Protective HLA class I alleles: investigation of viral control and lack of control in chronic HIV-1 subtype C infection

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Protective HLA class I alleles: investigation of viral control and lack of control in chronic HIV-1 subtype C infection

By

Catherine Kegakilwe Koofhethile

Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (Immunology) in the School of Laboratory Medicine and Medical Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal 2015

DECLARATION

I, Catherine Kegakilwe Koofhethile, declare that this is my original work where others have made contributions it has been acknowledged in the thesis. The work presented in this thesis has not been submitted in any other form to another University. The experimental work described in this thesis was performed in the HIV Pathogenesis Programme (HPP) Laboratory, Hasso Plattner Research Laboratory, and Africa Centre Laboratory, in the Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Professor Thumbi Ndung'u and co-supervisor Dr. Christina Thobakgale.

Catherine Kegakilwe Koofhethile

Signed: _____ Date: 12th February 2016

Professor T. Ndung'u

Signed: _____ Date: _____

Dr. C. Thobakgale

Signed: _____ Date: _____

As the candidate's supervisor I agree to the submission of this thesis.

Professor T. Ndung'u

Signed: _____ Date: _____

PLAGIARISM DECLARATION

I, Catherine Kegakilwe Koofhethile declare that:

i. The research reported in this dissertation, except where otherwise indicated, is my original work.

ii. This dissertation has not been submitted for any degree or examination at any other university.

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PUBLICATIONS AND CONFERENCE PRESENTATIONS

First author manuscript

1. **Catherine K. Koofhethile**, Zaza M. Ndhlovu, Christina Thobakgale, Julia J. Prado, Nasreen Ismail, Zenele Mncube, Lungile Mkhize, Mary van der Stok, Bruce D. Walker, Philip J.R. Goulder and Thumbi Ndung'u LONGITUDINAL ANALYSES OF $CD8^+$ T RESPONSES REVEAL DIFFERENCES IN MECHANISMS ASSOCIATED WITH HIV-1 VIREMIC CONTROL AMONG INDIVIDUALS WITH PROTECTIVE AND NON-PROTECTIVE HLA CLASS I ALLELES**.** ***Manuscript in preparation.***

Conference presentations

1. **Catherine K. Koofhethile**, Zaza M. Ndhlovu, Christina Thobakgale, Julia J. Prado, Nasreen Ismail, Zenele Mncube, Lungile Maphumulo, Mary van der Stok, Bruce D. Walker, Philip J.R. Goulder and Thumbi Ndung'u. Protective HLA alleles: Investigation of viral control and lack of control in chronic HIV-1 subtype C infection. Keystone HIV Immune Activation Conference, 2nd - 8th April 2013, Colorado, USA. **POSTER presentation**.

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5. **Catherine K. Koofhethile**, Zaza M. Ndhlovu, Christina Thobakgale, Julia J. Prado, Nasreen Ismail, Zenele Mncube, Lungile Maphumulo, Mary van der Stok, Bruce D. Walker, Philip J.R. Goulder and Thumbi Ndung'u. Reduction in the breadth of $CD8^+$ T cell responses is associated with loss of virologic HIV control in individuals with protective HLA alleles. HIV Research 4 Prevention conference 2014, Cape Town, South Africa, 27th – 31st October 2014. **ORAL presentation.**

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Thanks to the almighty God for the wonderful blessings and guidance throughout the years. 'I can do all things through Christ who strengthens me' (Philippians 4:13)

ABBREVIATIONS

AIDS: Acquired Immune Deficiency Syndrome

ANOVA: Analysis of variance

APC: Antigen presenting cell

APOBEC3G: Apolipoprotein B messenger RNA editing catalytic subunit-like protein 3G

ART: Antiretroviral therapy

ARV: Antiretroviral

bp: Base pair

CCR5: C-C chemokine receptor 5

CD: Cluster of differentiation

CO_2: Carbon dioxide

CTL: Cytotoxic T Lymphocytes

CRF: Circulating recombinant form

CXCR4: CXC chemokine receptor 4

DC: Dendritic cell

DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DEPC: Diethyl pyrocarbonate

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

EC: Elite controller

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

ELISpot: Enzyme-linked immunosorbent spot

Env: Envelope glycoprotein

FACS: Fluorescent activated cell sorting

FBS: Foetal bovine serum

FCS-A: Forward sides scatter area

FSC-H: Forward sides scatter height

FI: Entry/Fusion inhibitor

Gag: Group specific antigen

IFN-γ: Interferon gamma

IL-2: Interleukin 2

IN: Integrase

IQR: Interquartile range

HIV: Human immunodeficiency virus

HAART: Highly active anti-retroviral treatment

HLA: Human leukocyte antigen

KIR: Killer cell immunoglobulin-like receptor

LTNP: Long term non-progressor

LTR: Long terminal repeat

MHC: Major histocompatibility complex

MIP-1β: Macrophage inflammatory protein-1-beta

MOI: Multiplicity of infection

NK: Natural killer

Nef: Negative regulatory factor

NNRTI: Non-nucleoside reverse transcriptase inhibitor

NRTI: Nucleoside reverse transcriptase inhibitor

OLP: Overlapping peptides

PBMC: Peripheral mononuclear cells

PCR: Polymerase chain reaction

PD-1: Programmed Death 1

Pol: Polymerase

PHA: Phytohaemagglutinin

PI: Protease inhibitor

PR: Protease

REV: Regulator of gene expression

RNA: Ribonucleic acid

rpm: Revolutions per minute

RT: Reverse transcriptase

RT-PCR: Reverse transcriptase polymerase chain reaction

TAT: Trans-activator of transcription

TNF-α: Tumor necrosis factor alpha

TRIM5α: Tripartite motif-containing motif 5 alpha

SFC: Spot-forming cells

SK: Sinikithemba

SIV: Simian immunodeficiency virus

ss: Single-stranded

TCR: T cell receptor

VC: Viremic controller

VIF: Viral infectivity factor

VL: Viral load

VPU: Viral protein U

VPR: Viral protein R

WHO: World Health Organization

Mg: Microgram

U: Unit

V: Volt

α: Alpha

β: Beta

γ: Gamma

µl: Microlitre

µM: Micromolar

°C: Degrees Celsius

kb: Kilobase

ml: Millilitre

mm: Millimetre

mM: Millimolar

ng: Nanogram

x g: Times gravity

ETHICS

The research study for this thesis is registered with the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and was approved by this committee. **(BREC reference number: E028/99).**

The Academic Leader for Research at the School of Laboratory Medicine and Medical Sciences, granted approval of the research project towards studying for a PhD degree.

ABSTRACT

Some individuals with chronic HIV-1 subtype C infection are able to suppress viral infection without antiretroviral drugs. However, viral control can be temporary, with subsequent rise in viremia and associated disease progression. The immunological mechanisms associated with control or loss of viral control are not fully understood. We characterized longitudinally the immunological and virological features that may explain the divergence in disease outcome in 70 HIV-1 C-clade infected antiretroviral therapy (ART)-naïve South African adults from Durban, 35 of whom possessed protective HLA class I alleles.

We demonstrate that in this cohort, some individuals with protective HLA class I alleles lost viral control upon longitudinal follow up while the majority of individuals without protective HLA class I alleles maintained viral control. Sustained HIV-1 control in viremic controllers with protective HLA class I alleles (VC+) was related to the breadth of HIV-1 $CD8^+$ T-cell responses against Gag and enhanced ability to suppress viral replication in an *ex vivo* viral inhibition assay. The loss of virological control among some individuals with protective HLA class I alleles (failing viremic controllers- fVC+) was associated with reduction in the total breadth of $CD8^+$ T-cell responses in the absence of differences in HIV-1-specific $CD8^+$ cell polyfunctionality or proliferation. At the time point of virological control, fVC+ subjects already displayed a reduced capacity to suppress viral replication in an *ex vivo* viral inhibition assay, compared to others who did not subsequently experience loss of control. Additionally, we showed that $CD8^+$ T cell escape mutations were common in the fVC+ subgroup and in some instances the loss of $CD8^+$ T cell responses could be explained by immune escape, however, in some cases the loss of $CD8^+$ T cell responses could not be explained by viral escape. Interestingly some VC+ maintained

viral control throughout despite the presence of escape mutations within Gag, and persistence of $CD8^+$ T cell responses irrespective of sequence variation appeared to be a correlate of viral control. Controllers without protective HLA class I alleles (VC-) showed low breadth of HIV-1 $CD8^+$ T cell responses and a reduced ability (compared to viremic controllers with protective HLA class I alleles who never lost control) to suppress viral replication in an *ex vivo* viral inhibition assay. Furthermore, these VC- subjects had fewer escape mutations within Gag and maintained low viral load throughout.

Our data suggest that the control of HIV-1 in individuals with protective HLA class I alleles may be driven by broad $CD8^+$ T cell responses targeted towards Gag with potent viral inhibitory capacity while control among individuals without protective HLA class I alleles may be linked to alternative non-$CD8^+$ T cell mechanisms. These alternative mechanisms require further investigation. The data further suggest that the loss of virologic control is related to the loss of $CD8^+$ T cell responses. However, viral escape does not fully explain the loss of $CD8^+$ T cell responses in chronic HIV infection and therefore, alternative mechanisms require further investigation.

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CHAPTER 1: INTRODUCTION TO THE HISTORY, EPIDEMIOLOGY AND IMMUNE RESPONSES TO CHRONIC HUMAN IMMUNODEFICIENCY VIRUS (HIV) TYPE 1 INFECTION

1.1 THE DISCOVERY AND GLOBAL PANDEMIC OF HIV/AIDS

The first cases of acquired immune deficiency syndrome (AIDS) were recognised in 1981 in the United States. This syndrome was first identified amongst young homosexual men who presented with similar symptoms of unusual opportunistic infections and a rare skin malignancy called Kaposi's sarcoma (1, 2). A few years later the human immunodeficiency virus (HIV) was isolated and identified as the causative agent of AIDS (3–5). Thirty-four years since its discovery, HIV remains a public health threat having claimed the lives of an estimated 35 million people (6). An estimated 75 million people have been infected since the beginning of the epidemic and 36.9 million people were reported to be living with HIV in 2014, Figure 1.1. Sub-Saharan Africa still bears the brunt of the epidemic accounting for 70% (25.8 million) of the infected population worldwide (7, 8).

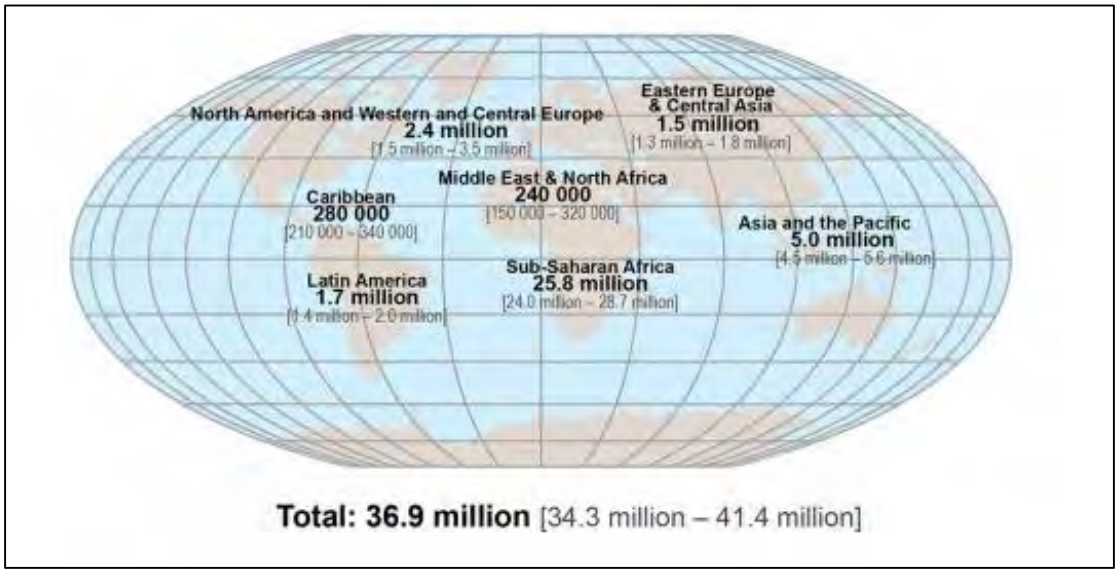

Figure 1.1. A global view of the adults and children estimated to be living with HIV/AIDS in 2014 (7)

Although there is still no cure for AIDS, the introduction, implementation and accessibility of antiretroviral therapy (ART) from the mid-1990s onwards has proven to be successful in prolonging the lives of those infected and reducing mortality and

morbidity rates caused by the HIV epidemic. This is clearly evident as the number of people estimated to have died from AIDS related illnesses has reduced from 2.0 million in 2009 to 1.5 million in 2013 (7). New infections have declined by 35% since the year 2000 where by worldwide, 2 million (1,9 million – 2.2 million) people became newly infected with HIV in 2010, a decrease from 3.1 million (3.0 million – 3.3 million) documented in 2000 (8). New infections among children have also declined by 58% where by worldwide, 220,000 (190,000 – 260,000) children became newly infected with HIV in 2014, a decline from 530,000 (470,000 – 580,000) in 2000 (8). Despite efforts being made to combat HIV, there is still no effective HIV vaccine available.

1.2 HIV PREVALENCE IN SOUTH AFRICA

South Africa is one of the hardest hit countries in the world with an HIV prevalence rate of 12.2% recorded amongst adults in 2012, an increase compared to 10.6% prevalence rate reported in 2008. Additionally, an estimated 6.4 million people were living with HIV in 2012 (9). Furthermore, HIV prevalence by province showed that KwaZulu-Natal continues to have the highest HIV prevalence rate of 16.9% when compared to other provinces in South Africa. Mpumalanga and Free State provinces follow with 14.1% and 14.0% respectively. The Western Cape province has the lowest HIV prevalence rate of 5%, Figure 1.2.

Figure 1.2. HIV prevalence rates by province in South Africa (9)**.** Key: KZN- KwaZulu-Natal, MP- Mpumalanga, FS- Free State, NW- North West, GP- Gauteng Province, EC- Eastern Cape, LP- Limpopo, NC- Northern Cape, WC- Western Cape.

In South Africa, female HIV prevalence is on the rise with a rate of 14.4% compared to males (9.9%) and has reached a peak in the age group 30 – 34 years with a prevalence of 36.1% higher than their male counterparts whose HIV prevalence was recorded to be 25.6% in 2012 (9). The highest HIV prevalence among males is in the age group 35 – 39 years (28.8%), still lower than their female counterparts with a rate of 31.6%, (Figure 1.3). The introduction of ART has increased survival of people living with HIV (PLWH), with 31.2% of PLWH having access to ART (9).

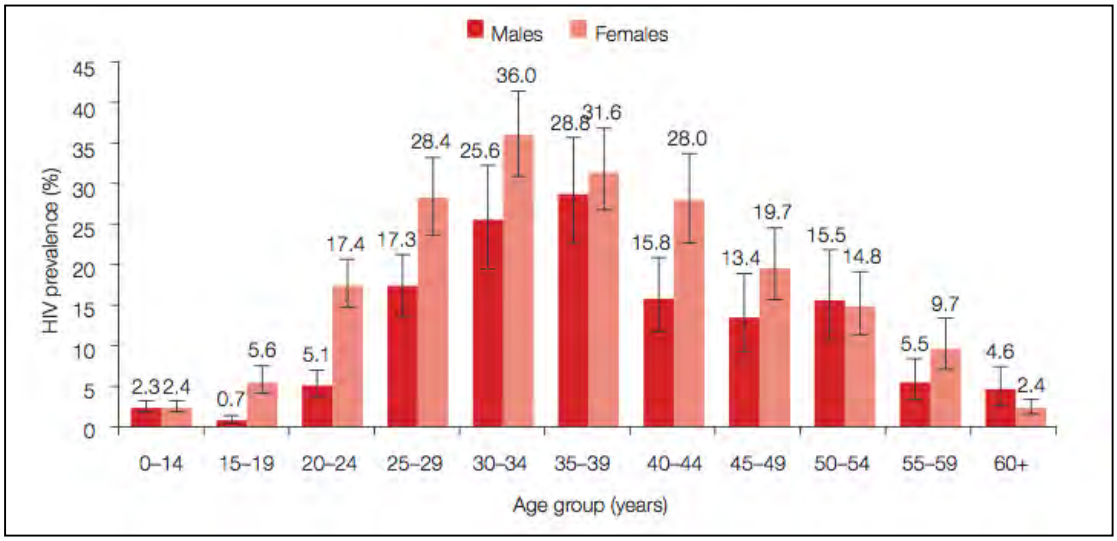

Figure 1.3. HIV prevalence rates by age and sex in South Africa, 2012. (9)**.**

1.3 THE RETROVIRUS

1.3.1 HIV Classification

HIV is classified as a Lentivirus, a subgroup of the *Retroviridae* family. This classification is based on the morphological, genetic and biological properties of the virus. In particular HIV is enveloped, contains reverse transcriptase and comprises of two identical copies of positive sense, linear ribonucleic acid (RNA) genome (10, 11). There are two types of HIV described so far; HIV-1 (3, 4) and HIV-2 (12). HIV-2 is commonly found in West Africa and is known to be a less transmissible and less pathogenic virus than HIV-1, which is responsible for the global pandemic. Previous studies have demonstrated that HIV-2 infected patients survived longer than HIV-1 infected patients, the transmission rate was lower than that of HIV-1 and that HIV-2 infected individuals had lower viral load and slower rate of $CD4^+$ T cell decline (13–15), supporting the view that HIV-2 is less pathogenic. Because HIV-2 is known to be less transmissible compared to HIV-1 (16), this implies that fewer individuals exposed to HIV-2 will be infected. In this thesis, the use of the term 'HIV' will refer to HIV-1.

HIV has four phylogenetically distinct groups, namely: M (the Main group), O (the Outlier group), N (non-M, non-O group) (17) and finally P, recently isolated from a woman in Cameroon (18). Groups O, P and N are very rare and mostly common in Central-West Africa (Cameroon) (19, 20) while group M is the most prevalent and is responsible for the global HIV/AIDS pandemic (21, 22). Group M comprises of eleven clades; A – K, denoted subtype A, B, C, D, F, G, H, J and K and this group accounts for about 90% of the global pandemic (17, 23, 24). There are also recombinant forms, which derive from a combination of different subtypes (22, 25). Figure 1.4 shows the global distribution of the major subtypes and recombinants of

HIV-1, (26). The most dominant HIV-1 subtypes are B and C, with subtype C being the predominant subtype found in southern Africa, parts of eastern Africa, India, and parts of China and is responsible for the AIDS pandemic in the region while subtype B is most commonly found in Europe, the Americas, Japan, and Australia (22, 26, 27).

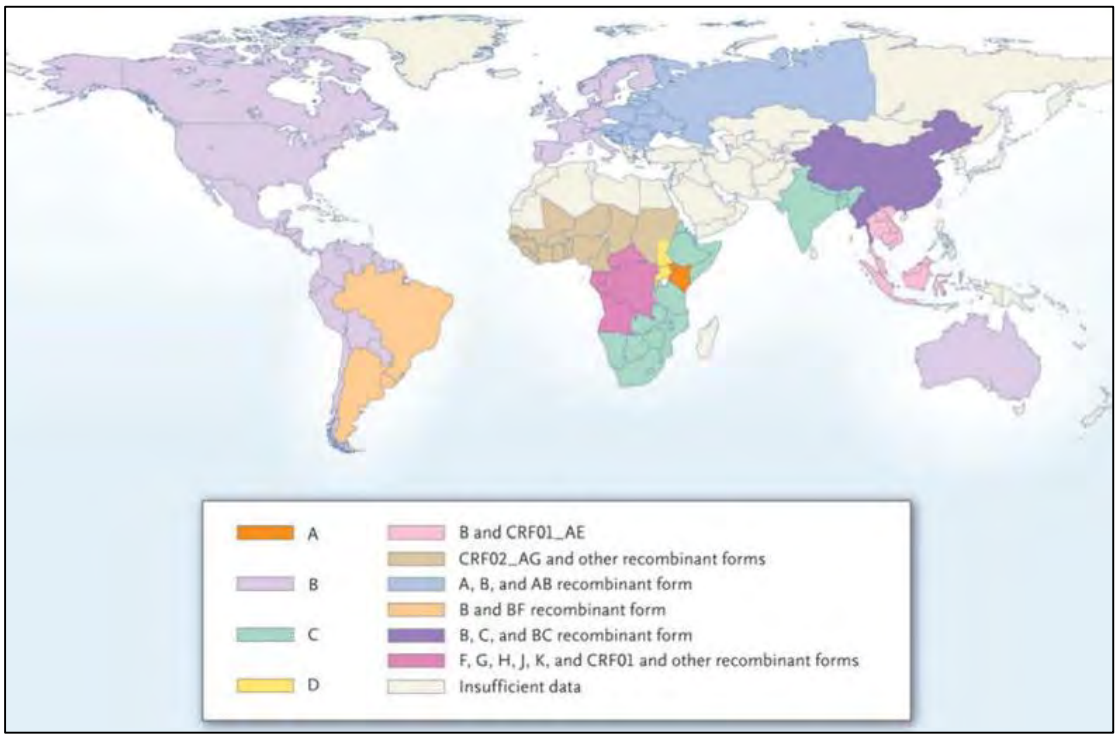

Figure 1.4. Global distribution of the major subtypes and recombinants of HIV-1 (28)**.**

1.3.2 HIV structure

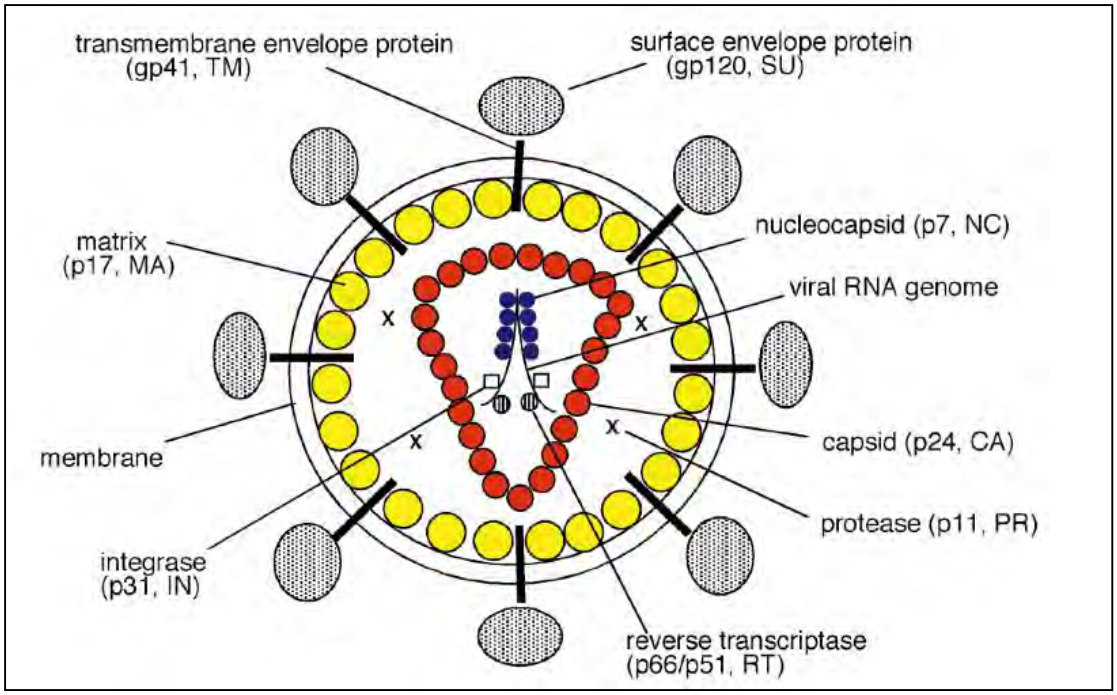

Figure 1.5. A schematic representation of the structure of HIV virion (29)**.**

Retroviruses are enveloped viruses that replicate inside the host cell. HIV is roughly spherical in shape with a diameter of about 120nm (30). A schematic representation of the virion structure is shown in Figure 1.5. HIV is characterised by 2 identical copies of positive sense single stranded RNA that codes for the virus's nine genes enclosed by a conical capsid (CA) that comprises of about 2,000 of the viral protein p24 (29). HIV, just like any other lentivirus has an inner and outer membrane. The outer membrane comprises of the lipid bilayer where surface glycoprotein (gp120 or SU) is anchored through its interaction with trans-membrane glycoprotein (gp41 or TM) (31, 32). Other host cellular proteins such as major histocompatibility antigens, actin and ubiquitin also contribute to the formation of the outer membrane (33). On the other hand, the inner membrane comprises of the matrix proteins (MA or p17). The nucleocapsid protein (CA or p24) encompasses the two RNA molecules, which

are anchored together in a ribonucleoprotein complex formed by several nuclear capsid proteins (NC or p7), integrase (IN or p31), reverse transcriptase (RT or p66/p51) and protease (PR or p11) (29).

Upon entry in to the host cell, the RNA is reverse transcribed into complimentary DNA by the viral reverse transcriptase, which is contained in the infecting virion. The DNA is then transported into the nucleus with host proteins as part of a pre-integration complex and integrated into the host genome with the help of another viral enzyme integrase and other cellular factors. The viral DNA can now function as a template to produce many copies of the virus inside the host cell (29, 34).

1.3.3 HIV genome

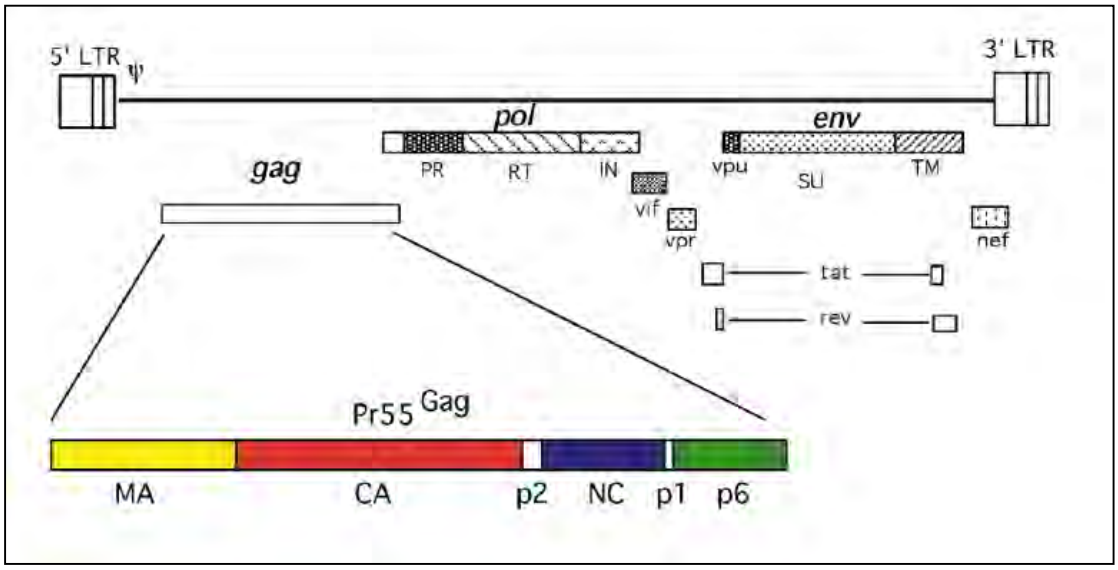

Figure 1.6. A schematic diagram of the HIV genome showing the long terminal repeats that flank either side of the genome and the 9 open reading frames. The *gag* gene encodes the viral proteins that form the capsid; the *pol* gene encodes the three enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN); the *env* gene encodes the envelope surface protein. The remaining six open reading fames encode the accessory proteins; Vif, Vpr, Vpu, Tat, Rev and Nef (29)**.**

The HIV genome, approximately 9.5 kilobases long comprises of nine genes. The HIV genome is flanked by long terminal repeat (LTR) sequences on either ends (five prime and 3 prime ends) (32, 35). There are three major structural proteins; Gag (group specific antigen polyprotein) that comprises the core structural proteins, Pol (polymerase) polyprotein that is processed into three viral enzymes and Env (envelope) that consists of the gp120 and gp41 proteins. These major structural proteins are essential for the formation of new viral particles. Furthermore, there exist six additional proteins namely two regulatory proteins; Tat (transcriptional transactivator) and Rev (regulator of virus protein expression) required for viral replication (36, 37) and four accessory proteins; Nef, Vif, Vpr and Vpu (Figure 1.6) required for regulatory functions and virion assembly (35, 37, 38).

1.3.4 Viral proteins and function

Gag – codes for the core structural proteins of the virus; capsid protein p17 (Matrix), p24 (capsid), p7 (nucleocapsid) and p6. The latter protein mainly associates and interacts with the plasma membrane to allow assembled virions to be released from the cell surface (39) as well production of the Vpr protein (40, 41).

Pol – codes for the viral enzymes; Protease (PR), Reverse Transcriptase (RT) and Integrase (IN). The RT is responsible for the conversion of the single stranded RNA in to single copy DNA (29, 42), which is then integrated into the host genome with the help of the IN. The IN facilitates the insertion of the linear double stranded DNA into the host cell chromosome (42). The PR then cleaves the Gag and Gag-Pol polyproteins to facilitate conformational changes required for the production of mature infectious viruses (35).

Env – the envelope is the most variable protein and comprises of the viral envelope glycoproteins; i. Subunit (SU) gp120. ii. Transmebrane (TM) gp41. The two derive from the cleavage of the highly glycosylated polyprotein gp160 by cellular proteases (43, 44). Both the gp120 and gp41 glycoproteins are essential for virus attachment and fusion to the target cell during the early stages of infection (29).

Tat – the *transactiva*tor of HIV *gene expression* activates initiation of transcription (45, 46).

Rev – a trans-activating protein that codes for a 19kD phosphoprotein, which functions include promotion of nuclear export, stabilization and usage of the viral mRNAs (45, 47).

Vif - the *viral infectivity factor* codes for a cytoplasmic protein required for subsequent infectivity of new viral particles. It disrupts the antiviral function of the human apolipoprotein B mRNA-editing enzyme-cytidine deaminase enzymes (APOBEC) enzyme hence preventing APOBEC3G protein from entering the virion during budding from a host cell by targeting it for proteasomal degradation (48–52).

Vpr – the Viral Protein R is a 96 amino acid 14-kDa protein which is required for virus replication in non-diving cells such as macrophages, regulation of the nuclear import of the HIV pre-integration complex and induces arrest of host cells at the G2/M phase of the cell cycle (43, 45).

Vpu – the Viral protein U is mainly involved in the late stages of infection and is known to enhance virion release from the plasma membrane of infected cells by counteracting host restriction by tetherin (45, 53, 54).

Nef – the negative regulatory factor is known to promote viral spread by down regulation of CD4 through endocytosis from plasma membrane and Major histocompatibility complex (MHC) class I molecules from the cell surface hence protecting the infected cells from cytotoxic T lymphocyte (CTL) recognition (45).

1.4 HIV REPLICATION

1.4.1 HIV tropism

HIV has the ability to infect a number of cell types including $CD4^+$ T cells as their main target and several others such as macrophages and microglial cells. Entry into $CD4^+$ T cells and macrophages involves an interaction between the CD4 molecule on the target cell and the virion envelope glycoprotein (gp120) together with chemokine co-receptors differentially expressed on $CD4^+$ T cells (55, 56). The different virus

strains can exhibit different affinities for either of the following chemokine receptors for entry into the target cell resulting in distinct tropisms: i. The β-chemokine receptor, chemokine receptor type 5 (CCR5), hence the viruses using this route are known as R5 isolates. ii. The α-chemokine receptor, chemokine receptor type 4 (CXCR4), hence the viruses using this route of entry are known as X4 isolates (57–59). Both the R5 and X4 isolates can infect and replicate in primary $CD4^+$ T cells. However, some viruses can use both chemokine receptors for entry into the host cell and these are known as R5X4 or dual tropic isolates. HIV has a bias towards utilizing CCR5 during acute infection which has been documented to facilitate rapid spread of infection, however during the course of the disease progression, there tends to be a switch either to dual tropism or CXCR4 co-receptor usage (56, 60, 61).

Other immune cells that HIV can infect are the myeloid dendritic cells in which virus entry occurs via either the CD4-R5 route or another route using mannose-specific C-type lectin receptors such as the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (62, 63). The infection of dendritic cells is very critical for maintenance of the viral reservoir in cases where the number of target cells ($CD4^+$ T cells) has declined to very low levels (62). In addition, other types of DCs such as plasmacytoid DCs and Langerhans can be infected by HIV (64). Furthermore, monocytes and other cell types may also be infected by HIV (65).

It is important to note that there exist a group of individuals who are resistant to HIV infection. These few individuals have a 32 base pair deletion on their CCR5 receptor sequence; a mutation known as CCR5 delta 32 (CCR5Δ32). Individuals with this mutation are resistant to infection with R5 - using viruses which are then unable to bind or enter the target cell and establish successful infection (61, 66–68). There are

very few people with this mutation. The frequency of individuals with this mutation is as low as 0.10 recorded in a cohort in the USA (69) and 0.01 in a cohort in Western Europe (66).

1.4.2 HIV infection

Once HIV comes into contact with a target cell, infection and further production of new infectious virions take place in a process known as viral replication. Viral replication steps include the following; virus attachment, fusion and entry, uncoating and reverse transcription, importation of the pre-integration complex into the nucleus, integration, transcription and translation, assembly and budding and finally maturation (29). These steps are discussed in detail below and summarized in Figure 1.7.

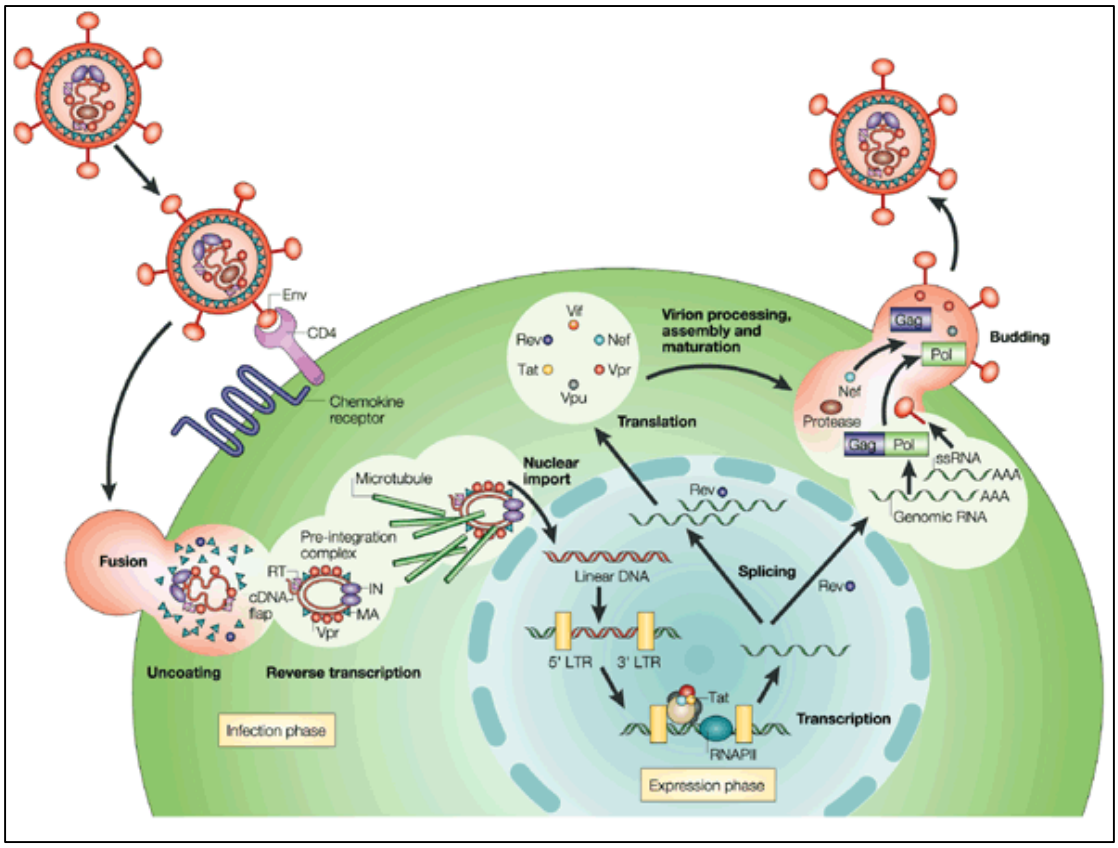

Figure 1.7. Schematic representation of the life cycle of HIV inside a host cell. HIV replication begins with the interaction of a virus particle with a receptor on the cell surface, resulting in the fusion of the viral and cellular membranes and transfer of the viral contents into the cell. The viral RNA genome is reverse transcribed resulting in

the production of a DNA copy, which is then imported into the nucleus. Inside the nucleus, the viral DNA is integrated into the host-cell genome. This is followed by transcription process from within the host genome, which is then exported from the nucleus. The translation of the viral proteins by host-cell machinery begins and the major structural protein, Gag, is transported to the plasma membrane where it directs assembly of the viral coat and incorporates other viral proteins and the viral genome. The virus buds through the cell membrane, and Gag is cleaved by the viral protease leading to a structural change of the virion into its mature infectious form, which is capable of fusing with a new susceptible cell (figure adapted from (70)**.**

1.4.2.1 Virus fusion and entry

The CD4 molecule expressed on the surface of the target cell, serves as a binding receptor for HIV, which interacts and binds with high affinity to gp120 found on the surface of the virus (71–74). This interaction triggers a conformational change of the envelope glycoprotein facilitating the virus to fuse with the target cell membrane (75, 76). However, the interaction between CD4 and gp120 alone is not sufficient to trigger conformational change adequate enough for virus entry. Therefore, co-receptors (CCR5 or CXCR4) must be expressed on the surface of the target cell in order to provide a much stronger conformational change of the glycoprotein necessary for successful virus entry (77–79). The conformational changes that occur following interaction of the CD4 with gp120 allow exposure of the chemokine binding domains of gp120 hence leading to their interaction with the target cell chemokine receptors (44, 58, 60, 75, 80). The interaction between CD4-gp120 and co-receptors leads to a prolonged attachment facilitating the N-terminal fusion peptide gp41 to penetrate the cell membrane therefore allowing fusion and entry to occur (72).

1.4.2.2 Uncoating and reverse transcription

Following virus entry is the uncoating and delivery of the viral core into the cytoplasm of the target cell (29, 81–83). During this process the nucleocapsid protein (CA) is lost and the viral core is retained which comprises of the viral RNA genome, tRNA primers and enzymes; reverse transcriptase (RT), integrase and protease (29).

The virus RT then facilitates the conversion of the virus RNA into a single copy of double stranded deoxyribonucleic acid (DNA) by a process known as reverse transcription (29, 84, 85). The virus RT has a low fidelity rate and has no proofreading capabilities, meaning it is extremely error-prone leading to high mutation rates of the HIV reverse transcription process (86–88), which may also later cause drug resistance and evasion of the virus from the host's immune responses (85). The newly sysnthesized DNA and the rest of the viral core contents are retained and form what is known as the pre-integration complex (PIC) (89, 90).

1.4.2.3 Integration

The PIC is then transported to the nucleus of the target cell. This is where the viral DNA is incorporated into the host genome catalyzed by the viral enzyme integrase (IN) in a process known as integration. The integrated viral DNA now known as the provirus will act as a template for further synthesis of viral RNA (29, 43, 85). The provirus may remain latent and inactive for many years before being activated via the transcription process (91, 92).

1.4.2.4 Transcription and translation

The integrated DNA is then transcribed into messenger RNA (mRNA), by a process known as transcription. This process is tightly controlled by Tat (transcription factor) and host cell's own machinery used for transcription of genes (93). The mRNAs are then spliced into smaller pieces and later exported out of the nucleus into the cytoplasm where they will undergo translation into proteins. The spliced mRNAs can be divided into 3 groups: i. Full length mRNA that encodes for structural proteins Gag-Pol polyproteins. ii. Singly spliced mRNA that encodes for Env, Vif, Vpr and Vpu. iii. The multiply spliced mRNA that encodes for the early regulatory proteins (Tat, Nef and Rev) (94, 95).

1.4.2.5 Assembly and release

The viral proteins formed from the spliced mRNA now move towards the outer cell membrane where they assemble. This process occurs in a series of steps, firstly the Env gp160 is transported via the endoplasmic reticulum to the Golgi complex where it is cleaved in the gp120 and gp41 by furin (96). Both the gp120 and gp41 then move to the plasma membrane where gp41 facilitates anchoring of gp120 to the membrane of the infected cell. Gag and Gag-Pol together with the viral genomic RNA associate with the inner surface of the plasma membrane and form a virion that will eventually bud out of the infected cell. Vif is also required for the stability of the viral core and its interaction with the antiviral restriction factor APOBEC3G hence preventing the latter from incorporation into the virion (49, 97, 98). The interaction between Gag and cellular proteins is critical for virion release. Viral protein U (Vpu) is also involved in the budding and interacts with tetherin, a membrane-associated protein that has the ability to block release of the virus particle (53, 54, 98).

1.4.2.6 Maturation

Shortly after release of an immature virus particle, the maturation process occurs. This process involves cleavage of the immature Gag and Gag-Pol polyproteins precursors by viral proteases to produce functional mature Gag and Gag-Pol proteins leading to the generation of a mature infectious HIV particle capable of initiating infection (29, 99).

1.4.3 HIV modes of transmission

Transmission of HIV occurs via direct transfer/exchange of bodily fluids; blood and blood products, semen, genital secretions and breast milk from infected mother to the newborn. The primary means of HIV transmission is via unprotected sexual contact with an infected person. In the 1990s it was documented that there was an increasing

incidence of HIV infection in the US and Europe amongst gay men and ethnic minority people and such infection rates accounted for 50% of new infections in the US (100). The majority of HIV infections worldwide is via heterosexual intercourse (101, 102) while non-sexual HIV transmission may occur through occupational exposure through needle sticks, blood transfusion with HIV contaminated blood products, injection drug use and mother to child (103).

Mother to child transmission cases in the absence of antiretroviral therapy have been documented in 25% of births from HIV infected mothers (104). Breastfeeding is another mode of mother to child transmission (105). However, with the implementation and availability of ART, the rate of perinatal HIV transmission has been reduced by 50% (106–108). Low viral load has also been associated with reduced HIV transmission (109).

In addition, HIV has been isolated from bodily fluids including; blood, semen, cerebral spinal fluid, tears, vaginal secretions, breast milk and saliva of HIV infected people (110–113). Although low HIV concentrations have been isolated from saliva and tears, there is no evidence that contact with these fluids can cause infection (114).

1.4.4 HIV transmission

The biology of HIV transmission is a complex process. Most transmissions occur heterosexually at the epithelial layer of the mucosal surfaces especially the genital and rectal mucosal surface (101, 115). At the epithelial layer, the founder virus undergoes a genetic bottleneck, resulting in only one virus being able to establish successful infection. Although the mechanisms by which infection occurs are not fully understood, previous work has shown that in about 80% of the cases, a single founder virus is responsible for successful infection (116). Furthermore, these founder viruses

are capable of infecting $CD4^+$ T cells to much greater extent as opposed to infecting monocytes and macrophages (117) and show resistance to type I interferons (118). Previous studies suggest that the first cellular targets of SIV and HIV reside in the lamina propria of the cervicovaginal mucosa. Two days following infection, the cervix is infected and the virus then spreads most likely through dendritic cells and $CD4^+$ T cells, which then migrate to regional lymph nodes and subsequently into the blood stream (119). HIV is also able to infect Langerhans cells, in addition to other dendritic cells and $CD4^+$ T cells that reside in the lamina propria of the cervicovaginal mucosa. These cell types express CD4 and CCR5; both essential for viral entry (120, 121). Studies on rhesus macaques have confirmed that successful infection arose from a single infecting virus when the animals were inoculated intra-rectally with a complex SIV quasispecies thus further supporting the use of SIV infection of rhesus macaques as a model for HIV-1 transmission and vaccine studies (122, 123).

1.4.5 Course of HIV infection

HIV infection is a chronic disease and progressively causes damage to the immune system from the time of infection to the manifestation of severe immunologic damage represented by a number of opportunistic infections, neoplasms, wasting, or low CD4 lymphocyte count that define AIDS (Figure 1.8). There are 3 main phases that define the course of HIV infection; acute phase, chronic phase and AIDS.

1.4.5.1 Acute Phase

Acute HIV infection is characterized by a burst of a to more than a million copies per ml of blood and a dramatic but temporal decline of $CD4^+$ T cells prior to detectable antibody responses or seroconversion. The acute phase normally lasts for up to several weeks and newly infected individuals may present with flu-like symptoms such as fever, disseminated lymphadenopathy, headache, myalgia, anorexia, rash,

and/or diarrhoea (124). Although there is a slight recovery of peripheral $CD4^+$ T cell counts in the blood following peak viremia, massive depletion of $CD4^+$ T cells occurs in the gut associated lymphoid tissue (GALT) where most of the $CD4^+$ T cells reside (125). Additionally, peripheral blood $CD4^+$ T cell counts eventually decline to a level that allows opportunistic infections (126, 127).

The peak in viremia is followed by a significant reduction to a viral set point. The viral set point predicts disease progression; the higher the viral set point, the faster the disease progression (128–131). Coincidental to the reduction of viral load to a set point is the emergence of HIV-specific $CD8^+$ T cells. HIV specific $CD8^+$ T cell activity has been shown to be involved in the reduction of viremia to a viral set point (132–134). Subsequent to $CD8^+$ T cell responses is the emergence of antibody responses. However, antibody responses do not appear to play a major role in reducing viremia during cute infection (135–138). Increased viral load (VL) is associated with increased risk of transmission, therefore persons with acute HIV-1 infection are more likely to transmit the virus to uninfected individuals (139–141).

1.4.5.2 Chronic Phase

This phase is asymptomatic and may last for years with viral load remaining relatively stable followed by a steady increase until the onset of AIDS (142, 143). However, persistent viral replication especially in the lymph nodes, fluctuating plasma viral load and a steady decline of the main target cell population is observed during chronic HIV infection (129, 144, 145). During this phase, HIV is able to trigger or activate potent host inflammatory and adaptive immune responses including CTL and antibody responses (146). However, the virus is able to evade these responses hence maintaining a significant level of viral replication that in turn sustains the state of

chronic immune activation, which eventually causes progressive immune dysfunction finally leading to progression to AIDS.

1.4.5.3 AIDS

AIDS is the end stage of disease usually characterized by a number of opportunistic infections and malignancies that eventually lead to death due to the immune-compromised state of the infected individual. The immune system is no longer able to control opportunistic pathogens culminating in life-threatening AIDS-defining diseases (147, 148). The $CD4^+$ T cell count below 200 cells per mm^3 of blood is a clinical indication of the onset of AIDS.

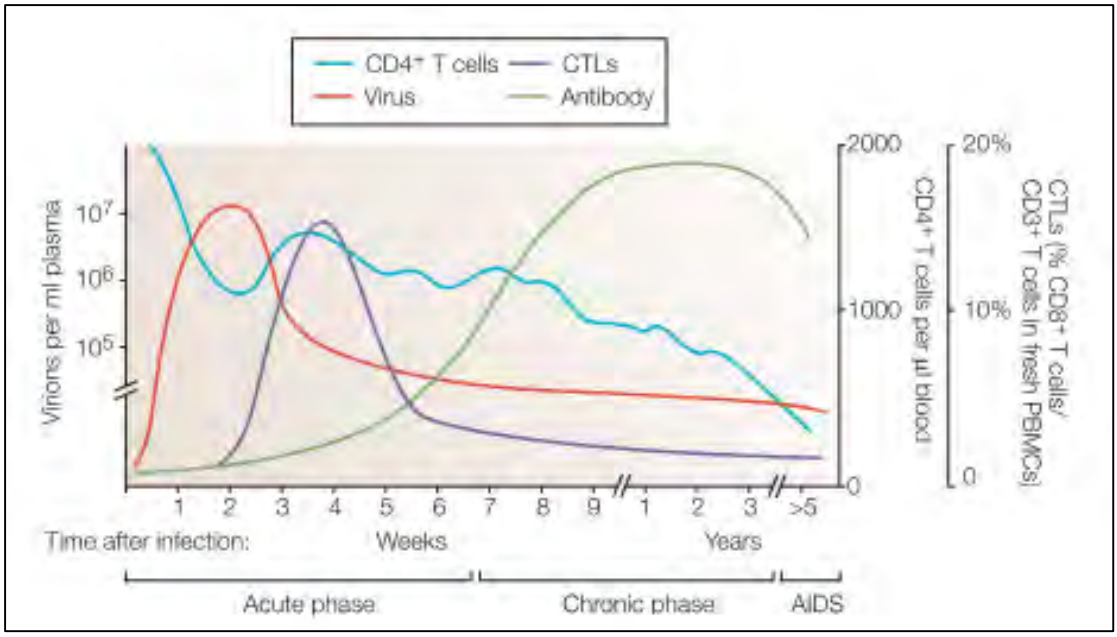

Figure 1.8. A schematic representation of the course of HIV/SIV infection showing changes in viral load, antibodies, CD4 and CD8 T-cell counts in the absence of antiretroviral treatment. Figure adapted from (149)**.**

1.5 ANTIRETROVIRAL TREATMENT

1.5.1 The role of ARVs

There is currently no cure or effective HIV vaccine available. However, there is treatment available for HIV, which consists of highly active antiretroviral treatment (HAART). This treatment has proven to be effective at controlling the epidemic since its introduction in the 1990s as demonstrated by a reduction in the death rate of HIV infected people (150–152). There are 25 antiretroviral drugs (ARVs) approved to date for the treatment of HIV infection and successful treatment is associated with the continued durable suppression of HIV viremia. Standard antiretroviral treatment (ART) regimens consist of a combination of at least 3 drugs belonging to at least two classes of ARVs. The main role of ART is to stop viral replication and restore immune function by inhibiting crucial HIV replication steps as summarised in Figure 1.9 below, and therefore preventing the onset of AIDS, AIDS-related illnesses, improving the quality of life and prolonging the life expectancy of those infected and finally preventing further transmission to the uninfected.

The six different classes of ARV agents currently approved by the US Food and Drug Administration (US-FDA) for the treatment of HIV infection are shown below.

a. Nucleoside reverse transcriptase inhibitors (NRTIs) target the reverse transcription step by interrupting the HIV replication cycle via competitive inhibition of HIV RT and termination of the DNA chain. Because they have a structure that is similar to that of the DNA nucleoside bases, they can be incorporated into the proviral DNA (153).

b. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) also target the reverse transcription step by binding to the RT at a site distant to the active

site and therefore inducing a conformational change that alters the active site of the enzyme and limiting its activity (153).

c. Protease inhibitors (PIs) function by binding competitively to the substrate site of the viral protease; an enzyme responsible for the post-translational processing and cleavage of polyprotein precursors that will generate core proteins and enzymes of mature virions during the budding from the infected cell. Inhibition of this process results in budding of immature viral particles (153, 154).

d. Integrase inhibitors (IIs) function by inhibiting the strand transfer reaction of the proviral integration step through binding of the metallic ions in the enzyme's active site (155, 156).

e. Fusion inhibitors (FIs) function by interfering with the HIV fusion step by competitively binding to the gp41 and preventing the conformational changes required for gp41 to complete the fusion process (157).

f. Chemokine receptor antagonists (CRAs) also known as CCR5 antagonists bind to the CCR5 co-receptor hence blocking the gp120-CCR5 interaction. However these drug types only inhibit CCR5 tropic HIV strains and not CXCR4 or dual tropic HIV strains (158, 159).

World Health Organization (WHO) has new recommendations that treatment should be initiated at 500 $CD4^+$ cells or fewer per mm^3 of blood of HIV infected individuals (160). However the main challenge is the high number of people eligible for treatment. There is still a great challenge of making ARVs accessible to everyone who is eligible and infected with HIV especially in low and middle-income countries,

which bear the brunt of the HIV/AIDS epidemic. In South Africa alone, only about 20% of the people who are HIV positive and eligible for treatment have access to treatment (152). The increasing evidence of resistance to treatment regimens available is of great concern. Additionally, HIV has a high mutation rate also contributing to the development of resistance to treatment (161, 162). Therefore, there are still milestones to be reached in order to combat the epidemic.

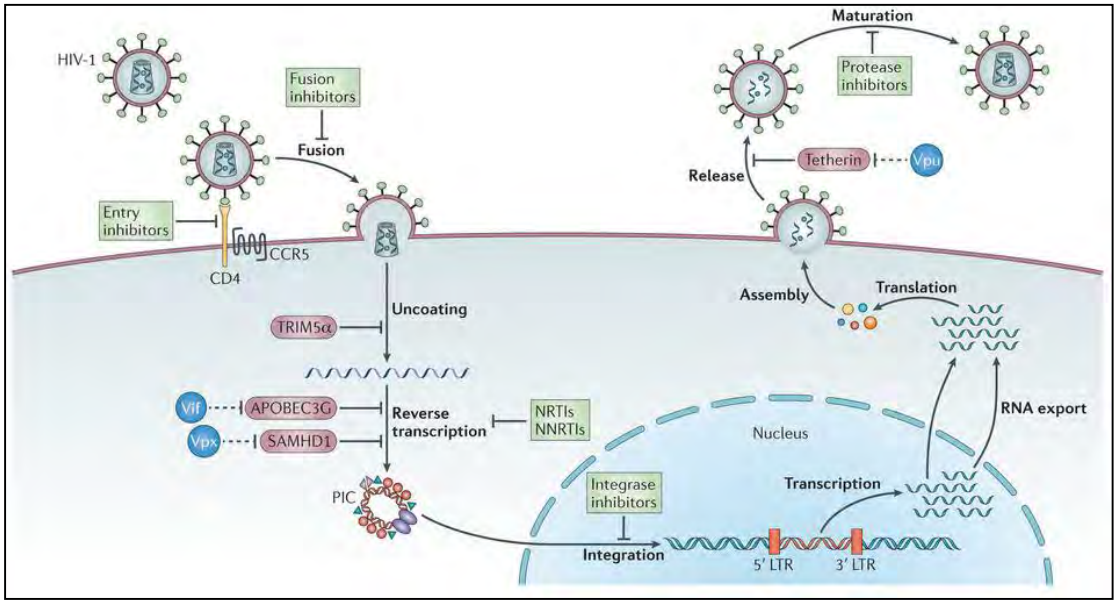

Figure 1.9. Schematic representation of the replication cycle of HIV showing crucial steps targeted by the current antiretroviral treatment drugs (163)**.**

1.5.2 HIV Reservoirs

Although HAART has proven effective at controlling HIV infection and reducing AIDS-related mortalities, the current drug regimens are not able to completely eradicate the virus from the body. The majority of HIV infected individuals on HAART achieve full suppression as indicated by the undetectable viral load levels for a long period of time. However, upon withdrawal the viral load rebounds rapidly and this is accompanied by a decline in $CD4^+$ T cell count (164). Previous studies have shown the existence of the provirus in latently infected resting memory $CD4^+$ T cells

(165) and these HIV reservoirs can later produce infectious virus even after successful treatment as a result of cellular activation (166–168). Because HIV DNA is already integrated into the host genome, it is believed that it can lie dormant for as long as the infected individual is alive. Some studies have attempted to see if activating the latently infected cells with reactivation drugs such as histone deacetylase (HDAC) inhibitors followed by ARVs would clear the virus from the body. Viral clearance was achieved when the HDAC inhibitor valproic acid was administered together with ART; the two studies assessed a small number of HIV infected individuals and hence provided proof of concept that HIV can be eliminated from persistent reservoirs and that HDAC inhibitors can be therapeutically used in man against HIV (169, 170). In parallel with treatment strategies and other measures for preventing infection and spread, more studies are needed to further explore the use of these inhibitors on clearing the viral reservoirs. An effective HIV vaccine is urgently needed.

1.6 HIV VACCINE STRATEGIES

The only way to combat the HIV/AIDS epidemic is through an effective HIV vaccine. An ideal HIV vaccine must completely block infection and provide sterilizing immunity and should be able to stimulate both broadly neutralizing antibodies and cell-mediated immune responses (171). However, a more realistic goal for an effective HIV vaccine would be one that is able to significantly reduce VL and delay clinical disease progression after infection hence reducing transmission to uninfected individuals (172). Several vaccine concepts have been studied with no success mainly due to the high genetic variability of HIV, lack of knowledge of the immune correlates of protection, lack of generation of broadly neutralizing antibodies and the absence of a relevant animal model (149). There are no clear correlates of immune protection. Therefore, more research is still required to identify what immune

responses an effective HIV vaccine should elicit. The correlates of immune control of HIV infection still need to be clearly defined.

1.6.1 The STEP and Phambili trials

The STEP and Phambili vaccine trials assessed the efficacy of Merck Ad5 gag/pol/nef subtype B HIV-1 preventive vaccine in South Africa (Phambili trial) and in the Americas, Caribbean, and Australia (STEP trial) and was aimed at eliciting T-cell-mediated immune responses that would provide complete or partial protection from HIV infection or a decrease in viral load after acquisition (173, 174). The trial was a was a double-blind, placebo-controlled, randomised trial that recruited HIV-1 uninfected, sexually active adults and eligible participants were randomly assigned placebo or vaccine group by a computer program. Disappointingly, the MRKAd5 HIV-1 vaccine in the Phambili trial did not prevent HIV-1 infection or lower viral load set point but managed to elicit interferon-γ (IFN-γ) - secreting T cells that were capable of recognizing both clade B (89%) and C (77%) antigens (174). In the STEP trial, the vaccine was also highly immunogenic as seen by induction of HIV-specific $CD8^+$ T cells (175). However, there was increased HIV acquisition amongst the vaccinated group in both trials and so because of futility in the STEP trial, both vaccine trials were discontinued (174, 176–178).

1.6.2 The RV144 HIV Vaccine trial

In 2009 the first ever HIV vaccine reported to have shown a modest efficacy was the Thai HIV Vaccine efficacy trial also known as the RV144 trial conducted in Thailand. This vaccine combination, which was based on HIV strains commonly circulating in Thailand, showed a 31.2% reduction in the incidence of HIV infection. The trial was conducted as a community-based, randomized, multicenter, double-blind, placebo-controlled efficacy trial, and evaluated four priming injections of a recombinant

canarypox vector vaccine (ALVAC-HIV [vCP1521]) including two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) (179). Following the exciting results of the Thai vaccine trial, a case-control analysis was conducted in order to identify antibody and cellular immune correlates of infection risk. It was concluded from the analyses that V1V2 antibodies may have contributed to protection against HIV-1 infection, whereas high levels of Env-specific IgA antibodies may have mitigated the effects of protective antibodies (180). Individuals presenting with high plasma levels of Env-specific IgA antibodies had a 54% increased HIV infection rate; same rate as for individuals in the placebo group suggesting that these antibodies disrupted the protective effect of the vaccine. Therefore, these findings suggest that an effective HIV vaccine with improved efficacy should elicit higher levels of V1V2 antibodies and lower levels of Env-specific IgA antibodies (180).

1.6.3 The CMV vector based SIV vaccine

The negative human vaccine-trial results raised questions about the whole concept of a CTL-inducing vaccine against HIV. In the recent years another study took a different approach. In an animal model setting, a rhesus monkey cytomegalovirus (RhCMV) vector was employed together with recombinant SIV genes to act as immunogens that would elicit strong and persisting effector memory CTL responses in vaccinated animals (181, 182). Of the vaccinated monkeys that were challenged with pathogenic SIVmac239 virus, 50% cleared the virus soon after peak vireamia during the acute infection phase. This type of vaccine elicited atypical $CD8^+$ T cell responses that were broad. However, it is not clear whether protection was dependent on these atypical $CD8^+$ T cell responses (171). The *in vivo* removal of $CD8^+$ T cells of the monkeys that had cleared the virus did not cause rebound of vireamia in these animals (181, 182), raising more questions about the immune correlates of protection

in these animals. It would be useful to further study the reasons for protection especially in the 50% of the monkeys that cleared the virus upon challenge. Nevertheless, these data further provide an opportunity to identify the correlates of protection and design an improved human HIV vaccine that would elicit strong and protective $CD8^+$ T cell responses.

1.7 IMMUNITY TO HIV

1.7.1 Innate immune responses to HIV

The innate immune response marks the very first line of defence against invading pathogens. Previous work has suggested that innate immune responses play an important role in the control of HIV infection especially during the early phase of infection as well as determining the establishment of infection and the subsequent rate of disease progression (183, 184). The reduction in viral replication observed during the acute phase often occurs prior to the induction of adaptive immune responses such as $CD8^+$ T cell responses confirming the involvement of innate responses at this stage of infection (185).

The innate immune system utilizes a number of cellular and soluble factors that work in order to elicit a rapid response following the recognition of pathogen associated molecular patterns (PAMPs) (186). There are several classes of pattern recognition receptors (PRR) and these including the three best known: the (RIG-I)-like receptors (RLRs), the toll-like receptors (TLRs), and the nucleotide oligomerization domain (NOD)-like receptors (NLRs) (186). Different combinations of these receptors can be activated primarily on innate immune cells hence leading to the induction of a nonspecific antiviral environment including release of cytokines such as interferons

(IFNs) (186). The IFNs can then blocking viral growth and finally initiate the induction of adaptive immune responses (186).

The innate immune system comprises of a number of cell subsets which all derive from the bone marrow. These include phagocytes (monocytes, macrophages, dendritic cells (DCs)), cytolytic cells (Natural killer (NK) cells and neutrophils) and professional antigen-presenting cells (DCs). The phagocytes are responsible for antigen clearance, while the cytolytic cells are responsible for the destruction of pathogen infected cells and finally the antigen presenting cells function by recognizing antigens and presenting them to the adaptive immune cells for the induction of an immune response and memory (187). The innate immune response is not only vital in the early HIV infection phase, but is also required for the activation of the adaptive immune responses. The main innate cell players involved in the control of HIV infection are the NK and DC cell subsets.

1.7.1.1 Natural killer cells

Natural killer (NK) cells recognize and kill virally infected cells and do not require prior activation in order to perform this function. They have the ability to produce cytokines and chemokines that attract and activate cells of the adaptive immune system (183). NK cells, in particular KIR3DS1$^+$ and KIR3DL1$^+$ NK cells have been shown to be involved in the acute control of HIV infection as demonstrated by their expansion during the peak of vireamia prior to seroconversion and the appearance of HIV-specific CD8$^+$ T cell responses (188). There is another small subset of T lymphocytes expressing surface markers that are characteristic of both T cells and NK cells known as NKT cells. These are now known to form a link between the innate and adaptive immune responses (183, 185).

HIV has evolved and developed mechanisms to escape from the early innate immune responses suggesting that the NK cells are indeed involved during acute HIV infection and are able to mount pressure on the virus (185, 189). However, HIV Nef protein is able to down-regulate major histocompatibility complex (MHC) class I molecules, specifically the Human leucocyte antigen (HLA)-A and HLA-B molecules which allows escape from CTL responses, but does not down-regulate HLA-C molecules which are the dominant ligands for NK KIR2D receptors thus allowing the virus to escape NK responses (190–192). Therefore, there is still more work to be done to better understand NK responses in the control of HIV and also develop ways of harnessing NK cellular responses in effective HIV vaccine design.

1.7.1.2 Dendritic cells

Dendritic cells (DCs) are the professional antigen presenting cells. Their main role is to recognize and present pathogen products to T cells in order for an immune response to be elicited. They respond rapidly following infection through recognition of viral products via pattern recognition receptors (187). Human plasmacytoid DCs (pDCs) produce cytokines such as interferons which mediate direct effector and immune-regulatory functions, which stimulate adaptive immune responses as well as mediate rapid antimicrobial effector functions (193, 194).

Conventional DCs can be found in the skin, blood and mucosal surfaces. Because they can be found at the mucosal surfaces, they are able to come in to direct contact with the HIV. Following this encounter, they engulf HIV and are triggered to migrate to the draining lymph nodes where they can perform their function of eliciting the adaptive immune responses by presenting antigens to T and B cells (194, 195). The activation of pDCs by HIV occurs via upregulation of the expression of costimulatory and MHC molecules, maturation markers, increasing T cell stimulatory activity, and

induction of the production of type I interferons and tumour necrosis factor alpha (TNF-α) (195).

A rapid decline of pDCs during HIV infection has been observed during both acute and chronic phases of infection and this decline was associated with high viral load and low $CD4^+$ T cell count (196–198). In addition, HIV has evolved and developed mechanisms to prevent degradation by DCs in order to gain access to its primary target cells $CD4^+$ T cells. DCs are also involved in the transmission of HIV to $CD4^+$ T cells (199). Thus DCs are a key target to investigate the ways of preventing HIV transmission.

1.7.2 Humoral immune responses to HIV

HIV infected individuals have the ability to develop antibodies against HIV early during infection soon after the onset of $CD8^+$ T cell responses and establishment of viral set point following acute HIV infection. However, these antibodies are not able to neutralize HIV or resolve natural HIV infection (200–203) but can mount selective pressure on the virus hence causing it to evolve rapidly (138, 202, 203). The ideal neutralizing antibodies are usually detected several months later to 2 years following seroconvertion (201, 204). We know from other viral infections that the most effective vaccines produced to date elicit neutralizing antibodies (nAbs). Since the envelope mediates HIV entry, it is ideal to design vaccines that act against the Env region in order to prevent viral entry and further infection (205). Most of the recent HIV vaccine research studies have focused on finding and understanding the possible mechanisms of virus inhibition and the development of strategies to prevent infection (206). In order to achieve full viral neutralization, HIV specific antibodies must recognize functional sites on the HIV-1 envelope spike, gp160, which is a hetero-trimer composed of gp120 and gp41 protein subunits (207). Although there is still no

effective vaccine against HIV, the RV144 study showed some modest protection and demonstrated that antibodies raised against the variable loop 2 of gp120 in vaccinees were the main positive predictive factor for protection (180).

Only a small percentage of HIV infected individuals known as 'elite neutralizers' are able to develop broadly neutralizing antibodies (bnAbs) (201, 208–210). These bnAbs are active against hundreds of different cell-free viral quasi-species from various HIV-1 subtypes (211). However, the bnAbs are not able to completely suppress viral replication in infected persons mainly due to viral escape (202, 203). Therefore, antibodies against HIV may play an important role by reducing the risk of infection and vertical transmission (211, 212).

1.7.3 Host restriction factors

Host restriction factors that are known to be potent inhibitors of HIV replication include APOBEC3G, TRIM5-alpha and Tetherin (213) and how they perform their function is discussed below.

APOBEC3G (apolipoprotein B mRNA-editing enzyme-cytidine deaminase enzymes) is a strong inhibitor of HIV replication and is counteracted by the viral Vif protein (49). However, in the absence of Vif, APOBEC3G is able to convert cytosine bases of the viral minus- strand DNA to uracil, which results in a G to A hypermutation in the complementary positive sense DNA strand (214–216). The U-rich transcripts can either be degraded by activation of the cellular uracil-DNA glycosylase hence causing failure of the reverse transcription or can yield non-functional proviruses as a result of the G to A hypermutation (216, 217). HIV has evolved and developed some escape mechanisms and is then able to use its protein Vif, which then interacts with APOBEC3G and causes its degradation via the ubiquitin-dependent proteosomal

pathway (49, 97, 218) and therefore disrupting the inhibitory properties of APOBEC3G and allowing the virus to replicate (214, 217).

Tetherin, which is also known as BST-2, CD317 or HM1.24 is a potent antiviral factor that inhibits HIV replication (53). It functions by inhibiting the release of newly formed virions from the host cell surface through binding to the cell surface at the virion budding site (53). HIV has evolved and developed some escape mechanisms and is then able to use its protein Vpu, which interacts directly with the transmembrane domain of tetherin and mediates down-regulation and degradation of tetherin levels at the cell surface (53). The exact mechanism of how tetherin functions is not fully understood.

TRIM5α - tripartite motif-containing motif 5 alpha, the most studied TRIM family member is known to mediate species-specific and early block of retrovirus infection (219, 220). TRIM5α was first identified in rhesus monkeys (rh) as a factor that blocks HIV replication (220). It has been shown that the rhTRIM5α is a lot more potent than the human TRIM5α at inhibiting virus replication (220). The exact mechanism of how TRIM5α inhibits HIV replication is not fully understood. However, it is known that TRIM5α trimers interact with hexameric capsids and block replication (221).

1.7.4 The major histocompatibility complex (MHC) class I molecules

Genome-wide association studies (GWAS) have identified HLA class I molecules as the major determinant of HIV control (222, 223). The major histocompatibility complex (MHC) or in humans known as Human Leucocyte Antigen (HLA) class I molecules, are highly polymorphic and map to the short arm of chromosome 6. They are responsible for processing and presenting antigens to T cells (224). Class I molecules present endogenous peptides to $CD8^+$ T cells while class II molecules

(HLA-DR, -DQ, and –DP) bind to and present antigens of extracellular origin to $CD4^+$ T cells (225). There are three classical human MHC class I alleles (HLA-A, -B and -C), which encode for cellular surface molecules responsible for processing and presenting antigens usually from intracellular pathogens to $CD8^+$ T cells. The peptide-binding groove of the HLA molecule is the most variable region and therefore dictates the binding motif for the peptides presented by each HLA allele. There are 6 pockets within the binding groove (A-F) and these differ in size. Of the six pockets, 2 exhibit a particular preference to one or two amino acids while the other 4 are flexible and function as secondary anchors (226).

HLA typing may be performed serologically and by molecular methods. HLA nomenclature is usually resolved to 4 digits. For example, in the HLA-A*24:02, 'A' represent the locus, this is followed by an asterisk, the first two digits (number 24) represents the allele family/group mostly determined serologically. This is also referred to as the antigen level resolution. Finally the second set of digits (the number 02) specifies the allele. For example, HLA-A*24:02 represents the second allelic member of the A*24 group. This is also known as the allele or subtype level resolution (227). HLA alleles can also be classified into supertypes that comprise HLA alleles of different types/subtypes that share peptide-binding specificities. The HLA B58 supertype, for example, comprises subtypes belonging to the B*57 and B*58 groups, among others (227). The HLA B7 supertype comprises subtypes belonging to the B*07:02, B*39:10, B*42:01, B*42:02 and B*81:01 groups. These alleles have similar peptide-binding motifs that can frequently bind the same peptides (228).

The HLA class I loci has been shown to display the strongest effect on HIV disease control (222). HLA molecules are known to contribute to HIV disease control

primarily through $CD8^+$ T cells. Additionally, HLA molecules also act as ligands for killer cell immunoglobulin-like receptors (KIRs), which are involved in controlling the NK cell activity. Furthermore, polymorphisms within the KIRs in particular KIR3DS1 have been associated with disease progression (229).

1.7.5 Adaptive immune responses to HIV

Adaptive immune responses or cell-mediated immune responses have been implicated in the control of HIV replication. Unlike the innate responses that occur rapidly, the adaptive immune responses are delayed and dependent on presentation of pathogen-derived peptides in the context of HLA molecules by professional antigen presenting cells (APCs). The main players of the adaptive immune responses are the T cells including $CD4^+$ and $CD8^+$ T cells. HIV preferentially infects $CD4^+$ T cells (230) and $CD8^+$ T cell recognise viral peptides presented by antigen presenting cells in the context of MHC class I molecules in order for an immune responses to be elicited, which occurs when $CD8^+$ T cells kill the virus infected cells. $CD8^+$ T cells are known as effector cells due their ability to recognize and kill virus-infected cells.

1.7.5.1 $CD8^+$ T cells

Immunological processes in the early phase of HIV infection shape the outcome of disease progression. During the acute phase infected subjects experience a high level of vireamia, followed by a temporal reduction to a viral set point coincident with the appearance of HIV specific $CD8^+$ T cells, and these cells are said to peak 1-2 weeks later following the decline in vireamia (133, 231–233). HIV-specific $CD8^+$ T cells can recognise and kill virus infected cells by releasing soluble factors that suppress viral replication. $CD8^+$ T cells respond in several ways during infection in order t clear virus infected cells. These include the production and release of various cytokines and chemokines and the exocytosis of pre-formed cytolytic granules containing perforin

and granzyme. The main functions known to be relevant in antiviral immunity are proliferative capacity, IL-2, IFN-γ, TNF-α, and MIP-1β cytokine and chemokine secretion and cytotoxicity as measured by perforin/granzyme B release and CD107a/b mobilization as a marker of degranulation (234–236).

Subsequent to the initial CTL response is the emergence of an antibody response soon after the vireamia has been reduced to lower levels (149, 231). Previous animal model studies have shown that in simian immunodeficiency virus (SIV) infection, depletion of $CD8^+$ T cells led to a rapid and marked increase in vireamia, and reconstitution of $CD8^+$ T cells led to a decline in viral load (237, 238). In addition, several previous studies also demonstrated that vaccine-induced $CD8^+$ T cell responses were able to protect macaques challenged with a SIV-HIV chimera from progression to AIDS (239–242). Furthermore, $CD8^+$ T cells also play an important role in the control and limitation of viral replication during chronic HIV/SIV infection (243–247). These findings support the view that $CD8^+$ T cell responses play a central role in the containment of vireamia in both HIV and SIV infection.

It is clear that not all CTL responses are effective in mediating viral control since the majority of HIV-infected persons eventually progress to disease. In addition, lack of control has been partially explained by the emergence of escape mutations that evade CTL mediated immune control (248, 228). Previous studies have shown that escape occurs early during HIV infection and is driven by $CD8^+$ T cell responses (234, 249–251). The immune responses to the conserved Gag polyprotein in particular have been associated with favourable disease outcomes (252–257). On the other hand, the selection of escape mutations within functionally important Gag epitopes has been shown to occur at a cost to the virus hence reducing viral replication capacity and resulting in maintenance of low viral loads as a consequence of viral variants with

reduced viral replication capacity (258). Ultimately, compensatory mutations and immune relaxation occur and there is loss of viral control (259).

1.7.5.2 $CD4^+$ T cells

$CD4^+$ T cells also play a role in HIV infection. They are the main targets for HIV infection hence their decline during the course of infection through killing by the virus itself or via induced apoptosis (230, 260, 261). Activated $CD4^+$ T cells function by mediating T helper 1 responses via secretion of cytokines (IL-2 and IFNγ); also required for the maintenance of the $CD8^+$ T cell responses (253, 262–265). These effector functions of the $CD4^+$ T cells are evident and persist during acute and chronic HIV infection (266). Because high frequencies of dual IFNγ and IL-2 secreting $CD4^+$ T cells have been seen in controllers, the suppression of viral replication observed in controllers was then associated with the presence of these polyfunctional HIV specific $CD4^+$ T cell with proliferative capacity (267–269) However, $CD4^+$ T cells may lose the functional ability to produce IL-2 and to proliferate resulting in the lack of viral control (268, 270, 271).

1.8 HIV NATURAL CONTROLLERS

Durable control of viral replication in the absence of ART is achieved in a small percentage of HIV infected individuals. These individuals can be divided into different categories depending on their viral load and $CD4^+$ T cell count and are termed long-term nonprogressors (LTNPs). LTNPs can be further divided into viremic controllers (VCs) and elite controllers (ECs).

1.8.1 Long-term nonprogressors (LTNPs)

Although different studies have varying definitions of LTNPs, most studies define this group as HIV seropositive individuals who lack disease progression, with a stable

to elevated $CD4^+$ T cell count for a duration of 7 – 10 years of symptom free HIV infection (272). These individuals have never been on ART and some of the individuals are known to have been infected for 20 – 25 years (273). They account for 2-5% of the HIV infected population (274)

1.8.2 Elite controllers (ECs)

Elite controllers (ECs) or elite suppressors (ESs) represent a small subset of the LTNPs that maintain HIV viral load of below the limit of detection (less than 50 HIV RNA copies/ml of blood) of the viral load assay for long periods in the absence of ART. They account for 1% of the infected population (274).

1.8.3 Viremic controllers (VCs)

Viremic controllers on the other hand display low but detectable viral load levels of between 50 and 2,000 HIV RNA copies/ml of blood in the absence of ART (274). They also display stable $CD4^+$ T cell counts.

1.9 FACTORS ASSOCIATED WITH NATURAL CONTROL OF HIV

Studying the natural controller groups of HIV infected population can provide some invaluable information regarding HIV pathogenesis, effective HIV vaccine strategies and development of novel therapeutic agents to combat HIV. A number of studies have attempted to determine the possible mechanisms associated with the natural control of HIV in the absence of ART in LTNPs, ECs and VCs. The factors associated with viral control or disease non-progression include host factors (HLA alleles, mutations in co-receptors, restriction factors), viral factors (mutations and deletions) and immunological factors (cellular immune responses, protective HLA class I alleles, innate and humoral responses).

1.9.1 Viral factors

Infection with attenuated virus strains has remained one of the reasons for non-progression amongst LTNPs and ECs. Previous studies on these groups on individuals have demonstrated that LTNPs compared to progressors harboured replication incompetent virus strains that were less evolved and less capable of evading host immunological responses (275, 276) and attenuating gene deletions known to be associated with viral control (277, 278). In another study, it was demonstrated that 5 out of 6 HIV infected individuals became LTNPs following blood transfusion from 1 donor who was also a LTNP and it was concluded that they had been infected with a less virulent strain of HIV (279). In yet another study, 6 blood recipients from 1 donor became LTNPs and remained asymptomatic for 14 to 18 years following infection. Sequencing of the full HIV genome showed that they had been infected with a defective virus strain that contained a large deletion in the long-terminal repeat of the Nef gene (280). Additionally, the virus isolated from other LTNPs was found to replicate a lot slower and infection of the $CD4^+$ T cells was less effective (281). These data may suggest that non-progression observed in LTNPs and ECs is due to their infection with attenuated viruses. However, in contrast, some studies have been able to demonstrate that some LTNPs harboured replication competent virus strains (282–284) and are rarely infected with similar Nef-deleted attenuated strains of HIV (282, 285, 286). The first study to sequence the whole HIV genome from ECs found some minor mutations that did not seem to affect HIV function (282) and another study showed that virus isolates from ECs were able to grow normally in $CD4^+$ T cells (287). Furthermore, some data suggest that low viral load observed in some individuals may have been as a result infection with drug resistant HIV which may have had a fitness cost on the transmitted virus (288). Overall, the data remain

inconclusive and has not ruled out attenuated viruses as a possible explanation for disease non-progression observed in some LTNPs and ECs.

1.9.2 HLA B*57/B*27 and other protective HLA class I alleles

The majority of HIV specific $CD8^+$ T cell responses are restricted by HLA-B alleles. Furthermore the expression of different HLA alleles has been found to be associated with differential control of vireamia where a number of HLA class I alleles are associated with low viral load (HLAs B*27, B*57, B*58:01, B*81:01 and A*74:01) while others are associated with disease progression or high viral load (HLAs B*18:01, B*58:02) (222, 223, 254, 289–293).

HLA B*57 variants have been strongly associated with the control of HIV infection through the induction of cross-reactive responses that are mounted against immunodominant Gag epitopes (294–296). This allele has also been found to be overrepresented in HIV controller study groups where for example, in a cohort of 30 or more individuals, between 8 and 63% of either LTNP or ECs carried the allele (272, 297), with ECs having the highest frequency of this allele. The actual mechanism by which control occurs amongst HLA B*57 positive individuals is not fully understood. However, in addition to B*57 restricting broad and protective $CD8^+$ T cell responses within the Gag region of HIV, these responses can put pressure on the virus leading to escape, which in turn has a significant negative impact on viral fitness (298, 299).

HLA B*27 is another allele that has been associated with viral control (294, 300). Previous studies suggest that protection from non-progression amongst HIV subtype B clade B*27 positive individuals was achieved by responses to the KK10 Gag epitope (KRWIILGLNK, Gag263-272) (248, 301–305) while B*57 positive

individuals mainly responded to epitopes KF11 (KAFSPEVIMF) and TSW10 (TSTLQEQIGW) (306).

Other protective HLA class I alleles include HLAs B*13 (307), B*51:01 (292), B*58:01 (290), B*81:01 (258) and A*74:01 (293). In most of the cohorts, 90-95% of LTNPs carry at least one of the protective HLA class I alleles (297). The mechanism of control amongst individual with protective HLA class I alleles still remain understudied.

1.9.3 The role of $CD4^+$ T cells in viral control

The role of $CD4^+$ T cells in the control of HIV replication has not been very clear. However, robust, polyclonal and Gag specific $CD4^+$ T cell responses have been detected in LTNPs and correlated directly with low viral load (308–311). These cells have been found to have a robust T helper 1 phenotype as evidenced by their production of cytokines; IL-2 and IFNγ (312, 313) and proliferative capacity (267–269). HIV-specific $CD4^+$ T cells may also have a cytotoxic role (314, 315). Therefore, the role and contribution of $CD4^+$ T cell in the control of viral replication amongst LTNPs and ECs is still debatable and needs to be resolved.

1.9.4 The role of HLA and $CD8^+$ T cells in viral control

$CD8^+$ T cell are the most important mediators of viral control especially amongst LTNPs (316). Previous work suggests that control of vireamia can be attributed to $CD8^+$ T cell responses against Gag (252–256, 317–320) and these effective responses are mainly restricted by protective HLA class I alleles such as B*57/B*27 (317). Protective HLA class I alleles tend to present epitopes that are within the conserved region Gag p24 (321–323). In addition, $CD8^+$ T cells from LTNPs were found to be more polyfunctional and to have a higher proliferative capacity compared to

progressors (235, 302, 324, 325). These data suggest that a more polyfunctional $CD8^+$ T cell phenotype is associated with viral control. However, not all $CD8^+$ T cell responses are truly protective, this is because these responses do not clear the infection and the individuals eventually progress to AIDS. $CD8^+$ T cell exhaustion, viral mutational escape and non-mutational viral escape have been suggested as possible explanations for immune failure to clearing infection (203, 248, 249, 303, 326). Hence, there is still a need to further identify CTL characteristics associated with viral control.

1.10 GAPS IN KNOWLEDGE

There are gaps in knowledge as to what really accounts for the control of HIV replication especially amongst individuals with protective HLA class I alleles. There is conflicting data regarding viral control amongst LTNPs and ECs, which still requires resolving. Although protective HLA class I alleles have been associated with control of viral replication, the majority of individuals expressing these protective HLA class I alleles eventually progress to disease. There is need to clearly identify and define the correlates of immune protection from disease progression in individuals with protective HLA class I alleles. Furthermore, amongst controllers with protective HLA class I alleles, the infection is so dynamic in that the loss of control is encountered in some individuals who were previous controllers. It is unclear whether immunological or virological mechanisms of viral control and lack of control are involved in such cases.

In addition, there are some limitations to the findings from previous studies of HIV controllers; most of the studies have been cross-sectional in terms of study design such that the long-term outcome of infection was never investigated, some only analyzed individuals following acute HIV-1 infection when most persons are not

controlling viral replication, or have only investigated one specific T cell parameter. Here, we had an opportunity to study individuals with and without protective HLA class I alleles in a longitudinal study design and investigated multiple T cell properties (breadth and magnitude of $CD8^+$ T cell responses, ability of $CD8^+$ T cells to induce escape within the conserved Gag region, $CD8^+$ T cell proliferation and polyfunctionality and ex-vivo ability of the $CD8^+$ T cells to inhibit virus replication) all at the same time.

1.11 THE STUDY RATIONALE AND HYPOTHESIS

There is compelling evidence linking the control of HIV replication to many host and viral factors including the expression of protective HLA class I alleles, strong immune pressure as represented by $CD8^+$ T cells responses on the virus that targets conserved regions of the virus and selection of immune escape mutations that are associated with viral fitness cost. Although individuals with protective HLA class I alleles are overrepresented among elite and HIV controllers, loss of viral control and disease progression is also encountered among these individuals. However, the exact events that lead to this loss of control have not been fully described. We aimed to study a group of individuals who possess protective HLA class I alleles in an attempt to explain the mechanisms associated with viral load control in these individuals and loss of viral control in some individuals by assessing the virological and immunological events that may be associated with control or loss of viral control amongst these individuals. A control group of individuals without protective HLA class I alleles was included to compare and contrast mechanisms of control and lack of control.

We hypothesised that a combination of the following factors will be responsible for the viral control in individuals with protective class I alleles:

1. Immune responses targeted towards specific epitopes located in highly conserved Gag regions,

2. Viral evolution and/or unusual polymorphisms that affect viral fitness,

3. And highly polyfunctional $CD8^+$ T cells that are capable of inhibiting viral replication *ex vivo*.

1.12 SPECIFIC OBJECTIVES

1. To characterize viral load dynamics and clinical disease progression in ARV-naïve HIV-1 subtype C chronically infected participants with and without protective HLA class I alleles.

2. To study and describe the magnitude and breadth of HIV specific $CD8^+$ T cell immune responses in individuals with and without protective HLA class I alleles and associate those with disease progression.

3. To study and describe the evolution of HIV specific immune responses in individuals with and without protective HLA class I alleles and associate those with disease progression.

4. To study viral evolution within Gag and its impact on disease progression.

5. To study the function (in terms of polyfunctionality and proliferative capacity) of $CD8^+$ T cells amongst individuals with controlled viremia and associate these $CD8^+$ T cell properties with viral control or lack of control upon longitudinal follow up.

6. To study the *ex vivo* ability of $CD8^+$ T cells to suppress HIV replication associate this function with disease progression or the lack thereof.

1.13 OUTLINE OF THE THESIS

Chapter 1 gives an overview of HIV epidemiology, replication, mechanisms of HIV control covering the role of innate, HLA and adaptive immunity in the control of HIV replication during chronic HIV-1 subtype C infection.

Chapter 2 gives an overview of the methodology and techniques employed in the research study.

Chapter 3 gives a comprehensive cohort description and defines and characterizes the immunological parameters (breadth and magnitude of $CD8^+$ T cell responses) associated with differential control of vireamia amongst individuals with and without protective HLA class I ales at enrolment (at baseline).

Chapter 4 describes the evolution of $CD8^+$ T cell responses and how they associate with divergent disease progression patterns observed in individuals with and without protective HLA class I alleles. The chapter further describes the evolution of Gag and determines whether $CD8^+$ T cell responses induced escape in the individual with and without protective HLA class I alleles and further associated escape with disease progression in these individuals.

Chapter 5 provides the functional ability of $CD8^+$ T cells to perform more than 1 function (polyfunctionality), proliferate and suppress viral replication and associated these functions with disease progression in individuals with and without protective HLA class I alleles.

Chapter 6 gives an overall discussion of the findings of this research project and highlights the findings' contribution to the body of knowledge of mechanisms of HIV control.

CHAPTER 2: MATERIALS AND METHODS

2.1 STUDY COHORT

Seventy HIV-1 subtype C chronically infected participants from the Sinikithemba (SK) cohort in Durban, South Africa (254, 258, 290) were included in the current study. Participants were ART-naïve at all time points analysed. Subtype had been determined previously by one of the researchers Dr. Jaclyn Mann. The details can be found in the following publication (258). The date of HIV infection is unknown for all participants. Viral loads (VL) for all subjects were measured every 6 months by the Roche amplicor version 1.5 and CD4 T cell counts were measured every 3 months by TruCount technology using flow cytometry. The University of KwaZulu-Natal's Biomedical Research Ethics Committee approved the study and all subjects provided written informed consent for participation in the study. The characteristics and demographic data for the 70 subjects studied are presented in Table 2.1 below.

Table 2.1. Clinical and demographic characteristics for the HIV-1 Clade C chronically infected individuals studied.

Characteristic	Median (interquartile range) or percentage (%)
Gender	78.5% female, 21.4% male
Age (at baseline)	32 (28 - 37) years
Viral load (at baseline)	109,000 (637 – 189,000) HIV RNA copies/ml
$CD4^+$ T cell count (at baseline)	446 (324.5 – 586.3) cells/mm^3
Median follow up time	48.50 (36 – 66.25) months

2.2 DEFINITION OF GROUPS AND SUB-GROUPS

We divided the 70 participants studied into two groups based on their VL at enrolment:

i. Baseline viremic controllers (bVC) were enrolled with a VL of equal to or below 2,000 HIV RNA copies/ml.

ii. Baseline progressors (Prog) were enrolled with a VL equal to or above 100,000 HIV RNA copies/ml.

Longitudinal follow-up of bVCs allowed us to further distinguish this group in to 2 subgroups based on progression stage:

i. Viremic controllers (VC) were defined as individuals who were enrolled with a VL of less than 2,000 HIV RNA copies/ml and maintained this low viral load for the entire period of enrolment.

ii. Failing viremic controllers (fVC) were defined as bVC that were enrolled with a VL of less than 2,000 HIV RNA copies/ml but lost virological control. We defined the loss of viral control as an increase in VL to more than 10,000 HIV RNA copies/ml at a minimum of 2 time points during the time of follow-up.

These groups are illustrated in the following Figure 2.1.

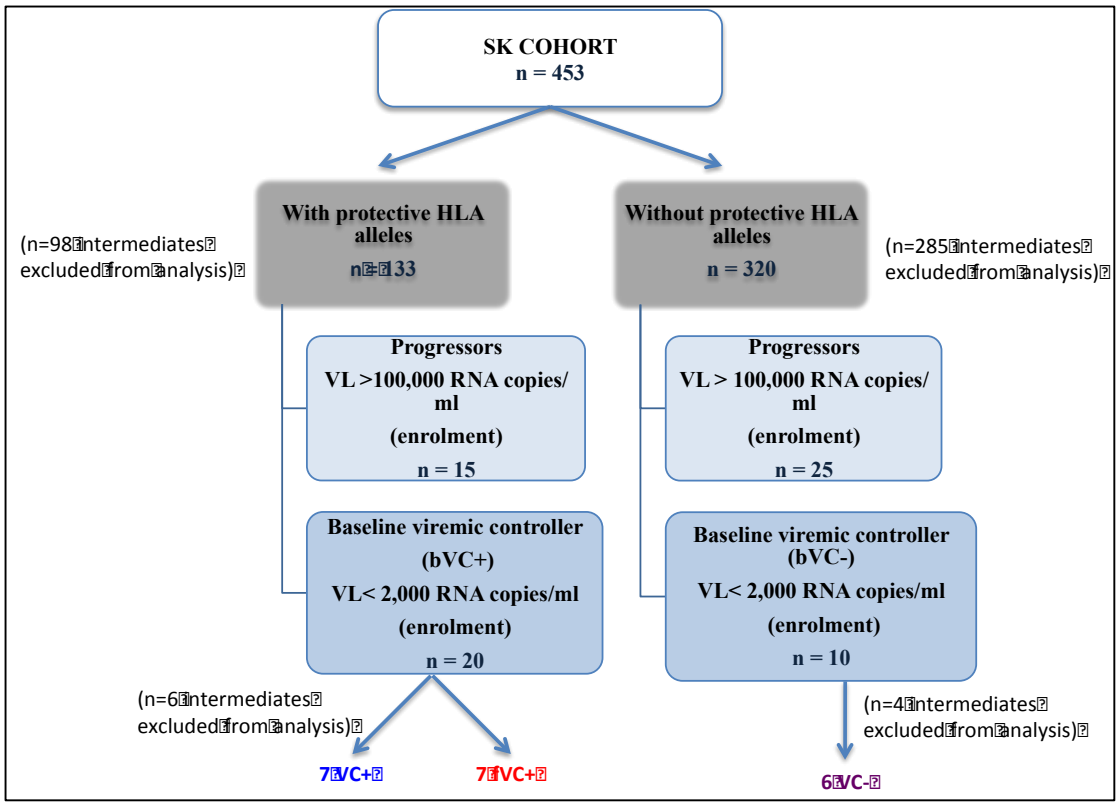

Figure 2.1. Showing the distribution of study participants in to different groups and subgroups. The 70 HIV-1 subtype C chronically infected ARV naive participants were from the Sinikithemba (SK) cohort in Durban, South Africa. The 70 subjects were selected based on availability of samples over a minimum of 2-year follow up period. Viremic controllers (VC+/-) were enrolled with a VL of below 2,000 HIV RNA copies/ml and maintained this low VL for the entire enrolment duration. Failing VC (fVC) were enrolled with a VL of below 2,000 HIV RNA copies/ml and later VL increased to more than 10, 000 HIV RNA copies/ml at a minimum of 2 time points. Of the 20 bVC with protective HLA class I alleles, 7 were VC and 7 were fVC, while the remaining 6 did not fall in either of the 2 subgroups. Of the 10 bVC without protective HLA class I alleles, 6 were VC and none lost viral control while the remaining 4 did not fall in either of the 2 subgroups.

2.3 IMMUNOLOGICAL METHODS

2.3.1 Thawing of cryopreserved PBMCs

Peripheral blood mononuclear cells (PBMCs) that had previously been cryopreserved in freezing solution (10% dimethyl sulfoxide (DMSO) in filtered heat inactivated fetal calf serum (FCS) (Gibco, NY, USA) were thawed quickly using a water bath set at 37°C and immediately transferred in to a 15ml falcon tube containing 9ml of R10 medium (RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10%

FCS, 1% L-Glutamine (Sigma), and 1% penicillin/streptomycin (Gibco). This was followed by centrifugation at 1,800 rpm (Eppendorf centrifuge 5810R, Merck, Germany) for 10 minutes after which the supernatant was discarded into a waste bucket containing 2% Virkon (DuPont de Demours, South Africa). The cells were washed twice and left to rest in R10 medium at 37°C with 5% CO_2.

2.3.2 Cell Counts

Cell count was carried out using a hemocytometer in a ratio of 1:9 with trypan blue stain solution (Sigma). In brief, 10 µl of the cell suspended in 10ml of R10 was added to a centrifuge tube containing 90 µl of the trypan blue stain solution and mixed well. Ten µl of the cells plus trypan blue solution was dispensed on to the hemocytometer and viewed under an electronic microscope (Olympus CH20 Upright microscope) for counting. The blue stain solution selectively penetrates the membranes of dead cells hence colouring them blue, so when used one is able to differentiate live from dead cells. A magnification of x10 allowed easy recognition of the desired cell types, which in our case were the PBMCs. The hemocytometer has 9 large squares and within each larger square are 9 squares. We counted round live cells in 3 large squares (top left, middle center and bottom right). The average of the 3 squares gave the number of million cells per 10 ml. The viability of the cells ranged from 0% to 96.61%. The % cell viability was determined by a formula (number of live cells)/(number of live cells+ number of dead cells)*100. The median viability was 86.36% (IQR) (64.86 – 92.06). Only cells with a viability of 65% or more were assessed.

2.3.3 HLA typing

HLA class I typing was performed on genomic DNA samples extracted from blood at the time of enrolment as described previously (290). The genomic DNA for HLA

typing was extracted using the Puregene DNA isolation kit for blood (Gentra Systems, Minneapolis, Minn.) according to the manufacturer's instructions. Genomic DNA samples were initially typed to an oligo-allelic level using Dynal RELITM reverse Sequence Specific Oligonucleotide (SSO) kits for the HLA-A, -B and -C loci (Dynal Biotech). Refining the genotype to the allele level was performed using the Dynal Biotech sequence-specific priming kits in conjunction with the previous SSO type. In cases where the alleles were still not defined to the allele level, bespoke sequence-specific priming primer mixes were then utilised. All HLA class I alleles in the IMGT allele release 2.4.0 were considered in the typing (327). Of the 70 patients identified for this study, 35 possessed protective HLA class I alleles (HLAs A*74:01, B*57:01/:02/:03, B*58:01 or B*81:01) (223, 289–291, 293, 328).

2.3.4 Synthetic HIV peptides

A panel of 410 peptides (18-mers overlapping by 10 amino acid residues), spanning the entire HIV-1 clade C consensus sequence together with optimal peptides with known HLA class I restriction patterns, were synthesized on an automated peptide synthesizer (MBS 396; Advanced ChemTech, Louisville, Ky. USA) and used in the Enzyme-linked immunosorbent spot (ELISpot) assay (329).

2.3.5 ELISpot assay

HIV immune responses were enumerated from freshly isolated whole PBMCs by the gamma interferon (IFNγ) ELISpot assay as previously described (290). PBMCs were stimulated with 410 consensus clade C 18-mer overlapping peptides (OLPs) covering the entire proteome followed by confirmations with individual peptides within a reactive pool at a final concentration of 2 μg/ml and optimal peptides corresponding

to each patient's HLA type, also at a final concentration of 2 µg/ml per peptide. The following 3 tables 2.2, 2.3 and 2.4 show the matrix layout.

Table 2.2: Positioning of OLP used in the Megamatrix in rows A and B

Pool A	OLPs	No of Peptides in pool	Pool B	Peptides												No Peptides in pool
A1	1-11	11	B1	1	13	25	37	49	61	73	85	97	121	x	x	10
A2	12-22	11	B2	2	14	26	38	50	62	74	86	98	122	x	x	10
A3	23-33	11	B3	3	15	27	39	51	63	75	87	99	111	123	x	11
A4	34-44	11	B4	4	16	28	40	52	64	76	88	100	123	124	x	11
A5	45-55	11	B5	5	17	29	41	53	65	77	89	101	113	125	x	11
A6	56-66	11	B6	6	18	30	42	54	66	78	90	102	114	126	x	11
A7	67-77	11	B7	7	19	31	43	55	67	79	91	103	115	127	x	11
A8	78-88	11	B8	8	20	32	44	56	68	80	92	104	116	128	x	11
A9	89-99	11	B9	9	21	33	45	57	69	81	93	105	117	129	x	11
A10	100-107	8	B10	10	22	34	46	58	70	82	94	106	118	130	x	11
A11	111-121	11	B11	11	23	35	47	59	71	83	95	107	119	131	x	11
A12	122-131	10	B12	12	24	36	48	60	72	84	96	x	120	x	x	9

Table 2.3: Positioning of OLP used in the Megamatrix in rows C and D

Pool	OLPs	No. Peptides in pool	Pool	Peptides												No. Peptides In pool
C1	145-156	12	D1	145	157	169	181	193	205	217	229	241	253	265	277	12
C2	157-168	12	D2	146	158	170	182	194	206	218	230	242	254	266	278	12
C3	169-180	12	D3	147	159	171	183	195	207	219	231	243	255	267	279	12
C4	181-192	12	D4	148	160	172	184	196	208	220	232	244	256	268	280	12
C5	193-204	12	D5	149	161	173	185	197	209	221	233	245	257	269	281	12
C6	205-216	12	D6	150	162	174	186	198	210	222	234	246	258	270	282	12
C7	217-228	12	D7	151	163	175	187	199	211	223	235	247	259	271	283	12
C8	229-240	12	D8	152	164	176	188	200	212	224	236	248	260	272	284	12
C9	241-252	12	D9	153	165	177	189	201	213	225	237	249	261	273	285	12
C10	253-264	12	D10	154	166	178	190	202	214	226	238	250	262	274	286	12
C11	265-276	12	D11	155	167	179	191	203	215	227	239	251	263	275	287	12
C12	277-288	12	D12	156	168	180	192	204	216	228	240	252	264	276	288	12

Table 2.4: Positioning of OLP used in the Megamatrix in rows E and F

Pool	OLPs	No. Peptides in pool	Pool	Peptides												No. Peptides In pool
E1	289-300	12	F1	289	301	313	325	337	349	361	373	385	397	409	421	12
E2	301-312	12	F2	290	302	314	326	338	350	362	374	386	398	410	422	12
E3	313-324	12	F3	291	303	315	327	339	351	363	375	387	399	411	423	12
E4	325-336	12	F4	292	304	316	328	340	352	364	376	388	400	412	424	12
E5	337-348	12	F5	293	305	317	329	341	353	365	377	389	401	413	425	12
E6	349-359	11	F6	294	306	318	330	342	354	366	378	390	402	414		11
E7	360-370	11	F7	295	307	319	331	343	355	367	379	391	403	415		11
E8	371-381	11	F8	296	308	320	332	344	356	368	380	392	404	416		11
E9	382-392	11	F9	297	309	321	333	345	357	369	381	393	405	417		11
E10	393-403	11	F10	298	310	322	334	346	358	370	382	394	406	418		11
E11	404-414	11	F11	299	311	323	335	347	359	371	383	395	407	419		11
E12	415-425	11	F12	300	312	324	336	348	360	372	384	396	408	420		11

The 96 well polyvinylidene difluoride-backed ELISpot plates (MAIP S45, Millipore, Bedford, Mass., USA) were pre-coated with 100 µl of anti-IFNγ antibody (1-D1k, 0.5 µg/ml, MabTech, Stockholm, Sweden) and incubated overnight at 4°C before use. Following incubation, the wells were washed 6 times with blocking buffer (1% fetal calf serum (FCS) (Gibco) in phosphate-buffered saline (PBS)) before addition of peptides at a final concentration of 2 µg/ml to a plate with 100 µl of R10 medium at

100,000 cells/well. For the controls, cells were added to 100 μl of media only (R10) in 4 wells as negative controls and an additional 2 wells containing phytohemagglutinin (PHA) at a concentration of 1 μg/ml/well were used as positive control. The plates were incubated at 37°C and 5% CO_2 overnight for 16 hours. Following this incubation, the wells were washed 6 times with 100 μl of PBS and 0.5 μg/ml of biotinylated anti-IFNγ antibody (7-B6-1, MabTech, Stockholm, Sweden) was added into each well and the plate incubated in the dark for 90 minutes at room temperature. The wells were then washed 6 times with PBS and 0.5 μg/ml of streptavidin-alkaline phosphatase conjugated antibody (MabTech) was added and the plate incubated for 45 minutes in the dark at room temperature. The wells were washed with PBS followed by addition of the alkaline phosphatase colour reagents (Bio-Rad Laboratories, Hercules, California, USA), allowing development of spots, which could be visualised directly. The plates were then finally washed with distilled water, blotted and left to dry prior to counting of spots.

The IFNγ-secreting cells were quantified by counting the number of spots per well using an automated ELISpot plate reader (AID ELISpot reader system; AutoimmunDiagnostika GmbH, Strasburg, Germany). The magnitude of responses was expressed as the number of spot-forming cells (SFC) per million PBMCs after subtractions of values from background wells. A response was defined as positive if greater or equal to 100 SFC/million PBMCs and greater than or equal to 3 standard deviations above the mean of four background wells containing PBMCs but no peptide. The breadth of responses was determined by the number of peptides/epitopes that had a magnitude of greater than or equal to 100 SFC/million PBMCs.

2.3.6 Intra cellular cytokine staining (ICS)

Freshly thawed cryopreserved PBMCs were left to rest at 37°C with 5% CO_2 for 4 hours. A total of 200,000 PBMCs per well, resuspended in R10 were stimulated with consensus clade C Gag peptide pools at a final concentration of 2 μg/ml/peptide and PHA (2 μg/ml) as a positive control. Anti-CD107a-PE-Cy5 (BD-Biosciences, USA) antibody, Golgi stop and Golgi plug (BD-Biosciences) were added in each well and cells incubated overnight at 37°C with 5% CO_2.

Cells were then washed and stained with a live/dead marker (fixable blue dead cell stain) (Invitrogen, Paisley, United Kingdom) for 10 minutes, followed by surface stain with anti-CD3 APC H7, anti-CD4 Alexa 700, anti-CD8 HV 500 (BD-Biosciences) and dump channel comprising of anti-CD14 pacific blue, anti-CD19 pacific blue and anti-CD16/56 pacific blue antibodies (Biolegend, San Diego Cal. USA). The cells were incubated in the dark at room temperature for 20 minutes. Following 2 washes, the cells were then permeabilized using cytoperm/cytofix (BD-Biosciences) to allow for intracellular staining with anti-IFNγ–PE Cy7, anti-interleukin-2 (IL-2)–FITC, or anti-tumor necrosis factor alpha (TNF-α)-APC and anti- macrophage inflammatory protein 1 beta (MIP1β)-PE (all from BD Biosciences). Following a 30 minute incubation at room temperature, the cells were washed twice with 2% FCS/PBS. The cells were resuspended in 2% FCS/PBS and acquired on the flow cytometer (LSR II, BD Biosciences). Between 30,000 and 100,000 events were acquired per well (sample). The acquired data was analysed by Flowjo version 9.6.2 (Tree Star, San Carlo, CA, USA). The initial gating was set on lymphocytes followed by the forward scatter height (FCS-H) versus forward scatter area (FSC-A) to eliminate doublets. We then gated on the live $CD3^+$ T cell population followed by gating of $CD8^+$ and $CD4^+$ T cell populations. The individual gating for

respective functions followed (set based on the negative control (unstimulated cells)), these were used to identify positive responses. Boolean gating was performed in order to allow creation of a full array of possible combinations of up to 32 response patterns. Positive responses were reported after background correction and the percentage of epitope-specific $CD8^+$ T cell responses had to be at least two times higher than background for each tested marker. PESTLE (version 1.6.2) and SPICE 5.0 (Mario Roederer, ImmunoTechnology Section, Vaccine Research Center, NIH, Bethesda, MD, USA) were used to analyse the multifunctional data.

Table 2.5 Antibody panel

Antigen	Fluorochrome	Clone	Isotype	Company	Product code	Volume used (µl)
CD3	APC-H7	SK7	Mouse IgG1	BD	560176	1
CD4	Alexa 700	RPA-T4	Mouse IgG1	BD	557922	1
CD8	HV 500	RPA-T8	Mouse IgG1	BD	560774	1
CD19	Pacific blue	HIB19	Mouse IgG1	Biolegend	302224	1
CD16	Pacific blue	3G8	Mouse IgG1	Biolegend	302037	1
CD14	Pacific blue	HCD14	Mouse IgG1	Biolegend	325612	1
MIP1β	PE	D21-1351	Mouse IgG1	BD	550078	1
TNFα	APC	6401.111	Mouse IgG1	BD	340543	1
CD107a	PE Cy5	H4A3	Mouse IgG1	BD	555802	2
IFNγ	PE Cy7	B27	Mouse IgG1	BD	557643	1
IL-2	FITC	5344.111	Mouse IgG1	BD	340448	2

2.3.7 CFSE proliferation assay

Freshly thawed cryopreserved PBMCs were left to rest at 37°C with 5% CO_2 for 4 hours and then stained with carboxyfluorescein succinimidyl ester (CFSE) dye (Invitrogen) for 7 minutes at 37°C with 5% CO_2. CFSE labelling was then stopped with 2 ml of FCS and washed 3 times with R10. A total of 400,000 CFSE-labelled PBMCs per well from each individual, resuspended in 200 µl R10 medium were then stimulated with Gag peptide pools at a final concentration of 2 µg/ml and PHA at a final concentration of 2 µg/ml as a positive control, followed by incubation at 37°C with 5% CO_2 for 7 days. After 7 days, cells were then washed and stained with a live/dead marker (fixable blue dead cell stain) (Invitrogen) for 10 minutes, followed by surface stain with anti-CD3 APC H7, anti-CD4 Alexa 700, anti-CD8 HV 500 (BD-Biosciences) and dump channel panel comprising of anti-CD14 pacific blue, anti-CD19 pacific blue and anti-CD16/56 pacific blue antibodies (Biolegend). The cells were incubated in the dark at room temperature for 20 minutes. The cells were then washed twice with 2% FCS/PBS and resuspended in 2% FCS/PBS. Between 30,000 and 100,000 events were acquired per well (sample). The acquired data was analysed by Flowjo version 9.6.2 (Tree Star, San Carlo, CA, USA). The initial gating was on lymphocytes followed by the forward scatter height (FCS-H) versus forward scatter area (FSC-A) to eliminate doublets. We then gated on the live $CD3^+$ T cell population followed by gating of $CD8^+$ and $CD4^+$ T cell populations. The next gates were set on the $CD8^+$ CFSE negative in order to identify proliferated $CD8^+$ T cell populations. PRISM graph pad version 5.0a software (GraphPad software, San Diego, California, USA) was used to analyse group data sets.

2.3.8 Viral inhibition assay

An *ex vivo* viral inhibition assay was carried out to determine the ability of $CD8^+$ T cells to suppress virus replication by measuring the amount of HIV p24 antigen as previously described elsewhere (330).

2.3.8.1 Separation of $CD4^+$ and $CD8^+$ T cells

Cryopreserved whole PBMCs were thawed and $CD4^+$ and $CD8^+$ T cells separated using the $CD8^+$ T cell Isolation kit (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In brief, PBMCs were thawed as already described. After the cells were counted they were washed once with 10ml of cold MACS buffer, which was prepared from Bovine Serum Albumin (BSA) and PBS (Whitehead scientific). To prepare BSA solution, 10g of BSA concentrate was added to 100 ml of distilled water and placed in the refrigerator (4 ºC) for 24 hours to dissolve. MACs buffer was prepared by adding 49 ml of PBS (Whitehead Scientific) to 1 ml of BSA solution giving a concentration of 0.5% BSA/PBS and this buffer was placed on ice. R10/50 was prepared by adding 25 µl of interleukine-2 (IL-2) to 50 ml of R10 and kept on ice until used.

Following addition of MACS buffer to the cells, the suspension was centrifuged at 300 x g for 10 minutes and the supernatant was aspirated completely. The cell pellet was resuspended with cold MACS buffer at a concentration of 40 µl of the buffer per 10 million cells. Ten microliters of the $CD8^+$ T cell Biotin antibody cocktail (MACS; Miltenyi Biotech) per 10 million cells was added to the cell suspension and mixed well by pipetting. This was followed by a 10 minute incubation at 2-8°C. Following the incubation, 30 µl of the cold MACS buffer was added plus 20 µl of the $CD8^+$ T cell microbead cocktail MACS; Miltenyi Biotech) per 10 million cells. The cell suspension was mixed well and incubated for an additional 15 minutes at 2-8°C. The

cells were washed with 5ml of the cold MACS buffer, centrifuged at 300 x g for 10 minutes at 4°C. The cells were resuspended in 500 µl of the cold MACS buffer.

MACS LS separation columns (MACS; Miltenyi Biotech) were used to magnetically separate CD8 and $CD4^+$ T cells. Firstly, the columns were placed on the magnetic field of the MACS separator and rinsed with 3ml of the cold MACS buffer. Once the 3 ml of the MACS buffer has passed through the column, the cell suspension was then applied to the column. The unlabelled cells ($CD8^+$ T cells) passed through the column in to the collection tube while the magnetically labelled cells ($CD4^+$ T cells) remained magnetically stuck on the column. The column was washed three times using the same 3 ml of the cold MACS buffer. The unlabelled cell fraction represented the enriched $CD8^+$ T cells while the magnetically labelled fraction represented the enriched $CD4^+$ T cells. The column was carefully removed from the magnetic separator and placed over a new collection tube. A volume of 5ml of cold MACS buffer was pipetted on to the column and the magnetically labelled cells were immediately flushed out by firmly pushing the plunger into the column. The 2 cell types were washed and counted as described before. The $CD8^+$ T cells were resuspended in R10 media while the $CD4^+$ T cells in R10/50.

The enriched $CD4^+$ T cells were activated with CD3/CD8-bispecific antibody at a concentration of 0.5 µl/ml (generous gift from Dr. Johnson Wong, Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, Boston, Massachusetts) for 3 days and later used as target cells in the inhibition assay. The enriched $CD8^+$ T cells were also cultured for 3 days in R10 medium for use as the *ex vivo* $CD8^+$ effector cells in the inhibition assay.

2.3.8.2 Infection of the enriched $CD4^+$ T cells

The stimulated $CD4^+$ T cells were infected with the NL4-3 laboratory adapted HIV strain (258) at a multiplicity of infection (MOI) of 0.001. The NL4-3 virus stocks used in this assay were kindly donated by Dr. Jaclyn Wright and Dr. Ravesh Singh from our laboratory (HIV Pathogenesis Programme, Durban, South Africa). In brief, the generated recombinant stocks had been titrated so as to determine the concentration of virus. One million GXR-cells were infected with 400 µl of each of the stock for 4 hours. Following this, the infected cells were washed and 100,000 cells/well plated in duplicate in a 96-well plate for 3 days. An aliquot of 100,000 uninfected GXR-cells/well were also plated in duplicate alongside the infected cells as negative control. The percentage infectivity was used to determine the MOI. After titration of each stock, the following formula was then used to calculate the desired MOI used in the subsequent assays for each of the virus stock generated:

Volume of virus to be used per 1 million cells = Desired MOI/Titre value * 400

The virus stocks generated were then used to infect the $CD4^+$ T cells that had been rested for 3 days after being separated from the $CD8^+$ T cells; for 4 hours at 37°C with 5% CO_2. Following the 3 days, $CD4^+$ T cells were counted and a proportion (300,000 $CD4^+$ T cells) left aside to be utilized in the 3 negative control wells. The rest of the $CD4^+$ T cells were infected with the NL4-3 virus and incubated for 4 hours at 37°C with 5% CO_2, flicking the tube every 30 minutes. Following infection, the cells were washed twice with R10 and co-culturing of infected $CD4^+$ T cells with $CD8^+$ T cells followed.

2.3.8.3 Co-culturing of effector and target cells

Following infection step, the infected $CD4^+$ T cells were cultured with or without effector cells in an effector - to - target cell ratio of 1:1 and incubated over a period of 7 days at 37°C with 5% CO_2. This experiment was done in triplicates, that is, 3 wells for the negative control wells whereby each well contained 100,000 uninfected $CD4^+$ T cells in 200 µl of R10/50; the positive control wells had 100,000 infected $CD4^+$ T cells also in 200 µl R10/50 per well and finally the 3 test wells contained 100,000 infected $CD4^+$ T cells plus 100,000 $CD8^+$ T cells in a total volume of 200 µl of R10/50. On days 3, 5 and 7 a volume of 90 µl of the culture supernatant was harvested without disturbing the adherent cells at the bottom of the well and 10 µl of the Triton X-100 solution added to the supernatant. The cells were fed with 90 µl of fresh R10/50 after each harvest and then placed back in to the incubator. The supernatants were cryopreserved at -80°C for later p24 antigen quantification.

2.3.8.4 Enzyme Linked Immunosorbent assay (ELISA)

The p24 antigen was quantified from the cryopreserved supernatants by enzyme-linked immunosorbent assay (ELISA) using the Quick Titre HIV Lentivirus Quantitation kit (HIV p24 ELISA) (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions.

In brief, the standard curve was prepared by serially diluting the recombinant HIV-1 p24 antigen (Cell Biolabs) in the concentration range of 100 ng/ml – 1 ng/ml by diluting the p24 stock solution in assay diluent (Cell Biolabs). There were 8 tubes; Tube 1 containing 990 µl of assay diluent and 100 ng/ml of the p24 HIV-1 p24 antigen, Tube 2 (500 µl assay diluent + 50 ng/ml p24 antigen), Tube 3 (500 µl assay diluent + 25 ng/ml p24 antigen), Tube 4 (500 µl assay diluent + 12.5 ng/ml p24 antigen), Tube 5 (500 µl assay diluent + 6.25 ng/ml p24 antigen, Tube 6 (500 µl assay

diluent + 3.125 ng/ml p24 antigen), Tube 7 (500 µl assay diluent + 1.5625 ng/ml p24 antigen) and finally Tube 8 contained 500 µl assay diluent only.

For the samples, 3 serial dilutions ranging from 10 to 1000 folds were prepared using R10 media. This was followed by transferring 225 µl of each of the samples into a 96 well plate. These were mixed well and incubated at 37°C for 30 minutes to inactivate the virus. Each sample, HIV p24 standard, blank and control medium were assayed in duplicate. A volume of 110 µl of each of the inactivated samples and p24 standard tubes were added to an anti-p24 antibody coated plate. The plate was covered and incubated at 37°C for at least 4 hours after which the plate was emptied and microwell strips washed 3 times with 250 µl of X1 Wash buffer (Cell Biolabs) per well with thorough aspiration between each wash. After the last wash, the wells were emptied and microwell strips tapped on an absorbent paper to remove excess X1 wash buffer. This was followed by addition of 100 µl of diluted FITC-conjugated anti-p24 monoclonal antibody (1:1000) (Cell Biolabs) to each well. The plate was then covered and incubated at room temperature for 1 hour. After which the plate was emptied and microwell strips washed 3 times with 250 µl of X1 Wash buffer (Cell Biolabs) per well with thorough aspiration between each wash. A volume of 100 µl of the diluted HRP-conjugated anti FITC monoclonal antibody (1:1000) (Cell Biolabs) was added to each well and plate covered and incubated at room temperature for 1 hour. After which the plate was emptied and microwell strips washed 3 times as already described. This was followed by an addition of 100 µl of warm (room temperature) substrate solution (Cell Biolabs) to each well; plate was covered and incubated at room temperature for about 2 to 30 minutes. The reaction was stopped by adding 100 µl of the stop solution (Cell Biolabs). The absorbance of each microwell was read immediately on a spectrophotometer using 450nm as a primary wave length.

Log inhibition values were calculated by subtracting log_{10} p24 values of the infected $CD4^+$ T cells co-cultured with $CD8^+$ T cells from log_{10} p24 values of the infected $CD4^+$ T cells without $CD8^+$ T cells at day 7. PRISM graph pad version 5.0a software (GraphPad software, San Diego, California, USA) was used to analyse group data sets.

2.4 MOLECULAR METHODS

2.4.1 DNA extraction

DNA was extracted from whole blood as previously described (298). In brief, 3 ml of whole blood was mixed with 9 ml of Red Blood Cell Lysis solution (Qiagen, Valencia, CA, USA) and left to stand at room temperature for 10 minutes. The mixture was centrifuged at 2,000 rpm (Eppendorf centrifuge 5810R, Merck) for 10 minutes and the supernatant was discarded in a waste bucket containing 2% Virkon (DuPont de Demours, South Africa) followed by resuspension of the cell pellet in 3 ml of cell lysis buffer (Qiagen). This cell suspension was left to stand at room temperature for at least 3 days. Following the 3-day incubation period, 1 ml of the protein precipitation solution was added and mixed well by inverting the tube several times. The mixture was centrifuged at 2,000 rpm (Eppendorf centrifuge 5810R, Merck) for 10 minutes. The supernatant was harvested and mixed with 3 ml of isopropanol by inverting the tube at least 20 times in order to precipitate the sample DNA. This mixture was centrifuged at 2,000 rpm for 3 minutes followed by washing the DNA with 5 ml of 70% ethanol and then left to dry. The DNA was then resuspended in 50 µl of elution buffer (Qiagen) and stored at minus 20°C till further use. One microlitre (1 µl) of the DNA was used for quantification using the Nanodrop (Thermo Fischer Scientific, USA).

2.4.2 RNA extraction

Viral RNA was extracted from plasma using a QIAamp Viral RNA Mini Kit from Qiagen (Qiagen) according to the manufacturer's instructions. The eluded RNA was stored at minus 80°C till use. One microlitre (1 µl) of the RNA was used for quantification using the Nanodrop (Thermo Fischer Scientific).

2.4.3 PCR primers for amplification of *gag*

The following table (Table 2.6) is a list of the primers used for the nested PCR to amplify the HIV *gag* region.

Table 2.6. PCR primers for the amplification of the *gag* region

Sequence	HIV clade	Region	Direction
5'-CACTGCTTAAGCCTCAATAAAGCTTGCC-3'	C	Gag	Forward
5'-TTTAACCCTGCTGGGTGTGGTATYCCT-3'	C	Gag	Reverse
5'-GAGGAGATCTCTCGACGCAGGAC-3'	C	Gag	Forward
5'- GGAGTGTTATATGGATTTTCAGGCCCAATT-3'	C	Gag	Reverse

2.4.4 First (1st) round RT-PCR

Complementary DNA (cDNA) synthesis and 1st round PCR were performed using the Invitrogen Superscript III One-step RT PCR kit (Invitrogen, Carlsbad, CA, USA). This kit allowed synthesis of cDNA and production of 1st round PCR product all in one step. The primers *gag*-specific primers used to generate 1st round PCR products; (5'-CACTGCTTAAGCCTCAATAAAGCTTGCC-3' and 5'-TTTAACCCTGCTGGGTGTGGTATYCCT-3') as listed on Table 2.2 above. The reaction mixture was made up of the following proportions giving a total of 40 µl:

14.4 µl	DEPC water
20.0 µl	2x reaction buffer
0.8 µl	Forward primer 5’ UTR 2 (10 µM)
0.8 µl	Reverse primer 3’2cRx (10 µM)
0.8 µl	Taq enzyme
4.0 µl	RNA template

The reaction was performed on a thermocycler (GeneAmp PCR system 9700, Applied Biosystems, Foster City, USA) using the PCR conditions described in the steps below:

1. 30 minutes 55°C
2. 2 minutes 94°C
3. 35 cycles of;
    i. 15 seconds 94°C
    ii. 30 seconds 55°C
    iii. 2 minutes 68°C
4. 5 minutes 68°C
5. Cooling at 4°C.

2.4.5 Second (2nd) Round PCR

A second/nested PCR mixture totalling 50 µl was prepared using the TaKaRa Ex *Taq* HS enzyme kit (Takara, Shiga, Japan) using the primers (5'-GAGGAGATCTCTCGACGCAGGAC-3', and 5'-GGAGTGTTATATGGATTTTCAGGCCCAATT-3') as listed on Table 2.2 and the following proportions:

37.0 µl	DEPC water
5.0 µl	10x Ex Taq buffer
4.0 µl	dNTPs
0.8 µl	Forward primer 5' Gag+1 (10 µM)
0.8 µl	Reverse primer 3' RVP (10 µM)
0.25 µl	Ex Taq enzyme
2.0 µl	first round PCR product

The reaction was performed on a thermocycler (GeneAmp PCR system 9700, Applied Biosystems) using the PCR conditions described in the steps below:

1. 2 minutes 94°C

2. 35 cycles of;

    i. 15 seconds 94°C

    ii. 30 seconds 55°C

    iii. 1 min 30 seconds 72°C

4. 7 minutes 72°C

5. Cooling at 4°C.

2.4.6 PCR product

Gag amplification was confirmed by running 5 µl of the product on a 1% polyacrylamide gel, showing a band of 1.5 kb.

2.4.7 PCR product purification

Purification of the PCR products was carried out using either of the following 2 methods:

i. QiaQuick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

ii. Illustra^{TM} GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Pittsburg, USA) according the manufacturer's instructions.

2.4.8 Sequencing primers

The following table (Table 2.7) lists the primers used for the sequencing of the *gag* region.

Table 2.7. Sequencing primers for the *gag* region

Sequence	HIV clade	Region	Direction
5'-CTTCAGACAGGAACAGAGGA-3'	**C**	**Gag**	**Forward**
5'- TAG AAG AAA TGA TGA CAG-3'	**C**	**Gag**	**Forward**
5'- GGAGCAGATGATACAGTATT-3'	**C**	**Gag**	**Forward**
5'- CTTGTCTAGGGCTTCCTTGGT-3'	**C**	**Gag**	**Reverse**
5'- GGTTCTCTCATCTGGCCTGG-3'	**B**	**Gag**	**Reverse**
5'- CAACAAGGTTTCTGTCATCC-3'	**B**	**Gag**	**Reverse**
5'- CCT TGC CAC AGT TGA AAC ATT T-3'	**C**	**Gag**	**Reverse**
5'- CAG CCA AGC TGA GTC AA-3'	**C**	**Gag**	**Reverse**

2.4.9 Sequencing reaction

The purified products were then sequenced using the ABI sequencing machine as described previously using Big Dye Terminator ready reaction mix V3 (Applied Biosystems, Foster City, CA, USA) (258) and the sequencing primers shown above on Table 2.3. A sequencing reaction mixture was prepared using the following proportions:

0.4 µl	Big Dye Terminator mix
2.0 µl	5x sequencing buffer
2.6 µl	Primer (2 µM)
4.0 µl	DEPC water
1.25 µl	template

The following thermocycler (GeneAmp PCR system 9700, Applied Biosystems) conditions were used:

1. 1 minute 96°C

2. 25 cycles: 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes.

3. Cooling at 4°C for at least 10 minutes.

The sequencing reaction clean-up was carried out on the same day.

2.4.10 Sequencing reaction clean up

The sequencing product was purified using the sodium acetate (NaOAC) (Sigma-Aldrich) precipitation method. In brief, 1 µl of 125 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 (Sigma-Aldrich) was added to the reaction, followed by addition of 1 µl of NaOAC pH 5.2 and 25 µl of 100% ethanol and mixed well by pipetting. The mixture was then centrifuged at 3,000 x g for 20 minutes. The supernatant was discarded and the pellet washed by adding 35 µl of cold 70% ethanol followed by centrifugation at 3,000 x g for 5 minutes. The supernatant was carefully discarded and samples dried at 50°C for 5 minutes using a thermocycler (GeneAmp PCR system 9700, Applied Biosystems).

The purified products were then sequenced using the ABI 3130xl genetic analyzer (Applied Biosystems). Escape was determined by evaluating the sequence variation within the known HLA epitopes and in the 5 amino acids on either side of the epitopes according to each participant's HLA profile.

2.4.11 Phylogenetic analysis

In order to check for any contamination between the samples, a phylogenetic tree was constructed using the Neighbour-Joining method utilizing ClustalX or bootstrap and visualized using Figtree.

2.5 DATA ANALYSIS AND STATISTICS

Sequence data were aligned to the HIV-1 subtype B reference strain HXB2 (Genbank accession number K03455) and insertions with respect to HXB2 were stripped before further analysis. Editing and alignment of sequences were carried out using Sequencher version 5 (Gene Codes, Corp., Ann Arbor, Michigan, USA) and SeAl version 2.0a11, (available online http://tree.bio.ed.ac.uk/software/seal/) (A. Rambaut, Department of Zoology, University of Oxford, Oxford, UK) software respectively.

GraphPad Prism version 5.0a was used to analyse the results. Statistical analysis was performed on Prism using Mann-Whitney U test, Wilcoxon paired test and student t-test as appropriate and detailed in the results for each analysis. Comparison between groups was corrected for multiple comparisons. The p value of below or equal to 0.05 was considered significant.

CHAPTER 3: COHORT CHARACTERISTICS AND BASELINE CD8$^+$ T CELL RESPONSES IN INDIVIDUALS WITH AND WITHOUT PROTECTIVE HLA CLASS I ALLELES

3.1 Introduction

Previous studies have linked the control of HIV clade C viral replication to a number of factors including protective HLA class I alleles. Certain HLA class I alleles are protective (e.g. B*57, B*27, A*74:01, B*81:01, B*58:01) against disease progression, have been strongly associated with viral load control and are found to be overrepresented amongst HIV controller groups (289–291, 293, 328, 331). However, disease progression is often encountered in some individuals with these protective HLA class I alleles and the mechanisms behind this progression are not well understood. We aimed to assess this by studying the factors behind viral control and loss of virologic control in individuals with protective HLA class I alleles during chronic HIV-1 subtype C infection. Individuals without protective HLA class I alleles were also included in this study for comparative purposes. Study participants were from a cohort of ARV naïve chronically HIV-1 subtype C infected adults in Durban, South Africa. The initial study aims were to:

1. Characterize viral load dynamics and clinical disease progression in ARV-naïve HIV-1 subtype C chronically infected participants with and without protective HLA class I alleles.
2. Study and describe the magnitude and breadth of HIV specific $CD8^+$ T cell immune responses in individuals with and without protective HLA class I alleles and associate these parameters with disease progression.

3.2 Viral load dynamics and clinical disease progression in study participants.

A total of 70 individuals with HIV-1 C-clade infection were studied in order to understand the $CD8^+$ T cell-based mechanisms of viral control and loss of control

amongst individuals with or without protective HLA class I alleles. The HLA types of the participants studied are shown in the Table 3.1 below. The number of females was 55 (78.5%) and there were 15 (21.4%) males. The median age at the time of enrolment was 32 years, interquartile range (IQR) (28-37).

Table 3.1. HLA types data of study participants

PID	Gender	Age	HLA Class 1 A	HLA Class 1 A2	HLA Class 1 B	HLA Class 1 B2	HLA Class 1 CW	HLA Class 1 CW2
SK-013	F	27	29:02	31:01	15:03	15:10	08:01	18:00
SK-014	F	29	29:02	29:02	44:03	**57:03**	07:01	07:01
SK-022	F	32	24:02	68:02	07:02	42:01	07:02	17:01
SK-023	F	41	66:01	80:01	18:01	58:02	02:02	06:02
SK-024	F	32	29:02	66:01	44:03	58:01	06:02	07:01
SK-031	M	32	26:02	**74:00**	08:01	15:10	02:02	08:01
SK-041	F	32	30:01	66:02	15:03	42:02	02:02	17:01
SK-049	M	36	23:01	31:00	15:10	35:01	04:01	16:01
SK-051	F	38	03:01	29:02	13:03	42:01	06:02	17:01
SK-055	F	38	30:01	30:02	08:01	42:02	07:01	17:01
SK-067	F	34	68:01	**74:00**	42:01	58:02	06:02	17:01
SK-070	F	32	03:01	68:01	08:01	15:03	07:02	18:01
SK-073	F	28	23:01	26:01	15:10	51:01	07:01	16:01
SK-079	F	30	02:05	29:02	44:03	**58:01**	07:01	07:01
SK-086	F	24	23:01	29:02	08:01	15:10	02:02	03:04
SK-089	F	26	23:01	**74:00**	15:03	**58:01**	02:02	03:02
SK-123	F	29	02:06	68:02	15:10	**58:01**	03:04	07:01
SK-135	F	28	34:02	68:01	41:03	44:03	04:01	17:01
SK-158	F	26	**74:00**	**74:00**	15:03	35:01	02:02	04:01
SK-165	F	27	33:01	68:01	42:01	58:02	06:02	17:01
SK-170	F	32	02:02	66:01	18:01	58:02	05:01	06:02
SK-175	M	42	02:01	30:02	18:01	39:10	12:03	12:03
SK-180	M	31	02:14	**74:00**	44:03	**57:03**	04:01	07:01
SK-187	F	25	30:02	68:02	08:01	**58:01**	03:02	07:01
SK-188	F	35	03:01	**74:00**	**57:03**	58:02	06:02	07:01
SK-199	F	27	29:02	**74:00**	13:02	**57:03**	06:02	07:01
SK-202	F	41	68:01	**74:00**	35:01	58:02	04:01	06:02
SK-208	F	22	02:01	23:01	08:01	58:02	06:02	07:01
SK-209	F	31	03:01	30:01	08:01	42:01	07:02	17:01
SK-224	F	31	02:05	68:02	07:02	**57:02**	03:04	18:00
SK-235	M	37	01:01	66:01	39:10	39:00	12:03	18:00
SK-242	F	28	01:01	66:01	**81:00**	39/67	12:03	18:00
SK-251	F	54	02:05	30:01	42:01	**58:01**	07:01	17:01
SK-275	F	26	03:01	30:01	08:01	39:10	07:02	12:03
SK-276	F	39	02:11	24:02	40:06	51:01	14:02	15:02
SK-282	M	46	02:05	34:02	44:03	**58:01**	04:01	07:01
SK-283	M	28	43:01	68:02	41:01	44:03	02:02	07:01
SK-292	F	34	02:01	30:02	39:10	40:01	03:04	12:03
SK-307	F	33	33:03	**74:00**	15:03	53:01	02:02	04:01
SK-315	F	28	30:01	30:02	42:01	42:01	17:01	17:01
SK-317	F	24	23:01	33:01	42:01	44:03	03:03	17:01
SK-321	M	25	23:01	80:01	18:01	**81:01**	02:02	04:01
SK-322	M	62	29:02	30:02	44:03	58:02	06:02	07:01
SK-324	F	53	02:01	02:05	45:01	**58:01**	06:02	16:01
SK-334	F	25	30:01	68:02	42:02	**58:01**	03:04	17:01
SK-342	F	25	29:02	30:01	42:01	45:01	06:02	17:01
SK-343	F	33	23:01	30:09	**58:01**	**81:00**	04:01	06:02
SK-346	F	24	30:04	68:01	**58:01**	82:02	03:02	06:02
SK-348	M	23	01:01	02:05/02:08	**58:01**	**81:00**	07:01	17:00
SK-354	F	38	30:01	**74:00**	35:01	**81:00**	04:01	04:01
SK-358	F	39	02:02	23:01	08:01	**57:01**	07:01	07:01
SK-362	F	30	03:01	**74:00**	15:03	15:10	02:02	04:01
SK-363	F	42	23:01	30:09	15:03	**81:00**	02:02	04:01
SK-374	F	30	23:01	34:02	14:02	44:03	04:01	08:02
SK-375	F	29	26:01	34:02	08:01	15:03	07:01	07:01
SK-378	F	35	33:03	68:01	15:10	53:01	03:04	04:01
SK-381	F	23	02:01	03:01	15:10	45:01	04:01	16:01
SK-382	F	28	03:01	29:02	42:01	**81:00**	04:01	17:00
SK-385	F	46	29:02	29:02	39:10	44:03	07:01	12:03
SK-387	F	48	02:02	29:11	13:02	15:16	06:02	14:02
SK-402	M	36	02:05	29:02	44:03	**58:01**	07:01	07:01
SK-404	M	34	23:01	29:01	15:03	15:03	02:02	04:01
SK-405	M	38	23:01	29:02	14:02	15:03	04:01	08:02
SK-407	F	32	01:01	**74:00**	35:01	**81:01/02**	04:01	18:01/02
SK-420	F	33	66:01	**74:00**	15:03	**57:03**	07:01	17:00
SK-422	M	29	30:02	30:02	15:10	42:01	03:04	17:00
SK-424	F	31	30:01	**74:00**	15:03	42:02	02:02	17:00
SK-425	F	37	03:01	30:02	15:10	58:02	03:04	06:02
SK-435	M	28	23:01	30:02	15:10	**57:03**	16:01	18:01
SK-445	F	33	23:01	33:01	42:01	58:02	06:02/7/10	17:00

Characteristics of the study subjects are shown in Table 3.2. Thirty-five participants had protective HLA class I alleles (+), of which 20 were viremic controllers at baseline (bVC+) whereas 15 were progressors (Prog+). The median time of follow-up of the bVC+ group of individuals was 5 years (4-6); and 4 years (3-6) for Prog+. The remaining 35 participants did not have protective HLA class I alleles (-) and of these, 10 were baseline viremic controllers (bVC-) and 25 were progressors (Prog-). The median follow-up time for individuals without protective HLA class I alleles was 5 years (4-6) for bVC- and 3 years (2.5-4) for Prog- (Table 3.2).

Table 3.2. Clinical characteristics for study subjects

Groups	Protective HLA allele +/-	n	Median years of follow up (IQR)	Median VL HIV RNA copies/ml (IQR)	p-value	Median CD4 count (cells/mmm^3) (IQR)	p-value
Baseline viremic controllers	+	20	5 (4 - 6)	487 (283 -1,252)	**0.77**	509 (459- 684)	**0.09**
Baseline viremic controllers	-	10	5 (4 - 6)	613 (399 - 1,213)		669 (550 - 865)	
Progressors	+	15	4 (3 - 6)	156,000 (118,000 - 192,000)	**0.26**	329 (257 – 377)	**0.56**
Progressors	-	25	3 (2.5 - 4)	209,000 (132,000- 330,000)		373 (260– 478)	

Protective HLA alleles: HLAs B*57, B*58:01, B*81:01 and A*74:01

There were no significant differences in the median VL between baseline viremic controllers with and without protective HLA class I alleles (p-value 0.77; Mann-Whitney test). There was a trend towards higher absolute $CD4^+$ T cell counts at baseline in viremic controllers without protective HLA class I alleles (bVC-) than baseline viremic controllers with protective HLA class I alleles (bVC+). Similarly, the median viral load and $CD4^+$ T cell count differences were not significant between

progressors with and without protective HLA class I alleles (p-value 0.26; (Mann-Whitney test) for viral load and p-value 0.56 (Mann-Whitney test) for $CD4^+$ T cell count) (Figure 3.1).

Study participants with baseline virologic control were followed longitudinally to assess viral load dynamics according to whether or not they expressed protective HLA class I alleles. Longitudinal follow-up of the 20 individuals with protective HLA class I alleles who controlled viremia at baseline (bVC+) allowed identification of two subgroups based on the VL dynamics: a subgroup of viremic controllers (VC+) that maintained viral load below 2,000 HIV RNA copies/ml for the entire study period and a second subgroup of failing viremic controllers (fVC+) who started with controlled viremia but eventually experienced virologic failure, with VL increasing up to more than 10,000 HIV RNA copies/ml in at least two consecutive time points during follow-up. There were some individuals whose viral load upon follow-up was above 2,000 but below 10,000 copies/ml and these individuals were excluded from some analysis as specified hereafter. Thus, the two subgroups showed divergent viral load dynamics despite viral load similarities at baseline (Figure 3.2A). Thirty five percent (n=7) of the bVC+ group maintained virologic control and were termed VC+ while another 35% (n=7) lost virologic control during follow up and were termed fVC+. The remaining 30% (n=6) fell into a third subgroup in which plasma VL were neither maintained below 2,000 nor increased to more than 10,000 HIV RNA copies/ml at any time point. We focussed on the 2 subgroups with extreme divergent outcomes in order to better understand the factors responsible for the control among the VC+ individuals and lack of viral control amongst fVC+ subgroups. Clinical parameters showed no differences in the $CD4^+$ T cell counts over time between the VC+ and fVC+ subgroups (Figure 3.2B). In each of the 2 subgroups (VC+ and

fVC+), 2 individuals maintained an absolute $CD4^+$ T cell count of less than 350 cells/mm^3 and the remaining 5 maintained an absolute $CD4^+$ T cell count of 350 cells/mm^3 or more during the entire time of follow up.

Longitudinal follow up of 10 bVC without protective HLA class I alleles allowed identification of a subgroup (VC-) defined as individuals who maintained virologic control (VL< 2,000 HIV RNA copies/ml) throughout the follow up period as shown in Figure 3.2C. Remarkably, sixty percent of the bVC- maintained virologic control (VC-, n=6) while none of the bVC- lost virologic control. The remaining 4 subjects (40%) did not fall in either of the 2 subgroups as their plasma VL were neither maintained below 2,000 nor increased to more than 10,000 HIV RNA copies/ml at any time point and these were excluded from further analysis. The changes in the absolute $CD4^+$ T cell count within the VC- subgroup are shown in Figure 3.2D. In this subgroup, there were 2 individuals maintaining an absolute $CD4^+$ T cell count of less than 350 cells/mm^3 whereas the remaining 4 maintained a count of 350 cells/mm^3 or more during the entire time of follow up.

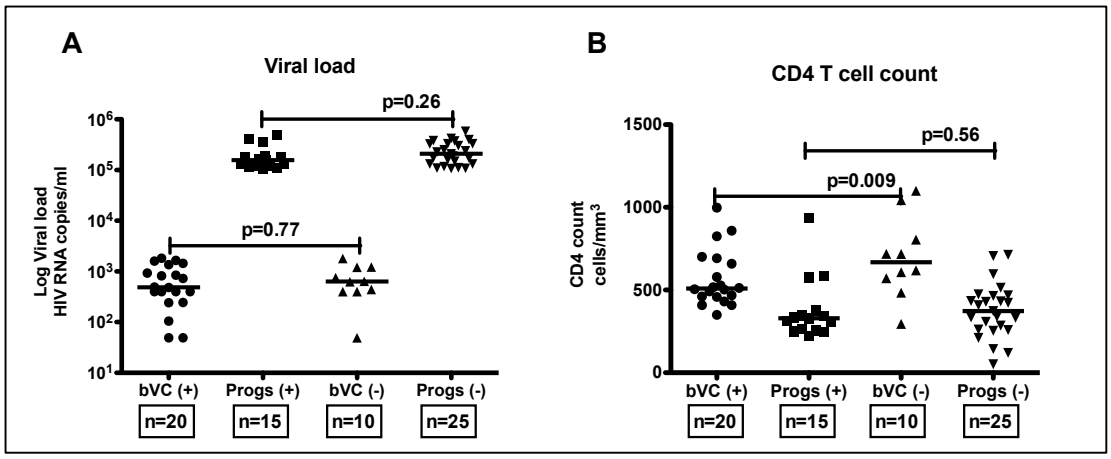

Figure 3.1. Viral load (A) and absolute CD4 T cell count (B) amongst individuals with and without protective HLA class I alleles; baseline viremic controllers (bVC+/-) with/without protective HLA class I alleles (enrolled with a viral load of < 2,000 HIV RNA copies/ml) and progressors (Prog+/-) with/without protective HLA class I alleles (enrolled with a viral load of > 100,000 HIV RNA copies/ml). Statistical analysis was performed on Prism using One-way ANOVA to compare all groups and Mann-Whitney U test to compare 2 groups.

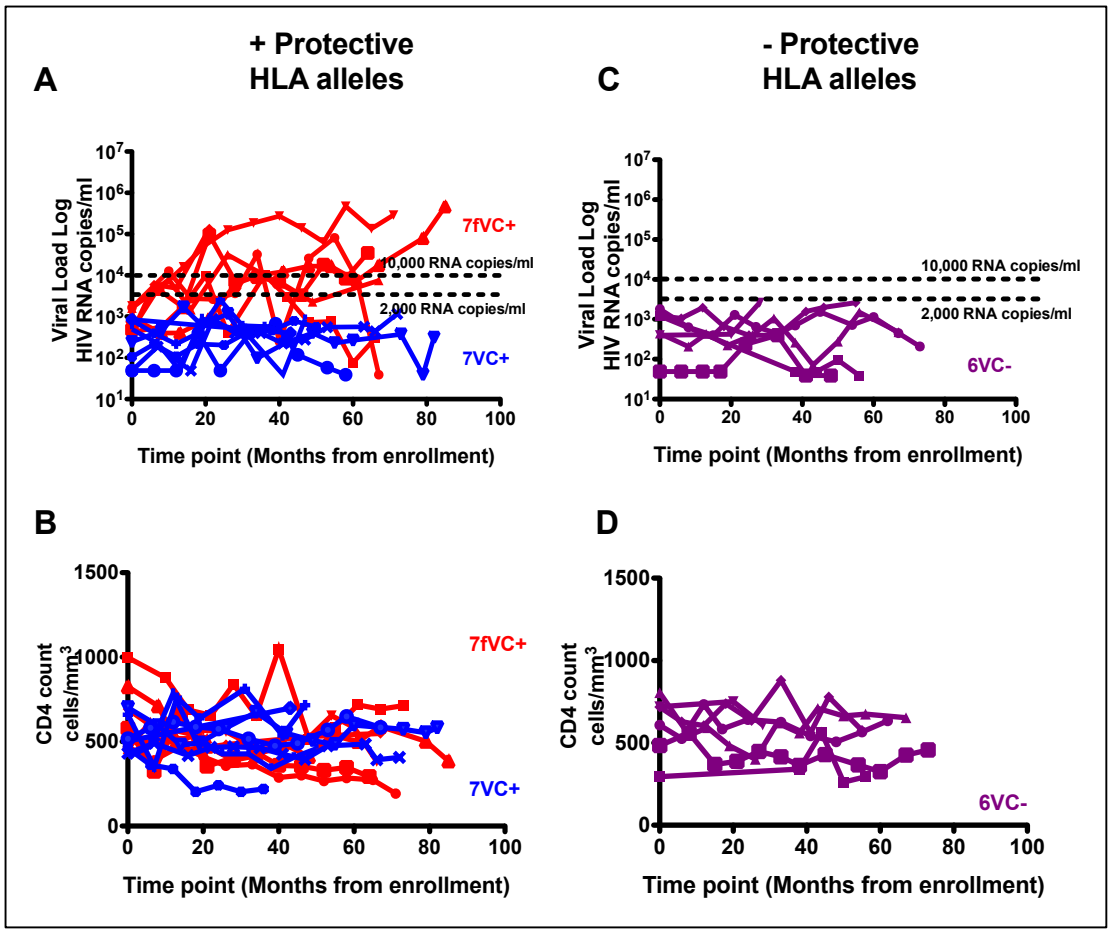

Figure 3.2. Longitudinal viral load (A, C) and absolute CD4 T cell count (B, D) patterns amongst baseline viremic controllers with and without protective HLA class I alleles respectively. Failing viremic controllers with protective HLA class I alleles (fVC+) were enrolled with a viral load of < 2,000 HIV RNA copies/ml and later lost control, n = 7; loss of control is defined by an increase in viral load to > 10,000 HIV RNA copies/ml at 2 or more time points during follow up. Viremic controllers with or without protective HLA class I alleles (VC+/-) maintained viral load < 2,000 HIV RNA copies/ml for the entire course of follow up.

We went on to determine whether there were viral load and $CD4^+$ T cell count differences at enrolment between viremic controllers (VC+) and failing viremic controllers (fVC+) with protective HLA class I alleles that could explain or possibly predict the later loss of control among some individuals. There was a trend towards higher baseline viral load in fVC+ individuals compared to VC+ individuals (p-value 0.06; Mann-Whitney test; Figure 3.3A) and we noted no significant differences in $CD4^+$ T cell counts between the two subgroups at baseline (p-value 0.26; Mann-Whitney test; Figure 3.3B). Taken together, these results reveal divergent viral load patterns amongst viremic controllers with protective HLA class I alleles.

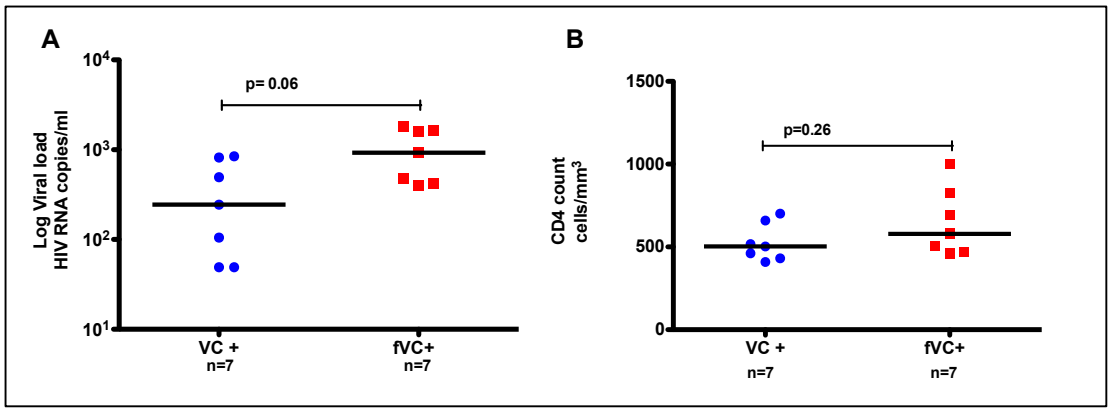

Figure 3.3. Log viral load (A) and absolute CD4 T cell count (B) at baseline amongst viremic controllers (VC+) and failing viremic (fVC+) individuals with protective HLA class I alleles. Statistical analysis was performed on Prism using Mann-Whitney U test to compare 2 groups.

3.3 HIV specific CD8$^+$ T cell responses at baseline amongst study participants

We next sought to understand whether the differences in viral load outcomes were attributed to CD8$^+$ T cell responses. We characterized the breadth and magnitude of HIV-specific CD8$^+$ T cell immune responses in study participants with the ELISpot matrix assay. PBMCs were stimulated with consensus subtype C peptides spanning the entire proteome followed by confirmations with individual overlapping peptides within a reactive pool. Comparison of the overall responses across the entire HIV proteome between the bVC and Prog groups with or without protective HLA class I alleles showed no significant differences between the groups, with a median breadth of 7.5 responses for both bVC+ and Prog+ and a median of 8 and 8.5 for bVC- and Prog- respectively (Figure 3.4A). There were no significant differences in the overall magnitude of CD8$^+$ T cell responses across the entire HIV proteome when comparing bVC+ to Prog+, with a median of 5,779 and 6,220 SFC/million PBMCs respectively; whereas the median magnitude for bVC- and Prog- were 2,763 and 5,574 SFC/million PBMCs respectively (Figure 3.4B). However, we observed a significant difference in the breadth of Gag-specific CD8$^+$ T cell responses between the 4 groups (p-value 0.01; Kruskal-Wallis), further analysis showed that the bVC with protective HLA class I alleles targeted a significantly higher number of Gag epitopes than Prog with protective HLA class I alleles (p-value 0.004; Mann-Whitney test) (Figure 3.4C). There were no significant differences between the groups when we analysed the magnitude of Gag-specific CD8$^+$ T cell responses (Figure 3.4D).

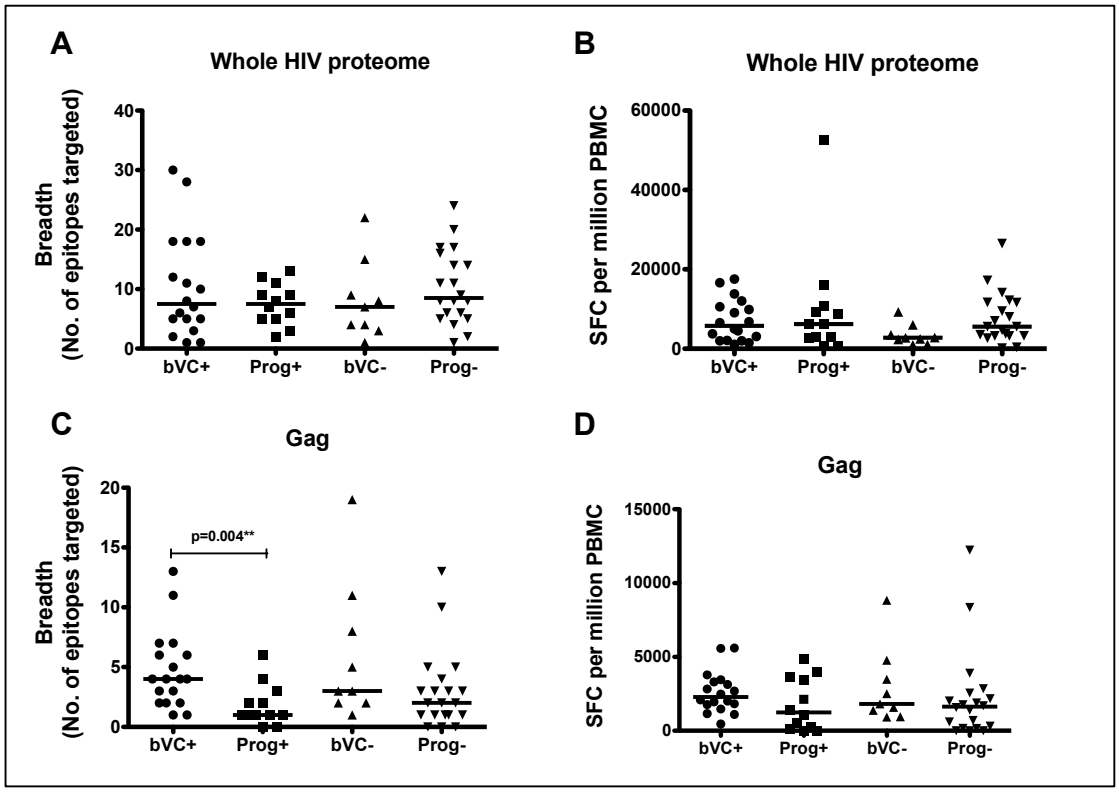

Figure 3.4. ELISpot screening of HIV-specific T cell responses at baseline; Breadth (A) and magnitude (B) of the whole HIV proteome; breadth (C) and magnitude (D) of Gag specific $CD8^+$ T cell responses amongst individuals with or without protective HLA class I alleles (bVC+/- and Prog +/-). An ELISpot matrix assay followed by confirmations with overlapping peptides spanning the entire HIV-1 clade C proteome was used on thawed PBMCs. Gag overlapping peptides were represented by OLPs 1 to 66. bVC+ (baseline viremic controllers with protective HLA class I alleles, n =18), Prog+ (progressors with protective HLA alleles, n = 12), bVC- (baseline VC without protective HLA class I alleles, n = 9), Prog- (progressors without protective HLA class I alleles, n = 20). Statistical analysis was performed on Prism using One-way ANOVA to compare all 4 groups in each graph and where significant differences were found, Mann-Whitney U test was used to compare 2 groups that indicated significant differences.

No significant differences between the groups were found when comparing other proteins individually; Nef, Env, Pol, and Accessory (Vpr, Vpu and Vif) and Regulatory (Rev and Tat) proteins, however progressors without protective HLA class I alleles had a significantly higher magnitude of Nef-specific $CD8^+$ T cell responses compared to baseline VC without protective HLA class I alleles (p-value 0.006; Mann-Whitney test) (Figure 3.5).

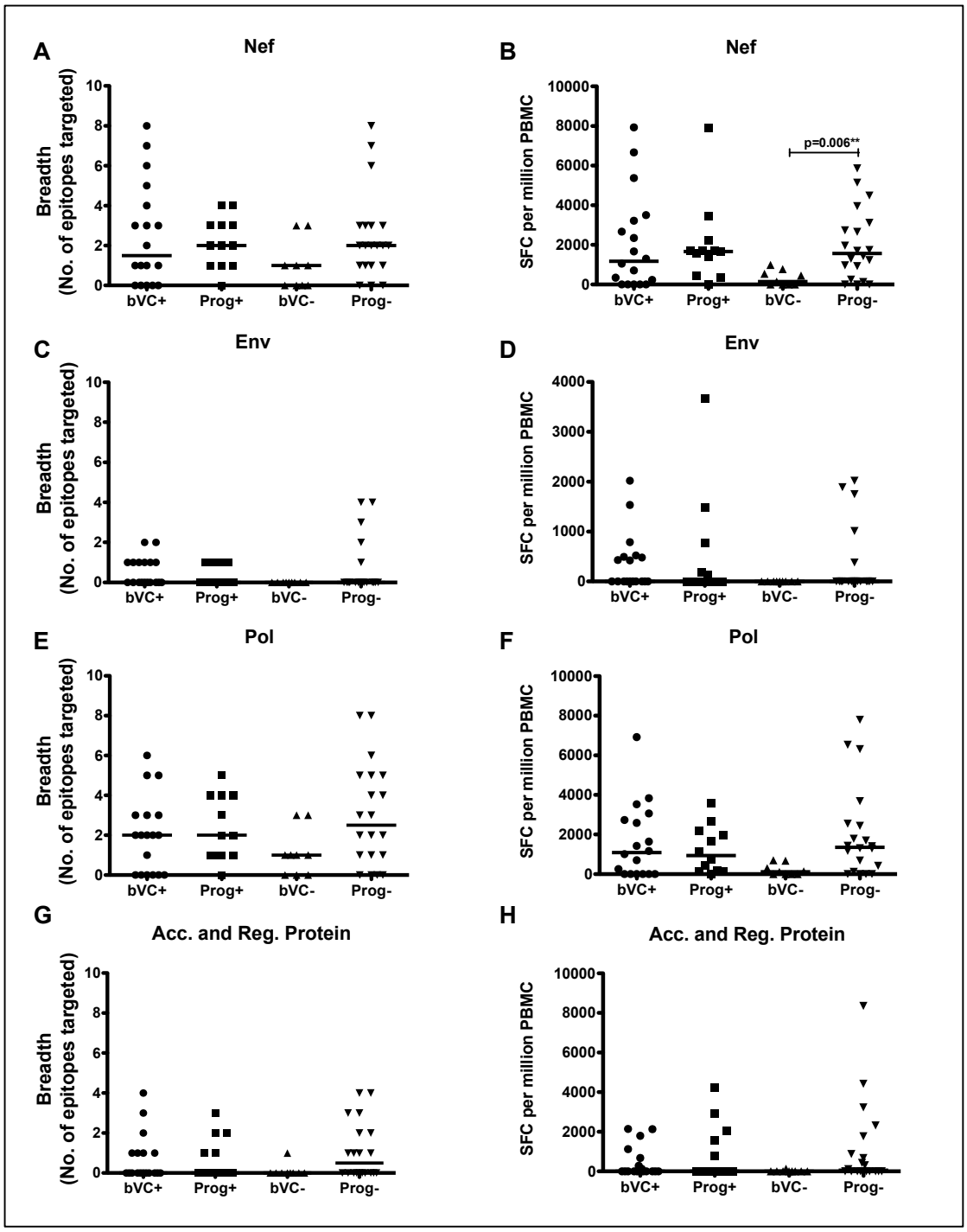

Figure 3.5. ELISpot screening of HIV-specific T cell responses at baseline; Breadth and magnitude of Nef (A, B), Env (C, D), Pol (E, F) and Accessory and Regulatory proteins (G, H) specific $CD8^+$ T cell responses amongst individuals with or without protective HLA class I alleles (bVC+/- and Prog +/-). An ELISpot matrix assay followed by confirmations with overlapping peptides spanning the entire HIV-1 clade C proteome was used on thawed PBMCs. Overlapping peptides were represented by OLPs 67-93 for Nef peptides; OLPs 289 - 401 for Env peptides; OLPs 145 – 277 for Pol peptides; Accessory and Regulatory proteins were grouped together and represented Rev peptides OLPs 94 -107; Tat peptides 111-122; Vpu peptides 123-131; Vpr peptides 278-288; and Vif peptides 402-425. bVC+ (baseline viremic controllers with protective HLA class I alleles, n =18), Prog+ (progressors with protective HLA class I alleles, n = 12), bVC- (baseline VC without protective HLA class I alleles, n = 9), Prog- (progressors without protective HLA class I alleles, n = 20). Statistical analysis was performed on Prism using

One-way ANOVA to compare all 4 groups in each graph and where significant differences were found, Mann-Whitney U test was used to compare 2 groups that indicated significant differences.

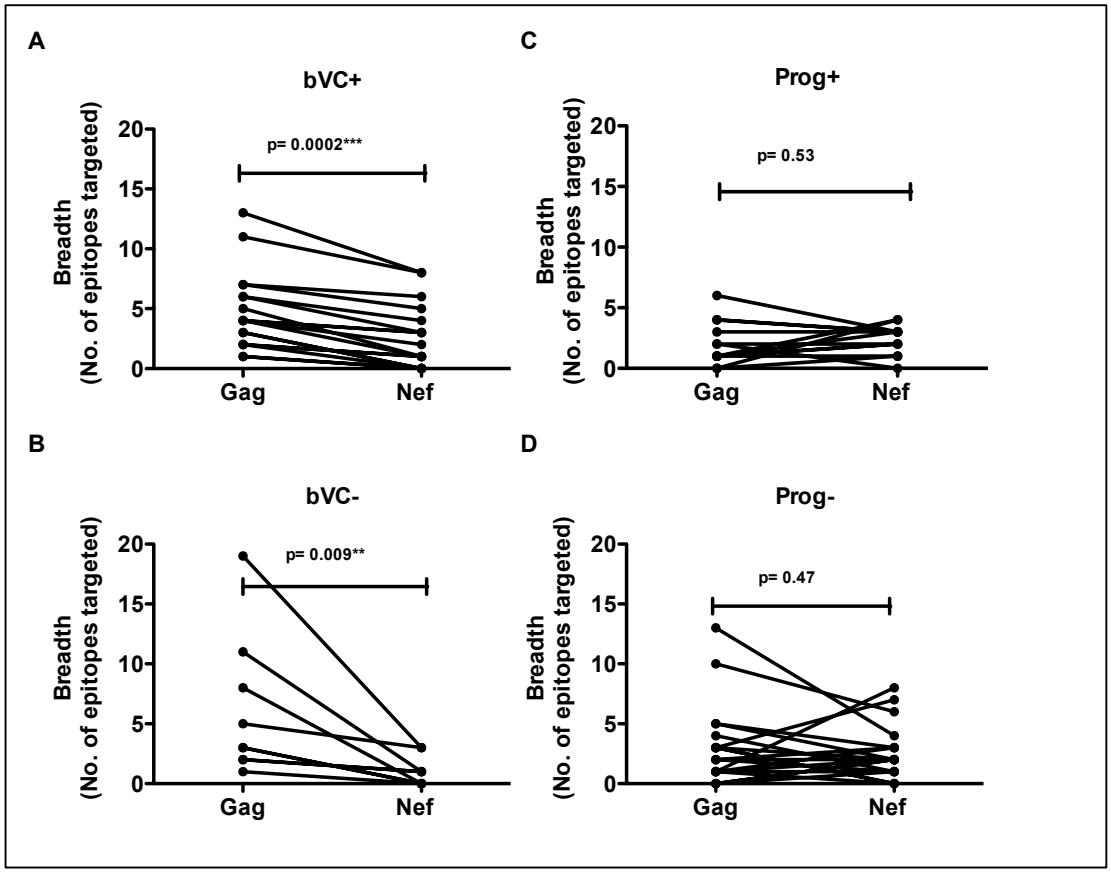

Figure 3.6. ELISpot screening of HIV-specific T cell responses at baseline- Gag versus Nef; An ELISpot matrix assay followed by confirmations with overlapping peptides spanning the entire HIV-1 clade C proteome was used on thawed PBMCs. The breadth of Gag versus Nef responses were assessed amongst the study groups (A, B, C, D); bVC+ (baseline viremic controllers with protective HLA class I alleles, n =18), Prog+ (progressors with protective HLA class I alleles, n = 12), bVC- (baseline VC without protective HLA class I alleles, n = 9), Prog- (progressors without protective HLA class I alleles, n = 20). Statistical analysis was performed on Prism using Wilcoxon paired test.

$CD8^+$ T cell responses to Gag have previously been associated with low viral load (252, 254) while responses to Nef have been previously associated with high viral load (233, 332). In order to further study the importance of targeting Gag as opposed to other HIV proteins amongst viremic controllers we first investigated the patterns of immunodominance of Gag responses compared with Nef responses in the 4 study groups. We found that there was a bias towards targeting of higher number of Gag epitopes (median breadth = 4) compared to Nef (median = 1.5) (p-value 0.0002; Wilcoxon matched pairs test) in baseline viremic controllers (bVC+) with protective HLA class I alleles (Figure 3.6A). This observation was similar to that in baseline viremic controllers without protective HLA class I alleles where a bias towards higher Gag breadth (median = 3) compared to Nef (median = 1) (p-value 0.009; Wilcoxon matched pairs test) was noted (Figure 3.6B). This bias towards higher Gag breadth than Nef was not observed amongst progressor groups with or without protective HLA class I alleles where no differences were found between the breadth of Gag and Nef (Figure 3.6C, D). Taken together these observations suggest that broad targeting of Gag may contribute to HIV control among viremic controllers.

Additionally, to further investigate the importance of targeting Gag as opposed to other HIV proteins amongst viremic controllers we went on to investigate the patterns of immunodominance of Gag responses compared with other HIV proteins in the 4 study groups. We examined Gag responses versus Env, Pol and accessory and regulatory protein responses. We observed a bias towards targeting of higher Gag versus Env breadth in all of the 4 groups of individuals with and without protective HLA class I alleles (p-value 0.002; Wilcoxon matched pairs test) for bVC+ subjects (Figure 3.7A), (p-value 0.009; Wilcoxon matched pairs test) for bVC- subjects (Figure 3.7B), (p-value 0.02; Wilcoxon matched pairs test) for Prog+ subjects (Figure

3.7C) and (p-value 0.009; Wilcoxon matched pairs test) for Prog- subjects (Figure 3.7D).

However when we analysed responses to Gag versus Pol, similar observations of higher targeting of Gag epitopes was noted in the baseline viremic controllers with and without protective HLA class I alleles (p-value 0.0007; Wilcoxon matched pairs test; for bVC+ and p-value 0.017; Wilcoxon matched pairs test for bVC- individuals) (Figure 3.8A, B). Among the progressors with and without protective HLA class I alleles, this bias was not evident (Figure 3.8C, D).

Furthermore, we observed a bias towards targeting of higher Gag versus Accessory and Regulatory proteins among bVC+ subjects (p-value 0.0002; Wilcoxon matched pairs test) (Figure 3.9A), bVC- subjects (p-value 0.009; Wilcoxon matched pairs test) (Figure 3.9B), a trending bias among Prog+ subjects (p-value 0.08; Wilcoxon matched pairs test) (Figure 3.9C) and (p-value 0.008; Wilcoxon matched pairs test) for Prog- subjects (Figure 3.9D).

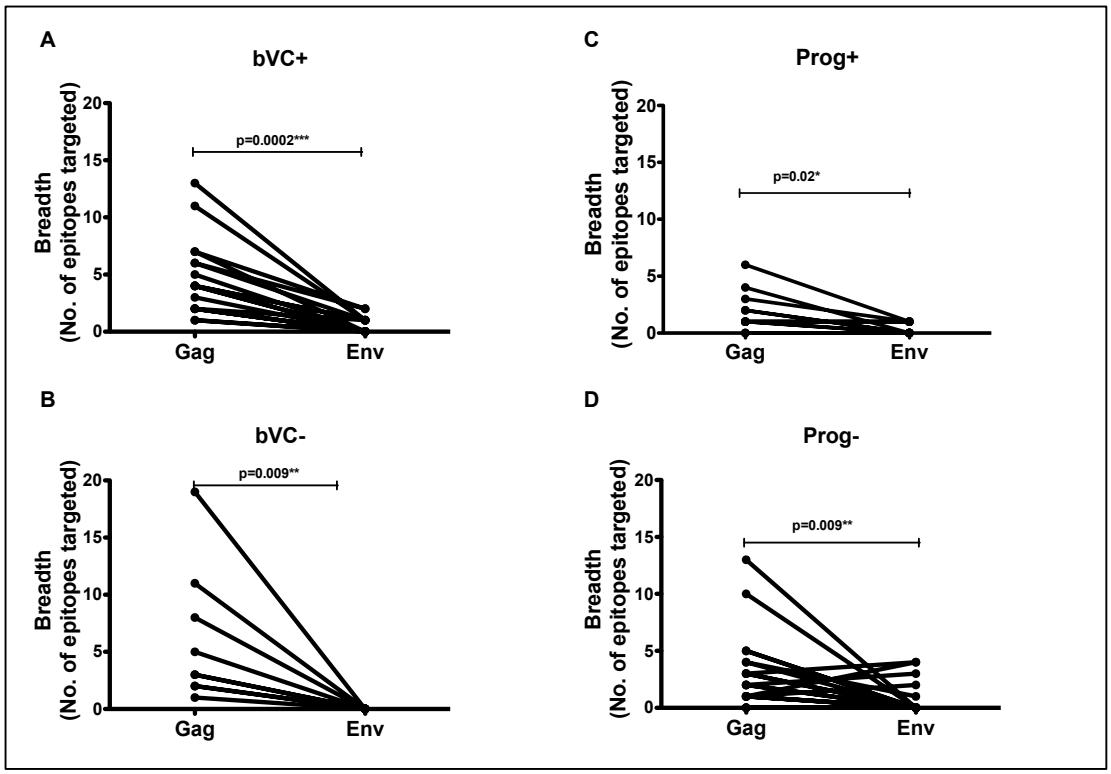

Figure 3.7. ELISpot screening of HIV-specific T cell responses at baseline- Gag versus Env; An ELISpot matrix assay followed by confirmations with overlapping peptides spanning the entire HIV-1 clade C proteome was used on thawed PBMCs. The breadth of Gag versus Env responses were assessed amongst the study groups (Fig A, B, C, D); bVC+ (baseline viremic controllers with protective HLA class I alleles, n =18), Prog+ (progressors with protective HLA class I alleles, n = 12), bVC- (baseline VC without protective HLA class I alleles, n = 9), Prog- (progressors without protective HLA class I alleles, n = 20). Statistical analysis was performed on Prism using Wilcoxon paired test.

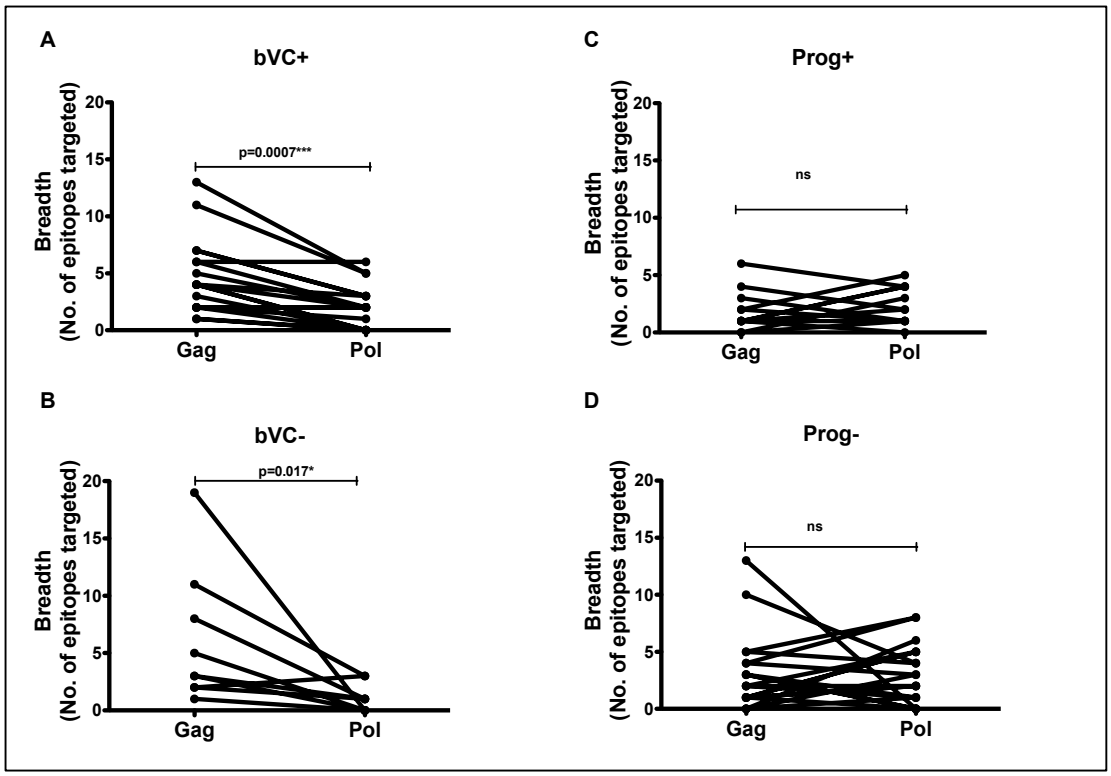

Figure 3.8. ELISpot screening of HIV-specific T cell responses at baseline- Gag versus Pol; An ELISpot matrix assay followed by confirmations with overlapping peptides spanning the entire HIV-1 clade C proteome was used on thawed PBMCs. The breadth of Gag versus Pol responses were assessed amongst the study groups (Fig A, B, C, D); bVC+ (baseline viremic controllers with protective HLA class I alleles, n =18), Prog+ (progressors with protective HLA class I alleles, n = 12), bVC- (baseline VC without protective HLA class I alleles, n = 9), Prog- (progressors without protective HLA class I alleles, n = 20). Statistical analysis was performed on Prism using Wilcoxon paired test.

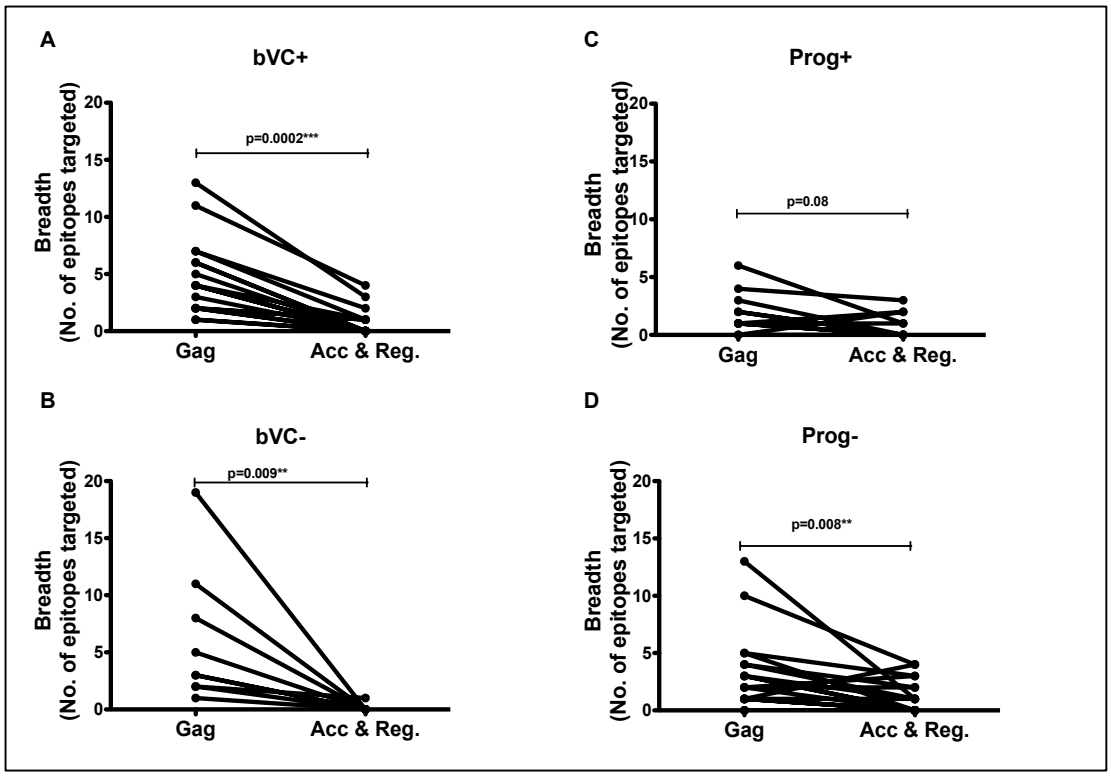

Figure 3.9. ELISpot screening of HIV-specific T cell responses at baseline- Gag versus Acc & Reg proteins; An ELISpot matrix assay followed by confirmations with overlapping peptides spanning the entire HIV-1 clade C proteome was used on thawed PBMCs. The breadth of Gag versus Accessory and regulatory responses were assessed amongst the study groups (Fig A, B, C, D); bVC+ (baseline viremic controllers with protective HLA class I alleles, n =18), Prog+ (progressors with protective HLA class I alleles, n = 12), bVC- (baseline VC without protective HLA class I alleles, n = 9), Prog- (progressors without protective HLA class I alleles, n = 20). Statistical analysis was performed on Prism using Wilcoxon paired test.

3.4 Summary of results

In Chapter 3, we characterized the baseline and longitudinal viral load and CD4 T cell dynamics in 35 chronic HIV-1 clade C infected individuals with protective HLA class I alleles and 35 individuals without protective HLA class I alleles. We show differential patterns in disease outcome amongst the individuals with protective HLA class I alleles despite similar viral load and $CD4^+$ T cell counts at baseline time point. Furthermore, we demonstrate that virological control was associated with biased targeting of Gag compared to other proteins. In particular, baseline viremic controllers with protective HLA class I alleles targeted significantly higher breadth of Gag specific $CD8^+$ T cells responses compared to progressors with protective HLA class I alleles. To our surprise baseline viremic controllers without protective HLA class I alleles displayed limited Gag specific $CD8^+$ T cell responses suggesting that mechanisms other than $CD8^+$ T cells may mediate control. An alternative hypothesis is that among individuals without protective HLA class I alleles, the $CD8^+$ T cells targeting only a few epitopes mediated very effective antiviral effects. Furthermore, we showed that there was a bias towards targeting of Gag versus Nef, Pol, Env, Accessory and Regulatory proteins amongst baseline viremic controllers. Taken together these data emphasize the importance of broad targeting of Gag among viremic controllers and confirm previous studies documented in HIV-1 subtype C infection (252, 254) on the beneficial role of Gag-specific $CD8^+$ T cell responses in HIV control.

CHAPTER 4: LONGITUDINAL ANALYSIS OF $CD8^+$ T CELL RESPONSES AND IMPACT OF VIRAL EVOLUTION ON DISEASE PROGRESSION IN HIV-1 SUBTYPE C CHRONICALLY INFECTED INDIVIDUALS WITH AND WITHOUT PROTECTIVE HLA CLASS I ALLELES

4.1 Introduction

HIV-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) have been demonstrated to play an important role in HIV control (237, 254, 290, 297). However, virus-specific $CD8^+$ T cell immune responses are not equally effective in HIV control as the majority of infected individuals progress to disease despite the presence of these cells. One possible explanation is that HIV is able to evade immune responses by developing mutations that mediate escape from CTL recognition (149, 234, 248, 333–336).

In the previous chapter we demonstrated that individuals with protective HLA class I alleles lost viral control and that Gag specific $CD8^+$ T cells responses were related to the control of viremia at baseline. Although the majority of previous studies have associated $CD8^+$ T cell responses with disease outcomes, there are some caveats: most of these studies characterized immune responses at a single time point which is usually the enrolment time point despite the study subjects displaying divergent disease progression pathways thereafter. In order to address this gap in knowledge, our study design afforded a unique opportunity to characterize $CD8^+$ T cell responses of the individuals longitudinally in an attempt to determine whether the $CD8^+$ T cell responses played any role in the control or loss of virologic control among individuals with and without protective HLA class I alleles. We also sought to determine whether viral evolution and escape due to CTL pressure explained the loss of viral control in these groups.

Therefore we aimed to:

1. Study and describe the evolution of HIV-specific immune responses and associate those with disease progression.

2. Study viral evolution within Gag in individuals with and without protective HLA class I alleles and determine the impact of viral evolution on disease progression

4.2 Study participants

Here we focused on individuals with and without protective HLA class I alleles but divergent disease progression status. These are described below:

iii. Viremic controllers (VC) were defined as individuals who were enrolled with a VL of less than 2,000 HIV RNA copies/ml and maintained this low viral load for the entire period of follow up.

iv. Failing viremic controllers (fVC) were defined as bVC that were enrolled with a VL of less than 2,000 HIV RNA copies/ml but lost virological control. We defined the loss of viral control as an increase in VL to more than 10,000 HIV RNA copies/ml at a minimum of 2 time points during the time of follow-up.

We also included some progressors with protective HLA class I alleles depending on sample availability as a control group. The schematic representation of these subgroups and HLA typing and general characteristics are illustrated on the flow chart (Figure 4.1) and Table 4.1 below. A total of 7 fVC+, 7 VC+, 6 VC- and 6 Prog+ individuals were studied.

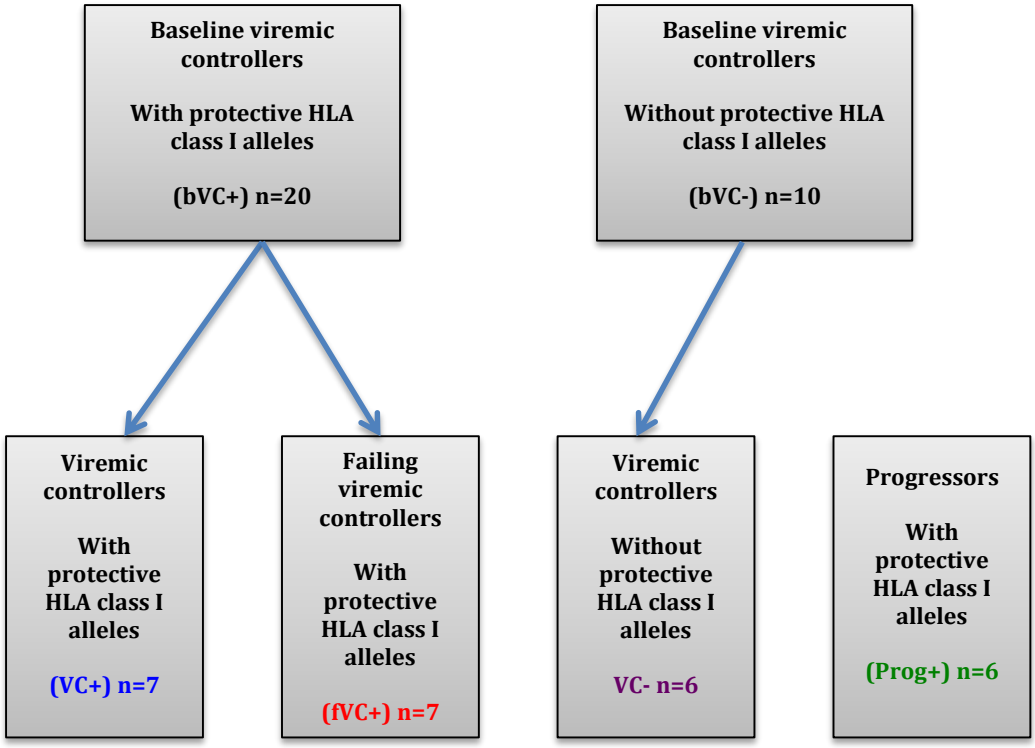

Figure 4.1 Flow chart showing the breakdown of study groups in to subgroups.

Table 4.1. HLA typing and general characteristics of study participants that were studied longitudinally.

PID	Gender	Age	HLA Class1 A	HLA Class1 A2	HLA Class1 B	HLA Class1 B2	HLA Class1 CW	HLA Class1 CW2	Role
SK-024	F	32	2902	6601	4403	5801	602	701	fVC+
SK-079	F	30	205	2902	4403	5801	701	701	fVC+
SK-187	F	25	3002	6802	801	5801	302	701	fVC+
SK-188	F	35	301	7400	5703	5802	602	701	fVC+
SK-435	M	28	2301	3002	1510	5703	1601	1801	fVC+
SK-224	F	31	205	6802	702	5702	304	1800	fVC+
SK-242	F	28	101	6601	81	39/67	1203	1800	fVC+
SK-199	F	27	2902	7400	1302	5703	602	701	VC+
SK-282	M	46	205	3402	4403	5801	401	701	VC+
SK-235	M	37	101	6601	3910	39	1203	1800	VC+
SK-348	M	23	101	0205/0208	5801	8100	701	17	VC+
SK-354	F	38	3001	7400	3501	8100	401	401	VC+
SK-362	F	30	301	7400	1503	1510	202	401	VC+
SK-089	F	26	2301	7400	1503	5801	202	302	VC+
SK-209	F	31	301	3001	801	4201	702	1701	VC-
SK-342	F	25	2902	3001	4201	4501	602	1701	VC-
SK-292	F	34	201	3002	3910	4001	304	1203	VC-
SK-275	F	26	301	3001	801	3910	702	1203	VC-
SK-315	F	28	3001	3002	4201	4201	1701	1701?	VC-
SK-317	F	24	2301	3301	4201	4403	303	1701	VC-
SK-334	F	25	3001	6802	4202	5801	304	1701	Prog+
SK-324	F	53	201	205	4501	5801	602	1601	Prog+
SK-307	F	33	3303	7400	1503	5301	202	401	Prog+
SK-358	F	39	202	2301	801	5701	701	701	Prog+
SK-363	F	42	2301	3009	1503	8100	202	401	Prog+
SK-382	F	28	301	2902	4201	8100	401	1700	Prog+

Key: fVC= failing viremic controllers; VC= viremic controllers; + = with protective HLA class I alles; - = without protective HLA class I alleles.

4.3 Loss of virologic control is associated with the loss of $CD8^+$ T cell responses

In order to identify HIV-specific $CD8^+$ T cell immune response changes associated with control and loss of viral control, the ELISpot assay was used to determine $CD8^+$ T cell responses following stimulation with HLA-matched optimal peptides at the time point of virological failure. Thus, for VC+, VC- and Prog+ participants, samples were assessed by an ELISpot assay at baseline (earliest time point) and the latest time point available, whereas for failing viremic controller (fVC+) participants, samples were evaluated at baseline (viremic control time point) and at the time point of first increase of VL to above 10,000 copies/ml (post loss of virologic control) and thereafter.

Firstly, we assessed the $CD8^+$ T cell response profiles between the subgroups at baseline in order to determine predictors of loss of viral control. We found that there was a significant difference in the overall breadth of HIV specific $CD8^+$ T cell responses across the entire HIV proteome between the 4 subgroups (p-value 0.005; Kruskal-Wallis). Further analysis showed that the progressors with protective HLA class I alleles targeted a significantly higher number of total epitopes than VC without protective HLA class I alleles (p-value 0.006; Mann-Whitney test) (Figure 4.2A). Similarly we further observed significant differences in the overall magnitude of $CD8^+$ T cells responses between the 4 subgroups (p-value 0.002; Kruskal-Wallis), with further analysis showing that fVC+ subjects displayed a significantly higher overall magnitude of $CD8^+$ T cells responses compared to VC without protective HLA class I alleles (p-value 0.005; Mann–Whitney test). Furthermore, Prog+ displayed a significantly higher magnitude of overall $CD8^+$ T cell responses when compared to VC without protective HLA class I alleles (p-value 0.004; Mann-Whitney test) (Figure 4.2B). There were no significant differences in the breadth and

magnitude of Gag specific $CD8^+$ T cell responses between the subgroups (Figure 4.2C, D).

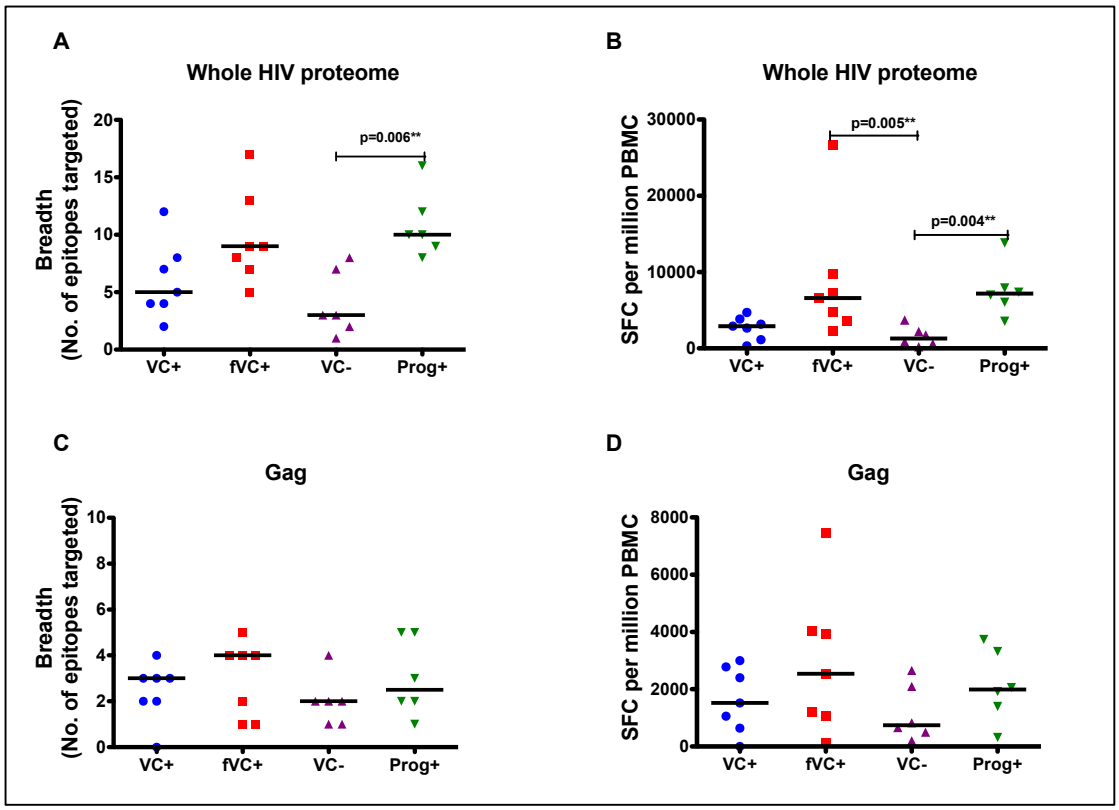

Figure 4.2. ELISpot screening of HIV-specific T cell responses at baseline amongst the 4 subgroups- Overall and Gag; ELISpot assay was used on thawed PBMCs to determine $CD8^+$ T cell responses following stimulation with HLA-matched optimal peptides spanning the entire HIV-1 clade C proteome. Breadth and magnitude to the whole HIV proteome (A, B) and the breadth and magnitude of Gag specific $CD8^+$ T cell responses (C, D). VC+; (viremic controllers with protective HLA class I alleles, n =7), fVC+; (failing viremic controllers with protective HLA class I alleles, n=7), VC-; (viremic controllers without protective HLA class I alleles, n=6), Prog+; (progressors with protective HLA class I alleles, n = 6). Statistical analysis was performed on Prism using Kruskal-Wallis test to compare all 4 groups in each graph and Mann-Whitney U test to compare 2 groups that indicated significant differences.

We extended the analysis to all other proteins; Nef, Env, Pol, accessory and regulatory proteins (Figure 4.3). Here we found a significant difference in the breadth of Nef between the subgroups (p-value 0.003; Kruskal-Wallis), further analysis showed that the difference was between fVC+ and VC+ with fVC+ displaying a significantly higher breadth of Nef specific $CD8^+$ T cell response than VC+ (p-value 0.005; Mann–Whitney) (Figure 4.3 A). Similarly we observed a significant difference in the magnitude of Nef between the subgroups (p-value 0.0007; Kruskal-Wallis), further analysis showed that Prog+ and fVC+ subgroups had significantly higher magnitude of Nef responses compared to VC+/- protective HLA class I alleles. The differences were as follows: between VC+ and fVC+ (p-value 0.006; Mann–Whitney test); VC+ and Prog+ (p-value 0.007; Mann–Whitney test); fVC+ and VC- (p-value 0.004; Mann–Whitney test) and between VC- and Prog+ subgroups (p-value 0.005; Mann–Whitney test) (Figure 4.3B).

No significant differences were observed between the subgroups when we analysed the breadth and magnitude of $CD8^+$ T cell responses to the Env and accessory and regulatory proteins (Figure 4.3C, D, G, H). We observed significant differences between the subgroups when we assessed responses to Pol optimal peptides (breadth, p-value 0.03, Kruskal–Wallis; magnitude, p-value 0.007, Kruskal–Wallis), with further analysis revealing that Prog+ targeted higher breadth (p-value 0.02; Mann–Whitney test) and magnitude (p-value 0.007, Mann–Whitney test) of Pol specific $CD8^+$ T cell responses than VC without protective HLA class I alleles (Figure 4.3E, F).

Taken altogether these data may suggest that broad targeting of Nef by viremic controllers with protective HLA class I alleles is a predictor of loss of virologic control. Only 2 VC+ subjects targeted Nef epitopes at baseline compared to all 7

fVC+ subjects. It is noteworthy that all individuals either targeted very few epitopes within the envelope, accessory and regulatory regions or none at all. Viremic controllers without protective HLA class I alleles targeted very few numbers of epitopes suggesting a possible alternative mechanism for the control of viremia in these individuals.

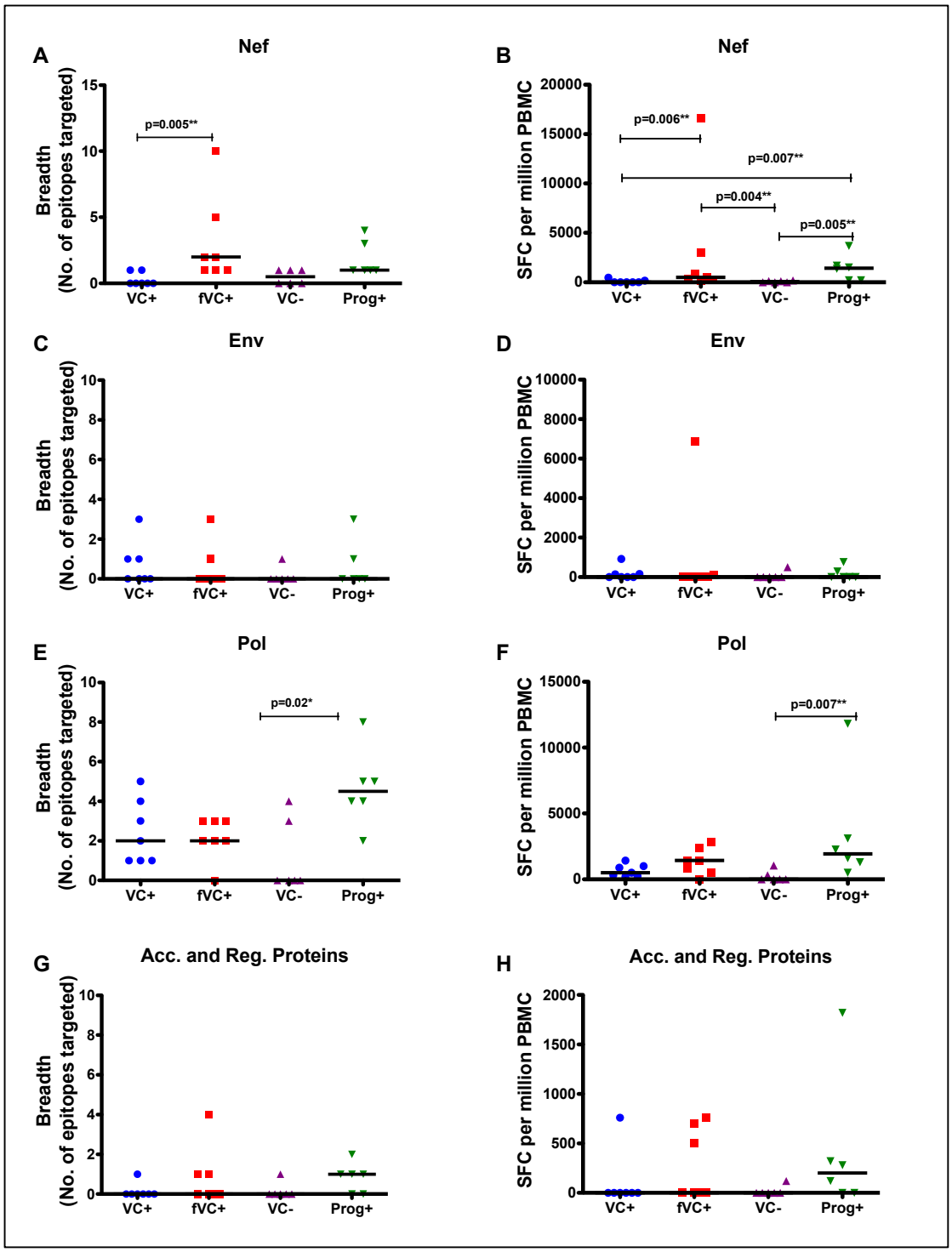

Figure 4.3. ELISpot screening of HIV-specific T cell responses at baseline among the 4 subgroups- Nef, Env, Pol, Acc & Reg proteins; ELISpot assay was used on thawed PBMCs to determine $CD8^+$ T cell responses following stimulation with HLA-matched optimal peptides spanning the entire HIV-1 clade C proteome. Breadth and magnitude of Nef (A, B), Env (C, D), Pol (E, F) and accessory and regulatory proteins (G, H) specific $CD8^+$ T cell responses amongst individuals with or without protective HLA class I alleles (bVC+/- and Prog +/-). VC+ (viremic controllers with protective HLA class I alleles, n =7); fVC+ (failing viremic controllers with protective HLA class I alleles, n=7); VC- (viremic controllers without protective HLA class I alleles, n=6); Prog+ (progressors with protective HLA class I alleles, n = 6). Statistical analysis was

performed on Prism using Kruskal-Wallis test to compare all 4 groups in each graph and Mann-Whitney U test to compare 2 groups that indicated significant differences.

We next studied the evolution of $CD8^+$ T cell responses among the subgroups. Figure 4.4A and B represent the evolution of responses among the VC+ subgroup where no significant changes in the overall responses across the entire HIV proteome and Gag specific $CD8^+$ T cell responses were observed. However, we noted a significant reduction in the overall breadth of $CD8^+$ T cell responses across the entire HIV proteome amongst the fVC+ over time (p-value 0.02; Wilcoxon matched pairs test) and a significant reduction of Gag targeted epitopes over time (p-value 0.003; Wilcoxon matched pairs test) (Figure 4.4C, D). This change was associated with increases in viral load in these individuals. No changes in overall responses across the entire HIV proteome but a trend in reduction of Gag targeted epitopes were observed in VC- subgroup (Figure 4.4E, F). Prog+ subgroup showed a trend in reduction of overall breadth of $CD8^+$ T cell responses across the entire HIV proteome (p-value 0.06; Wilcoxon matched pairs test) and a trend in reduction of Gag targeted epitopes over time amongst Prog+ (p-value 0.09; Wilcoxon matched pairs test) (Figure 4.4G, H). These changes were associated with further increases in viral load in these individuals. This analysis was performed while taking into account the duration of time between the tested time points for all participants since this time differed from participant to participant.

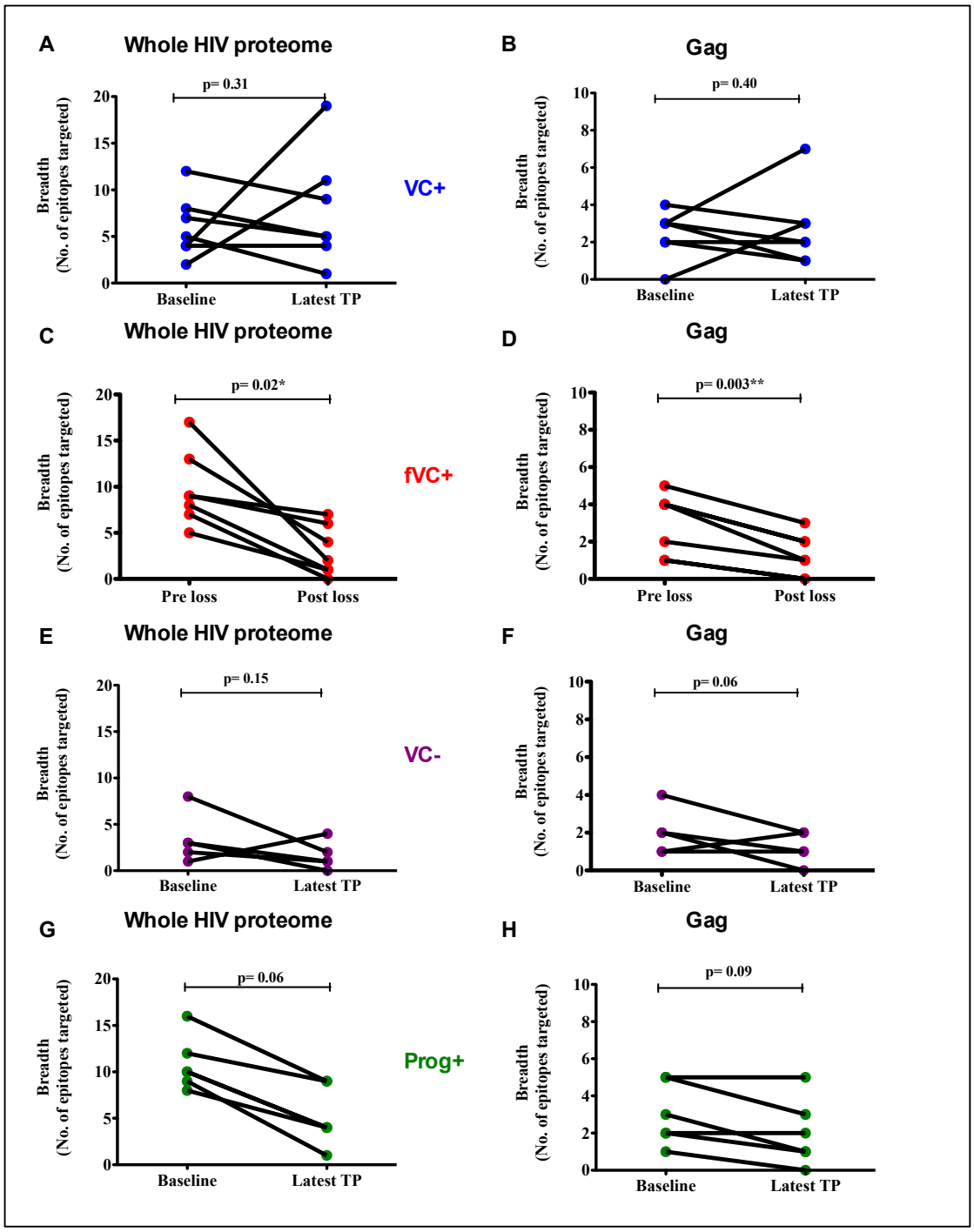

Figure 4.4. Longitudinal ELISpot screening of HIV-specific T cell responses amongst viremic controllers (VC+, n = 7, A-B), failing viremic controllers (fVC+, n = 7, C-D) and progressors (Prog+, n = 6, E-F) with protective HLA class I alleles and viremic controllers (VC-, n = 5, G-H) without protective HLA class I alleles – Overall and Gag. PBMCs were stimulated with optimal peptides restricted only to individuals HLA-B alleles and spanning the whole HIV proteome. Here we show overall responses across the entire HIV proteome and Gag specific $CD8^+$ T cell responses over time for the 4 subgroups. Latest TP represents latest time point. Statistical analysis was performed on Prism using the Wilcoxon matched pairs test to compare responses between the two time points. Time between the time points was taken in to account.

This longitudinal analysis of the evolution of $CD8^+$ T cell responses was extended to other proteins. No significant differences were observed in the breadth of Nef and Env amongst the subgroups. However we noted a trend towards a reduction in the breadth of Nef-specific $CD8^+$ T cell responses among the fVC+ over time (p-value 0.09; Wilcoxon matched pairs test) and Prog+ subgroup over time (p-value 0.09; Wilcoxon matched pairs test) (Figure 4.5). This analysis was performed taking in to consideration differences in follow up duration for participants. The VC- subgroup did not have any detectable $CD8^+$ T cell responses to Env.

No significant differences were observed in the breadth of Pol amongst the VC+ and VC- subgroups overtime. However, there was a significant reduction in the breadth of Pol-specific $CD8^+$ T cell responses among the fVC+ over time (p-value 0.03; Wilcoxon matched pairs test) and Prog+ subgroup over time (p-value 0.04; Wilcoxon matched pairs test) (Figure 4.6). No significant differences were observed in the breadth of responses to accessory and regulatory proteins amongst the subgroups. This analysis was performed while controlling for participant length of follow up. The VC- subgroup did not have any detectable $CD8^+$ T cell responses to accessory and regulatory proteins. Taken together these data demonstrate that the immunological parameter most strongly associated with of loss of viral control is the loss of overall responses across the entire HIV proteome, and in particular Gag specific $CD8^+$ T cell responses.

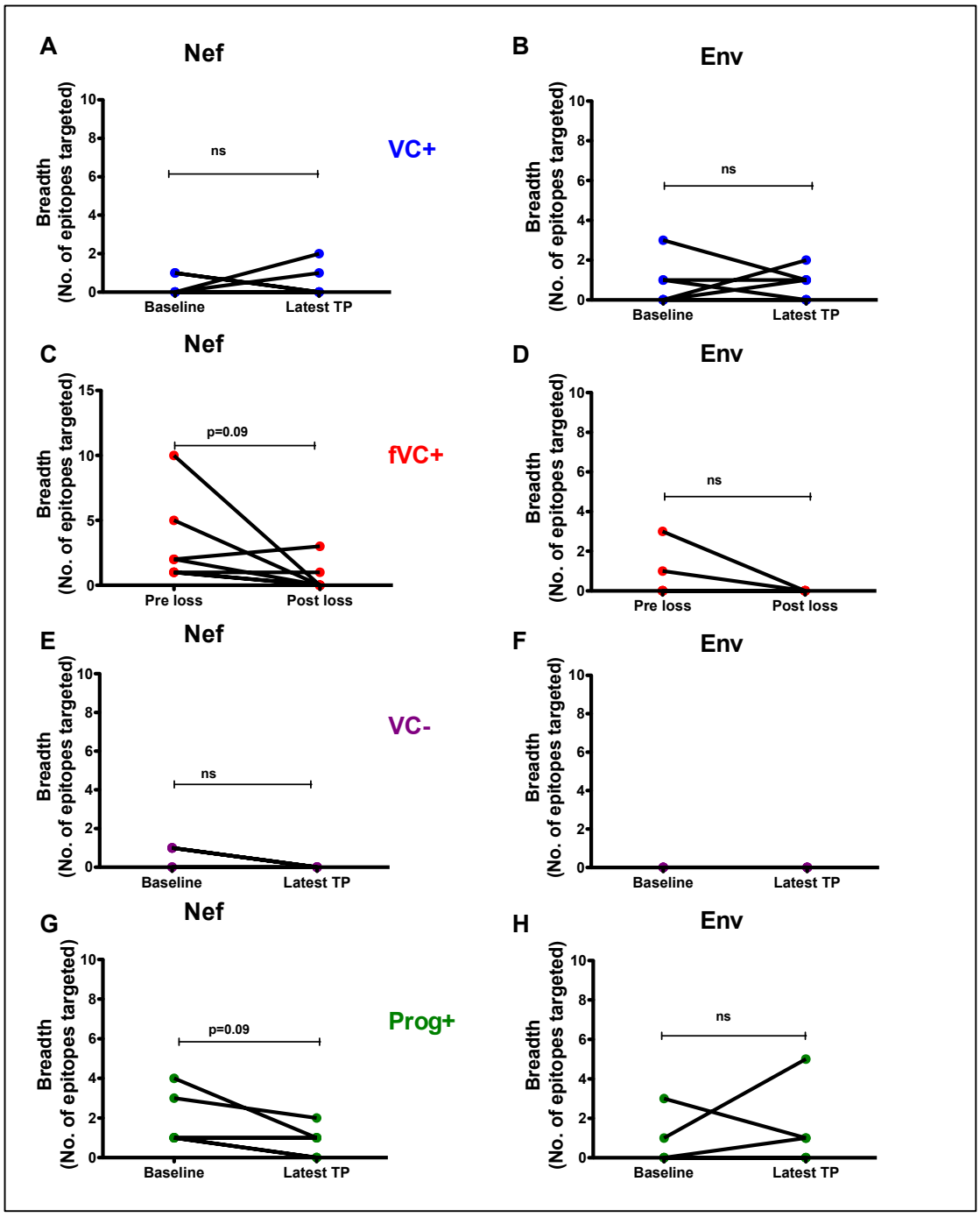

Figure 4.5. Longitudinal ELISpot screening of HIV-specific T cell responses amongst viremic controllers (VC+, n = 7), failing viremic controllers (fVC+, n = 7) and progressors (Prog+, n = 6) with protective HLA class I alleles and viremic controllers (VC-, n = 5) without protective HLA class I alleles – Nef and Env. PBMCs were stimulated with optimal peptides restricted only to individuals HLA-B alleles and spanning the whole HIV proteome. Here we show Nef and Env specific $CD8^+$ T cell responses over time for the 4 subgroups. Latest TP represents latest time point. Statistical analysis was performed on Prism using the Wilcoxon matched pairs test to compare responses between the two time points. Time between the time points was taken in to account.

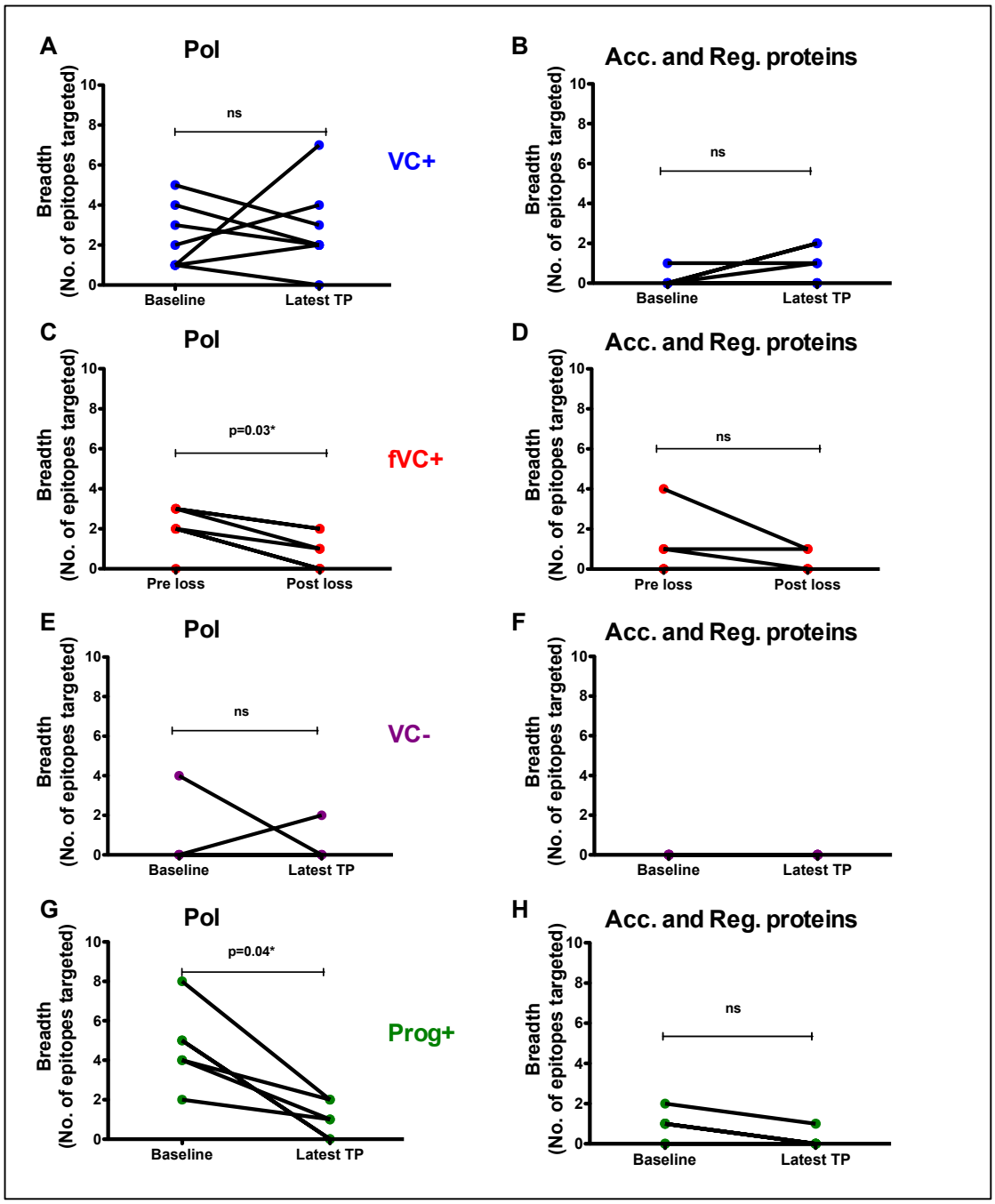

Figure 4.6. Longitudinal ELISpot screening of HIV-specific T cell responses amongst viremic controllers (VC+, n = 7), failing viremic controllers (fVC+, n = 7) and progressors (Prog+, n = 6) with protective HLA class I alleles and viremic controllers (VC-, n = 5) without protective HLA class I alleles – Pol and Acc & Reg proteins. PBMCs were stimulated with optimal peptides restricted only to individuals HLA-B alleles and spanning the whole HIV proteome. Here we show Pol and Accessory and regulatory protein specific $CD8^+$ T cell responses over time for the 4 subgroups. Latest TP represents latest time point. Statistical analysis was performed on Prism using the Wilcoxon matched pairs test to compare responses between the two time points. Time between the time points was taken in to account.

In order to further characterize factors that may be associated with loss of virologic control amongst individuals with protective HLA class I alleles, we focused on the fVC+ subgroup and identified the exact HIV-specific $CD8^+$ T cell responses lost over time. Table 4.2 shows longitudinal changes in HIV-specific $CD8^+$ T cell responses highlighting optimal peptides targeted at baseline, those no longer detectable at the time of virologic failure and those that persisted over time.

We noted that within the fVC+ subgroup, the majority (73%) of the total $CD8^+$ T cell responses were no longer detectable by ELISpot at the time of virological failure, and these responses were never regained despite the emergence of a few new $CD8^+$ T cell specificities at this latter time point in 3 individuals (e.g. SK-435, SK-224 and SK-079 gained responses to B*15:10-WI9 Vif, B*57-KAF9 Nef and B*57-QY10 Rev epitopes respectively) (Table 4.2). The decrease in the overall breadth of $CD8^+$ T cell responses was noted across the whole proteome post loss of control. Taken together these findings further demonstrate that virological failure in individuals with protective HLA class I alleles is associated with loss of overall $CD8^+$ T cell responses across the entire HIV proteome.

Table 4.2. Optimal HIV peptides targeted by failing viremic controllers before and after loss of viral control.

PID	HLA	BASELINE TIME POINT			POST LOSS OF VIROLOGIC CONTROL TIME POINTS					
		EPITOPES TARGETED			EPITOPES LOST			EPITOPES TARGETED		
		Epitopes targeted	Total Breadth	Total Magnitude (SFU/Mil PBMCs)	Epitopes lost	Total Breadth lost	Total Magnitude lost (SFU/Mil PBMCs)	Epitopes targeted	Total Breadth	Total Magnitude (SFU/Mil PBMCs)
SK-187	B*58:01/ B*08:01	[B8-DI8 (p24), -FL8 (Nef), -GL9 (RT), -EL8 (p15), -NL11 (gag)], [B58:01-TW10 (p24), -QW9 (p24), -IAW9 (RT), -SW10 (Int)]	9	2240	[B8-EL8 (p15), -NL11 (gag)], B58:01-SW10 (Int)	3	460	[B8-DI8 (p24), -FL8 (Nef), -GL9 (RT)], [B58:01-TW10 (p24), -QW9 (p24), -IAW9 (RT)]	6	3188
SK-435	B*57:03/ B*15:10	[B15:10-TIL9 (RT), -RI11 (Vif)], [B57-WF9(p17), -ISW9(p24), -KF11(p24), -TW10(p24), -AW9(Vpr), -VF9(Vif), -KAF9(Nef), B57-AF10 (RT), -QL11 (gp120), -LW9 (Vif), -VI9 (POL)]	13	6580	[B15:10-RI11 (Vif)], [B57-WF9(p17), -TW10(p24), -AW9(Vpr), -VF9(Vif), -KAF9(Nef), -AF10 (RT), -QL11 (gp120), -LW9 (Vif), -VI9 (POL)]	10	2300	[B15:10-TIL9 (RT)], [B57-ISW9(p24), -KF11(p24), B15:10-WI9(Vif)]	4	6128
SK-224	B*57:02/ B*07:02	[B7-GL9(p24), -RM9 (Nef), -TL10(nef)], [B57-ISW9(p24), -TW10(p24), -QW9 (p24), -IW9(RT), -AF10 (RT), -KI13 (RT)]	9	7280	B7-GL9(p24), [B57-TW10(p24), B57-KI13 (RT)]	3	540	[B7-RM9 (Nef), -TL10(nef)], [B57-ISW9(p24), -QW9 (p24), -IW9(RT), -AF10 (RT), -KAF9(Nef)]	7	7600
SK-242	B*81:01/ B*67	[B39-GL9(p24), -TL9 (p24), -NL11 (p24)], [B81-TL9(p24), -RM9 (Nef), -RGF9(nef), -LI9(Int), -SL10 (RT)]	8	9740	[B39-GL9(p24), -NL11 (p24), -TL9(p24)], [B81-RM9 (Nef), -RGF9(nef), -LI9(Int), -SL10 (RT)]	7	6520	B39-TL9 (p24)	1	323
SK-024	B*58:01/ B*44	[B44-SL9(P24), -KY11 (NEF), -QY9 (NEF)], [B58:01-SW10(Int), -YY8 (Nef), -YT9 (Nef), -KY11 (Nef)]	7	4740	[B44-SL9(P24), -KY11 (NEF), -QY9 (NEF)], [B58:01-SW10(Int), -YY8 (Nef), -YT9 (Nef), -KY11 (Nef)]	7	4740	-	0	0
SK-079	B*58:01/ B*44	[B44-MY9 (gp120), -KY11 (NEF), -QY9 (NEF)], [B58:01-TW10(p24), -HW9(nef), -IAW9(RT), -SW10(Int), -KW11(Env), -VF9(Vif), -KAF9(Nef), -QL11, -YY8 (Nef), -YT9 (Nef), -NW9 (Nef), -KAF9(Nef), -KY11 (Nef), -HQ10 (Nef)]	17	26700	[B44-MY9 (gp120), -KY11 (NEF), -QY9 (NEF)], [B58:01-TW10(p24), -HW9(nef), -SW10(Int), -KW11(Env), -VF9(Vif), -KAF9(Nef), -QL11, -YY8 (Nef), -YT9 (Nef), -NW9 (Nef), -KAF9(Nef), -KY11 (Nef), -HQ10 (Nef)]	16	24680	B58:01-IAW9(RT), B58:01-QY10(Rev)	2	456
SK-188	B*57:03/ A7401	[B57-ISW9(p24), -KF11(p24), -HW9(nef), -AF10 (RT), -FF9 (RT)]	5	3640	[B57-ISW9(p24), -HW9(nef), -AF10 (RT), -FF9 (RT)]	4	1480	B57-KF11(p24)	1	110
TOTAL			68	60920		50	40720		21	17805

4.4 Escape mutations in Gag only partially explain the loss of virological control in fVC with protective HLA class I alleles

The ability of HIV to evade CTL recognition by developing mutations that escape recognition and undermine the immune response is well documented in HIV infection (149, 333–339). We next investigated whether the observed loss of virologic control associated with loss of immune responses was linked to the presence of mutations that potentiate immune escape. We hypothesized that the loss of $CD8^+$ T cell responses will primarily be due to the presence of escape mutations. In the current study, longitudinal *gag* population sequencing was performed from plasma samples at baseline and post loss of control on the fVC+ subjects and at baseline and available later time points for the viremic controllers with and without protective HLA class I alleles (VC+ and VC-). We assessed mutations within epitopes restricted by the patients' protective HLA class I alleles plus epitopes restricted by the other HLA-B alleles that the subjects possessed. HIV-1 clade C Gag consensus sequence was used as reference for comparison in this analysis.

For the fVC+ subjects, we assessed a cumulative total of 67 epitopes at baseline and 69 at the first time point when the subjects had a viral load increase to more than 10, 000 HIV RNA copies/ml (post loss of viral control) (Figure 4.7). Of the 67 epitopes assessed at baseline, 28% (19 of 67) induced detectable $CD8^+$ T cell responses. Furthermore, still at baseline, 51% (34 of 67) of the epitopes evaluated had variant sequences either within the epitope or in the 5 flanking regions and of these, 32% (11 of 34) elicited detectable $CD8^+$ T cell following stimulation with peptides corresponding to the wild type epitopes. Forty nine percent (33 of 67) of the epitopes had wild type sequences and of these, 24% (8 of 33) were capable of inducing a detectable $CD8^+$ T cell response. At the post loss of viral control time point we

analysed a cumulative total of 69 epitopes. Of these, 53% (37 of 69) epitopes had variant sequences either within the epitope or in the 5 flanking regions with 11% (4 of 37) having elicited detectable $CD8^+$ T cell following stimulation with peptides corresponding to the wild type epitopes that persisted.

The number of epitopes with wild type or variant sequences did not change much pre- and post-loss of control, however we observed a drop in the number of epitopes that induced detectable $CD8^+$ T cell responses post loss of control (Figure 4.7). We observed that of the 11 variant epitopes that elicited a $CD8^+$ T cell response at baseline, 7 responses were no longer detectable post loss of viral control. In addition we observed that of the 8 wild type epitopes that elicited a $CD8^+$ T cell response at baseline, 6 responses were lost at post loss of control time point. Of these 6, 3 epitopes did not elicit a $CD8^+$ T cell response as a result of escape while the other 3 remained wild type despite failure to elicit a detectable $CD8^+$ T cell response. This data may suggest that the accumulation of these escape mutations may have abrogated the immune responses in some individuals while the reason for the loss of responses in some individuals are not known.

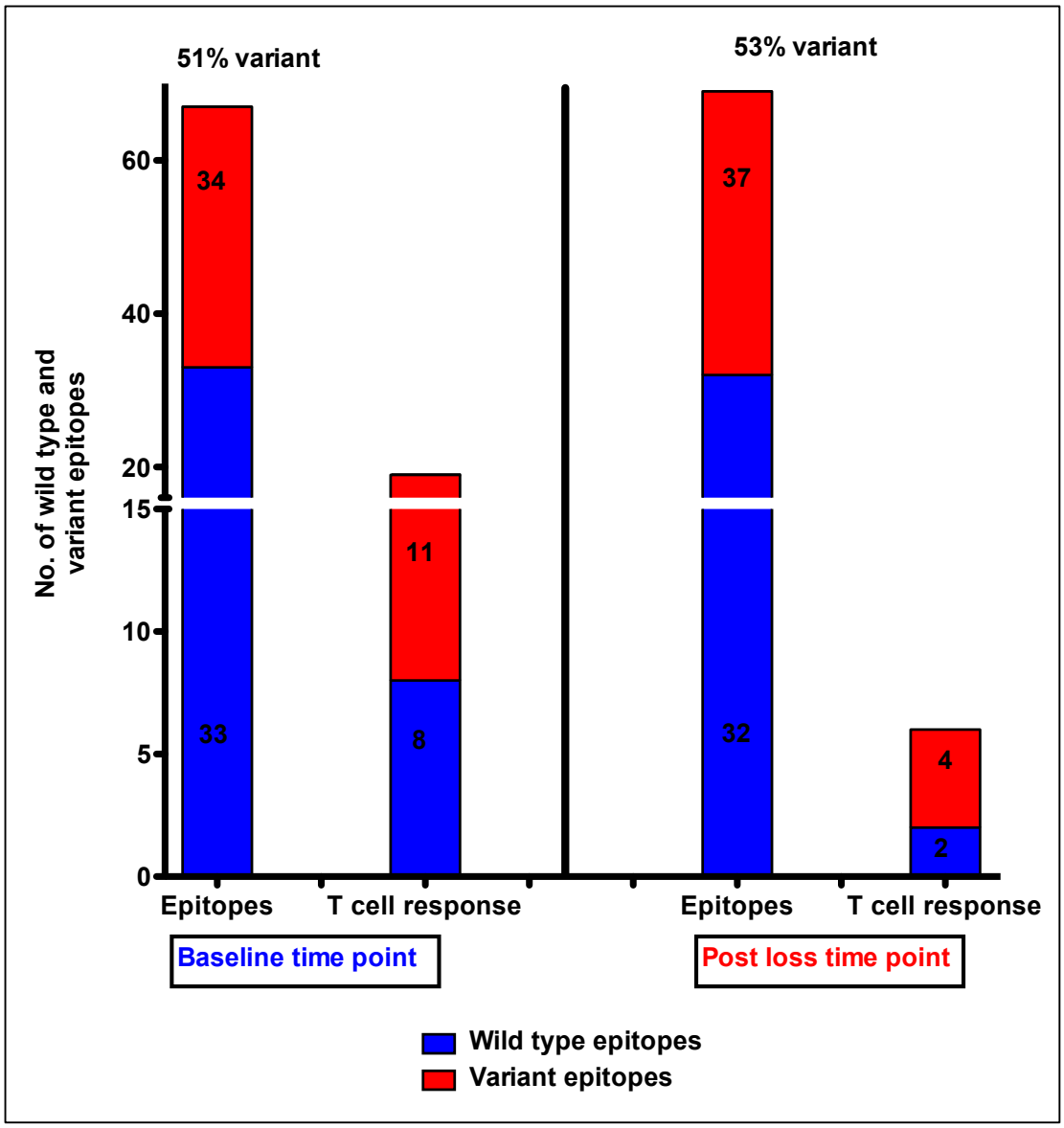

Figure 4.7. Immunogenicity and escape of fVC+ subjects before and after loss of viral control. We evaluated if the epitopes within the Gag region had variant sequences either within the epitope or in the 5 flanking regions. HIV-1 clade C Gag consensus sequence was used as reference for comparison in this analysis.

We narrowed down the analysis and focussed on escape mutations in known immunodominant epitopes. Of the 7 fVC+, there are 2 B*57:03-, 1 B*57:02-, 3 B*58:01- and 1 B*81:01-positive individuals. We evaluated the presence or absence of escape mutations within the 4 well-defined B*57:02/03-restricted immunodominant Gag epitopes; TW10, ISW9, KF11 and QW9 among subjects possessing HLAs B*57:02/03 and B*58:01 over time (Table 4.3). We observed that the most common mutation was the T242N and reversion to wild type occurred in 2 subjects with no detectable $CD8^+$ T cell responses. Escape in the KF11 epitope was seen in only 1 subject.

For one of the subjects we evaluated escape mutations within the 3 B*81:01-restricted immunodominant epitopes; SV9, TL9 and HA9. Amongst individuals possessing HLA B*81:01, increase in viremia may be associated with escape mutations in the immunodominant TL9 and HA9 epitopes resulting in the loss of these $CD8^+$ T cell responses. However, in HIV-1 subtype C, mutations in the TL9 epitopes, unless accompanied by compensatory mutations, may severely compromise viral fitness resulting in clinical benefit to the patient (258). In our study we had only 1 fVC (SK-242) possessing HLA B*81:01 (Table 4.3). This subject had one wild type epitope (SV9) with no detectable $CD8^+$ T cell response while 2 of the 3 had variant sequences with a detectable $CD8^+$ T cell response induced by stimulation with wild type epitopes. We noted sequence variations within the TL9 with a detectable $CD8^+$ T cell response at baseline; the mutation was present after the loss of control and reverted back to wild type in the subsequent time point following post loss. We also noted a mutation (H219Q) within the HA9 epitope with no detectable $CD8^+$ T cell response at baseline; the mutation was still present post loss of control time point. Furthermore, this subject did not respond to SV9 epitope at baseline and no mutation was observed

post loss of control time point. The overall observation of the fVC+ subgroup supports the view that accumulation of escape mutations within Gag may abrogate immune responses in some individuals. The reasons for the loss of responses in the absence of escape require further investigation.

Table 4.3. Sequence variation within HLAs B*57:02/03/58:01 and B*81:01 restricted immunodorminant epitopes

Patient ID	HLA Type	Months from enrollment	Epitope KF11(gag 162-172) KVIEEKAFSPEVIPMFTALSE	CD8+ T cell response	Epitope ISW9 (gag 147-155) MVHQAISPRTLNAWVKVIE	CD8+ T cell response	Epitope TW10 (gag 240-249) DIAGTTSTLQEQIAWMTSNP	CD8+ T cell response	Epitope QW9 (gag 296-304) TLRAEQATQDVKNWMTDLL	CD8+ T cell response
B*57 subjects										
SK-188 fVC	B*57:03	0	.....	+	....PL.....	+	.....N.....G..	–	.....	–
		10	.....	+	....PL.....	–	.....N.....G..	–	.....	–
SK-224 fVC	B*57:02	0	.....	–	....P.....	+	.....N.....	+	.....E..	+
		7	.....	–	....P.....	+	.....N....T.....	–	.....E..	+
		14	.....	–	....P.....	+	.....N....T.....	–	.....E..	+
		20	.....	–	....P.....	–	.....N....T.....	–	.....E..	+
		71	.....	–	....P.....	–	.....N....T.....	–	.....E..	+
SK-435 fVC	B*57:03	0	.....	+	....VL.....	+	.....N....T.....	+	.....	–
		6	.....	–	....VL.....	+	.....N....T.....	–	.....	–
		12	.....	+	....VL.....	+	.....N....T.....	–	.....	–
		21	..V.....S....	+	.....V.	+	.....G...R..	–	.....	–

B*58:01 subjects	HLA Type	Months from enrollment	Epitope KF11(gag 162-172) KVIEEKAFSPEVIPMFTALSE	CD8+ T cell response	Epitope ISW9 (gag 147-155) MVHQAISPRTLNAWVKVIE	CD8+ T cell response	Epitope TW10 (gag 240-249) DIAGTTSTLQEQIAWMTSNP	CD8+ T cell response	Epitope QW9 (gag 296-304) TLRAEQATQDVKNWMTDLL	CD8+ T cell response
SK-024 fVC	B*58:01	0	.....	–	.....	–	.....N..	–	.....E.....	–
		21	.....	–	.....	–	.....N..	–	.....E.....	–
SK-079 fVC	B*58:01	0	.....	–	.....T.....	–	n/a	+	n/a	+
		21	.....	n/a	.....	n/a	.....N.....	n/a	.....S.E.....E..	n/a
		34	.....	n/a	.....	n/a	.....N.....	n/a	.....S.E.....E..	n/a
		41	.....	–	.....	–	.....N.....	–	.....S.E.....E..	–
SK-187 fVC	B*58:01	0	.....	–	....P.....	–	.....N....T.....	+	.....	+
		10	.....	–	.....	–	.....	–	.....	+
		26	n/a	–	n/a	–	n/a	–	n/a	–
		55	.....	–	.....	–	.....I.G..	–	.....	–

B*81:01 subjects	HLA Type		Epitope SV9 (gag 148-156) VHQAISPRTLNAWVKVIEE	CD8+ T cell response	Epitope TL9 (gag 180-188) LSEGATPQDLNTMLNTVGG	CD8+ T cell response	Epitope HA9 (gag 216-224) EWDRLHPVHAGPIAPGQMR	CD8+ T cell response
SK-242 fVC	B*81:01	0	.....	–	.....Y.....A...	+	.....Q.....	–
		20	.....	n/a	.....Y.....A...	n/a	.....Q.....	n/a
		26	...P.....	n/a	.....	n/a	.....Q...V.....	n/a

4.5 Maintenance of low viral load despite the presence of Gag escape mutations among viremic controllers with protective HLA class I alleles

We also assessed epitope sequences of the VC+ and VC- subjects at baseline and later time point. However due to limited sample availability only 2 subjects were sequenced at both baseline and later time points. Evaluation of epitope sequences allowed us to assess a cumulative total of 67 epitopes at baseline for the VC+ subgroup. Of the 67, 30% (20 of 67) were wild type with 20% (4 of 20) inducing a positive $CD8^+$ T cell response while 70% (47 of 67) had variant sequences and 21% (10 of 47) elicited detectable $CD8^+$ T cell following stimulation with peptides corresponding to the wild type epitopes (Figure 4.8). Furthermore, in the VC- subgroup, a cumulative total of 47 epitopes were evaluated. Fifty one percent (51%) (24 of 47) were wild type sequences and 33% (8 of 24) induced a positive $CD8^+$ T cell response, while 47% (23 of 47) had variant sequences with 13% (3 of 23) having elicited detectable $CD8^+$ T cell responses following stimulation with peptides corresponding to the wild type epitopes.

Tables 4.4 and 4.5 show the sequences of the immunodominant epitopes restricted by the patients' HLA B alleles of VC with and without protective HLA class I alleles at baseline. Although the number of subjects studied here is limited, these data demonstrate that VC+ maintained viral control despite having the highest prevalence of variant sequences either within the epitope or in the 5 flanking regions when compared to individuals lacking protective HLA class I alleles.

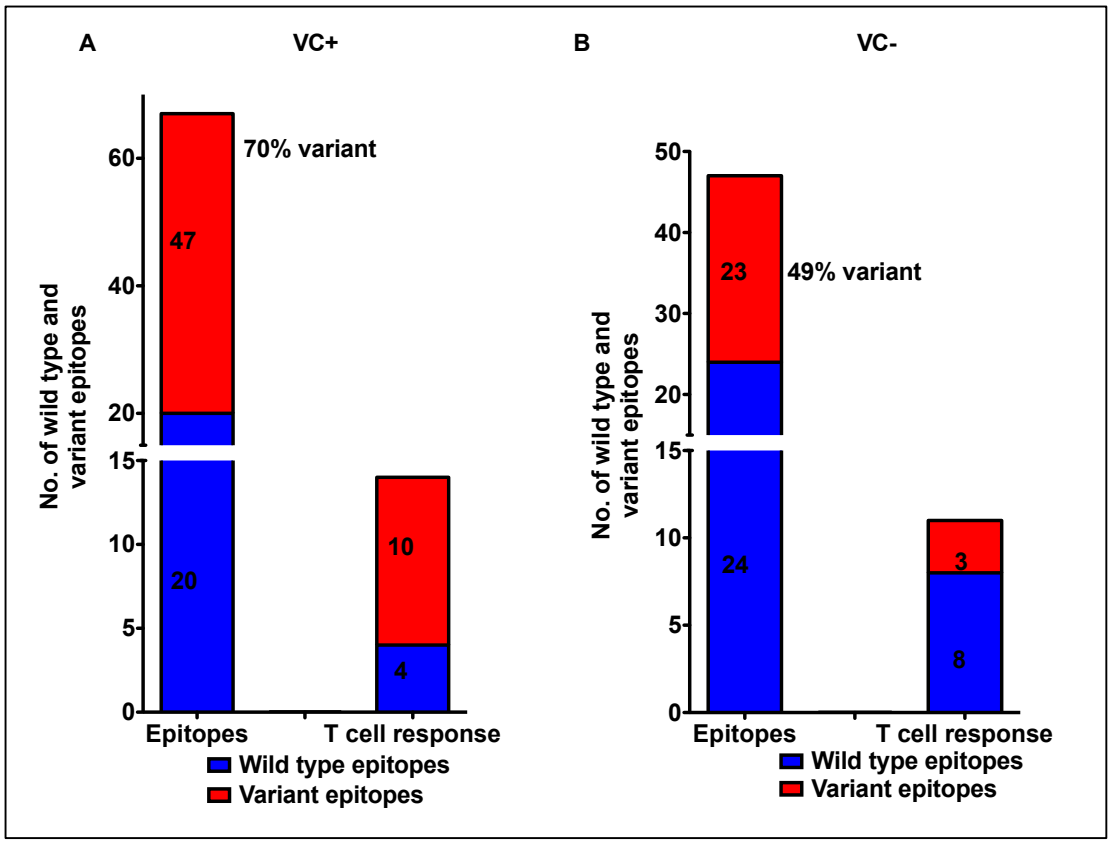

Figure 4.8. Immunogenicity and escape of VC+ and VC- subjects at baseline. We evaluated if the epitopes within the Gag region had variant sequences either within the epitope or within 5 amino acids flanking the epitope. HIV-1 clade C Gag consensus sequence was used as reference for comparison in this analysis.

Table 4.4. Sequence variation within HLAs B*57:03/58:01, B*81:01 and A*74:01 restricted immunodominant epitopes

Patient ID	HLA Type	Months from enrollment	Epitope KF11(gag 162-172) KVIEEKAFSPEVIPMFTALSE	CD8+ T cell response	Epitope ISW9 (gag 147-155) MVHQAISPRTLNAWVKVIE	CD8+ T cell response	Epitope TW10 (gag 240-249) DIAGTTSTLQEQIAWMTSNP	CD8+ T cell response	Epitope QW9 (gag 296-304) TLRAEQATQDVKNWMTDLL	CD8+ T cell response
B*57 subjects										
SK-199 VC	B*57:03/ A*74:01	0	.....G.....	+	....PM.....	+	.....N....V.....	–	.....	+
		82	N/A	+	N/A	+	N/A	–	N/A	+
B*58:01 subjects	HLA Type	Months from enrollment	Epitope KF11(gag 162-172) KVIEEKAFSPEVIPMFTALSE	CD8+ T cell response	Epitope ISW9 (gag 147-155) MVHQAISPRTLNAWVKVIE	CD8+ T cell response	Epitope TW10 (gag 240-249) DIAGTTSTLQEQIAWMTSNP	CD8+ T cell response	Epitope QW9 (gag 296-304) TLRAEQATQDVKNWMTDLL	CD8+ T cell response
SK-089 VC	B*58:01/ A*74:01	0	.....I.....	–	.....	–	.....N.....	–	.....	–
		43	N/A	–	N/A	–	.....N.....T.....	–	.....	+
SK-282 VC	B*58:01	0	.....I.....	–	.....	–	.....N.....N..	+	....E.....	+
		67	.....G.....	–	....P.....	–	.....N.....	–	.....C.....	–
SK-348 VC	B*58:01/ 81:01	0	.....	–	.....	–	.....N..	+	.....	+
		47	N/A	–	N/A	–	N/A	–	N/A	–
B*81:01 subjects	HLA Type		Epitope SV9 (gag 148-156) VHQAISPRTLNAWVKVIEE	CD8+ T cell response	Epitope TL9 (gag 180-188) LSEGATPQDLNTMLNTVGG	CD8+ T cell response	Epitope HA9 (gag 216-224) EWDRLHPVHAGPIAPGQMR	CD8+ T cell response		
SK-348 VC	B*81:01/ 58:01	0	.....	–	.....	+	.....V...I.	–		
		47	N/A	–	N/A	–	N/A	–		
SK-354 VC	B*81:01/ A*74:01	0	....L.....	–	.....G.....	+	.....V.....	+		
		36	N/A	–	N/A	+	N/A	+		
A*74:01 subjects	HLA Type		Epitope KK9 (gag 18-26) LDKWEKIRLRPGGKKHYML	CD8+ T cell response	Epitope RH9 (gag 20-28) KWEKIRLRPGGKKHYMLKH	CD8+ T cell response	Epitope RLY10 (gag 20-29) KWEKIRLRPGGKKHYMLKHL	CD8+ T cell response		
SK-362 VC	A*74:01	0	..R...N.....K.R.	–	R....N.....K.R...	–	R....N.....K.R...I	–		
		50	N/A	–	N/A	–	N/A	–		
SK-354 VC	B*81:01/ A*74:01	0	.....S.....R...	–	.....S.....R.....	–	.....S.....R.....	–		
		36	N/A	–	N/A	–	N/A	–		
SK-089 VC	B*58:01/ A*74:01	0	.....R.....Q.R.	–	...R.....Q.R...	–	...R.....Q.R...I	–		
		43	N/A	–	N/A	–	N/A	–		
SK-199 VC	B*57:03/ A*74:01	0	.....R.K.....K...	–	...R.K.....K.....	–	...R.K.....K.....	–		
		82	N/A	–	N/A	–	N/A	–		

Table 4.5. Sequence variation within immunodominant epitopes among VC subjects without protective HLA alleles

B*42:01/39:10 subjects	HLA Type		Epitope SV9 (gag 148-156) VHQAISPRTLNAWVKVIEE	CD8+ T cell response	Epitope TL9 (gag 180-188) LSEGATPQDLNTMLNTVGG	CD8+ T cell response	Epitope HA9 (gag 216-224) EWDRLHPVHAGPIAPGQMR	CD8+ T cell response
SK-292 VC	B*39:10/ 40:01	0	....	-	....	+	....	-
		18	....	-	....	+	.....Q.....	-
SK-342 VC	B*42:01/ 45:01	0	....	-	.....G.....	+	....V.	-
		44	N/A	N/A	N/A	N/A	N/A	N/A
SK-209 VC	B*42::01 /08:01	0	....	-	.....T.....	-	.....Q...V.	-
		38	N/A	-	N/A	-	N/A	-
SK-315 VC	B*42:01	0	....	-	....	+	.....V.....V.	-
		28	N/A	-	N/A	+	N/A	-
SK-317 VC	B*42:01/ 44:01	0	....	-	....	+	....V.	-
		43	N/A	-	N/A	+	N/A	-

4.6 Summary of results

In Chapter 4 we studied longitudinal $CD8^+$ T cell responses and sequence variation within CTL epitopes and associated these parameters with divergent disease progression and loss of CD8+ T cell responses in the 4 subgroups; VC+, fVC+, VC- and Prog+ subjects. We showed that the loss of viral control seen in the failing viremic controller subgroup with protective HLA class I alleles (fVC+) was related to a significant loss of overall $CD8^+$ T cell responses across the whole HIV proteome and of Gag specific $CD8^+$ T cell responses. Additionally we demonstrated that fVC+ subjects targeted significantly higher Nef epitopes at baseline compared to VC+ subjects suggesting that targeting of Nef may be a predictor of loss of viral control. We also showed that the viral control seen among the viremic controller subgroup with protective HLA class I alleles (VC+) was associated with the maintenance of the $CD8^+$ T cell responses over time. Interestingly VC- subjects targeted fewer epitopes despite maintaining viral control throughout the time of follow up suggesting an alternative mechanism of viral control for these individuals.

We also studied $CD8^+$ T cell immune-driven sequence variation within Gag and associated escape with divergent disease progression. We demonstrated that escape and potential escape mutations were evident in the fVC+ subgroup and in some instances the loss of $CD8^+$ T cell responses were as a result of escape in some individuals while the loss of some of the responses could not be explained by escape. Interestingly some VC+ maintained viral control throughout despite the presence of escape mutations within Gag, and persistence of responses irrespective of sequence variation appeared to be a better correlate of viral control. VC- on the other hand had fewer escape mutations within Gag and maintained low viral load throughout.

CHAPTER 5: $CD8^+$ T CELL FUNCTIONAL ANALYSIS AND ASSOCIATION WITH DISEASE PROGRESSION IN CHRONICALLY HIV-1 SUBTYPE C INFECTED INDIVIDUALS WITH AND WITHOUT PROTECTIVE HLA CLASS I ALLELES

5.1 Introduction

The maintenance of a highly polyfunctional $CD8^+$ T cell profile has been well documented elsewhere amongst elite controllers (ECs) and long-term non-progressors (LTNPs) where $CD8^+$ T cells from these individuals have demonstrated to be more polyfunctional, to have a higher proliferative capacity and greater ability to suppress viral replication compared to progressors (302, 324, 325, 340, 341). These data suggest that a more polyfunctional $CD8^+$ T cell phenotype is associated with viral control. However, there are some caveats; firstly, the extent to which this highly polyfunctional and proliferative $CD8^+$ T cell phenotype is maintained in some individuals or lost in some individuals who experience loss of viral control is not fully understood. Secondly, most of the previous studies characterized the functional properties of $CD8^+$ T cells at only one time point. To address this gap in knowledge, we characterized longitudinally the functional properties of $CD8^+$ T cells for polyfunctionality, proliferation and ability to suppress viral replication from study participants in an attempt to determine whether these functional properties of $CD8^+$ T cells played any role in the control or loss of virologic control among individuals with and without protective HLA class I alleles.

We hypothesised that viremic controllers with or without protective HLA class I alleles will present with highly polyfunctional and proliferating $CD8^+$ T cells able to suppress viral replication in an *ex vivo* viral inhibition assay compared to progressors. We further hypothesized that the $CD8^+$ T cells amongst failing viremic controllers (individuals that were controlling vireamia and later experienced loss of viral control) with protective HLA class I alleles will show reduced polyfunctionality, reduced proliferative capacity and reduced ability to suppress viral replication post loss of virologic control.

In order to test these hypotheses we aimed to:

1. Study the polyfunctionality of $CD8^+$ T cells amongst individuals with controlled vireamia and associate these $CD8^+$ T cell properties with viral control or lack of control upon longitudinal follow up.

2. Study the proliferative capacity of $CD8^+$ T cells amongst individuals with controlled vireamia and associate these $CD8^+$ T cell properties with viral control or lack of control upon longitudinal follow up.

3. Study the *ex vivo* ability of $CD8^+$ T cells to suppress HIV replication and associate this function with disease progression or the lack thereof.

5.2 Study participants

For this sub-study, we focused on individuals with divergent disease progression status with and without protective HLA class I alleles. These are described below:

i. Viremic controllers (VC) were defined as individuals who were enrolled with a VL of less than 2,000 HIV RNA copies/ml and maintained this low viral load for the entire period of enrolment.

ii. Failing viremic controllers (fVC) were defined as bVC that were enrolled with a VL of less than 2,000 HIV RNA copies/ml but lost virological control. We defined the loss of viral control as an increase in VL to more than 10,000 HIV RNA copies/ml at a minimum of 2 time points during the time of follow-up.

We also included some progressors with protective HLA class I alleles as a control subgroup.

We studied 7 fVC+, 7 VC+, 6 VC- and 6 Prog+ as illustrated in the flow chart below:

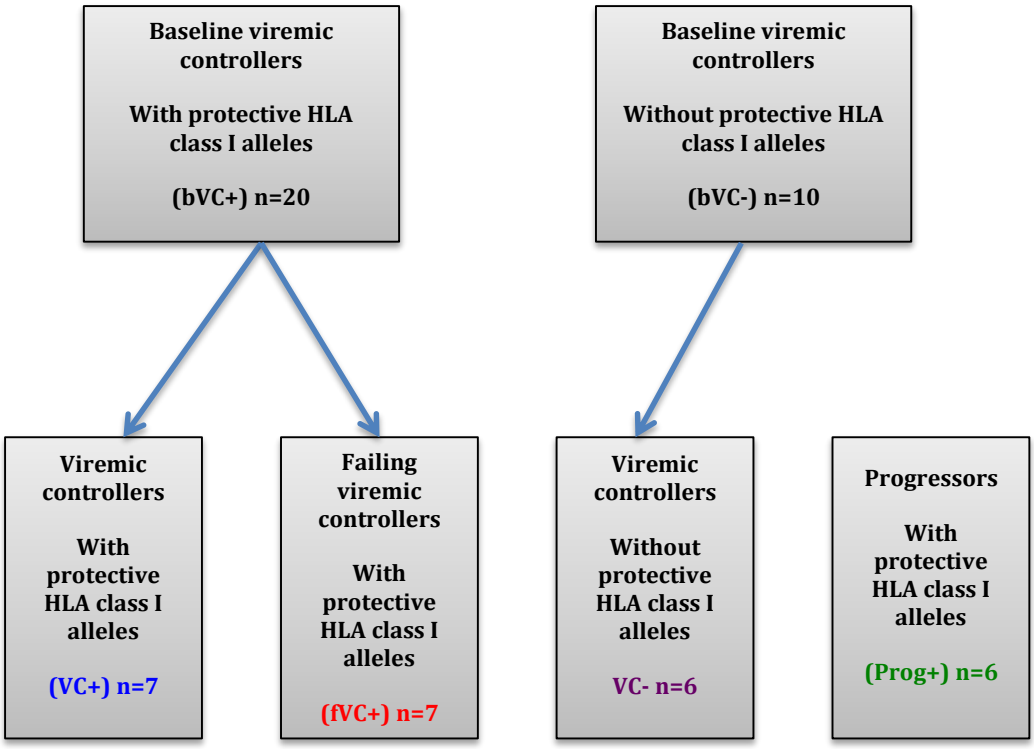

Figure 5.1 Flow chart showing the breakdown of study groups in to subgroups.

5.3 No significant differences in the polyfunctionality of $CD8^+$ T cells between the 4 subgroups; VC+, fVC+, VC- and Prog+

We first studied the ability of $CD8^+$ T cells to produce diverse cytokines as previously described (340). This is for consistency with previously studies so that we can compare our findings. We assessed 5 functions; interferon gamma (IFNγ), interleukin-2 (IL-2), tumour necrosis factor alpha (TNF-α), macrophage inflammatory protein 1 beta (MIP1β) and CD107a at baseline and later time points for VC+, VC- and Prog+; and pre and post loss of viral control time points for fVC+ subjects. The gating strategy for this analysis is summarized on Figure 5.2. Our data show that there were no significant differences in $CD8^+$ T cell polyfunctionality to explain the divergence in disease progression amongst the subgroups (VC+, fVC+, VC- and Prog+) at baseline and later time points (Figure 5.3). Although the profiles of the different

subgroups seem different, we were underpowered by the small sample size to make conclusions.

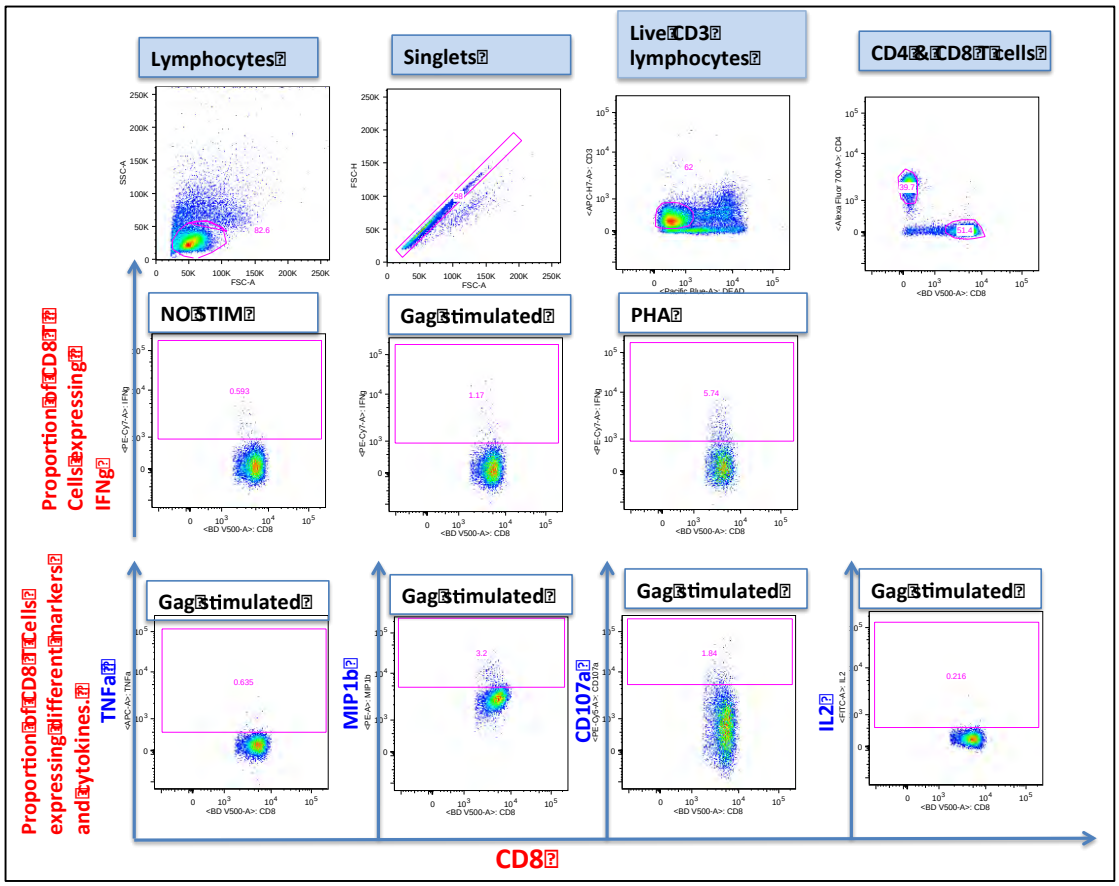

Figure 5.2. Polyfunctionality of CD8$^+$ T cells upon stimulation with Gag peptide pools – gating stratergy. The initial gating was on lymphocytes followed by the forward scatter height (FCS-H) versus forward scatter area (FSC-A) to eliminate the doublets. Subsequently live CD3$^+$ T cell population was gated on followed by gating of CD8$^+$ and CD4$^+$ T cell populations. The individual gating for respective functions followed (set based on the negative control ('NO STIM')), these were used to identify positive responses.

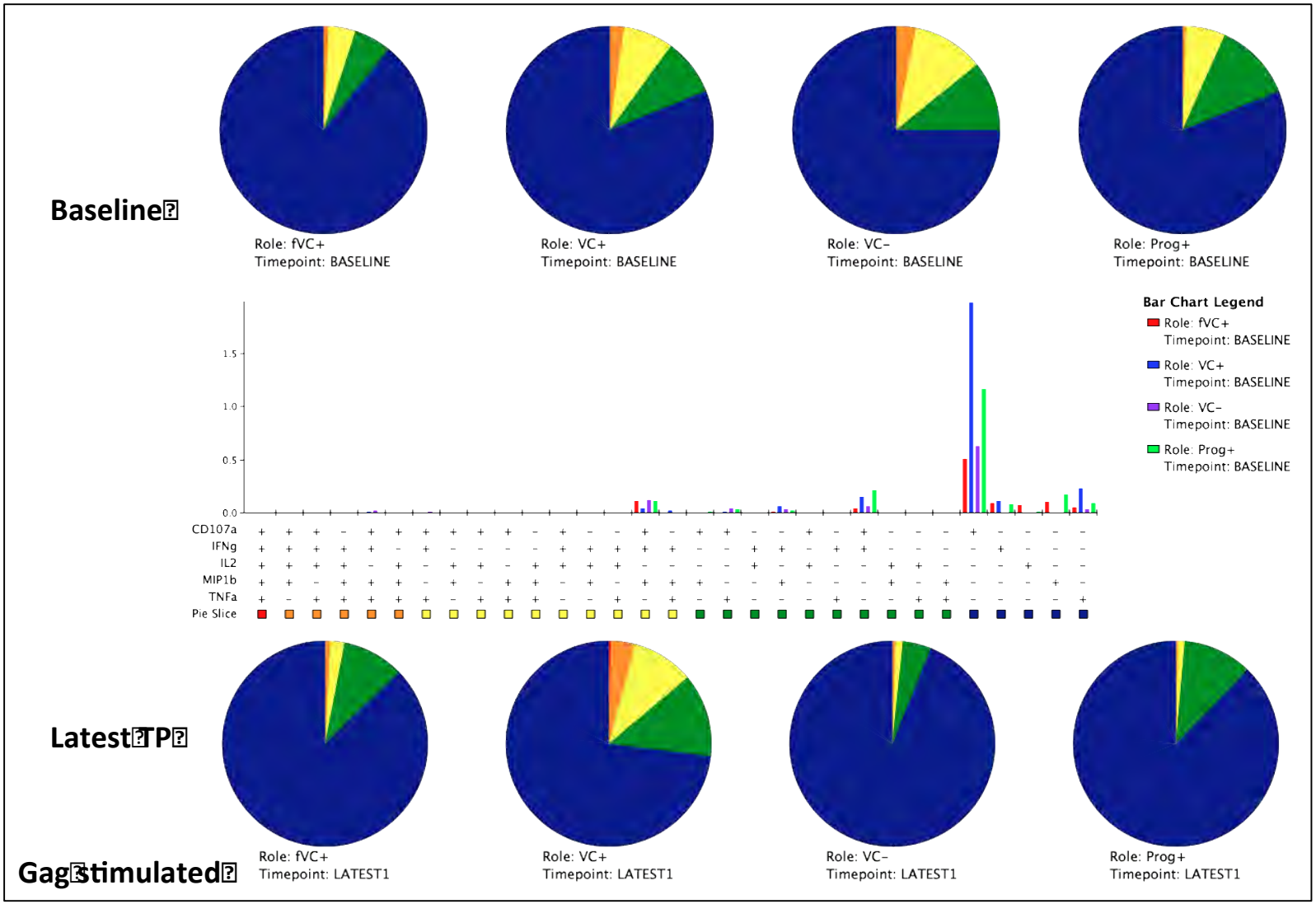
Baseline
Role: fVC+
Timepoint: BASELINE
Role: VC+
Timepoint: BASELINE
Role: VC−
Timepoint: BASELINE
Role: Prog+
Timepoint: BASELINE
Bar Chart Legend
Role: fVC+
Timepoint: BASELINE
Role: VC+
Timepoint: BASELINE
Role: VC−
Timepoint: BASELINE
Role: Prog+
Timepoint: BASELINE
1.5
1.0
0.5
0.0
CD107a
IFNg
IL2
MIP1b
TNFa
Pie Slice
Latest TP
Gag stimulated
Role: fVC+
Timepoint: LATEST1
Role: VC+
Timepoint: LATEST1
Role: VC−
Timepoint: LATEST1
Role: Prog+
Timepoint: LATEST1

Figure 5.3. Polyfunctionality of $CD8^+$ T cells upon stimulation with Gag peptide pools - amongst viremic controllers (VC+), failing viremic controllers (fVC+), progressors (Prog+) with protective HLA class I alleles and viremic controllers without protective HLA class I alleles; At baseline and at later time points. The 5 functions studied were CD107a, IFNg, IL2, MIP1b and TNFa. On the pie charts, red represents 5 functions, orange; 4 functions, yellow; 3 functions, green; 2 functions and blue; 1 function. Gating strategy is shown in Figure 5.1. Boolean gating was performed in order to allow creation of a full array of possible combinations of up to 32 response patterns. Positive responses were reported after background correction and the percentage of epitope-specific $CD8^+$ T cell responses had to be at least two times higher than background for each tested marker. PESTLE (version 1.6.2) and SPICE 5.0 (Mario Roederer, ImmunoTechnology Section, Vaccine Research Center, NIH, Bethesda, MD) were used to analyze the multifunctional data. Later TP represents later time point.

We extended our analysis to determine whether there were any similarities of differences in the expression of single/mono functional $CD8^+$ T cells upon stimulation with Gag pool peptide between the 4 subgroups at baseline that may possibly predict the later divergence of disease progression observed. We did not observe any significant differences in the mono expression any of the five markers at baseline; CD107a, IL-2, IFN-γ, MIP1-β and TNF-α (Figure 5.4). This analysis may have been underpowered by the small sample numbers.

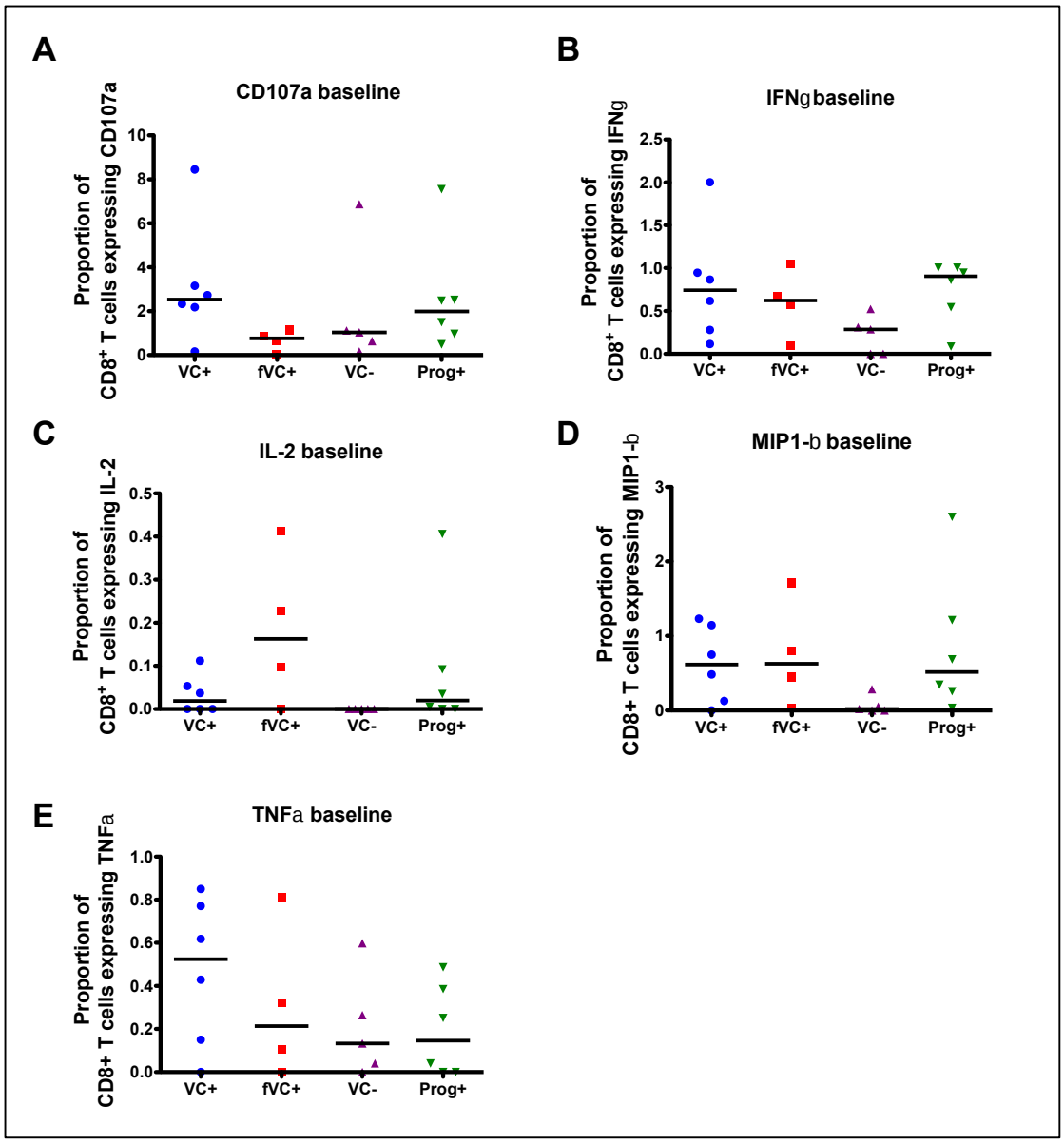

Figure 5.4. The expression of single functions by CD8$^+$ T cells upon stimulation with Gag peptide pools - amongst viremic controllers (VC+), failing viremic controllers (fVC+), progressors (Prog+) with protective HLA class I alleles and viremic controllers without protective HLA class I alleles at baseline. The 5 functions studied were CD107a, IFNγ, IL-2, MIP1-β and TNF-α. Gating strategy is shown in Figure 5.1. Positive responses were reported after background correction by subtracting the proportion of unstimulated CD8$^+$ T cells from the Gag stimulated CD8$^+$ T cells. Statistical analysis was performed on Prism using Kruskal-Wallis test to compare all 4 groups in each graph and Mann-Whitney U test to compare 2 groups that indicated significant differences.

We also determined whether there were any changes in the single function of $CD8^+$ T cells over time, that is, for VC+, VC- and Prog+ subjects we assessed these responses at baseline and later time points. Expression of these markers was assessed at baseline (pre loss time point) and post loss of viral control time point for the fVC+ subjects. We did not observe any significant differences in the mono expression of the 5 markers over time in all the 4 subgroups (data shown Appendix A) and neither did we find any $CD8^+$ functionality patterns that could explain the loss of viral control in the fVC+ subjects.

5.4 No significant differences in the proliferative capacity of $CD8^+$ T cells between the 4 subgroups

We further studied the ability of $CD8^+$ T cells to proliferate upon stimulation with Gag peptide pool. We assessed the subjects' whole PBMCs at baseline and later time points for VC+, VC- and Prog+; and pre- and post-loss of viral control time points for fVC+ subjects. The gating strategy is summarized in Figure 5.5. Our data show that there were no significant differences in proliferative capacity of the $CD8^+$ T cell to explain the divergence in disease progression amongst the compared subgroups (VC+, fVC+, VC- and Prog+) at baseline and later time points (Figure 5.6). However, at baseline (when all were controlling) there was a trend towards a higher proliferative capacity of $CD8^+$ T cells from viremic controllers (VC+) compared to those who subsequently lost control (fVC+) among individuals with protective HLA class I alleles (p-value 0.09; Mann-Whitney test). No significant differences were observed at later time points for all subgroups (Figure 5.6 B).

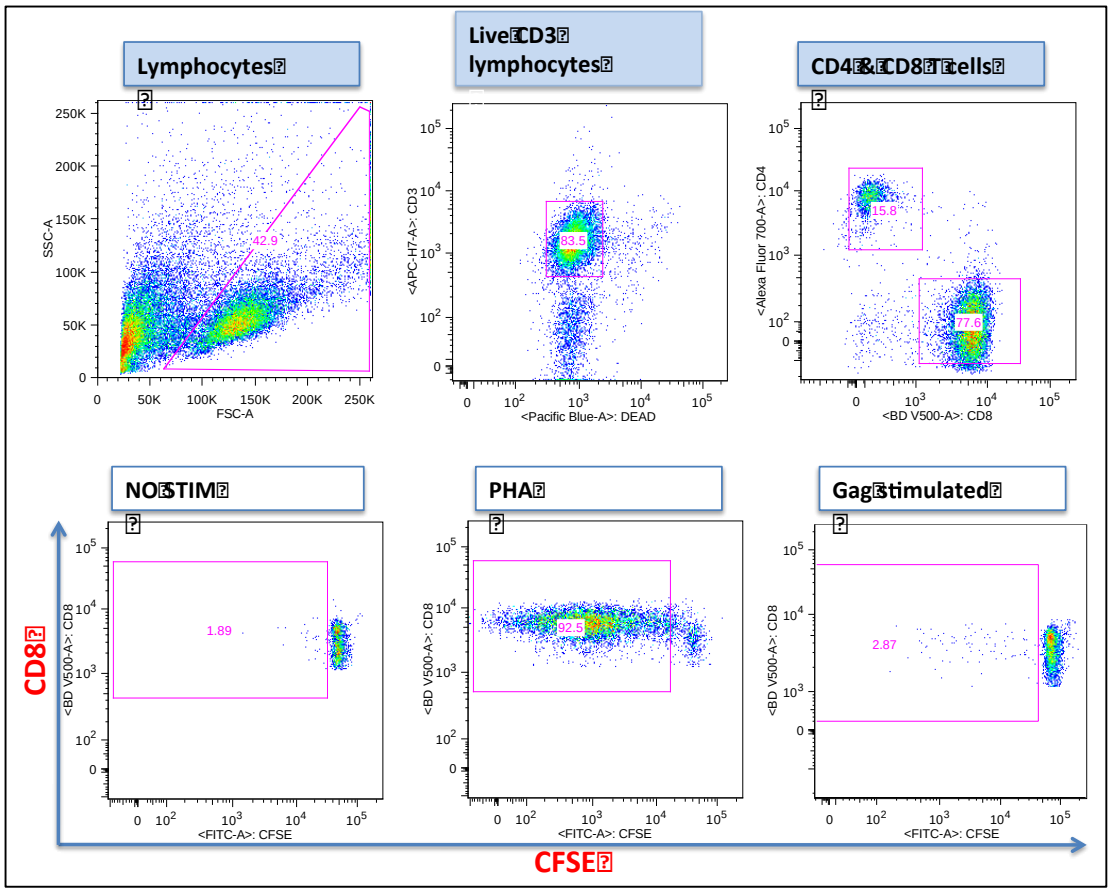

Figure 5.5. The proliferative capacity of CD8$^+$ T cells upon stimulation with Gag peptide pools – gating stratergy. The initial gating was on lymphocytes followed by gating on the live CD3$^+$ T cell population and further followed by gating of CD8$^+$ and CD4$^+$ T cell populations. The next gates were set on the CD8$^+$ CFSE negative in order to identify proliferated CD8$^+$ T cell populations. The individual gating for proliferating cells was set based on the negative control (no stimulation), these were used to identify positive responses by subtraction from stimulated proliferating population.

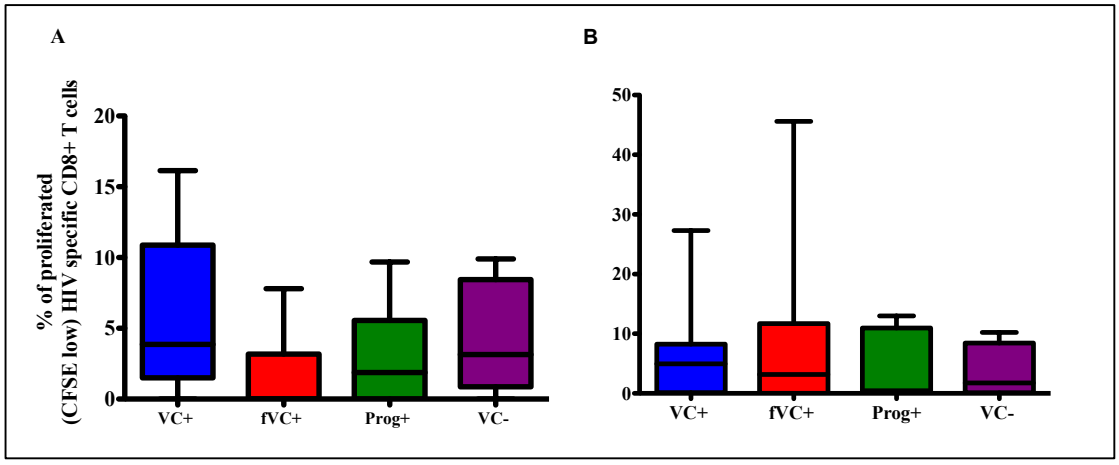

Figure 5.6. The proliferative capacity of CD8$^+$ T cells upon stimulation with Gag peptide pools - amongst viremic controllers (VC+; n=7), failing viremic controllers (fVC+, n=7), progressors (Prog+, n=6) with protective HLA class I alleles and viremic controllers (VC-, n=6) without protective HLA class I alleles at baseline (A) and latest time point (B). The gating strategy is shown on Figure 5.5. Statistical analysis was performed on Prism using Kruskal-Wallis test to compare all 4 groups in each graph and Mann-Whitney U test to compare 2 groups that indicated significant differences. These box

and whisker plots show the median and the error bars represent the minimum and maximum values.

Taken together these data showed that polyfunctionality and proliferative capacity of $CD8^+$ T cells did not play a role in the maintenance of low viral load among VC+/- individuals or loss of control in the fVC+ subjects, suggesting that other mechanisms may be responsible for the observed divergent disease progression outcomes in our cohort.

5.5 Viremic controllers without protective HLA class I alleles display limited *ex vivo* $CD8^+$ T cell inhibition capacity compared to viremic controllers with protective HLA class I alleles.

We further investigated the ability of $CD8^+$ T cells to suppress viral replication *ex vivo* as a possible mechanism of viral control. Viral inhibition assay was used to directly compare the virus inhibitory activity of $CD8^+$ T cells *ex vivo* amongst VC+, fVC+, VC- and Prog+ subgroups at baseline and later time points. Figure 5.7 shows representative data for the 5 VC+ subjects studied at baseline and 4 subjects at later time points (due to limitations of sample availability, fewer samples were analysed at more than one time point). We show here that VC+ displayed enhanced *ex vivo* $CD8^+$ T cell inhibition capacity. This phenotype was maintained at a later time point suggesting that this particular $CD8^+$ T cell function may be important for viral suppression in VCs with protective HLA class I alleles.

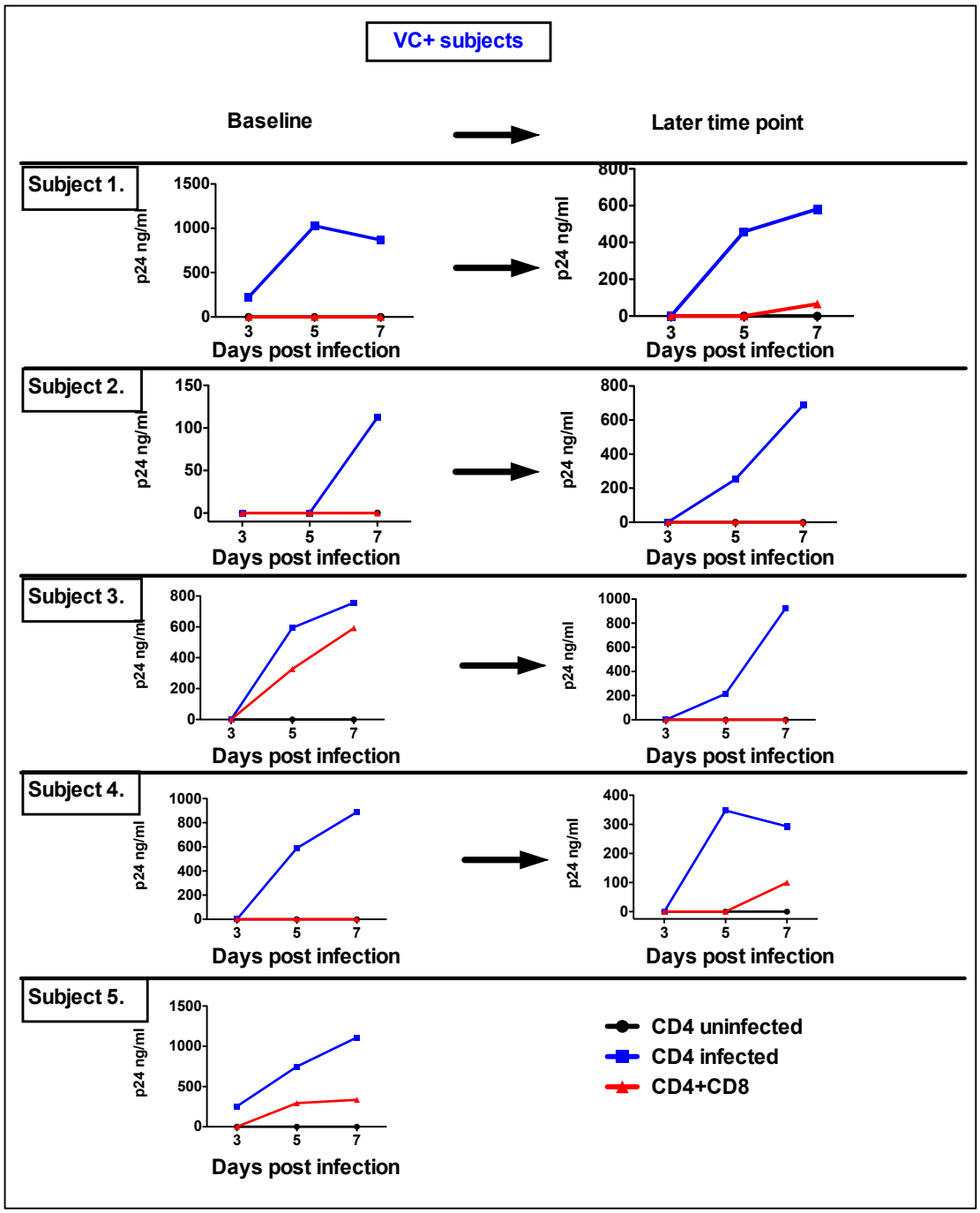

Figure 5.7. Viral inhibition of the NL4-3- infected autologous CD4$^+$ T cells by *ex vivo* CD8$^+$ T cells - VC+ subjects. Infected CD4$^+$ T cells were cultured with *ex vivo* CD8$^+$ T cells at a ratio of 1:1. Blue lines represent infected CD4$^+$ T cells alone, black lines represent the negative control; uninfected CD4$^+$ T cells alone and the red lines represent coculture of infected CD4$^+$ T cells with *ex vivo* CD8+ T cells. Representative data are displayed for 5 VC+ at baseline (n=5) and later time points (n=4).

For the fVC+ subjects, we observed a reduced inhibitory capacity by the $CD8^+$ T cells for the 3 individuals assessed (Figure 5.8). Furthermore, one fVC+ subject studied over time (i.e. pre- and post-loss of viral control), demonstrated a reduced *ex vivo* $CD8^+$ T cell inhibitory capacity at baseline with a further reduction in T cell inhibitory capacity following loss of viral control. These data may suggest that the loss of viral control may be associated with reduced capacity of the $CD8^+$ T cells to suppress viral replication. For the two other fVC+ subjects, samples were not available at the post loss of control time point.

For the 5 VC- subjects, we observed a reduced inhibitory capacity of the $CD8^+$ T cells (Figure 5.9). In addition, in the 4 VC- subjects studied over time (baseline and latest time point), despite the subjects displaying a reduced *ex vivo* $CD8^+$ T cell inhibitory capacity at baseline, we saw a similar reduction in the *ex vivo* $CD8^+$ T cell inhibitory capacity at a later time point. These data may suggest that the $CD8^+$ T cells may not be involved in the control of viral replication among VC without protective HLA class I alleles.

A representative data set for one Prog+ subject showed a reduced inhibitory capacity of the $CD8^+$ T cells. In addition, in the one Prog+ subject studied over time (baseline and latest time point), the subject displaying a reduced *ex vivo* $CD8^+$ T cell inhibitory capacity at baseline, this low *ex vivo* $CD8^+$ T cell inhibitory capacity was maintained at a later time point. These data may suggest that the $CD8^+$ T cells of the progressors are not as functional as $CD8^+$ T cells from viremic controllers.

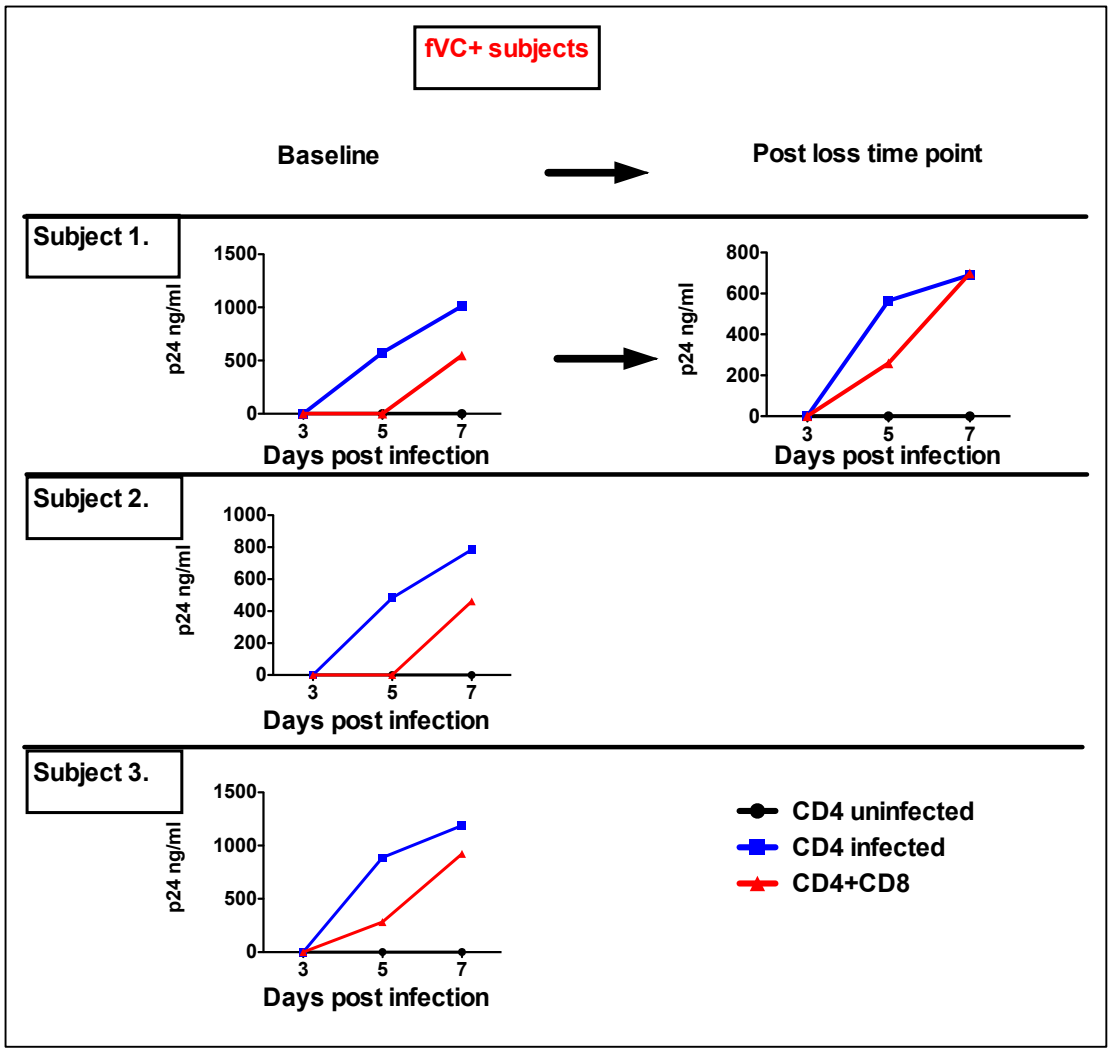

Figure 5.8. Viral inhibition of the NL4-3- infected autologous CD4$^+$ T cells by *ex vivo* CD8$^+$ T cells – fVC+ subjects. Infected CD4$^+$ T cells were cultured with *ex vivo* CD8$^+$ T cells at a ratio of 1:1. Blue lines represent infected CD4$^+$ T cells alone, black lines represent the negative control; uninfected CD4$^+$ T cells alone and the red lines represent coculture of infected CD4$^+$ T cells with *ex vivo* CD8+ T cells. Representative data for 3 fVC+ at baseline (n=3) and post loss of viral control time point (n=1) are displayed.

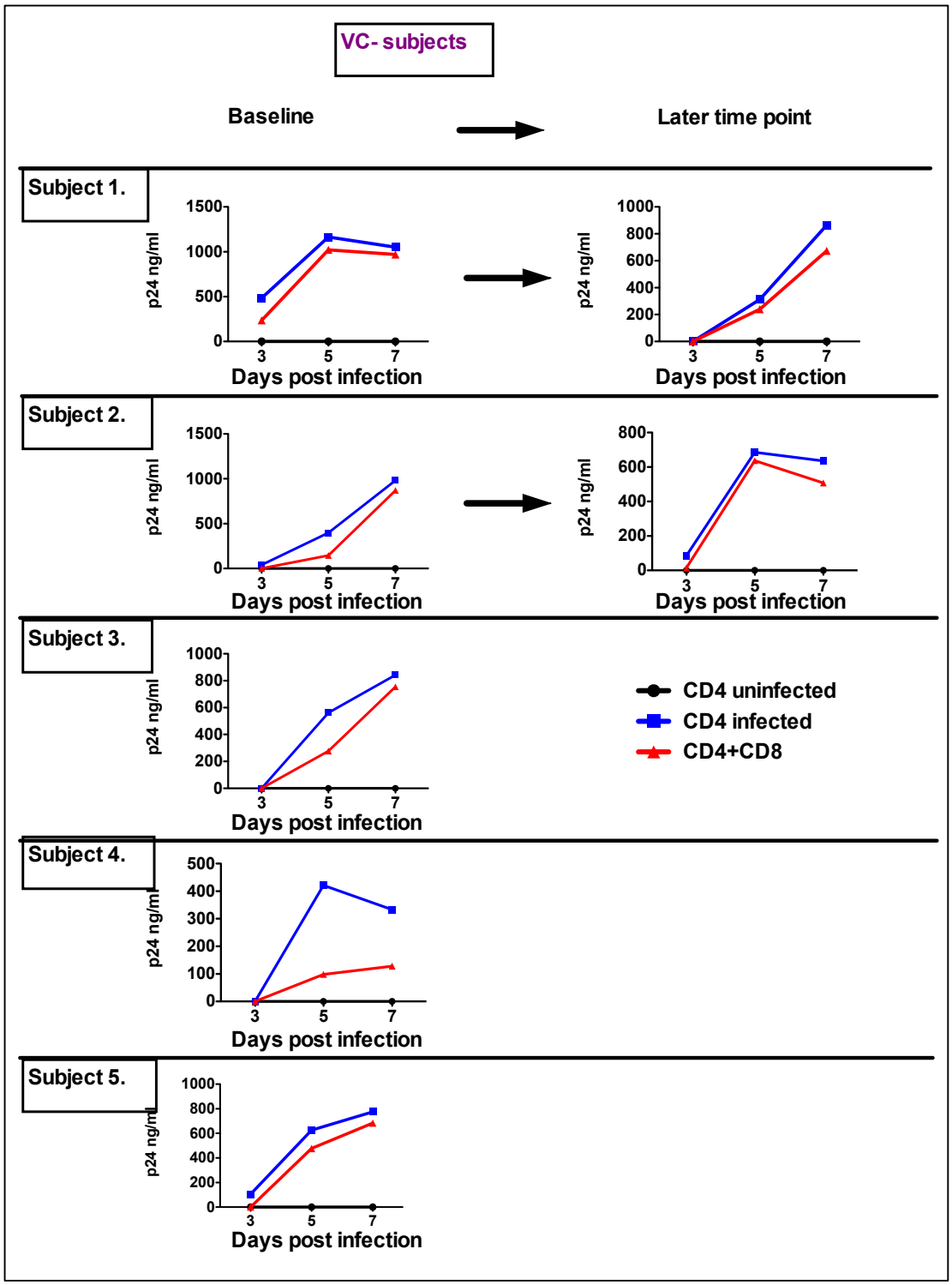

Figure 5.9. Viral inhibition of the NL4-3- infected autologous $CD4^+$ T cells by *ex vivo* $CD8^+$ T cells – VC- subjects. Infected $CD4^+$ T cells were cultured with *ex vivo* $CD8^+$ T cells at a ratio of 1:1. Blue lines represent infected $CD4^+$ T cells alone, black lines represent the negative control; uninfected $CD4^+$ T cells alone and the red lines represent coculture of infected $CD4^+$ T cells with *ex vivo* $CD8^+$ T cells. Representative data are displayed for 5 VC- at baseline (n=5) and later time points (n=2).

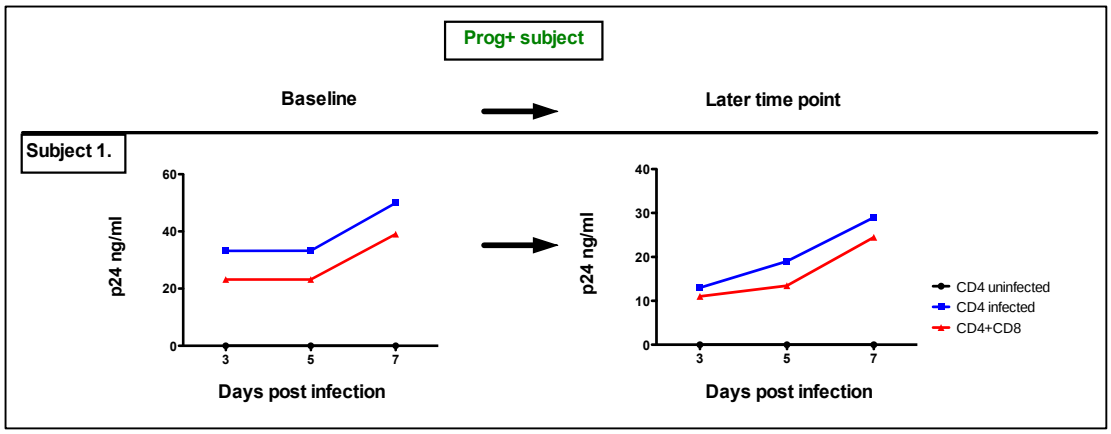

Figure 5.10. Viral inhibition of the NL4-3- infected autologous CD4$^+$ T cells by *ex vivo* CD8$^+$ T cells – Prog+ subject. Infected CD4$^+$ T cells were cultured with *ex vivo* CD8$^+$ T cells at a ratio of 1:1 Blue lines represent just infected CD4$^+$ T cells alone, Red lines represent the negative control; uninfected CD4$^+$ T cells alone and the black lines represent coculture of infected CD4$^+$ T cells with *ex vivo* CD8$^+$ T cells. Representative data are displayed for 1 prog+ subject at baseline (n=1) and later time points (n=1).

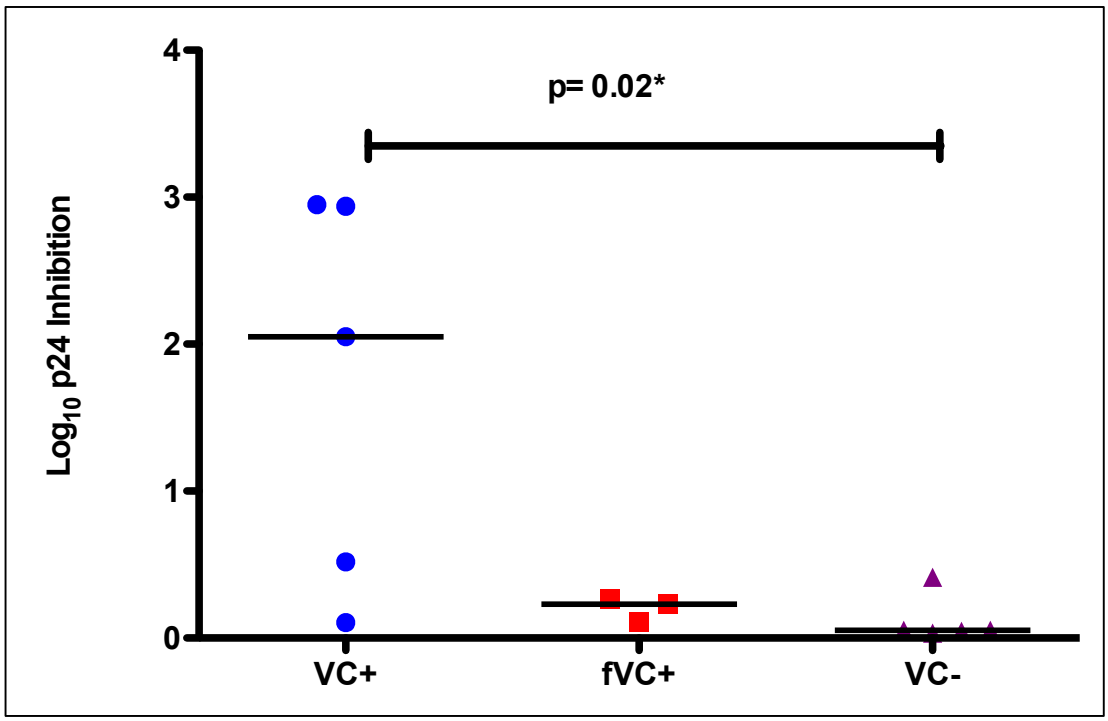

Figure 5.11. Log_{10} p24 viral inhibition of the NL4-3- infected autologous CD4$^+$ T cells by *ex vivo* CD8$^+$ T cells. Infected CD4$^+$ T cells were cultured with *ex vivo* CD8$^+$ T cells at a ratio of 1:1. Log_{10} p24 inhibition of individual subjects was calculated by subtracting log_{10} p24 values of CD4$^+$ T cells co-cultured with CD8$^+$ T cells from log_{10} p24 values of CD4$^+$ T cells without CD8$^+$ T cells at day 7, here we compared data from 5 VC+, with 3 fVC+ and 5 VC- individuals. Statistical analysis was performed on Prism using Kruskal-Wallis test to compare all 3 subgroups in each graph and Mann-Whitney U test to compare 2 groups that indicated significant differences.

For a combined assessment of the impact of *ex vivo* $CD8^+$ T cell inhibition capacity on disease outcome, we evaluated the subgroups at baseline. Interestingly, we noted a significant difference in the log_{10} p24 inhibition between the viremic controllers with protective HLA class I alleles and viremic controllers without protective HLA class I alleles, with the former having $CD8^+$ T cells with greater viral inhibition capacity (p-value 0.02; Mann- Whitney test; Figure 5.11). Overall, viremic controllers with protective HLA class I alleles (VC+) displayed significantly higher *ex vivo* $CD8^+$ T cell inhibition capacity compared to viremic controllers without protective HLA alleles (VC-) and failing viremic controllers with protective HLA class I alleles (fVC+). The VC+ participants tended to maintain this inhibitory capacity to suppress virus replication over time. Taken together these data may suggest that whereas $CD8^+$ T cells from viremic controllers with protective HLA class I alleles are associated with control of viremia, those of viremic controllers without protective HLA class I alleles display less ability to inhibit virus replication. These results are consistent with our hypothesis that among viremic controllers without protective HLA class I alleles there is an alternative mechanism of viral control other than CD8+ T cells.

5.6 Summary of results

In chapter 5, we characterized the function of $CD8^+$ T cells and associated the function with divergent disease progression that we observed in the 4 subgroups; VC+, VC-, fVC+ and Prog+. We found that there were no significant differences in the polyfunctionality of $CD8^+$ T cells between the subgroups at baseline and later time points. We also observed no significant changes in the polyfunctionality of $CD8^+$ T cells over time in the subgroups. Additionally, similar findings were reported when we assessed the proliferative capacity of $CD8^+$ T cells in the same subgroups at

baseline and longitudinally. The statistical insignificance of these data may have been largely due to our very small sample numbers.

We went further to investigate the *ex vivo* ability of $CD8^+$ T cells to suppress HIV replication and associated this function with disease progression. Here we demonstrated that VC+ displayed enhanced *ex vivo* $CD8^+$ T cell inhibitory capacity to suppress viral replication while fVC+, VC- and Prog+ subjects displayed a reduced *ex vivo* $CD8^+$ T cell inhibitory capacity at baseline with a further reduction in T cell inhibitory capacity at later time points (for VC- and Prog+ subjects) and following loss of viral control (for the fVC+ subjects). Overall these data reveal that the viral control observe in VC subjects with protective HLA class I alleles is primarily driven by the $CD8^+$ T cells and their ability to suppress virus replication. These data also suggest that VC without protective HLA class I alleles may possess an alternative mechanisms to maintain viral control. Future studies will require investigation of these possible alternative mechanisms.

CHAPTER 6: DISCUSSION

6.1 Discussion

Few HIV infected individuals are able to control viremia naturally without the need for treatment. Studying HIV infected individuals who naturally control virus replication can provide some invaluable information to better understand HIV pathogenesis, virus-host interactions and inform vaccine design. HIV controllers serve as an ideal model for effective HIV vaccine development. Research on HIV has undergone some considerable milestones in the past years to better define the mechanisms of viral control of HIV. Although the natural control of HIV has been linked to a number of factors including immunological factors, host genetics and viral factors in the HIV controller groups (viremic controllers, LTNPs and ECs), the evolution of such factors and their association with divergent disease progression patterns have rarely been characterized longitudinally in chronic HIV infection. Additionally, the cross-sectional findings regarding the mechanisms of viral control in HIV controllers have been inconclusive and therefore need resolving. There is need to clearly identify and define the correlates of immune protection from disease progression in individuals who naturally control HIV.

In this thesis, we explored the mechanisms of viral control and lack of viral control amongst individuals with protective HLA class I alleles. We investigated viremic controllers with similar baseline clinical characteristics but progressing to divergent viral load status with one subgroup maintaining control, while the other subgroup lost viral control. Both immunological and viral factors were addressed in a longitudinal study design.

6.2 Protective versus non-protective HLA class I alleles and alternative mechanisms of viral control

HLA class I alleles have previously been associated with differential control of viremia, where the expression of certain HLA class I alleles such as HLAs B*57, B*81:01, B*58:01 and B*27 have been strongly associated with low viral load hence termed 'protective HLA class I alleles', while other alleles have been termed 'non-protective HLA class I alleles' because of their strong association with high viral load (222, 223, 254, 289–293). However, the exact mechanisms of viral control amongst individuals with protective HLA class I alleles are not fully understood. Furthermore, the majority of individuals with protective HLA class I alleles experience progressive infection suggesting that protective HLA class I alleles alone are not sufficient to account for the control of HIV replication. In chapter 3 we identified chronically HIV infected individuals with controlled viremia at enrolment (VL<2,000 HIV RNA copies/ml), some possessing protective HLA class I alleles while others did not and these were termed baseline viremic controllers (bVC). These individuals were followed over time to assess viral load dynamics based on whether they expressed protective HLA class I alleles or not. Interestingly we observed that the loss of control was only evident amongst baseline viremic controllers with protective HLA class I alleles, while none of the viremic controllers without protective HLA class I alleles lost viral control despite the two groups displaying viral load similarities at baseline. This unexpected finding suggests that viremic controllers without HLA class I alleles are less likely to subsequently progress in disease status. The question still remains as to why only those with protective HLA class I alleles lost viral control. At this point, it was not clear whether immunological or other mechanisms of viral control were involved in viral control or loss of control in these individuals. Previous studies have

shown that HLA class I alleles present viral peptides on the surface of antigen presenting cells to CTLs, and therefore protective HLA class I alleles are thought to mediate their effects primarily through CTLs. Our data shows that protective HLA class I alleles alone do not account for viral control in all controllers. This is consistent with data from subtype B infection elite controllers, among whom it has been shown that whereas protective HLA class I alleles are overrepresented in this group, not all such individuals possess the protective alleles (272, 297). Our data also suggest that the mechanisms responsible for viremic control may differ between those with versus those without protective HLA class I alleles. Furthermore, the mechanisms of control in those without protective HLA class I alleles may be more durable than in those with protective HLA class I alleles. We next characterized HIV-specific $CD8^+$ T cell immune responses in the study participants with the ELISpot assay using overlapping consensus subtype C peptides spanning the entire proteome followed by confirmations with individual peptides within a reactive pool.

6.3 The Gag responses in individuals with protective HLA class I alleles

There is mounting evidence that Gag specific $CD8^+$ T cells responses play a beneficial role in HIV disease outcome. $CD8^+$ T cell responses to Gag have previously been associated with low viral load (252, 254) whereas broad or high magnitude responses to Nef correlate positively with viral load (233, 332). In chapter 3 we showed that baseline viremic controllers with protective HLA class I alleles had significantly higher breadth of Gag specific $CD8^+$ T cell responses compared to progressors with protective HLA class I alleles confirming what other studies have previously shown that targeting of Gag is associated with viral control in chronic HIV infection. We further observed that there was a bias towards targeting of Gag compared to Nef amongst baseline viremic controllers regardless of whether they

possessed protective HLA class I alleles or not. This was not evident amongst progressors with and without protective HLA class I alleles as we did not observe significant difference between the breadth of Gag versus Nef $CD8^+$ T cell responses. Further analysis showed a bias towards targeting of Gag than other proteins such as Pol, Env, Accessory and Regulatory proteins in the bVC with and without protective HLA class I alleles. These findings suggest that targeting epitopes within regions of low entropy (highly conserved regions of the virus such as Gag) in HIV as opposed to high entropy proteins (highly variable regions of the virus such as Nef) may be protective. Furthermore, Nef targeting irrespective of HLA class I alleles appears to be detrimental and is associated with high viral load.

We did not observe any significant differences when we assessed the breadth of Gag specific $CD8^+$ T cell responses between baseline viremic controllers and progressors without protective HLA class I alleles. This finding suggests that mechanisms other than Gag specific $CD8^+$ T cells may mediate control in baseline viremic controllers without protective HLA class I alleles. It is possible that these individuals may have robust innate immunity or cytolytic $CD4^+$ T cell responses, which may compensate for the ineffective $CD8^+$ T cell responses, however we did not characterize these alternative potential mechanisms in the present study. Previous studies have described cytotoxic $CD4^+$ T cell responses to be associated with HIV control and suggested that less effective $CD8^+$ T cell responses can be compensated for by robust $CD4^+$ T cell responses (315, 342). However in those studies, strong $CD4^+$ T cell responses were maintained through into the chronic phase but ultimately lost which suggests that if the individuals are still maintaining control, other mechanisms other than $CD4^+$ and $CD8^+$ T cell may take over the control of chronic HIV infection (315). Furthermore, central memory T cells have been associated with viral control which can be detected

using the peptide based cultured ELISpot (330), which were not investigated in this study. Overall, our data on individuals with protective HLA class I alleles support the 'Gag hypothesis' that responses to Gag are protective and associate with controlled viremia which is consistent with other reports (252, 254). However, we provide new evidence in controllers without protective HLA class I alleles, Gag $CD8^+$ T cell responses do not appear to play a significant role in viral control.

6.4 Evolution of $CD8^+$ T cell responses

Many studies of chronic HIV infection have assessed $CD8^+$ T cell responses at only one time point (cross-sectionally) and associated these responses with contemporaneous viral load. Here we analyzed $CD8^+$ T cell responses longitudinally using optimal peptides allowing us to assess the evolution of these responses and associate them with viral load dynamics (chapter 4). We were able to assess not only the specificity of the responses but also their persistence. Here we focused our analysis on 4 subgroups described in detail in chapter 2; VC+/VC-: viremic controllers with and without protective HLA class I alleles who controlled their viremia throughout the time of follow-up; (maintaining a VL< 2,000 HIV RNA copies/ml), fVC+: failing viremic controllers with protective HLA class I alleles; individuals who had controlled viremia at baseline and later lost viral control (VL increased to >10,000 HIV RNA copies/ml at a minimum of 2 time points) and Prog+ subjects; progressors with protective HLA class I alleles. We assessed responses at baseline and later time points for VC+, VC- and Prog+ subjects and baseline and post loss time point for the fVC+ subjects.

We first determined whether there were differences in the $CD8^+$ T cell responses at baseline. The reason for this analysis was to determine if there were differences in $CD8^+$ T cell responses at baseline that may be predictive of the divergent disease

progression patterns observed in the 4 subgroups. No significant differences were observed when comparing overall $CD8^+$ T cell responses across the whole HIV proteome and Gag specific $CD8^+$ T cell responses between VC+ and fVC+ subgroups despite the individuals displaying similar VL at baseline. Failing VC+ displayed a trend towards higher breadth of overall $CD8^+$ T cell responses across the whole HIV proteome and Gag specific $CD8^+$ T cell responses. The reasons for this observation are not known. However, these responses may not be effective in restricting virus replication in subsequent time points since high viral load was noted in these fVC+ subjects. Further analysis revealed that fVC+ subjects targeted significantly higher breadth of Nef at baseline compared to VC+ despite similarities in viral load at this point. Nef has previously been shown to be associated with increased viral load (233, 332, 343). Our observation in this study suggests that targeting of Nef while still displaying controlled viremia may predict subsequent virologic failure. However, this needs further investigation. It is also possible that the high Nef responses observed in the fVC+ subjects might be driving the elevated overall responses observed in this subgroup at baseline.

Longitudinal analysis of $CD8^+$ T cell responses among VC with and without protective HLA class I alleles (VC+/-) revealed that there was maintenance of overall $CD8^+$ T cell responses across the whole HIV proteome (Gag, Pol, Nef, Env and accessory and regulatory proteins) over time. These data suggest that the maintenance of $CD8^+$ T cell responses over time is important for sustained viral control during chronic HIV infection. Further analysis of the longitudinal $CD8^+$ T cell responses of the fVC+ subgroup revealed that the overall $CD8^+$ T cell responses across the whole HIV proteome, Pol and Gag specific $CD8^+$ T cell responses were lost over time (comparing baseline to first time point of viral load increase to more than 10,000 HIV

RNA copies/ml (post loss). These observations suggest that the loss of $CD8^+$ T cell responses may be related to the loss of viral control in these individuals. However, we are unable to determine whether the loss of responses arose as a result of the increase in viral load or the other way round. This phenomenon was also seen in progressors with protective HLA class I alleles with near-significant p-values. We also observed a trend in the reduction of Nef specific $CD8^+$ T cell responses with increasing viral load in the fVC+ and Prog+ subgroups. Although previous studies have linked targeting of Nef with high viral load (233, 332) suggesting that Nef responses are driven by antigenemia, in our study we observed that as the viral load was increasing, there was a reduction in the Nef responses suggesting that the increase in viremia may ultimately lead to impairment of $CD8^+$ T cell responses to Nef. These data also suggest that Nef responses do not seem to play a role in viral control, as they appeared to wane with disease progression. Overall, so far our data suggest that maintenance of $CD8^+$ T cell responses is important for sustained viral control especially in individuals with protective HLA class I alleles while the loss of $CD8^+$ T cell responses is associated with loss of viral control. Increasing viremia is related to the impairment of the $CD8^+$ T cell responses.

It is important to note that the *ex vivo* ELISpot assay can underestimate the true breadth and magnitude of $CD8^+$ T cell responses (232) and the fact that we stimulated the cells with optimal peptides based on HIV reference strain as opposed to autologous viruses may have resulted in non-detection of some responses because autologous virus sequences are different from reference strains. Additionally, the ELISpot assay may not have detected low frequency $CD8^+$ T cell responses or the control may be associated with central memory T cells which are usually low in frequency and can only be assessed by a cultured ELISpot (330). In future, the use of

autologous optimal peptides to stimulate cells or the employment of a cultured ELISpot assay may provide more information.

6.5 Viral escape and $CD8^+$ T cell responses

Gag specific responses are known to contribute significantly to viral control by reducing virus replication. Previous studies have consistently demonstrated that HIV is able to evade CTL recognition by developing escape mutations (149, 333–339). Here we hypothesized that the loss of $CD8^+$ T cell responses we observed in the failing viremic controllers with protective HLA class I alleles (fVC+) subgroup will primarily be as a result of escape mutations. In the second half of chapter 4, longitudinal population *gag* sequencing was performed from plasma samples at baseline and post loss of control on the fVC+ subjects and at baseline and available later time points for the viremic controllers with and without protective HLA class I alleles (VC+ and VC-). Here, we determined whether the changes in the $CD8^+$ T cell responses were a result of sequence evolution on targeted Gag epitopes or their flanking regions.

Analysis of the fVC+ subjects showed that escape was already present in 51% of the Gag epitopes and of these, 32% (11 of 34) elicited detectable $CD8^+$ T cell following stimulation with peptides corresponding to the wild type epitopes. At post loss time point only 53% (an additional 2%) of the Gag epitopes had escaped and of these, 11% (4 of 37) had detectable $CD8^+$ T cell responses to the wild type epitopes that persisted, representing a 21% reduction in the number of T cell epitopes with a detectable $CD8^+$ T cell response. These data suggested that only a minority of $CD8^+$ T cell responses were lost as result of sequence variation (viral escape) within the cognate epitopes. Further analysis showed that 7 $CD8^+$ T cell responses were no

longer detectable at the post loss time point. At this point it is not very clear why these responses were lost. It is possible that the loss of these $CD8^+$ T cell responses represents a natural waning of responses to the wild type antigenic peptide following escape. It is also possible that these cells eventually died due to lack of continued stimulation because they were no longer seeing the antigen (cognate epitope). These data also reveal that the loss of viral control did not occur as a result of escape. This is because there were no significant differences in the level of escape at baseline versus post loss of control time point, suggesting that the loss of control is primarily related to the loss of $CD8^+$ T cell responses. Furthermore, previous studies have described mutations such as T242N, A163G and T186S as reverting mutations in HIV infection possibly due to their fitness cost (344, 345). In our study, the T242N mutation was the most common mutation and reversion to wild type occurred in only 2 fVC+ subjects with no detectable $CD8^+$ T cell response indicating that reversion in these 2 subjects resulted from the loss of CTL pressure. However, in some individuals there was no reversion observed despite the loss of $CD8^+$ T cell responses. We know from previous studies that some mutations within Gag have a fitness cost to the virus (258, 346), however compensatory mutations can develop and restore viral fitness or reversion can occur especially when the CTL pressure is lost. In our study, it is possible that the fVC+ individuals may have developed compensatory mutations that restored viral fitness hence the increasing viral load in these individuals. We also observed that 4 $CD8^+$ T cell responses were maintained although escape was evident in those epitopes. This is clear evidence that the subjects responded to the wild type optimal peptides that the cells were stimulated with.

In addition, there were 8 wild type epitopes that elicited detectable $CD8^+$ T cell responses at baseline and 6 of these responses were not detected at the post loss time

point. We observed that in 3 of these 6 epitopes, sequence variation had occurred at time of virologic failure, which may explain that the loss of these responses was due to viral escape. However the other 3 epitopes remained wild type post loss of control and yet did not elicit detectable $CD8^+$ T cell responses. It remains unclear why these 3 responses were lost in the absence of escape. Overall, we observed that some $CD8^+$ T cell responses in the fVC+ subgroup were lost as a result of viral escape but escape cannot explain the loss of some of the responses. The mechanisms that underlie the loss of $CD8^+$ T cell responses in the absence of viral sequence changes requires further investigation, however we can speculate that we may not have been able to detect these responses because of their low frequencies. More sensitive assays such as cultured ELISpot, intracellular staining or tetramer staining may have given a more accurate result to check whether these responses were actually lost or not. It might be that the frequency was reduced below the level of detection of a conventional ELISpot. It is also possible that the cells may be prone to apoptosis or were exhausted. Nevertheless, our data unequivocally show that these $CD8^+$ T cell responses underwent functional or phenotypic changes and the mechanisms involved will require further investigation.

We also assessed the presence or absence of escape in the VC+ and VC- subgroups. Interestingly we show that VC+ subjects had a high level of escape, with 70% of the epitopes at baseline having variant sequences. Viral control was sustained in these individuals despite ongoing evolution in plasma viruses and this is in agreement with some previous studies that showed that some elite controllers maintained viral control despite ongoing viral replication and evolution in plasma viruses and they were able to develop *de novo* immune responses to variant peptides (347, 348). However, not all escaped epitopes were targeted. One possible explanation is that responses to the wild

type peptides were lost and could not be elicited by stimulation with the peptide. We cannot rule out that in these cases *de novo* $CD8^+$ T cell responses to the viral variant epitopes were present since we did not test for these using autologous peptides in this thesis. Future work will be needed to assess whether *de novo* responses to variant epitopes were elicited in some of these cases.

Overall these data reveal that while as expected viral escape was associated with the loss of $CD8^+$ T cell responses and this is consistent with other studies (149, 339, 349). However, not all loss of response could be explained by viral escape. Deciphering the underlying mechanisms for loss of these responses (not explained by viral escape) will require further investigation.

6.6 The impact of $CD8^+$ T cell functionality on disease progression

The ability of $CD8^+$ T cell to produce diverse cytokines (polyfunctionality) and proliferate upon stimulation has been extensively studied in HIV infection. Previous studies have shown that a highly polyfunctional and proliferative $CD8^+$ T cell phenotype play a significant role in HIV control especially in LTNPs and ECs (235, 325, 350, 351). However, whether or not this phenotype plays a role in the divergent disease progression patterns in chronic HIV infection remains understudied, as most previous studies have been cross-sectional. In chapter 5 we describe longitudinal analysis of the functional properties of $CD8^+$ T cells, proliferation and ability to suppress viral replication *in vitro*. The goal of this analysis was to determine whether these functional properties of $CD8^+$ T cells played any role in the sustained viral control observed in a subset of individuals and loss of viral control in some individuals.

In our study we observed no significant differences in the polyfunctionality and proliferative capacity of $CD8^+$ T cells at enrolment amongst the subgroups studied; VC+, VC-, fVC+ and Prog+ subjects. Further analysis of the longitudinal polyfunctionality data revealed no differences between the subgroups and at different time points. We did not observe significant changes in the polyfunctionality of the $CD8^+$ T cells over time or as the viral load was changing in some individuals. We could not link this function to the differences in divergent disease progression patterns observed in the subgroups. Therefore we concluded that polyfunctionality of $CD8^+$ T cells might not be playing a role in disease progression patterns in our cohort. Analysis of the proliferative capacity of the $CD8^+$ T cells also revealed no significant differences between the subgroups at baseline and longitudinally. However VC+ subjects had a trend towards displaying a higher proliferative capacity compared to fVC+ subjects. This could signal a role proliferation plays in the control of viremia as demonstrated in previous studies (350). Some limitations of our study are worth highlighting here, first, limited sample numbers may have reduced our statistical power to detect differences and secondly due to study limitations, polyfunctionality and $CD8^+$ T cell proliferation were studied in bulk and not virus-specific $CD8^+$ T cells. Further investigations with a larger sample size and focusing on HIV-specific $CD8^+$ T cell may yield additional insights on these $CD8^+$ T cell properties.

One of the main functions of the $CD8^+$ T cells is to kill virally infected cells. We hypothesized that viral control will be associated with $CD8^+$ T cells capable of blocking viral replication in an *ex vivo* viral inhibition assay. Our data revealed that VC+ subjects displayed enhanced ability of $CD8^+$ T cells to suppress viral replication in an *ex vivo* viral inhibition assay while fVC+ and VC- subjects displayed reduced ability of $CD8^+$ T cells to suppress viral replication. The VC+ subjects maintained this

function throughout while the fVC+ subject studied showed a further reduction in the ability of the $CD8^+$ T cells to suppress viral replication. These data suggest that fVC+ possessed $CD8^+$ T cells that are dysfunctional or impaired in their function compared to $CD8^+$ T cells from the VC+ subjects. It is important to note the fVC+ subjects displayed reduced ability to suppress viral replication at the time when they had controlled viremia (at baseline when their VL was below 2,000 HIV RNA copies/ml). These data suggest that the loss of viral inhibitory capacity of $CD8^+$ T cells prior to the rise in viremia indicates dysfunction even before the subsequent clinical consequences. Our data is partly in agreement with previous studies which demonstrated that the $CD8^+$ T cells from controllers and elite controllers differed to those of progressors in their ability to inhibit viral replication (330, 352).

However, it is important to note that not all viremic controllers in our cohort exhibited this effective $CD8^+$ T cell viral inhibition capacity. In chapter 4 we showed that VC- targeted fewer epitopes and these findings led us to hypothesize that either $CD8^+$ T cell responses do not play a major role in viral control in these individuals or that the few virus-specific $CD8^+$ T cells in these persons are highly suppressive despite their limited overall breadth. In chapter 5 we showed that the VC- subjects displayed significantly lower ability of $CD8^+$ T cells to suppress viral replication in an *ex vivo* viral inhibition compared to VC+. These data therefore indicate that mechanisms other that $CD8^+$ T cells are responsible for sustained viral control in these individuals. It is possible that these individuals may have strong $CD4^+$ T cell responses that are capable of displaying cytolytic activity as previously shown in some studies (315, 342). Other mechanisms of control in these individuals may involve innate immunity or differential expression of host restriction or replication cofactors, attenuated and defective viruses. Overall our data support the view that $CD8^+$ T cells play an

important role in the sustained control of HIV replication, particularly in individuals with protective HLA class I alleles, whereas in those without protective HLA class I alleles, alternative mechanisms of viral control may be at play.

6.7 Conclusion

In this thesis we explored the possible mechanisms of viral control and lack of viral control in individuals possessing protective HLA class I alleles and those without protective HLA class I alleles as control groups. We utilized a longitudinal study design in an attempt to overcome some of the bias inherent in cross-sectional studies where the long-term outcome of progression is unknown. We explored longitudinally the immunological and virological factors that may explain the divergent disease progression patterns observed in our cohort. We first showed that there was loss of viral control amongst individuals who had controlled viremia at enrolment and this phenomenon was only evident in those individuals possessing protective HLA class I alleles. Our findings strengthen the important role $CD8^+$ T cell responses play in sustained viral control as we showed that Gag specific $CD8^+$ T cells were associated with low viral load. We went further to demonstrate that the loss of viral control in some individuals was related to the loss of Gag specific $CD8^+$ T cell responses while sustained viral control was related to persistence of Gag specific $CD8^+$ T cell responses. Interestingly, only a minority of Gag specific $CD8^+$ T cell responses that did not persist could be explained by viral escape. This data therefore suggest that mechanisms other than viral sequence may be playing a more important role in lack of persistence of $CD8^+$ T cell responses than previously appreciated.

Additionally, we found no evidence of differences in the polyfunctionality and proliferative capacity of $CD8^+$ T cells and these parameters did not play a role in the loss of viral control. However, individuals with protective HLA class I alleles

possessed $CD8^+$ T cells with high virus inhibition capacity, and loss of viral control was associated with diminution of virus inhibition capacity. In contrast viremic controllers without protective HLA class I alleles had $CD8^+$ T cells with poor virus inhibition capacity, suggesting that $CD8^+$ T cell-independent mechanisms may potentiate viral control in these individuals.

Taken together, the finding in this thesis highlight the importance of Gag specific $CD8^+$ T cells in mediating viral control particularly in individuals with protective HLA class I alleles. These data suggest that maintenance of $CD8^+$ T cell responses is crucial for control even in the face of viral escape. However, viral escape does not fully explain the loss of $CD8^+$ T cells in chronic HIV infection and alternative mechanisms require further investigation. Finally, in viremic controllers without protective HLA class I alleles viral control occurred in the absence of broad $CD8^+$ T cell responses and poor virus inhibition capacity of $CD8^+$ T cells highlighting the need to study alternative mechanisms in these individuals. More work still needs to be done in order to further determine the correlates of immune protection. Other factors such as protective antibodies may play an important role in the control of viremia however we do not know how. There may be other unknown immunological factors associated with control.

We had initially hypothesized that the control of viremia in viremic controllers with protective HLA class I alleles will be as a result of a combination of the following 3 factors:

1. Immune responses targeted towards specific epitopes located in highly conserved Gag regions,

2. Viral evolution and/or unusual polymorphisms that affect viral fitness,

3. And highly polyfunctional $CD8^+$ T cells that are capable of inhibiting viral replication *ex vivo*.

We were able to show that indeed HIV control was associated with $CD8^+$ T cell responses to Gag; which is the region of the virus that is highly conserved. Although polyfunctionality of $CD8^+$ T cells was not associated with viral control in our study, the small sample numbers hampered the statistical significance in this analysis. We were able to demonstrate that the control of viremia was associated with increased viral mutation rates among viremic controllers with protective HLA class I alleles but we did not test whether these mutations affected viral fitness. In agreement with our hypothesis we were able to show that viral control was associated with $CD8^+$ T cells capable of inhibiting viral replication as demonstrated in an *ex vivo* assay.

6.8 Limitations of the study

There were some noteworthy limitations in this thesis study. The overall frequency of the $CD8^+$ T cell response may have been underestimated in our study since we only used consensus viral peptides rather than autologous ones, and as noted, this has been shown to underestimate the frequency of virus-specific $CD8^+$ T cells. The cohort was relatively small and limited sample availability at key time points prevented extensive investigations. For example, polyfunctionality, proliferation and viral inhibition assays could only be performed with bulk instead of virus-specific $CD8^+$ T cells due to sample limitations. We also had technical challenges amplifying the *gag* gene from plasma samples with undetectable viral loads.

6.9 Future work

There are a number of directions that one could take from the findings in this thesis.

i. **The loss of $CD8^+$ T cells in the absence of viral escape**. It would be interesting to determine why these responses were lost despite no evidence of escape. Mechanisms may include changes in the T cell receptor sequences as has been previously documented (352, 353), antigen processing defects of antigen presenting cells (337, 354) and changes in the virus sequence that affect antigen processing (333, 355). Other mechanisms may include T cell exhaustion or continuous immune activation leading to clonal deletion or apoptosis. It is also possible that these $CD8^+$ T cells persist but become functionally defective and unable to secrete IFNγ. $CD8^+$ T cells may also become defective by failure to express molecules associated with long-term survival and memory such as the IL-7Rα (356–358). Tetramer staining may help clarify whether this is indeed the case. Other markers that should be studied include T cell exhaustion markers (HLA DR and CD38). To study T cell apoptosis, Annexin V and Propidium Iodide assay may be used.

ii. **The persistence of $CD8^+$ T cells despite viral escape**. It would be interesting to investigate why some $CD8^+$ T cell response to the wild type viral peptide persisted long after escape. It is possible that some wild type viral sequences persist as minor variants and continually stimulate an immune response. Alternatively, the variant peptides may themselves stimulate *de novo* $CD8^+$ T cell response that cross-react with the wild type peptide sequence. Whatever the mechanisms, our data suggest that these

persistent $CD8^+$ T cells are important and mediate viral control even in the face of viral escape. Our data underline persistence of $CD8^+$ T cell responses as an important correlate of viral control among individuals with protective HLA class I alleles.

iii. **Further studies on the mechanisms of viral control among viremic controllers without protective HLA class I alleles**. There is need to investigate the role of cytotoxic and cytolytic activity of $CD4^+$ T cells in these individuals (315, 342). It is possible that HIV host restriction factors or differentially expressed replication cofactors may be playing a role in the control of viremia in these individuals. Other innate mechanisms of viral control cannot be ruled out. Therefore further investigations are required.

iv. **To isolate and study the virus and check for infectivity (infectiousness) and competence**. It is also possible that the virus in the viremic controllers may be defective virus or have lowered replication fitness (275, 276). This requires further investigation in order to fully determine mechanisms of viral control especially in individuals without protective HLA class I alleles.

6.10 Final remarks

Although ART has been very effective reducing HIV related deaths and morbidities, better strategies to prevent infection are needed and an HIV vaccine would be ideal in cutting the spread of the virus. However, there is still no efficacious preventative vaccine available. For an effective HIV vaccine to be developed, we need to

accelerate our efforts and better understand HIV pathogenesis, host-virus interactions, and define clearly the correlates of viral control. A lot can be leant from the long-term non-progressors, elite controllers and viremic controllers. Currently, the mechanisms of natural HIV control without antiretroviral therapy are incompletely understood. The general consensus is that the mechanisms of viral control are multifactorial and may differ between cohorts or populations. However, $CD8^+$ T cell responses to Gag have consistently been shown to be a correlate of control in chronic HIV infection. These data suggest that an effective HIV vaccine should elicit $CD8^+$ T cell responses to Gag. However, it should be noted that despite association of Gag responses with better control, the majority of HIV infected people eventually progress to full blown disease. Therefore, it is important to understand the mechanisms that lead to loss of Gag specific responses and also how these responses can be enhanced or augmented. We also cannot rule out other important factors, which may enhance the partially effective natural immune responses. This may lead to design of better vaccines.

This thesis contributed to the body of knowledge by providing data on the evolution of immunological and virological events associated with viral control and lack of viral control in chronic HIV-1 subtype C infection in the presence or absence of protective HLA class I alleles. The data indicated that not all $CD8^+$ T cell responses among viremic controllers are effective at suppressing viral replication. We were able to demonstrate that while viremic control in individuals with protective HLA class I alleles was associated with broad $CD8^+$ T cell responses, viremic control in those without protective HLA class I alleles seemed dependent on alternative mechanisms. Further investigations of what mechanisms these individuals use to sustain controlled viremia are warranted as it may lead to design of better immunogens.

CHPATER 7: REFERENCES

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M, **Henn MR**, **Le Gall S**, **Streeck H**, **Haas DW**, **Kuritzkes DR**, **Robbins GK**, **Shafer RW**, **Gulick RM**, **Shikuma CM**, **Haubrich R**, **Riddler S**, **Sax PE**, **Daar ES**, **Ribaudo HJ**, **Agan B**, **Agarwal S**, **Ahern RL**, **Allen BL**, **Altidor S**, **Altschuler EL**, **Ambardar S**, **Anastos K**, **Anderson B**, **Anderson V**, **Andrady U**, **Antoniskis D**, **Bangsberg D**, **Barbaro D**, **Barrie W**, **Bartczak J**, **Barton S**, **Basden P**, **Basgoz N**, **Bazner S**, **Bellos NC**, **Benson AM**, **Berger J**, **Bernard NF**, **Bernard AM**, **Birch C**, **Bodner SJ**, **Bolan RK**, **Boudreaux ET**, **Bradley M**, **Braun JF**, **Brndjar JE**, **Brown SJ**, **Brown K**, **Brown ST**, **Burack J**, **Bush LM**, **Cafaro V**, **Campbell O**, **Campbell J**, **Carlson RH**, **Carmichael JK**, **Casey KK**, **Cavacuiti C**, **Celestin G**, **Chambers ST**, **Chez N**, **Chirch LM**, **Cimoch PJ**, **Cohen D**, **Cohn LE**, **Conway B**, **Cooper DA**, **Cornelson B**, **Cox DT**, **Cristofano M V**, **Cuchural G**, **Czartoski JL**, **Dahman JM**, **Daly JS**, **Davis BT**, **Davis K**, **Davod SM**, **DeJesus E**, **Dietz CA**, **Dunham E**, **Dunn ME**, **Ellerin TB**, **Eron JJ**, **Fangman JJW**, **Farel CE**, **Ferlazzo H**, **Fidler S**, **Fleenor-Ford A**, **Frankel R**, **Freedberg KA**, **French NK**, **Fuchs JD**, **Fuller JD**, **Gaberman J**, **Gallant JE**, **Gandhi RT**, **Garcia E**, **Garmon D**, **Gathe JC**, **Gaultier CR**, **Gebre W**, **Gilman FD**, **Gilson I**, **Goepfert PA**, **Gottlieb MS**, **Goulston C**, **Groger RK**, **Gurley TD**, **Haber S**, **Hardwicke R**, **Hardy WD**, **Harrigan PR**, **Hawkins TN**, **Heath S**, **Hecht FM**, **Henry WK**, **Hladek M**, **Hoffman RP**, **Horton JM**, **Hsu RK**, **Huhn GD**, **Hunt P**, **Hupert MJ**, **Illeman ML**, **Jaeger H**, **Jellinger RM**, **John M**, **Johnson JA**, **Johnson KL**, **Johnson H**, **Johnson K**, **Joly J**, **Jordan WC**, **Kauffman CA**, **Khanlou H**, **Killian RK**, **Kim AY**, **Kim DD**, **Kinder CA**, **Kirchner JT**, **Kogelman L**, **Kojic EM**, **Korthuis PT**, **Kurisu W**, **Kwon DS**, **LaMar M**, **Lampiris H**, **Lanzafame M**, **Lederman MM**, **Lee DM**, **Lee JML**, **Lee MJ**, **Lee ETY**, **Lemoine J**, **Levy JA**, **Llibre JM**, **Liguori MA**, **Little SJ**, **Liu AY**, **Lopez AJ**, **Loutfy MR**, **Loy D**, **Mohammed DY**, **Man A**, **Mansour MK**, **Marconi VC**, **Markowitz M**, **Marques R**, **Martin JN**, **Martin HL**, **Mayer KH**, **McElrath MJ**, **McGhee TA**, **McGovern BH**, **McGowan K**, **McIntyre D**, **Mcleod GX**, **Menezes P**, **Mesa G**, **Metroka CE**, **Meyer-Olson D**, **Miller AO**, **Montgomery K**, **Mounzer KC**, **Nagami EH**, **Nagin I**, **Nahass RG**, **Nelson MO**, **Nielsen C**, **Norene DL**, **O'Connor DH**, **Ojikutu BO**, **Okulicz J**, **Oladehin OO**, **Oldfield EC**, **Olender SA**, **Ostrowski M**, **Owen WF**, **Pae E**, **Parsonnet J**, **Pavlatos AM**, **Perlmutter AM**, **Pierce MN**, **Pincus JM**, **Pisani L**, **Price LJ**, **Proia L**, **Prokesch RC**, **Pujet HC**, **Ramgopal M**, **Rathod A**, **Rausch M**, **Ravishankar J**, **Rhame FS**, **Richards CS**, **Richman DD**, **Rodes B**, **Rodriguez M**, **Rose RC**, **Rosenberg ES**, **Rosenthal D**, **Ross PE**, **Rubin DS**, **Rumbaugh E**, **Saenz L**, **Salvaggio MR**, **Sanchez WC**, **Sanjana VM**, **Santiago S**, **Schmidt W**, **Schuitemaker H**, **Sestak PM**, **Shalit P**, **Shay W**, **Shirvani VN**, **Silebi VI**, **Sizemore JM**, **Skolnik PR**, **Sokol-Anderson M**, **Sosman JM**, **Stabile P**, **Stapleton JT**, **Starrett S**, **Stein F**, **Stellbrink H-J**, **Sterman FL**, **Stone VE**, **Stone DR**, **Tambussi G**, **Taplitz RA**, **Tedaldi EM**, **Theisen W**, **Torres R**, **Tosiello L**, **Tremblay C**, **Tribble MA**, **Trinh PD**, **Tsao A**, **Ueda P**, **Vaccaro A**, **Valadas E**, **Vanig TJ**, **Vecino I**, **Vega VM**, **Veikley W**, **Wade BH**, **Walworth C**, **Wanidworanun C**, **Ward DJ**, **Warner DA**, **Weber RD**, **Webster D**, **Weis S**, **Wheeler DA**, **White DJ**, **Wilkins E**, **Winston A**, **Wlodaver CG**, **van't Wout A**, **Wright DP**, **Yang OO**, **Yurdin DL**, **Zabukovic BW**, **Zachary KC**, **Zeeman B**, **Zhao M**. 2010. The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. Science **330**:1551–1557.

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CHAPTER 8: APPENDICES

The following graphs represent changes in the proportion of CD8+ T cells expressing CD107a, IFNγ, MIP1-β and TNF-α over time, that is, for VC+, VC- and Prog+ subjects, we assessed these responses at baseline and later time points. Expression of these markers was assessed at baseline (pre loss time point) and post loss of viral control time point for the fVC+ subjects.

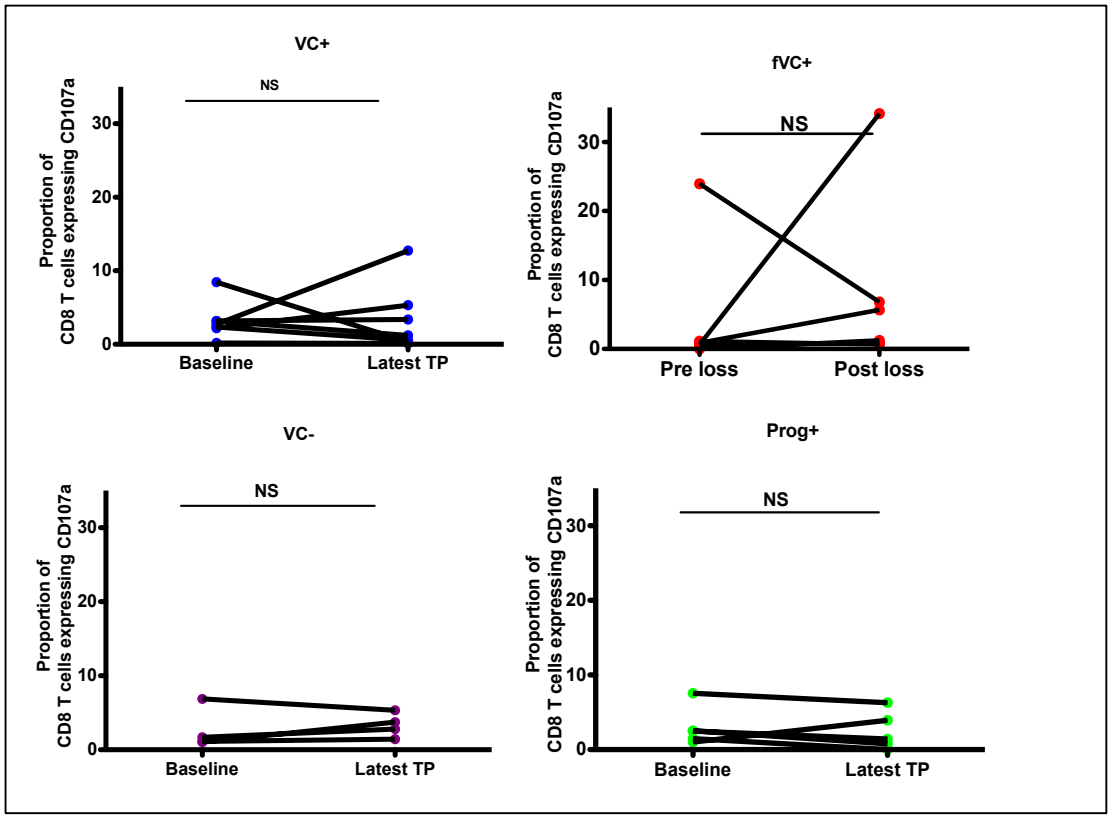

Figure A1. Longitudinal results showing the proportion of $CD8^+$ T cells expressing CD107a upon stimulation with Gag peptide pools - amongst viremic controllers (VC+), failing viremic controllers (fVC+), progressors (Prog+) with protective HLA class I alleles and viremic controllers without protective HLA class I alleles at baseline and later time point. Gating strategy is shown in Figure 5.1. Positive responses were reported after background correction by subtracting the proportion of unstimulated $CD8^+$ T cells from the Gag stimulated $CD8^+$ T cells. Later TP – later time point.

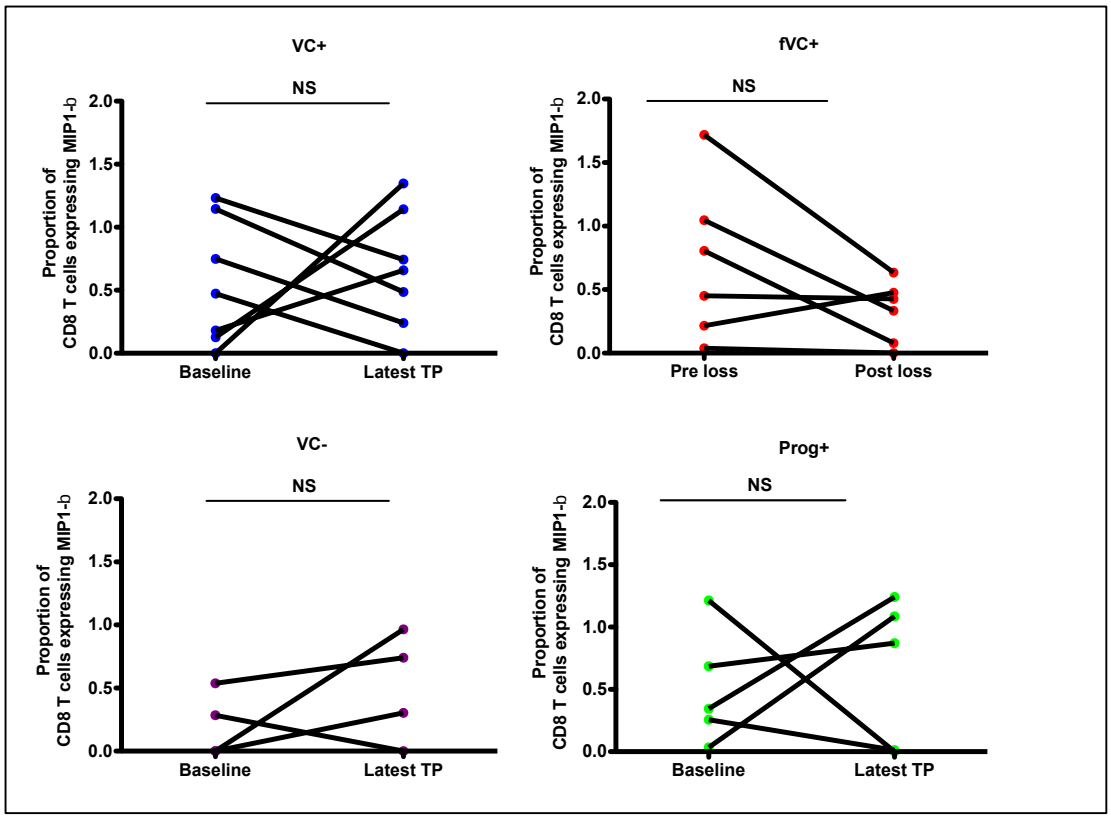

Figure A2. Longitudinal results showing the proportion of $CD8^+$ T cells expressing MIP1-β upon stimulation with Gag peptide pools - amongst viremic controllers (VC+), failing viremic controllers (fVC+), progressors (Prog+) with protective HLA class I alleles and viremic controllers without protective HLA class I alleles at baseline. Gating strategy is shown in Figure 5.1. Positive responses were reported after background correction by subtracting the proportion of unstimulated $CD8^+$ T cells from the Gag stimulated $CD8^+$ T cells. Later TP – later time point.

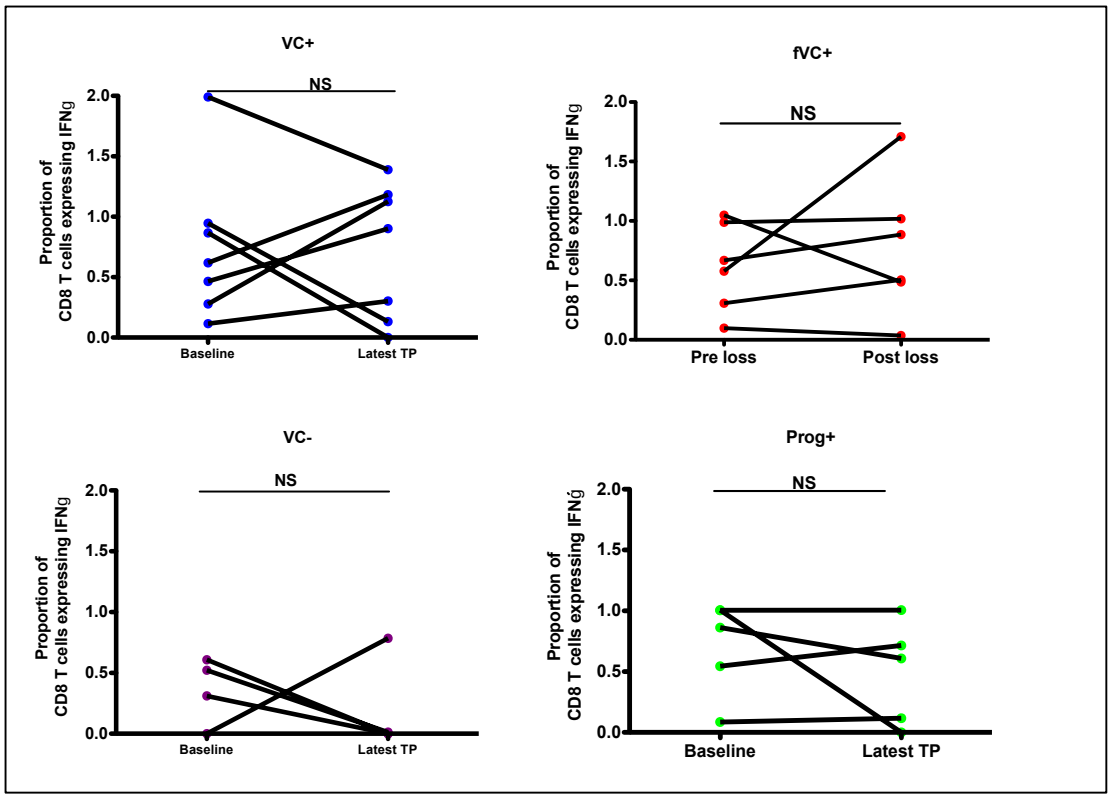

Figure A3. Longitudinal results showing the proportion of $CD8^+$ T cells expressing IFNγ upon stimulation with Gag peptide pools - amongst viremic controllers (VC+), failing viremic controllers (fVC+), progressors (Prog+) with protective HLA class I alleles and viremic controllers without protective HLA class I alleles at baseline. Gating strategy is shown in Figure 5.1. Positive responses were reported after background correction by subtracting the proportion of unstimulated $CD8^+$ T cells from the Gag stimulated $CD8^+$ T cells. Later TP – later time point.

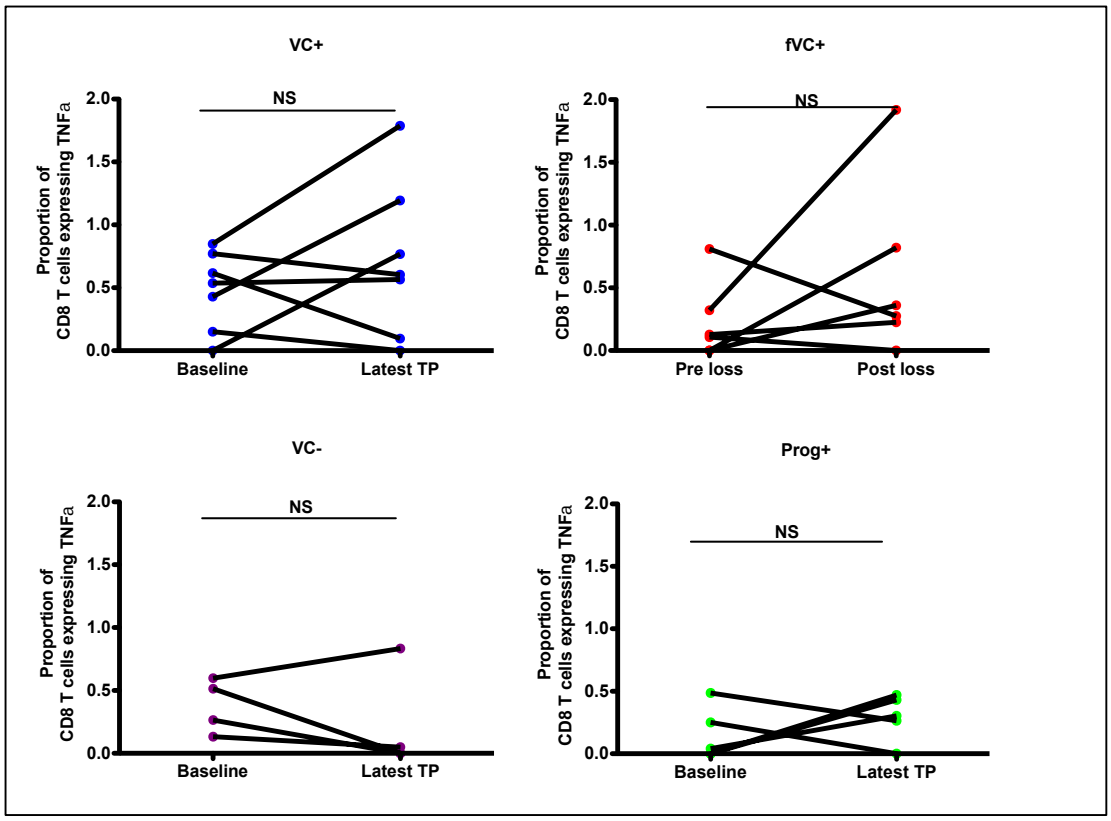

Figure A4. Longitudinal results showing the proportion of $CD8^+$ T cells expressing TNF-α upon stimulation with Gag peptide pools - amongst viremic controllers (VC+), failing viremic controllers (fVC+), progressors (Prog+) with protective HLA class I alleles and viremic controllers without protective HLA class I alleles at baseline. Gating strategy is shown in Figure 5.1. Positive responses were reported after background correction by subtracting the proportion of unstimulated $CD8^+$ T cells from the Gag stimulated $CD8^+$ T cells. Later TP – later time point.