CD4+ T cell subsets, with a variety of consequences including increased turnover of memory T cells (Brenchley *et al*. 2004).

1.7.2 NATURAL HISTORY OF HIV INFECTION IN CHILDREN

The vast majority of paediatric HIV infections are acquired through MTCT (WHO 2014) although infection via contaminated blood products or tissue, unsafe injection or incision practices and sexual abuse also occurs (Lackritz *et al*. 1998; Lindegren *et al*. 1998; Schmid *et al*. 2004). Amongst adolescents, horizontal spread through sexual contact and injection drug use are also significant modes of transmission (UNAIDS 2013).

The overall risk of MTCT in the absence of interventions is 15-30% in Europe and USA (WHO 2014) and 25-40% in sub-Saharan Africa (WHO 2014) due to differences

in population characteristics, obstetric practice and method of infant feeding. This can be effectively reduced to <2% with no breastfeeding and <5% with very high coverage of effective ARV drugs, depending on the duration of breastfeeding among HIV-exposed infants (WHO 2014). Overall, in the absence of interventions to prevent transmission, two thirds of MTCT occurs in the peripartum period. In the absence of ART, this can either occur *in utero* (approx 1/3), mostly late in the third trimester, or intrapartum (2/3) (Prendergast *et al.* 2007). MTCT also arises postpartum during breastfeeding (National Department of Health 2015).

Progression to AIDS is much more rapid in paediatric compared to adult HIV infection. In USA/Europe, 20% of infants will become rapid progressors, defined as those that develop AIDS within the first year of life. Beyond infancy, approximately 5% children per year progressing to AIDS in the absence of ART (European Collaborative Study 2012). In resource limited areas, such as sub-Saharan Africa, progression to AIDS is even more rapid, without treatment, approximately 50% of HIV-1 infected children develop AIDS within a year and 50% will die before their second birthday (Prendergast *et al*. 2012). Contributing factors include a higher prevalence of other infections, malnutrition and micronutrient deficiencies (Prendergast *et al*. 2007). However, paediatric progression is clearly biphasic: after the first 2 years, by which time approximately 80% of children have either died or developed AIDS, progression is much slower, and ~15% of infected children reach 5 years with absolute CD4 counts of >350 cells/mm^3. Overall, 5-10% of ART naïve, HIV infected children remain clinically healthy and maintain normal-for-age CD4 counts throughout childhood (Mphatswe *et al*. 2007; Blanche *et al*. 1997; Paul *et al.*

2005). The mechanisms of non-progression in children have not previously been defined.

1.7.3 PREVENTION OF MTCT (pMTCT)

A recent report from the WHO (2014) quantified that 89% of all new paediatric HIV infections in 2013 occurred in sub-Saharan Africa. However, there has been an overall decline in MTCT due to substantial efforts to increase coverage of available drugs. In 2013 there was an estimated overall transmission rate of 17% (14-19%) in low-middle income countries, which is a significant drop from transmission rates in 2009 which were reportedly 26% (23-28%) (WHO 2014).

South Africa have reported a 90% coverage of ART for pregnant women in 2013 which has resulted in an overall transmission rate of 10% or less (WHO 2014). However, a lack of accurate data on ART adherence during the postpartum period hinders an accurate quantification of MTCT rates. In addition, complete data on outcomes of pMTCT are not yet available and 'lost to follow up' (LTFU) status remains a major issue for HIV exposed infants.

1.7.4 DIAGNOSIS AND TREATMENT OF PAEDIATRIC HIV-1 INFECTION

Identification of HIV-infected children by reliable, affordable techniques is essential to enable early initiation of treatment. Although HIV antibody testing is suitable over the age of 18 months, up to this age transplacental maternal antibody persists and a positive result is therefore diagnostic only of maternal infection. Children

under 18 months of age require virological testing by PCR (Owens *et al*. 1996), however virological testing still remains unaffordable or unavailable in much of sub-Saharan Africa.

CD4 count and viral load are surrogate markers for disease progression in children as in adults (Palumbo *et al*. 1998). The normal absolute CD4 count varies widely with age, so CD4% is a preferred marker in children under the age of 5 years. However, unlike adults, there are no good markers during infancy to predict disease progression and deaths can occur suddenly even in infants with well preserved CD4 counts (Violari *et al*. 2008).

At the onset of these studies, antiretroviral therapy (ART) was started in children when they met WHO 2010 treatment initiation criteria which may be summarised as thus: (i) age (all those diagnosed at <1yr are recommended to initiate ART irrespective of CD4 count) (ii) CD4 criteria to initiate ART: a CD4%<25% in children aged 1-4yrs or an absolute CD4<350/mm^3 in children aged $\geq$5yrs; (iii) clinical criteria to start ART (WHO clinical disease stage 3 or 4).

A current day cohort of this type would not be possible to study because, since 2008, WHO guidelines have recommended ART initiation in all HIV-infected infants, irrespective of CD4% (Violari *et al.* 2008; WHO Antiretroviral therapy for infants and children 2008) and since 2013 ART initiation in all children aged <5yrs, irrespective of CD4%. Indeed a recent study in adult HIV infection has shown a significant benefit in starting immediate ART, regardless of CD4+ T cell count (The INSIGHT START Study Group 2015). It is likely therefore that, in the near future, all countries will follow suit for HIV-infected children, even those who are the paediatric 'non-progressors' who form the subject of much of this thesis.

1.8 OVERVIEW OF THIS THESIS

The aim of this thesis is to describe the immunological and viral influences on HIV disease progression in adults and children. The particular focus of this work is on subtype C infection, as this accounts for 50% of global HIV disease burden (Hemelaar *et al*. 2011). Taking advantage of several well characterized cohorts of HIV-infected individuals from the UK and South Africa, this thesis will address several key questions in immunopathogenesis of HIV infection.

Chapter 3 will investigate the impact of viral replicative capacity on disease progression in mother-to-child-transmission of HIV. It will also consider the impact of HLA class I alleles and viral escape on disease progression in both adults and children.

Chapter 4 will explore the efficacy of anti retroviral therapy in restoring T cell function in children. T cell function will also be comparatively investigated in both progressing and non-progressing ART-naïve children.

Chapter 5 will investigate the immunophenotype of group of non-progressing children who are able to maintain high CD4 counts in the setting of high viraemia. Contributing factors such as immune activation, immune exhaustion, T cell function and the potential role of broadly neutralizing antibodies will be investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 PATIENT COHORTS

2.1.1 KIMBERLEY COHORT, SOUTH AFRICA

Treatment-naïve HIV-infected children who were not eligible for ART according to WHO (2008) guidelines, and mothers with C clade HIV infection were recruited from Kimberley Hospital, South Africa. Paediatric slow-progressors (PSP) were defined as those children who failed to meet 2010 WHO criteria to initiate ART. These criteria may be summarised thus: (i) age (all those diagnosed at <1yr are recommended to initiate ART irrespective of CD4 count) (ii) CD4 criteria to initiate ART: a CD4%<25% in children aged 1-4yrs or an absolute CD4<350/mm^3 in children aged $\geq$5yrs; (iii) clinical criteria to start ART (WHO clinical disease stage 3 or 4). Paediatric non-progressors (PNP) are defined as children with an absolute CD4>750/mm^3 in children aged $\geq$5yrs. Paediatric progressors (PP) were defined as those who had reached a CD4%<25% by 24 months of age. HIV positive, ART treated children were also recruited regardless of age and stage of disease. HIV negative siblings of enrolled HIV positive children were also recruited as healthy control children. Informed consent was obtained from all individuals. Blood samples were processed within Kimberley Hospital. Viral load and CD4 counts were supplied

by the NHLS in Kimberley Hospital. The study was approved by the Ethics Committee of the Faculty of Health Sciences, University of the Free State and the Clinical Head of Kimberley Hospital.

Viral load measurement was undertaken using the BioMérieux NucliSens Version 2.0 Easy Q/ Easy Mag (NucliSens v2.0) assay (dynamic range 20-10m) prior to 2010 and using the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test version 2.0 by Roche (CAP/CTM v2.0) (dynamic range 20-10m) subsequent to 2010. CD4+ T cell counts were measured by flow cytometry.

2.1.2 THAMES VALLEY COHORT, UK

ART-naïve adult subjects attending HIV outpatient clinics in the Thames Valley region were recruited. Participating centers were Oxford, Northampton, Reading and High Wycombe. The majority of these subjects were chronically HIV infected. These subjects were predominantly infected by MSM and heterosexual transmission. All subjects provided informed consent for participation. For each subject, 30-50ml of whole blood was collected in EDTA at enrollment, and, when available, at follow-up time points of 6 month intervals. VL and CD4 count data were provided by the recruiting centre. The study was approved by local Research and Development Committees at each recruitment site and by the Oxford Research Ethics Committee.

2.1.3 PAEDIATRIC COHORT, UK

HIV-infected ART naïve children were recruited from Great Ormond Street Hospital, London and St Mary's Hospital, London. Following written informed consent, blood was collected during routine venepuncture, transported by courier and processed within 4 hours of collection. Ethical approval was granted by the Barnet and Haringey Research Ethics Committee.

2.1.4 DURBAN COHORT, SOUTH AFRICA –

HIV-infected children were enrolled from the Ithembalabanthu Clinic in Umlazi, KwaZulu-Natal, South Africa. Eligible children were those who were ART-naïve at enrolment. Although the majority of these therefore were children aged >5yrs who did not meet the WHO 2010 criteria for ART initiation, principally a CD4 count of >350 cells/mm^3, the children enrolled also included those who were enrolled immediately prior to ART initiation, having CD4 counts of <350 cells/mm^3. The cohort in Ithembalabanthu therefore mirrored the one in Kimberley in comprising mostly slow –progressor or 'non-progressor' children, but also some children progressing to CD4 counts that meant they met criteria to start ART. Ethics approval was obtained through the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal.

Antiretroviral therapy-naïve adults with C-clade HIV infection were recruited from Durban, South Africa. Subjects received a voluntary testing and counseling in either antenatal or medical outpatient clinics at Cato Manor Clinic, Sinikithemba Clinic, St Mary's Hospital and King Edward VIII Hospital. Subjects were almost

exclusively of Zulu or Xhosa origin and were infected by heterosexual transmission. Informed consent was obtained from all individuals. Blood samples were processed at the HIV pathogenesis programme (HPP) laboratory, Durban. CD4 counts were measured by flow cytometrty on BD FACS Calibur and viral load was measured from the plasma using the Roche Amplicor version 1.5 assay. The study was approved by the University of KwaZulu-Natal BREC, and the Oxford Research Ethics Committee..

2.2 PROCESSING OF BLOOD SAMPLES

2.2.1 BLOOD COLLECTION

Whole blood was collected in Ethylenediaminetetraacetic acid (EDTA) blood collection tubes and processed within 6 hours of blood draw.

2.2.2 SEPARATION OF PLASMA AND PBMC

Whole blood was spun at 2000rpm for 10 minutes to separate the plasma layer. 4 cryovials containing 1.5ml plasma each are collected and frozen at -80°C. PBS was added to the remaining blood and layered slowly on top of Lymphoprep (Axis-Shield, Norway) at a Lymphoprep:PBS:Blood ratio of 1:1:1. This was then centrifuged at room temperature (RT) for 30 minutes at 2000rpm with no brake. The mononuclear cell layer was then transferred into a 50ml falcon tube using a Pasteur pipette and topped up to 50ml with R10 media to wash. The cells were then

spun at 2000rpm for 10 minutes. The supernatant was then discarded and the cell pellet resuspended in 50ml R10 and spun at 2000rpm for 5 minutes. This wash step was repeated again. PBMC's were counted by haemocytometer and resuspended in R10 for further use in cellular assays or cryopreserved as described in section 2.2.3.

2.2.3 CRYOPRESERVATION AND THAWING OF PBMC

To freeze, PBMC pellets of 5-10million cells were slowly resuspended in freezing media (2.9.1) dropwise using a Pasteur pipette. They were transferred into a cryovial and placed into a Freezing container, 'Nalgene Mr Frosty' (Sigma-Aldrich) at -80°C for a minimum of 80 minutes. Cryovials were then transferred into liquid nitrogen for long-term storage within 12 hours of cryopreservation.

To thaw frozen cells from liquid nitrogen vials were placed immediately into a water bath pre-heated at 37°C until the suspension becomes loose enough to transfer into a falcon tube. R10 media was added dropwise by Pasteur pipette. Cells are then spun at 2000rpm for 10 minutes. The supernatant was then discarded and the cell pellet resuspended in R10 and spun at 2000rpm for 5 minutes. This wash step was repeated again. The cells were then left to rest in a 37°C 5% CO_2 incubator for 1 hour before use.

2.2.4 DNA EXTRACTION

From 3ml whole blood, 9ml Red Blood Cell Lysis (Qiagen) is added and left to incubate for 10 minutes at room temperature and inverted periodically to mix. The suspension was spun at 2000rpm for 10 minutes to pellet. Supernatant was poured off and the pellet was resuspended in 3mls Cell Lysis Solution (Qiagen) The DNA can be left like this for up to 18 months. 1ml Protein Precipitation Solution (Qiagen) was added and vortexed for 20 seconds. The suspension was then spun at 2000rpm for 10 minutes. The supernatant was then removed into 3ml isopropanol. This was inverted 50 times until white threads of DNA could be seen. This was then spun at 2000rpm for 5 minutes. The supernatant was then discarded and the tube was blotted on a paper towel to dry. 3ml 70% ethanol was added, the tube inverted and spun at 2000rpm for 3 minutes. The supernatant was again discarded and the tube dried on a paper towel. 250ul RNase/DNase-free water was added to the DNA before being stored at -20°C.

2.2.5 VIRAL RNA EXTRACTION

Viral RNA was extracted from plasma or viral stocks using RNA extraction Minikit (Qiagen UK) in accordance with the manufacturer's instructions. In addition, 10ul DNAse per sample (RNAse free DNAse set, Qiagen UK) was added prior to washing the column with AW1 buffer and 1ul RNAse inhibitor (RNAsin Plus, Promega) was added per column prior to elution of RNA. RNA was eluted in RNase/DNase-free H2O (Sigma) and stored at -80°C.

2.3 HLA CLASS I TYPING

Samples from study subjects were HLA-A, -B, and –C Sequence Based Typed in the CLIA/ASHI accredited laboratory of William Hildebrand, PhD, D (ABHI) at the University of Oklahoma Health Sciences Center using a locus specific PCR amplification strategy and a heterozygous DNA sequencing methodology for exon 2 and 3 of the class I PCR amplicon.

DNA sequence analysis and HLA allele assignment were performed with the software Assign-SBT v3.5.1 from Conexio Genomics.

2.4 MOLECULAR BIOLOGY TECHNIQUES

2.4.1 QUANTIFICATION OF DNA

DNA was quantified using a ND-100 spectrophotometer (Labtech International). 2ul of DNA was used for quantification. DNA concentration was subtracted from background using the ND1000-V3.5.2.

2.4.2 AGAROSE GEL ELECTROPHORESIS

PCR products were visualized by agarose gel electrophoresis. PCR products were mixed 1:1 ratio with a gel loading buffer (Bioline UK). This was then loaded onto a 1% agarose gel (Sigma-Aldrich UK) in TBE buffer (Tris-Borate-EDTA, Promega, UK). Ethidium bromide solution was added at 5μl per 500ml of gel. Samples were run in parallel with Hyperladder I (Bioline UK) to allow an estimation of the size and

concentration of the DNA fragments. The gel was run by electrophoresis at 180V for 40 minutes. The DNA in the gel was then visualized under UV light using a Gel Doc 2000 (Biorad).

2.5 PLASMID PREPARATION

2.5.1 PLASMID pNL4-3

The plasmid pNL4-3 was obtained through our collaborators, Zabrina Brumme and Mark Brockman, originally acquired from the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH, from Dr Malcolm Martin (GenBank ref AF324493). It is a full-length replication competent DNA of HIV-1 subtype B derived from NY5 (5') and LAV (3') (Adachi, *et al.* 1986). The Ampicillin resistant plasmid was provided as DNA (100**µ**l of 630**µ**g/mL) in TE buffer, transformed into Stbl3 E. Coli and grown in LB/Amp Agar and broth.

Modification of this well described laboratory strain virus has led to the development of its related plasmid DNA construct, pNL4-3**Δ***gag-protease*, permitting generation of chimeric viruses (see below) (Miura, *et al*. 2009). A diagram of the plasmid, identifying relevant restriction sites is presented in Figure 2.1. The original plasmid do not contain any BstEII restriction enzyme (RE) sites (purple dotted line, Figure 2.1), and mutagenesis are required to engineer and introduce the two BstEII RE sites into pNL4-3 genome and create deletion over *gag-protease* gene fragment (see below).

2.5.2 PLASMID pNL4-3Δ*gag-protease*

The pNL4-3Δ*gag-protease* was obtained through our collaborators, Zabrina Brumme and Mark Brockman, who contributed to the engineering of the plasmid construct (Miura, *et al*. 2009). The pNL4-3Δgag-protease is essentially a pNL4-3 plasmid deleted with its original gag-protease gene, and is replication incompetent unless recombined with correctly amplified gag-protease PCR product (from autologous product, for example).

The pNL4-3Δgag-protease is engineered based on serial mutagenesis of pNL4-3 that leads to introduction of two unique restriction enzyme (BstEII) sites: one at the 5' end of the gag gene and another one at 45 bases downstream from the 3' end of the protease gene of pNL4-3. The BstEII enzyme digest results in deletion of HIV gag-protease and self ligation of the remaining plasmid (pNL4-3Δ*gag-protease*). The circular pNL4-3Δgag-protease plasmid with self-ligated BstEII site can be digested (and therefore linearised) during future transfection experiments, and recombines with co-transfected HIV *gag-protease* PCR product containing BstEII sites at its end, through homologous recombination.

2.5.3 PLASMID DNA EXTRACTION AND PURIFICATION

Plasmid DNA was extracted using Qiagen Miniprep Kit or Qiagen Maxiprep Kit, according to manufacturer's instruction (Qiagen). The quality and yield of the purified plasmid DNA are assessed by nanodrop, and stored at -200C.

2.5.4 RESTRICTION ENZYME DIGESTION OF PLASMID

The pNL4-3 and pNL4-3Δ*gag-protease* differ in their genetic sequences surrounding gag-protease region, and each plasmid possess a different fragmentation pattern following restriction enzyme digestion using Hind III and BstE II (New England Bio-Lab, UK, according to manufacturer's instruction) (Figure 2.1). Unique restriction sites in DNA were checked using Sequencher software (version 4.8, Gene Codes Corporation and its licensors), which detects all restrictions sites of restriction enzymes in a given uploaded sequence.

The circular pNL4-3 contains 5 Hind III sites and no BstE II sites, in contrast to the circular pNL4-3Δ*gag-protease* that contains 4 Hind III sites and 1 BstE II site. The differences in restriction enzyme sites is due to the deliberate modification of pNL4-3 that derived pNL4-3Δ*gag-protease*, two BstEII sites were introduced via mutagenesis at either end of gag-protease genes (Figure 2.1). The subsequent BstE II digestion then removes the gag-protease gene from pNL4-3, leading to self ligation and hence formation of pNL4-3Δ*gag-protease*.

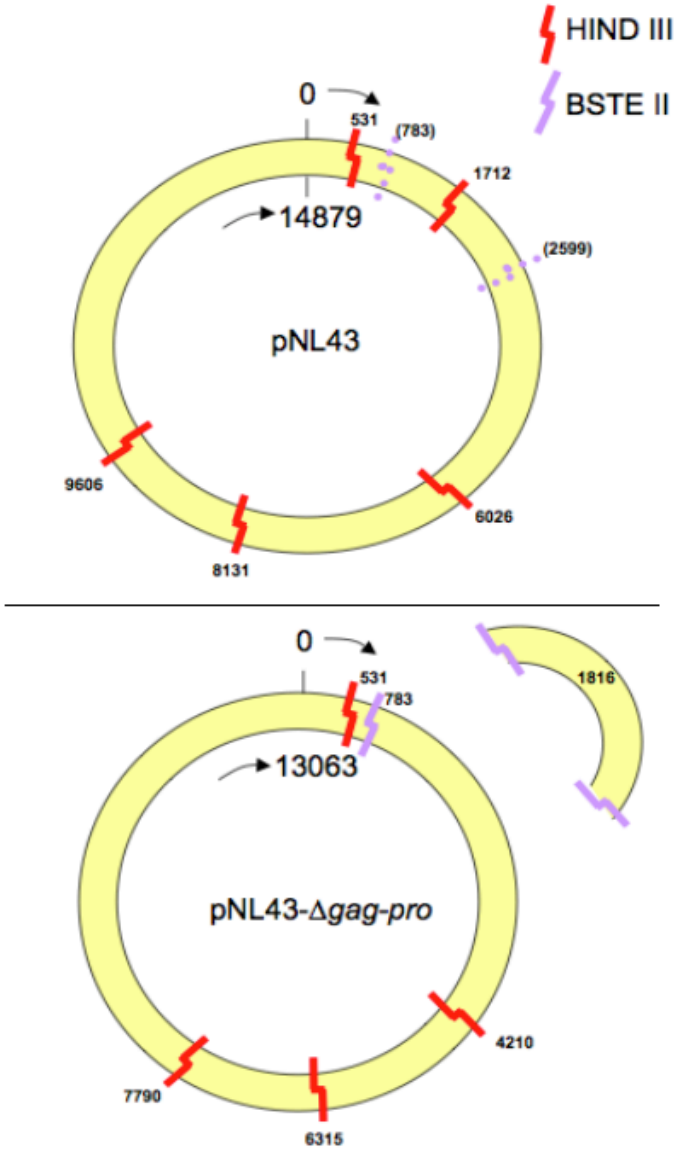


Fig 2.1: Schematic map of plasmid pNL4-3 and pNL4-3Δ*gag-protease* and restriction enzyme digestion sites. Upper panel: circular plasmid, pNL4-3, with HIND III restriction enzyme (RE) sites (in red). The numbers demarcate the nucleotide position of RE digestion. The size of RE digested product is confirmed by gel electrophoresis. In wildtype pNL4-3, there are no BstE II RE sites. However, mutagenesis introduces novel BstE II sites (dotted purple lines) at either end of the gag-protease region of pNL4-3. The subsequent BstE II digestion then removes the gag-protease gene from pNL4-3, leading to self ligation and hence formation of pNL4-3Δ*gag-protease* (Lower panel). The circular pNL4-3Δgag-protease contains a different number of HIND III sites (red) and BstE II site (in purple solid line), and therefore differing length polymorphisms by gel electrophoresis. Adapted from Huang *et al.* 2011.

2.6 AMPLIFICATION OF HIV GENES BY POLYMERASE CHAIN REACTION (PCR)

2.6.1 AMPLIFICATION FROM PROVIRAL DNA

Genomic DNA was extracted from PBMC and amplified by nested PCR to obtain population sequences. Regions of the virus amplified were the three most immunogenic proteins, Gag, Pol and Nef. A mastermix was generated for each PCR reaction using 20pmol/ul unmodified oligonucleotide primers (MWG Biotech, Germany) as below (Table 2.1 and Table 2.5).

The PCR reaction was performed using the Taq DNA polymerase enzyme, Biotaq (Bioline UK) in a total volume of 50ul.

Volume	Reagent	Supplier
35ul	H2O (DNAse/RNAse free)	Sigma
5ul	10x NH4 buffer	Bioline
3ul	MgCl2 (50mM)	Bioline
0.5ul	DNTP's (25mM)	Bioline
1ul	5' primer (20pmol/ul)	MWG Biotech
1ul	3' primer (20pmol/ul)	MWG Biotech
0.5ul	BioTaq (5U/ml)	Bioline
4ul	DNA template (minimum 50ng/ul)	

Table 2.1. Components of PCR reaction.

The PCR reaction was performed on a Peltier Thermal Cycler PTC-200 (MJ Research UK) using the procedure shown in Table 2.2. Specific primer annealing temperatures are indicated in Table 2.3.

PCR Conditions			
1	Denaturation	94°C	2 min
2	Denaturation	94°C	2 min
	Annealing	x1	30 sec
	Elongation	72°C	1 min
3	Repeat step 2, 19 times		
4	Denaturation	94°C	2 min
	Annealing	x2	30 sec
	Elongation	72°C	1 min
5	Repeat step 4, 19 times		
6	Elongation	72°C	5 min
7	Storage	4°C	

Table 2.2. PCR cycle. Temperatures x1 and x2 defined in Table 2.3.

PCR Reaction	Temperature 1(°C)	Temperature 2(°C)
Gag (B) OP	63	60
Gag (B) IP	55	52
Pol (B) OP	58	56
Pol (B) IP	60	58
Nef (B) OP	60	57
Nef (B) IP	60	57
Gag (C) OP	60	57
Gag (C) IP	57	54
Pol (C) OP	61	58
Pol (C) IP	60	57
Nef (C) OP	61	58
Nef (C) IP	61	58

Table 2.3. Annealing temperature for PCR

2.6.2 AMPLIFICATION FROM RNA

RNA was extracted as described in section 2.2.5. Reverse-transcription of RNA to cDNA was undertaken using Superscript III One-Step Reverse Transcriptase kit (Invitrogen) as a one-step reaction combined with outer PCR according to the manufacturers instructions. (Table 2.4). 7ul RNA was added per reaction.

	RT-PCR Conditions		
1	cDNA synthesis and pre-denaturation	45-60°C	15-30min
2	Denaturation	94°C	15 sec
	Annealing	55-60°C	30 sec
	Extension	68°C	1 min/kb
3	Repeat step 2, 39 times		
4	Extension	68°C	5 min
5	Storage	4°C	

Table 2.4. RT-PCR cycle. Annealing temperature defined as 5°C less than the lowest melting temperature of the two primers (range 55-60°C, Table 2.3).

2.6.3 PRIMERS

Primers were designed previously by A. Leslie and K. Pfafferott and subsequently optimized to amplify fragments of the HIV genome of approximately 1200 base pairs. These primers were designed to bin conserved regions of the genome and were checked for binding specificity and melting temperatures using the freeware PCR program Amplify (http://engels/genetics/wise/edu/amplify). All primers were unmodified oligonucleotide primers purchased from MWG Biotech, Germany.

Clade	Gene	Primer	Sequence
B	Gag	5'OP	AAATCTCTAGCAGTGGCGCC
		3'OP	TGTTGGCTCTGGTCTGCTCT
		5'IP	ACTAGCGGAGGCTAGAA
		3'IP	GCCACAATTGAAACACTT
B	Pol	5'OP	CTGAAAACAGGAAAATATGCAAG
		3'OP	TACTTGCCACACAATCATCACC
		5'IP	GAGGGGTGCCCACACTAATGA
		3'IP	CTTTTCTTCTTGGCACTACTTTTA
B	Nef	5'OP	TGCTCTGGAAAACTCATTTGCACC
		3'OP	CAGGCTCAGATCTGGTCTAACCA
		5'IP	AACATGACCTGGATGGAGTGGGAAA
		3'IP	TACAGGCAAAAAGCAGCTGCTTATATG
C	Gag	5'OP	CTAGCAGTGGCGCCCGAACA
		3'OP	GCAGTCTTTCATTTGGTGTCCTTC
		5'IP	ACTCGGCTTGCTGAAGTGC
		3'IP	CAATTTCTGGCTATGTGCCC
C	Pol	5'OP	CATGGTGGACAGACTATTGGC
		3'OP	CCTGCCATCTGTTTTCCATA
		5'IP	CCTCCCCTAGTAAAATTATGG
		3'IP	GCTGTCTCTGTAATAAACCCG
C	Nef	5'OP	GATCCATTCGATTAGTGAACGGA
		3'OP	CTTATATGCAGCATCTGAGG
		5'IP	TTCAGCTACCACCGATTGAGA
		3'IP	TGAGGGTTGGCCACTCC
C	Gag-Pro	5'OP	AAATCTCTAGCAGTGGCGCCCGAACAG
		3'OP	TTTAACCCTGCTGGGTGTGGTATYCCT
		5'IP	GACTCGGCTTGCTGAAGCGCGCACGGCAAGAGG
			CGAGGGGCGGCGACTGGTGAGTACGCCAAAAAT
			TTTGACTAGCGGAGGCTAGAAGGAGAGAGATGGG
		3'IP	GGCCCAATTTTTGAAATTTTTCCTTCCTTTTCCAT
			TTCTGTACAAATTTCTACTAATGCTTTTATTTTTT
			CTTCTGTCAATGGCCATTGTTTAACTTTTG

Table 2.5. PCR Primers for amplification of HIV gag, pol, nef and gag-pro genes.

OP = outer primer (first round of PCR) IP = inner primer (second round of PCR).

2.6.4 VISUALISATION OF PCR PRODUCT

Following amplification by PCR, 5ul of the PCR product was loaded onto a 5% agarose gel (Sigma, UK) in TBE buffer (Tris-borate-EDTA, Promega, UK). Ethidium bromide was added at 5ul/400ml gel. To determine the size of the amplified fragment, samples were run in parallel with Hyperladder I (Bioline, UK). After running at 180V for 40 minutes, bands were visualized under UV light.

At least one blank (negative) control was run for each PCR reaction. Following attainment of sequence data, taxa were assembled into a phylogenetic tree to ensure each sequence was distinct.

2.6.5 PCR PURIFICATION

The PCR product was purified by using a QIAquick PCR purification kit (Qiagen, UK) according to manufacturers instructions.

2.6.6 SEQUENCING

Sequencing of purified PCR products was performed using six overlapping primers binding both the sense and non-sense strands of DNA, for increased coverage and accuracy. The inner primers (second round) of the nested PCR reaction were two of the sequencing primers and four additional primers were designed (Table 2.8). Sequencing was performed using the Big Dye Terminator v3 reaction mix (Applied Biosystems UK). The reaction mix components are outlined in Table 2.6.

Volume	Reagent
3.7ul	ddH20
1.5ul	Big Dye buffer
2ul	Primer (0.625uM)
0.8ul	BigDye Terminator v3 reaction mix
2ul	PCR template

Table 2.6. Sequencing reaction mix.

Temperature	Time
960C	10 seconds
500C	5 seconds
600C	2 minutes
Repeat for 30 cycles	

Table 2.7. Thermal Cycler conditions for sequencing reaction

Sequence data were analysed using Sequencher v.4.8 (Gene Codes Corporation). Nucleotides for each gene were aligned manually in Se-Al v.2.0a11 (A. Rambaut, Department of Zoology, University of Oxford).

Clade	Gene	Primer	Sequence
B	Gag	Gag 5' IP	ACTAGCGGAGGCTAGAA
		Gag_2	CCTGGAGGTTCTGCAC
		Gag_3	GACACCAAGGAAGCTTTAG
		Gag_4	ATCCCAGGATTATCCATC
		Gag_5	CCAAGGGAAGTGACAT
		Gag 3' IP	GCCACAATTGAAACACTT
B	Pol	Pol 5' IP	GAGGGGTGCCCACACTAATGA
		Pol_2	GATTCACTTTTATCTGGTTGTG
		Pol_3	CAGACTCACAATATGCATTAGG
		Pol_4	ACTGGCCATCTTCCTGCTA
		Pol_5	AGTGGATATATAGAAGCAGAAGT
		Pol 3' IP	CTTTTCTTCTTGGCACTACTTTTA
B	Nef	Nef 5' IP	AACATGACCTGGATGGAGTGGGAAA
		Nef_2	TCCACCAATATTTGAGGACTTCCC
		Nef_3	CCACCGCTTGAGAGACTTACTCT
		Nef_4	GTCCCCCCTTTTCTTTTAAAAAGTGGCT
		Nef_5	TTCCAGTCAGACCTCAGGTACCTTT
		Nef 3'IP	TACAGGCAAAAAGCAGCTGCTTATATG
C	Gag	Gag 5' IP	ACTCGGCTTGCTGAAGTGC
		Gag_2	CTGCACTATAGGATAATTTTGAC
		Gag_3	GACACCAAGGAAGCCTTAG
		Gag_4	CTCCCACTGGAACAGGTG
		Gag_5	GGAACAAATAGCATGGATGAC
		Gag 3' IP	CAATTTCTGGCTATGTGCCC
C	Pol	Pol 5' IP	CCTCCCCTAGTAAAATTATGG
		Pol_2	TCACTAGCCATTGCTCTCC
		Pol_3	ATTGGAGGAAATGAACAAGTAGA
		Pol_4	GCCCACCAACAGGCTGC
		Pol_5	GAAACAGGACAAGAAACAGC
		Pol 3' IP	GCTGTCTCTGTAATAAACCCG
C	Nef	Nef 5' IP	TTCAGCTACCACCGATTGAGA
		Nef_2	ACCTGAGGTCTGACTGGAAAG
		Nef_3	AGATAAACATGGGGCACTTAC
		Nef_4	AGCAGTCTTTGTAATACTCCG
		Nef_5	ACCTCAGGTGCCTTTAAGAC
		Nef 3'IP	TGAGGGTTGGCCACTCC

Table 2.8. HIV gag, pol, and nef sequencing primers

2.6.7 CAVEATS AND QUALITY CONTROL OF PCR

Each set of PCR reactions included a negative control containing no template DNA. This was to ensure that the DNA contamination risk during preparation of the PCR reaction was low. In addition, following sequence editing and alignment a

Maximum-Likelihood phylogenetic tree was generated using PHYm131 (http://www.hiv.lanl.gov) and visualised using Figtree v.1.2.2 (http://tree.bio.ed.ac.uk/software/figtee/).

Taq DNA polymerase is a thermostable enzyme derived from the thermophilic organism *Thermus acquaticus*. BioTaq (Bioline, UK) was used in this study for amplification of HIV-1 *gag* genes. BioTaq is not a proof-reading DNA polymerase since it lacks 3'-5' exonuclease activity. The error rate of this DNA polymerase during a PCR reaction cycle has been estimated at between 2x10^-5 and 2x10^-4 errors/base pair. This translates to approximately 10 errors per 10,000 base pairs. PCR errors can also occur as a result of frameshifts during amplification, which have been estimated at 2.0x10^5 per base pair or 1 per 10,000 base pairs (Eckert and Kunkel 1991; Bracho *et al.* 1998). Frameshifts are easily detectable during sequence analysis. Finally, bias during amplification due to a primer binding preference is a consideration in population sequencing and may result in the over representation of particular viral quasispecies. The PCR errors described here are of low frequency in the context of amplification of 1200 fragments of the HIV-1 genome. In addition, analysis of large study populations reduces the effect of these errors on statistical analysis.

2.7 CELLULAR CULTURE

2.7.1 MEDIA AND REAGENTS

Media	Reagents	Company
FCS (Foetal calf serum)	Heat inactivated for 30 minutes at 560C, filtered through a 0.45um filter	Hyclone
R10	RPMI	Sigma
	10% heat inactivated FCS	Hyclone
	2mM L-glutamine	Sigma
	100U/ml penicillin	Sigma
	100ug/ml streptomycin	Sigma
H10	RPMI	Sigma
	10% heat inactivated human serum	Sigma
	10% T-stim	Helvetica
	2mM L-glutamine	Sigma
	100U/ml penicillin	Sigma
	100ug/ml streptomycin	Sigma
Blocking Buffer	PBS	Gibco
	1% heat-inactivated FCS	Hyclone
Freezing Media	Heat-inactivated FCS	Hyclone
	10% DMSO	Sigma
Peptide Dilution Solution	RPMI	Sigma
	100U/ml penicillin	Sigma
	Amphotericin	Sigma

Table 2.9. Reagent components used in cellular culture

2.7.2 IMMORTALISED CELL LINES

GXR Cells are a CEM reporter T cell line that encode green fluorescent protein (GFP) which is expressed following HIV-1 infection via a tat-dependent manner. GXR cells express CD4, CXCR4 and CCR5 (Brockman *et al.* 2006). These cells were cultured in R10 media (outlined in section 2.9.1) and split every 2 - 3 days to maintain exponential growth phase.

MT4 Cells are an HTLV-I- transformed T-cell line which support growth of HIV-1. MT4 cells were originally derived from a Japanese patient with human T cell leukaemia. The MT4 cells were obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH. MT4 cells were cultured in R10 media and split every 3-4 days to maintain exponential growth phase.

2.7.3 CELL COUNTING

Cells were counted 1:1 in Trypan Blue (Sigma) and loading 10ul onto a plastic counting chamber (Immune systems). Two grids are counted, one from the top of the slide and one from the bottom and cell concentrations were obtained as follows:

Number of cells per ml = (mean count for two squares) x dilution factor (2) x 10,000.

2.8 CELLULAR ASSAYS

2.8.1 IFNγ ELISpot ASSAY

ELISpot assays were performed using sterile 96 well plates with a Polyvinylidene Fluoride membrane (Millipore.) A minimum of 24 hours prior to use the plate was pre-coated with anti-human IFN-γ monoclonal antibody (Mabtech) and incubated at 4°C. Prior to use the plate was washed 6 times with blocking buffer (5% FCS in PBS) and 50ul R10 was added to each well. Peptide was added at a final concentration of 2ug/ml per well. For each plate, two positive wells containing Phytohaemagglutinin

(PHA) at 250ug/ml (Roche) and four negative wells containing cells but no peptide/PHA were included. PBMC's were added at 50,000/well.

Following 12-14 hours incubation at 37°C, the wells were washed 6 times with PBS. The plate was processed by addition of biotinylated IFN-γ antibody at 1ug/ul (Mabtech) and incubated for 90 minutes at room temperature in the dark, followed by re-washing and addition of 100ul streptavidin-alkaline phosphatase (ALP) conjugate (Mabtech) and incubated for a further 45 minutes. Development was performed by addition of substrate colour solution (Bio-Rad Laboratories.) The plates were read using AID ELISpot plate reader (ELISpot v.4.0 Autoimmune Diagnotiska, Germany.) Positive responses were calculated by subtracting the background (mean of the values from the four negative control wells.) A significant positive response was then defined as anything above the background and (3 x standard deviation) of the control wells and the final results were expressed as number of spot forming cells (SFC's) per million PBMC.

2.8.2 SCREENING FOR HIV-SPECIFIC RESPONSES BY MEGAMATRIX

To screen subjects for CD8+ T cell responses to any HIV proteins, a panel of 410 overlapping 18-mer peptides (OLP's) spanning the entire HIV proteome were synthesized based on the 2001 B and C clade consensus sequences (provided by Massachusetts General Hospital Peptide Core Facility.) These were used in a matrix system of 11-12 peptides/pool in 72 pools with each peptide represented in 2 different pools. The pools were pre-mixed and stored at 4°C.

To confirm CD8+ T cell responses to individual OLP's (suggested by positive responses to both pools containing the individual peptide) a confirmation was undertaken using individual OLP's in place of peptide pools. In addition, optimal peptides of the relevant HLA type restriction were added to the megamatrix or confirmation plate at a final concentration of 2ug/ml. For the purposes of data analysis, subjects making ELISpot responses to adjacent OLP's, only one of these was counted as a positive response (the bigger the magnitude response.)

2.8.3 WHOLE BLOOD ICS ASSAY

The whole blood ICS assay was designed to quantitate the frequency of specific CD4+ and CD8+ T cells in small volumes of whole blood from infants in developing countries. The assay is performed in two steps and was designed by Hanekom *et al.* 2004. For each antigen investigated, 1ml sodium heparinised whole blood was incubated within 2 hours after collection in the presence of anti-CD28 (BD Bioscience) and anti-CD49d (BD Bioscience). Antigens used included Gag peptide pool (2ug/ml), Pol peptide pool (2ug/ml), Nef peptide pool (2ug/ml), CMV lysate (NIH AIDS Reagent Programme, 5mg/ml) and non-infected cell extract (NIH AIDS Reagent Programme, 5mg/ml). After 5 hours incubation at 37°C, 5ug/ml BFA (Sigma) was added, and incubated for a further 5 hours at 37°C. 2mM EDTA was added for 15min at room temperature. 9ml of 1xFACS Lysing solution (BD Biosciences) is added and incubated for 10 minutes at room temperature. The solution is spun at 1500RPM for 7 minutes and washed with 10ml PBS, twice, before being placed in 1ml freezing medium and stored at -80°C.

Cryopreserved white cells are thawed (as in section 2.2.3), pelleted and washed first in PBS, then in FACS buffer (BD Biosciences) before being stained with fluorescence-conjugated antibodies against the following surface molecules: CD3 (Alexa Fluor 700, BD Pharmingen), CD4 (Qdot605, Life Technologies), CD8 (Pacific Blue, BD Biosciences). Following a 20 min incubation at room temperature in the dark, the cells were washed twice with FACS buffer and resuspended in Fix/Perm solution (BD Biosciences) for 20 minutes at 4°C. Cells were then washed twice with Perm Buffer (BD Biosciences) and resuspended in the ICS antibody mix with fluorescence-conjugated antibodies against the following molecules: IFN-γ (PECy7 ebioscience), IL-2 (PE Beckman Coulter), MIP1β (FITC R&D Systems), TNFα (Alexa Flour 647, Biolegend) for 30 mins at room temperature in the dark. Cells were again washed twice with Perm Buffer and the pellet suspended in 2% paraformaldehyde in PBS. Flow cytometric acquisition was performed on an LSR II (BD) flow cytometer within 12 hours of staining and analyzed using FlowJo version 8.8.6.

2.8.4 CELL PROLIFERATION ASSAY

CFSE (Carboxyfluorescein succinimidyl ester) proliferation assays were performed on freshly isolated PBMCs as previously described (Ndhlovu *et al.* 2013). Briefly, cells were resuspended in pre-warmed PBS with 0.1% BSA and CFSE (Invitrogen) was added to a final concentration of 0.5μM. Cells were incubated at 37°C for 10 minutes, quenched with 10ml ice-cold H10 media (containing 10% human serum), incubated for 5 minutes on ice and then washed 3 times in fresh H10 media. Cells were stimulated with Gag peptide pools and PHA as a positive control. The cell cultures were fed twice during the following week and on day 7 the cells were

harvested and stained with surface antibodies against CD3, CD4, CD8 and live/dead marker. Samples were immediately acquired on an LSR II (BD) flow cytometer and analyzed using FlowJo version 8.8.6. The proliferative response was calculated as the fraction of $CFSE_{low}$ CD4+ or CD8+ T-cells minus background. Only samples with less than 1% $CFSE_{low}$ CD4+ or CD8+ T-cells in the unstimulated control wells were included for analysis.

2.8.5 PLASMA LEVELS OF IMMUNE ACTIVATION AND INFLAMMATION

Plasma levels of sCD14 were quantified using a commercially available Luminex kit (R&D systems, Minneapolis, MN). Plasma samples were used at a 100-fold dilution in duplicate as per manufacturers recommendations. Results of sCD14 were expressed in pg/ul. Plasma levels of iFABP were quantified using a commercially available ELISA kit (R&D systems, Minneapolis, MN). Plasma samples were diluted 1:10 and run in duplicate. Results are calculated in pg/ml.

2.8.6 ANTIBODY NEUTRALISATION ASSAY

Neutralization was measured as a reduction in luciferase gene expression after a single round of infection of JC53bl-13 cells, also known as TZM-bl cells (NIH AIDS Research and Reference Reagent Program), with a multi-subtype panel of 16 Env-

pseudotyped viruses (Montefiori *et al.* 2004) with breadth defined as neutralization of ≥50% of heterologous viruses.

2.9 VIRAL CULTURE

2.9.1 GENERATION OF HIV VIRUS

HIV-1 virus was generated by electroporation cotransfection of a plasmid carrying the 5' half of the HIV-1 NL4-3 strain (p83-2) and a plasmid carrying the 3' half of the genome of HIV-1 NL4-3 (p83-10eGFP) (obtained from NIH AIDS Research and Reference Programme) into MT4 cells. The plasmid p83-10 contains a GFP-reporter gene in frame with nef (Weber *et al.* 2006).

2.9.2 GENERATION OF VIRAL STOCKS

Virus stocks are generated by cotransfection electroporation of the plasmids p83-2 and p83-10eGFP. 5ug of p83-10eGFP and p83-2 plasmid DNA was linearized by EcoRI digestion and electroporated into 5 million MT4 cells using the following electroporation conditions; 250mV, 950uF, 4mm. The MT4 cells were then cultured in R10 and 1 million MT4 cells were added to the culture after 24 hours. Approximately 100,000 cells were removed from the culture every 24-48 hours, fixed in 2% formaldehyde and monitored for GFP expression as a mark of productive infection using an LSR II (BD) flow cytometer. When peak infection was reached (80-90% GFP positive cells), the culture supernatant was harvested, filtered through a 0.45um filter and stored in 500ul aliquots at -800C. The sequence of NL4-

3 Wildtype was confirmed by viral RNA extraction (2.2.5) followed by RT-PCR (2.6.2) and viral sequencing (2.6.6).

2.9.3 CALCULATION OF TCID50 OF VIRAL STOCKS

The tissue culture infectious dose of viral stocks necessary to achieve 50% infection, (TCID50) was calculated using the following method; 100ul R10 was added to 60 wells of a 96 well flat bottomed plate. 25ul of viral stock was added to 6 wells of the first lane. This was then serially diluted at a ratio of 1:5. 100ul of MT4's (30,000 cells) is then added to each well and the plate was incubated for 5 days at 37°C, 5% CO_2. GFP positive cells were identified using a Motic Trinocular AE31 inverted fluorescent microscope. The titre or TCID50/ml was calculated using the Spearman-Karber method.

2.9.4 MULTIPLICITY OF INFECTION (MOI)

Tissue Culture Infective Dose (TCID50): number of infectious particles required to produce pathological change in 50% of cells (log units/ml).

Multiplicity of infection (MOI): ratio of infectious particles to target cells.

To calculate volume of viral stock to achieve a given MOI:

MOI=volume x (TCID50)/(number of cells to be infected)

2.10 VIRAL REPLICATION ASSAY

2.10.1 LINEARIZATION OF pNL4-3Δ*gag-pro* PLASMID

A deleted version of pNL4-3 was constructed (2.5.2) that lacks the entire Gag and Protease region (Stratagene Quick-Change XL kit) replacing this region with a BstE II (New England Biolabs) restriction site at the 5' end of Gag and the 3' end of protease. To linearize the plasmid the following digestion reaction was set up and incubated at 600C for 2 hours:

Digestion reaction (for 50 co-transfections):

Plasmid DNA (~1000 ug/ml)	~500ul (=500ug DNA)
10x NEB #3 Buffer	65ul
100x BSA	6ul
BstE II Enzyme (10U/ul)	100ul (=1000 Units, or ~2 U/ug DNA)

2.10.2 CO-TRANSFECTION OF PLASMID AND GAG-PRO PCR BY ELECTROPORATION

To generate recombinant viruses, 10ug of BstEII-linearized plasmid plus 50ul of the second-round amplicon (approximately 2.5ug) were mixed with 2 x 10^6 cells of a Tat-driven green fluorescent protein (GFP) reporter T cell line (GXR 25 cells) in 800ul of R10 medium (2.9.1) and transfected by electroporation using a Bio-Rad

GenePulser II instrument (300 V and 500 uF). Following transfection, cells were rested for 45 min at room temperature, transferred to T25 culture flasks in 5ml warm R10 and fed with 5ml R10 on day 4.

2.10.3 CULTURE TRANSFECTED GXR CELLS

GFP expression was monitored by flow cytometry (FACS Calibur; BD Biosciences) every 48 hours, and once GFP expression reached >30% among viable cells supernatants containing the recombinant viruses were harvested and aliquots stored at -80°C.

2.10.4 DETERMINE VIRAL TITRES USING GXR CELLS

Viral stocks were titred by incubating 400ul of viral stock with 1 million GXR cells in a 24 well plate (500ul total volume). After 12 – 16 hours, 1ml R10 medium was added. After 48 hours %GFP positive cells are measured by flow cytometry by removing 1 ml culture, pelleting cells and resuspending in 2% formaldehyde.
To calculate the amount of virus required for inoculation at an moi of 0.003 the following calculation is used;

The amount of virus required (in ul) = 0.003 x (titre%/400) x 1000

2.10.5 VIRAL PROPOGATION

1 million GXR cells (resuspended in 100ul R10) were inoculated with each chimeric virus at an moi of 0.003 in a total of 500ul (viral stocks were diluted with R10 media to a final volume of 400ul). One well of NL4-3 infected GXR cells were used as a

positive control and one well of uninfected GXR cells as a negative control. The % GFP positive cells was measured daily from days 2 to 7 by flow cytometry by removing 500ul of the culture, pelleting the cells and fixing in 2% formaldehyde. 500ul fresh R10 media was added to the cultures each day.

The mean slope of exponential growth from day 2 to day 7 was calculated using the semi log method in Excel:

[=1n(LOGEST({x1,x2,x3},{y1,y2,y3},0,0))]

This was divided by the slope of growth of the wild-type NL4-3 control included in each assay to generate a normalized measure of replication capacity. Replication assays were performed in triplicate, and results were averaged.

2.11 FLUORESCENT ACTIVATED CELL SORTING (FACS)

2.11.1 CELL SURFACE STAINING

Patient PBMC's were thawed (2.2.3) in R10 medium, rested for 1h at 37°C, 5% CO_2; and stained with a combination of the following cell surface markers;

Antibody	Fluorochrome	Company
CD3	Pacific Orange	Invitrogen
CD4	Qdot 605	Invitrogen
CD8	Pacific Blue	BD Biociences
Live/Dead	A750	Invitrogen
HLA-DR	FITC	BD Biociences
CD38	PECy7	BD Biosciences
PD-1	APC	eBioscience
CD244	PECy5	Biolegend
CCR7	PE	R&D
CD45RA	A700	Biolegend
CD27	FITC	BD Biosciences
CCR5	FITC	Invitrogen
Tim 3	PECy7	Invitrogen
CD95	APC	Biolegend
CD25	PECy5	BD Biosciences

Table 2.10. Summary of cell surface antibodies used in this study

Cells were stained for 30 minutes at RT in the dark. Cells were then washed twice in PBS and fixed in 2% paraformaldehyde. Samples were acquired on an LSR II (BD) flow cytometer within 12 hours of staining and analyzed using FlowJo version 8.8.6.

2.11.2 INTRACELLULAR STAINING

Following surface staining for markers such as CD3, CD4 and CD8, cells were washed twice with FACS buffer and resuspended in Fix/Perm solution (BD Biosciences) for 20 minutes at 4°C. Cells were then washed twice with Perm Buffer (BD Biosciences) and resuspended in the relevant ICS antibody mix with a combination of the following fluorescence-conjugated antibodies:

Antibody	Fluorochrome	Company
IFNγ	PECy7	eBioscience
IL-2	PE	Beckman Coulter
MIP1β	FITC	R&D Systems
TNFα	Alexa Flour 647	Biolegend

Table 2.11. Intracellular antibodies used in this study

Cells were stained for 30 mins at room temperature in the dark. Cells were again washed twice with Perm Buffer and the pellet suspended in 2% paraformaldehyde in PBS.

2.11.3 ACQUISITION AND ANALYSIS

Flow cytometric acquisition was performed on an LSR II (BD) flow cytometer within 12 hours of staining and analyzed using FlowJo version 8.8.6.

2.12 STATISTICAL ANALYSIS AND PHYLOGENETICS

2.12.1 STATISTICAL TESTS USED IN CHAPTER 3

The VRCs of rapid progressor and slow progressor children were compared using the Student's t test. These data were normally distributed as determined by the D'Agostino and Pearson omnibus normality test. The Mann-Whitney U Test was used to compare differences in VRC resulting from protective or disease-susceptible

HLA allele expression as these data were not normally distributed (D'Agostino and Pearson omnibus normality test). The relationship between maternal VRC and child VRC and CD4 and VRC were assessed using the Spearman's correlation. The relationship between VRC and number of selected Gag mutations was also assessed by determination of the Spearman rank correlation coefficient between the two variables. The selected mutants comprised (Carlson *et al*. 2012; Matthews *et al*. 2008; Payne *et al.* 2014; Kloverpris *et al.* 2012): A146X and I147X within the HLA-B*57 restricted epitope ISPRTLNAW (ISW9, Gag 147-155); A163X and S165X within the HLA-B*57:03-restricted epitope KAFSPEVIPMF (KF11, Gag 162-172); E177X, Q182X and T186S within the HLA-B*81:01-restricted epitope TPQDLNTML (TL9, Gag 180-188); T242X and I247X within the HLA-B*57/58:01-restricted epitope TSTLQEQIAW (TW10, Gag 240-249); T310X within the HLA-B*58:01-restricted epitope QATQDVKNW, (QW9, Gag 308-316). In the analyses of the impact of HLA type on CD4 count and viral load in paediatric study subjects, we undertook power calculations based on previously published adult data (Leslie *et al.* 2010; Carlson *et al*. 2012) via bootstrap experiments where we sampled with replacement from the adult data. For example, to test the difference in viral loads between the subjects expressing Protective HLA alleles and those expressing Disease-susceptible patients, for each study group sample size, 1000 experiments were undertaken to determine the proportion where the difference in viral loads between Protective and Disease-susceptible groups remained significant at $p<0.05$. The number in each group required to provide 80% power to show this significance difference between the groups was a group size of 27; having as many as 46 in each group provided 97% power. These calculations demonstrate that we had ample statistical power to

demonstrate whether there was an HLA type effect in children that was equivalent to that observed in adults.

2.12.2 STATISTICAL TESTS USED IN CHAPTER 4

Correlations were analysed using the Spearman Correlation test unless otherwise specified. Comparisons of clinical parameters in groups of ART-treated children were performed using the Kruskal-Wallis test. Comparisons of the presence or absence of protective HLA alleles were performed with a two proportion z test. Correlations between groups of children were analysed using the ANOVA test.

2.12.3 STATISTICAL TESTS USED IN CHAPTER 5

We used a generalized linear model (GLM) to assess the influence of several predictor variables on the absolute CD4+ T cell count (McCullagh & Nelder, 1989; Fahrmeir & Tutz, 2001).

Due to the comparatively small sample size in relation to the number of regarded covariates and considerable correlations within the covariates, we adopted a *Least Absolute Shrinkage and Selection Operator (LASSO)* approach, (Tibshirani *et al.* 1996*)* All computations were carried out in the statistical software R (R Development Core Team, 2014). We adopted three different LASSO methods; the glmnet (Friedman *et al*. 2010), the penalized (Goeman, 2010) and the grplasso (Meier *et al.* 2008). For all three methods, the optimal tuning parameter λ, which reflects the amount of penalization and, hence, controls variable selection, is determined via 10-fold cross-validation based on the model's deviance.

Other statistical tests are described where appropriate and were performed using Graphpad Prism v.5.0 unless otherwise stated.

2.12.4 PHYLOGENETIC ANALYSIS

Maximum-Likelihood phylogenetic trees were generated using PHYm131 (http://www.hiv.lanl.gov) and visualised using Figtree v.1.2.2 (http://tree.bio.ed.ac.uk/software/figtee/).

2.12.5 CORRGRAM

A correlation matrix (Friendly 2002) was used to visualize the Spearman correlations between selected clinical (CD4 count, CD4%, viral load), phenotypic (frequencies of T naïve (Tn) and T effector memory (Tem), activated (CD38+HLADR+) and PD-1 high (PD1+) CD4+ and CD8+ T-cells), plasma levels of sCD14 and functional (CD4+ and CD8+ T-cell responses upon SEB-stimulation) variables. A dataset of n=49 vertically HIV-infected ART-naïve children above 5 years of age for whom all measurements were available from the same study visit was used for analysis. Variables are grouped based on principal component analysis using the R package 'corrgram'.

CHAPTER 3

DISCORDANT IMPACT OF HLA ALLELES ON VIRAL REPLICATIVE CAPACITY AND DISEASE PROGRESSION IN PAEDIATRIC AND ADULT HIV INFECTION

3.1 INTRODUCTION

HIV-infected children typically progress more rapidly to AIDS than adults (Prendergast *et al* 2012). Without treatment, approximately 50% of HIV-1 infected children develop AIDS within a year and 50% will die before their second birthday in sub-Saharan Africa (Prendergast *et al* 2012). However, a minority of perinatally infected children remain asymptomatic through childhood despite being antiretroviral therapy (ART) naive. The reasons for this variation in paediatric disease progression remain largely unknown. Although paediatric HIV infections have accounted for only approximately 10% of those arising in the global epidemic (http://www.avert.org/global-hiv-aids-epidemic.htm), they provide the potential to understand new mechanisms by which HIV disease can be avoided following infection. This remains an important goal of HIV research, with direct relevance to vaccine design.

The central role of HLA class I molecules in control of adult HIV infection is underlined by several genome-wide association studies (Pereyra *et al*. 2010; Fellay *et al* 2009; Fellay *et al* 2007). These are consistent with earlier work showing

characteristic rates of disease progression, depending upon the particular HLA molecules expressed (Goulder *et al*. 2012). In African populations, HLA-B*57, HLA-B*58:01 and HLA-B*81:01 are the class I molecules most consistently associated with slow progression to HIV disease and HLA-B*18:01, HLA-B*45:01 and HLA-B*58:02 the HLA alleles most strongly associated with high viral load and rapid disease progression (Pereyra *et al*. 2010; Costello *et al.* 1999; Kiepiela *et al.* 2004; Leslie *et al*. 2010; Tang *et al.* 2011; Lazaryan *et al*. 2011; Carlson *et al.* 2012).

Several distinct mechanisms are likely to contribute to the immune control or lack of immune control of HIV mediated by these particular HLA-B alleles (Goulder *et al* 2012). One important mechanism is believed to be the impact of selection pressure imposed on HIV by Gag-specific CD8+ T cell responses. Viral escape mutants selected to evade the Gag-specific responses restricted by protective HLA alleles tend to reduce viral replicative capacity (Leslie *et al*. 2004; Martinez-Picado *et al*. 2006; Schneidewind *et al*. 2007; Matthews *et al*. 2008; Crawford *et al*. 2009; Miura *et al*. 2009; Wright *et al* 2010; Wright *et al*. 2012; Boutwell *et al.* 2013). Although in some cases compensatory mutations have been selected that almost cancel out the fitness cost of the escape mutant (Schneidewind *et al.* 2009), in most cases the compensatory mutations only partially recover the reduction in viral replicative capacity resulting from selection of the escape mutant (Schneidewind et al. 2007; Wright et al. 2012; Boutwell *et al*. 2013; Crawford *et al*. 2007; Schneidewind *et al*. 2008). In contrast with protective HLA molecules, those that mediate lack of immune control typically restrict dominant epitopes outside of Gag, in proteins such as Env and Nef (Kiepiela *et al*. 2007), and escape mutants are either not selected by these CTL responses or they have no discernible effect on viral replicative capacity

(Kiepiela *et al.* 2007; Ngumbela *et al.* 2008; Troyer *et al.* 2009). Thus the mechanism of HLA allele-mediated immune control or lack of control is strongly linked to the impact that the relevant escape mutants have on viral replicative capacity.

The role of HLA alleles and the impact of viral replicative capacity on disease progression has been less well studied in paediatric HIV infection. Although CD8+ T cell responses against HIV are detectable in the first days of life (Luzuriaga *et al*. 1995; Thobakgale *et al*. 2007), a viral setpoint is not established for years (Prendergast *et al.* 2007), unlike adult infection where viral load reaches a setpoint 6 weeks after infection (Prendergast *et al*. 2012). The evidence that HLA class I plays an important role in immune control of HIV in infected children is mixed and somewhat contradictory (Goulder *et al*. 2001; Feeney *et al.* 2004; Feeney *et al*. 2006; Thobakgale *et al*. 2009; Schneidewind *et al*. 2009; Prado *et al.* 2010; Prendergast *et al.* 2011). In the largest of these studies, which followed 61 HIV-infected mother-child pairs from birth, there was no overall impact of HLA alleles on rate of progression in children, but if either the child or mother carried a protective HLA allele (HLA-B*57/58:01/81:01), progression in the child was significantly delayed, provided that the protective allele was not shared by mother and child. This supported earlier work (Goulder *et al*. 2001; Feeney *et al*. 2004), and was reinforced by subsequent studies in adults showing that disease progression in the recipient is related to the viral replicative capacity of the transmitted virus (Goepfert *et al*. 2008; Hollingsworth *et al*. 2010; Hecht *et al.* 2010; Prince *et al*. 2012). Nonetheless, findings from a smaller study of 11 Jamaican children suggested that infected children did not benefit from having an HLA-B*57-positive mother (Schneidewind *et al*. 2009). However, in none of these studies were direct measurements of viral

replicative capacity made in infected children and their transmitting mother, with the exception of one study (Prado *et al.* 2010) that measured VRC in 8 children only, and therefore was unable to examine the impact of HLA alleles on VRC in pediatric infection.

To better define the relationship between viral replicative capacity (VRC), HLA allele expression, and disease outcome in paediatric infection, we here studied a cohort of >350 HIV infected children in South Africa. These studies are the first to measure viral replicative capacity both in infected children and in their transmitting mothers, enabling the impact of transmitted virus and maternal HLA type on paediatric VRC and disease outcome to be directly assessed. In addition, these investigations are the first to be undertaken in sufficient numbers of study subjects to analyze with statistical rigor the impact of the VRC and HLA alleles in both mother and child on paediatric disease outcome. We aimed, first, to determine the relationship between viral replicative capacity and disease progression in paediatric infection; second, to assess the impact of maternal HLA alleles and maternal VRC on VRC in the child; third, to compare the impact of both protective and disease-susceptible HLA alleles, respectively, on the VRC of viruses in adults and children; and, finally, to compare the impact of protective and disease-susceptible HLA alleles on disease outcome adults and children.

3.2 RESULTS

3.2.1 LOWER VIRAL REPLICATIVE CAPACITY IS ASSOCIATED WITH SLOW PROGRESSION IN CHILDREN

Recombinant viruses were generated from 43 mother-child pairs, and from an additional 41 children for whom the mother was unavailable for study and a further 4 mothers for whom the child was unavailable for study. All mother and child samples described in this chapter were derived from the Kimberley cohort, South Africa. Fifty-five of these children were defined as slow-progressor (ART-naïve, absolute CD4 count >350/mm^3, age ≥5yrs, did not meet 2010 WHO criteria to commence ART, mean age 7.5 years) and 29 were defined as rapid-progressor (CD4 ≤25% by 24 months of age, mean age 14 months). Chimeric viruses generated by co-transfection of the amplified patient-derived gag-protease genes with the linearized pNL4-3Δgag-protease were used to infect a GFP reporter cell line as outlined in section 2.10 and as previously described (Wright *et al*, 2010; Wright *et al.* 2012; Brockman *et al.* 2006; Payne *et al.* 2014; Miura *et al.* 2009; Brockman *et al*. 2010; Huang *et al*. 2011; Nomura *et al*. 2013; Juarez-Molina *et al*. 2014). Fig 3.1 shows representative FACS plots of viral replication measured daily by flow cytometry.

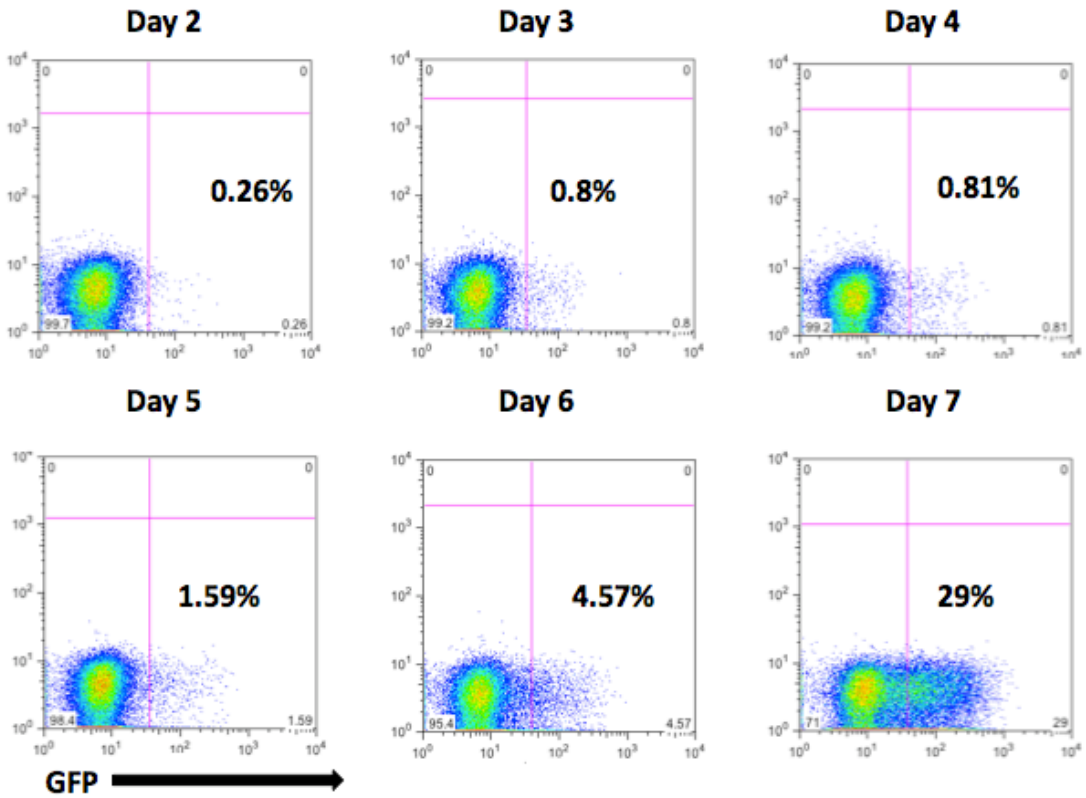


Fig 3.1. Representative FACS plots showing daily cell to cell spread of viral infection. Gated on live GXR cells. x axis shows % GFP expression, the y axis represents forward scatter (FSC-A).

Several previous studies adopting this methodology (Wright *et al.* 2010; Payne *et al.* 2014; Miura *et al*. 2009; Brockman *et al*. 2010) have demonstrated 99-100% amino acid sequence identity between plasma virus and the gag-pro-chimeric viruses, after exclusion of codons containing mixed bases. The same comparison was undertaken here in a subset of the study subjects (n=33), confirming amino acid identity between plasma and gag-pro-chimeric viruses sequence at a median of 99.4% residues (range IQR 99-100%) (Fig 3.2). Thus the gag-pro chimeric viruses that were generated are indeed representative of the plasma virus in each study subject.

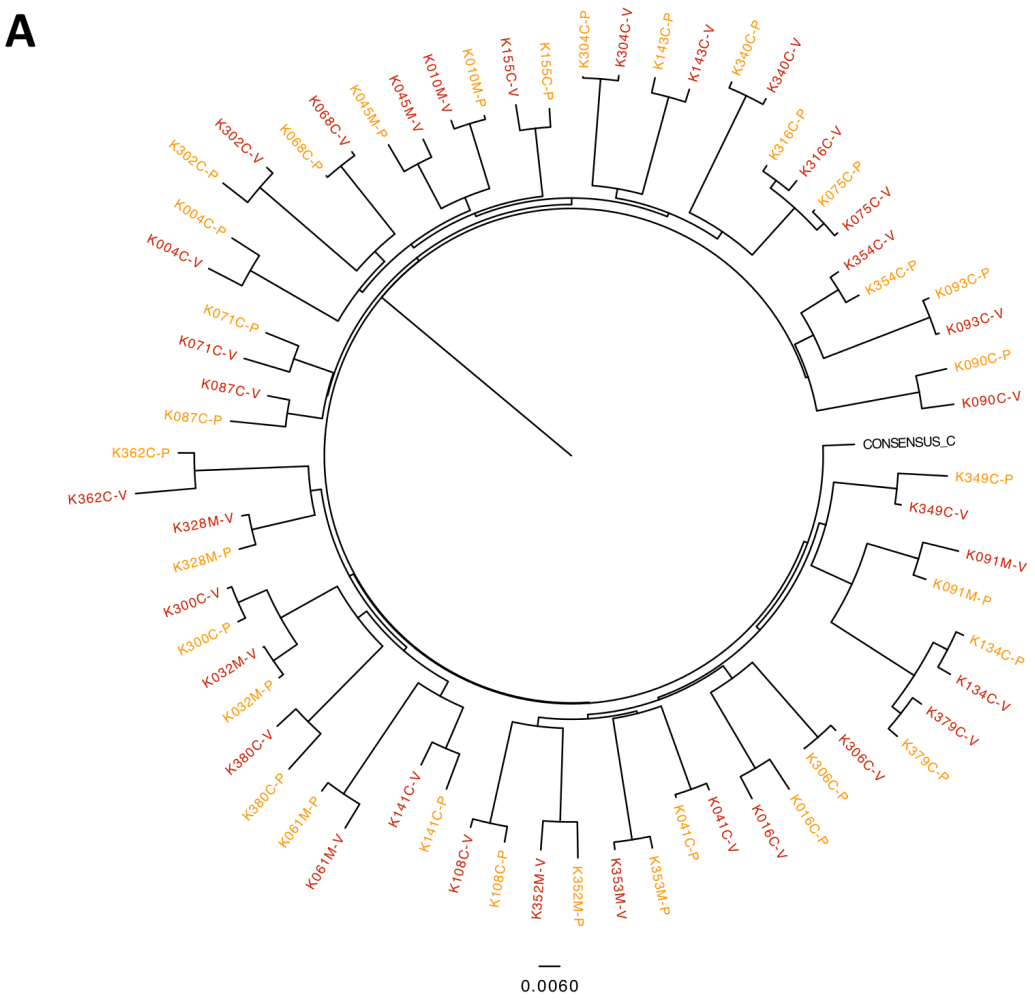

A
K304C-P
K304C-V
K143C-P
K143C-V
K340C-P
K340C-V
K316C-P
K316C-V
K075C-P
K075C-V
K354C-V
K354C-P
K093C-P
K093C-V
K090C-P
K090C-V
CONSENSUS_C
K349C-P
K349C-V
K091M-V
K091M-P
K134C-P
K134C-V
K379C-V
K379C-P
K306C-V
K306C-P
K016C-P
K016C-V
K041C-V
K041C-P
K359M-V
K359M-P
K352M-V
K352M-P
K108C-P
K108C-V
K141C-P
K141C-V
K061M-V
K061M-P
K380C-P
K380C-V
K032M-P
K032M-V
K300C-P
K300C-V
K328M-P
K328M-V
K362C-V
K362C-P
K087C-P
K087C-V
K071C-V
K071C-P
K004C-V
K004C-P
K302C-P
K302C-V
K068C-P
K068C-V
K045M-P
K045M-V
K010M-V
K010M-P
K155C-V
K155C-P
0.0060
B
100
98
96
% amino acid identity
Plasma and chimeric virus sequence pairs

Figure 3.2: Phylogenetic tree of Gag sequences derived from 33 patient plasma samples and Gag-Pro chimeric viruses. A. A maximum-likelihood tree was constructed using PHYm131 (http://www.hiv.lanl.gov). "C" denotes a child isolate and "M" donates a maternal isolate. Each mother-child pair carries the same number, for example K140C and K140M represent the K140 mother-child pair. The scale represents substitutions per site. V represents chimeric virus sequence, P represents plasma virus sequence. B. The deduced Gag amino acid sequences determined from plasma virus and from the Gag-Pro-chimeric virus were compared for each of the 33 subjects. The percent amino acid identity was determined for each subject after exclusion of codons including mixed bases, as previously described (Wright *et al*. 2010; Payne *et al*. 2014; Miura *et al*. 2009; Brockman *et al.* 2010).

Gag-protease chimeric viruses derived from slow-progressor children displayed significantly reduced replication capacity to those derived from rapid-progressors (p=0.02; Student's t test; Fig 3.3A). Furthermore, VRC correlated significantly with decreasing absolute CD4 count in the slow-progressor children at 5 yrs of age (r=-0.33 p=0.026, Fig 3.3B) and at 7.5 yrs of age (r=-0.42, p=0.008, Fig 3.3C). There was no correlation between VRC and absolute CD4 count in the rapid progressor children, The plasma samples from which the chimeric viruses were generated were obtained from slow progressors aged a mean of 7.5 years, and thus the stronger correlation with CD4 count at 7.5 years than with CD4 count at 5yrs is consistent with this. Similarly, the measured VRC also correlated with the CD4 count exactly coincident with sample obtained for generation of the chimeric viruses from which VRC was determined (r=-0.34, p=0.01; Fig 3.3D).

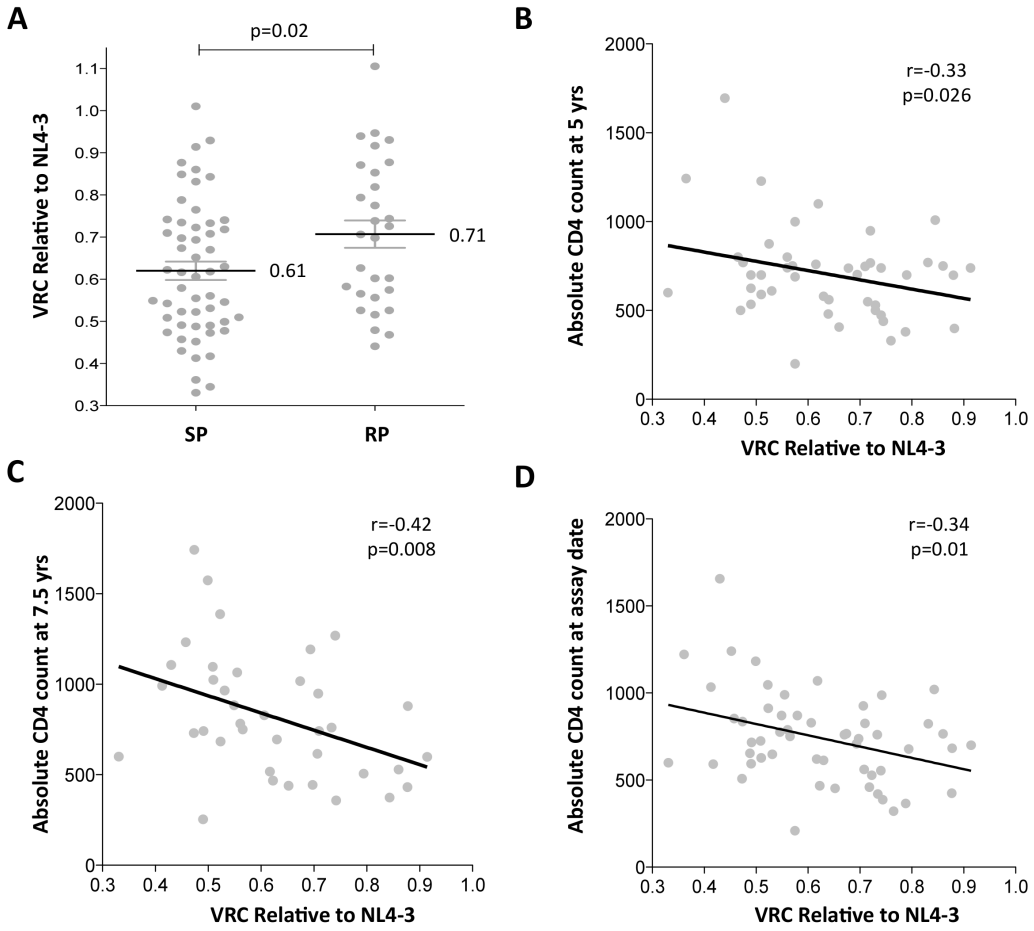


Figure 3.3: Lower viral replicative capacity is associated with slow progression in children. A. Replication capacities, normalized to NL4-3 comparator virus, were significantly lower in slow progressor children, median 0.61 (n=55) compared to rapid progressor children, median 0.71 (n=29) (p=0.02, Students t test). B. VRC correlation with decreasing absolute CD4 count in slow-progressor children at 5 years of age (n=46). C. VRC correlation with decreasing absolute CD4 count in slow-progressor children at 7.5 years of age (n=38). D. VRC correlation with decreasing absolute CD4 count in slow-progressor children at the age of assay date (n=55). Spearman correlations shown.

Together these data suggest that VRC influences disease progression in paediatric infection, with CD4 count being closely associated with disease progression in HIV-infected children.

Of note, in contrast to HIV-infected adults, viral load and absolute CD4 are not significantly correlated, except in older children as they approach adolescence. Here, in the slow progressor children aged 5yrs, the correlation between VL and CD4 is weak ($r=-0.25$, $p=0.32$) and in the 7.5yo children, the correlation approaches significance ($r=-0.31$; $p=0.09$) (Fig 3.4). For this reason, there is no correlation observed between VRC and viral load in the slow progressor children (Fig 3.4). In contrast to absolute CD4 count, viral load is a poor marker of disease progression in children and is not part of WHO criteria for ART initiation in children.

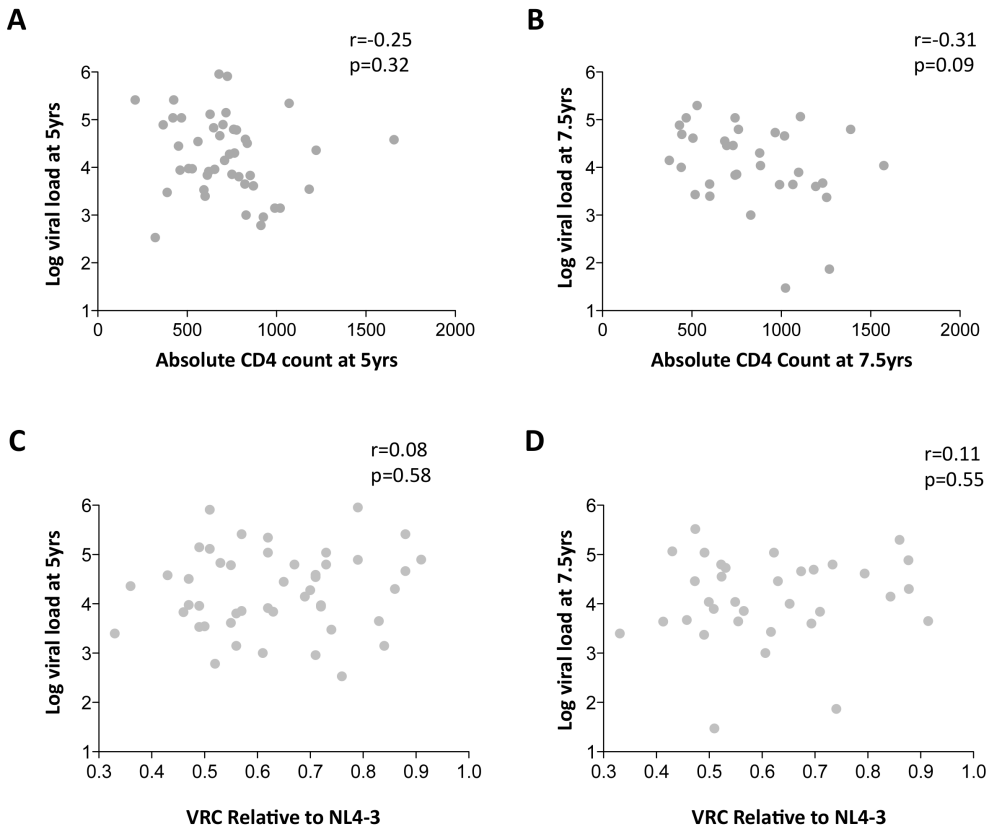


Figure 3.4. Relationship between viral load and VRC and absolute CD4 count in paediatric slow progressors. A. Viral load versus absolute CD4 count in ART-naïve children of 5 yrs age. B. Viral load versus absolute CD4 count in ART-naïve children of 7.5 yrs age. C. Viral load versus VRC using viral loads at 5yrs age in paediatric slow progressors. D. Viral load versus VRC using viral loads at 7.5yrs age in paediatric slow progressors. Spearman correlations shown.

3.2.2 IMPACT OF THE MATERNALLY TRANSMITTED VIRUS

First, the authenticity of the mother-child transmission pairs was validated by phylogenetic analysis of all the viral sequences (Fig 3.5).

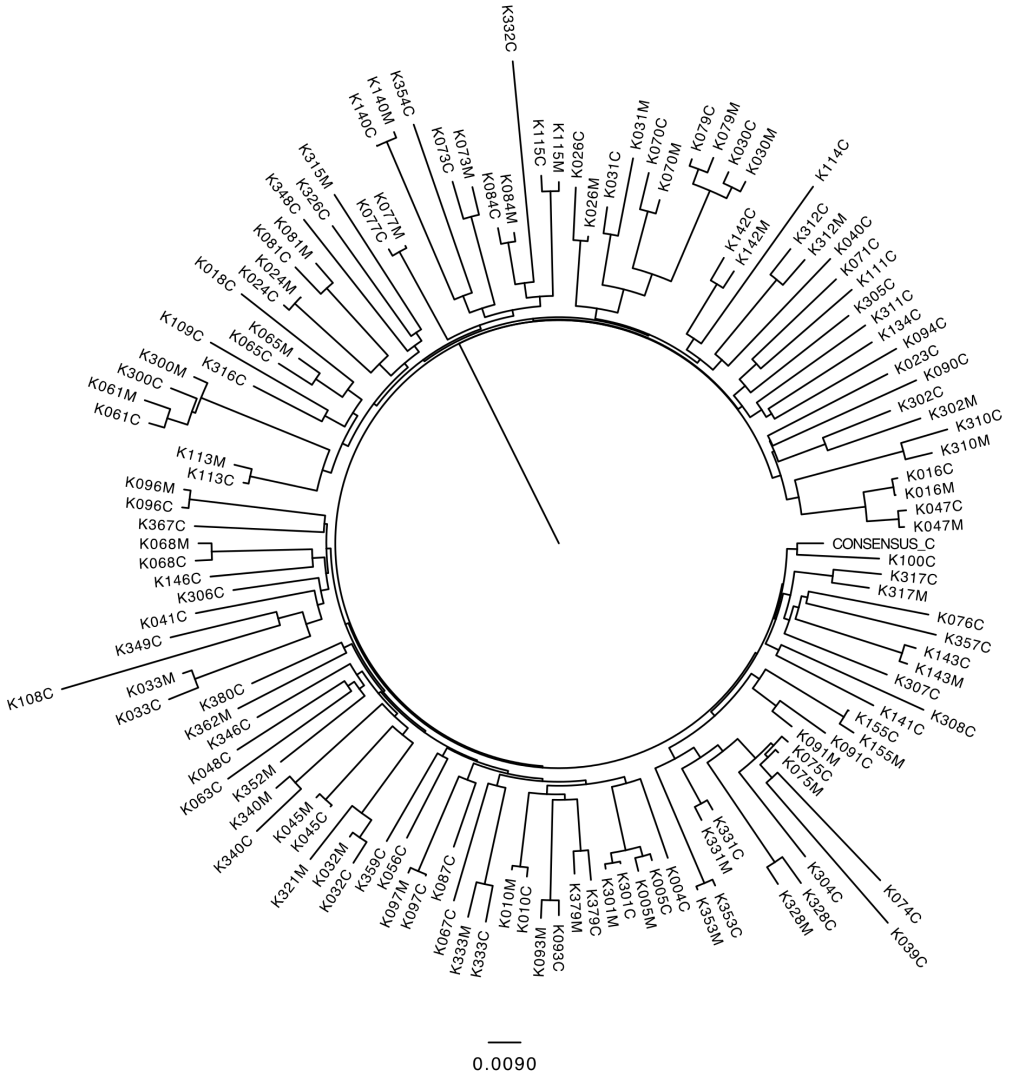


Figure 3.5. Phylogenetic tree of Gag sequences derived from patient plasma samples. A maximum-likelihood tree was constructed using PHYm131 (http://www.hiv.lanl.gov). "C" denotes a child isolate and "M" donates a maternal isolate. Each mother-child pair carries the same number, for example K140C and K140M represent the K140 mother-child pair. The scale represents substitutions per site.

The VRC of chimeric viruses derived from the children was then compared with those of the respective mothers. Overall, somewhat surprisingly, the VRC in the mothers tends to be slightly higher than in the children (mean VRC 0.69 versus 0.62; p=0.056, paired t test, Fig 3.6A,B). This result is discussed further below. However, considerable variation in this pattern was observed. As expected, overall, maternal and child VRCs were significantly correlated (r=0.43, p=0.004; Fig 3.6C), in spite of the fact that on average 7.5yrs had elapsed since transmission in the case of the slow progressors. In keeping with the fact VRC in the rapid progressors was determined from children and mothers a mean of only 14 months post transmission, the Spearman correlation coefficient between VRC in mothers and children was higher for the rapid progressors than for the slow progressors (r=0.55 versus r=0.36, respectively; Fig 3.6D,E).

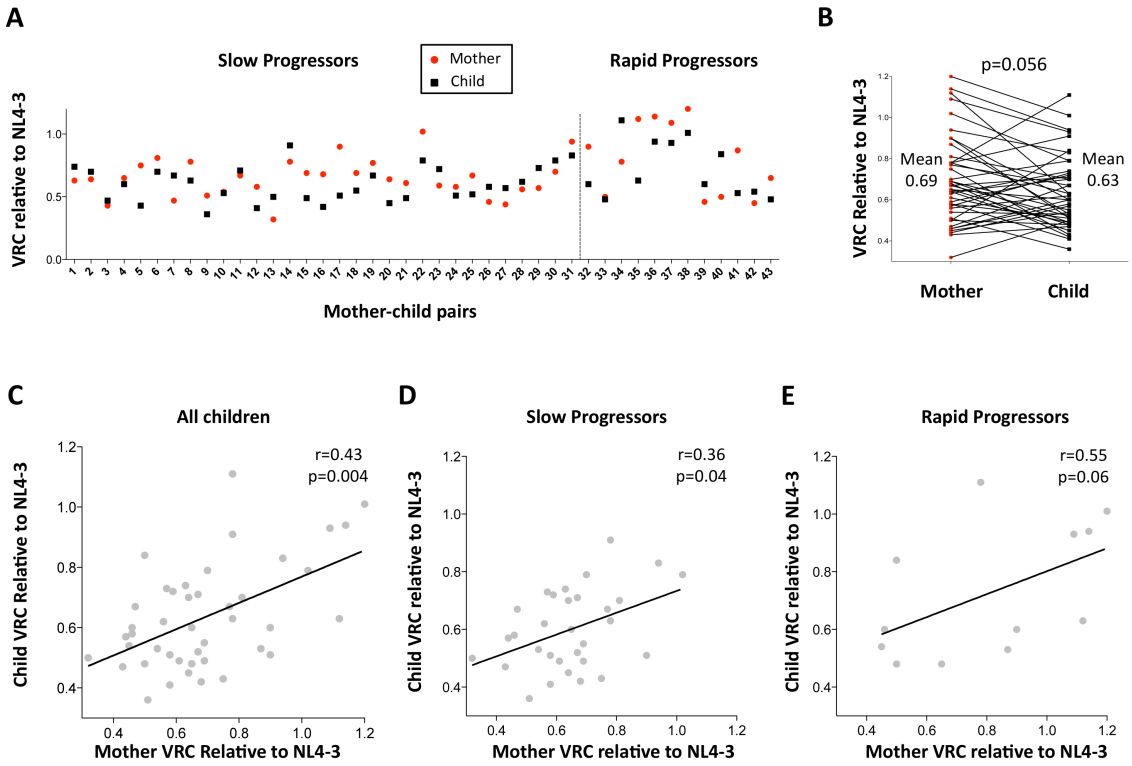


Figure 3.6: Relationship between maternal VRC and paediatric VRC. A. Maternal VRC (red circles) and child VRC (black squares) for each mother-child transmission pair. B. Comparison between VRC in mother and child: maternal mean VRC 0.69 versus VRC in the child 0.63; p=0.056, paired t test. C. Correlation between VRC in mother and child for all children (n=43, r=0.43, p=0.004). D. Correlation between VRC in mother and child for slow progressor children (n=31, r=0.36, p=0.04). E. Correlation between VRC in mother and child for rapid progressor children (n=12, r=0.55, p=0.06). Spearman correlations shown.

3.2.3 DIFFERENTIAL IMPACT OF HLA CLASS I EXPRESSION ON VRC IN ADULT AND PAEDIATRIC INFECTION

In order to better understand the observed differences in VRC within mother-child pairs we next investigated the relative impact on VRC in mother and child of the protective HLA alleles in sub-Saharan African populations, HLA-B*57, HLA-B*58:01 and HLA-B*81:01, previously shown to significantly reduce VRC (Costello *et al* 1999; Kiepiela *et al* 2004; Lazaryan *et al* 2006; Leslie *et al* 2010; Tang *et al* 2010; Lazaryan *et al* 2011, Carlson *et al* 2012). The VRC of chimeric viruses was substantially lower (VRC 0.52 vs 0.77) in the mothers who expressed any of these protective HLA alleles, compared with the mothers who did not ($p<0.0001$, Mann-Whitney test, Fig 3.7A). In contrast, there was a marginal impact on VRC of these protective HLA alleles in children (VRC 0.59 versus 0.66; $p=0.08$). Similarly, in mothers who expressed one or more of the sub-Saharan African HLA alleles associated with more rapid disease progression, HLA-B*18:01, HLA-B*45:01, and HLA-B*58:02 (Kiepiela *et al* 2004; Leslie *et al* 2010; Tang *et al* 2010; Carlson *et al* 2012) - HLA-B*35 was not included as this allele is not associated with rapid progression in C clade infection (Matthews *et al* 2012) - VRCs were significantly higher than in those who did not express these alleles (VRC 0.87 vs 0.64; $p=0.007$) whereas in children the VRC was not increased in those with disease-susceptible HLA alleles (VRC 0.62 vs 0.64, $p=0.41$, Fig 3.7B). Thus the effects of the protective HLA alleles and disease-susceptible HLA alleles on VRC were striking among the adult subjects and minimal to absent in the children.

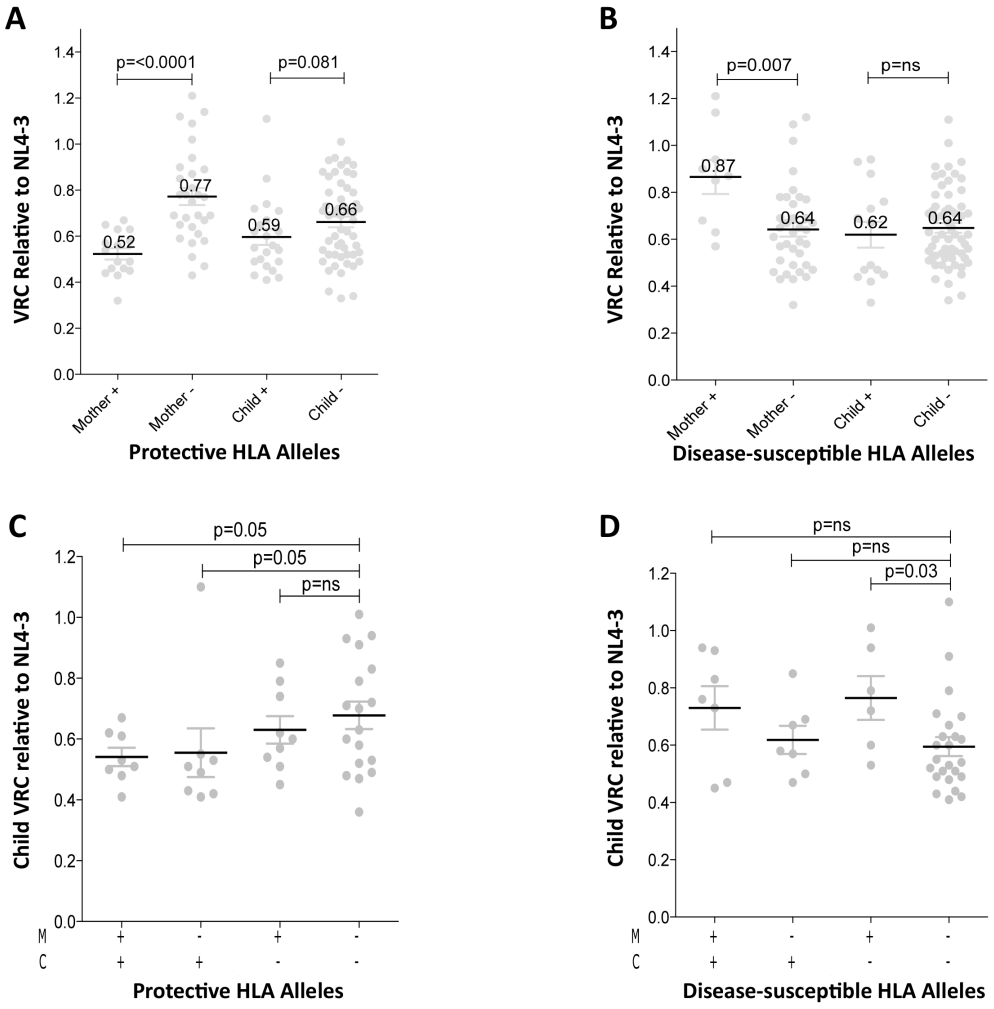


Figure 3.7. Relative contribution of HLA-B alleles in VRC in mothers and children. A. VRC in mothers expressing protective HLA alleles (n=16) and not (n=30); and in children expressing protective HLA alleles (n=22) and not (n=54). B. VRC in mothers expressing disease-susceptible HLA alleles (n=9) and not (n=37); and in children expressing disease-susceptible alleles (n=14) and not (n=62). C. VRC in the child according to expression of protective HLA alleles in mother or child (+/+ n=8; -/+ n=8; +/- n=9; -/- n=18). D. VRC in the child according to expression of disease-susceptible alleles in the mother and/or child (+/+ n=7; -/+ n=7; +/- n=6; -/- n=23). Mann-Whitney U Tests shown.

To further investigate the previous finding that slow progression is significantly associated with the mother or the child possessing one of the protective HLA-B alleles, but less so if the protective allele was shared by mother and child (Goulder *et al*. 2001; Thobakgale *et al*. 2009) we analyzed the VRC in children according to the presence or absence of protective alleles (HLA-B*57, HLA-B*58:01 or HLA-B*81:01) in mother and/or child. We observed here that, in the group where children and mothers both lacked protective alleles, the VRC was higher than the other three groups, significantly so where the mother and child share a protective allele and when the child alone carries the protective allele (p=0.05) (Fig 3.7C). These data suggest that, although protective HLA alleles have a greater impact on VRC in adults than in children (Fig 3.7A), the VRC in children is more influenced by the protective alleles when they are expressed in the children themselves than when expressed in the mothers (Fig 3.7C). In contrast, the impact of the disease-susceptible alleles HLA-B*18:01/45:01/58:02 on the child's VRC was greatest when the mother carried any of these (Fig 3.7D). Disease-susceptible alleles in the children therefore appeared to have little impact on VRC in the child.

3.2.4 STRONG CORRELATION BETWEEN GAG ESCAPE MUTANT NUMBER AND VRC

To determine whether the differences in VRC observed were indeed related to the occurrence of escape mutants in Gag selected by these protective HLA alleles as indicated by previous work (Carlson *et al.* 2012; Leslie *et al.* 2004; Martinez-Picado *et al.* 2006; Schneidewind *et al.* 2007; Matthews *et al.* 2008; Crawford *et al.* 2009;

Miura *et al.* 2009; Wright *et al*. 2010; Wright *et al*. 2012; Boutwell *et al*. 2013; Payne *et al.* 2014; Kloverpris *et al.* 2012) we sought a correlation between VRC and number of Gag escape mutants associated with these HLA alleles in each of the chimeric viruses studied, irrespective of the study subject.

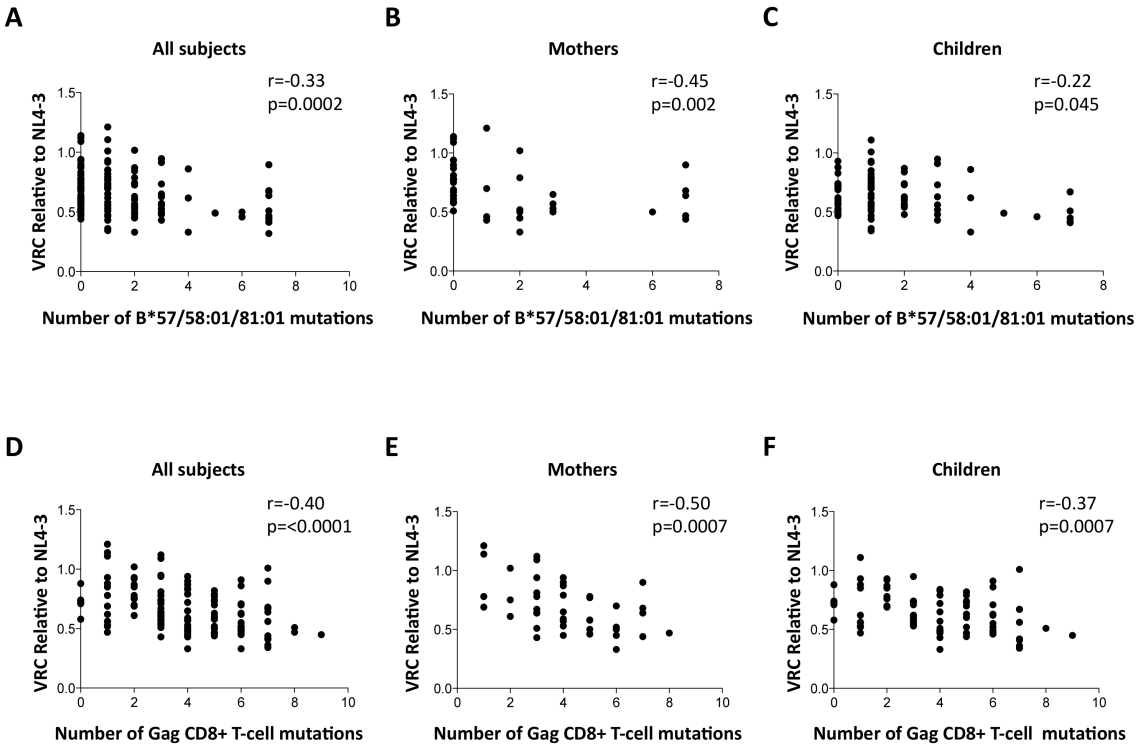


Figure 3.8. Impact of Gag mutations on VRC. A-C: HLA-B*57/58:01/81:01 mutations. (A146X, I147X, A163X, S165X, E177X, Q182X, T186S, T242X, I247X and T310X). A. All subjects; B. Mothers; C. Children. **D-F: Gag CD8+ T-cell mutations** (listed in Table 3.1). DS. All subjects. E. Mothers. F. Children. Spearman rank correlation coefficients shown.

As expected, a highly significant correlation was observed between the number of Gag escape mutants associated with the protective HLA alleles and decreasing VRC ($r=-0.33$, $p=0.0002$, Fig 3.8A). Breaking these data down into the mothers and children, the impact on VRC of these Gag mutants associated with the protective HLA molecules appeared greater in the mothers ($r=-0.45$, $p=0.002$, Fig 3.8B) than the children ($r=-0.22$, $p=0.045$, Fig 3.8C). However the impact of these Gag escape mutants on VRC in the slow progressor children was similar to that in the mothers ($r=-0.37$, $p=0.006$); only in the rapid progressor children, where the number of Gag mutants associated with protective HLA alleles was low, was there no evidence of any impact of these HLA molecules on VRC (Fig 3.9).

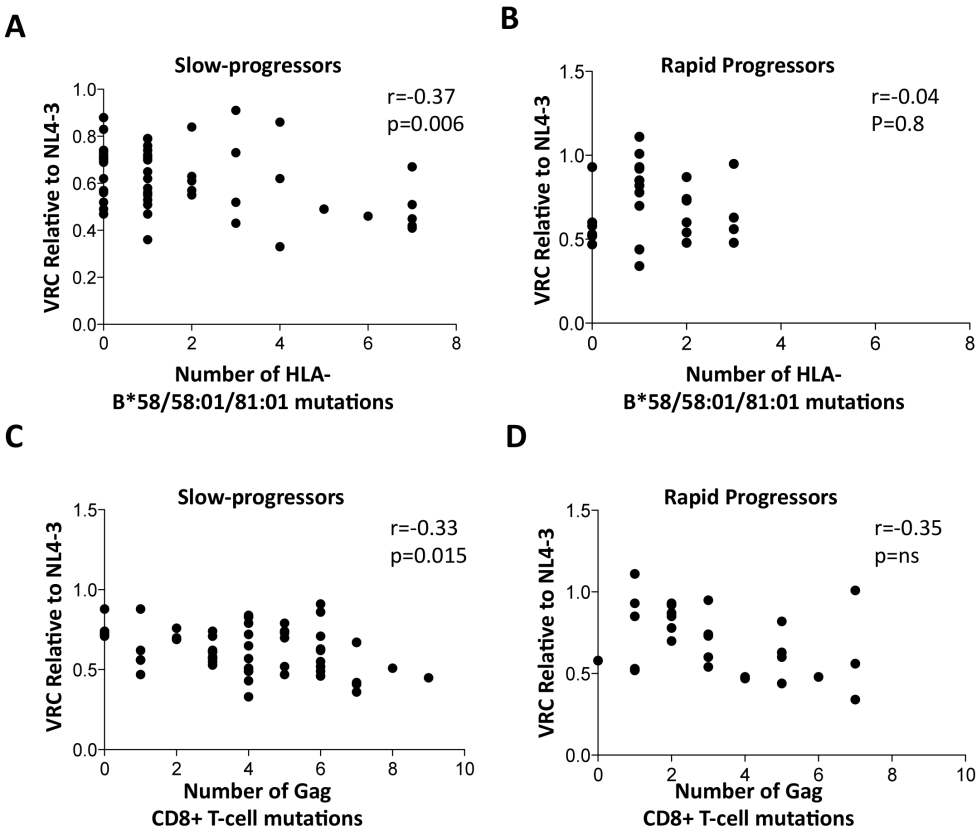


Figure 3.9. VRC compared with number of Gag-specific CD8+ T-cell escape mutations in Rapid and Slow progressor children. A-B: HLA-B*57/58:01/81:01-associated Gag mutants. A. Slow progressors. B. Rapid progressors. **C-D. Gag-specific CD8+ T-cell mutations within defined epitopes (**listed in Table 3.1**).** A. Slow progressors B. Rapid progressors. Spearman rank correlation coefficients shown.

These analyses were repeated to assess the impact, not only of the Gag mutants associated with the protective HLA molecules HLA-B*57/58:01/81:01, but of all 20 Gag escape mutants within defined Gag CD8+ T-cell epitopes (Carlson *et al.* 2012; Matthews *et al*. 2008; Payne *et al.* 2014; Kloverpris *et al*. 2012) (Table 3.1).

HLA restriction	Epitope	Gag variant
HLA-B*42:01	RPGGKK**H**YM	H28X
HLA-B*42:01	RPGGKKHY**M**	M30X
HLA-B*57	**A**ISPRTLNAW	A146X
HLA-B*57	A**I**SPRTLNAW	I147X
HLA-B*57:03	K**A**FSPEVIPMF	A163X
HLA-B*57:03	KAF**S**PEVIPMF	S165X
HLA-B*81:01/42:01	TP**Q**DLNTML	Q182X
HLA-B*81:01	TPQDLN**T**ML	T186X
HLA-B*81:01	TPQDLNTMLN**T**	T190X
HLA-B*57/58:01	TS**T**LQEQIAW	T242X
HLA-B*57:03	TSTLQEQ**I**AW	I247X
HLA-B*35:01	NPPIPVG**D**IY	D260E
HLA-B*27:05	K**R**WIILGLNK	R264X
HLA-B*27:05	KRWII**L**GLNK	L268X
HLA-B*14:01	DRFF**K**TLRA	K302R
HLA-B*58:01	QA**T**QDVKNW	T310S
HLA-B*44:03	AEQATQ**D**VKNW	D312E
HLA-B*08:01	NANPDC**K**TIL	K331X
HLA-B*39:10	NPDCK**T**ILRAL	T332X
HLA-B*07:02	GP**S**HKARVL	S357X

Table 3.1. Gag-specific CD8+ T-cell mutations within defined epitopes. (Carlson *et al.* 2012; Matthews *et al*. 2008; Payne *et al.* 2014; Kloverpris *et al.* 2012).

These include a number of mutants that it may be inferred reduce viral replicative capacity from evidence that these revert in the absence of the relevant HLA molecule (Leslie *et al.* 2004; Martinez-Picado *et al*. 2006; Matthews *et al*. 2008) and reflect the impact of HLA molecules such as HLA-B*42:01, HLA-B*39:10 and HLA-B*14:01 that offer some degree of protection against disease progression, though not as much as provided by HLA-B*57/58:01/81:01 (Pereyra *et al.* 2010; Kiepiela *et*

al. 2004; Leslie *et al.* 2010; Carlson *et al.* 2012). In contrast, the disease-susceptible HLA molecules make no contribution to the selection of Gag mutants. These analyses suggest that the additional Gag mutants also contribute to VRC, with overall a strong correlation between VRC and number of Gag CD8+ T-cell epitope mutants (r=-0.40, p<0.0001, Fig 3.8D); similar in mothers (r=-0.50, p=0.0007, Fig 3.8E) as in children (r=-0.37, p=0.0007, Fig 3.8F).

3.2.5 LOW GAG MUTANT NUMBER IN ADULTS WITH HIGH VRC EXPRESSING DISEASE-SUSCEPTIBLE HLA ALLELES

The observation of a clearcut increase in VRC in mothers expressing disease-susceptible alleles HLA-B*18:01/45:01/58:02 (shown in Fig 3.7B) prompts the question of what drives the VRC up in these subjects; or whether, alternatively, the VRC is driven down in those who do not express these alleles. Analysis of the number of Gag variants, using all 20 Gag escape mutants, in subjects who do and who do not express these disease-susceptible alleles, respectively, shows a substantially lower number of Gag escape mutants in the mothers expressing disease-susceptible alleles (mean 2.4 versus 4.6; p=0.001, Student *t* test; Fig 3.10).

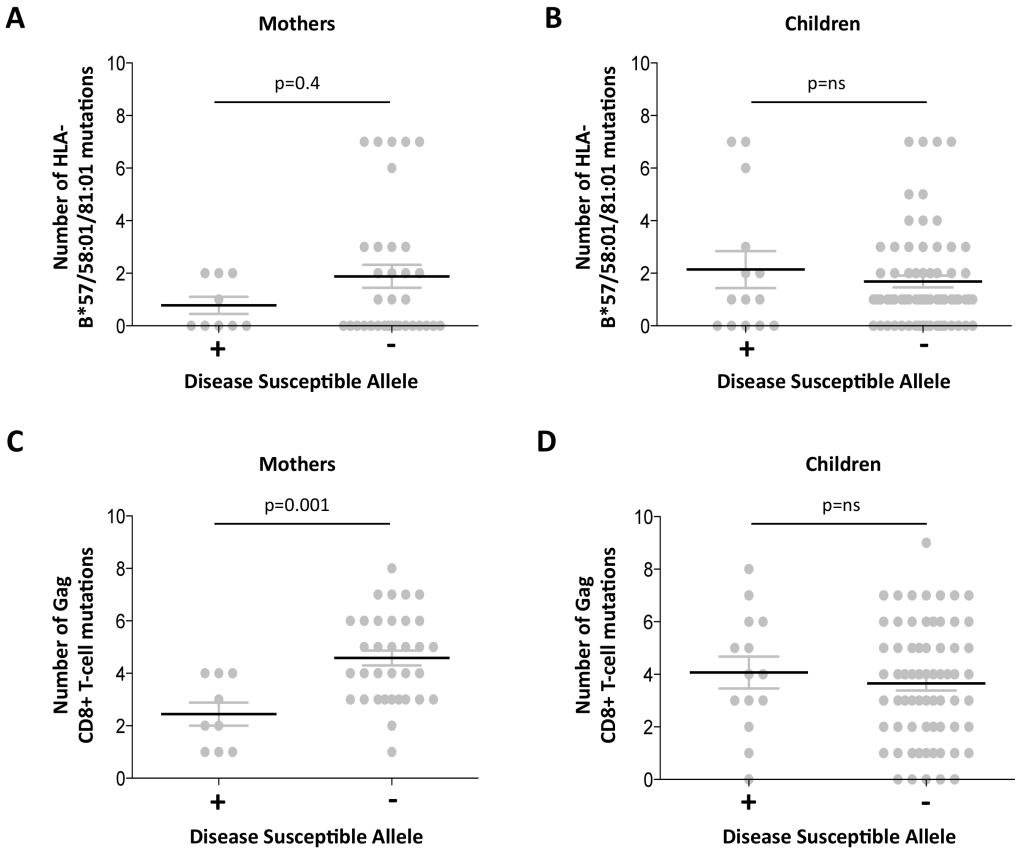


Figure 3.10. Gag mutant number in adults with high VRC expressing disease-susceptible HLA alleles. A-B: HLA-B*57/58:01/81:01 mutations. A. Mothers. B; Children. **C-D: Gag CD8+ T-cell mutations.** C. Mothers. D. Children. Mann-Whitney U Tests shown.

This difference persisted even when subjects expressing the protective HLA alleles were omitted (p=0.003, Fig 3.11).

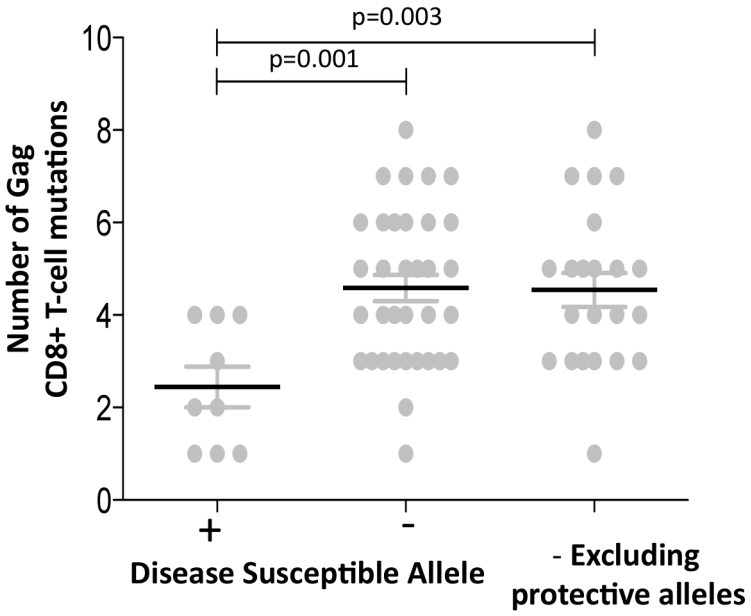


Figure 3.11 Impact of Gag CD8+ T-cell mutations (listed in Table 3.1) in adults expressing disease susceptible HLA alleles. Number of gag CD8+ T-cell mutations in mothers expressing disease-susceptible HLA alleles (HLA-B*18:01/45:01/58:02, left hand panel), mothers who do not express HLA-B*18:01/45:01/58:02 (centre panel), and mothers excluding those that express either disease-susceptible or protective HLA alleles (HLA-B*57/58:01/81:01, right hand panel). Mann-Whitney U Tests shown.

These differences were also marked when the analysis was limited to the Gag mutants associated with the protective HLA alleles (mean 0.8 versus 1.9 in subjects who did and who did not express disease-susceptible alleles, respectively) although in this case this did not approach statistical significance (p=0.4; Fig 3.10). In summary, these data are consistent with high VRC in adult subjects expressing disease-susceptible allele as the result principally of these HLA molecules failing to drive the selection of Gag CD8+ T-cell escape mutants. In contrast to the adults, in

children the effects of these disease-susceptible alleles is apparently nil, with Gag escape mutants equally distributed between children who do and who do not express these HLA molecules.

3.2.6 DISCORDANT IMPACT OF HLA ALLELES ON PROGRESSION IN HIV-INFECTED CHILDREN AND ADULTS

To compare the impact of protective and disease-susceptible HLA alleles on progression in adults and children we first analyzed the expression of these alleles in chronically infected, ART-naïve South African adults, analyzed according to absolute CD4 count at enrollment (Fig 3.12AB).

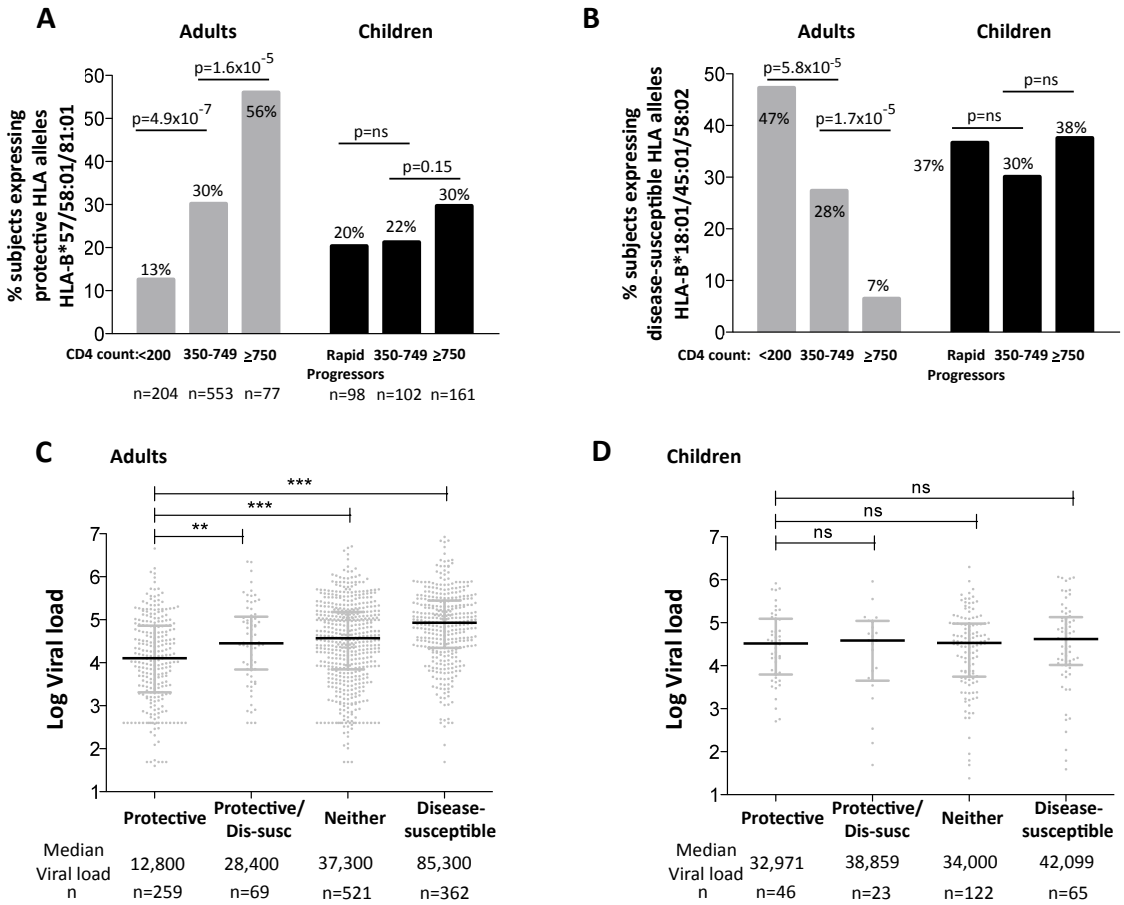


Figure 3.12. Impact of Protective and Disease-susceptible HLA alleles on disease progression in adults and children. A. The frequency of ART-naïve adults and children stratified according to CD4 count and disease progression expressing one or more of the protective HLA alleles HLA-B*57/58:01/81:01. Adult study numbers: absolute CD4<200/mm^3, n=204; CD4 350-749/mm^3, n= 553; CD4≥750/mm^3 n= 77. Children study numbers: Rapid Progressors, n = 98; CD4 350-749/mm^3, n= 102; CD4≥750/mm^3, n=161 (Fishers exact test). B. As in panel A, but the frequency of subjects expressing one or more of the disease-susceptible HLA alleles HLA-B*18:01/45:01/58:02 (Fishers Exact test). C. Viral load in ART-naïve adults (total, n=1,211) according to the presence of protective HLA alleles and/or disease-susceptible alleles. ** represents p<0.001, *** represents p<0.0001. Not shown on the figure are significant differences between viral loads in the disease-susceptible group and each of the other groups (in each case p<0.0001, Mann Whitney U test). D. As in panel C, but in ART-naïve children (total n=256, age ≥5yrs).

As expected, protective HLA alleles were highly enriched in adults with high CD4 counts and disease-susceptible alleles highly enriched in adults with low CD4 counts. In HIV-uninfected children during the first five years of life, absolute CD4 counts change dramatically according to age, declining from a median of 2500-3000/mm^3 during the first year to a stable median of approximately 750-1000 cells/mm^3 from age 5yrs through adulthood (Shearer *et al.* 2003). Comparing ART-naïve, HIV-infected children aged >5yrs with CD4 counts of ≥750 cells/mm^3 with those with CD4 counts of 350-749 cells/mm^3, we observed no significant difference in the frequency of protective or disease-susceptible HLA alleles in these groups. Similarly, in a rapid progressor group of HIV-infected children (median age at ART initiation 6m, median absolute CD4 count 584/mm^3, median CD4% 13%, and median viral load 1.1m), the frequency of protective and disease-susceptible alleles also did not differ significantly from the 'slow progressor' groups of infected

children. Thus, the impact of the protective and disease-susceptible HLA alleles on CD4 count is marked in adult infection, but is barely discernible in paediatric infection. Similarly, analysis of the impact of the protective and disease-susceptible HLA alleles on viral loads in ART-naïve adults and children (Fig 3.12CD) showed significant effects among the adults (a median 0.8 log HIV copies/ml difference between adults with protective alleles versus those with disease-susceptible alleles), and insignificant effects among the slow progressor children. Although, as described above, viral load is less helpful as a proxy for disease progression in paediatric versus adult infection, it is clear that in terms of the biological markers of disease progression, absolute CD4 count and viral load, the HLA alleles that have the strongest effects on outcome in adult infection have minimal impact in paediatric infection.

3.2.7 LACK OF SELECTION PRESSURE MEDIATED BY PROTECTIVE HLA ALLELES IN RAPID PROGRESSOR CHILDREN

In order to help explain the diminished impact of particular HLA allele expression in infected children, we sought to test the hypothesis that the CD8+ T-cell responses restricted by protective HLA molecules such as HLA-B*57/58:01/81:01 generally have less antiviral efficacy in children compared to adults. The selection of viral escape mutants to evade the dominant Gag-specific CD8+ T-cell responses restricted by these alleles is evidence of immune selection pressure, and is observed in 60-80% of unselected, chronically adults expressing these alleles (Carlson *et al.* 2012; Leslie *et al.* 2004; Martinez-Picado *et al.* 2006; Kloverpris *et al.* 2012). Hypothesizing that anti-HIV CD8+ T-cell efficacy would be significantly lower, and therefore that

the frequency of these escape mutants would also be substantially lower, in rapidly progressing infants expressing any of these 'protective' HLA molecules, we studied a historical cohort of 20 ART-naïve, HIV-infected infants born in Durban, South Africa, between 2002-2005 (Mphatswe *et al*. 2007), who were followed from birth. Antenatal, third trimester samples in each case were obtained from the transmitting mothers, providing close approximations to the transmitted virus. A current day cohort of this type would not be possible to study because since 2008 WHO guidelines have recommended ART initiation in all HIV-infected infants, irrespective of CD4% (Violari *et al.* 2008; WHO 2008). In this historical cohort, the CD4 criteria for ART initiation in infants followed the prevailing WHO guidelines, namely a confirmed CD4% of <20% (WHO 2008).

Of these 20 children, 17 met the CD4 criteria for ART initiation within the first year of life (confirmed CD4%<20%), thereby also meeting the criteria in this current study for rapid progression (CD4%<25% within the first 24m of life). Five of these 17 rapid progressor children carried one of the protective HLA molecules HLA-B*57/58:01/81:01. For convenience, these children are referred to here as P1-P5. Children P1-P2 each expressed HLA-B*58:01 and the mothers did not express HLA-B*58:01; as expected, therefore, in each case, the transmitted virus encoded no mutations within the dominant HLA-B*58:01-restricted Gag epitope, TSTLQEQIAW ('TW10'; Gag 240-249). In P1, a strong CD8+ T-cell response was detectable towards 66 pooled overlapping 18mer Gag peptides when first assayed on day 10 of life (Fig 3.13AB); individual testing of the overlapping Gag peptides demonstrated that the single Gag-specific response was towards the two 18mer peptides (Gag-33/Gag-34) containing the TW10 epitope in the 10 amino acid overlap (Fig 3.13CD). Other than

the short-lived drop in viremia (Goulder *et al*. 2004) resulting from single dose Nevirapine that each child received on the day of birth, and the mother received during labour, as per prevailing prevention of mother-to-child transmission guidelines at the time (Guay *et al*. 1999), viral load in this child increased from 6.5m copies/ml on day 1 of life to >10m copies/ml over the first 6 months of life, coincident with maintenance of the TW10/Gag-specific response at the same level of 0.73-1.14% CD8+ T-cells. However, throughout this time there was no evidence of selection of TW10 or other Gag escape mutants (Fig 3.13D).

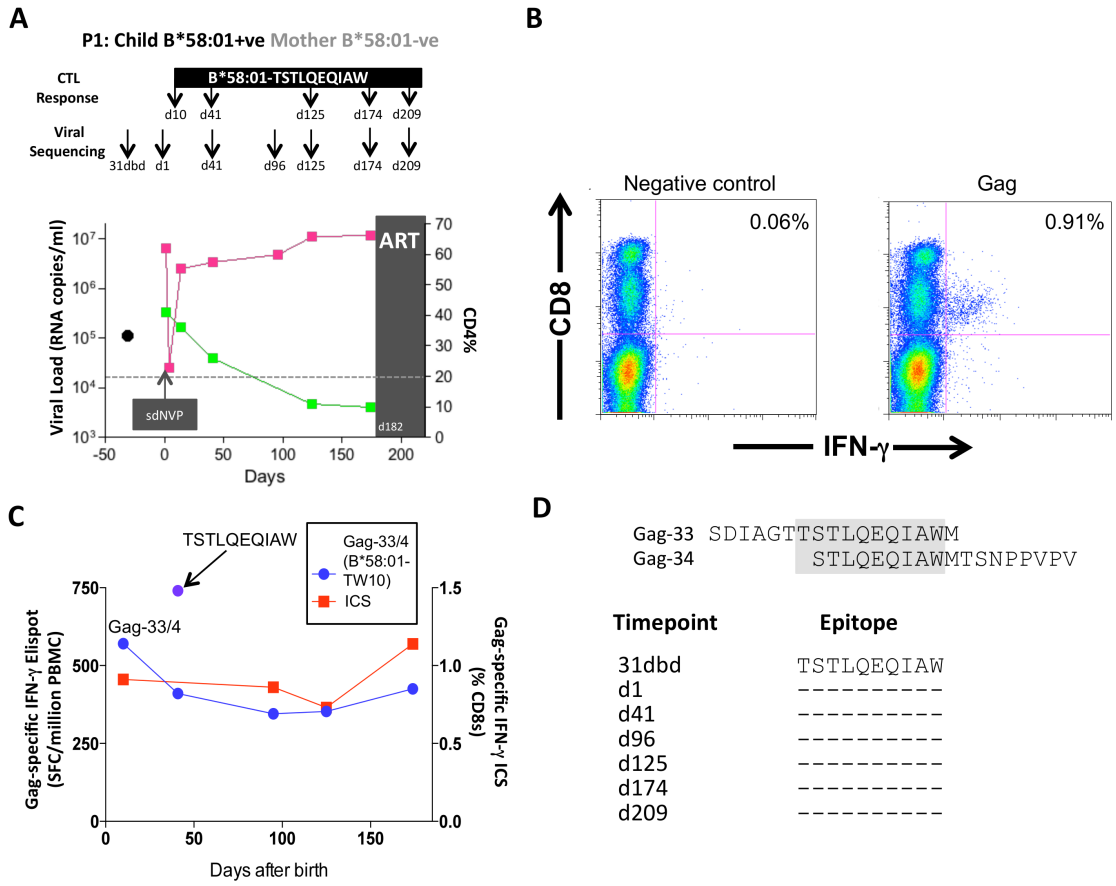


Figure 3.13. Gag-specific CD8+ T-cell responses and autologous viral sequences in a rapid progressing child, P1. A. Viral load and CD4% changes, CTL assays

and viral sequencing timepoints. Closed red squares: viral load. Closed green squares: CD4%. Horizontal dotted lie: threshold for ART initiation (confirmed CD4<20%). Black closed circle: timepoint of maternal viral sequencing. Dbd: days before delivery. sdNVP: single dose nevirapine, given during labour and on the first day of life to the infant. D182: age of child at ART initiation. B. Gag-specific intracellular interferon-gamma staining assay undertaken when P1 was 10 days of age. C. Interferon-gamma elispot responses to Gag-33 and Gag-34 (two of 66 overlapping 18mers spanning the Gag protein), and on day 41 to the HLA-B*58:01-retricted Gag epitope TSTLQEQIAW contained within overlapping peptides Gag-33/4; and Gag-specific IFN-g ICS data, showing responses to 66 pooled overlapping Gag peptides. D. Maternal viral sequence 31 days before delivery and autologous viral sequence in P1 showing lack of escape in all time points analyzed from day 1 to day 207, 25 days after ART initiation.

No HLA-B*58:01-TW10 or Gag escape mutants were selected in P2, although in this case no CD8+ T-cell responses were detected to any of the same set of Gag peptides (Fig 3.14A, Table 3.2).

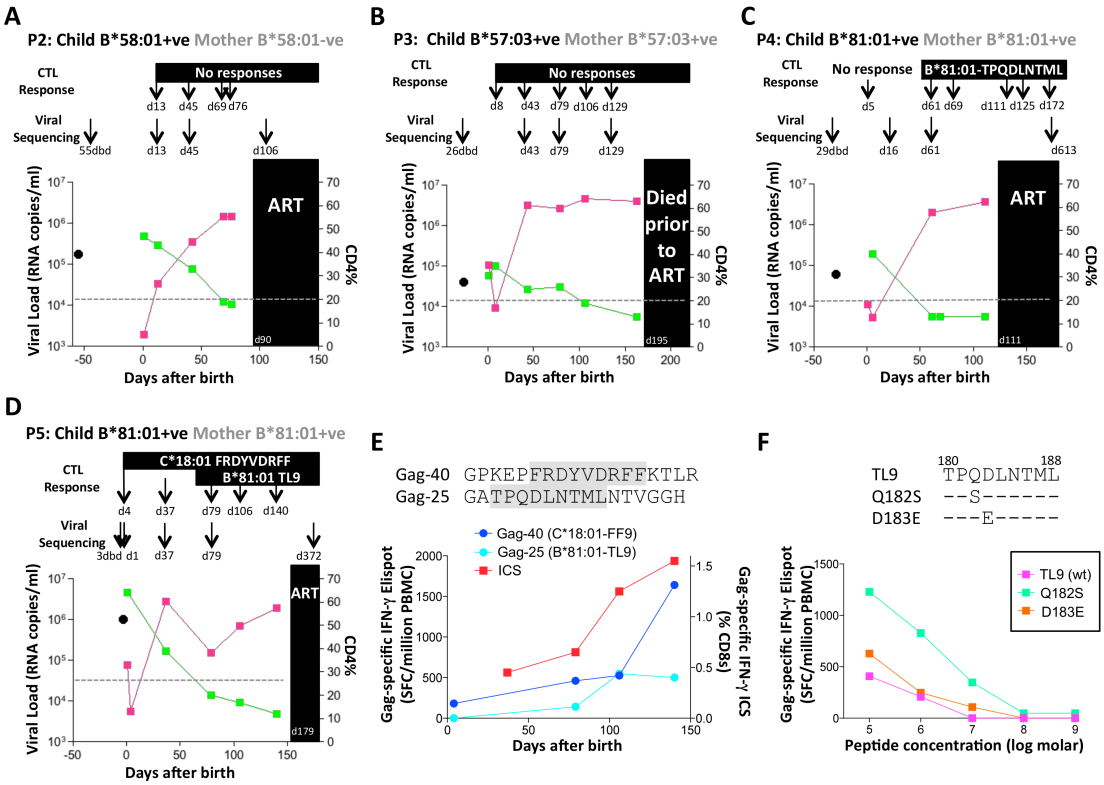


Figure 3.14. Rapid progression and lack of CTL escape in 4 additional rapid progressor expressing protective HLA-B*57/58:01/81:01. A-D. Clinical data for subjects P2-P5. E. In child P5, elispot IFN-g responses to 17-18mer Gag peptides Gag-40 (from day 4 of life) and Gag-25 (from day 79 of life) and Gag ICS responses to the pool of 66 Gag overlapping peptides. F. In child P5, recognition in IFN-g elispot assays of autologous TL9 variants TPSDLNTML (Q182S) and TPSELNTML (D183E), compared to recognition of wildtype TL9. PBMC were tested at day 79 and day 141, with the pattern; data are shown from PBMC at day 141.

In the HLA-B*57:03-positive child, P3, although the mother also expressed HLA-B*57:03, the transmitted virus did not carry escape mutants in the HLA-B*57:03-restricted Gag epitope KAFSPEVIPMF ('KF11', Gag 162-172). In this child, no KF11 CD8+ T-cell responses were detected and no escape mutants selected (Fig 3.14B, Table 3.2). In the HLA-B*81:01-positive child, P4, the transmitted virus did not carry

escape mutants in the HLA-B*81:01-restricted Gag epitope TPQDLNTML ('TL9', Gag 180-188). In this case, strong TL9-specific CD8+ T-cell responses were consistently detectable at >1000 SFC/million PBMC (1162 and 1073 SFC/million PBMC on day 61 and day 69, respectively), but progression to CD4%<20% with rising viremia occurred without selection of TL9 escape mutants (Fig 3.14C, Table 3.2).

Progressor Pair		**Timepoint**[a]	**Epitope & HLA-allele Restriction**				
			B*57:03 AISPRTLNAW	**B*57:03** KAFSPEVIPMF	**B*81:01** TPQDLNTML	**B*57/58:01** TSTLQEQIAW	**C*18:01** FFRDYVDRF
P2 Mother	B*58:01-ve	55dbd	N/A	N/A	N/A	----------	N/A
P2 Child	B*58:01+ve	d13	N/A	N/A	N/A	----------	N/A
		d45	N/A	N/A	N/A	----------	N/A
		d106	N/A	N/A	N/A	----------	N/A
P3 Mother	B*57:03+ve	26dbd	PL--------	----------	N/A	--N-------	N/A
P3 Child	B*57:03+ve	d43	PL--------	----------	N/A	--N-------	N/A
		d79	PL--------	----------	N/A	--N-------	N/A
		d123	PL--------	----------	N/A	--N-------	N/A
P4 Mother	B*81:01+ve	29dbd	N/A	N/A	---------	N/A	N/A
P4 Child	B*81:01+ve	d18	N/A	N/A	---------	N/A	N/A
		d61	N/A	N/A	---------	N/A	N/A
		d613	N/A	N/A	---------	N/A	N/A
P5 Mother	B*81:01+ve	3dbd	N/A	N/A	--SX------	N/A	---------
P5 Child	B*81:01+ve	d1	N/A	N/A	---E------	N/A	---------
		d37	N/A	N/A	--S-------	N/A	---------
		d79	N/A	N/A	--S-------	N/A	---------
		d372	N/A	N/A	--S-------	N/A	---------

Table 3.2. Autologous virus sequences in children P2-P5 and their mothers encoding the relevant Gag-specific CD8+ T-cell epitopes restricted by HLA-B*57/58:01/81:01 and HLA-C*18:01. [a] timepoint: dbd = days before delivery of child; X: mixed bases; N/A: not applicable - relevant HLA allele not expressed

The fifth case, P5, was more complex to evaluate, because both mother and child expressed HLA-B*81:01, and the virus sequenced from the mother 3 days prior to delivery encoded a mixture of TL9 variants (Fig 3.14D-F, Table 3.2). The dominant variant in the child switched from D183E on day 1 of life to Q182S by day 37. A response to the Gag 18mer (Gag-25) containing TL9 was not detectable until day 79 (Fig 3.14D-F), and assays at this time point and at day 141 just prior to ART initiation showed the same pattern, namely, no evidence that Q182S was an escape mutant, but that Q182S was better recognized than both the D183E variant and the wildtype TL9 peptide (Fig 3.14F). The evidence that TL9 escape occurred in child P5 is therefore equivocal, at best. It is clear however that the dominant Gag response in this child, that was detectable from day 4 in life, was to the HLA-*18:01-restricted epitope FRDYVDRFF ('FF9', Gag 293-301) (Honeyborne *et al*. 2006). This response, although like the TL9-Q182S response, present at >1000 SFC/million PBMC in elispot assays, also did not drive selection of escape mutants over the 6 months of disease progression towards ART initiation.

In summary, these 5 cases of HIV-infants followed from birth progressed rapidly to CD4% of <20%, in the setting of persistent viremia of >10^6-10^7 copies/ml, in spite of expression of the protective HLA alleles HLA-B*57/58:01/81:01, and did so in a manner notable by the absence of the selection of escape mutants. In some cases these children did not generate detectable CD8+ T-cell responses, but in others these responses were maintained at relatively high frequencies.

3.3 DISCUSSION

These studies of HIV-infected children and mothers show, first, a significant correlation between the replicative capacity of viruses in infected children and their rate of progression. Second, despite the fact that, on average, 7.5yrs had elapsed since transmission, there remained a highly significant correlation between the VRC in mothers and children within transmission pairs. Unexpectedly, the VRC was typically lower in the child than in the mother within each pair. Third, the impact of protective HLA alleles HLA-B*57/58:01/81:01 and disease-susceptible HLA alleles HLA-B*18:01/45:01/58:02 on VRC was marked among the adults but non-significant or absent, respectively, among the children. However, the impact of maternal alleles on the child's VRC was greatest when the mother carried any of the disease-susceptible alleles HLA-B*18:01/45:01/58:02. The impact of protective and disease-susceptible alleles on VRC was strongly linked to the number of Gag CD8+ T-cell escape mutations within the virus. Finally, the impact of protective and disease-susceptible HLA alleles on disease progression, using CD4 count or viral load as a marker, was strikingly discordant between children and adults, with minimal HLA allele impact discernible among children. Longitudinal analysis from birth of 5 HIV-infected children who progressed rapidly in spite of expressing protective HLA alleles showed that even when high frequency responses restricted by the relevant protective HLA alleles were generated, no evidence for any impact on viral replication was evident. Together, therefore, these data are consistent with a substantially diminished impact of HLA alleles on disease outcome in pediatric infection compared to the marked effect observed in adults, although the influence

of maternal HLA alleles, principally via VRC of the transmitted virus to the child, does also affect disease progression in children.

The central part HLA alleles play in immune control of adult HIV infection has long been evident, and the more recent GWAS studies have served to highlight the exclusivity of HLA allele polymorphism among human genetic variation in shaping HIV disease outcome (Pereyra *et al.* 2010; Fellay *et al*. 2010; Fellay *et al.* 2007). Numerous mechanisms by which HLA alleles may mediate these effects have been proposed (Goulder *et al* 2012), prominent among which is the hypothesis that protective HLA alleles such as HLA-B*27/57/58:01/81:01 reduce viral replicative capacity (VRC) through driving the selection of escape mutants in conserved regions of the proteome such as Gag (Leslie et al. 2004; Martinez-Picado *et al.* 2006; Schneidewind *et al.* 2007; Matthews *et al.* 2008; Crawford *et al.* 2009; Miura *et al.* 2009; Wright *et al*. 2010; Wright *et al*. 2012; Boutwell *et al*. 2013). The high throughput assay developed to measure VRC using Gag-Protease recombinant viruses (Miura *et al.* 2009) is therefore well-suited to examine the impact of HLA alleles on VRC through the selection of escape mutants largely arising in Gag.

Although in previous studies the relationship between VRC as measured by this assay and the viral escape mutants selected in response to Gag CD8+ T-cell activity has been somewhat inconsistent (Wright *et al.* 2010; Payne *et al*. 2014; Huang *et al*. 2011; Juarez-Molina *et al*. 2014), here the correlation between the number of Gag escape mutants and VRC has been very clear (in adults, $r=-0.50$, $p<0.001$ when including all Gag CD8 T-cell escape mutants; and $r=-0.45$, $p=0.002$ when including only the HLA-B*57/58:01/81:01-associated Gag escape mutants). Taking into

account that the 20 Gag CD8+ T-cell escape mutants are only those that represent the strongest HLA allele footprints on the virus, and therefore meet the statistical criteria (usually $q<0.05$ or $q<0.2$) (Carlson *et al.* 2012; Matthews *et al*. 2008; Payne *et al.* 2014) to be included, the likelihood is that the selection of Gag escape mutants by CD8+ T-cell responses has a substantially greater impact on VRC even than these data would indicate. Furthermore, the correlations between VRC and number of Gag escape mutants would diminish and ultimately disappear if in the majority of cases compensatory mutants were successfully selected by the virus to nullify any adverse impact on VRC. On the basis of these data, the ability of compensatory mutants to restore VRC appears limited. If so, this would be consistent with recent data suggesting that immune pressure mediated by protective alleles such as HLA-B*57/58:01 is contributing to a decline in HIV virulence over time (Payne *et al*. 2014).

The present studies address the role of HLA alleles and of VRC in adult and paediatric HIV infection. It is known that VRC affects outcome in adult infection, as previously shown (Goepfert *et al*. 2008; Prince *et al*. 2012). Consistent with the only other study that has examined VRC in paediatric HIV infection, in a small study of 8 infected children (Prado *et al*. 2010), here we show that VRC is also related to paediatric disease outcome (Fig 3.3), being on average 15% lower in slow progressors compared to rapid progressors, and correlating with CD4 count at 5yrs and 7.5yrs age, despite the VRC assays in the slow progressor children being determined on average 7.5yrs after transmission.

Unexpectedly, the VRC in mothers of the 43 mother-child pairs studied tended to be somewhat higher than in the children (mean VRC 0.69 vs 0.63; p=0.056). In some cases, this result might be explained by the accumulation of costly escape mutants in the child's virus post-transmission; however, the same result was obtained from a separate study of VRC in 39 mother-child pairs in which the VRCs were determined from samples taken from mother and child within 6 weeks of birth (Naidoo *et al.* 2013). In that study, the VRC of the infected neonates was on average 20% lower than that in the mothers (median VRC 0.62 vs 0.77; p<0.0001). The fact that both these results are consistent in indicating that the virus in the child has lower VRC than in the mother suggests either that intense selection pressure is imposed on HIV in the initial weeks on perinatal infection, which seems unlikely (Muenchhoff *et al*. 2014); or that lower VRC viruses are preferentially transmitted. If the latter, this would be the exact opposite of adult sexual transmission, in which a transmission bias exists against viruses with low VRC (Carlson *et al*. 2014). This discrepancy remains unexplained, and requires further investigation, but may indicate a distinct bottleneck in operation in perinatal mother-to-child-transmission compared to adult horizontal transmission.

The discordant relationship between HLA alleles and VRC in infected adults and children (Fig 3.7) highlights the substantial impact of HLA alleles on VRC in adults but not in children. Although protective alleles in the child appear to effect a modest reduction in VRC (Fig 3.7AC), the protective alleles in the mother do not appear to have significant influence on the VRC in the child, consistent with one previous study (Schneidewind *et al.* 2009) and in contrast to another (Thobakgale *et al* 2009). However the existence of disease-susceptible alleles in the mother had a significant

impact on VRC not only in the mother but also in the child. The mechanism by which these disease-susceptible HLA alleles might operate is suggested by the data showing a significantly lower number of Gag CD8+ T-cell escape mutants in mothers expressing these alleles than those not. This supports the hypothesis that disease susceptible alleles are ineffective against HIV principally because they fail to contribute to Gag-specific immune pressure on the virus, rather than because they actively mediate immune responses that prevent effective antiviral control (Huang *et al*. 2009; Bashirova *et al*. 2014).

Comparison of the relationship between HLA alleles and disease outcome in infected adults and children (Fig 3.12) serves to contrast the striking influence of HLA alleles in adults with the relative lack of impact in children. Protective and disease-susceptible HLA alleles had no statistically significant effect on disease progression in infected children, on CD4 counts or on viral loads. Although previous studies have anecdotally described slow disease progression in HIV-infected children expressing protective HLA alleles such as HLA-B*27 or HLA-B*57 (Goulder *et al*. 2001; Feeney *et al.* 2004, Schneidewind *et al.* 2009), less prominently the same result has also been observed in children expressing no protective alleles (Feeney *et al*. 2006; Prendergast *et al.* 2011). It seems clear that, in a minority of slow progressor children expressing the classical protective HLA alleles, HLA-B*57/58:01/81:01, the same Gag-specific CD8+ T-cell responses are generated as have been well-described in adults, driving the same immune selection pressure, and in some cases these appear to contribute to slow progression in these children. There is evidence for this in the published literature (Goulder *et al*. 2001; Feeney *et al*. 2004; Schneidewind *et al*. 2009), and also in the current study, in which we observed a modest impact on

VRC in children expressing HLA-B*57/58:01/81:01 (Fig 3.7A and Fig 3.7C), a moderate increase in children expressing these alleles in the slow progressors with the highest CD4 counts (Fig 3.12), and examples of HLA-B*57/58:01/81:01 CD8+ T-cell pressure driving the selection of Gag escape mutants that reduce VRC (Table 3.3), just as in adults. However, it is equally clear from these studies that the impact of these protective and also of the disease-susceptible HLA alleles is substantially less in paediatric than adult HIV infection.

Subject	HLA	VRC	146	147	148	149	150	151	152	153	154	155	162	163	164	165	166	167	168	169	170	171	172	180	181	182	183	184	185	186	187	188	240	241	242	243	244	245	246	247	248	249
			A	**I**	**S**	**P**	**R**	**T**	**L**	**N**	**A**	**W**	**K**	**A**	**F**	**S**	**P**	**E**	**V**	**I**	**P**	**M**	**F**	**T**	**P**	**Q**	**D**	**L**	**N**	**T**	**M**	**L**	**T**	**S**	**T**	**L**	**Q**	**E**	**Q**	**I**	**A**	**W**
K-084-C	B*57:03-pos	0.49	P	L	-	-	-	-	-	-	-	-	-	G	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-
K-084-M	B*57:03-neg	0.61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-
K-077-C	B*58:01-pos	0.55	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-
K-077-M	B*58:01-neg	0.69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K-075-C	B*81:01-pos	0.51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-
K-075-M	B*81:01-neg	0.90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.3. Representative autologous mother (M) and child (C) Gag sequences within epitopes restricted by protective HLA-B alleles B*57/B*58:01/81:01 to demonstrate occurrence of escape within selected slow progressor children. Mutations shown; A146X and I147X within the HLA-B*57 restricted epitope ISPRTLNAW (ISW9, Gag 147-155); A163X and S165X within the HLA-B*57-restricted epitope KAFSPEVIPMF (KF11, Gag 162-172); Q182X and T186S within the HLA-B*81:01-restricted epitope TPQDLNTML (TL9, Gag 180-188); and T242X and I247X within the HLA-B*57/58:01-restricted epitope TSTLQEQIAW (TW10, Gag 240-249).

Potential reasons for diminished HLA allele effect in children are suggested by the data presented showing rapid progression in children in spite of expressing the protective HLA-B*57/58:01/81:01 alleles and, at least in some cases, in spite of the classical Gag-specific CD8+ T-cell responses being detectable at highly respectable levels. The precise reasons why these CD8+ T-cell responses appear to have been ineffective in these children would be of interest to define further. However, a number of more general factors have been identified that mitigate against effective CD8+ T-cell responses being generated in infancy, foremost amongst these being the consequences of immune ontogeny (Muenchhoff *et al.* 2014), and the bias of CD4 T cells in infancy to make Th2 type responses. This in turn may reflect the inability of innate immune cells in the infant to produce IL-12, in contrast with high production of the immunomodulatory cytokine IL-10 (Muenchhoff *et al.* 2014).

It is important also to consider the limitations of this study. One of these is the fact that we assessed VRC in the rapid progressor children at mean age 14 months and in the slow progressor children at mean age 7.5 years. Ideally we would have measured VRC in the slow progressor children at the same age as in the rapid progressors. However this would have necessitated follow up of a cohort of HIV-infected children from birth, and most of these children were not identified until >5yrs of age. However, a small longitudinal study was previously undertaken by our group, of VRC in 8 HIV-infected children followed from birth, comprising 3 'progressors' and 5 'slow progressors' (Prado *et al*. 2010). The conclusions of that longitudinal study, that VRC is higher in progressors than in slow progressors, and that the lower VRC observed in slower progressors is

lower from early in the course of infection, are consistent with those of the current study. However, it would be useful to undertake longer follow up of VRC over the course of infection to determine whether any predictable pattern of VRC changes over time exists. On the one hand it might be expected that escape mutants accumulating over the course of infection might decrease VRC, whilst on the other hand the accumulation of compensatory mutants might increase VRC over time.

With regard to the other potential limitations of this study, it is clear that viral replicative capacity is not solely dependent upon mutations within *gag* and *pro* but also in genes elsewhere in the genome. Furthermore, it is important to note that a range of factors, in addition to CD8+ T-cells, operate to impose selection pressure on the virus, including neutralizing antibodies and influences affecting viral tropism. However, the justification for focusing on the Gag-Pro region as we have done here is as follows. First, there is strong evidence that Gag-specific CD8+ T-cell epitopes play a central role in immune control of HIV, and that the fitness cost of Gag escape mutants contributes to the impact of protective HLA molecules such as HLA-B*57 (Goulder *et al*. 2012; Leslie *et al*. 2004; Martinez-Picado *et al.* 2006; Schneidewind *et al.* 2007; Matthews *et al.* 2008; Crawford *et al.* 2009; Miura *et al.* 2009; Wright *et al*. 2010; Wright *et al*. 2012; Boutwell *et al*. 2013; Miura *et al.* 2009). Although studies of the fitness cost of CD8+ T-cell escape mutations outside of Gag-Pro are less extensive than within Gag, the available evidence suggests that escape mutants in proteins such as Env are less likely to carry a fitness cost (Kiepiela *et al.* 2007; Ngumbela *et al.* 2008; Troyer *et al.* 2009).

Second, in several studies, the biological relevance of the Gag-Pro assay has been demonstrated by the correlations with markers of disease progression (namely, absolute CD4 count and viral load) (Wright *et al.* 2010; Payne *et al*. 2014; Miura *et al.* 2009; Brockman *et al.* 2010; Huang *et al.* 2011; Nomura *et al*. 2013; Juarez-Molina *et al.* 2014). Thus, in order to examine the impact of specific HLA allele-associated sites within Gag, the optimal way to do that would using an isogenic backbone to generate Gag-Pro recombinant chimeric viruses as we have done here.

The third point in favor of this assay is that, in practical terms, generating a full-length replication competent clone from each subject isolate is a labor-intensive and costly process. This illustrates why, when we previously adopted that approach, in the only report to date to measure directly VRC in infected children, the study was limited to n=8 (Prado *et al.* 2010). In contrast, here we have determined VRC in more than 15 times that number of subjects. Finally, at the end of the process of isolating full-length viruses, unless many hundreds of virus clones are assayed, there are too may mutations across the whole viral genome to determine which has contributed to differences in replication capacity. A practical approach therefore to address the impact of HLA alleles on VRC is the one adopted here.

Together these data suggest that VRC, and the impact that HLA alleles have on VRC, plays an important role in disease progression in adult and paediatric infection. However, these data also would indicate that VRC is by no means the

only factor that influences rate of disease progression in paediatric infection. Furthermore, in infected children, the effect of HLA alleles on outcome that is independent of VRC appears to be limited. The difference in HLA allele impact between adults and children may reflect the fact that distinct mechanisms may operate to limit disease. In adults, the route to immune control and consequent disease limitation appears to lie, at least in part, in an effective CD8+ T-cell response, such as that restricted by protective HLA alleles, that can bring down viral load to low levels. In paediatric infection, non-pathogenicity is weakly related to HLA allele and viral load (Adland *et al*. International AIDS Society, Melbourne, Australia 2014). In contrast to adult infection, persistently high viral loads in paediatric slow progressors are observed in association with low levels of immune activation (Adland *et al.* International AIDS Society, Melbourne, Australia 2014), with similarities to the natural hosts of SIV infection (Silvestri *et al*. 2003). Paediatric slow progressors thus now provide the opportunity to address in humans a central, unanswered question in relation to HIV pathogenesis, namely, the mechanisms underlying low immune activation in the face of persistently high viremia.

CHAPTER 4

RECONSTITUTION OF FUNCTIONAL HIV-SPECIFIC CD4+ T-CELL ACTIVITY FOLLOWING ART IN PAEDIATRIC INFECTION

4.1 INTRODUCTION

The challenges posed currently by paediatric HIV infection differ increasingly from those presented in the earlier decades of the global epidemic. In sub-Saharan Africa, where 90% of HIV infected children live, strategies to prevent mother-to-child transmission (MTCT) have developed to the point where MTCT rates are now down to 1-2% from approximately 40% in the pre-antiretroviral therapy (ART) era (Moodley *et al*. 2014). The numbers of new paediatric infections are therefore decreasing (UNAIDS Global Reports). Furthermore, increased use of ART has made an especially dramatic impact in paediatric infection, since without ART approximately 50% of HIV-infected children have died by 2 years of age. These successes bring the new prospect of a lifetime of ART dependence for children. Aside from the cost and potential toxicity of long-term ART taken throughout childhood, the reality is that ART adherence is highly problematic during adolescence (Jaspan *et al*. 2011) and resources that have been invested into ART initiation may not be sufficient to maximise uninterrupted maintenance of treatment.

These factors prompt the need to consider whether there may be alternative options for HIV-infected children other than the prospect of a lifetime on ART. Recent reports of HIV Cure or post-treatment remission of HIV infection in children receiving ART early in life (Persaud *et al*. 2013; Frange *et al*. 2015) have highlighted the fact that there are fundamental differences from adults that may open up unique possibilities for immune-based therapies in children. These differences include the impact of immune ontogeny (Muenchhoff *et al*. 2014) and more active thymopoiesis. A number of studies have demonstrated more rapid immune reconstitution in ART-treated children compared to adults (Sleasman *et al.* 1999; Gibb *et al*. 2000; Walker *et al*. 2004; Resino *et al*. 2006; COHERE/Sabin *et al*. 2008), and in part this has been related to increased thymic output (De Rossi *et al*. 2002). However many of these studies have focused principally on recovery of CD4 T-cell numbers as opposed to functional reconstitution (Sutcliffe *et al*. 2008; Sauvageot *et al*. 2010).

Previous studies that have investigated immune reconstitution of T-cell function in children have, with rare exceptions (Vrisekoop *et al*. 2008) indicated that clear-cut functional differences exist between infected adults and children on ART. Children aged >5yrs achieving suppression of viraemia on ART showed strong Gag-specific CD4+ T-cell proliferative capacity. In contrast, chronically infected adults who also achieved suppression of viraemia on ART typically showed no Gag-specific proliferative response (Feeney *et al*. 2003). Consistent with this, a separate study showed CD4 proliferation limited by ongoing

viraemia, but in these settings the ability of CD4 T-cells to secrete IFN-γ persisted (Scott *et al*. 2003). A subsequent study examining functional patterns of HIV-specific CD4 responses in ART-treated children aged 10yrs showed that the dominant CD4 Gag-specific response in children achieving viraemia suppression was IL-2 production, whereas children with uncontrolled viraemia showed predominant IFN-γ CD4 T-cell activity (Correa *et al*. 2007). These findings would be consistent with a more differentiated CD4 T-cell compartment in the setting of persistent viraemia, with effector memory responses characterized by predominantly IFN-γ production, limited proliferative activity and high expression of T-cell exhaustion markers (Sallusto *et al*. 1999; Day *et al*. 2006). In contrast, in the absence of viraemia, these findings suggest a less differentiated CD4 T-cell compartment, comprising a greater proportion of naïve and central memory CD4+ T-cells, functionally designed for IL-2 production and high proliferative capacity, with relatively low expression of exhaustion markers.

Together these studies suggest a rapid recovery of CD4 count in ART-treated children, and a degree of normalization of CD4 T-cell function in children aged >5yrs in whom viral replication was successfully suppressed. However, there are few data describing HIV-specific immune responses or reconstitution of CD4 function in HIV-infected children in whom ART was initiated prior to 5 years. Since WHO recommendations are that all HIV-infected children aged <5yrs should receive ART irrespective of their health of CD4 count, it is likely that this group represent the majority of HIV-infected children. We therefore undertook a study here of two distinct groups of HIV-infected children to determine the time

to recovery of functional CD4 activity in children initiating ART at ~2yrs of age. The first group of 58 subjects comprised ART-naïve children, in whom we defined the HIV-specific CD4 responses that were associated with maintenance of normal CD4 counts for uninfected age-matched children. The second study group comprised 84 ART-treated children, categorized according to duration of ART-mediated suppression of viraemia.

4.2 RESULTS

This study was carried out, firstly, to investigate associations between T cell functionality and paediatric non-progression; and, secondly, to assess the impact of successful ART on the restoration of T cell functionality. It is important to highlight that this is a cross-sectional data study of groups of children receiving ART for different periods of time and must be interpreted as such. This does not assess the same group of children assessed at different intervals.

4.2.1 Functional profile of CD4 and CD8 T-cell in ART-naive HIV-infected children

To define the functional profile of CD4 and CD8 T-cells in non-progressing compared to progressing children, we first analysed a cohort of HIV vertically infected, ART naive children. All children were aged $\geq$ 5yrs, with a mean age of 9.9yrs. The children selected did not differ significantly by age, sex or viral load.

We first evaluated the CD4 and CD8 T-cells in these subjects on the basis of their ability to secrete IFNγ, IL-2, MIP1β and TNFα after stimulation with a pool of HIV-1 overlapping Gag peptides (Fig 4.1).

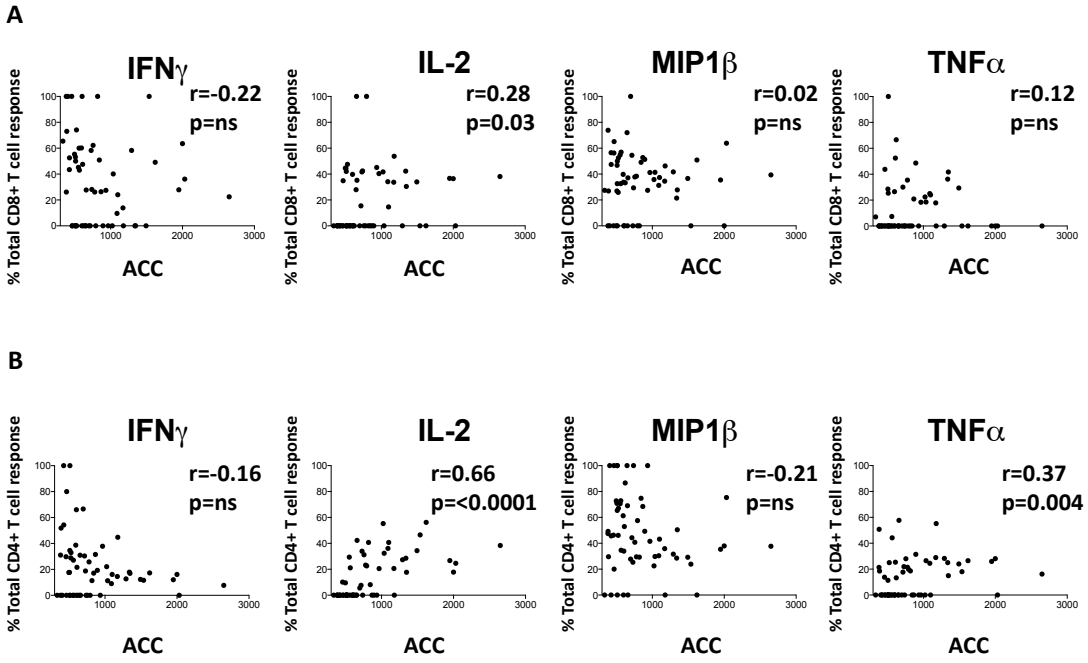


Fig 4.1. Increasing absolute CD4 count (ACC) is associated with an increase in IL-2 and TNFα production in CD4+ T cells in HIV infected ART naïve children. % contribution of the indicated functional response toward the total [A] CD8+ T cell and [B] CD4+ T cell responses to HIV Gag peptide pool stimulation. Spearman correlations shown.

For CD8+ T-cells, the percentage contribution to the total CD8+ T-cell response of IFNγ, MIP1β and TNFα responses was not significantly correlated with absolute CD4 count (ACC) however the contribution of IL-2 production to the total CD8+ T-cell response increased in association with increasing ACC (Fig

4.1A). For CD4+ T-cells, the contribution of the IFNγ and MIP1B production to the total CD4+ T cell response was not significantly correlated with ACC, whilst the contribution of IL-2 and TNFa production to the total CD4+ T-cell response increased in association with increasing absolute CD4 count (r=0.66, p=<0.0001, r=0.37, p=0.004 respectively Fig 4.1B). Expressed in terms of absolute magnitude of response, IL-2 production, MIP1B and TNFα on CD8+ T-cells and IL-2 and MIP-1β production on CD4 T-cells increased in association with increasing absolute CD4 count (r=0.72, p<0.0001; r=0.28, p=0.03; r=0.48, p=0.0001; and r=0.37, p=0.003; r=0.27, p=0.04; respectively, Fig 4.2). However, no variation was observed in the magnitude of IFNγ produced with increasing absolute CD4 count in either the CD8+ T-cells or the CD4+ T-cells (Fig 4.2).

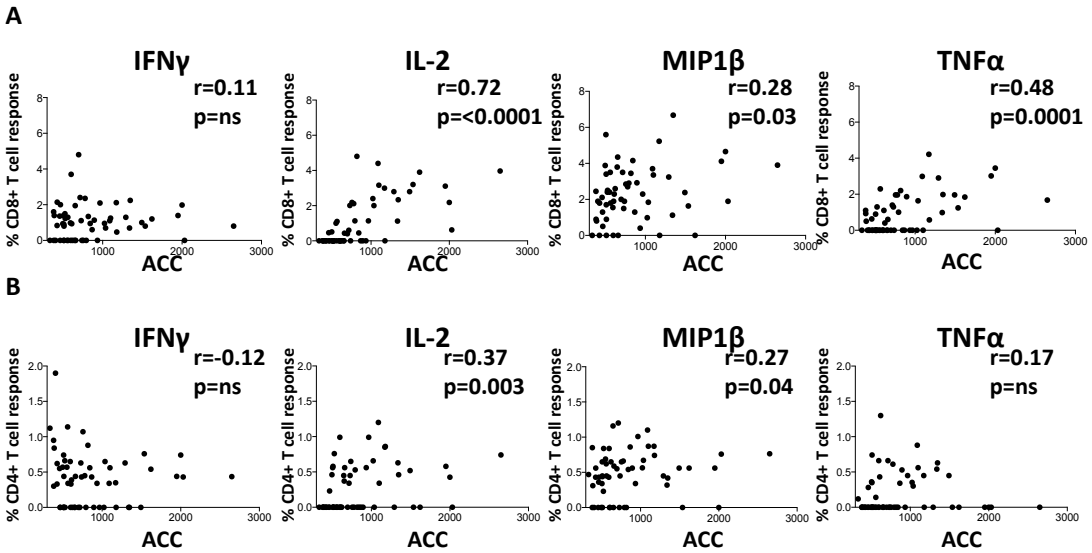


Fig 4.2. Increasing ACC is associated with an increase in the magnitude of IL-2, and MIP1β production in both CD8+ and CD4+ T-cells and an increase in TNFα production in CD8+ T cells in HIV infected ART naïve children. [A] Percentage of CD8+ T cells and [B] Percentage of CD4+ T cells producing the indicated function in response to HIV Gag peptide pool stimulation. Spearman correlations shown.

We next evaluated, in relation to absolute CD4 count, T cell polyfunctionality in these ART-naive children. Polyfunctionality is defined as the ability of HIV-specific CD8+ and CD4+ T cells to produce multiple cytokines and chemokines in response to viral antigen and has previously been associated with viral control in adults (Betts *et al*. 2006; Rehr *et al.* 2008; Nemes *et al*. 2010; Nemes *et al*. 2011). Responses were defined by the number of cytokines/chemokines of IFNγ, IL-2, MIP1β and TNFα produced by each cell in response to the Gag peptide pool. Increasing polyfunctionality in both CD8+ T cells and CD4+ T cells was associated with increasing absolute CD4 T cell count ($r=0.34$, $p=0.008$; $r=0.56$, $p=<0.0001$; Fig 4.3). Similar findings were observed in response to HIV-Pol and Nef peptide pools (data not shown).

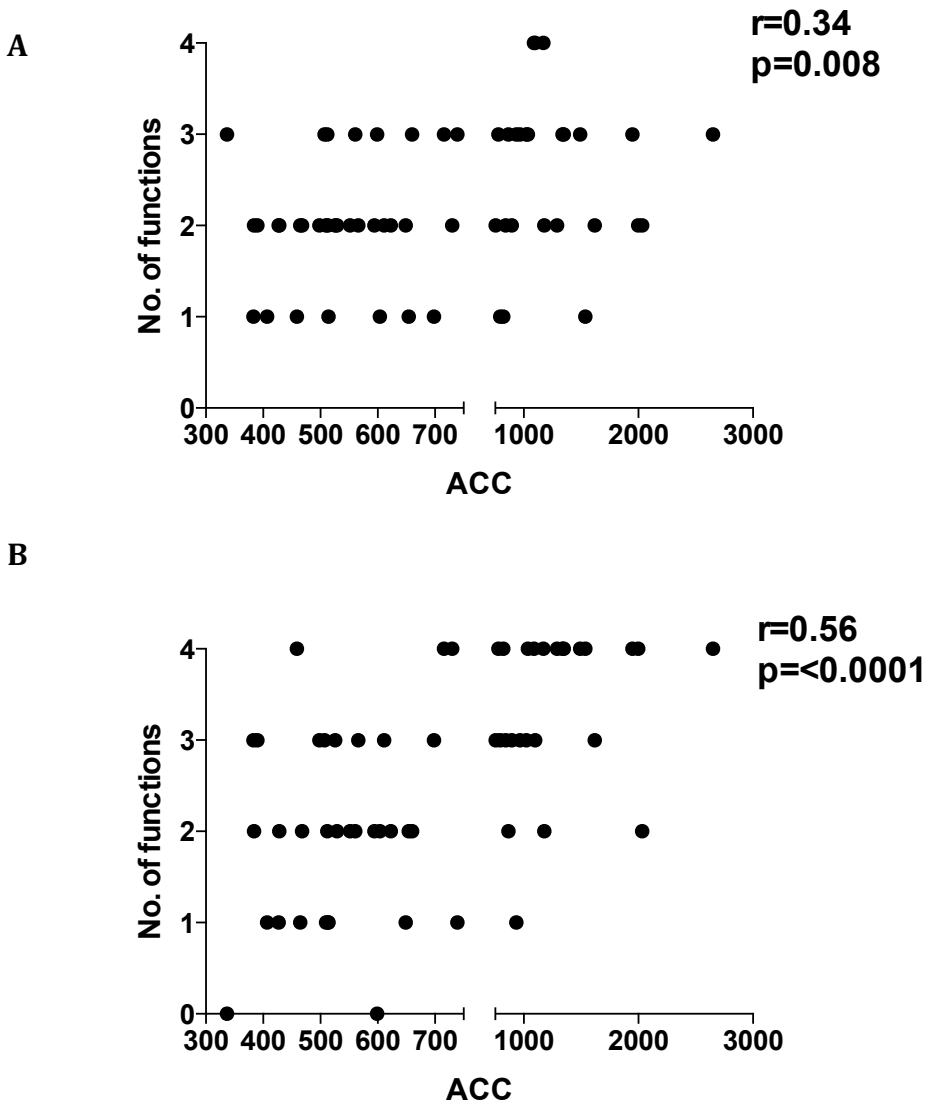


Fig 4.3. Polyfunctional T cells are associated with increasing ACC. y axis indicates the number of functional responses (IFNγ, IL-2, MIP1β and TNFα) made by each subject in response to HIV gag stimulation in (A) CD8+ T cells and (B) CD4+ T cells. X axis indicates absolute CD4 count (ACC). Spearman correlations shown.

4.2.2. Viraemia is positively related to CD4 IFNγ, and inversely related to CD4 IL-2, TNFα and MIPβ responses to HIV gag stimulation

Having observed enhanced functionality in the non-progressing children with high CD4 counts, we next assessed the relationship between viral load and CD8+ and CD4+ T-cell function. The total HIV-specific CD8+ T-cell response to HIV Gag was not correlated with viral load (r=0.19, p=ns, Figure 4.4A). Similarly, CD8 responses stratified by IFN-γ, IL-2, MIP1β, and TNFα function were also unrelated to viral load (data not shown). In contrast, the total Gag-specific CD4+ T-cell response was significantly related to decreasing viral load (r=-0.26, p=0.04, Fig 4.4B). A positive correlation between viral load and IFNγ production was observed (r=0.73, p=<0.0001, Fig 4.4C) and negative correlations between viral load and IL-2, TNFα and MIP1β responses (r=-0.53, p=<0.0001; r=-0.42, p=0.001; r=-0.39, p=0.002, respectively, Fig 4.4C-F). Similar relationships were observed in response to stimulation of CD4 and CD8 T-cells with HIV-1 Pol and Nef peptide pools (data not shown). Thus, the functionality of the HIV-specific CD4+ T-cell response, but not the CD8+ T-cell response, is associated with viral load in this paediatric cohort.

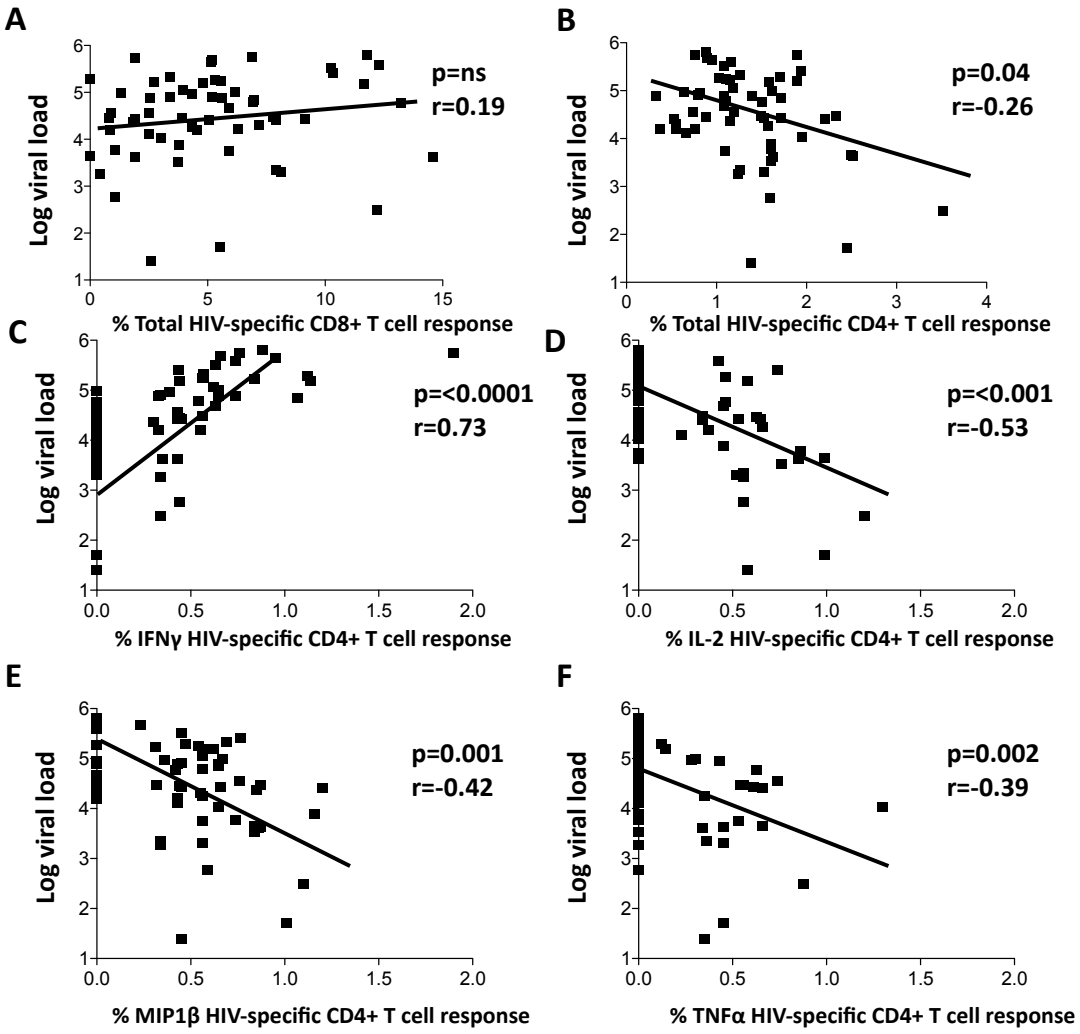


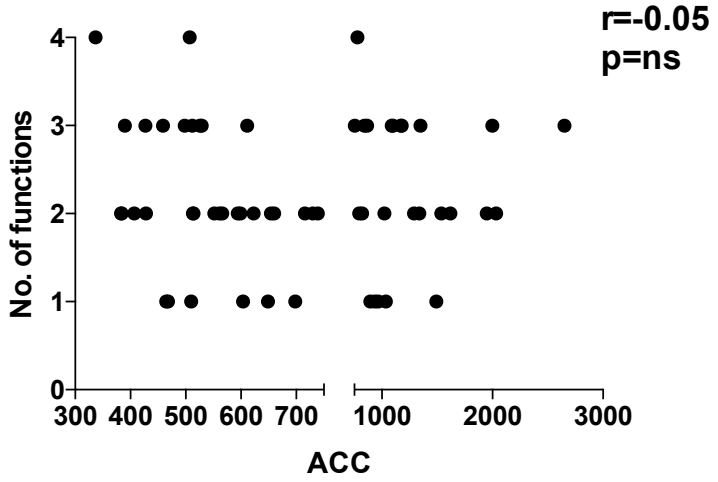
Fig 4.4. Viraemia is positively related to CD4 IFNγ, and inversely related to CD4 IL-2, TNFα and MIPβ responses to HIV Gag peptide pool stimulation.

Viral load is [A] not associated with the total % Gag-specific CD8+ T cell response (p=ns, r=0.19) [B] inversely correlated with the total % Gag-specific CD4+ T cell response (p=0.04, r=-0.26); [C] positively correlated with % CD4+ T cell IFNγ production (p=<0.0001, r=0.73); and inversely correlated to % CD4+ T cell [D] IL-2 production (p=<0.0001, r=-0.53); [E] MIP1β production (p=0.001, r=-0.42); and [F] TNFα production (p=0.002, r=-0.39); n=58. Spearman correlations shown.

4.2.3 Functionality of CMV-specific CD8+ and CD4+ T cells in HIV infection

Limited T cell functionality in the HIV infected children that are progressing (as classified by their absolute CD4 T cell count) could result from a number of factors including generalized immunosuppression and the direct effect of viral load. We tested the hypothesis that the progressing children would also have limited functionality against non-HIV antigens, such as cytomegalovirus (CMV) by stimulating PBMC both with a CMV derived lysate and a pp65 peptide pool. However, rather than limited in nature, the functionality of the CMV-specific CD8+ and CD4+ T cells was quite high in the groups with the lowest absolute CD4+ T cell counts and did not differ significantly with increasing absolute CD4 T cell counts (Fig 4.5). In all HIV infected children, regardless of their CD4+ T cell count, the majority of CMV-specific CD8+ T cells were positive for 3 or 4 functions and the CMV-specific CD4+ T cells for 2 or more functions (Fig 4.5).

A



B

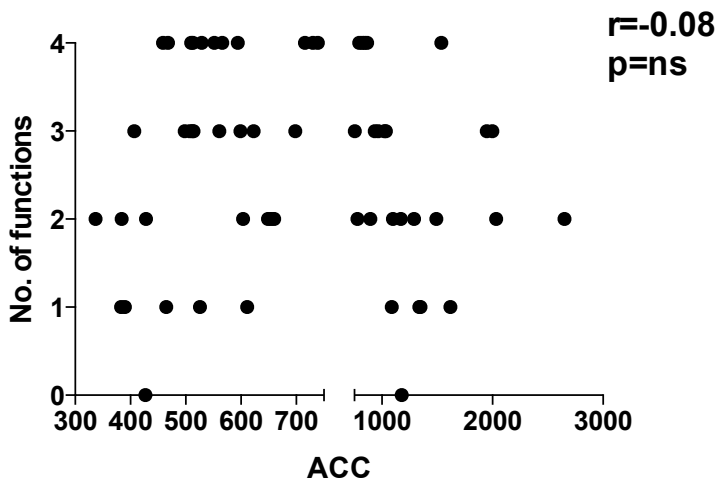


Fig 4.5. Consistent CMV-specific T cell functionality, regardless of ACC. y axis indicates the number of functional responses (IFNγ, IL-2, MIP1β and TNFα) made by each subject in response to CMV lysate stimulation in (A) CD8+ T cells and (B) CD4+ T cells. X axis indicates absolute CD4 count (ACC). Spearman correlations shown.

Thus, limited functionality of HIV-specific CD4+ and CD8+ T cells is restricted to the HIV-specific CD4+ and CD8+T cells and is not a generalized characteristic of all CD4+ and CD8+ T cells in HIV-infected individuals.

4.2.4 Influence of HLA type on functionality

Rates of HIV disease progression have been well characterised, depending upon the particular HLA molecules expressed (Goulder & Walker 2012). In African populations, HLA-B*57, HLA-B*58:01 and HLA-B*81:01 are the class I molecules most consistently associated with slow progression to HIV disease (Pereyra *et al*. 2010; Costello *et al.* 1999; Kiepiela *et al*. 2004; Leslie *et al*. 2010; Tang *et al.* 2011; Lazarayan *et al*. 2011; Carlson *et al.* 2012). However there were no functional differences between those children who expressed protective HLA alleles and those that did not (Fig 4.6).

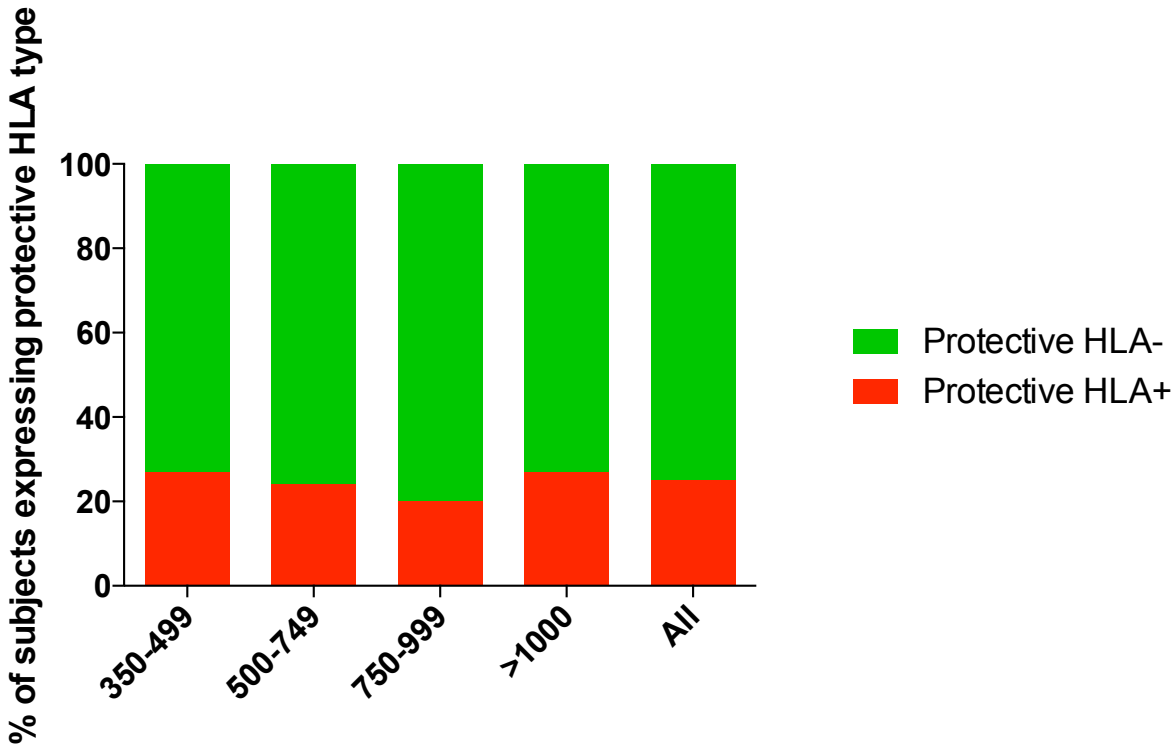


Fig 4.6. Enhanced functionality in PNP's is not due to the presence of protective HLA alleles. % of subjects expressing a protective allele shown in red, absence of a protective allele shown in green. Protective alleles are HLA-B*57/B*58:01/B*81:01. ACC 350-499 (n=11), ACC 500-749 (n=21), ACC 750-999 (n=10), ACC >1000 (n=16). No significant differences found between groups, p values obtained from the two proportioned z test.

These results indicate that enhanced functionality in the non-progressing children is not associated with the presence of HLA-B*57/B*58:01/B*81:01 as a presenting allele.

4.2.5. In ART-treated children, increased duration of viral suppression in cross-sectionally studied children is associated with increased IL-2 and decreased IFN-g production in CD8+ and CD4+ T cells

To determine whether ART-treated children achieve immune reconstitution to the same degree of functionality among HIV-specific CD8+ and CD4+ T cells as in ART-naïve paediatric non-progressors, we recruited 84 HIV-positive, ART-treated children, subdivided into 6 groups according to duration of viraemic suppression (Table 4.1).

Group Duration of VC	1 No VC	2 Blippers	3 <12mnths	4 13-24mnths	5 25-60mnths	6 >60mnths	p value
n	20	9	10	10	17	18	ns
male (n)	7	4	5	6	5	12	ns
female (n)	13	5	5	4	12	6	ns
Age at ART initiation (months)	24.4 (9-29.5)	23.2 (4.5-34.5)	22.8 (9.5-28.5)	32.6 (10-54)	21.8 (6-19)	21.8 (5.5-45)	ns
Age at suppression of viraemia (months)	na	na	54.3 (39.8-62)	54.8 (41.5-63.3)	33.7 (14-41.5)	30.8 (11.5-54)	0.011
Time to suppression of viraemia (months)	na	na	31.5 (25.5-40.5)	22.2 (6-33.8)	11.8 (6-12)	8.2 (6-12)	0.0008
Duration of therapy (months)	35.6 (11.5-54.8)	78.3 (62.5-102)	35.7 (23-42.8)	31.4 (21.8-42)	58.4 (52.5-65)	87.8 (72.3-100)	<0.0001
VL at ART initiation (copies/ml)*	853700 (117,344-2,150,000)	850000 (350,000-1,250,000)	628250 (145,750-3,125,000)	160000 (112,810-516,500)	190000 (65,250-2,000,000)	655000 (195,000-925,000)	ns
CD4% at ART initiation	22.3 (14.8-35.3)	17.1 (8.5-23)	21.9 (16.3-29)	18.3 (13.5-23.5)	26.6 (17.8-39.8)	15.1 (8.3-22.8)	0.029
ACC at ART initiation	1252 (489-1626)	706 (326-988)	594 (284-775)	865 (364-1413)	1107 (434-1466)	776 (286-1053)	ns
Age at evaluation (months)	59.9 (36.5-80)	101.6 (74-134)	58.5 (43-66.3)	64 (48.8-83.3)	80.2 (58.5-88)	109.6 (79.5-133.8)	<0.0001
VL at evaluation (copies/ml)*	17199 (333.5-295645)	627 (40-5691)	40 (25-65)	40 (25-72.5)	36.5 (25-40)	32.5 (25-44.8)	ns
CD4% at evaluation	26.2 (19.8-30)	33.4 (28-40)	31.7 (24.3-38.3)	29.9 (26-33.5)	36.8 (32-41)	30.7 (24-37.5)	ns
ACC at evaluation	1300 (689.8-1725)	1203 (792.5-1365)	1291 (911.3-1624)	1890 (1023-2460)	1669 (1150-1860)	1014 (726.5-1287)	0.028

Table 4.1. Clinical parameters in the 6 groups of HIV infected, ART treated children characterised by duration and degree of virological control (VC). VC defined as two consecutive VLs <50 copies/ml following ART initiation and maintaining VL's <400 copies/ml. Intermittent viraemia for isolated 'blips' <1000 copies/ml was allowed. Group 1 did not achieve VC. Group 2 had multiple consecutive 'blips' of viraemia >400 copies/ml. (ACC) absolute CD4 count; (VL) viral load. Values shown are the mean of the group and IQR in brackets. * median values shown. p values obtained from Kruskal-Wallis test comparing groups 3 to 6 (aviraemic children) only.

Virological control was defined as maintaining viral loads beneath the level of detection (<50 copies/ml or lower, depending on the assay used; see Methods) for two consecutive measurements and at <400 copies/ml thereafter. Viral loads were obtained every 6 months and there were no differences in clinical data availability between the groups of children. Isolated single 'blips' of viraemia of <1000 copies/ml were allowed. One group of children (Group 1) was defined as Viraemic Non-Suppressors (viral loads not maintained at <1000 copies/ml). Children in whom repeated 'blips' of viral load to <1000 copies/ml were observed comprised a second group. The four remaining groups (Groups 3-6) of viraemic suppressors were defined according to duration of aviraemia (Table 4.1). There was no difference between the groups in age or in viral load at ART initiation (overall median age at ART initiation was 24 months). The groups did vary somewhat by CD4% at treatment initiation, however this was not related to duration of viraemic suppression. Children who achieved suppression of viremia for longer reached undetectable viral loads sooner ($p<0.0001$).

Functional T-cell responses were evaluated as described above for ART-naïve children. In response to the Gag peptide pool, the relative contribution of IFNγ production to the total T-cell response, in both CD8+ and CD4+ T-cells, decreased in association with increasing duration of viral suppression ($p=0.0001$; and $p=<0.0001$, respectively, Fig 4.7AB). In contrast, the relative contribution of IL-2 production to the total T-cell response, both in the CD8+ and CD4+ T-cells ($p=<0.0001$; and $p=<0.0001$ respectively, Fig 4.7AB) increased in association with increasing duration of viraemic suppression. We also observed moderate

increases in CD4+ T-cell TNFα production in association with increasing duration of aviraemia (p=0.01; Fig 4.7B).

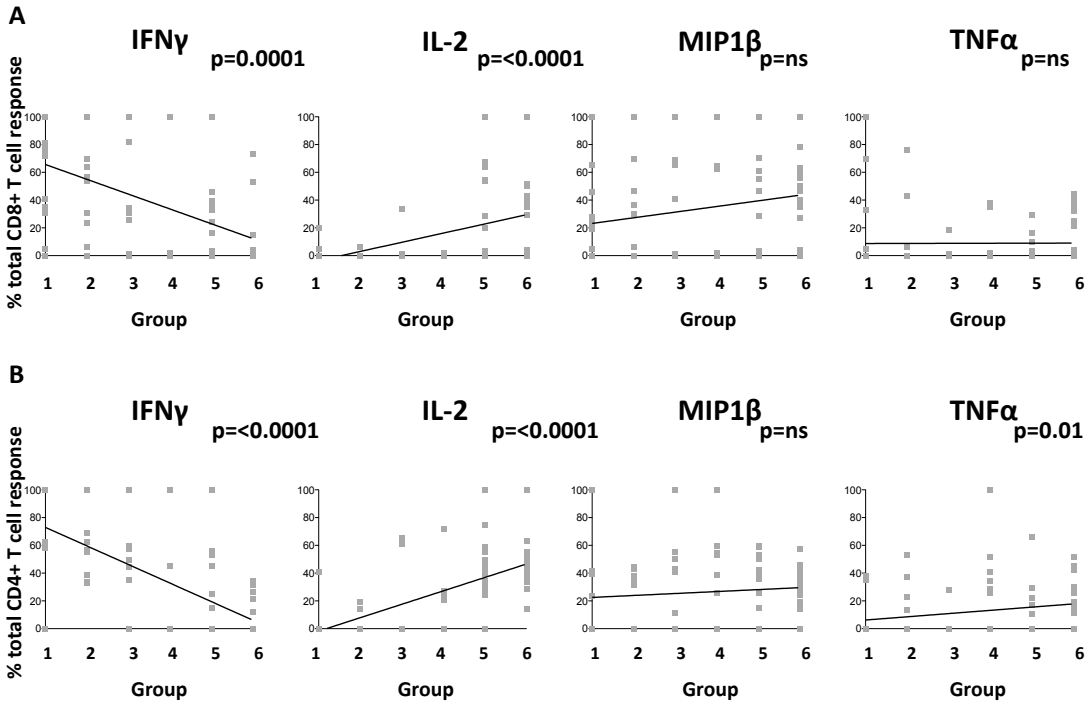


Fig 4.7. An increase in the duration of ART is associated with a decrease in IFNg production and an increase in IL2 production in CD8+ and CD4+ T cells in HIV infected ART treated children. % contribution of the indicated functional response toward the total [A] CD8+ T cell and [B] CD4+ T cell responses to HIV Gag peptide pool stimulation. Group 1, no VC n= 20, Group 2, 'blippers' n=9; Group 3, VC <12mnths n=10; Group 4, VC 13-24mnths n=10; Group 5, VC 25-60 mnths n=17; Group 6 VC >60 mnths n=18. p values obtained from ANOVA.

Similar observations were made when analysing children in groups 2-6 only, i.e. those children that had been classified as achieving virological control and stratified according to the duration of VC achieved. In response to the Gag peptide pool, the relative contribution of IFNγ production to the total T-cell response, in both CD8+ and CD4+ T-cells, decreased in association with increasing duration of viral control (r=-0.48, p=0.0002; and r=-0.39, p=0.003,

respectively, Fig 4.8). In contrast, the relative contribution of IL-2 production to the total T-cell response, both in the CD8+ and CD4+ T-cells (r=0.33, p=0.01; and r=0.49, p=0.0002 respectively, Fig 4.8) increased in association with increasing duration of viraemic suppression. We also observed moderate increases in both CD8+ and CD4+ T-cell TNFα production in association with increasing duration of aviraemia (r=0.26, p=0.05; r=0.33, p=0.01 respectively. Fig 4.8).

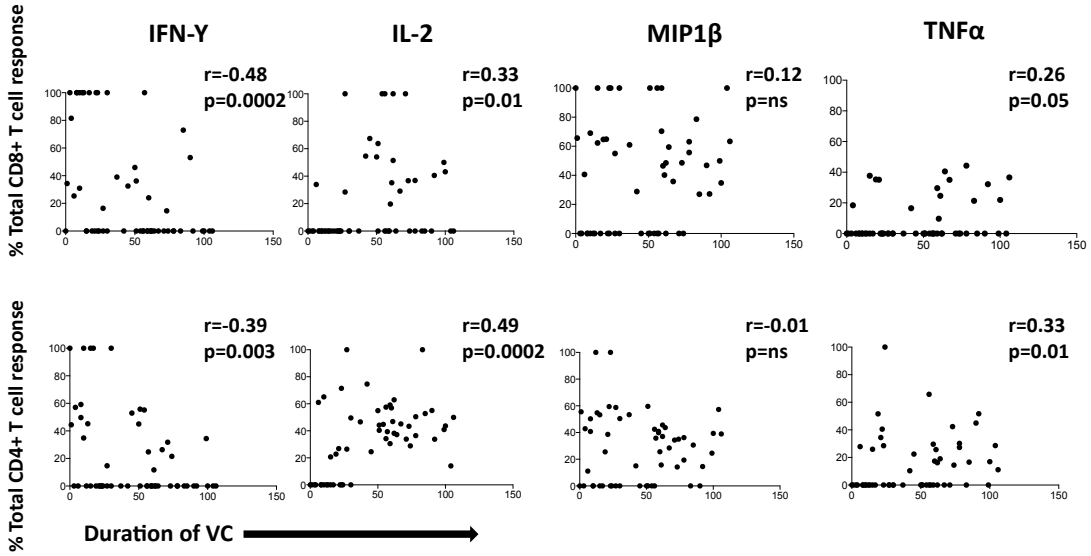


Fig 4.8. An increase in the duration of VC is associated with a decrease in IFNg production and an increase in IL2 production in CD8+ and CD4+ T cells in HIV infected ART treated children. % contribution of the indicated functional response toward the total [Top Panel] CD8+ T cell and [Bottom Panel] CD4+ T cell responses to HIV Gag peptide pool stimulation. ART treated children stratified according to duration of viraemic control (VC). Duration of VC is shown in months. Spearman correlations shown.

In terms of the absolute magnitude of responses to the Gag peptide pool, similar findings were observed, with decreasing IFNγ, and increasing IL-2 responses in both the CD8+ and CD4+ T cells and increasing MIP1β and TNFα responses in the CD4+ T-cells only in association with increasing duration of viraemic suppression (Fig 4.9AB).

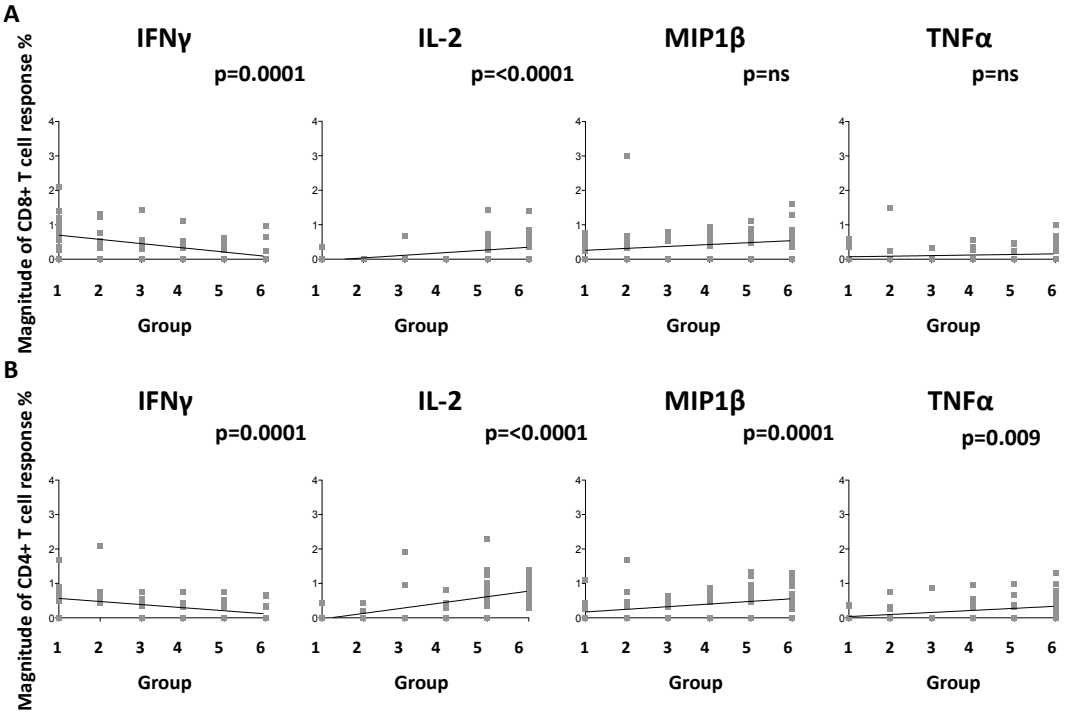


Fig 4.9. An increase in the duration of ART is associated with a decrease in IFNγ production and an increase in IL2 production in CD8+ and CD4+ T cells in HIV infected ART treated children. Magnitude (%) of the indicated functional response of [A] CD8+ T-cell and [B] CD4+ T-cell responses to HIV Gag peptide pool stimulation. Group 1, no VC n= 20, Group 2, 'blippers' n=9; Group 3, VC <12mnths n=10; Group 4, VC 13-24mnths n=10; Group 5, VC 25-60 mnths n=17; Group 6 VC >60 mnths n=18. p values obtained from ANOVA.

Similar observations were made when analysing children in groups 2-6 only, i.e. those children that had been classified as achieving virological control and stratified according to the duration of VC achieved. In response to the Gag peptide pool, the relative contribution of IFNγ production to the total T-cell response, in both CD8+ and CD4+ T-cells, decreased in association with increasing duration of viral suppression (r=-0.438 p=0.004; and r=-0.29, p=0.03, respectively, Fig 4.10) and the relative contribution of IL-2 production to the total T-cell response, both in the CD8+ and CD4+ T-cells increased in association with increasing duration of viraemic control (r=0.34, p=0.01; and r=0.58, p=<0.0001 respectively, Fig 4.10) . We also observed an increase in CD4+ T-cell TNFα production in association with increasing duration of aviraemia (r=0.39, p=0.003 Fig 4.10).

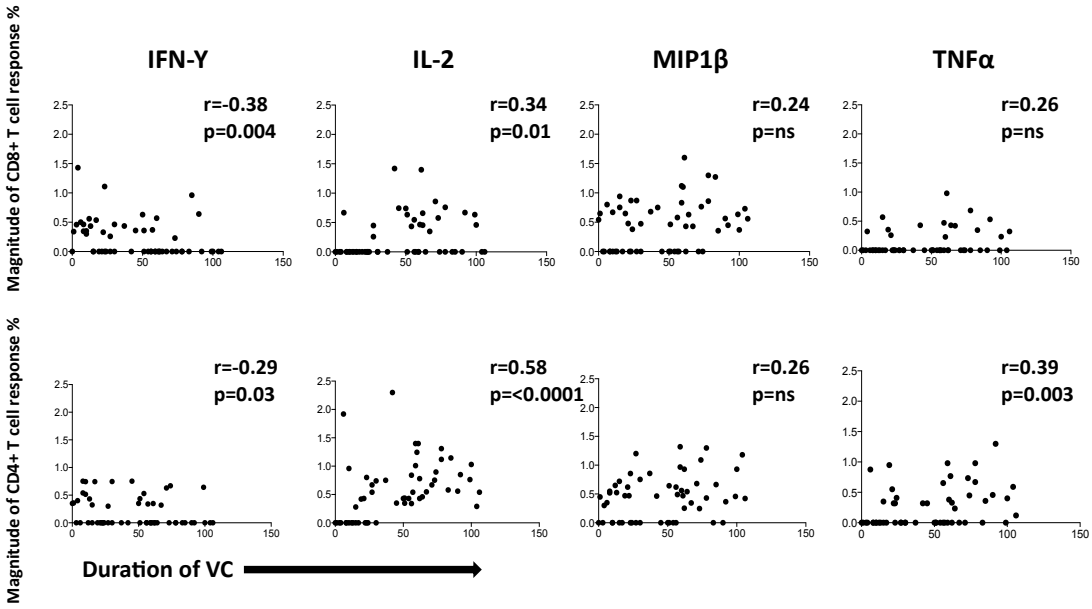


Fig 4.10. An increase in the duration of VC is associated with a decrease in IFNg production and an increase in IL2 production in CD8+ and CD4+ T cells in HIV infected ART treated children. % contribution of the indicated functional response toward the total [Top Panel] CD8+ T cell and [Bottom Panel] CD4+ T cell responses to HIV Gag peptide pool stimulation. ART treated children stratified according to duration of viraemic control (VC). Duration of VC is shown in months. Spearman correlations shown.

With respect to polyfunctionality in ART-treated children, there was a strongly significant increase in CD4+ but not CD8+ T-cell polyfunctionality in association with increasing duration of viraemic suppression (r= 0.55, p=<0.0001 and r=0.26, p=ns, respectively, Fig 4.11). Similar findings were observed following stimulation of CD4+ and CD8+ T-cells by Pol and Nef peptide pools (data not shown).

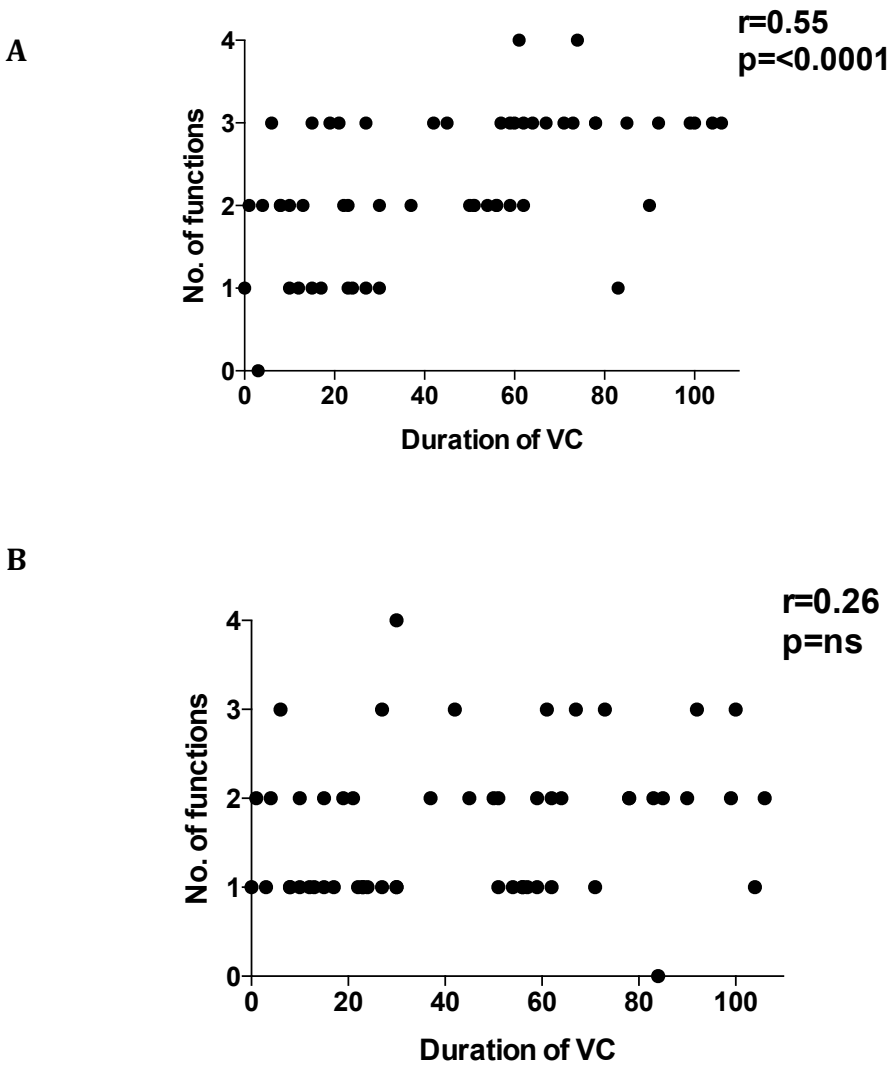


Fig 4.11. Association of ART duration with restoration of CD4 but not CD8 polyfunctionality. y axis indicates the number of functional responses (IFNγ, IL-2, MIP1β and TNFα) made by each subject in response to HIV gag stimulation in (A) CD4+ T cells and (B) CD8+ T cells. X axis indicates duration of viraemic control (VC) in months. Spearman correlations shown.

Restoration of the CD4+ T cell function in ART-treated children is suggested by the responses observed in these children compared to the quality of response

that is observed in HIV-infected children maintaining normal CD4 counts in the absence of ART. As stated above, it should be noted that restoration of the response in HIV-infected children receiving long-term ART can only be unequivocally demonstrated via longitudinal studies of the same children on ART.

4.2.6. Enhanced T cell functionality is associated with low immune activation and low exhaustion

To assess the degree of residual immune activation in ART-treated children, we first determined HLA-DR/CD38 expression in PBMC from these study subjects. Reduced immune activation (HLA/DR/CD38 expression) on both CD8+ and CD4+ T-cells was associated with increasing duration of viraemic control ($p=<0.0001$; and $p=<0.0001$, respectively Fig 4.12A). We next looked at the levels of programmed death 1 (PD-1), a cell surface negative regulator of T cells that is upregulated in the context of immune activation and a marker of immune exhaustion. Similar to immune activation, PD-1 expression on both CD8+ and CD4+ T-cells was reduced with increasing ART duration ($p=<0.0001$; and $p=<0.0001$, respectively, Fig 4.12B). Levels of a second immune checkpoint molecule, T-cell immunoglobulin mucin-3 (Tim-3), like PD-1, a negative regulatory receptor associated with T-cell exhaustion, were inversely related to duration of viraemic suppression in both the CD8+ and CD4+ T-cell subsets ($p=<0.0001$; and $p=<0.0001$ respectively, Fig 4.12C). The expression of a third inhibitory receptor, CD244, also implicated in T-cell exhaustion, was likewise strongly correlated with PD-1 and Tim-3 expression (data not shown). Finally,

we examined CD95 expression in these ART-treated children, the CD95/APO-1/Fas receptor (R)/ligand (L) system, being critically involved in induction of apoptosis in mature T cells. As anticipated, we observed a significant decrease in the percentage of cells expressing CD95 in association with increasing duration of viraemic control in both the CD8+ and CD4+ T-cell subsets ($p=<0.0001$; and $p=<0.0001$ respectively, Fig 4.12D).

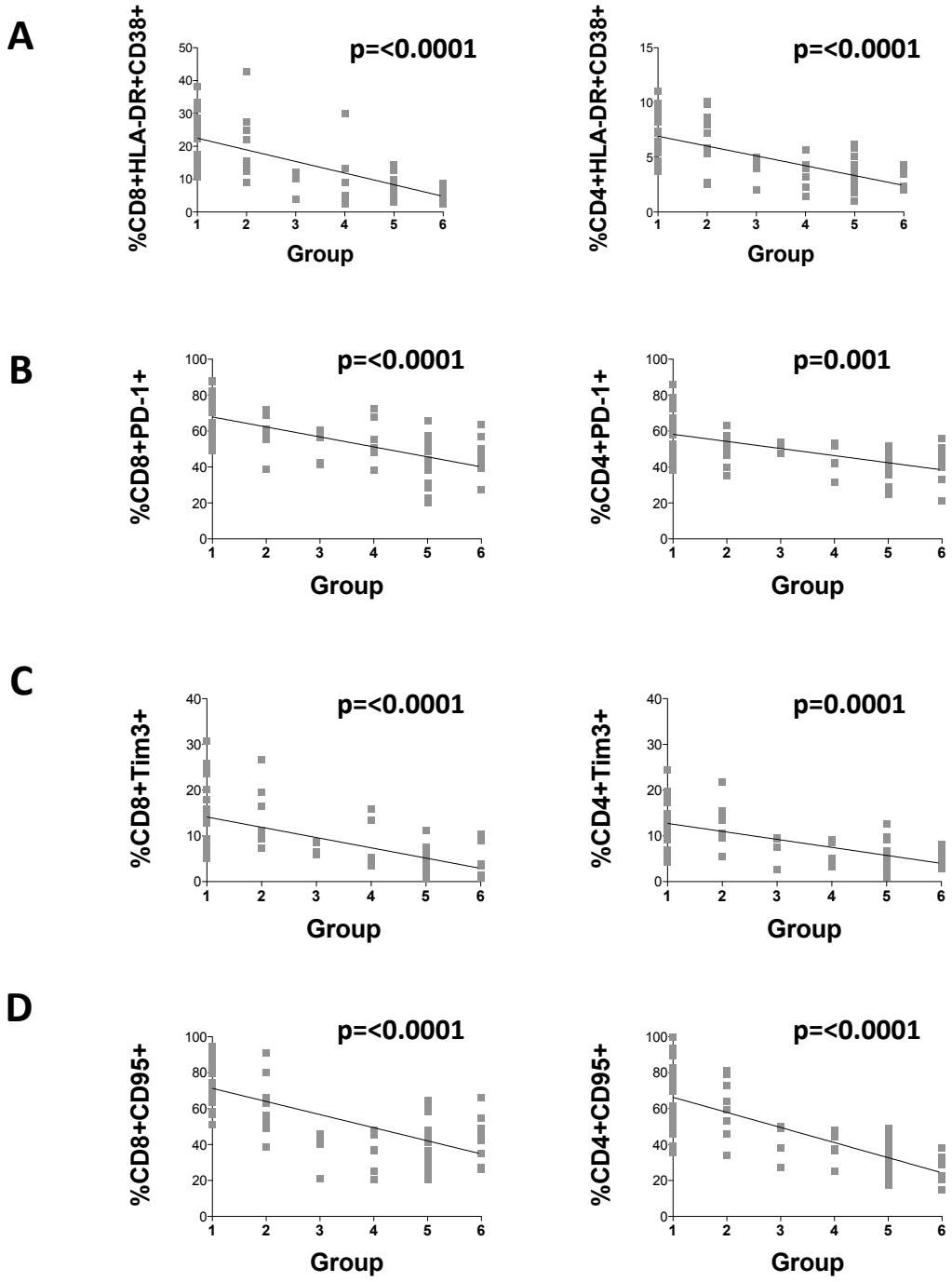


Figure 4.12. Enhanced T cell functionality is associated with low immune activation and low exhaustion. Correlations between the proportion of baseline HLA-DR+ CD38+ T-cells [A], PD-1+ T-cells [B], Tim3+ T-cells [C], and CD95+ T-cells [D], with duration of viraemic control are shown. Correlations for both CD8+ T-cells and CD4+ T-cells are shown. Group 1, no VC n= 20, Group 2, 'blippers' n=9; Group 3, VC <12mnths n=10; Group 4, VC 13-24mnths n=10; Group 5, VC 25-60 mnths n=17; Group 6 VC >60 mnths n=18. p values obtained from ANOVA.

In order to investigate whether these correlations remained statistically significant in the aviraemic groups 3-6, these analyses were repeated in the absence of groups 1-2. Only the levels of immune activation on CD8+ T-cells, Tim-3 expression on CD8+ T-cells, and CD95 expression on CD4+ T-cells remained significantly associated to duration of aviraemia on ART ($r=-0.31$, $p=0.05$; $r=-0.38$, $p=0.02$; and $r=-0.47$, $p=0.003$, respectively, Fig 4.13). These data highlight the impact of persistent viraemia in maintaining immune dysfunction, and the rapid, albeit incomplete, reversal towards restored function brought about even by a short period of viraemic suppression.

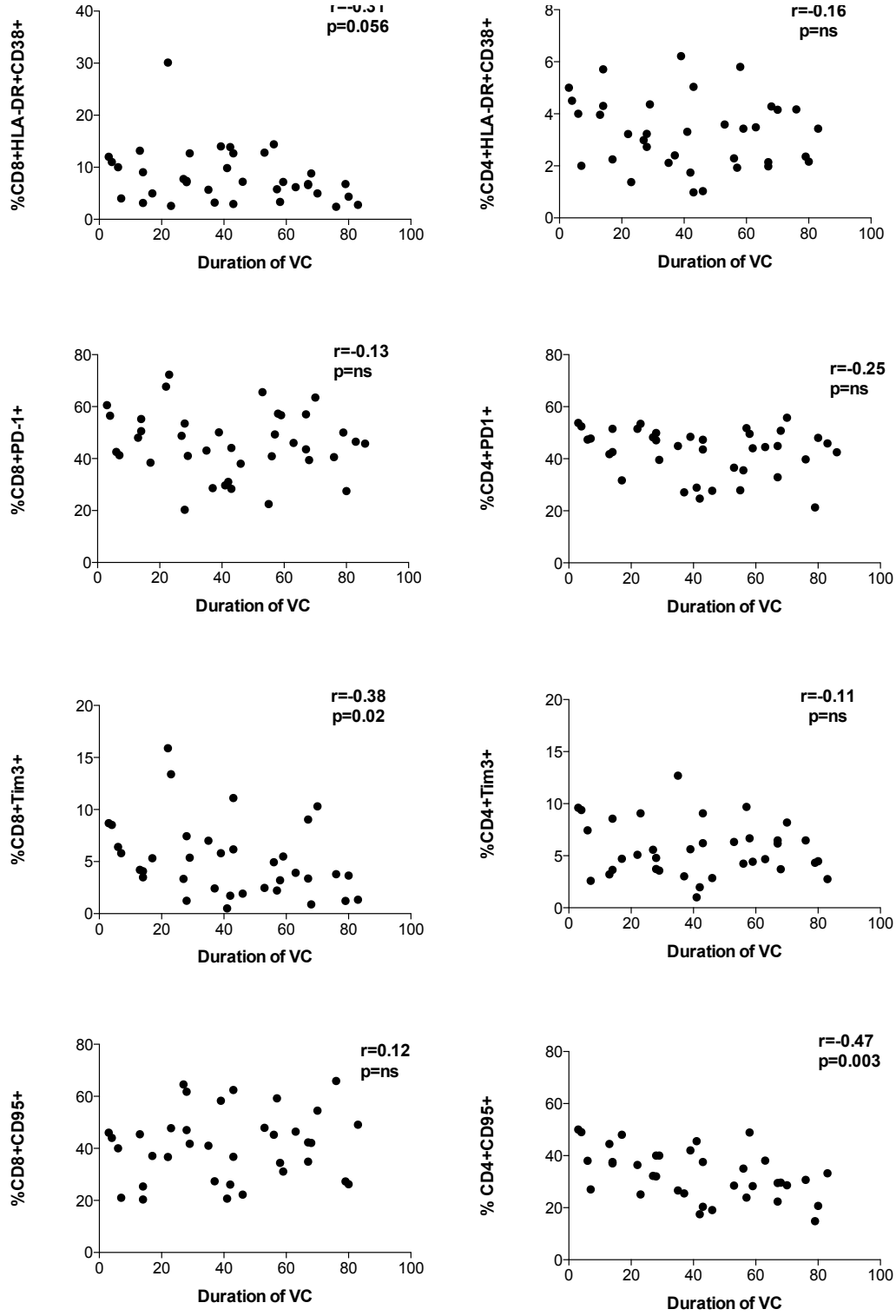


Fig 4.13 **Enhanced T cell functionality is associated with low immune activation and low exhaustion in aviraemic children.** Correlations between the proportion of HLA-DR+ CD38+ T-cells, PD-1+ T-cells, Tim3+ T-cells, and CD95+ T-cells, with duration of viraemic control in months are shown. Correlations for both CD8+ T-cells and CD4+ T-cells are shown. . ART treated children stratified according to duration of viraemic control (VC). Spearman correlations shown.

4.3 DISCUSSION

These analyses of CD4 T-cell function in ART-naïve and ART-treated HIV-infected children, although a cross-sectional study, suggest a rapid recovery of CD4+ T-cell function within 12 months of viraemic suppression on ART. In ART-naïve children, a high absolute CD4 count was strongly correlated with a predominant IL-2 Gag-specific CD4+ T-cell response and with CD4+ and CD8+ T-cell polyfunctionality. Viral load in the same children was correlated with CD4+ but not CD8+ T-cell function, with high IFN-γ Gag-specific responses directly related, and IL-2, TNFα and MIP1β responses inversely related to viraemia. In ART-treated children, a similar and consistent binary pattern emerged, with viraemic children maintaining predominantly IFN-γ responses and those with suppression of viraemia showing mainly IL-2 responses. In ART-treated children, longer duration of aviraemia was associated with increasing polyfunctionality on CD4+ but not CD8+ T-cells, and with decreasing expression levels of immune activation and inhibitory receptors such as PD1 and Tim-3. A small longitudinal study carried out on 20 ART-treated, HIV –infected children in our cohort in Durban, South Africa displayed strikingly concordant results with our cross sectional study. The percentage of IL-2 production by CD4+ T cells in these children increased significantly with the duration of time on ART ($p=0.026$, data not shown).

These findings therefore are, broadly, highly consistent with previous studies (Sleasman *et al.* 1999; Gibb *et al*. 2000; Walker *et al*. 2004; Resino *et al*. 2006; COHERE/Sabin *et al*. 2008) that have shown recovery of CD4 T-cell function in children, but not in adults, who achieve aviraemia on ART. Here, however, excluding the two groups of children in whom ART failed to suppress viraemia, the evidence that increased duration of aviraemia improved function further in CD4+ T-cells was modest. Thus, albeit in a cross-sectional study, these data suggest that a relatively short period of aviraemia was sufficient to largely restore CD4+ T-cell function in ART-treated children. In contrast, after as much as >5yrs on ART, polyfunctionality in CD8+ T-cells was apparently not restored to the levels observed even in ART-naïve children whose CD4 counts were at the current ART initiation level of <500 cells/mm^3. The two groups of ART-treated children who failed to suppress viraemia, viraemic non-suppressors (VNS) and 'blippers', appeared similar in terms of failure to reconstitute T-cell function.

The data presented for the ART-naïve children are especially striking in demonstrating the discordant relationship between CD8+ T-cells and viral load, versus that between CD4+ T-cells and viral load. Whilst for the CD8+ T-cells there is no correlation with viral load (r=0.19), for CD4+ T-cells there is a statistically significant *inverse* correlation with viral load (r=-0.26, p=0.04). What drives this inverse relationship is the combination of IL-2 , MIP1β and TNFα production. In contrast, production of IFN-γ by CD4 T-cells is strongly associated with increasing viraemia. These data are consistent with largely passive CD8+ T-cell responses being driven by viral load, while control of viraemia is related to

effective CD4+ T cell function featuring in particular IL-2 production. This would be consistent with naïve and central memory CD4 T-cells predominating in settings of high CD4 counts, low viral loads, low immune activation and low expression of immune checkpoint markers such as PD-1 and Tim-3; whereas in the context of declining CD4 counts and high viraemia, one observes a more differentiated CD4 effector memory T-cell compartment, with high immune activation, high expression of exhaustion markers and functionality limited to IFN-γ production.

These findings support the concept that CD4 T-cell function is central to maintenance of non-pathogenesis in paediatric infection and that CD8+ T-cells play a somewhat passive part. Recent studies by our group highlighted the fact that HLA class I plays a much smaller part in non-progressing paediatric HIV infection compared to non-progressing adult infection. Protective HLA class I molecules such as HLA-B*27/57/58:01/81:01, are strongly associated with slow progression in adult but not in paediatric infection. Similarly, in adults the CD8+ T-cell response restricted by these protective HLA class I alleles appears to perform a critical function in killing virus-infected cells and thereby reducing viraemia (Kiepiela *et al*. 2007, Goulder & Walker 2012). The part played by HIV-specific CD8+ T-cells in non-progressing paediatric infection is less clear.

These studies were undertaken in children in whom ART was initiated at approximately 2 years of age. Current WHO guidelines are such that all children

aged < 5yrs who are HIV-infected should initiate ART, irrespective of CD4 count. Thus the findings described here may not apply to HIV-infected children in whom ART is initiated very early in life and aviraemia established within weeks of infection, as in the Mississippi child (Persaud *et al*. 2013). However it should be noted, first, that most in utero infected babies have viral loads of ~10^6 in the first days and weeks of life, and therefore prior to ART initiation (Mphatswe *et al.* 2007); and, second, that the majority of in utero infected babies have detectable HIV-specific CD8+ T-cell responses at birth (Thobakgale *et al.* 2007, Luzuriaga *et al.* 1995). Thus, in most cases, even early ART would not prevent priming of HIV-specific responses by HIV. It would nonetheless be of importance to evaluate HIV-specific CD4 and CD8 T-cell responses in this group of early-treated in utero infected infants, who have received years of ART from days or hours of birth, to assess the potential for immune-based therapies in childhood either to augment pre-existing responses, or to prime novel and possibly more effective ones and pave the way for ART discontinuation.

CHAPTER 5

SOOTY MANGABEY-LIKE PHENOTYPE IN NON-PROGRESSING HIV-INFECTED CHILDREN

5.1 INTRODUCTION

While viral set point in adult HIV infection is strongly predictive of disease progression, immune activation (CD38+ HLA-DR+ expression) has emerged as a stronger correlate of progression (Giorgi *et al.* 1999). The establishment of a CD8+ T cell activation set-point in acute HIV-infection is a strong independent predictor of the rate of CD4+ T cell decline (Deeks *et al.* 2004). As in adult infection, immune activation is a better predictor than viral load of disease progression in children. Even as early as 2 months of age, levels of immune activation can predict immunological progression in HIV infected children (Paul *et al.* 2005). However, the relationship between viral load and immune activation differs in children and adults. In adult HIV infection, immune activation correlates with viral load (Day *et al.* 2006). In contrast, immune activation in children does not appear to be driven by viral load (Prendergast *et al* 2011). This highlights the poor predictive value of viral load in paediatrics, particularly in infancy when there is considerable overlap in levels of vireamia between rapid and slow progressors (Shearer *et al.* 1997).

The mechanism by which immune activation is proposed to operate in HIV infection is via depletion of CD4 T cells in the gut mucosa, creating a breach in the mucosal barrier, opening the way to translocation of microbial products such as LPS into the circulation (Brenchley *et al.* 2006). The consequence of this process, so-called 'microbial translocation', is immune activation, and activation induced apoptosis. This model explains the disparity between the relatively small number of CD4 cells infected with HIV, and the much larger number of CD4 cells dying from bystander effects and pyroptosis (Finkel *et al.* 1995; Doitsh *et al.* 2014), leading to the observed CD4 decline and ultimately, AIDS.

The nonpathogenic nature of SIV infection in natural hosts of SIV, such as sooty mangabeys (SM) or African green monkeys (AGM), who maintain high CD4 T cell counts in the setting of high viral loads, has been attributed to the low level of immune activation in these animals (Silvestri *et al*. 2003). This contrasts with the high levels of immune activation observed in HIV-infected humans and SIV-infected rhesus macaques (RM). Rhesus macaques are Asian primates, and, unlike sooty mangabeys or African green monkeys, are not naturally infected with SIV. Indian rhesus macaques experimentally infected with SIV represent the best-studied model of HIV. In acute infection in SM/AGM, there is a robust - albeit transient - innate immune response (Bosinger *et al*. 2009; Harris *et al.* 2010, leaving unresolved high levels of viraemia (setpoint $\sim 10^5$ copies/ml). In contrast, resolution of high viraemia occurs in acute SIV infection in RM, which is dependent upon the activity of the CD8+ T-cell response (Schmitz *et al*. 1999). Blocking of the CD8+ T-cell response using monoclonal anti-CD8 antibodies in SM does not affect viral load. The lack of impact on viral setpoint of the CD8+ T-

cell response in the SM/AGM compared to RM has been related to the early resolution of immune activation in SM/AGM, which is correlated with the early expression of PD-1 (Estes *et al.* 2008). In RM, immune activation is not more prominent in acute infection than in SM/AGMs but is maintained throughout the course of infection, and PD-1 expression is only observed in chronic infection. Decreased immune activation enables SIV-infected SM or AGM to avoid the bystander damage observed in pathogenic infections and protects them from developing AIDS (Silvestri *et al.* 2003).

The situation described in relation to SM/RM is somewhat similar to what we observe in HIV-1 infected paediatric non progressors (PNP) who maintain normal, or sometimes even high CD4 counts, indistinguishable to those of HIV-uninfected age-matched children, in the setting of high viraemia for many years. In the setting of PNP, viral load declines towards adult levels of ~30,000 copies/ml plasma after approximately 3-5 years of life. More recent work in humans has identified a rare subset of HIV infected individuals termed 'adult viremic non progressors (AVNP's) who remain asymptomatic and are able to maintain preserved CD4+ T cell counts despite persistently high viraemia (Klatt *et al.* 2014). Unlike non-progressing HIV-infected adults, in whom high CD4 counts are typically associated with very low or undetectable levels of viraemia, control of viraemia is very rare in non-progressing children. In fact there is no single case in the literature of an ART-naïve HIV-infected child whose viral load is undetectable.

Whilst the mechanism of non-progressive HIV infection in adults, with high CD4 counts accompanied by low viral loads, is strongly associated with expression of

particular HLA class I molecules, such as HLA-B*27 and HLA-B*57, HLA class I variation does not make a substantial contribution to non-progressive paediatric infection (Adland *et al*. 2015).

A recent study of AVNP's found no significant differences in levels of T cell activation between the AVNPs and putative progressors (PP's) but what they did find were decreased levels of infection in central memory CD4+ T cells in AVNPs compared to PPs which could therefore contribute to preservation of CD4 T cell homeostasis and lack of disease progression despite high viraemia (Klatt *et al*. 2014). Again this is a similar scenario to what has been previously observed in the SM model where low levels of SIV infection in central memory CD(4)(+) T cells are associated with limited CCR5 expression (Paiardini *et al.* 2011). We hypothesised that a similar mechanism may be identifiable in PNP that is allowing these children to maintain high absolute CD4 counts in the setting of high viraemia and hence protecting them from developing AIDS.

We here describe a relatively large cohort of HIV-infected children who are indistinguishable from age-matched uninfected children in terms of clinical health and CD4 counts, yet who have viral loads in the 10^4–10^5 copies/ml range.

5.2 RESULTS

5.2.1 Paediatric Non-Progressors maintain high CD4 counts despite persistently high viraemia

We studied a total of 276 vertically HIV-1 C clade infected children recruited from paediatric clinics in Kimberley Hospital, Kimberley, South Africa, the Ithembalabantu Clinic in Umlazi, Durban, South Africa, and Great Ormond Street Hospital, London, United Kingdom who have remained antiretroviral therapy (ART)-naïve.

Based on normal absolute CD4 counts in HIV-uninfected children (Shearer *et al.* 2003), we here defined paediatric non-progressors (PNP) as ART-naïve, HIV-infected children with CD4 counts of >750 cells/mm^3 at age $\geq$5yrs, who have not met the CD4 or clinical criteria for ART initiation. The median age of the PNPs (n=168) was 8.25years (IQR 6.625-10.48), median absolute CD4 count (ACC) 886cells/ul (IQR 815-1020), median CD4% 18% (IQR 24-31.2) and median viral load 25913cp/ml (IQR 5317-76000).

An example of one such PNP child followed from birth shows that absolute CD4 count and CD4% stay within the physiological range of an uninfected child of the same age, without ART, over its 10-year follow up period (Fig 5.1AB). Although absolute CD4 count and CD4% are close to the 50^{th} centile for age-matched HIV-uninfected children, the viral loads reach a quasi setpoint of 10^4-10^5 copies/ml plasma (Fig 5.1C).

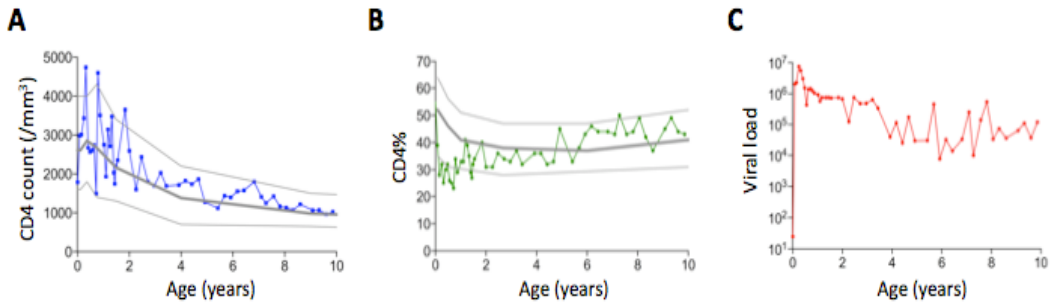


Fig 5.1. Changes with age in absolute CD4 count, CD4% and viral load in a representative Paediatric Non-Progressor and HIV-uninfected children.

Data from HIV-uninfected children (adapted from Shearer *et al*. 2003) are shown in grey (10th, 50th and 90th percentile). Absolute CD4 count (A), CD4% (B) and viral load (C) are shown for a representative ART-naïve HIV-infected paediatric non-progressor followed from birth.

In contrast to adult HIV-infection where a viral set point is reached within weeks of infection (Lyles *et al.* 2000), cross-sectional data from the presenting viral loads in the 170 PNPs, 27 of whom presented at age<5yrs, indicate a decrease in viral load over the first 5 years of life (r=-0.57, p=0.0005) to reach a quasi setpoint of 10^4-10^5 copies/ml plasma without further decrease with age (r=-0.04, p=ns) (Fig 5.2).

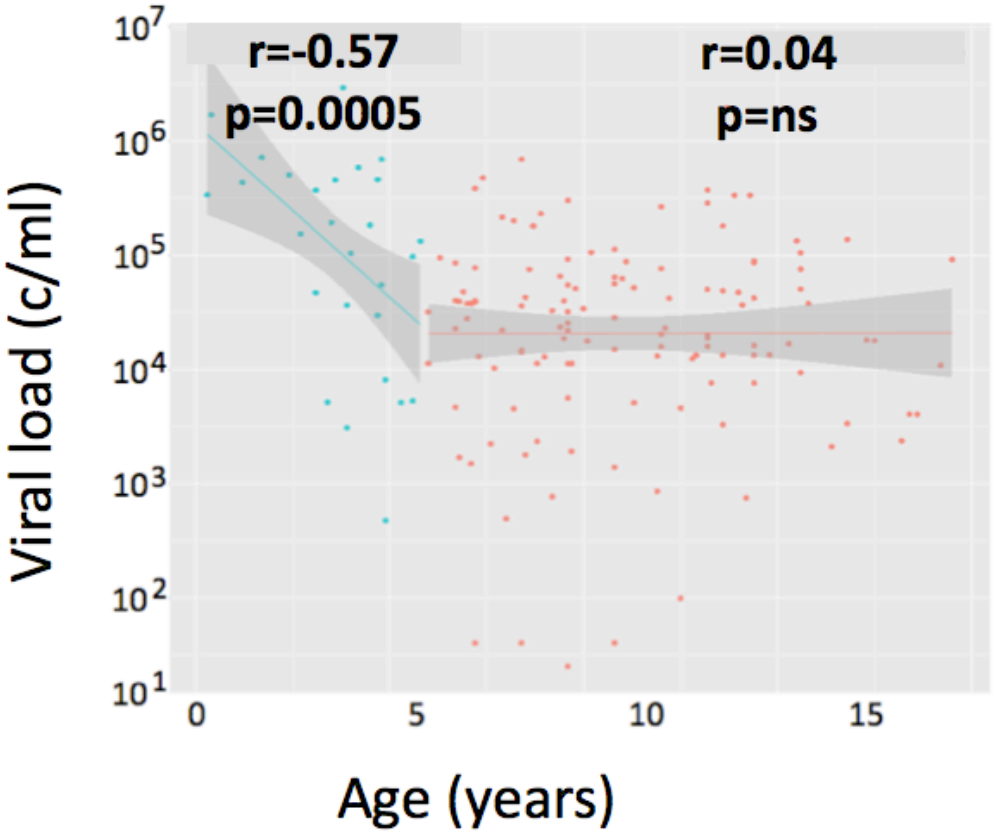


Fig 5.2 Presenting viral loads in HIV-positive ART-naïve children (n=170). A significant inverse correlation is observed in children <5 years of age (r=-0.57, p=0.0005) and no correlation >5 years of age (r=0.04, p=ns). Spearman correlations shown.

In contrast with ART-naïve non-progressor adults, in whom sustained high CD4 counts are typically found in association with very low or undetectable viral loads (Deeks & Walker 2007) our cohort of ART-naïve PNPs have high CD4 counts (median 1013 CD4 T-cells per µl) that are maintained in the setting of high viraemia (median 22,172 copies per ml) (Fig 5.3)

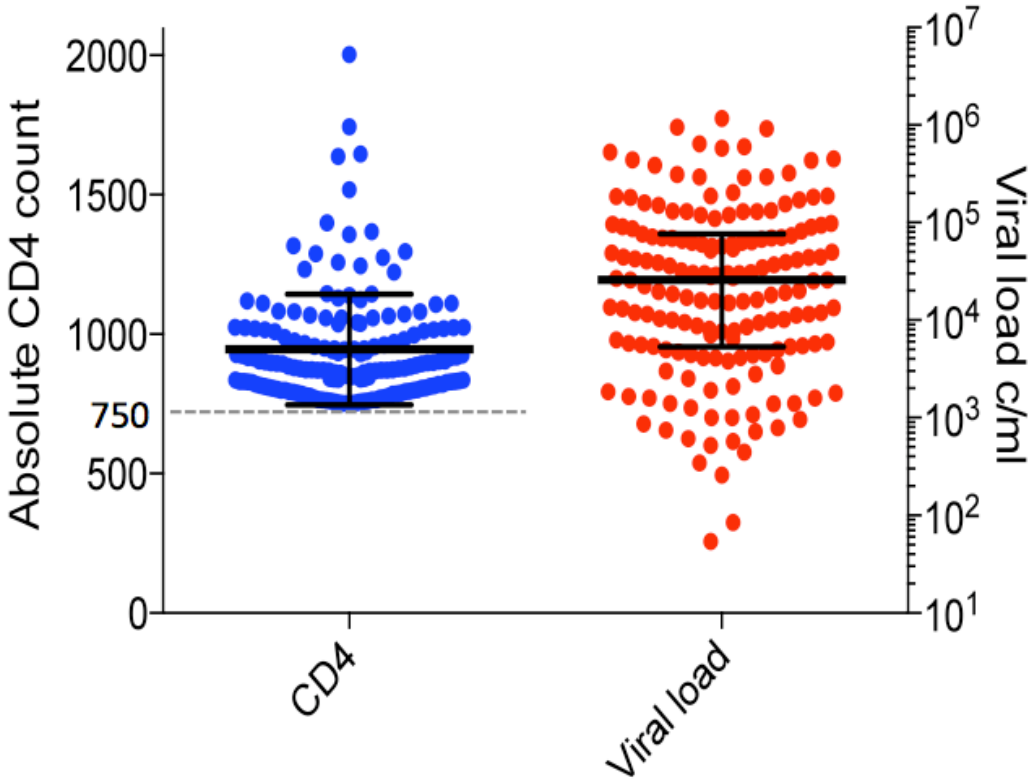


Fig 5.3 Presenting clinical data in ART-naive non-progressors (CD4>750/mm³) aged ≥5yrs (mean 6.4yrs, n=164). Median CD4: 1013/mm³, median VL: 22, 172.

Viral load itself is a poor predictive value of disease progression in HIV-infected children. We observe that there is no association between viral load and CD4 counts in HIV-infected children at 5years of age (Spearman r=-0.01, p=0.9) with a relatively weak correlation becoming apparent at 7.5 years of age (Spearman r=-0.18, p=0.0003) (Fig 5.4). This is of course in stark contrast to chronically HIV-infected adults where a strong correlation exists between HIV VL, CD4 count, and hence progression to AIDS (Spearman r=-0.50, p<0.0001) (Fig 5.4).

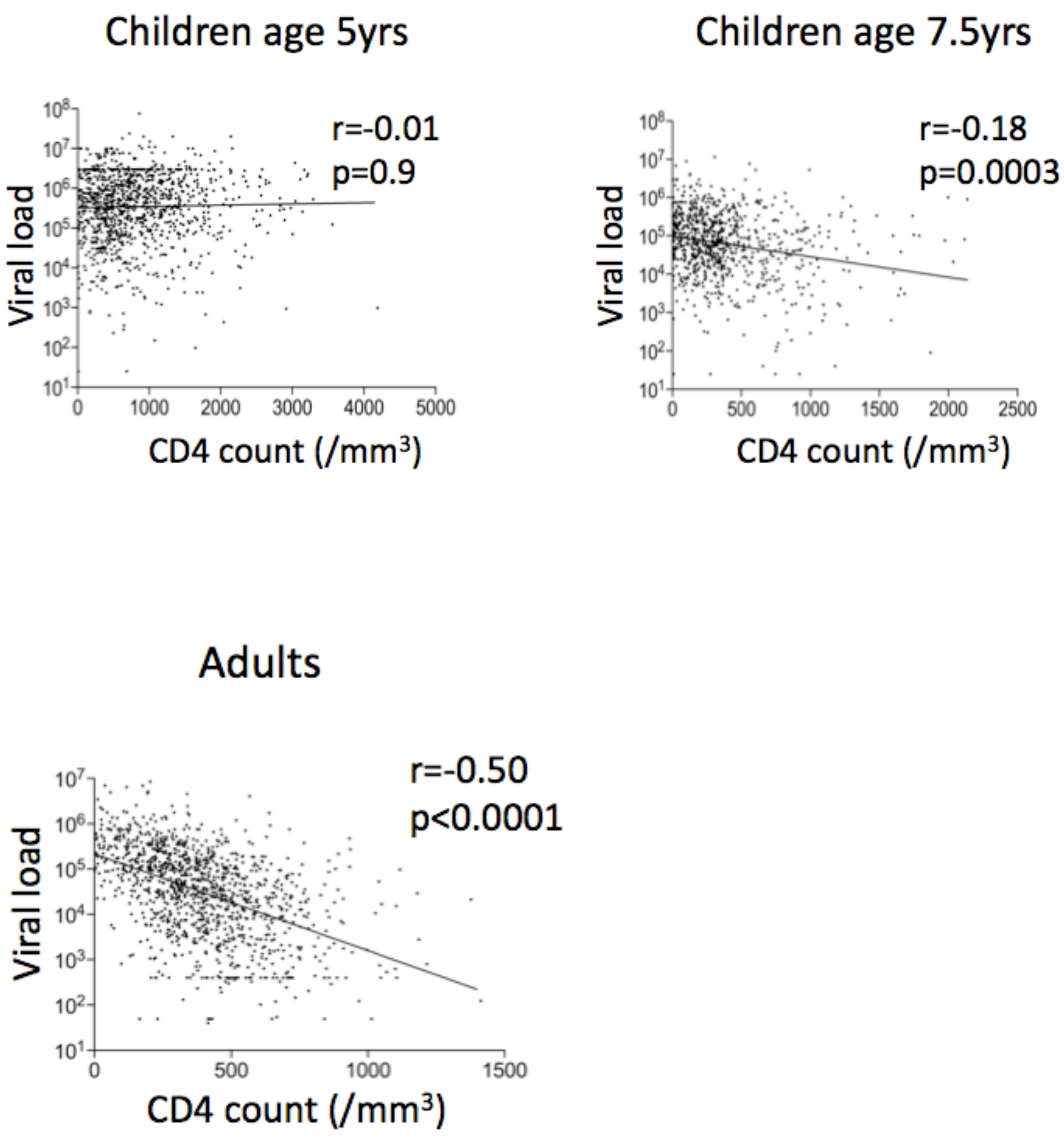


Fig 5.4. Viral load as clinical marker of disease progression in HIV-infected children and adults. Presenting viral loads in ART-naïve children (n=164) at 5 years (r=-0.01, p=0.9), 7.5 years (r=-0.18, p=0.0003) and a comparative cohort of chronically HIV-infected adults from Durban, South Africa (r=-0.50, p<0.0001). Spearman correlations shown.

These data suggest a similarity between PNP and the natural hosts of SIV infection, such as sooty mangabeys and African green monkeys, in whom

persistent high viral loads of 10^4-10^5 copies/ml are also associated with lack of disease and maintained CD4 counts (Silvestri *et al.* 2003).

5.2.2 Low levels of immune activation define PNPs

We next asked if non-pathogenic paediatric HIV-infection is determined by low levels of immune activation in the presence of persistently high viraemia, a similar mechanism to the non-pathogenic SIV-infection observed in sooty mangabeys. To explore this, we measured cellular markers of immune activation, CD38 and HLA-DR, in the CD4+ and CD8+ T-cell compartments in n=183 ART-naïve HIV-infected children above 5 years of age. We also recruited HIV-uninfected siblings of HIV-infected children to serve as HIV-uninfected controls (n=21). The HIV-uninfected children were age matched to the HIV-infected children (median 13.1 years). We observed that increasing levels of both CD4+ and CD8+ T cell immune activation (CD38/HLA-DR co-expression) correlate significantly with decreasing absolute CD4+ T cell count ($r=-0.63$, $p<0.0001$; $r=-0.55$, $p=<0.0001$, respectively) (Fig. 5.5). We also observe in the PNPs that levels of immune activation are only marginally higher than age-matched uninfected children, although this difference is statistically significant ($p= 0.048$) (Fig 5.5).

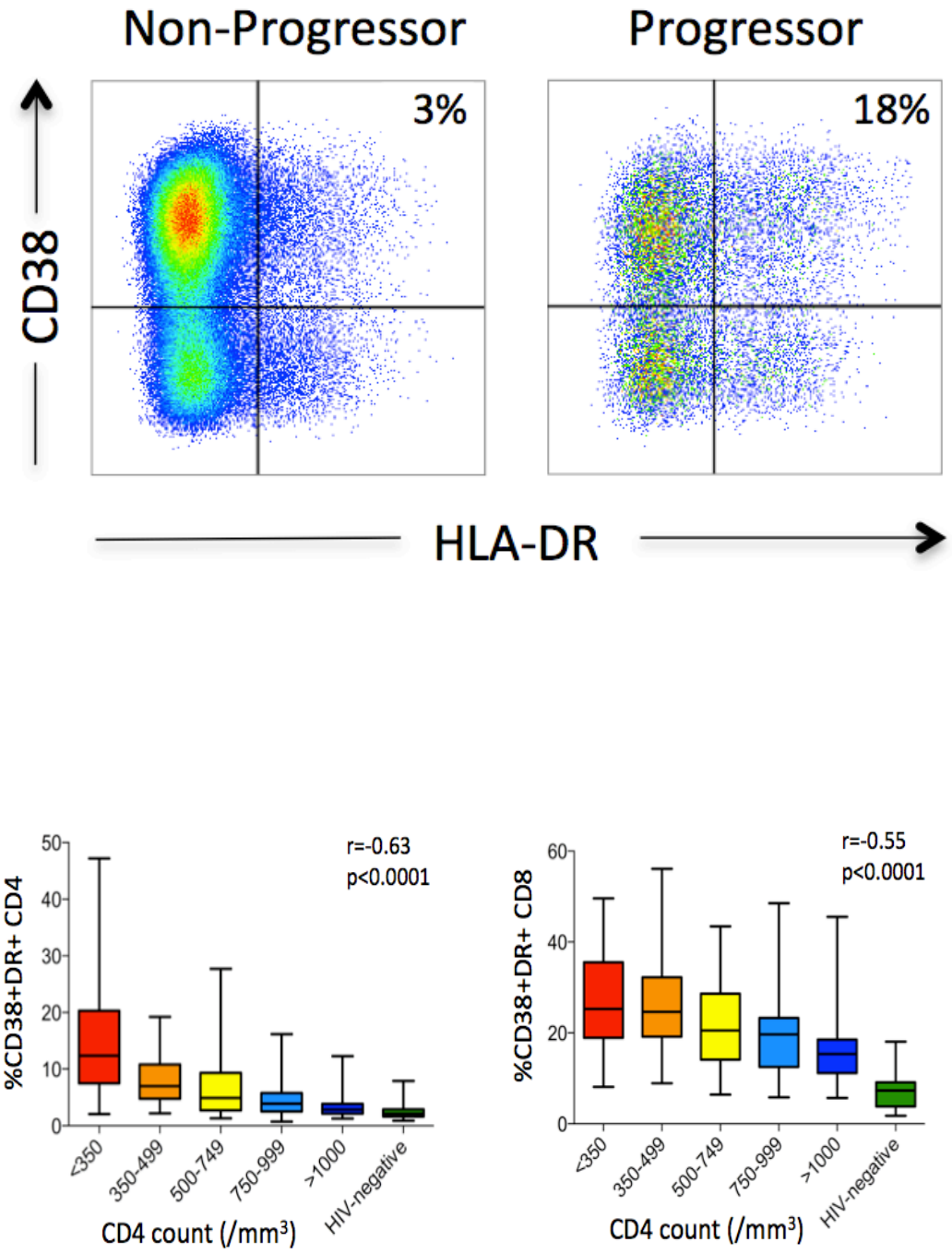


Fig 5.5. Low immune activation in Paediatric Non-Progressors. Top panel: Representative CD4+ T-cell immune activation FACS data from a Paediatric Non-Progressor (CD4>750/mm³) and a Paediatric Progressor (CD4<500/mm³). **Bottom Panels**: Immune activation in CD4+ and CD8+ T-cells in HIV-infected children aged >5yo stratified by absolute CD4 count and age-matched HIV-negative children (n=20 CD4<350, n=38 CD4=350-499, n=49 CD4=500-749, n=32 CD4=750-999, n=22 CD4>1000, n=21 HIV-negative). p values obtained from ANOVA

We also observed strong inverse correlations between both CD4+ and CD8+ T cell activation and CD4% (Fig 5.6)

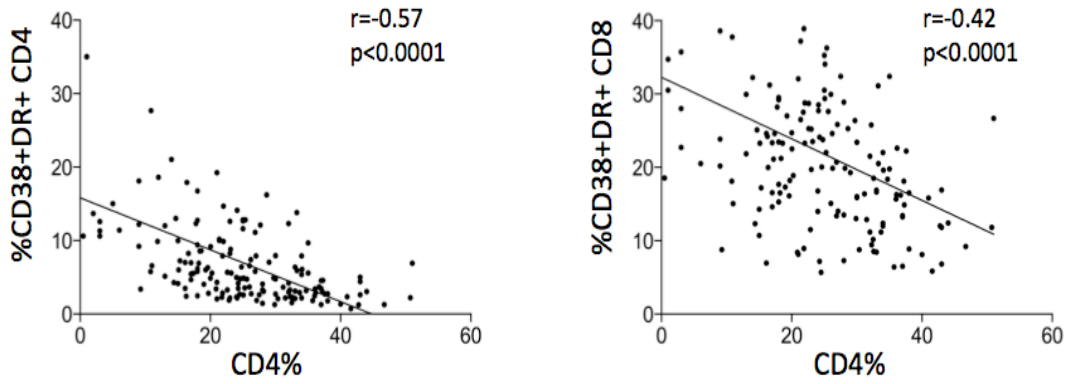


Fig 5.6. Correlation between CD4+ and CD8+ T-cell immune activation and CD4% in HIV-infected ART naïve children. (n=161). r=-0.57, p<0.0001 and r=-0.42, p<0.0001 respectively. Spearman correlations shown.

We next compared the relationship of HIV viral loads and T cell activation in PNPs to a control group of Paediatric Progressors (PP) that we define here as vertically HIV-infected children above 5 years of age with CD4 counts < 500 cells per µl. We observed that in PP, CD4+ T cell immune activation is driven by the viral load (Pearson r=0.36, p=0.0074, Fig 5.7), but interestingly we observe only a weak correlation between CD4+ T cell activation and viral load in PNPs (Pearson r=0.27, p=0.07) (Fig. 5.7).

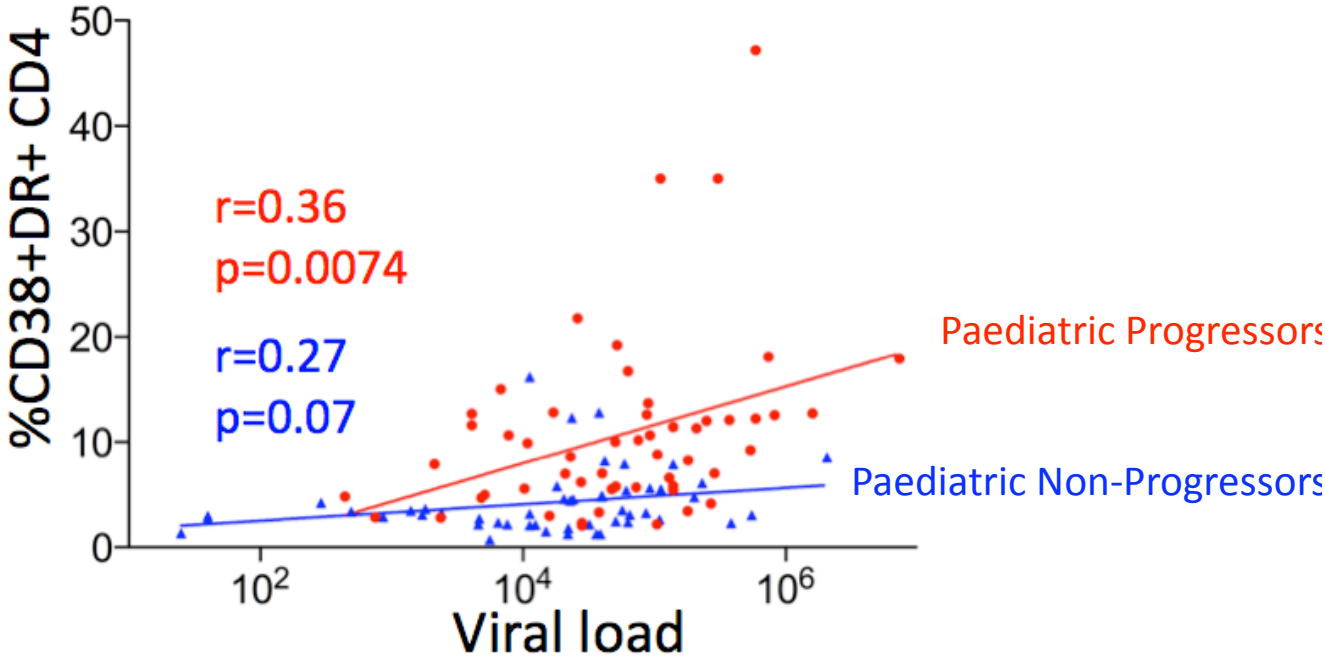


Fig 5.7 Relationship between CD4+ T cell immune activation and viral load in HIV-infected children. Stronger correlation between CD4+ T-cell immune activation and viral load in paediatric progressors (n=54, shown in red) than in paediatric non-progressors (n=50, shown in blue). Pearson r=0.36, p=0.0074 and r=0.27, p=0.07 respectively.

In pathogenic SIV and HIV infections, the translocation of microbial products from the gastrointestinal (GI) tract to portal and systemic circulation has been proposed as a major driver of the chronic immune activation that is associated with disease progression (Brenchley *et al* 2006). Microbial translocation, however, is not a feature of nonpathogenic SIV infections of natural host species (Favre *et al* 2009; He *et al* 2015). We first measured levels of soluble CD14

(sCD14) in our paediatric cohort, since sCD14 levels are characteristically raised in pathogenic SIV or HIV infection as a consequence of microbial translocation and chronic stimulation by LPS of monocytes/macrophages; indeed this has been identified to be the best predictor of death from AIDS mortality or non-AIDS events (Sandler *et al* 2011). We found sCD14 levels to be similar in PNPs and age-matched uninfected children, and significantly lower than in age-matched progressor children (Fig 5.8).

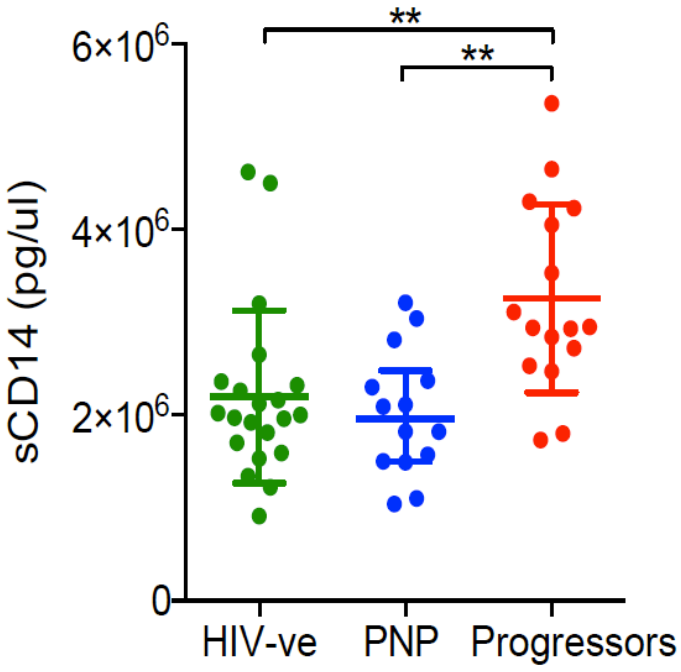


Fig 5.8. Levels of sCD14 in HIV-infected children. Comparison of sCD14 levels in HIV-negative children (n=20), PNP (n=13), and PP (n=24). P values from Mann Whitney test shown as * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

Levels of intestinal fatty acid binding protein (iFABP), a biomarker of intestinal mucosal damage, have also been used to correlate intestinal impairment and microbial translocation, and strongly predict AIDS-related mortality (Sandler *et al.* 2011; Mavinger *et al.* 2012). In our paediatric cohort we found levels of iFABP to be significantly lower in PNP compared to progressors. Unexpectedly, iFABP levels in PNP were also lower than in age-matched uninfected controls (Fig 5.9).

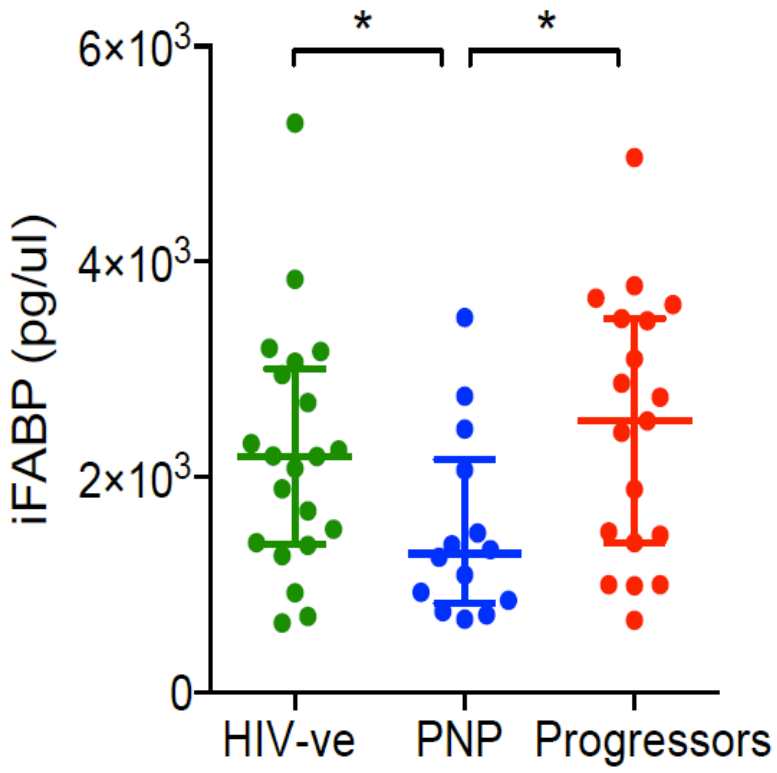


Fig 5.9. Levels of iFABP in HIV-infected children. Comparison of iFABP levels in HIV-negative children (n=20), PNP (n=14), and PP (n=17). P values from Mann Whitney test shown as * p<0.05, ** p<0.005, *** p<0.0005.

These data are consistent with a picture similar to that observed in the natural hosts of SIV infection, in which low immune activation in the face of persistent high viral loads is associated with evidence of low-level microbial translocation.

5.2.3 Loss of IL-2 responses distinguishes Paediatric Progressors from Non-Progressors

We next examined CD4 T-cell function in the children. We first evaluated, in PNP, Progressors (age-matched, CD4 count<500/ul) and age-matched uninfected children, intracellular production by CD4+ T-cells of Th1 cytokines IFN-g, IL-2 and TNF-a in response to staphylococcal enterotoxin B (SEB). IL-2 production in PNP did not differ from uninfected controls, but was significantly higher than in Progressors (Fig 5.10). IFN-g production was, conversely, significantly higher in Progressors than in the other two groups using intracellular cytokine staining (Fig 5.10).

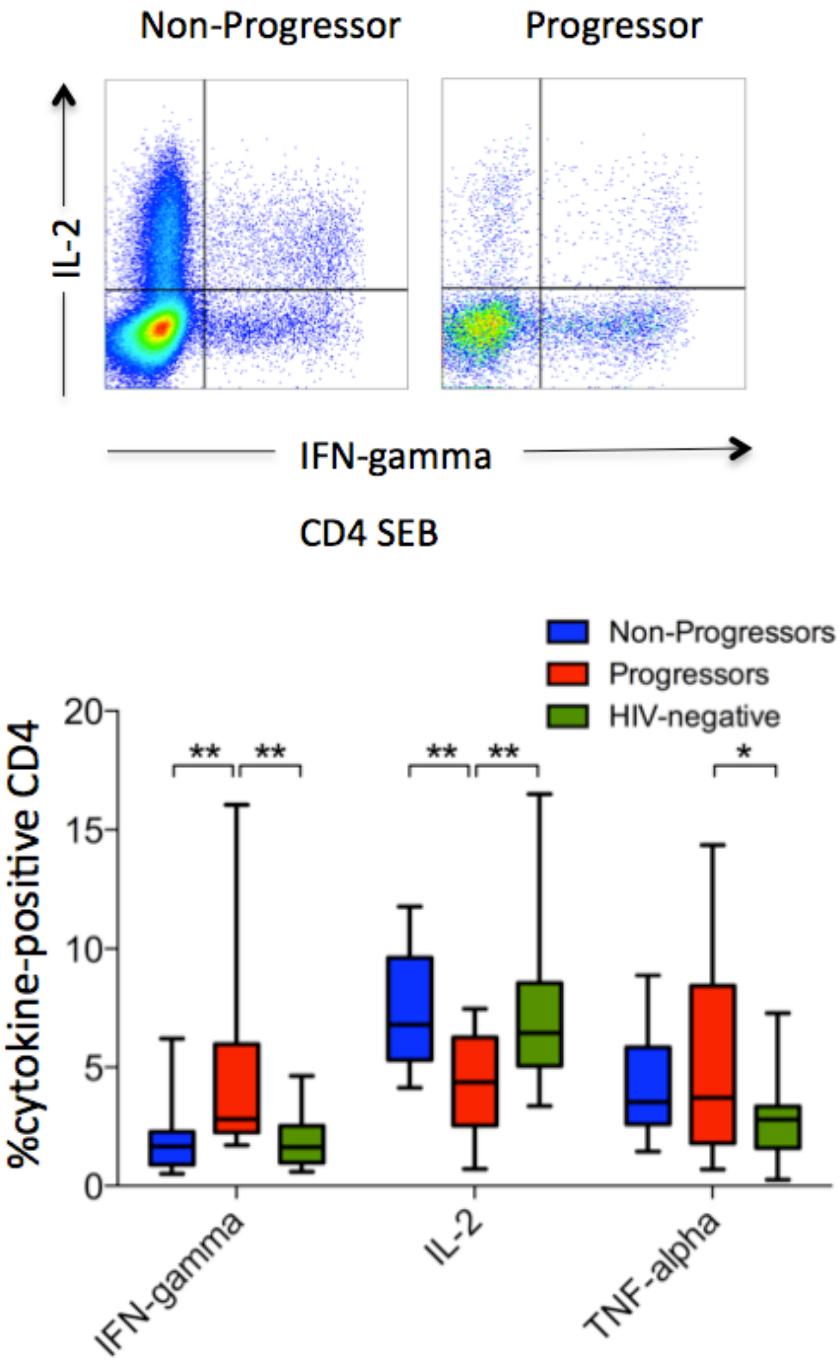


Fig 5.10. Th1 cytokine production by CD4 T-cells in response to SEB. Top panel: Representative CD4+ T-cell response upon SEB stimulation for a Non-Progressor and Progressor child. **Bottom Panel:** Increased IFN-gamma and decreased IL2 response in CD4+ T-cells upon SEB stimulation in progressors (n=18), but not in non-progressors (n=15) compared to HIV-uninfected age-matched children (n=19). P values from Mann Whitney test shown as * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

A similar pattern was observed for the CD8+ T cell response to SEB stimulation with a clear deficit in IL-2 production in paediatric progressors compared to non-progressors and HIV-uninfected children (p=0.0006 and p=0.004, respectively, Fig 5.11).

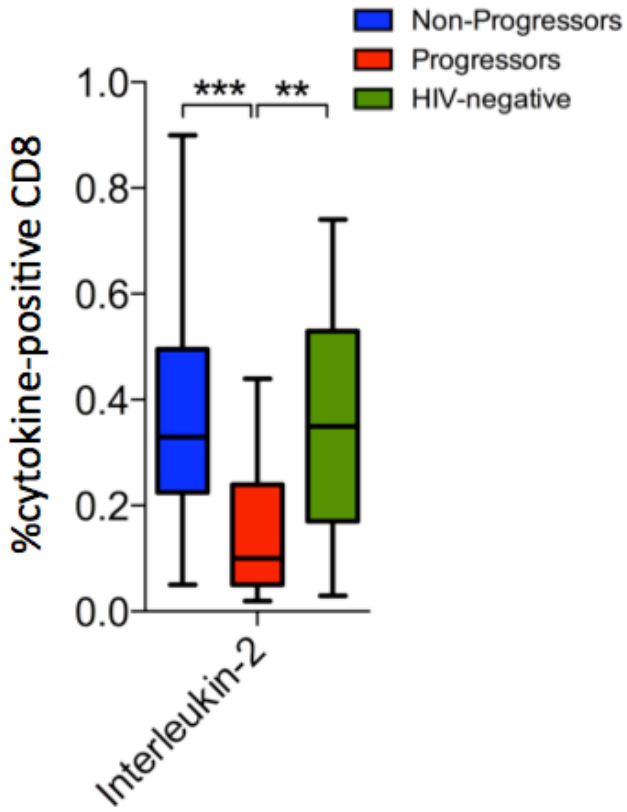


Fig 5.11. IL-2 production by CD8 T-cells in response to SEB. Decreased CD8 IL2 response upon SEB stimulation in Progressors (n=18) compared to Non-Progressors (n=15, p=0.0006) and HIV-uninfected children. P values from Mann Whitney test shown as * p<0.05, ** p<0.005, *** p<0.0005.

Interestingly, the CD8+ IFN-γ response to SEB stimulation compared to HIV-negative children was equally increased in both PNPs and PP's (Mann-Whitney test p=0.0001 and p=0.0024, respectively). Likewise, there was a marked increase in CD107a expressing CD8+ T cells upon SEB stimulation in both HIV-infected groups compared to HIV-uninfected children (Mann-Whitney test p<0.0001 for both groups), but with no statistical difference apparent between PNP's and PP's (Fig 5.12).

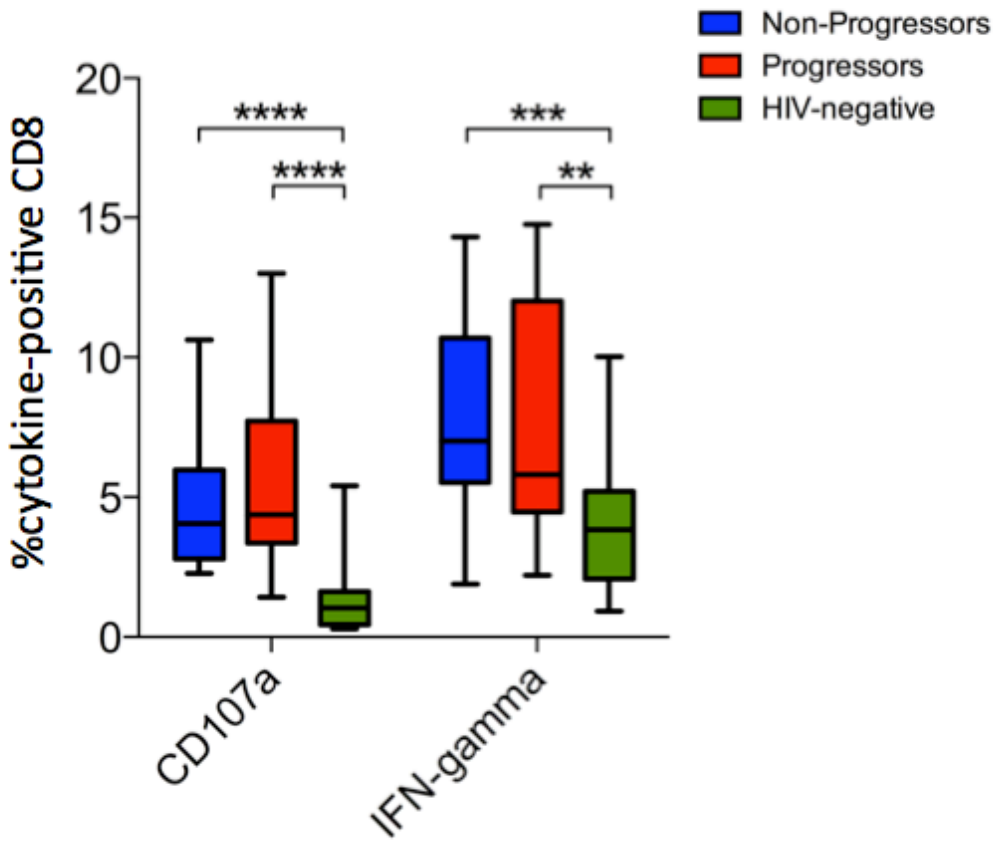


Fig 5.12. Increase in IFN-γ and CD107a expression in CD8+ T cells upon SEB stimulation in both PNP (n=18) and PP (n=15) compared to HIV-uninfected age-matched children (n=19). P values from Mann Whitney test shown as * p<0.05, ** p<0.005, *** p<0.0005.

Similarly, in response to a pool of overlapping Gag peptides, intracellular IL-2 responses in CD4+ T cells were significantly higher in PNP compared to progressors (p=0.0463, Mann-Whitney test) (Fig. 5.13) consistent with the generalized deficit in IL-2 production in progressors observed for SEB stimulation. The same patterns were observed for CD8+ T cells in response to Gag, and in CD4+ and CD8+ T-cell responses to pools of other HIV proteins including Pol and Nef (data not shown).

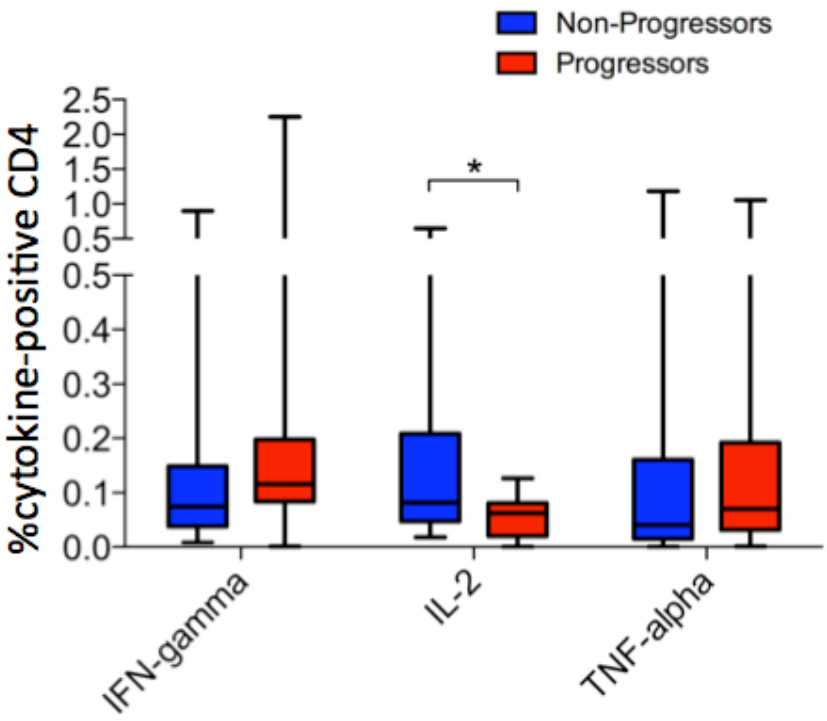


Fig 5.13: Decreased IL-2 production in Gag-specific CD4+ T-cells in progressors (n=18) compared to non-progressors (n=15); * = p=0.0463, Mann-Whitney test.

The capacity of CD4+ T cells to produce IL-2 upon SEB stimulation is positively correlated with CD4% (r=0.39, p=0.0069) and inversely correlated with viral load levels (r=-0.44, p=0.002) in all vertically HIV-infected children tested here

(n=46) (Fig. 5.14). Also for the CD4 Gag response we found a positive correlation with CD4% (r=0.040, p=0.0053) but no correlation with viral load (Fig. 5.15).

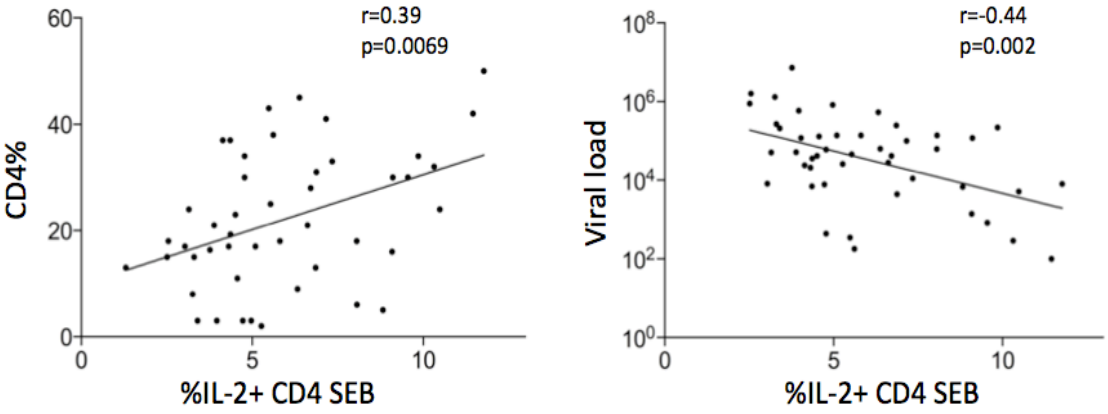


Fig 5.14 . IL-2 Production on CD4+ T cells is a marker of disease progression. Correlation between IL2-positive CD4+ T-cells upon SEB stimulation and CD4% and viral load. (r=0.39, p=0.0069, and r=-040, p=0.0059, respectively, Spearman correlations shown) (n=46).

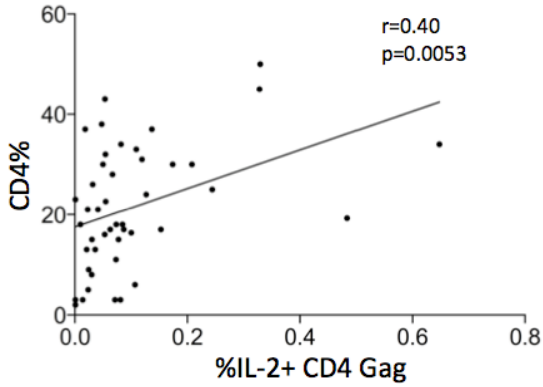


Fig 5.15. IL-2 Production by HIV-gag specific CD4+ T cells correlates with CD4%. Correlation between IL-2-positive CD4+ T-cells upon HIV-gag stimulation and CD4%. (r=0.40, p=0.0053, Spearman correlation shown) (n=46).

CD4 and CD8 proliferative activity, as measured by CFSE dilution, was substantially diminished in Progressors compared to PNP in response to Gag peptide pool stimulation (Fig. 5.16).

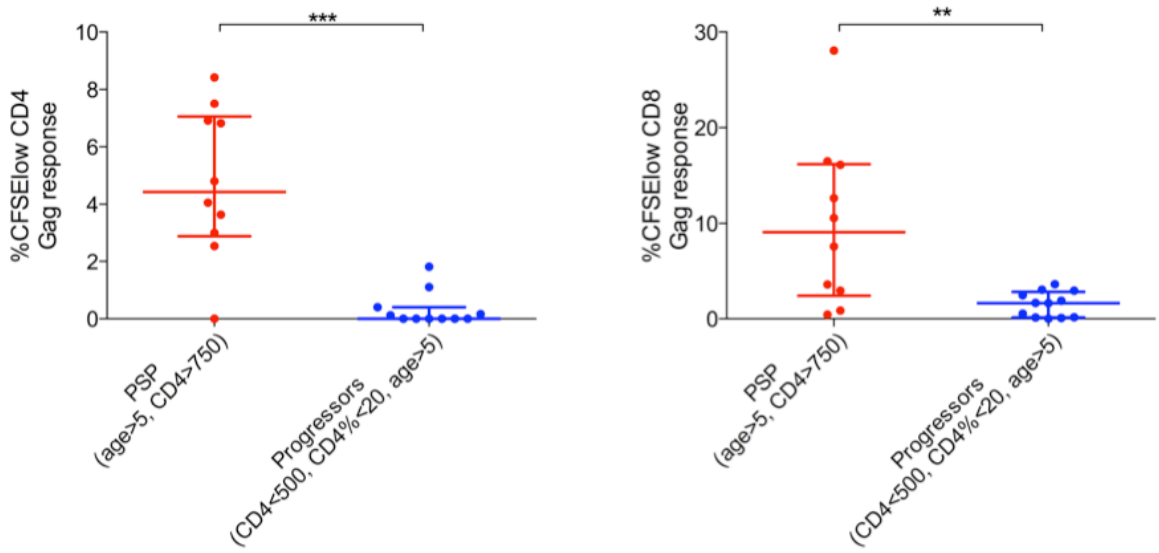
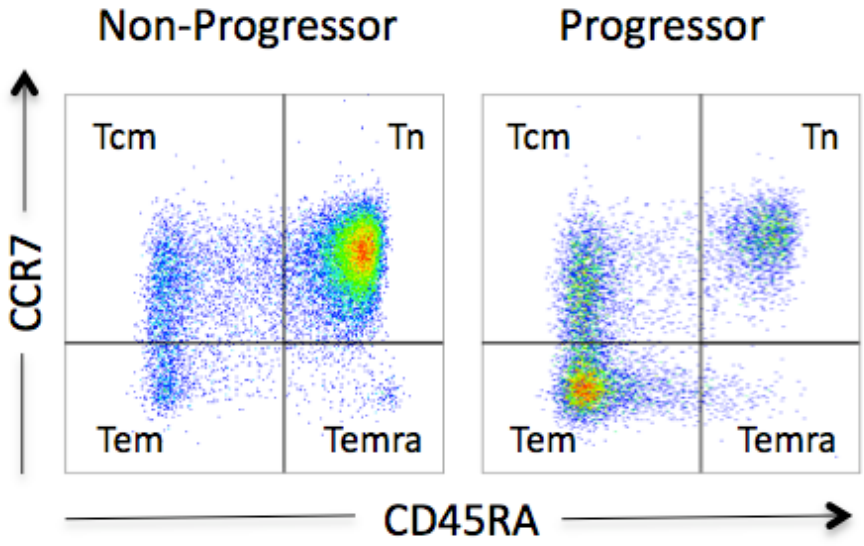


Fig 5.16. CD4 and CD8 proliferative activity is substantially diminished in Progressors compared to PNP. PNP n=10, progressors n=12, P values from Mann Whitney test shown as * p<0.05, ** p<0.005, *** p<0.0005.

These data indicate a degree of T-cell dysfunction in paediatric progressors, typical of exhaustion in the face of chronic high viral loads, and maintenance of broadly unaffected T-cell function in PNP, which would normally be unexpected in the face of persistent high antigen loads (Wherry & Ahmed 2007, Wherry and Kurachi 2015).

5.2.4 Chronic immune activation drives terminal memory differentiation in progressor children with a shift from IL-2 towards IFNγ dominated functional profiles

These functional differences between PNP and progressor children are reflected by distinct patterns of T cell differentiation. We studied T cell memory differentiation using the memory phenotype markers CD45RA and CCR7 to measure frequencies of naïve (Tn, CD45RA+CCR7+), central memory (Tcm, CD45RA-CCR7+), effector memory (Tem, CD45RA-CCR7-) and Temra (CD45RA+, CCR7-) CD4+ and CD8+ T cells in n=161 ART-naïve HIV-infected children above 5years of age. A strong correlation is observed between absolute CD4 count and the percentage of both CD4+ and CD8+ T-cells that are naïve in phenotype, and an inverse correlation with the percentage that are effector memory in phenotype (Fig 5.17).



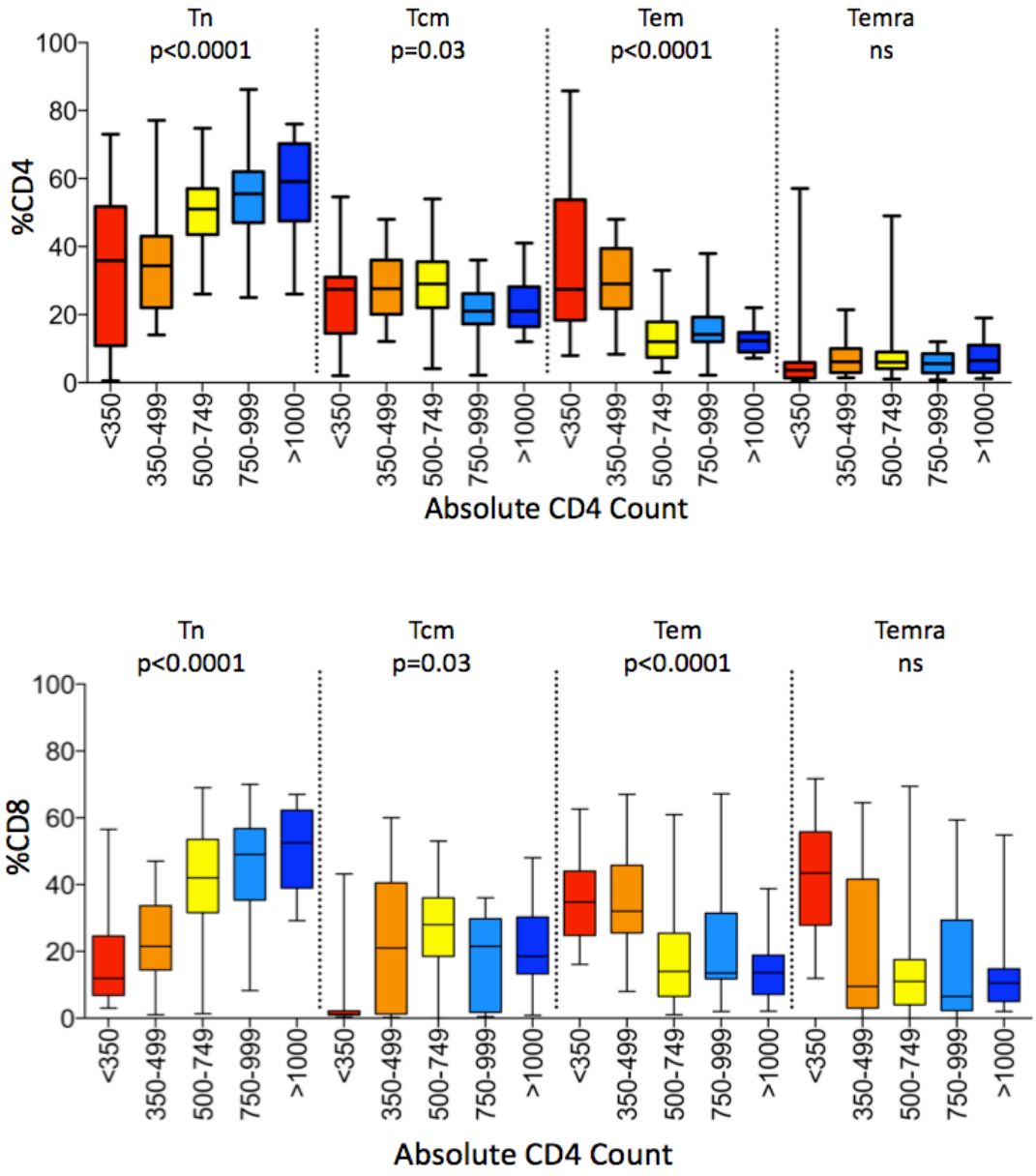


Fig 5.17. Terminal memory differentiation in the T cell compartment of Progressor children. **Top panel**: Representative FACS data for CD4 memory subsets of a progressing and a non-progressing child. **Middle Panel:** Decreased frequencies of T-naïve CD4+ T-cells and increased frequencies of effector memory CD4+ T-cells in progressor children (n=161). p values obtained from ANOVA. **Bottom Panel**: Decreased frequencies of T-naïve CD8+ T-cells and increased frequencies of effector memory CD8+ T-cells in progressor children (n=161). p values obtained from ANOVA.

Interestingly, the differentiation towards effector memory phenotypes in both compartments is more strongly correlated to immune activation than viral load levels. For example, the correlation between the frequency of Tem and activated CD4 T cells is stronger (Spearman r=0.51, p=0.0019) than the correlation with viral load (Spearman r=0.23, p=0.0035) (Fig. 5.18). Surprisingly, even in the CD8 compartment, the frequency of Tem is more strongly correlated with CD4 T cell activation (Spearman r=0.39, p<0.0001) than with viral load (Spearman r=0.25, p=0.0024) or CD8 T cell activation (Spearman r=0.28, p=0.0003). These data suggest that differentiation towards effector memory subsets is driven more strongly by CD4 T cell activation than HIV antigen levels.

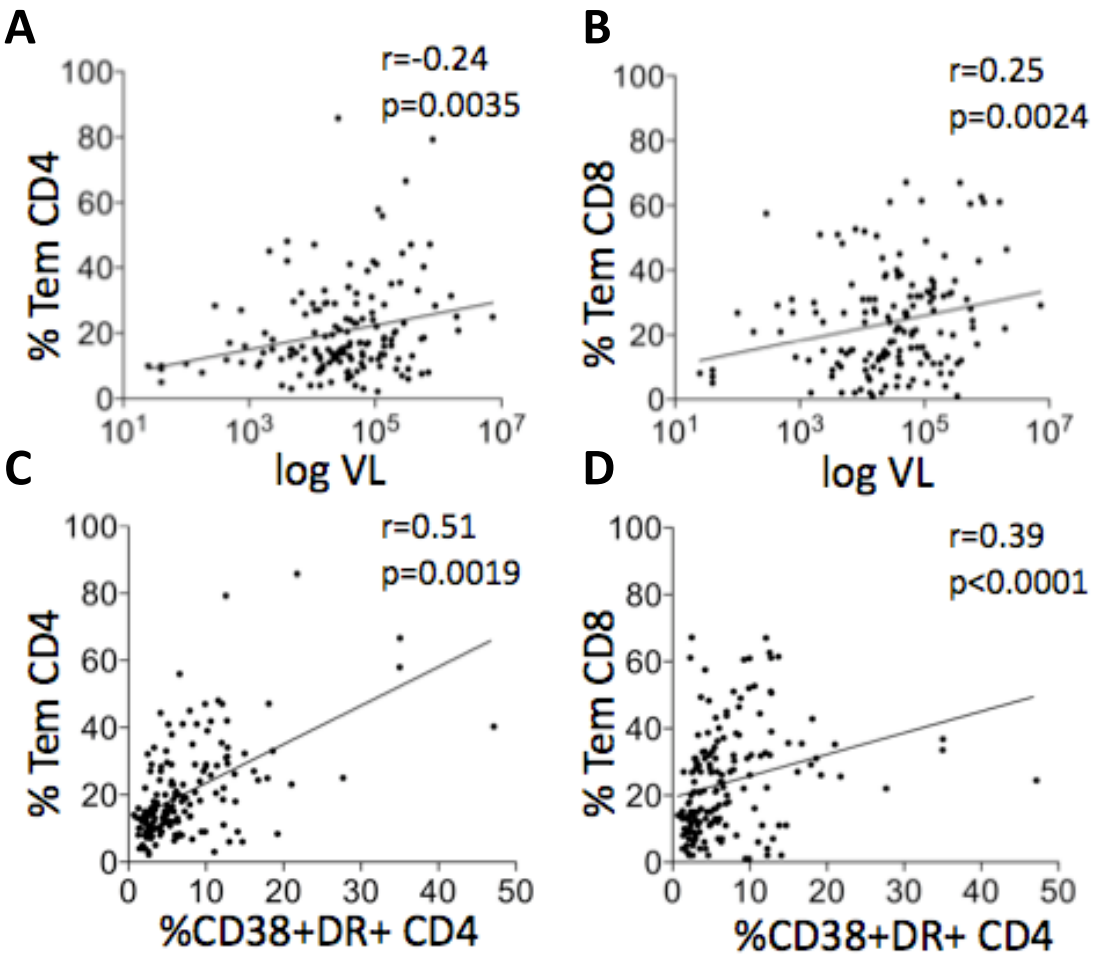


Fig 5.18. Correlations between effector memory T cell subsets, viral load and CD4+ T cell activation. Correlation between (A) % Tem CD4+ T cells and viral load, (B) % Tem CD8+ T-cells and viral load, (C) % Tem CD4+ T cells and immune activation (%CD38+ HLA-DR+) on CD4+ T-cells, and (D) % Tem CD8+ T cells and immune activation (%CD38+ HLA-DR+) on CD4+ T-cells, (n=161). Spearman correlations shown.

We next studied the functional profiles of different memory subsets in n=11 HIV-infected children. Naïve and central memory CD4 T cells almost exclusively respond with IL-2 upon SEB stimulation (Fig. 5.19). There is a clear decrease in the proportion of CD4 T cells responding with IL-2 and an increase in cells responding with pro-inflammatory cytokines IFN-γ and TNF-α for more terminally differentiated memory subsets (ANOVA p<0.0001).

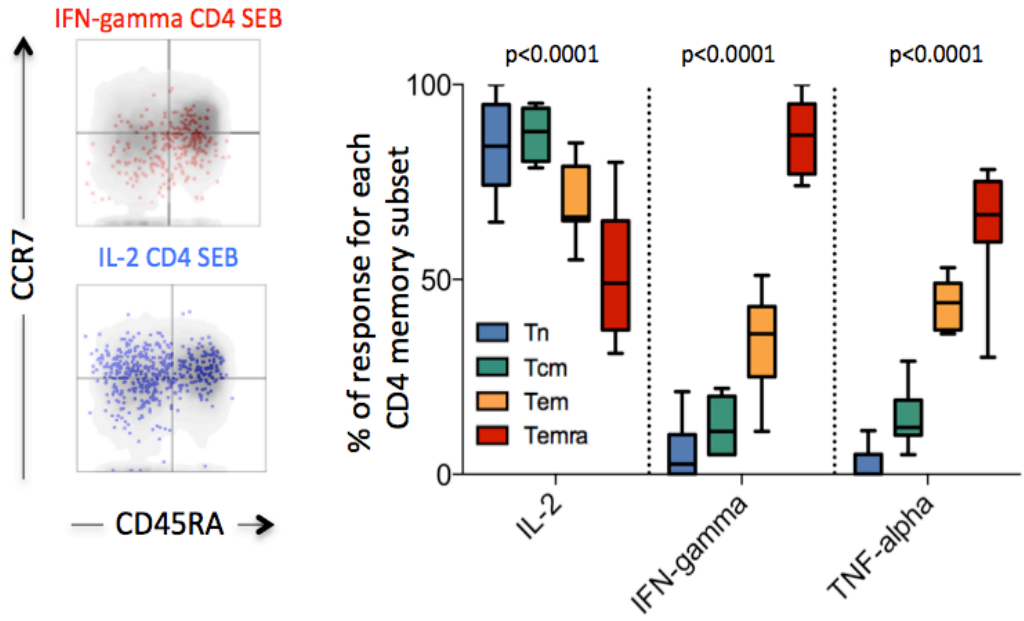


Fig 5.19. Terminal memory differentiation in the CD4+ T cell compartment of Progressor children is associated with decreased IL2-production and increased IFN-γ production upon SEB stimulation. Left hand panel: Representative FACS plots of memory phenotype of cytokine-positive CD4+ T cells after stimulation with SEB. **Right hand panel:** Functional profile of CD4 memory subset showing a decrease in IL2 and increased IFN-γ and TNF-α expression in more terminally differentiated memory subsets after stimulation with SEB. n=11, p values obtained from ANOVA

The same pattern was observed for CD8+T-cell functional profiles upon SEB stimulation (Fig 5.20) and for CD4 and CD8 T cell responses upon Gag stimulation.

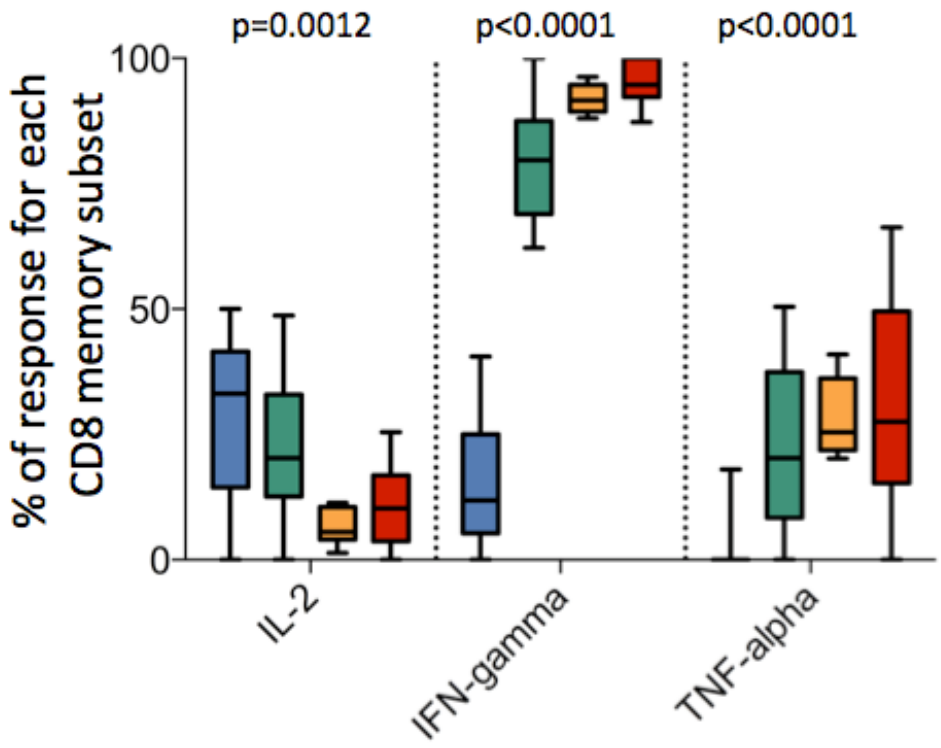


Fig 5.20. Terminal memory differentiation in the CD8+ T cell compartment of Progressor children is associated with decreased IL2-production and increased IFN-γ production. Functional profile of CD8+ memory subset showing a decrease in IL-2 and increased IFN-γ and TNF-α expression in more terminally differentiated memory subsets. n=11, p values obtained from ANOVA.

Taken together these data indicate that the observed differences in T cell function in progressor children with a deficit in IL-2 production and increased frequencies of IFN-γ producing CD4+ and CD8+ T cells are largely caused by a shift towards more terminally differentiated memory subsets. This appears to be driven primarily by T cell immune activation.

5.2.5 Immune exhaustion is driven by immune activation and discriminates progressors from non-progressors

To study the role of immune exhaustion in paediatric HIV-infection, we measured levels of PD-1-expression in HIV-infected children above 5 years of age (n=56) and HIV-uninfected age-matched controls (n=22). PD-1 expression was higher in progressors and non-progressors than in HIV-uninfected children (Mann-Whitney test $p<0.0001$ and $p=0.0006$, respectively), but there is a clear increase of PD-1 high cells in progressors compared to PNPs in the CD4+T cell populations (Mann Whitney test $p=0.0005$) (Fig. 5.21). The same pattern was observed for the CD8+ T cell compartment with higher levels of PD-1 expression in progressors and PNPs than HIV-uninfected children (Mann Whitney test $p<0.0001$ and $p=0.0011$, respectively) and higher frequencies of PD-1 high cells in Progressors than PNPs (Fig. 5.21).

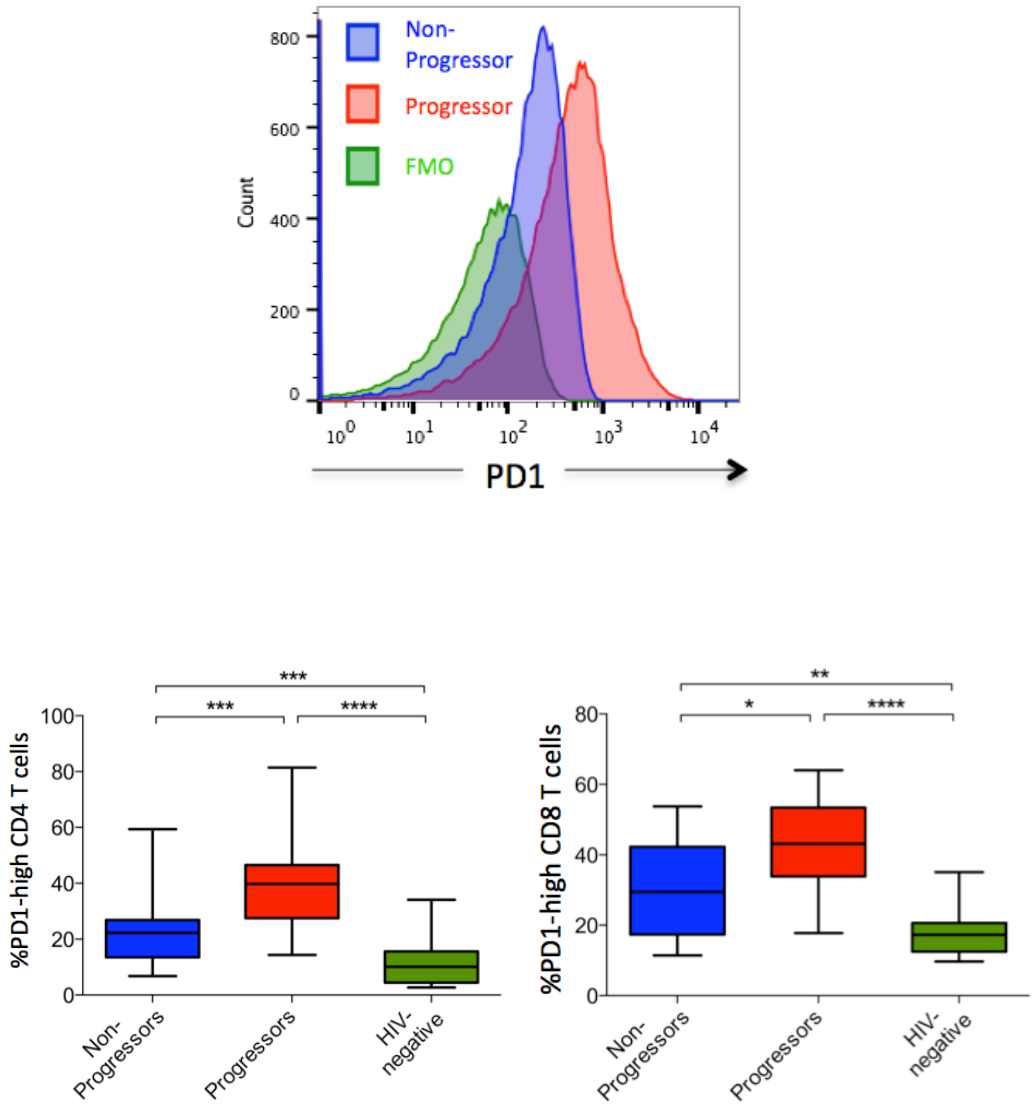


Fig 5.21. Low levels of immune exhaustion in Non-Progressor. Top panel: Representative PD-1 data on CD4+ T cells for a Non-Progressor and Progressor child. **Bottom panels:** Lower levels of PD-1 expression on CD4+ and CD8+ T cells in Non-Progressors compared to Progressors. P values from Mann Whitney test shown as * $p<0.05$, ** $p<0.005$, *** $p<0.0005$..

We next asked if the increased frequency of PD-1 high cells in progressor children is solely driven by higher frequencies of more terminally differentiated T cells that have previously been shown to express PD-1 at higher levels

(Yamamoto *et al.* 2011). We measured PD-1 expression on naïve, central, effector and effector RA memory subsets on both CD4+ and CD8+ T cells. Compared to HIV-uninfected controls, PD1 expression was higher in all memory subsets for both groups of HIV-infected children (Fig. 5.22).

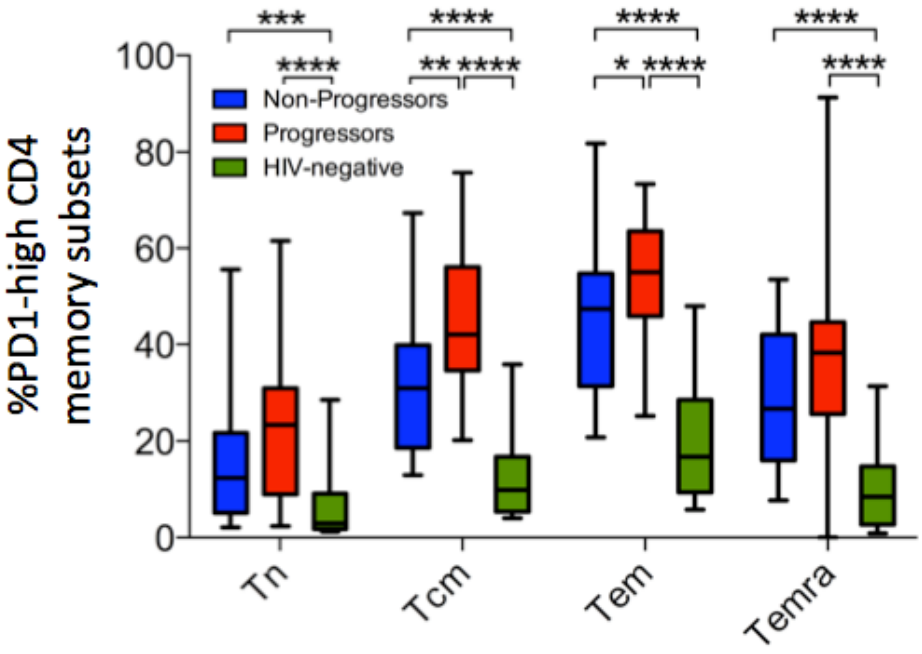


Fig 5.22 Higher levels of PD-1 expression on CD4+ Tcm and Tem in Progressors compared to PNP. P values from Mann Whitney test shown as * p<0.05, ** p<0.005, *** p<0.0005..

As expected, PD-1 expression was higher on more differentiated CD4+ T cells and therefore on CD4+ T-cells in Progressors. There was a significant increase in PD-1 expression on Tem cells in the progressors compared to the PNP (p=0.03) and most pronounced for the central memory subsets (p=0.0041). Interestingly, also in the CD8 compartment, differences in PD1 expression between

progressors and non-progressors were most pronounced for the central memory populations (p=0.026) (Fig 5.23).

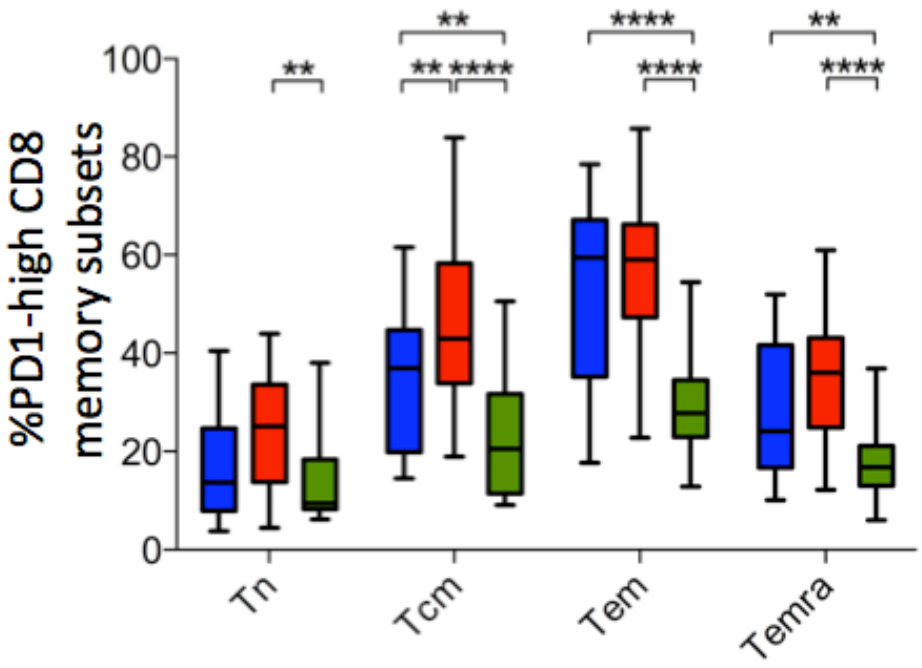


Fig 5.23 Higher levels of PD-1 expression on CD8+ Tcm in Progressors compared to PNP. Blue bars represent PNP, Red bars represent progressors, green bars represent HIV-negatives. P values from Mann Whitney test shown as * p<0.05, ** p<0.005, *** p<0.0005..

To study the impact of PD-1 expression on T cell function we next compared functional profiles of PD-1 low expressing with PD-1 high expressing cells in n=11 HIV-infected children (Fig. 5.24). The frequency of PD-1 high CD4+ T cells

responding with IL-2 upon HIV-gag stimulation was markedly lower than the frequency of PD-1 low IL-2 responding cells (Mann-Whitney test p=0.0018) (Fig. 5.24).

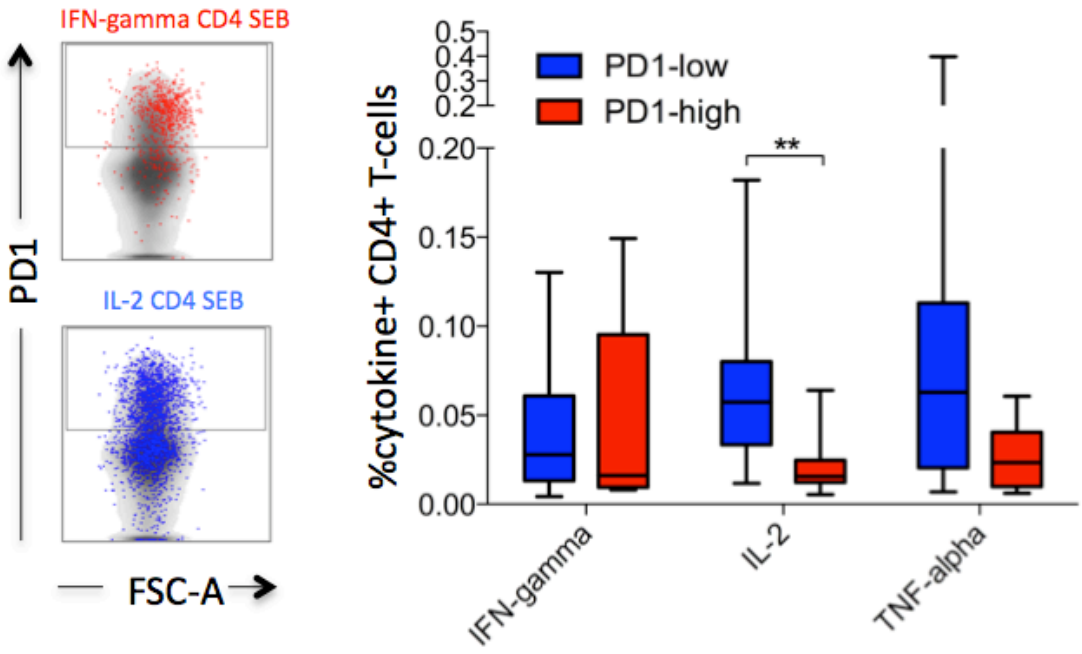


Fig. 5.24 Distribution of cytokine-positive CD4+ T cells and PD1-expression. Deficit of PD1-high Gag-specific CD4+ T cells in IL-2 production (n=11). P values from Mann Whitney test shown as * p<0.05, ** p<0.005, *** p<0.0005..

Analysis of polyfunctional profiles of Gag-specific CD4+ T cells shows that PD-1 high cells predominantly respond with IFN-γ only, and the fraction of IL2-responding cells is higher in the PD-1 low CD4+ population (Fig. 5.25).

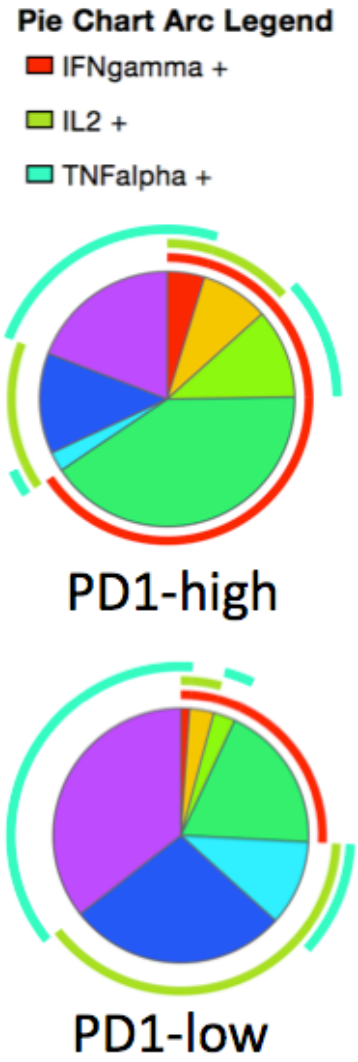


Fig 5.25 Polyfunctional profile of Gag-specific PD-1 high and PD-1 low CD4+ T cells in paediatric slow progressors. Each fragment represents the number of subjects responding to the one or more indicated cytokines. (n=11)

The same pattern was observed for SEB stimulated CD4+ T-cells with a deficit in IL-2 production in PD-1 high cells (p=0.0045) and increased frequencies of IFN-gamma (p=0.0023) and TNF alpha responding cells (p=0.0447) (**Fig. 5.26**).

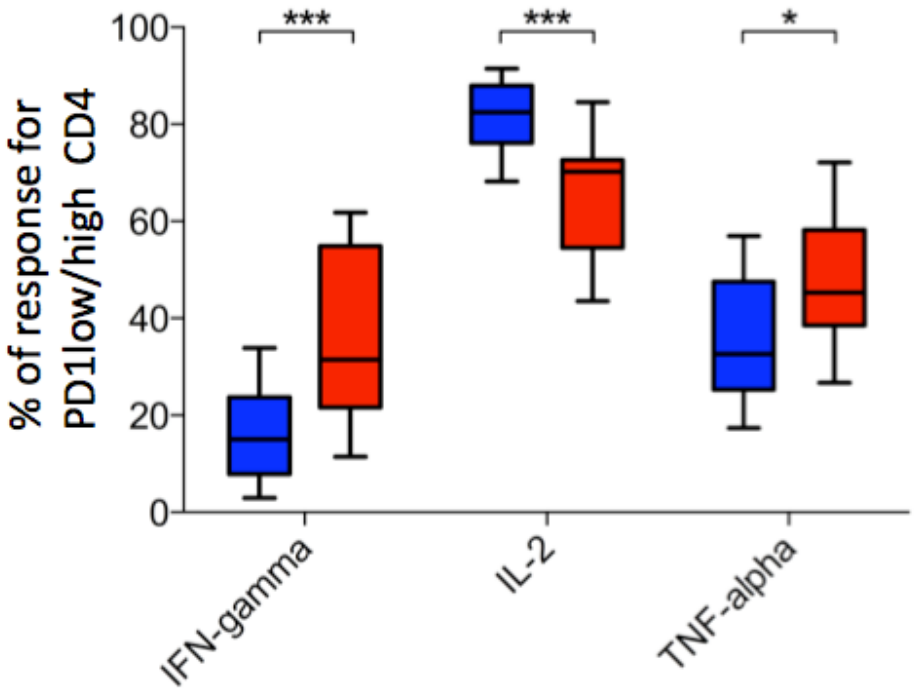


Fig 5.26 Increased IFN-γ and TNF-α and decreased IL-2 response of PD-1 high CD4+ T cells upon SEB stimulation. P values from Mann Whitney test shown as * p<0.05, ** p<0.005, *** p<0.0005.

Increased frequencies of PD-1 high CD4+ and CD8+ T cells are strongly correlated with low CD4% (r=-0.64, p<0.0001 and r=-0.40, p=0.0016 respectively) and high viral load levels (r=0.43, p=0.001 and r=0.43, p=0.0012)

(Fig. 5.27). Strikingly, however, the strongest correlation was observed between CD4+ T-cell activation and CD4+ PD-1 expression (r=0.73, p<0.0001). Of note, CD8+ T-cell PD-1 expression was more strongly correlated to CD4+ T-cell immune activation (r=0.47, p<0.0001) than viral load, but there was no correlation with CD8+ T-cell activation itself. These data suggest that immune exhaustion is more strongly driven by CD4 T cell activation than antigen levels.

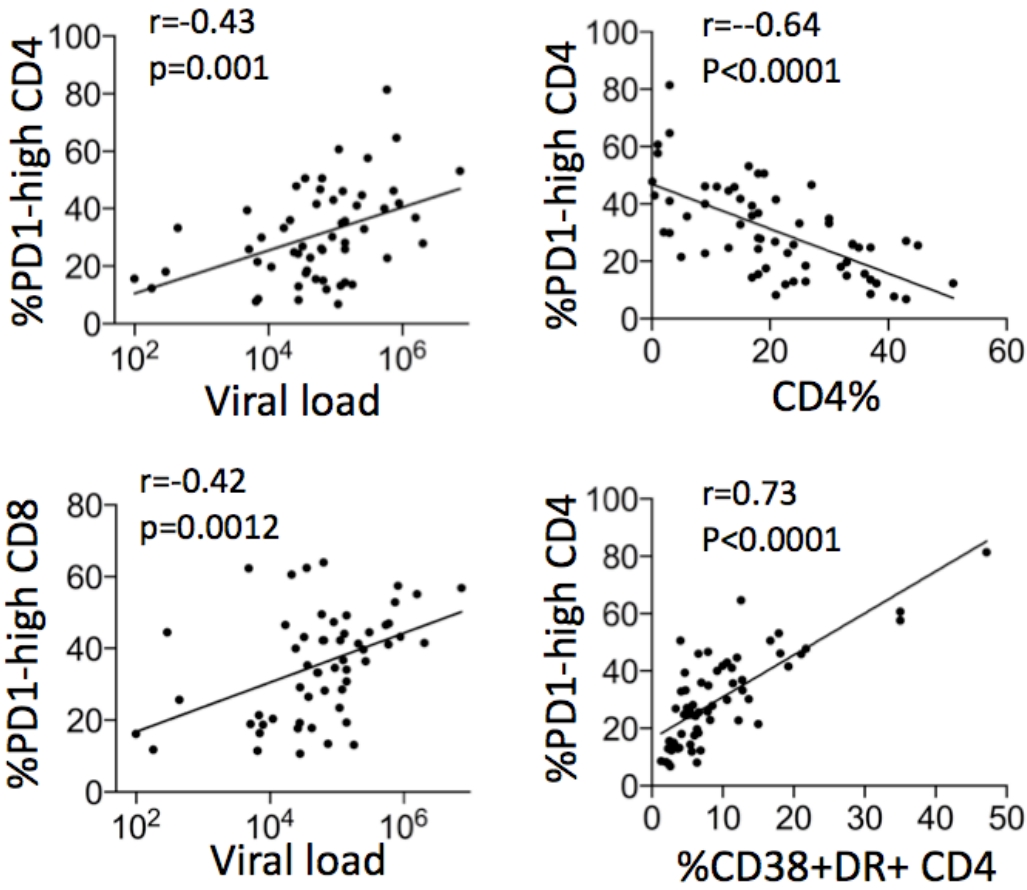


Fig 5.27 Correlation between PD-1 high expression and disease progression. Spearman correlations shown.

These findings are consistent with increased differentiation, exhaustion and dysfunction in CD4+ T-cells in the paediatric progressors, but lack of these features in PNP, whose CD4+ T-cells are comparable in terms of absolute number, differentiation and function to those of age-matched uninfected children.

5.2.6 CD4+ T-cell immune activation as the major driver of progression in paediatric HIV infection

Identification of several factors, including low immune activation, CD4+ T-cell differentiation, T-cell subset function, in CD4+ and CD8+ T-cells, in association with markers of progression or non-progression, such as absolute CD4 count and viral load, prompts the question: what is the primary driver of progression in paediatric infection? To begin to address this, a correlation matrix or 'Corrgram' was constructed (see Methods section 2.15.5) out of the multiple correlations observed between these factors (Fig 5.28). This Corrgram illustrates a clear dichotomy between factors associated with progression (upper left quadrant) and with non-progression (lower right quadrant).

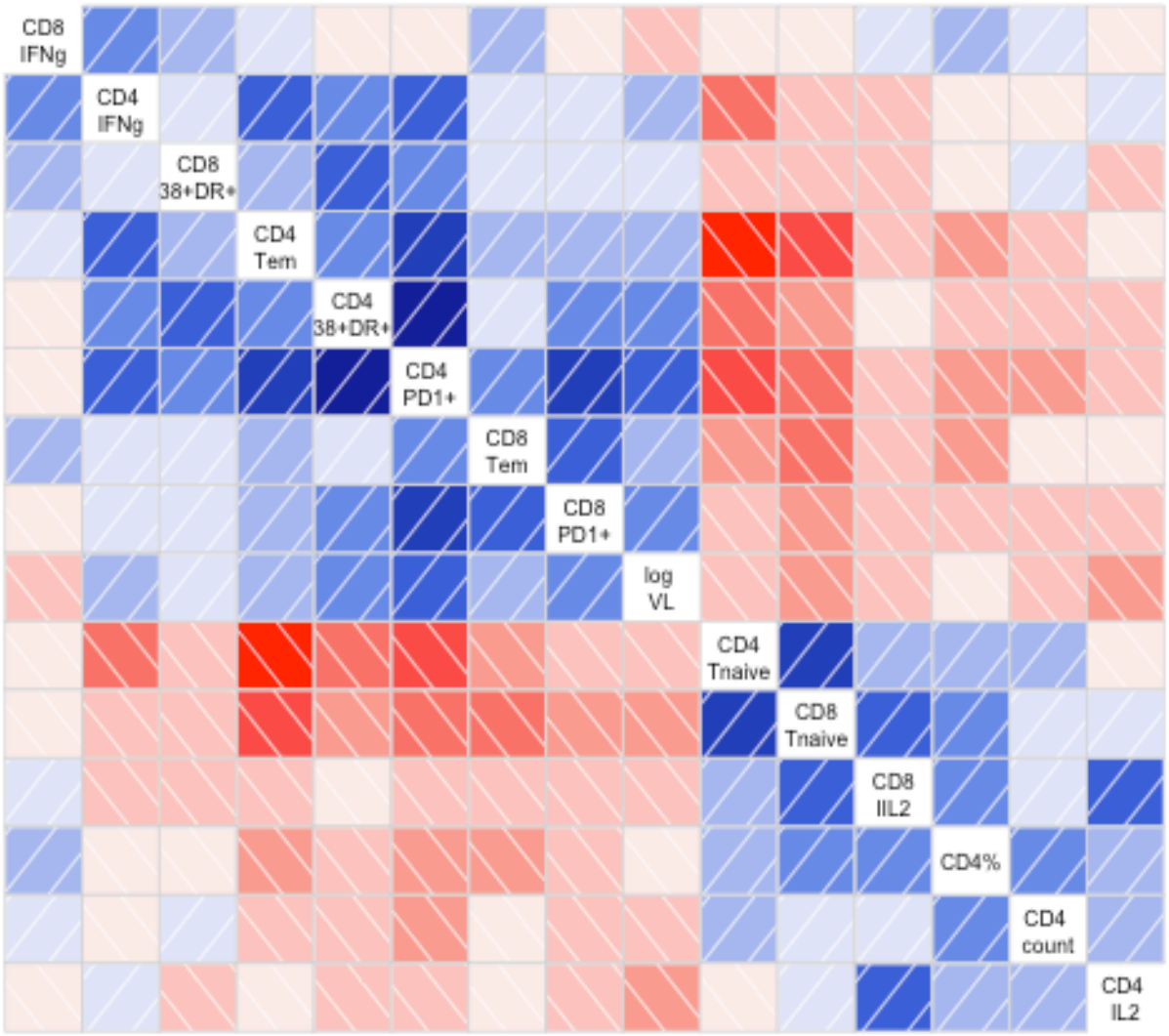


Fig 5.28. Correlation matrix of immunological variables associated with differential disease progression. Positive correlations are shown in blue, negative correlations are shown in red. Darker shades indicate stronger associations. Variables are ordered based on principal component analysis. Variables associated with disease progression such as CD4 immune activation and PD1-expression cluster together opposed to variables associated with non-progressive HIV-infection such as preserved T-naïve memory subsets and CD4 IL-2 responses. IFN-γ and IL-2 responses are shown for SEB stimulation.

To better determine the major driver of these effects linked by multicollinearity, we employed three LASSO generalized linear mixed model approaches to determine the regression parameter estimates for a selected set of covariates (Table 5.1). In each case, CD4 immune activation level (CD38/DR expression) had the strongest influence on paediatric progression, defined by decreasing CD4 count. Notably, viral load had no impact, consistent with our earlier findings of no correlation between CD4 and viral load in these HIV-infected children (Fig 5.4).

LASSO regression parameter estimates

Variable	grplasso	penalized	glmnet
CD4+ CD38+ DR+	-0.4322	-0.4305	-0.3988
CD4+ Tn	0.2479	0.2472	0.2597
CD4+ Tem	-0.1173	-0.1164	-0.0854
Age at visit	-0.1079	-0.1076	-0.1023
CD8+ Tem	0.0632	0.0619	0.0433
CD4+ Tcm	-0.0582	-0.0575	-0.0392
CD8+ PD-1+	0.0556	0.055	0.0449
CD8+ CD38+ DR+	-0.0363	-0.0361	-0.0361
Sex	-	-	-
CD4+ PD-1+	-	-	-
Viral Load	-	-	-
CD4+ Temra	-	-	-
CD8+ Tn	-	-	-
CD8+ Tcm	-	-	-
CD8+ Temra	-	-	-

Table 5.1: Selected regression parameter estimates with three LASSO approaches implemented in the *R* packages *grplasso, penalized and glmnet.*

Interestingly, in this model the only CD8 + T-cell covariates that were selected were CD8+ T cell activation, PD-1 expression and frequencies of effector memory subsets, but with a much lower effect than the CD4+ covariates. PD-1 expression on CD4+ T cells was not selected as a variable in this model, likely due to the strong multicollinearity with CD4+ T-cell activation and T-cell memory subsets. These findings were highly consistent between the three different algorithms used here.

To further validate these results we used this set of covariates selected by LASSO in an un-regularized conventional Generalized Linear Model using the R-function glm on the same dataset (**Table 5.2**). Also in this model, CD4 T cell activation is by far the covariate with the strongest impact on disease progression.

General Linear Mixed Model regression parameter estimates		
Variable	beta-coefficient	p-value
CD4+ CD38+ DR+	-0.536	$p<10^{-10}$
CD4+ Tn	0.2201	$p<10^{-10}$
CD4+ Tem	-0.2064	$p<10^{-10}$
Age at visit	-0.1221	$p<10^{-10}$
CD8+ Tem	0.1187	$p<10^{-10}$
CD4+ Tcm	-0.1084	$p<10^{-10}$
CD8+ PD-1+	0.0871	$p<10^{-10}$
CD8+ CD38+ DR+	-0.0341	p=0.0006
Sex	-	-
CD4+ PD-1+	-	-
Viral Load	-	-
CD4+ Temra	-	-
CD8+ Tn	-	-
CD8+ Tcm	-	-
CD8+ Temra	-	-

Table 5.2: Standardized regression parameter estimates for the selected set of covariates by an un-regularized conventional General Linear Mixed Model using the R-function glm.

Taken together these data support a model of HIV pathogenesis with CD4+ T-cell immune activation as a hallmark of disease progression driving CD4+ T-cell

depletion, terminal memory differentiation and functional immune exhaustion. Non-pathogenic paediatric HIV infection is marked by low levels of immune activation despite persistently high viraemia, resembling the phenotype of non-pathogenic SIV-infection in its natural hosts. The immune-regulatory mechanisms underlying this lack of immune activation despite constantly high antigen levels in PNPs will need to be further elucidated. Insights in the pathways involved in dampened immune activation in PNPs could elucidate novel targets for pharmacological and immunological interventions to reduce HIV-disease driven by chronic immune activation in a large population of people living with HIV well beyond HIV-infected children alone.

5.2.7 High titre, broadly neutralizing antibodies in PNP

The observation that PNP have normal or near-normal CD4 T-cell numbers and function, despite persistent high viral loads, suggested the hypothesis that they might also have broadly neutralising antibody (bNAb) responses, since bNAbs are associated with long-term high level viraemia in adult HIV infection. In contrast to PNPs, however, adults with high viral loads and bNAbs have rapidly declining CD4 numbers and function. We initially studied a mother-child pair followed from the child's birth. The CD4 and viral load data of the PNP child are shown in Fig 5.1.

PIDs	Timepoint	ID50																								
		SUBTYPE C									SUBTYPE B						SUBTYPE A							MuLV	% Neutralised
		ConC	Du151.2	Du156.2	Du172.17	CAP45.G3	ZM197M	CAP239.G3	CAP256.SU	ZM214M	PVO.4	6535.3	TR0.11	WITO.4160	AC10.0.29	QH0692.42	Q259.d2.17	Q23.17	Q168.a2	BG505	Q842.d12	Q769.d22	Q461.e2		
A2-43-517 (SK-459)	04/05/05	56	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	5%
A2-43-517 (SK-459)	03/07/06	107	40	40	40	40	40	46	40	40	40	91	40	40	40	40	355	40	40	40	40	40	40	40	18%
A2-43-517 (SK-459)	23/10/07	780	63	190	40	40	73	48	104	40	40	540	132	40	40	82	863	40	40	40	40	40	40	40	41%
A2-43-517 (SK-459)	10/07/08	212	40	152	40	40	60		40	40	40	356	79	40	40	75	309	40	60	40	40	40	40	40	36%
A2-43-517 (SK-459)	07/04/09	148	55	204	560	40	45	532	40	40	40	281	75	40	40	125	198	40	85	40	40	40	40	40	50%
A2-43-517 (SK-459)	06-Oct-09	175	445	1173	594	20	157	8349	354	20	20	275	231	20	20	20	205	58	66	20	76	20	20	<20	59%
A2-43-517 (SK-459)	12/01/10	297	1176	3445	353	406	257	10721	445	40	40	309	541	40	40	105	157	40	42	40	42	40	40	40	64%
A2-43-517 (SK-459)	05/07/11	3628	3267	8432	211	8840	1037	13568	933	763	204	350	2189	80	119	452	2200	1121	228	736	2986	2300	342	40	100%
A2-43-517 (SK-459)	12/07/12	1819	1869	3625	92	4548	565	4809	428	533	190	255	880	501	71	163	1332	1174	344	816	2286	1806	444	40	100%
A2-43-517 (SK-459)	19/08/13	3764	1121	6055	279	5561	747	7220	618	894	255	851	1163	474	65	626	1999	2317	1498	1871	8992	2768	989	40	100%
A2-43-517 (SK-459)	19-Aug-13	1221	1255	2740	261	1061	783	7538	726	282	400	923	1031	165	20	239	976	1092	1449	1697	2961	956	459	<20	95%
A2-43-517 (SK-459)	18/08/14	3807	988	4032	622	25754	701	5045	550	953	291	1324	1476	540	98	755	1118	1804	970	1226	3983	2540	671	40	100%

Child

P5-517 (SK-462)	04/04/05	21090	1677	458	138	2846	52	40	405	40	40	696	200	863	142	92	5357	14686	40	695	392	40	40	40	68%
P5-517 (SK-462)	03/07/06	10039	1042	437	504	4948	76	40	309	164	40	734	188	1094	190	47	3654	11593	73	458	238	40	40	40	82%
P5-517 (SK-462)	24/07/07	14288	1186	455	548	8209	129	40	349	40	47	490	130	797	254	40	3734	9753	126	293	367	40	63	40	91%
P5-517 (SK-462)	12-Jan-09	18533	3258	576	344	3373	166	101	1071	146	170	368	223	330	268	20	17340	11127	905	1377	1498	209	62	<20	95%
P5-517 (SK-462)	07/07/09	17834	2268	1109	442	5217	399	40	1387	151	98	573	221	433	486	93	6706	13954	623	763	2021	810	40	40	95%
P5-517 (SK-462)	13/07/10	31192	2900	2814	426	2640	176	53	2414	40	220	716	199	454	517	99	13543	21860	1318	2064	3465	1064	114	40	82%
P5-517 (SK-462)	19/08/13	18608	2196	3014	520	35570	366	180	2461	2402	201	1058	265	1260	656	285	17488	28179	1698	2225	7261	2381	97	40	100%
P5-517 (SK-462)	19-Aug-13	18409	1829	1872	422	5954	385	132	1686	1919	198	350	342	295	418	20	16110	17059	2192	3173	3294	691	20	<20	91%
P5-517 (SK-462)	18/08/14	15023	1926	3678	348	11323	181	273	1738	939	137	1061	273	914	614	243	18704	18207	1061	1845	4429	783	141	40	100%

Mother

Table 5.3 Mother and Child 517 Neutralization activity. Antibody neutralization activity of a paediatric non progressor (517) and his mother.
Neutralization was measured as a reduction in luciferase gene expression after a single round of infection of JC53bl-13 cells, with a multi-subtype panel of 16 Env-pseudotyped viruses (Montefiori *et al.* 2004) with breadth defined as neutralization of ≥50% of heterologous viruses. Colour coding represents: white – neutralisation titre <40; pale yellow: titre 40-99; light orange: titre 100-999; dark orange: 1000-9999; red titre: >10,000.

The mother also expresses the same viraemic non-progressor phenotype, being ART-naïve for >17years in spite of a viral setpoint of 85,000 copies/ml, maintaining CD4 counts at a median of 665 cells/mm^3 (IQR 588-728 cells/mm^3) over the 10-year period she has been followed. Plasma from both mother and child neutralised 100% of a panel of tier-2/3 clade A, B and C viruses (Table 5.3) and in both cases at unusually high titres of >1:10,000 in a number of instances. The ability of plasma from the child to neutralise increased from birth to broadly neutralise (defined as >50% of a panel of 22 viruses neutralised) at approximately 4yrs of age (Fig 5.29).

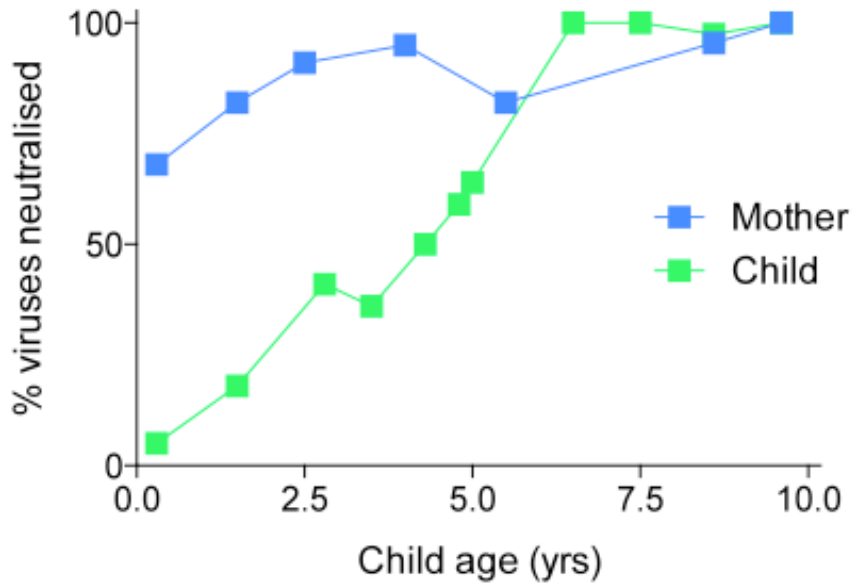


Fig 5.29 Neutralisation ability of mother and child 517. Antibody neutralization activity of PNP child and mother over the first 10 years of the childs life. Child age (years) shown on x axis, % viruses neutralized on y axis. Neutralisation defined as >50% Env-pseudotyped viruses neutralized (n=22 viruses).

We next extended these studies to test plasma from 67 additional PNPs against the same panel of viruses (Table 5.4). Overall, 85% of PNPs showed broadly neutralising activity, again in many cases with very high neutralisation titres. In contrast, only 20% of plasma samples from a cohort of 40 chronically infected South African adults were broadly neutralising, a figure consistent with that widely quoted in the literature. As expected, the breadth of virus neutralisation correlated with viral load in the PNP (Fig 5.30). These data demonstrate that the magnitude and frequency of broadly neutralizing antibodies in non-progressing children is considerably higher than in chronically infected adults.

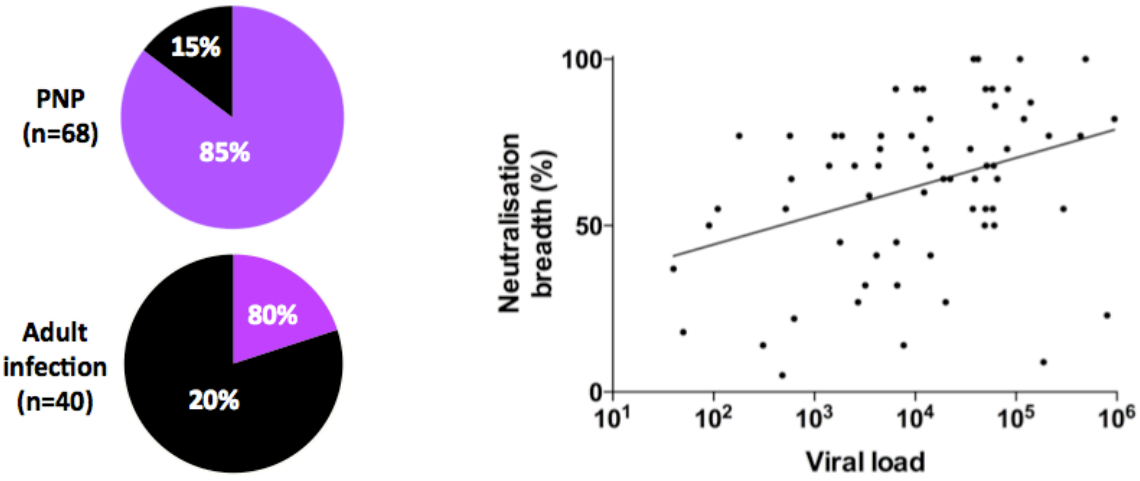


Fig 5.30 The magnitude and frequency of bNAb in PNP is higher than that of adults. **Left hand panel**: Comparison of subjects showing broadly neutralizing activity, PNP n=68, Adult n=40. **Right hand panel**: Neutralisation breadth correlates with viral load in PNP. Spearman correlation shown.

PIDs	ID50																						
	SUBTYPE C									SUBTYPE B						SUBTYPE A						
	ConC	Du151.2	Du156.2	Du172.17	CAP45.G3	ZM197M	CAP239.G3	CAP256.SU	ZM214M	PVO.4	6535.3	TR0.11	WITO.4160	AC10.0.29	QH0692.42	Q259.d2.17	Q23.17	Q168.a2	BG505	Q842.d12	Q769.d22	Q461.e2
A2-43-517 (SK-459)	3807	988	4032	622	25754	701	5045	550	953	291	1324	1476	540	98	755	1118	1804	970	1226	3983	2540	671
203-33-0004-1	1573	219	389	446	1011	345	538	698	166	74	119	201	2751	465	52	537	1639	324	193	441	118	52
203-33-0009-1	1081	1564	309	313	1060	734	391	639	92	170	54	239	186	81	57	570	83	290	73	194	87	43
203-33-0014-1	1792	1183	1822	664	426	1178	472	244	341	81	97	129	20	20	56	1062	82	20	86	20	45	20
203-33-0032-1	440	159	348	343	233	258	286	692	243	111	68	158	115	20	20	240	242	75	213	181	45	20
203-33-0011-1	139	80	53	186	221	266	78	20	187	155	62	102	143	20	20	704	70	103	49	53	20	61
203-33-0008-1	180	112	151	144	178	204	54	20	126	131	20	76	59	20	20	179	94	83	54	64	20	89
203-33-0002-1	1033	533	66	274	79	194	144	159	20	90	113	798	127	20	20	338	157	206	75	50	20	20
C2-47-385	267	161	152	262	319	338	49	63	20	96	85	20	46	20	20	129	83	48	92	20	20	20
A1-07-133	169	84	112	113	81	20	91	62	20	85	62	20	296	20	20	45	20	68	20	20	20	20
203-33-0039-1	325	103	103	153	20	20	553	63	20	266	116	20	20	20	20	96	99	51	339	20	20	20
203-33-0007-1	80	74	48	221	556	239	100	77	154	115	20	20	20	20	20	80	20	114	20	20	20	20
B1-63-021 (SK-467)	133	104	64	102	142	20	80	20	57	20	51	20	20	20	20	91	180	20	65	20	20	20
203-33-0024-1	120	201	125	20	164	146	155	20	75	120	20	20	20	20	20	60	80	20	20	20	20	20
203-33-0031-1	97	355	156	177	20	20	174	111	20	20	93	20	20	20	20	97	230	20	87	20	20	20
K017C	14402	1048	247	112	659	329	458	741	94	91	255	279	84	53	60	>540	501	40	241	155	191	40
K022C	391	720	40	144	194	121	81	1200	197	40	149	82	71	40	40	>540	81	40	40	40	129	40
K031C	801	398	295	213	583	5177	165	205	218	102	656	660	277	152	88	379	940	291	318	706	110	97
K039C	136	40	40	40	105	40	41	40	40	47	80	40	40	40	40	39	40	40	40	40	40	40
K040-C	840	318	95	110	1486	414	982	190	188	83	82	161	40	40	40	>540	301	489	347	370	423	40
K043C	791	294	119	69	88	40	357	160	223	40	296	689	40	40	40	132	182	40	75	40	40	40
K044C	616	40	40	40	40	40	41	40	40	40	120	40	40	40	40	95	77	40	133	54	40	40
K046C	2240	210	840	93	132	252	807	915	114	40	5021	731	40	219	40	1419	1710	40	64	52	40	40
K049C	100	40	40	40	40	40	40	40	40	40	40	40	40	40	40	23	40	40	40	40	40	40
K050C	191	40	110	61	54	40	208	85	40	40	141	40	40	40	40	>540	104	40	40	40	40	40
K054C	2870	121	74	75	844	104	623	192	246	46	293	115	597	204	40	>540	724	55	334	143	123	40
K060C	7127	912	65	40	163	116	152	91	86	40	432	87	409	63	40	>540	496	40	49	40	40	40
K061C	2464	271	64	49	179	191	457	219	239	93	57	171	196	40	42	>540	162	40	202	40	40	40
K062C	796	91	65	40	5908	57	399	124	221	40	63	120	920	225	40	>540	1009	251	395	167	40	40
K064C	1054	65	40	46	3659	110	561	872	40	40	55	40	1695	45	40	1729	67311	436	92	612	40	40
K065C	747	40	113	40	155	96	107	134	40	40	446	41	40	40	40	>540	543	40	49	40	40	40
K067C	602	869	224	82	1152	231	251	191	341	40	191	40	207	40	40	>540	494	40	48	212	40	40
K068C	1078	385	207	80	40	182	265	310	40	64	326	280	40	190	40	>540	436	87	117	40	40	40
K072C	72	40	40	40	40	45	163	40	40	40	40	40	40	40	40	160	40	40	40	40	40	40
K074C	888	432	591	269	320	102	390	237	102	78	122	126	225	41	40	>540	84	88	192	90		40
K082C	899	285	600	660	404	659	90	81	126	238	819	235	224	120	113	369	66	40	294	40		40
K083C	928	521	1309	119	430	102	90	59	181	40	66	40	40	40	40	108	487	40	40	40		40
K084C	795	2435	4509	202	2629	337	269	68	242	49	109	52	192	58	88	>540	288	40	110	40		40
K096C	2511	1239	888	119	179	115	440	775	40	40	1061	368	40	223	40	1808	2480	136	382	40		40
K097C	3901	403	412	145	1364	100	266	169	157	40	447	53	40	157	122	>540	338	40	156	40		40
K103C	2868	2426	512	789	3415	203	600	566	281	40	3508	283	17319	281	525	40	17511	40	684	40		40
K112C	186	40	192	40	220	40	66	234	40	40	40	129	40	40	76	167	124	40	52	40		40
K117C	1978	167	197	82	833	148	266	551	40	40	1068	76	40	106	44	>540	537	40	700	213		40
K120C	>87480	227	600	186	112	97	107	959	40	85	308	73	40	40	40	>540	946	40	576	157		40
K137C	2014	176	215	78	4154	125	662	1842	40	40	623	40	720	72	40	10598	>87480	686	368	5763		40
K138C	11003	40	388	329	358	519	204	819	72	40	4437	227	209	170	74	975	3075	40	177	40		40
K146C	1722	161	77	305	893	309	122	131	182	113	590	73	40	40	40	>540	40	40	40	40		40
K155C	1145	1859	3620	261	24330	40	211	331	40	40	661	86	80	46	40	935	8614	40	1713	501		40
K170C	898	328	252	53	242	89	158	303	40	40	246	60	85	40	40	109	314	40	40	40		40
K172C	1511	78	405	144	17007	69	114	100	384	343	180	42	40	40	139	453	176	164	197	295		79
K181C	4594	479	292	40	251	161	91	349	40	40	70	40	40	40	40	>540	583	40	81	40		40
K186C	2337	60	40	95	22147	218	40	40	40	40	107	44	833	42	40	1150	1009	40	40	40		40
K190C	40	40	294	40	136	40	141	70	40	40	233	40	40	40	118	354	40	40	87	40		40
K193C	12999	814	329	157	230	40	392	837	65	40	1723	266	40	390	106	3888	2290	40	445	51		40
K3013C	3161	715	446	222	668	843	154	263	40	133	412	299	199	88	393	>540	235	40	130	44		40
K3015C	1006	682	123	408	248	169	60	111	44	104	47	120	139	90	40	423	40	40	46	40		40
K3016C	224	73	40	40	151	40	58	40	40	40	40	40	40	40	40	>540	40	40	40	40		40
K3042C	119	40	40	40	123	40	40	40	40	40	40	40	40	40	40	99	40	40	40	40		40
K3044C	1004	40	40	40	40	40	40	40	40	40	40	40	46	40	40	289	40	40	40	40		40
K3051C	408	1148	90	88	576	84	127	93	126	40	40	40	40	40	40	274	295	40	223	69		40
K3054C	1083	611	531	200	40	55	82	313	40	40	139	165	40	92	40	148	133	40	74	40		40
K3056C	569	481	42	40	40	40	45	267	40	40	40	40	40	232	40	223	156	40	40	40		40
K3076C	101	40	40	40	40	40	40	40	40	40	40	40	40	40	40	301	50	40	40	40		40
K3092C	2890	188	674	40	197	152	1217	476	40	40	657	130	40	41	54	402	1055	40	301	40		40
GOS-033-C	1026	53	227	118	93	210	103	77	40	40	42	76	40	40	50	219	40	40	40	40		40
GOS-012-C	3224	1074	589	110	440	121	77	66	40	359	108	88	40	696	40	>540	40	77	149	40		40
GOS-041-C	298	40	40	40	40	40	118	40	40	40	74	40	40	40	40	56	40	40	40	40		40
GOS-038-C	528	40	78	40	40	40	133	49	40	40	40	49	40	40	40	43	40	40	40	40		40

Table 5.4 67 PNP Neutralization activity. Neutralization measured as a reduction in luciferase gene expression after a single round of infection of JC53bl-13 cells, with a multi-subtype panel of 16 Env-pseudotyped viruses. Breadth defined as neutralization of ≥50% of heterologous viruses. Colour coding represents: white – neutralisation titre <40; pale yellow: titre 40-99; light orange: titre 100-999; dark orange: 1000-9999; red titre: >10,000.

We finally examined the question of whether these high frequency and high magnitude bNAbs were dependent upon the maintenance of high CD4 counts in PNP or whether these levels of bNAbs are also evident in PNPs whose CD4 counts have declined. We identified 25 of these progressing children and compared neutralising antibody responses in this group with those an age-matched subset of 23 subjects in the PNP group whose viral loads also matched. We observed no statistical differences in the breadth of viruses neutralized in the two groups (not shown), suggesting that the bNAbs do not prevent CD4 decline but rather, as in adult HIV infection, are driven by persistent high levels of viraemia.

5.3 DISCUSSION

The mechanisms by which disease-free infection is achieved in HIV-infected elite controllers and in the natural hosts of SIV infection, such as the sooty mangabey and African green monkey, are diametrically opposed. Elite controllers suppress HIV replication to undetectable levels via immune responses mediated principally by protective HLA class I molecules such as HLA-B*27 and HLA-B*57. In contrast, non-pathogenic SIV infection is independent of virus-specific immunity, and viral replication continues unabated but without ill consequences. These current studies of non-progressing paediatric HIV infection demonstrate that, although – in contrast to the sooty mangabeys - strong virus-specific immune activity is present, it does not contribute to the maintenance of normal CD4 counts and absence of disease, consistent with observations in the natural hosts of SIV. The normal-for-age CD4 counts and low immune activation despite

persistent high viral loads seen in these non-progressing children are also characteristic of SIV infection in African non-human primates. In both cases CD4 count is unrelated to viral load.

Immune activation is central to the pathogenesis of HIV infection in both adults and children and predicts disease progression better than viral load (Bofill *et al.* 1996; Giorgi *et al.* 1999; Deeks *et al*. 2004; Paul *et al*. 2005). In this cohort, progressing children displayed higher levels of immune activation than non-progressing children and intriguingly only in the progressing children did immune activation correlate significantly with viral load. The dissociation between the degree of HIV viraemia and immune activation in the non-progressing children is consistent with previous studies in children (Resino *et al.* 2006; Ssewanyana *et al.* 2007; Prendergast *et al*. 2011) but is in contrast to HIV infected adults where strong correlations between immune activation and HIV viraemia exist. This finding may explain the poor predictive value of viral load in children where there is considerable overlap in levels of viraemia between rapid and slow progressors (Shearer *et al*. 1997).

The findings of low immune activation with normal CD4 counts, despite persistent high viraemia present a picture similar to that observed in the natural hosts of SIV infection. We also observed decreased levels of microbial translocation in PNP compared to that of progressing children. Unexpectedly, we also observed lower iFABP levels in the PNP compared to uninfected controls, suggesting that the PNP group may employ mechanisms to reduce even 'normal' levels of intestinal mucosal damage that might arise from, for example, non-HIV-related gastro-intestinal infections or inflammation. High levels of adenosine in

mucosal tissues maintaining Th17 cell numbers in the gut have been implicated in non-pathogenic SIV infection (He *et al* 2015) and one may speculate that similar mechanisms could operate among PNP.

In nonpathogenic SIV infection of sooty mangabeys, the mucosal barrier has been reported to remain intact so as to prevent microbial translocation and a reduction in LPS levels is observed compared to the pathogenic infection in rhesus macaques (Marchetti *et al.* 2013). Since the initial study by Brenchley *et al* in 2006, a range of studies have supported the association between microbial translocation, immune activation and disease progression, independent of viral load (Marchetti *et al* 2013). The findings that markers of gut epithelial barrier dysfunction (sCD14, iFABP) and innate immune activation (IL-6, D-dimer, CRP, T-cell CD38/DR expression) predict mortality in treated HIV infection (Hunt *et al.* 2014) suggest that increased translocation of gastrointestinal microbial products directly contribute to systemic immune activation and may ultimately determine the rate of progression to AIDS.

The unsolved question remains of why, or how, following acute infection in the natural hosts of SIV, the initial robust immune response is arrested, and damage to the gut with consequent microbial translocation limited to minimal levels. The fact that sooty mangabeys maintain normal, healthy frequencies of Th17 cells in blood and GI tract, while there is substantial loss of these cells in human HIV infection (Brenchley *et al.* 2008) suggest the possibility that Th17 cells may play

a key role in determining the level of microbial translocation. It is established that Th17 cells are critical in defence against bacteria and fungi at mucosal surfaces. Th17 cells produce that promote recruitment of neutrophils to areas of bacterial infection, induce proliferation of enterocytes and production of antibacterial defensins (Kolls *et al*. 2004; Kao *et al*. 2004; Liang *et al*. 2006), However, if preservation of Th17 cells is key to non-pathogenic SIV infection, the mechanisms by which these cells are maintained remain unknown.

Irrespective of the underlying cause of decreased levels of immune activation in our PNPs, similar to the non-pathogenic model of SIV infection in the sooty mangabey, these children do not experience the bystander consequences of a generalized state of immune activation that result in increased apoptotic loss of uninfected T cells. This is supported by the data presented here showing decreased levels of PD-1 expression, a marker of immune exhaustion, on the T cells of PNP compared to progressing children and a degree of T-cell dysfunction in paediatric progressors. Immune exhaustion would normally be expected in the face of persistent high antigen loads (Wherry & Ahmed 2007). However, in PNP, immune function appears broadly maintained. The depletion of naïve T cells during HIV progression is consistent with loss of long-lived central memory cells through direct infection, activation induced cell death or differentiation into effector memory cells, and the failure of replacement by naïve cells to keep pace with T-cell loss. At the same time increased activation results in T-cell dysfunction so not only are numbers of central memory cells down but so also is function. In contrast, the regenerative capacity of the CD4+ T cell compartment is

maintained in HIV-infected PNPs. Again the underlying mechanism are not entirely clear, but the absence of generalized immune activation and bystander immunopathology is likely of fundamental importance in explaining how these paediatric non-progressors avoid immunodeficiency following HIV infection.

It is possible that there may be some barrier to HIV infection of central memory or Tscm cells, as has been described in both the sooty mangabey, African green monkey, and adult viraemic non-progressors (Klatt *et al*. 2014; Beaumier *et al.* 2009; Paiardini *et al*. 2012; Buzin *et al*. 2014). However this is yet to be determined in the PNP group. In the sooty mangabey, reduced CCR5 expression in CD4+ central memory T cells (Paiardini *et al*. 2011) was associated with lower levels of infection in these cells compared to effector memory cells which could therefore protect the central memory population from virus-mediated depletion and thus favours the preservation of CD4+ T cell homeostasis. A recent study conducted in HIV-infected humans observed that the Tcm and Tscm in viraemic non-progressors (VNP) harbour less HIV DNA when compared to putative progressors (PP) (Klatt *et al*. 2014) which is concordant with another study in humans showing that VNPs have lower T cell immune activation than PP's in peripheral blood, yet higher T cell activation in the rectal mucosa where a much higher proportion of CD4+ T cells have an effector phenotype (Shaw *et al.* 2013). Preservation of CD4+ Tcm and Tscm from direct virus infection may be of particular importance during HIV and SIV infections as these cells are longer lived than CD4+ Tem and proliferation of Tscm feeds the CD4+ Tcm cell pool, which in turn is essential to maintain a sufficient number of CD4+ Tem in

mucosal tissues (Okoye *et al*. 2007). Indeed, preliminary studies in our PNP have shown a similar reduction in HIV DNA in the Tscm and Tcm cell subsets compared to the paediatric progressors, in whom we observe the majority of HIV DNA in the longer lived Tscm and Tcm cells compared to the effector memory cells (data not shown). Thus a mechanism based on preserving CD4+ Tscm and Tcm cells from viral infection may underlie the lack of disease progression in both PNP and SIV-infected SM.

In African-origin primates such as the African green monkey (AGM) and SM it has been shown that mother-to-infant-transmission (MTIT) is a very rare occurrence despite high blood and milk viral loads (Rey-Cuille *et al.* 1998; Pandrea *et al.* 2008; Fultz *et al.* 1990; Chahroudi *et al.* 2011; Otsyula *et al.* 1995). Even in the rare event of MTIT of SIV in SM it has been demonstrated that the transmission is associated with a 2-log reduction in viraemia compared to SM's that become infected as adults (Chahroudi *et al*. 2011). This contrasts with HIV-infected humans that readily transmit the virus to their infants. The reasons for the lack of MTIT in SIV infection has remained relatively elusive. There has been evidence of strong autologous virus-neutralizing antibody responses in the plasma and milk of chronically infected AGM's (Gicheru *et al*. 1999; Wilks *et al*. 2011; Amos *et al.* 2013) and more recently it has been shown that during acute SIV infection in AGM there is an earlier development of high-magnitude and more functional systemic gp120-specifc antibody responses and Env-specific memory B cell population evolution than is observed in the pathogenic infection of RM (Amos *et al*. 2015). Thus, although phenotype of high viraemia and low

immune activation without disease observed in the sooty mangabey phenotype may appear similar in the PNP group, there are clearly differences. Among these is the apparent ease with which HIV is transmitted by adult viraemic non-progressors to PNPs.

A second difference in PNPs from the natural hosts of SIV is in the broadly neutralising antibody (bNAb) responses. These bNAb responses do not appear to be a feature of natural SIV infection (Kilgore *et al*. 2015; Li *et al*. 2010; Fischer *et al*. 2012). In HIV infection, bNAbs typically arise after many of years of HIV-infection in humans and only occur in a small percentage of infected individuals (Dhillon *et al*. 2007; Simek *et al.* 2009; Gray *et al.* 2011; Tomaras *et al.* 2011; Hraber *et al*. 2014). In our data presented in this chapter we have demonstrated that the frequency of broadly neutralizing antibodies in non-progressing children is considerably higher than in chronically infected adults. As has previously been shown in SHIV infected monkeys and HIV-infected humanized mice, bNAbs isolated from HIV infected individuals can reduce the size of the latent virus reservoir and control systemic viremia (Barouch *et al*. 2013; Halper-Stromberg *et al.* 2014). Maintenance of normal CD4 counts in the PNPs however does not depend on the bNAbs, since these bNAb responses are no lower in the paediatric progressors. Thus the major value of the finding here of high frequency bNAbs and the high magnitude of these responses, is that these may be isolated and monoclonal broadly neutralising generated that will be highly effective in prevention and cure strategies.

Together these data suggest that the mechanisms that operate to achieve

disease-free paediatric HIV infection may have closer similarities to those that have evolved over thousands of years in African non-human primates as a consequence of SIV infection than to the well-described HLA mediated immune suppression of HIV observed in adult elite controllers.

Clear differences, however, are apparent between non-pathogenic SIV infection and nonprogressing paediatric HIV infection, in particular in relation to virus-specific immune responses observed. SIV-specific CD4+ and CD8+ T-cell responses in sooty mangabeys are relatively low in magnitude and breadth whereas the same cytokine responses – IFN-**γ**, IL-2 and TNF-**α** -measured here in response to HIV peptides in the non-progressing HIV-infected children, were of high magnitude and breadth. Similarly, neutralising antibody responses in sooty managbeys are detectable but relatively low-level whereas remarkably potent and broadly neutralizing antibody responses are observed in the paediatric non-progressors. Nonetheless, in common with the sooty mangabey, the virus-specific cellular and humoral responses detected in the nonprogressing children are not responsible for lack of immunodeficiency virus disease.

The contrast between non-pathogenic HIV infection achieved via suppression of viral replication in adult elite controllers and via mechanisms independent of HIV-specific immune responses in non-progressing children deserves further comment. Previous studies have shown that, whereas more than 50% of adult elite controllers express one of the main 'protective' HLA class I alleles, HLA-B*27 or HLA-B*57 in Caucasians, or HLA-B*57/58:01/81:01 in African populations, HLA class I differences do not significantly influence disease

progression in paediatric infection. Whereas suppression of viraemia as a result of strong CD8+ T-cell responses presented by these protective class I molecules can interrupt and terminate the vicious cycle of immune activation and CD4 decline in adult infection, in paediatric infection, reduction in viraemia via increasing potency of the HIV-specific CD8+ T-cell response coincides with increased immune activation and therefore comes at the cost of CD4 decline. The fact that no elite controllers of HIV infection have been described in ART-naive paediatric infection is itself evidence of the dangers of an immune strategy designed to suppress viraemia if at the same time this is associated with increased immune activation. Consistent with this are the reports of a subset of ART-naïve elite controller adults who succeed in suppressing viral replication to undetectable levels but have progressed to AIDS because of increased immune activation. Further studies are required to determine the degree to which specific mechanisms underlying non-progression in HIV-infected children are similar to AVNP and to natural SIV hosts, noting that even among the natural hosts of SIV infection distinct specific mechanisms have evolved to reach a similar phenotype. Further defining these mechanisms is relevant both to reaching a better understanding of HIV pathogenesis and therefore the development of new immunotherapeutic approaches to prevent HIV disease, but also to the HIV Cure field.

CHAPTER 6

DISCUSSION

6.1 INTRODUCTION

More than 30 years after the first descriptions of HIV/AIDS, the disease is pandemic, with an estimated 38 million people living with HIV and 2 million new infections every year (UNAIDS 2014). There are currently two HIV epidemics; a generalized epidemic in sub-Saharan Africa and an epidemic concentrated in high-risk groups elsewhere. With the introduction of new strategies to reduce mother-to-child transmission, the risk of transmission can be successfully reduced to <2% (WHO 2014; Moodley *et al*. 2013). However, in countries such as South Africa, where the burden of HIV-1 is highest, and where >0.3m babies are born to HIV-positive mothers each year, even these interventions are not able to prevent mother-to-child-transmission altogether. The consequences of these statistics are particularly devastating for children, over 50% of whom may die before 2 years of age in the absence of treatment (Newell *et al*. 2004). This thesis has primarily addressed paediatric HIV infection, focusing specifically on the differences in immunopathogenesis in children compared to adults.

6.2 THE RELATIVE CONTRIBUTION OF VIRAL FITNESS ON DISEASE PROGRESSION

In Chapter 3, the contribution of viral replicative capacity (VRC) to disease progression was investigated. The Kimberley paediatric cohort provided a valuable opportunity to study the impact of the transmitted virus, since disease progression was very well characterized in these children and the timing of transmission (in contrast to horizontal infection in adults) was known. Despite the fact that, on average, 7.5 years had elapsed since transmission, there remained a highly significant correlation between the VRC in the mothers and their children. Comparison of paediatric progressors and non-progressors demonstrated that overall there was a significant reduction in viral replicative capacity in the non-progressors and a significant correlation existed between the replicative capacity of viruses and rate of disease progression.

The major shortcoming of these studies was the unavailability of the virus that was transmitted by the mother. However, access to the Durban cohort of mother child pairs followed from birth (Mphatswe *et al*. 2007) would allow these analyses to be undertaken. Preliminary data from measurements we have done of the VRC in mother and child in this cohort indicate that the VRC of transmitted virus is in fact somewhat lower in the child compared to the mother. This unexpected finding contrasts with what has been described in adults, in which the fitter viruses in the donor or more likely to be transmitted (Carlson *et al* 2014). The main conclusion, however, is that, although VRC plays a part in rate

of progression in paediatric infection, it is by no means the only factor that determines disease outcome.

6.3 THE PROTECTIVE INFLUENCE OF HLA ALLELES IN CHILDREN AND ADULTS

Chapter 3 also presented data that addressed the influence of HLA alleles on control of HIV in both adults and children. The adult study was derived from a large C-clade infected South African cohort and demonstrated that the impact of HLA-B*57/58:01/81:01 was significant in reducing VRC, viral load and in maintaining higher CD4+ T-cell counts, thus delaying disease progression. Similarly the disease susceptible alleles HLA-B*1801/45:01/58:02 had a marked impact in the adult cohort with significantly higher VRC, viral loads and lower CD4+ T cell counts and hence a more rapid progression to AIDS. It has previously been reported that HLA-B alleles and Gag-specific CD8+ T-cell responses are each beneficial in viral containment (Pereyra *et al*. 2010; Costello *et al.* 1999; Kiepiela *et al.* 2004; Leslie *et al*. 2010; Tang *et al.* 2011; Lazaryan *et al*. 2011; Carlson *et al.* 2012) but the data presented here provide an explanation for these findings in that the impact of protective and disease-susceptible alleles on VRC was strongly linked to the number of Gag CD8+ T-cell escape mutations within the virus. This study therefore adds to the growing body of evidence that highlights the importance of escape mutations that reduce viral fitness (Leslie *et al*. 2004; Martinez-Picado *et al*. 2006; Schneidewind *et al*. 2007; Matthews *et al*. 2008; Crawford *et al*. 2009; Miura *et al*. 2009; Wright *et al* 2010; Wright *et al*. 2012; Boutwell *et al.* 2013).

The paediatric study in Chapter 3 describes the relative lack of influence of HLA alleles on HIV disease and progression in children. There was no significant difference in VRC in children either expressing protective or disease susceptible alleles. Mutations, particularly within the conserved Gag protein, that arise at a cost to viral fitness, have previously been shown to benefit both donor and recipient, both in adult transmission pairs (Chopera *et al.* 2008; Goepfert *et al*. 2008) and in mother-to-child-transmission. Surprisingly the impact of maternal alleles on the child's VRC was greatest when the mother carried any of the disease-susceptible alleles (HLA-B*18:01/45:01/58:02). Previous evidence that HLA class I plays an important role in immune control of HIV in infected children is mixed and somewhat contradictory (Goulder *et al*. 2001; Feeney *et al.* 2004; Feeney *et al*. 2006; Thobakgale *et al*. 2009; Schneidewind *et al*. 2009; Prado *et al.* 2010; Prendergast *et al.* 2011). In contrast to adult HIV infection we have shown here, for the first time, that there is no correlation between the presence of protective HLA alleles and disease progression in children, using CD4 count or viral load as markers of progression.

6.4 RECOVERY OF T CELL FUNCTION ON ART

In view of the rapid disease progression that is a hallmark of perinatal infection, and the recommendation that all HIV-infected infants are now started on ART regardless of CD4%, Chapter 4 addressed the efficacy of ART in restoring T cell function. These studies demonstrated a rapid recovery of CD4+ T-cell function

within 12 months of viraemic suppression on ART. This is an encouraging result, supporting the notion that, when HIV-specific T-cell responses are needed to eradicate viral reservoirs in HIV-infected people on long-term ART, they will be supported by effectively reconstituted CD4+ T-cell activity.

These findings therefore are consistent with previous studies (Sleasman *et al.* 1999; Gibb *et al*. 2000; Walker *et al*. 2004; Resino *et al*. 2006; COHERE/Sabin *et al*. 2008) that have shown recovery of CD4 T-cell function in children, but not in adults, who achieve aviraemia on ART. These studies also provide evidence that increased duration of aviraemia showed little improved function in CD4+ T-cells. Thus, a relatively short period of aviraemia was sufficient to largely restore CD4+ T-cell function in ART-treated children. In contrast however, no recovery of CD8+ T-cell function was observed, despite many years of successful ART, and unlike CD4+ T cell function, CD8+ T cell function did not have any impact on viral load in ART naïve PNP. These findings support the concept that CD4 T-cell function is central to maintenance of non-pathogenesis in paediatric infection and that CD8+ T-cells play a somewhat passive part.

There are two especially notable findings within this set of data. First is the result that the magnitude of HIV-specific CD4 response, but not the CD8 response, is related to control of viral load. The second especially striking result is contrast between the *direct* correlation between viral load and the magnitude of the IFN-gamma HIV-specific CD4 response ($r=0.73$, $p<0.0001$) and the *inverse*

correlation between HIV-specific CD4+ IL-2 production and viral load. Doubtless this relates to the types of CD4 T-cells prevalent in non-progressor children versus progressors: the abundance of effector memory CD4 T-cells and terminally differentiated CD4 T-cells in progressors compared with the abundance of naïve and central memory CD4+ T-cells in non-progressors would explain this dichotomy (Sallusto *et al*. 1999). However it does prompt the question of what the CD8+ T-cells are doing in the paediatric non-progressors, and why it is that they do not appear to be engaging with the virus.

6.5 CENTRAL ROLE OF IMMUNE ACTIVATION IN HIV PATHOGENESIS

Immune activation is central to the immunopathogenesis of HIV-1 infection and drives immune senescence and CD4 cell depletion. Whilst activation appears to be driven largely by HIV viral load in adults, there was no correlation between T cell activation and level of viraemia in our cohort of non-progressing children studied in Chapter 5. The causes of immune activation are likely to be multifactorial and although HIV replication is necessary, as in adults it is not the most important driver. Perhaps this is best illustrated in the non-progressor group of children in whom viral replication continues at high levels, and yet immune activation is almost as low as that in uninfected healthy controls.

The data presented in Chapter 5 demonstrates high absolute CD4 counts were observed in PNPs in association with high viral loads; there was no relationship

observed between viral load and absolute CD4+ T-cell count. In association with high CD4% in PNPs we observed (i) low levels of immune activation in both CD8+ and CD4+ T cells accompanied by low-level microbial translocation; (ii) an increase in the percentage of naïve CD4 T cells and a decrease in effector memory CD4 cells; (iii) an increase in IL-2 production and a decrease in IFNγ production upon antigen stimulation; (iv) decreased levels of PD-1 expression on T cells, and (v) high titre, broadly neutralising antibodies.

Levels of PD-1 on T cells were upregulated in paediatric progressors compared to non-progressing children and correlated positively with immune activation and viral load and negatively with CD4+ T-cell count. PD-1, a negative regulator of T cell activation, was originally described as an 'exhaustion' marker (Day *et al.* 2006; Petrovas *et al*. 2006; Trautmann *et al*. 2006). A study conducted by Velu *et al.* 2009 demonstrated that a PD-1 blockade in pathogenic SIV infection leads to lower viral loads and increased survival time, refocusing interest in PD-1 as an immunotherapeutic target in HIV infection.

These data show several similarities to observations made in relation to non-pathogenic SIV infection in the sooty mangabey. The maintenance of high CD4 counts in the setting of high viraemia and low immune activation, accompanied by evidence of low levels of microbial translocation, all describe the phenotype observed in the natural hosts of SIV infection. In the non-progressing HIV-infected children, these normal CD4 T-cell numbers are associated with

maintenance of a broadly unaffected T-cell function. This scenario suggested the possibility that these PNP, after years of being exposed to high levels of viraemia, would have broadly neutralising antibody responses; and indeed, 85% of PNPs tested showed broadly neutralizing antibody activity, in many cases with very high neutralization titres. As expected, the breadth of viruses neutralized correlated with HIV- viral load. This level of bNAb response compares to ~20% in adult infection (Gray *et al* .2011). Curiously, studies in the sooty mangabey show that these animals do not have high levels of autologous neutralising antibody (Li *et al.* 2010).

These non-progressor children therefore present an opportunity to identify potent, broadly neutralizing antibody responses, from which ultimately monoclonal antibodies could be generated that could be invaluable immunotherapeutic agents for prevention and cure. In addition, the fact that PNPs exhibit low immune activation in the setting of high viral loads provides a chance to define the underlying mechanisms of low immune activation. This has the longer term potential to identify new therapeutic options for the millions of HIV-infected adults on ART whose levels of immune activation remain relatively high despite the fact that viral replication is successfully suppressed. The consequence of persistent high immune activation in ART-treated individuals is an increased risk of diseases associated with ageing, such as coronary artery disease, strokes, and cancers. For these reasons findings ways to disconnect HIV infection from immune activation may be more broadly applicable, far beyond considerations of paediatric infection.

6.6 SUMMARY OF RESULTS

The mechanisms by which disease-free infection is achieved in HIV-infected elite controllers and in the natural hosts of SIV infection, such as the sooty manganey and African green monkey, are diametrically opposed. Elite controllers suppress HIV replication to undetectable levels via immune responses mediated principally by protective HLA class I molecules. In contrast, non-pathogenic SIV infection is independent of virus-specific immunity and viral replication continues unabated but without ill consequences. These studies of non-progressing paediatric HIV infection are the first to demonstrate that, although in contrast to the sooty managbeys – strong virus specific immune activity is present, it does not contribute to the maintenance of normal CD4 counts and absence of disease. These are the first studies to report that HLA class I differences do not significantly influence disease progression in paediatric HIV-infection. These are also the first studies to show that normal-for-age CD4 counts are associated with low levels of T cell immune activation despite persistent high viral loads observed in these non-progressing children. The potency and high frequency of the broadly neutralizing antibody responses in non-progressing children is an additional feature that is both novel and striking. The novel mechanisms described in this thesis that operate to achieve disease-free paediatric HIV-infection have close similarities to those that have evolved over thousands of years in African non-human primates as a consequence of SIV infection. These mechanisms in children therefore operate in entirely different manner to the well described HLA-mediated immune suppression of HIV observed in adult elite controllers.

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