Neutralizing Antibody Responses and Viral Evolution in a Longitudinal Cohort of HIV Subtype C Infected Antiretroviral-Naïve Individuals

By

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Submitted in partial fulfillment of the academic requirements for the degree of Doctor of Philosophy in the Department of Paediatrics & Child Health, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban

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Declaration

The experimental work described in this thesis was carried out in the following labs: HIV Pathogenesis Programme (HPP), Hasso Plattner Research Lab (HPRL), located in the Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, 4013, South Africa; Yerkes Vaccine Research Centre, Emory University, Atlanta, Georgia, USA, Ragon Institute of MGH, MIT and Harvard, Charlestown, Boston, Massachusetts, USA; National Institute for Communicable Diseases, Johannesburg, South Africa. This research was carried out from November 2006 to December 2011, under the supervision of Professor Thumbi Ndung'u (Durban), and Professor Hoosen M Coovadia (Durban). These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

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Statement

The following publication, fully referenced has been reproduced in part non-continuously throughout the thesis:

Derseree Archary, Michelle L. Gordon, Taryn N. Green, Hoosen M. Coovadia, Philip J.R. Goulder and Thumbi Ndung'u. HIV-1 subtype C envelope characteristics associated with divergent rates of chronic disease progression. *Retrovirology* 2010 Nov 4;7(1):92.

The candidate performed the experiments described in this paper, and where others made contributions it has been duly acknowledged in the text. The candidate drafted this publication in full and they were reviewed by co-authors.

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Professor HM Coovadia: _____ Date: _____

Professor T. Ndung'u: _____ Date: _____

Research Output

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Dedication

To my sons, Thashir and Shayur, whose magic continues to inspire me.

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Summary

Background: HIV-1 envelope (Env) diversity is arguably the most significant challenge for the development of an efficacious vaccine. An ideal vaccine would elicit the production of broadly neutralizing antibodies (nAb), capable of retaining potent activity against a diverse panel of viral isolates. The evolutionary forces that shape the diversity of envelope and ensuing nAb responses are incompletely understood in HIV-1 subtype C infection, the dominant subtype globally. Therefore there is an urgent need to define the patterns of envelope diversity, determine the correlates of immune protection and to discover subtype C immunogens in order to develop a globally relevant vaccine.

Methods: We applied the single genome sequencing strategy to study plasma derived viruses from four slow progressors and four progressors over a median of 21 months between study entry and study exit. The participants' samples were from the Sinikithemba cohort of antiretroviral therapy-naïve chronically infected individuals and were termed slow progressors or progressors based on CD4 T-cell counts and viral loads over two years. We analyzed *env* sequence diversity, divergence patterns and envelope characteristics across the entire HIV-1 subtype C gp160. We studied the evolution of autologous nAb (AnAb) and heterologous nAb responses in order to test the hypothesis that slow disease progression is associated with more potent autologous or heterologous nAb responses. Furthermore, genotypic *env* characteristics were correlated to potency of neutralization in order to understand possible differences in nAb responses with divergent rates of disease progression and to describe genotypic differences associated with differential nAb potencies. In addition, the binding affinities of HIV-specific immunoglobulins (IgGs) and the affinities of the IgGs

to various Fcγ receptors (both activating- FcγRI, FcγRIIa, FcγRIIIa; inhibitory- FcγRIIb) were assessed. These binding affinities were used as a surrogate for the recruitment of effector functions of cells of the innate immune system e.g. macrophages or natural killer cells to initiate antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody dependent cell-mediated viral inhibition (ADCVI) and these were correlated to markers of disease progression namely CD4 T-cell counts and viral loads.

Results: Intra-patient diversity was higher in slow progressors for regions C2 (p=0.0006), V3 (p=0.01) and C3 (p=0.005) compared to progressors. Consistent with this finding, slow progressors also had significantly increased amino acid length in V1-V4 with fewer potential N-linked glycosylation sites (PNGs) compared to progressors (p=0.009 and p=0.02 respectively). Similarly, in progressors, the gp41 region was significantly longer and had significantly fewer PNGs compared to slow progressors (p=0.02 for both parameters). Positive selection was prominent in regions V1, C3, V4, C4 and gp41 in slow progressors, whereas in progressors, it was prominent in gp41. Signature consensus sequence differences between the groups occurred mainly in gp41. Neutralizing antibodies (nAb) evolved over time in progressors, as evidenced by significantly higher nAb IC_{50} titers to baseline (study entry) viruses when tested against study exit time-point plasma compared to contemporaneous responses (p=0.003). In contrast, slow progressors' nAb titers did not differ significantly between study entry and study exit time points. nAb IC_{50} titers significantly correlated with amino acid lengths for C3-V5 (p=0.03) and V1-V5 (p=0.04) for slow progressors and V1-V2 for progressors (p=0.04). Slow progressors and progressors displayed preferential heterologous activity against the subtype C panel. There were no significant differences in breadth of responses between the groups for either subtype A or C. Neutralization breadth and titers to subtype B reference strains however, was significantly higher in progressors compared to slow progressors (both $p<0.03$) with increasing nAb

breadth from study entry to study exit in progressors. Progressors had cross-reactive neutralizing antibodies that targeted V2 and V3. Binding affinities of non-neutralizing antibodies to HIV-specific gp120, gp41 and p24 and to activating and inhibitory Fcγ receptors (FcγRs) were similar in both groups. However, in slow progressors, CD4 T-cell counts correlated inversely with antibody binding affinity for the activating FcγRIIa ($p=0.005$).

Conclusions: These data suggest that separate regions of Env are under differential selective forces, and the heterogeneity of *env* diversity and evolution differ with HIV-1 disease course. Single genome sequence analysis of circulating viruses in slow progressors and progressors indicate that diversity, length polymorphisms, sites under positive selection pressure, and PNGs consistently map to specific regions in Env. Cross-reactive neutralizing antibodies targeting epitopes in V2 and V3 indicate that nAb breadth may be dictated by a limited number of target Env epitopes. Certain key N-linked glycosylation sites were shown to be crucial for antibody neutralization. The potencies of autologous nAbs were directly affected by the amino acid lengths in certain regions of Env gp160 and by the numbers of PNGs. Target vaccine immunogens may have to be given over long periods of time and may have to include multiple subtype immunogens to elicit the production of potent, broad cross neutralizing antibodies with high binding affinity. Overall, the data suggest that neither nAbs nor non-neutralizing antibodies could be directly associated with disease attenuation in this cohort of chronically infected individuals. However, continuous evolution of nAbs was a potential marker of HIV-1 disease progression. Further studies on larger cohorts to identify people with potent nAbs and to identify specific targets of these antibodies are needed. Furthermore studies of non-neutralizing antibodies in HIV-1 infection using functional assays will be required in order to determine their role in HIV-1 pathogenesis.

Thesis Organization

The layout of this thesis is as follows:

1. Chapter One is the introduction to the thesis and covers the background, aims and objectives for the study undertaken.

2. Chapter Two details the materials and methods carried out to achieve the objectives of the study.

3. Chapter Three is the first results chapter. This chapter is in a journal article format that been reflected as a publication.

4. Chapter Four is the second results chapter. This chapter is in a journal article format, and this section is currently being prepared for manuscript submission.

5. Chapter Five is the overall discussion for the results in Chapters Three and Four. Included are the conclusions of the thesis and the future direction of the field.

6. Chapter Six contains the entire bibliography for the references used throughout the thesis.

7. Chapter Seven contains the appendices as referred to in the thesis.

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List of Abbreviations

μg	-	microgram
ng	-	nanogram
μl	-	microlitre
μM	-	micromolar
°C	-	Degrees Celsius
ADCC	-	Antibody Dependent Cell-Mediated Cytotoxicity
ADCVI	-	Antibody Dependent Cell-Mediated Viral Inhibition
AnAbs	-	Autologous Neutralizing Antibodies
APOBEC3G	-	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like
ARV	-	Antiretroviral
BEAST	-	Bayesian Evolutionary Analysis Sampling Trees
BSA	-	Bovine Serum Albumin
C1	-	Constant Region 1 of HIV-1 gp120
C2	-	Constant Region 2 of HIV-1 gp120
C3	-	Constant Region 3 of HIV-1 gp120
C4	-	Constant Region 4 of HIV-1 gp120
C5	-	Constant Region 5 of HIV-1 gp120
CCR5	-	C-C Chemokine Receptor 5
cDNA	-	Complementary Deoxyribonucleic Acid
CXCR4	-	C-X-C Chemokine Receptor 4
dH_2O	-	Distilled Water
DMEM	-	Dulbecco's Modified Eagles Medium
DNA	-	Deoxyribonucleic Acid
EC	-	Effective Concentration
ELISA	-	Enzyme-Linked Immunosorbent Assay
Env	-	Envelope (Protein)
env	-	Envelope (gene)

FBS	-	Fetal Bovine Serum
Fc	-	Fragment crystallizable
FcγR	-	Fc Gamma Receptor
HIV	-	Human Immunodeficiency Virus
GEE	-	Generalized Estimating Equations
Gp41	-	Glycoprotein 41
Gp120	-	Glycoprotein 120
Hour/s	-	hr/hrs
IC_{50}	-	50% inhibitory concentration
IgG	-	Immunoglobulin G
LB broth	-	Luria Bertani
Minute/s	-	min/mins
MPER	-	Membrane Proximal External Region
nAbs	-	Neutralizing Antibodies
NIHARRRP	-	NIH AIDS Research and Reference Reagent Program
nmAbs	-	Neutralizing human monoclonal antibodies
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction
PNGs	-	Potential N-Linked Glycosylation sites
Pol	-	Polymerase
RNA	-	Ribonucleic acid
RPM	-	Revolutions Per Minute
RLU	-	Relative Luminescence Units
RT	-	Room Temperature
RT-PCR	-	Reverse Transcriptase Polymerase Chain Reaction
SGA	-	Single Genome Amplification
SHIV	-	Simian Immunodeficiency Virus–HIV chimera
SIV	-	Simian Immunodeficiency Virus
SK	-	Sinikithemba

Tat	-	Transactivator of Transcription Factor
TM	-	Transmembrane
TRIM5α	-	Tripartite Motif -Containing 5 alpha
UKZN	-	University of KwaZulu-Natal
V1	-	Variable Loop 1 of HIV-1 gp120
V2	-	Variable Loop 2 of HIV-1 gp120
V3	-	Variable Loop 3 of HIV-1 gp120
V4	-	Variable Loop 4 of HIV-1 gp120
V5	-	Variable Loop 5 of HIV-1 gp120
Vif	-	Viral Infectivity Factor
Vpr	-	Viral Protein R
Vpu	-	Viral Protein U
VL	-	Viral Load
WebPSSM	-	Web Position Specific Scoring Matrix

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Chapter One - Introduction

1.1 HIV-1 Epidemiology and Life Cycle

Since the discovery of human immunodeficiency virus type 1 (HIV-1) in 1983 (Barre-Sinoussi et al., 1983), an estimated 33.3 million people were living with HIV-1 worldwide at the end of 2009 (UNAIDS, 2010). Additionally, approximately 2.6 million people became newly infected and approximately 1.8 million deaths occurred at the end of 2009 (UNAIDS, 2010). A disproportionately large majority- 68% of infections (22.5million) are estimated to be in sub-Saharan Africa (UNAIDS, 2010). The geographic distribution of HIV-1 varies between countries within sub-Saharan Africa, with South Africa bearing the largest HIV-1 burden with an estimated 5.6 million individuals living with the virus (UNAIDS, 2010). Development of a safe and efficacious preventative vaccine remains a high global health priority.

HIV-1 has been phylogenetically characterized into four groups, group M (major), group O (outlier), group N (non-M, non-O) and the most recently discovered group P (Vallari et al., 2011). HIV-2 is closely related to simian immunodeficiency virus (SIV) and is phylogenetically distinct from HIV-1 and is found certain parts of West Africa (Chen et al., 1996, Chen et al., 1997). The diverse HIV-1 group M lineage is responsible for most of the current worldwide pandemic (McCutchan, 2000, Peeters et al., 2003, Taylor et al., 2008). HIV-1 group M is further divided into 13 subtypes (A1-A4, B, C, D, F1-F2, G, H, J, K) (Taylor et al., 2008). Due to the predominance of HIV-1 subtype B infections in the West, much of the basic science research on HIV-1 has focused on this subtype. However, subtype C remains the most rapidly spreading and globally prevalent subtype accounting for more than 50% of all infections and is the dominant infecting strain in southern Africa (McCutchan, 2000, Esparza, 2005, Hemelaar et al., 2006, Hemelaar et al., 2011). There is a critical need therefore to place more research emphasis on this subtype, and to focus on

unraveling and improved understanding of the correlates of immune protection and the characteristics of cognate immunogens in order to develop a globally relevant vaccine.

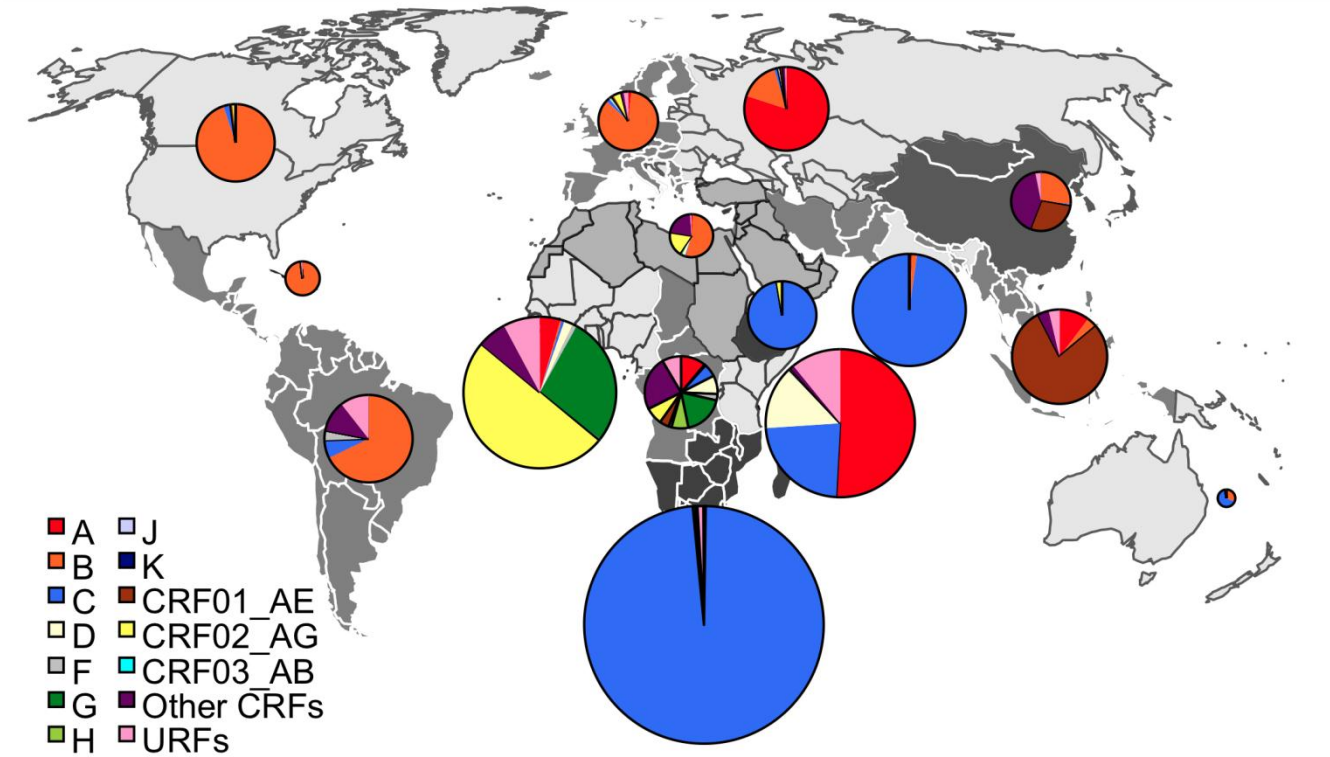

Figure 1.1. Global distribution of HIV-1 subtypes and recombinants in 2004. The colour key on the left-hand side of the figure represents the different HIV-1 subtypes (adapted from Hemelaar et al., 2011).

1.2 HIV-1 Genomic Structure

HIV-1 belongs to the lentivirus genus. It is an RNA retrovirus made up of nine genes which code for nine polyproteins which are further processed into fifteen protein subunits (Watts et al., 2009). Of these, three genes, *gag* (which codes for the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins), *pol* (which codes for the protease (PR), reverse transcriptase (RT) and integrase enzymes (IN)) and *env* (which codes for the surface glycoprotein- gp120 and transmembrane glycoprotein- gp41), contain information needed to

make structural proteins for new virus particles. The other six genes, known as *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*, code for regulatory or accessory proteins that control the ability of HIV-1 to infect a cell, produce new copies of virus, or cause disease (reviewed in (Frankel and Young, 1998)).

1.3 HIV-1 Life Cycle

When HIV-1 comes into contact with a target cell bearing a CD4 receptor, its Env gp120 spike becomes engaged and there is subsequent exposure of the chemokine coreceptor binding site (CCR5 or CXCR4) which leads to exposure of the fusion domain of gp41 (reviewed in (Eckert and Kim, 2001)). Thereafter, coreceptor binding also occurs which induces a conformational change within the transmembrane glycoprotein (gp41) region. This essential process enables fusion to occur between the viral and target cell membranes (as shown in the figure below) (reviewed in (Wyatt and Sodroski, 1998, Harrison, 2008)).

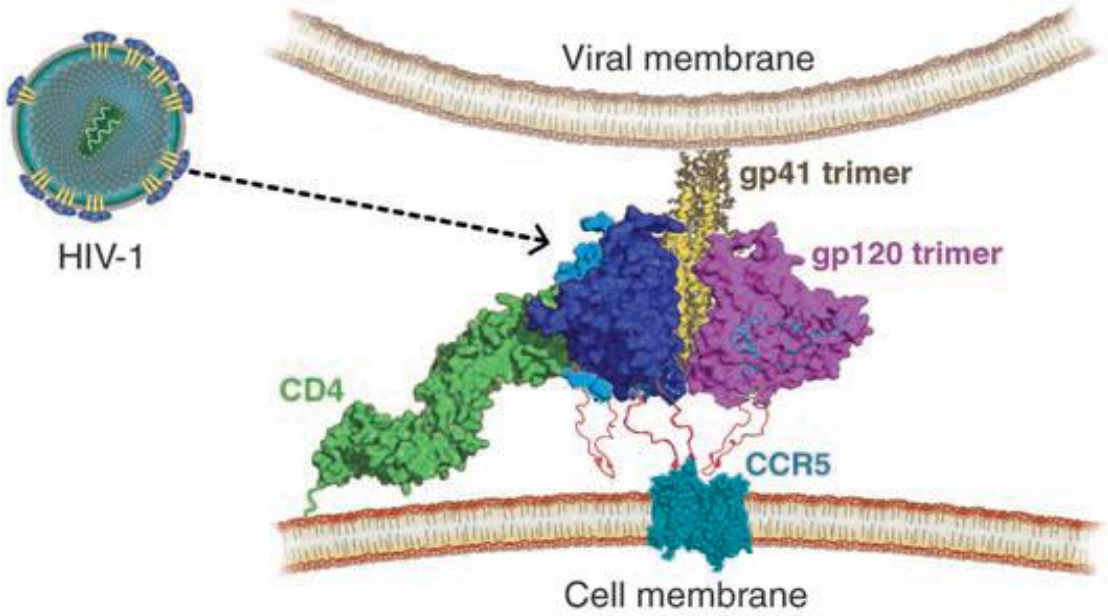

Figure 1.2. Schematic of the HIV-1 envelope spike interacting with its cellular receptors on a target cell. Gp120 of the envelope spike interacts with CD4 (shown in green) on target T cells leading to conformational changes that allow interaction with the chemokine coreceptor CCR5. Further conformational changes are induced in the spike leading to fusion of viral and target cell membranes and transmission of viral genetic material into the target cell (adapted from Walker and Burton, 2008).

Subsequently during this early phase, the viral core or capsid protein (p24) is released into the target cell, leaving the envelope behind and viral RNA integration is achieved through the reverse transcriptase enzyme which transcribes the viral RNA into complimentary DNA (cDNA). The enzyme integrase is then involved in splicing viral DNA into the host's DNA within the nucleus generating the provirus (reviewed in (Turner and Summers, 1999)). The messenger RNA, which contains the blue-print for the production of HIV-1 proteins, is then transported out of the cell's nucleus to the endoplasmic reticulum where ribosomes translate the mRNA into HIV-1-viral proteins, which can then be used to make new HIV-1 virions (figure 1.3). Specifically, the envelope spike is initially made up as a single polypeptide precursor, which undergoes modification through the addition of N-linked and O-linked high-mannose type oligosaccharides in the Golgi apparatus. The protein oligomerizes and becomes highly glycosylated producing a 160 kDa glycoprotein (gp160). The glycosylation process is important for proper protein folding and structural stability of the envelope glycoprotein. Endo-proteases e.g. furin cleave the gp160 into two non-covalently associated fragments i.e. the gp120 receptor binding fragment and the gp41fusion fragment expressed at the cell surface (Allan et al., 1985, Veronese et al., 1985). The gp120-gp41 complexes are then incorporated into the lipid bi-layer of the cell during HIV-1 budding to produce new viruses (Wyatt and Sodroski, 1998). These new virions are capable of infecting new target cells and perpetuating production of new virions, and eventually leading to the host immune system collapse.

1.4 Envelope Structure

Envelope (Env) is a heavily glycosylated protein and is structurally complicated. Env gp160 is a heterodimer comprising of a surface glycoprotein gp120 non-covalently linked to a

transmembrane gp41 stalk anchored within the viral membrane. The Env spike consists of three heterodimers making up a trimer and there is on average eight to ten trimers on the HIV-1 virus surface (Zhu et al., 2003). The Env trimer mediates viral tropism as well as viral entry and is a principal target for nAbs.

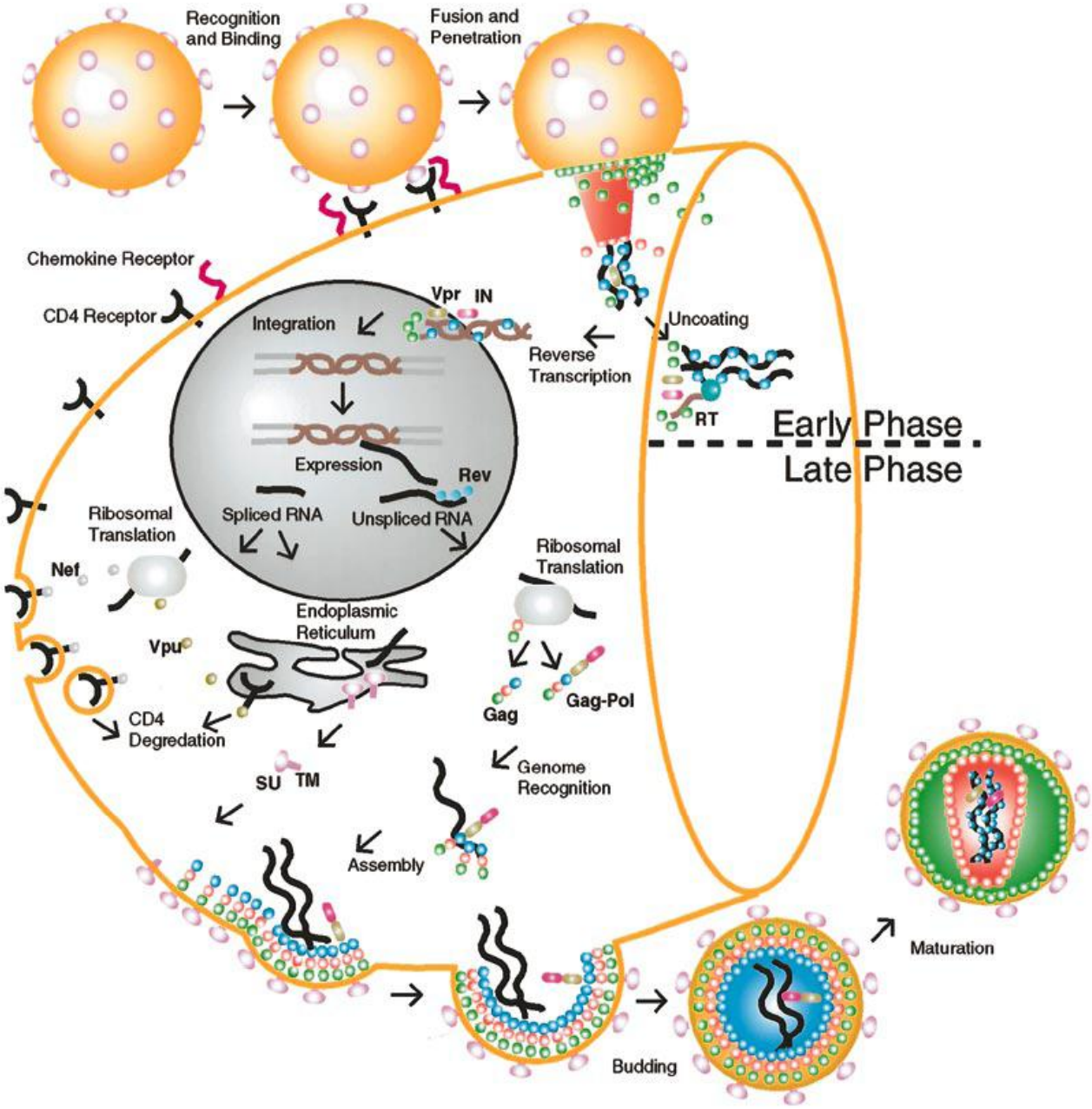

Figure 1.3. HIV-1 replication cycle divided into the early and late phase. The early phase involves the CD4 receptor and CCR5/CXCR4 coreceptor binding and includes the generation of provirus. The late phase involves the viral DNA transcription and translation leading to the production of new virions from the infected cell (adapted from Turner and Summers, 1999).

1.4.1 Structural Organization of Gp120

Gp120 is made up of approximately 511 amino acids. Gp120 is divided into 5 conserved regions (C1-C5) and five variable regions (V1-V5) based on comparative sequence analyses (Willey et al., 1986, Modrow et al., 1987). Based on the crystal structures of monomeric gp120, it was further elucidated that regions C1 and C5 formed mainly the inner domain. It was proposed therefore that the inner domain interfaces with gp41. In addition, C2, C3 and C4 were purported to be hidden within the hydrophobic core and may contain several epitopes sensitive to antibody neutralization. Unlike the inner domain, the outer domain is covered by N-linked glycans, and therefore shows an immunologically "silent face" (Wyatt et al., 1998). Up to 50% of the molecular mass of gp120 is carbohydrate (Wyatt et al., 1998, Kwong et al., 1998, Kwong and Wilson, 2009). The inner and outer domains of gp120 are linked by a bridging sheet, which alters conformationally according to a CD4-bound (liganded) or CD4-unbound (unliganded) state (reviewed in (Pantophlet and Burton, 2006)).

The variable loops V1-V2 in the unliganded state are thought to partially shield V3, which has both the coreceptor and the CD4 binding sites. V1-V2 and V4 can tolerate many changes to their sequences including insertions, deletions, and evolution of their potential N-linked glyscosylation sites (PNGs). V3 lies in the outer domain in the ribbon structure as shown in figure 1.4. and is downstream of C3. Upon CD4 binding the V3 loop protrudes from the tip of the trimeric spike to subsequently engage the coreceptor binding site (Chen et al., 2005, Huang et al., 2005). V3 is a particularly desirable target for nAbs, as anti-V3 antibodies are produced early in infection (Moore et al., 2008, Davis et al., 2009a, Davis et al., 2009b). The roles of V4 and V5 have been less well defined, although it has been postulated that deletion of the V4 loop may impair proper gp160 protein folding (Rong et al., 2007b, Moore et al., 2008).

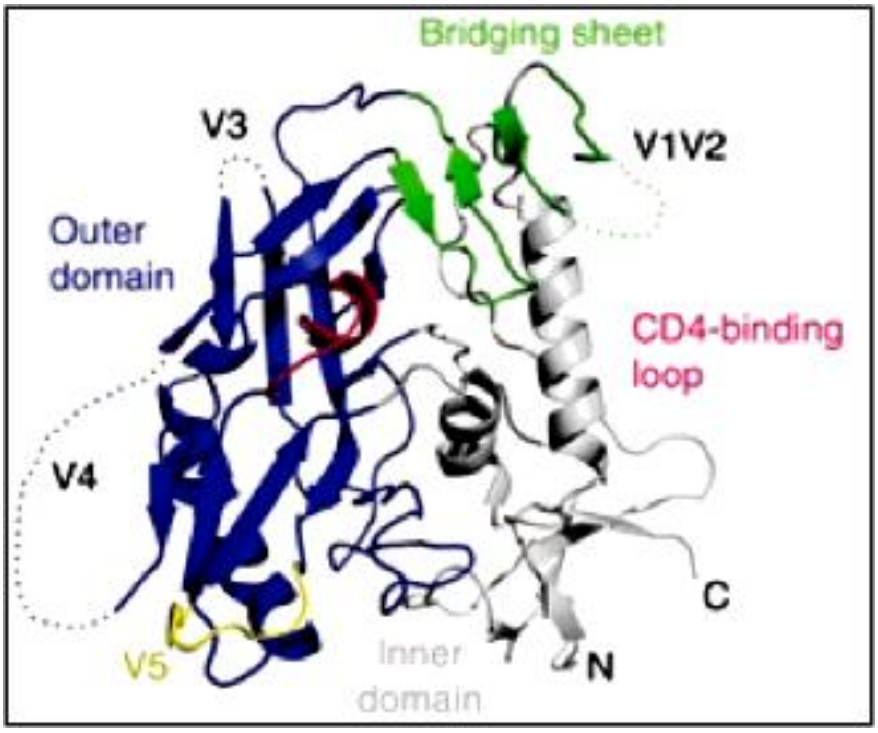

Figure 1.4. Schematic of the gp120 core as a ribbon representation of the template subtype C (CAP210) core gp120 structure from the Protein Data Bank (PDB) with variable loops and other features highlighted. In the orientation, the cellular and viral membranes would be located above and below the protein respectively (adapted from Diskin et al., 2010).

1.4.2 Structural Organization of Gp41

Gp41 is 341 amino acid-long glycoprotein divided into three main regions: the amino (N) terminal transmembrane (TM) ectodomain, a membrane spanning domain and an endodomain- a long cytoplasmic tail (C-terminal) that interacts with the matrix protein of the virus (Turner and Summers, 1999). The ectodomain is a trimer. The trimer consists of three monomers. Each monomer is made up of two anti-parallel α helices connected by an extended loop. The N-terminal helices form the central three-helix coiled coil while the C-terminal helices are arranged on the outside of the coiled coil.

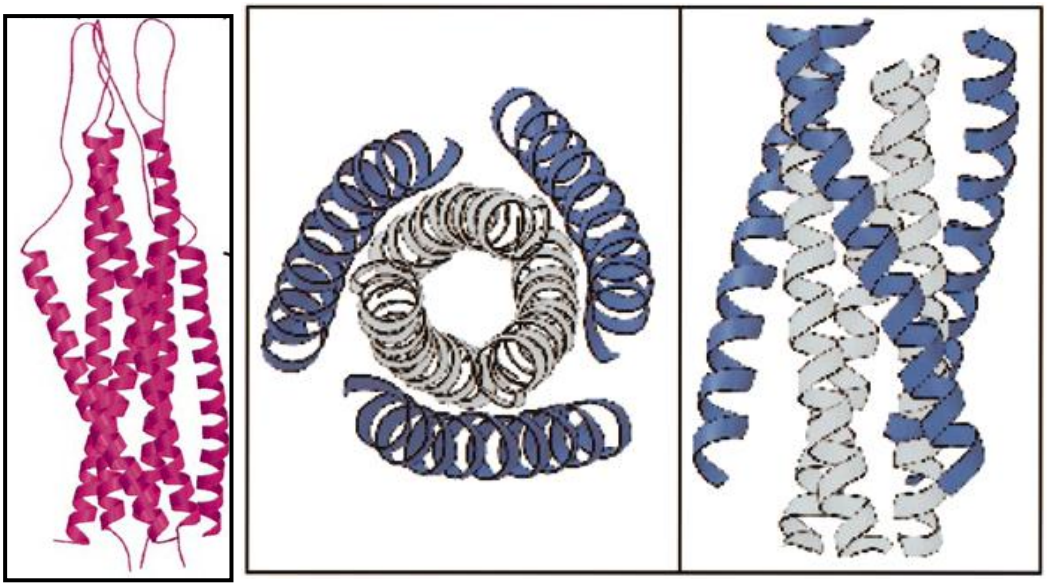

Figure 1.5. Ribbon representation of the trimeric coiled coil N- and C-helices joined by a loop. Helix packing of the trimeric coiled coil structure of the transmembrane (TM) ectodomain showing the N- (shown in gray) and C-helices (shown in blue)- (adapted from Turner and Summers, 1999).

Upon gp120-coreceptor binding, the N-terminal peptide of gp41 trans-locates and inserts itself into the target cell membrane, this gives rise to the pre-hairpin intermediate (reviewed in (Chan and Kim, 1998)). Subsequently, the C-helices then fold back into a hairpin formation, while the C-terminal transmembrane region of gp41 remains inserted in the viral membrane, creating the six-helix bundle of the N-helices and the C-helices. This process effectively brings the fusion peptide and the transmembrane segment in close proximity ultimately leading to viral and host membrane fusion (Melikyan et al., 2000). Therefore during membrane fusion gp41 has three conformations, a pre-fusion form, the pre-hairpin intermediate form and, the post-fusion form, with each form transiently revealing three distinct antigenic targets to the immune system.

1.5 Viral Diversity and the Impact on Vaccine Development

HIV-1 is highly diverse, with a 17-35% difference between subtypes and an estimated 8-17% difference within infected individuals depending on the genome regions under study (Korber et al., 2001). Env in particular is one of the most genetically diverse of the HIV-1 genome and exhibits a 35% inter-subtype diversity (reviewed in (Korber et al., 2001)). Diversity is a combined result of many factors: firstly, the high replicative rate of HIV-1; secondly the error-prone reverse transcriptase enzyme through the lack of a proof-reading mechanism induces changes to the viral sequence; thirdly, the *in vivo* recombination rate both within the individual and at the population level and lastly, immune selection pressure (reviewed in (Coffin, 1995)). As a result, the immune system with its repertoire of nAbs and CD8 T-cells is always one step behind a continuously evolving virus in accordance with the "original antigenic sin" paradigm (reviewed in (Brander et al., 2006, Forsell et al., 2005)). HIV-1 diversity therefore poses a significant challenge to HIV-1 vaccine development.

Within the viral genome, the *env* gene is of particular interest due to the concentration of genetic variation inherent to it (Korber et al., 2001). The HIV-1 envelope (Env) subunits gp120 and gp41 are the only surface-exposed viral proteins and are the main targets of antibodies. As a result Env is under continuous host selection pressure. Certain key Env sequence characteristics have been shown to influence antibody neutralization sensitivity, cell tropism, coreceptor usage and virus transmission. These include the overall amino acid diversity, the number of putative N-linked glycosylation sites (PNGs) and the length of variable loops (Resch et al., 2001, Wei et al., 2003, Rademeyer et al., 2007). Studies of Env are critical to delineate the selective forces that may significantly influence the rate of disease progression and to identify specific regions of the Env protein that are important targets of

effective immune pressure which may be important to include in rational HIV-1 vaccine design.

A number of studies in HIV-1 subtype B, illustrate that the relationship between HIV-1 Env diversity and disease progression is complex. These studies have shown increased diversity over time in the V3 loop (Nowak et al., 1991), direct associations between diversity for V3-V5 loops and patient survival and an inverse correlation with CD4 T-cell loss (Wolinsky et al., 1996). There is complex heterogeneity of sequences for C2-V5 at different stages of HIV-1 infection in men followed longitudinally over six to twelve years (Shankarappa et al., 1999). This study demonstrated that following seroconversion, there was an early stage of linear increases in divergence and diversity, followed by an intermediate stage with increase in divergence but stabilization or decline of diversity and a final stage showing stabilization or reduction in divergence and continued stability or decline in diversity (Shankarappa et al., 1999). Another study looking at C2-V5 Env sequences, demonstrated higher diversity but lower intra and inter-sample divergence in typical progressors compared to slow progressors (Bagnarelli et al., 1999). Additionally, this study showed that typical progressors exhibited lower host selective pressure and increases in both synonymous and non-synonymous substitutions over time while slow progressors exhibited an increase in non-synonymous substitutions only.

Although a number of studies have explored Env diversity and diversification within HIV-1 subtype C (Tscherning et al., 1998, Ping et al., 1999), data on this subtype remain relatively limited. There is accumulating evidence that HIV-1 subtype C may differ significantly from its subtype B counterpart in certain biological properties mediated by the Env protein (Ping et al., 1999, Tscherning et al., 1998, Ball et al., 2003, Gnanakaran et al., 2007, Abraha et al., 2009, Rong et al., 2009, Moore et al., 2009). The differences between subtypes B and C include distinct sequence characteristics and structural differences. The alpha 2 (α-2) helix of

the C3 region in HIV-1 subtype C is amphipathic compared to subtype B which hydrophobic. Additionally, the C3-V4 region is more exposed implicating C3 as a target for neutralization in subtype C (Gnanakaran et al., 2007, Moore et al., 2008). In addition, the hypervariable V4 loop is shorter in subtype C than it in subtype B (Gnanakaran et al., 2007). In particular, differences in Env diversity, divergence and selective pressures between HIV-1 subtype C-infected individuals with divergent rates of disease progression remain understudied.

In addition to nAbs, other factors responsible for diversification of Env include selection for CXCR4 viruses which plays a role in generating diversity of HIV-1 Env through changes in a few residues within the Env coreceptor binding domain in V3 (Fouchier et al., 1992, Hoffman et al., 2002, Pillai et al., 2003, Koning et al., 2003, Stalmeijer et al., 2004). Viral evolution in response to CTL-mediated pressure may also be responsible for the diversification of Env, although other cellular responses may be stronger and/or are more common to other proteins such as Gag, Nef and Tat, particularly during the early stages of infection (Addo et al., 2003, Cao et al., 2003, Lichterfeld et al., 2004, Navis et al., 2008). Innate immune factors such as cytidine deaminases like APOBEC3G also play a role in causing hypermutations in the viral Env sequence, inducing stop codons, which in turn alters the viral footprint rendering the Env glycoprotein non-functional (Sheehy et al., 2002). Recently, it has been demonstrated that natural killer cells mediated immune pressure may also contribute to viral sequence evolution (Alter et al., 2011).

1.5.1 Immune Responses in HIV-1 Infection

The immune system is classically divided into innate and adaptive immune systems. HIV-1 has to overcome many agents of the innate immune system and once the virus has breached the mucosal barrier, natural killer cells, macrophages, neutrophils and mast cells are the first

line of defense to any invading pathogen, and exert their effector function through a limited repertoire of germline-encoded receptors (reviewed in (Vivier et al., 2011)). Adaptive immunity is the second line of defense and is comprised of two types of lymphocytes; B and the T-cells. These cells express a large repertoire of antigen receptors that are produced in response to a specific pathogen (Vivier et al., 2011). B lymphocytes mature into plasma cells that produce antibodies (both neutralizing (nAb) and non-neutralizing) in response to particular viral antigens. CD4 T-cells and CD8 T-cells both originate in the thymus. CD8 T-cells recognize virus infected cells through antigen presentation on the cell surface complexed to a human leukocyte antigen (HLA) class I molecule (reviewed in (Walker and Burton, 2008)).

In the past decade, intrinsic immunity in HIV-1 infection has gained a lot of prominence. The discovery of APOBEC3G, a potent intracellular immune factor effecting the inhibition of HIV-1 replication has focused the spotlight on intrinsic immunity as a possible target for anti-HIV-1 drug therapies (Sheehy et al., 2002).

In addition, the cells of the innate immune system e.g. natural killer cells through their Fc gamma receptors (FcγRs) also synergistically interact with the Fc part of the antibodies to effect viral killing or inhibition through antibody dependent cell-mediated cytoxicity (ADCC) or through antibody dependent cell mediated viral inhibition (ADCVI) (reviewed in (Forthal and Moog, 2009)). This Fcγ receptor–Fc antibody interaction may play an important biological function of non-neutralizing antibodies modulating or delaying HIV-1 disease progression (Forthal and Moog, 2009). However, additional data is needed to better understand the contribution of non-neutralizing antibodies to prevention of transmission and viral control. Such validation of the role of these antibodies in viral pathogenesis would be a

rational approach in an attempt to understand whether they would need to be elicited in candidate HIV-1 vaccines.

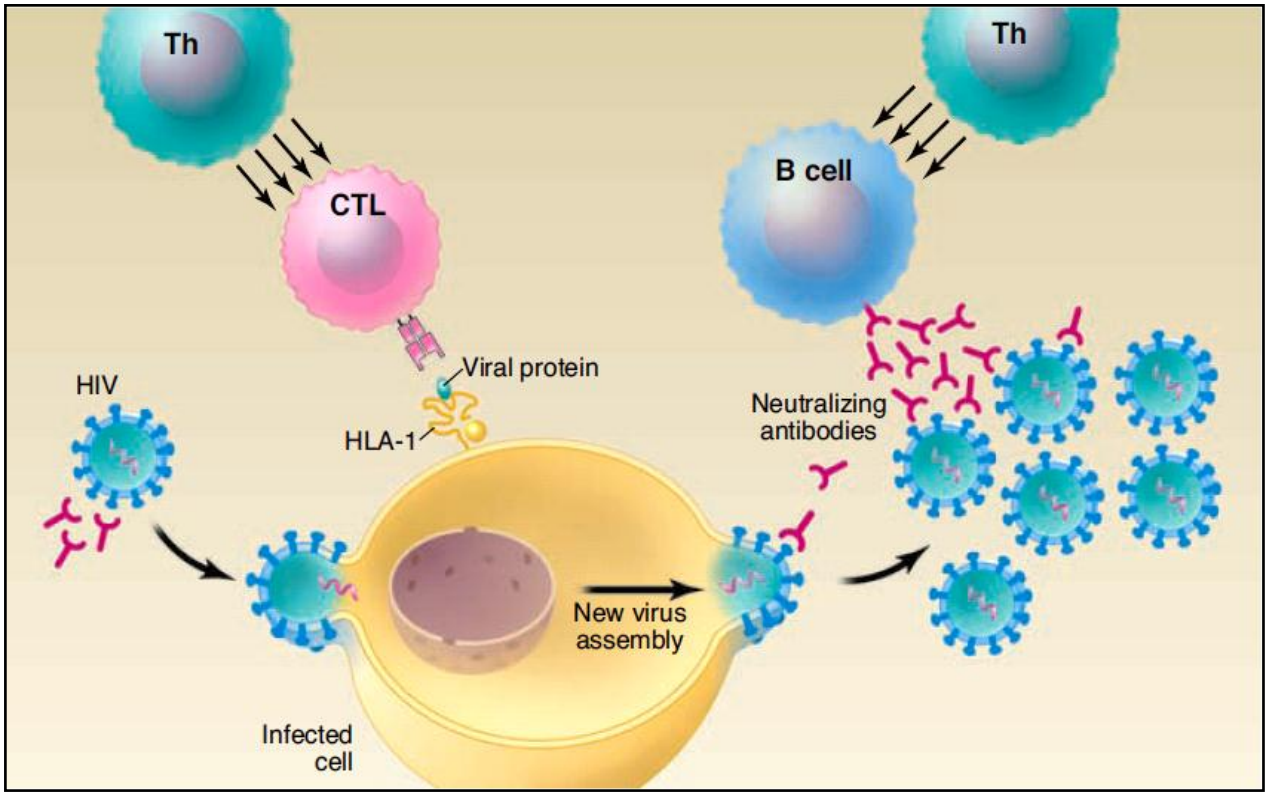

Figure 1.6. Adaptive immune responses in HIV infection. Two arms of the adaptive immune system are activated in response to HIV-1- the T cell response and the antibody response. CD8 T-cells act once a cell becomes infected by recognizing processed viral proteins presented in the context of HLA class I molecules at the cell surface through the T cell receptor of the CTL. Antibodies that can neutralize free virus, can neutralize newly released virions from infected cells, and can act against infected cells can prevent HIV-1 infection of cells. Coordination of CTL and neutralizing antibody responses is mediated by CD4+ T helper (Th) cells (adapted from Walker and Burton, 2008).

1.5.2 HIV-1 Strategies in Evasion of the Immune System

Envelope is heavily glycosylated and is therefore poorly immunogenic (Quinones-Kochs et al., 2002). Vulnerable target sites are protected within the non-immunogenic glycan shield (Burton et al., 2004). In addition trimerization of gp120-gp41 hides peptide-rich epitopes that are highly immunogenic, although antibodies are elicited against these sites (Wyatt et al.,

1998). Envelope baits the immune system with non-essential targets rendering the antibodies ineffective e.g. the variable loops of gp120 are a prime nAb target but often such nAbs have a narrow neutralization breadth against different strains of HIV-1 and anti V3 antibodies which appear in early infection do not contribute to autologous neutralization (Moore et al., 2008, Davis et al., 2009b). Additionally, the shed or non-functional forms of gp120 act as a decoy to divert the immune system away from vulnerable epitopes (Herrera et al., 2005). It has been demonstrated that antibodies are elicited to these forms of Env gp120 but they are ineffective at neutralizing native Env (Moore et al., 2006). Env is highly flexible in solution and may alter its structural configuration, e.g. in the CD4 liganded and unliganded states there are distinct structural differences between the two states, and this could make it challenging for the immune system to target putative epitopes (Liu et al., 2008a). Additionally, shifting of the N-linked glycans may also impose structural changes- resulting in an evolving glycan shield, thereby making Env a difficult target for the immune system (Wei et al., 2003, Chen et al., 2009).

1.5.3 Antibodies- How do they work?

Despite the structural complexities of the envelope (Env) glycoprotein it remains an attractive target for vaccine immunogens, as Env is a primary target for the generation of antibodies. Antibodies can act against both free virus and infected cells. The most effective way an antibody works is by neutralization of free virus *in vivo* to confer protection. Neutralization has been defined as the "loss of infectivity which ensues when antibody molecule(s) bind to a virus particle, and usually occurs without the involvement of another agent" (reviewed in (Dimmock, 1995)). Mechanistically, the process of antibody-virus neutralization is complex and involves cells and other agents of the innate immune system. Mostly, it is hypothesized

that *in vivo* antibodies work extra-cellularly through binding of one or more antibodies to the virus particle; antibodies may induce conformational changes to envelope that destabilizes the virus, or they could sterically hinder fusion of the viral and target cell membranes or they could inhibit the viral uncoating process by binding to nascent viruses (reviewed in (Reading and Dimmock, 2007)).

1.5.4 Vaccine Development

Hepatitis B, measles, mumps and polio are highly infectious viral pathogens to which effective vaccines that confer sterilizing immunity have been developed (Plotkin, 2008). The vaccine strategies involved either the attenuation of live viruses e.g. for measles and mumps, or killing of the virus in the case of polio, or the use of recombinant proteins in the case of hepatitis B. Despite the successes of these vaccines there is limited knowledge on exactly how protection against infection was conferred (Walker and Burton, 2008). All these strategies proved to be highly successful in preventing infection through the production of antibodies and immunological memory similar to that which arises during natural infection (Plotkin, 2008).

When the HIV-1 was first identified in 1983 and was confirmed as the causative agent for AIDS (Barre-Sinoussi et al., 1983), scientists had proposed that within a few years an effective HIV-1 vaccine will have been made, based on the largely empiric approach adopted with most other viral pathogens. However, much to the disappointment of scientists, HIV-1 vaccine strategies based on similar strategies with either recombinant gp120 (monomeric gp120) or killed or inactivated virus were unsuccessful in preventing infection, lowering viral loads or protecting against CD4 T-cell decline (Levine et al., 1996, Flynn et al., 2005, Gilbert et al., 2005). Possible reasons for the lack of success using these strategies include altered

structural conformation of the recombinant and/or killed antigens compared to the native conformation.

Over the ensuing years, numerous attempts have been made toward a vaccine that may elicit protective immune responses. Since 1987 more than 30 HIV-1 vaccines have been advanced to human trials based on vaccine efficacy results in non-human primate models (Mascola and Montefiori, 2010). Ideally an HIV-1 vaccine should be able to elicit both arms of the adaptive immune system, the antibody arm in order to prevent or block infection, and the T-cell arm to eliminate virus infected cells and thereby prevent disease. To date three HIV-1 vaccines based on either eliciting protective antibody or CD8 T-cell responses or a combination of the two have undergone completed testing in humans in phase III or phase IIb trials:

1. The AIDSVAX 120 phase III trials (Vax003 and Vax004) focused on eliciting protective antibodies with a recombinant bivalent gp120 in high-risk populations. Vax 003 included HIV-1 subtypes B and E gp120 and Vax004 included subtype B gp120 only. Both vaccines failed to elicit protective antibodies (Flynn et al., 2005, Pitisuttithum et al., 2006).
2. Scientists then focused on vaccines that may be able to elicit T-cell responses. The STEP phase IIb/Phambili vaccine trial comprised of three recombinant attenuated adenovirus serotype viruses expressing HIV-1 Gag, Pol and Nef (Buchbinder et al., 2008, McElrath et al., 2008, Gray et al., 2011b). CD8 T-cell responses were elicited but the vaccine failed to protect the vaccinated individuals against HIV-1 infection (Buchbinder et al., 2008, McElrath et al., 2008). The evidence indicated that the vaccinated population was significantly more likely to acquire HIV-1 infection compared to the control arm and there were also no differences in viral load among those who became HIV infected.

3. The RV144 phase III vaccine trial in Thailand comprised of a canary-pox viral vector expressing gp120, Gag and Protease (four doses) to prime the immune responses, and the AIDSVAX gp120 (two doses- Vax003) to boost the immune response. This vaccine achieved a moderate 31.2% efficacy in a low-risk heterosexual population (Rerks-Ngarm et al., 2009). The vaccinated individuals who became infected had no difference in their viral loads compared to the control group. However, this trial provided a glimmer of hope that incremental steps are being made toward a vaccine, and more detailed studies are ongoing to define the immune correlates of protection.

1.5.5 The Neutralizing Human Monoclonal Antibodies (nmAbs)

Currently, there are no immunogens that have been successful in stimulating the production of broadly neutralizing antibodies. This is despite the fact that several epitopes have been identified in conserved and variable regions of both gp120 and gp41that induce broadly neutralizing antibodies (Muster et al., 1993, Wyatt et al., 1998). Inducing nAbs to these epitopes using rationally designed immunogens has been difficult possibly due to subtle structural or conformational differences compared to native immunogens (Selvarajah et al., 2005, Law et al., 2007, Selvarajah et al., 2008). The human neutralizing monoclonal antibodies (nmAbs) with broad and potent neutralizing ability are b12, 2G12, 2F5, 4E10 and VRC01 (Roben et al., 1994, Stiegler et al., 2001, Zwick et al., 2001, Zhou et al., 2010). In addition, monoclonal antibodies PG9 and PG16 have been characterized relatively recently, and more recently- the PGT series of antibodies and CH01-04 nmAbs (Walker et al., 2009, Pejchal et al., 2011, Bonsignori et al., 2011). All of these nmAbs target different regions of Env (see Table 1.1) and they can work in one of two ways, either they bind to the mature trimer on the virion surface thereby acting as an entry inhibitor or they bind to the pre-hairpin

inter-mediate, that is after receptor and co-receptor binding thereby acting as a fusion inhibitor (Parren and Burton, 2001). More recently the focus has been on designing the immunogen based on the interaction between the antibody and its cognate site on Env using crystallographic and computed tomography studies (Liu et al., 2008a). This type of reverse engineering may be more effective when developing immunogens in the context of the native Env trimer-antibody interaction.

Table 1.1 List of nmAbs and the targeted Env sites

nmAb	Target Site on Env	Reference
b12	CD4 binding site	Roben et al. (1994)
VRC01	CD4 binding site	Zhou et al. (2010)
2G12	Discontinuous epitope on gp120	Trkola et al. (1996)
PGT Series- PGT 128	Glycans on gp120	Pejchal et al. (2011)
2F5	MPER	Stiegler et al. (2001)
4E10	MPER	Zwick et al. (2001)
PG9/PG16	V2-V3	Walker et al. (2009)
CH01-04	V2-V3	Bonsignori et al. (2011)

In 1992, b12 was one of the first nmAbs to be isolated and neutralizes about 40% of HIV-1 strains. b12 was selected from a phage-display library constructed from the bone marrow of an HIV-1-infected individual. b12 was mutated for high affinity binding to gp120, targets the CD4 binding site with high affinity and the epitope targeted by b12 is surrounded and protected by N-linked glycans (Roben et al., 1994). The CD4 binding site is one of the more conserved regions of Env gp120. b12 recognizes a discontinuous epitope on gp120 as they react with several residues in different regions of gp120 (Zhou et al., 2007). This nmAb occludes the CD4 binding site and acts primarily as an entry inhibitor. VRC01 is another nmAb recently characterized, and was isolated through binding on a rationally designed mimic of the CD4 binding site. VRC01 also targets a narrow site in the CD4-binding domain (Zhou et al., 2010) and neutralized 91% of Env pseudotyped primary isolates, potently

neutralized multiple HIV-1 subtypes and had the broadest neutralization activities compared to any antibody to date (Wu et al., 2010). In addition, this nmAb is highly desirable as it does not have autoreactive properties (Scheid et al., 2011, Li et al., 2011).

2G12 is an unusual nmAb as it targets a discontinuous epitope (conformational epitope) on gp120. 2G12 targets a unique conformational epitope of oligomannose-rich carbohydrate in gp120 that is non-immunogenic (Trkola et al., 1996) and it has an unusual shape. 2G12 represents proof that the carbohydrate-rich gp120 can be immunogenic. More recently however, the PGT series of nmAbs was discovered. Specifically, PGT128, which targets glycans, is highly potent and also displays tremendous breadth (Pejchal et al., 2011). Despite all the intense effort in immunogen design, it still remains unclear how to elicit such unique antibodies.

2F5 and 4E10 are nmAbs that target epitopes proximal to each other within the membrane proximal external region (MPER) of gp41 just N-terminal to the insertion of gp41 into the viral membrane (Stiegler et al., 2001, Zwick et al., 2001). The 2F5 epitope resides in amino acid (AA) position 662-668 (ELDKWAS) while the 4E10 epitope resides in amino acid position 671-676 (NWFDIT). Both these epitopes are in lipid-rich sites within the MPER. These nmAbs do not interfere with receptor binding but they inhibit the fusion between the viral and target cell membranes most likely through steric hindrance. Both 2F5 and 4E10 exhibit potent and broad neutralization of diverse HIV-1 isolates, however, due to their auto-reactive properties and membrane-lipid affinity, their potential for clinical application is a subject of ongoing debate. Although the epitopes that 2F5 and 4E10 target remain attractive vaccine immunogens, it is uncertain whether elicitation of such nmAbs may be detrimental and unsafe due to the autoreactive properties that the antibodies possess and this therefore remains a topic of ongoing research.

Two potent nmAbs, PG9 and PG16 were recently obtained using a high-throughput neutralization screen of antibody-containing culture supernatants from approximately 30,000 activated memory B cells from a subtype A-infected African donor (Walker et al., 2009). The nmAbs exhibit broad and potent neutralization profiles to genetically diverse HIV-1 isolates. PG9 and PG16 target conserved epitopes within the V2 and V3 loops, it is likely that their epitopes overlap because the two nmAbs are somatic variants. Interestingly, these nmAbs recognize epitopes on the native Env trimer, are able to bind monomeric gp120 and monomeric scaffold proteins presenting V1-V2 and neutralize about 80% of primary HIV-1 isolates (Walker et al., 2009, Pancera et al., 2010, McLellan et al., 2011). Both nmAbs do not have autoreactive properties and would therefore be desirable to elicit such antibodies. Similarly, CH01-04 nmAbs that have recently been characterized, are also conformationally dependent on the quartenary structure and target the V2 and V3 loops (Bonsignori et al., 2011). Another nmAb, 2909, targets a quaternary neutralizing epitope on parts of V2 and V3, however 2909 is strain specific and does not bind to monomeric gp120 (Gorny et al., 2005, Honnen et al., 2007).

Overall, these nmAbs have provided the opportunity to define vulnerable epitopes that exist across diverse strains. In addition, their conformational properties in conjunction with their cognate epitopes on the native Env trimer have been well characterized. Crystallographic and structure-function analyses are crucial to designing immunogens for efficacious HIV-1 vaccines.

1.5.6 Env Epitopes as Vaccine Targets

Epitopes should be: i) highly conserved across diverse HIV-1 strains in order to stimulate broadly neutralizing antibodies (Rong et al., 2009); ii) non-autoreactive, that is, their elicited

antibodies should not react to self-antigens (Rong et al., 2009); iii) available on the native Env trimeric spike (Pinter et al., 2004, Davis et al., 2009a, Kwong et al., 2002); and iv) immunogenic to elicit antibodies with high neutralizing titers (Rong et al., 2009). Neutralizing antibody titers have to be high in order to prevent infection as it has been demonstrated that one trimer is necessary to facilitate infection into a cell, therefore all eight to ten trimers have to be engaged to an antibody in order to abrogate infection (Yang et al., 2005). In addition, other studies observed that super-infection may occur in the presence of low-levels of cross-reactive pre-existing antibodies elicited against the initial infecting virus (Blish et al., 2008). Furthermore, antibody titers based on theoretical calculations from *in vivo* passive transfer studies in humans indicated that antibody titers in excess of 1:200 and 1:1,000 were required to protect during the acute and chronic infection stages respectively

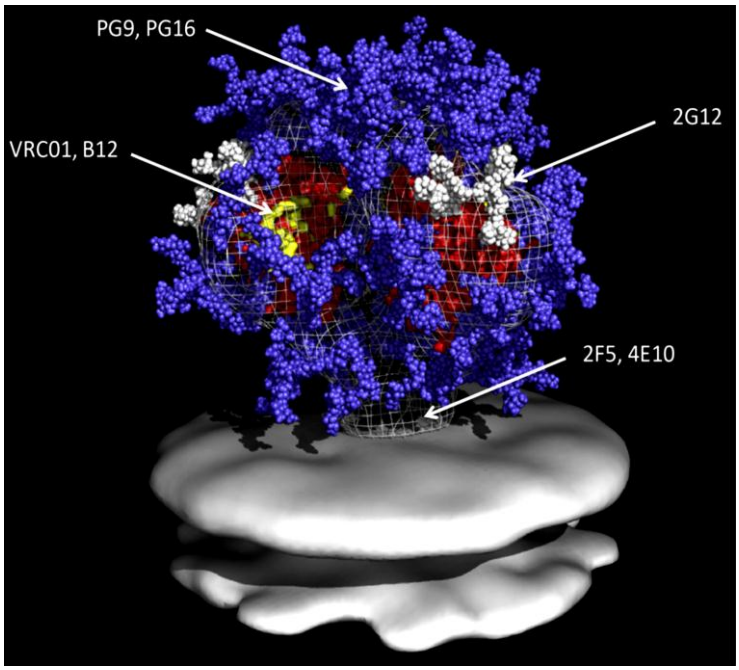

Figure 1.7. A model of the unliganded HIV-1 envelope trimer indicating the approximate location of epitopes recognized by broadly nmAbs. All glycans on gp160 are shown. Glycans are light blue and white, core gp120 is red, 2G12 epitopes are white (arrow), b12 and VRC01 epitopes are yellow (arrow), and the location of the quaternary epitope involving V2 and V3 loops of nmAbs PG9 and PG16 is indicated. The representation of gp41 is shown in grey proximal to the viral membrane. It is likely that the 4E10 and 2F5 epitopes are not available until the Env spike has engaged its cellular coreceptors (adapted from McElrath and Haynes, 2010).

(Trkola et al., 2008). However, animal studies by Hessell et al. (2009) demonstrated that macaques were protected from infection by physiologically relevant amounts (low titer) of nmAbs despite repeated low dose mucosal SHIV challenge (Hessell et al., 2009a, Hessell et al., 2009b).

1.5.7 Neutralizing Human Monoclonal Antibodies in Pre-Clinical and Human Studies

Pre-clinical studies using non-human primate models to test neutralizing human monoclonal antibodies and vaccine efficacy have proven to be highly successful in both pre-exposure and post-exposure prophylaxis studies. NmAbs used in different combinations have conferred sterilizing immunity to macaques when the animals were challenged intravenously, intra-vaginally, intra-rectally, and orally to SHIV (Shibata et al., 1999, Mascola et al., 1999, Mascola et al., 2000, Parren et al., 2001, Ferrantelli et al., 2004). These were proof-of-concept studies that nAbs are effective in preventing SHIV infection. Indeed, one of the caveats to many of the earlier studies, is the very high, non-physiological doses of SHIV administered to the animals. Subsequently, animal models that mimic natural HIV-1 infection have been developed using repeated low dose mucosal simian immunodeficiency virus–HIV chimera (SHIV) challenge (Hessell et al., 2009a, Hessell et al., 2009b). Burton and colleagues showed that b12 was able to confer sterilizing immunity to macaques against vaginal SHIV challenge compared to the weakly neutralizing b6 antibody which conferred no protection or non-neutralizing antibody F240 which conferred limited or no protection in some animals (Burton et al., 2011). Together, these animal models studies provided a compelling argument that antibodies are protective against SHIV infection and that HIV-1 vaccine research should focus on the induction of potent nAbs.

The nmAbs were also tested in adult human HIV-1 infected individuals. A phase 1 safety trial of seven HIV-1 infected individuals using a combination of nmAbs administered intravenously (2G12 and 2F5) (Armbruster et al., 2002) showed that these nmAbs were safe to use and well-tolerated with five of seven individuals experiencing transient reductions in viral loads. A subsequent trial including nmAb 4E10 plus 2F5 and 2G12 in the nmAb regimen again in seven individuals, showed that nmAbs were safe and well tolerated (Armbruster et al., 2004). Viral loads decreased and CD4 T-cells increased transiently in all individuals. However, there was development of viral resistance to nmAb 2G12.

Trkola et al. (2005) then tested the efficacy of a combination of intravenously administered nmAbs (2G12, 2F5 and 4E10) in HIV-1 acutely (n=6) and chronically (n=8) -infected individuals during structured treatment interruption and found that the viral loads were lower in four of the six acutely-infected individuals but only two of the eight chronically-infected individuals receiving the nmAbs (Trkola et al., 2005). These results indicated that perhaps these nmAbs may be more effective during the acute rather than in the chronic phase of infection and argues against the protective role of nmAbs in chronic infection. Viral rebound was evident when the nmAbs were stopped, and in 12 of 14 individuals there was viral escape to 2G12 only (Manrique et al., 2007). Again these nmAbs were safe to use and well tolerated. Although these trials provided proof-of-concept that nmAbs indeed do play a role in viral containment in HIV-1 infected humans, the sustainability, cost-effectiveness and practicality of such an application remains a big concern. It is therefore a high priority to design and develop vaccine immunogens that can elicit potent and broad neutralizing prophylactic antibodies.

1.6 Autologous Neutralizing Antibodies and Heterologous Responses during Acute and Chronic HIV-1 Infection

Developing and designing immunogens that can elicit potent neutralizing antibody (nAb) responses remains a high priority in the HIV vaccine field. It is well known that most HIV-infected individuals develop potent antibodies against their own viruses- termed autologous nAbs (AnAbs) within a few months of HIV-infection (Richman et al., 2003, Gray et al., 2007, Bunnik et al., 2008). AnAbs are generated early on in HIV-1 infection (Albert et al., 1990, Tomaras et al., 2008), however, they are usually strain specific and much of these studies have involved mainly subtype B infections. Only in a subset of individuals, about 20-30%, develop broadly, cross-neutralizing antibodies that neutralize heterologous viruses (Li et al., 2009, Sather et al., 2009, Simek et al., 2009, Stamatatos et al., 2009). A possible reason for this may be that during the earliest stages of HIV-1 infection there is follicular damage and germinal centre loss in the B cell generative microenvironment which may influence the development of broadly neutralizing antibodies (Levesque et al., 2009). Another possible reason is the 'cytokine storm' observed in acute HIV-1 infection that may irreversibly impair antiviral immune responses (Stacey et al., 2009). Examining the neutralization profiles using standard high throughput assays of HIV-1-infected plasma to neutralize autologous and heterologous viruses is a valuable tool in HIV-1 humoral immunity research. This method allows for the screening of nAb responses, to decipher the potency of AnAbs through heterologous virus neutralization, to characterize and better understand the nature of the cognate epitope at a genotypic level and thereby identify putative immunogens for vaccine development. AnAb and heterologous nAb responses in HIV-1 subtype C infection particularly in the context of chronic infection remains mostly unknown and the role of AnAbs in attenuating disease progression is not fully understood.

Specifically, in HIV-1 subtype C infection, AnAbs develop to higher titers than in subtype B infected individuals indicating that there could be antigenic or host genetic differences between the Env that are prompting these differences in antibody responses (Li et al., 2006a, Li et al., 2006b, Patel et al., 2008, Lynch et al., 2009). Several studies found that AnAbs mediate viral escape through humoral immune driven Env diversity during the early stages of infection (Delwart et al., 1997, Wei et al., 2003, Richman et al., 2003). Data from early subtype B and subtype C infections indicate AnAb-driven Env escape and it was found that contemporary viruses were less sensitive to the autologous plasma neutralization than earlier autologous viruses indicating escape (Wei et al., 2003, Richman et al., 2003, Rong et al., 2009, Moore et al., 2009). Furthermore, it was found that the AnAbs targeted very narrow specificities on Env, mainly in V1-V2 (Lynch et al., 2009) and in C3 (Moore et al., 2008). Several mechanisms of viral escape have been significantly associated with antibody resistance; these include insertions and deletions of amino acids, single amino acid substitutions and shifting the position of N-linked glycans on the Env glycoprotein. Some nmAbs for e.g., PG9 and PG16 are highly dependent on a glycan motif to bind to their cognate epitope, and positional glycan shifting confers viral escape as shown in a Zambian seroconverter (Lynch et al., 2011). This study found that a single amino acid mutation in V2 was enough to confer resistance without a replicative fitness cost to the virus. All these mechanisms may impose structural constraints on the ability of the antibody to engage and bind to its cognate epitope through altered Env protein folding and conformation.

1.6.1 Targets of Autologous Antibodies

The Env glycoprotein and in particular the variable loops of gp120 are generally targeted by the humoral immune system, however certain regions appear to induce more AnAb responses

than others. Sagar et al. (2006) found that V1-V2 are direct targets for autologous neutralization in early HIV-1-infection (Sagar et al., 2006). The role of V1-V2 in increasing neutralization resistance through addition of PNGs, V1-V2 length modification and insertions and deletions has been well defined in subtype B and C infection (Pinter et al., 2004, Sagar et al., 2006, Rong et al., 2007a, Lynch et al., 2011). Although V3 elicits antibodies very early on during infection, anti-V3 antibodies are not effective for autologous neutralization (Moore et al., 2008, Davis et al., 2009a, Davis et al., 2009b). The role of V4 and V5 as AnAb targets is not fully understood. Amino acid changes in the α-2 helix in C3 of HIV-1 subtype C mediates escape from AnAbs (Gnanakaran et al., 2007, Moore et al., 2009). Taken together, these studies provide many clues about Env targets for putative vaccine immunogens.

1.7 Fcgamma (Fcγ) Receptors and Viral Inhibition

Although the main aim of the HIV-1 vaccine field is the development of immunogens to induce broadly neutralizing antibodies, other inhibitory functions of non-neutralizing antibodies are now being considered as potentially protective. Non-neutralizing antibodies are defined as antibodies which do not show "classical neutralizing activity", rather, they inhibit virus replication through an FcγR-dependent mechanism (Peressin et al., 2011). FcγR are present on most cells of the innate immune system e.g. non-phagocytic natural killer cells and mast cells, and phagocytic cells e.g. macrophages, immature dendritic cells and neutrophils. There are five major Fcγ receptors with either an activating or inhibitory function: FcγRI, FcγRIIa, FcγRIIIa and FcγRIIIb (all activating) and FcγRIIb (inhibitory) (Forthal and Moog, 2009). The Fc portions of antibodies form a bridge between the cell bearing the target antigens and the effector cell bearing the Fc receptors. This then results in virus killing through antibody dependent cell-mediated cytotoxicity (ADCC) or through

antibody-dependant cell-mediated viral inhibition (ADCVI). Figure 1.8 illustrates how the various components of the humoral immune system work in concert to either effect direct killing of virus through nAbs, or through the complement activation, through opsonization and phagocytosis or through the Fc-receptor-mediated mechanisms (Huber and Trkola, 2007). These FcγR-bearing immune cells are involved in the capture and degradation of HIV-1 antigen-antibody complexes and induction of immune effector functions such as antigen presentation. Potent ADCC activity was found in the sera of long-term HIV-1-subtype B infected non-progressors suggesting that ADCC controls viraemia and therefore protects against disease progression (Alsmadi et al., 1997). Another study showed an inverse relationship between viral loads and ADCC activity in rapid progressors compared to non-rapid progressors (Baum et al., 1996). Interestingly, in subtype B-infected elite controllers, ADCC activity was significantly more potent than in viraemic individuals (Lambotte et al., 2009). This study however, did show that elite controllers had a heterogenous nAb response that was lower or no different to viraemic individuals (Lambotte et al., 2009). It has previously been demonstrated that interaction of FcR-expressing cells with antibody-complexed target cells appears to be more important as a mechanism of ADCVI than complement interaction with antibody-complexed cells (Hessell et al., 2007). Recently, it was demonstrated that non-neutralizing antibodies conferred limited or no protection to macaques against vaginal SHIV challenge (Burton et al., 2011). A series of studies have shown that the binding interaction between the Fc portion of the antibody and the FcγRIIIa and/or FcγRIIa potently increases ADCC activity, Fc binding affinity was altered through the manipulation of antibodies using deglycosylation or site-specific mutagenesis (Lazar et al., 2006, Richards et al., 2008, Shields et al., 2001, Jefferis, 2009). These studies therefore provide a rationale for assessing the binding affinities of the FcγRs as a surrogate indicator of ADCC/ADCVI activity *in vivo*.

V1-V2 binding antibodies were found to be protective against HIV-1 acquisition in vaccinated individuals of the RV144 trial[*]. However the exact mechanism by which these binding antibodies prevented infection is unknown. Harnessing the potential of these inhibitory FcγR-Fc antibody interactions may be important in improving HIV-1 vaccine efficacy. This may be particularly important given the recent results of the Thai trial (RV144 Trial) where the 30.5% efficacy (Rerks-Ngarm et al., 2009) was attributed to non-neutralizing antibodies, in the absence of finding nAbs or cytotoxic T cells (Tomaras and Haynes, 2009).

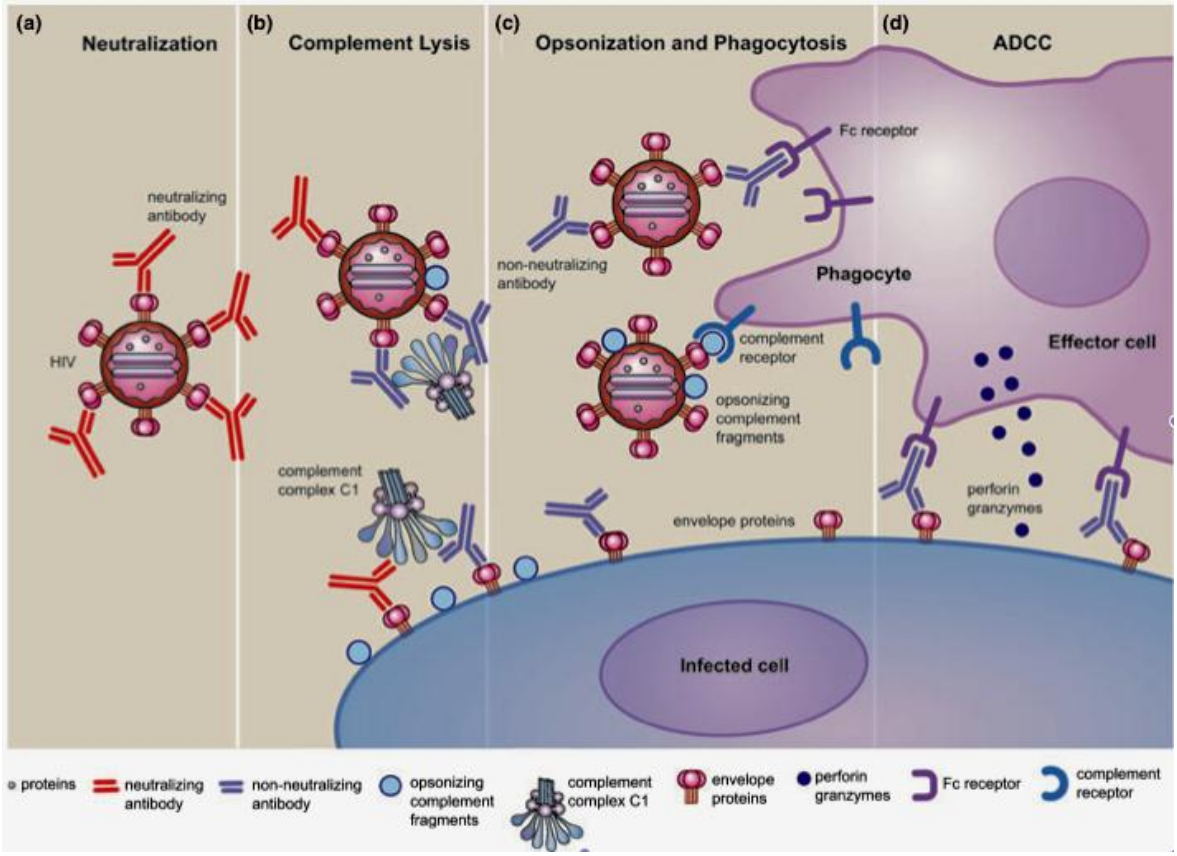

Figure 1.8. How antibodies- both neutralizing and non-neutralizing combat HIV-1. (a) Neutralization of free virus by antibodies, (b) complement-mediated lysis of free virus and infected cells through complement activation (c) opsonization through antibodies coating virus particles and phagocytosis of through Fc-mediated receptor binding to antibody or complement receptors, (d) antibody-dependent cell-mediated cytotoxicity (ADCC) against infected cells. nAbs are denoted in red, non-neutralizing antibodies- blue, Fc receptors- violet, complement – light blue, complement receptors- black (adapted from Huber and Trkola, 2007).

[*] Oral presentation by Barton Haynes at the 2011 AIDS Vaccine Conference, Bangkok, Thailand.

In the chronic infection phase, the contribution of FcγR-Fc antibody interactions in controlling against disease progression remains poorly defined, particularly in the context of subtype C infection.

1.8 Aims of the Thesis

This is a study in chronic HIV-1 subtype C infection that links both the envelope characteristics with neutralizing and non-neutralizing antibody patterns. This type of study is particularly important, as there are very large gaps in the field regarding the interplay between the viral evolution and the response of the humoral immune system. We hypothesized that durable neutralizing antibody responses against HIV-1 envelope and reduced viral diversification correlate with lack of disease progression in chronically HIV-1 subtype C infected individuals.

We focused on HIV-1 subtype C envelope evolution and neutralizing antibody responses during the course of natural chronic infection over a median of 21 months in order to gain a better understanding of the correlates of immune protection or disease progression. Two groups of participants with divergent rates of disease progression were analyzed and compared. These two groups (designated slow progressors and progressors) were chosen based on their immunological and virological status. They were immunologically and virologically matched at study entry; however, over the period of observation to their study exit time point, the progressors immunological status had declined and their viral load risen significantly compared to the study entry time point. It was therefore intriguing to study both the genotypic characteristics of the viruses in both groups as well as the phenotypic differences between both groups as we hypothesized that might be certain genotypic and virologic as well as phenotypic characteristics associated with the *env* gene and antibody responses that correlate with divergent rates of disease progression.

For the genotypic characteristics, we used single genome sequencing to improve detection through terminal dilution to detect minority variants at levels below 20-30% as is has been found that population based sequencing may not detect minor virus quasispecies. In addition, unless limiting dilution is used, there is a risk of PCR-generated recombination that may result in artifactual genomes that do not exist in real life. In addition, in our study the entire *env* gp160 was sequenced, whereas in other studies on subtype C, partial *env* sequences were done and therefore the data is limited in scope and interpretation. There are several points that need to be highlighted about the importance and justification of such a study in HIV-1 subtype C infection. Firstly, the relationship between the genotypic characteristics of HIV-1 subtype C envelope e.g. Env sequence diversity over time, length polymorphisms within gp160, numbers of potential N-linked glycosylation sites, positive selection as a surrogate for humoral immune pressure, and negative selection pressure and their relationship to divergent rates of HIV-1 subtype disease progression is not completely understood and was therefore explored. Secondly, we further defined the relationship between the autologous nAb responses in participants with divergent rates of disease progression in conjunction with genotypic characteristics and immunological parameters of disease progression namely CD4 T-cell count and viral load. Thirdly, the breadth of the autologous neutralizing antibody responses was tested against heterologous viruses of different subtype lineages (subtypes, A, B and C) in order to profile antibody potencies and evolution of neutralization breadth. We found that the breadth of response correlated to disease progression. Finally, we also correlated the IgG binding affinities of three activating and one inhibitory Fcγ receptors to disease progression, to establish a putative role for the activating/inhibitory function of non-neutralizing antibodies in divergent rates of disease progression. We chronicled the genotypic properties of HIV-1 subtype C Env gp160 and the autologous and heterologous

humoral immune responses over time in chronic infection to expand our current knowledge in the field and to better understand the correlates of disease progression.

1.9 Hypothesis

Durable neutralizing antibody responses against HIV-1 envelope and reduced viral diversification correlate with lack of disease progression in chronically HIV-1 subtype C infected individuals. We further hypothesized that non-neutralizing antibodies may contribute to disease attenuation in chronic HIV-1 subtype C infection.

1.10 Study Objectives

1.10.1 Primary Objectives

1.10.1.1 To determine the evolution of *env* viral diversity and diversification patterns in chronically HIV-1 subtype C-infected slow progressors and progressors over two years.

1.10.1.2 To determine neutralizing antibody activity and evolution in chronically HIV-1 subtype C-infected slow progressors and progressors over two years in order to assess the contribution of these responses to the rate of disease progression.

1.10.1.3 To determine differences in the IgG non-neutralizing antibodies binding affinities to HIV-1 specific antigens and various activating and inhibitory Fc gamma receptors in chronically HIV-1 subtype C-infected slow progressors and progressors over two years.

1.10.2 Secondary Objectives

1.10.2.1 To determine whether diversity and diversification of *env* are associated with the rate of disease progression.

1.10.2.2. To determine whether neutralization breadth exerts control over HIV-1 by investigating its association with CD4 T-cell count and viral loads.

1.10.2.3 To determine whether non-neutralizing IgGs or HIV-1 antigen specific IgGs exert control over HIV-1 by investigating their association with CD4 T-cell counts and HIV-1 viral loads.

Chapter Two - Materials and Methods

2.0 Materials and Methods

2.1 Study Design

This was a retrospective study. Ethics approval for this study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee - reference number E055/06 (see Appendix for scanned copies of approval letters pages 171-173). Of the nine participants who met the predetermined study inclusion criteria, one participant was excluded because their envelope sequences were found to be of subtype A lineage that was outside the scope of this PhD thesis. The current study focuses on subtype C infection only. Eight study participant samples therefore, from the original Sinikithemba cohort from McCord Hospital, Durban, KwaZulu-Natal (KZN), South Africa were used to complete the objectives of the study. These patients were chosen according to the following study inclusion criteria below.

Study subjects with longitudinal follow-up data were included in this study. All the patients were antiretroviral naïve for the period of evaluation. CD4 T-cell count over two years was chosen as the primary determinant of disease progression for stratification into slow progressor (n=4) and progressor (n=4) categories. Both slow progressors and progressors were selected on the basis of a CD4 T-cell counts >500 cells/µl at study entry time point. The CD4 T-cell counts did not differ significantly between the two groups at the study entry time-point. However, at study exit, slow progressors maintained a CD4 T-cell count above 500 cells/µl or a viral load less than 10,000 viral RNA copies/ml. In contrast, progressors declined in CD4 T-cell counts to below 500 cells/µl and had a viral load above 10,000 copies/ml. The overall median time was 21 months between study entry and study exit sampling. When the virological and immunological data became available beyond the study window (follow-up of an average of 39.8 months for slow progressors and 36.8 months for

progressors, we analyzed these parameters relative to the study entry criteria and they remained statistically different for the progressors only (p=0.03 for both CD4 T-cell count and viral load).

2.2 Sample Collection, CD4 T-cell Counts and Plasma Viral Load

CD4 T-cell counts were performed every three months and viral loads every six months. Blood was drawn from each subject into EDTA tubes and plasma was separated by centrifugation and stored at $-80^{\circ}C$ until use. Viral load was measured using the Amplicor Version 1.5 assay (Roche Diagnostics, Indianapolis, USA). CD4+ T-cell counts were enumerated by Trucount technology on a four colour FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

2.3 Single Genome Amplification (SGA) of Envelope

This protocol involves the dilution of viral cDNA to a point that, upon setting up multiple replicate PCR reactions with the cDNA as template, the product generated has a high probability of having been amplified from a single genome copy. In this case, according to Poisson's distribution, no more than 30%, or 28 out of a 96-well reaction plate with two negative controls, should be positive for amplification product to ensure that 6 of 7 amplified DNAs arise from a single-copy template (Rodrigo et al., 1997). Figure 2.1. illustrates the process of single genome amplification in order to achieve the desired dilution for sampling both the major and minor circulating viral quasispecies.

For example, if plasma viral load was known, a specified amount of plasma is used to extract viral RNA is purified from plasma using the QIAamp Viral RNA Mini Kit and it is transcribed at an approximate concentration of 200 copies per µl using a gene-specific primer with Invitrogen's Superscript III—a reverse transcriptase that has been genetically modified to reduce RNase H activity and to maintain cDNA synthesis activity at temperatures (i.e. 50-55°C) required to melt template secondary structure. The viral cDNA was serially diluted to obtain single copy in a two-step "nested" PCR procedure. Screening PCRs with differing dilutions were performed in order to achieve the desired 30% positivity rate.

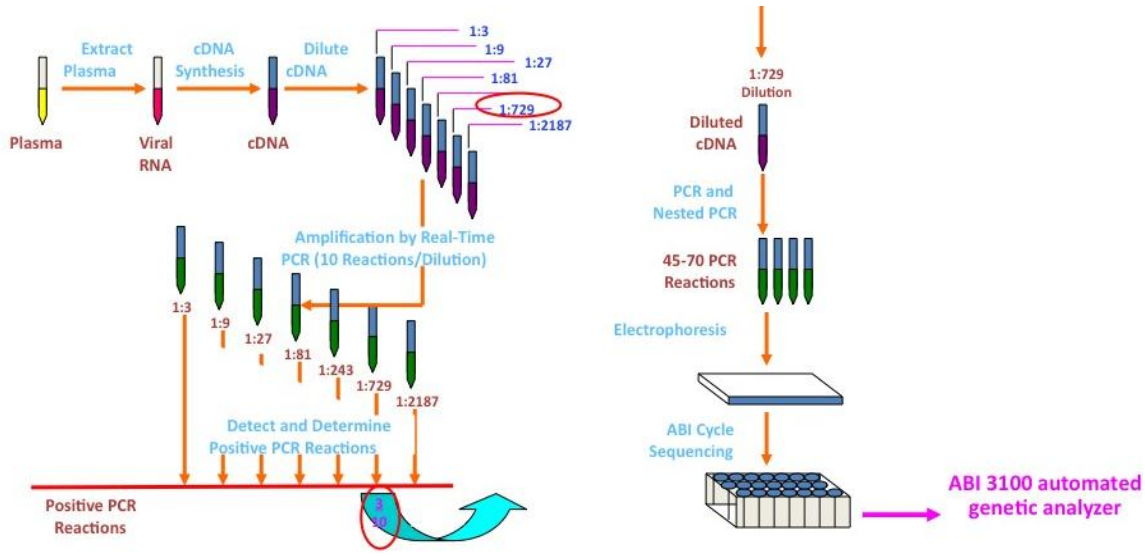

Figure 2.1. Experimental overview of single genome amplification where serial dilution of the cDNA was performed based on the viral load of the participant sample. By Poisson's distribution if for example the 1:729 dilution resulted in a ≤ 30% positive PCR reaction rate, for that particular participant the 1:729 dilution of cDNA was used subsequently for single genome analysis.

This was necessary to account for the variations in sample integrity, stability, plasma/serum components and viral concentration that can all affect PCR efficiency. Thereafter, a confirmatory PCR at the desired dilution of cDNA was performed and the amplicons from the first and second round PCR reactions were stored at -20°C.

To further confirm single-genome amplification, the entire envelope gene was sequenced in forward and reverse directions and any sequences containing double peaks were excluded from the analyses.

2.4 cDNA Synthesis from Viral RNA Extracted from Human Plasma

The QIAamp Viral RNA Mini Kit (Qiagen, Dusseldorf, Germany) was used according to the protocol as written in the manufacturer's handbook with the following adaptations:

A volume of 140 µl of the plasma sample was used, and based on a viral load of 100,000 copies/ml, this volume is estimated to contain the equivalent of 20,000 viral copies. The sample volume was determined by the participant's viral load.

If the participant's viral load >200,000 copies/ml then the volume of plasma was adjusted proportionately and in order to normalize to a volume of 140 µl, PBS was added accordingly. Or, if viral load was <1,000 copies/ml, 1ml of plasma virus was concentrated by centrifugation for 1 hr at 17,000 rpm. 860 µl of supernatant was removed and the viral pellet was re-suspended in the remaining 140 µl of plasma. The standard method of viral RNA isolation was followed as per manufacturer's guidelines. Briefly, 200 µl of plasma was reconstituted with 800 µl of the AVL buffer containing the carrier RNA from the kit into a 1.5ml eppendorf tube and pulse-vortexed for 15 sec. Thereafter the mixture was left to incubate at room temperature (RT) for 10 mins and thereafter briefly centrifuged. 800 µl of ethanol was added and the mixture was pulse vortexed briefly again and 650 µl of this mixture was added to a QiaAmp Mini spin column and then centrifuged at 6000 x g for 1 min, and this process was repeated until all the mixture was put through the column. After all the RNA was bound through repeated column centrifugation, 500 µl of the AW1 kit buffer

was added and then centrifuged at 6000 x g for 1 min. Thereafter, 500 µl of the AW2 kit buffer was added and then centrifuged at 20 000 x g for 3 min. The RNA was then eluted from the spin column with 55µl buffer (stock elution buffer is diluted in a 1:5 ratio in sterile RNase/DNase free H_2O) to achieve approximately 50 µl net recovery. cDNA synthesis was performed directly after RNA extraction.

2.5 Reverse Transcription and cDNA Synthesis

The Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, USA) was used in order to generate the cDNA through reverse transcription (components of the kit included: SSIII RT (200 U/µl), 5X First-Strand Buffer, 0.1 M DTT.) and 10 mM DNTP (Fermentas, Canada). This procedure was performed in a RNA-only clean room. The condensate from the previous step was spun down briefly, after the heat incubation steps.

The following components were added into a 0.2 ml RNase-free tube for each cDNA synthesis as depicted in table 2.1.

Table 2.1. Mastermix for the cDNA synthesis

Reagent	Volume(µl)/tube
Sterile Water	8.75
Primer: (OFM19)20 µM	1.25
dNTP Mix (10mM each)	5.0
RNA template	50
Final volume/tube	65

The tubes were placed in a thermo-cycler at 65°C for 3-5 mins, removed and placed on ice for 1 min. The following components were added from the kit to the mix from above according to the method depicted in table 2.2.

Table 2.2. Mastermix for the cDNA synthesis

Reagent from Kit(SSIII RT)	Volume(µl)/tube	Stock from Kit	Final Dilution
5x Buffer	20	5x	1x
DTT	5	100 mM	5
RNaseOUT	5	40 u/µl	2
SSIII RT	5	200 u/µl	10
Sterile water	65		
Final volume/tube	100		

The reaction mixture was then gently mixed and left to incubate at 50°C for 1 hr and then increased to 55°C for 1 hr. Inactivation of the SSIII RT was achieved by heating at 70°C for 15 mins. To each tube, 1 µl of RNase H was added and left to incubate at 37°C for 20 mins.

2.6 PCR Amplification

For single genome amplification of full-length envelope, nested PCR was implemented. PCR reaction mixes were made up and aliquotted in an area free of PCR amplified or plasmid DNA. The subtype C-specific *env* primers used were:

OFM19: 5'–GCACTCAAGGCAAGCTTTATTGAGGCTTA (HXB2 positions 9604-9632) and VIF1: 5'– GGGTTTATTACAGGGACAGCAGAG (HXB2 positions 4900-4923) were used for the first round of PCR.

ENVA: 5'– GGCTTAGGCATCTCCTATGGCAGGAAGAA (HXB2 positions 5955-5982) and ENV N: 5'– CTGCCAATCAGGGAAGTAGCCTTGTGT (HXB2 positions 9145-9171) were used for the second round PCR.

2.7 Establishment of the Appropriate cDNA Titration

2.7.1 cDNA Dilutions

Serial dilutions of cDNA (ten reactions per dilution) were performed to obtain 30% positivity based on Poisson's distribution as illustrated above in figure 2.1. This was the methodology used for the screening PCR in order to establish the optimal dilution of the cDNA to obtain the desired 30% positivity. Amplicons that were generated in the titration were screened using full-length envelope sequencing and used if they presented with single peaks for each nucleotide on the chromatogram, it was confirmed that the amplicons were generated from single *env* templates. Once the optimal dilution of cDNA was established confirmatory PCR amplification was then performed. If there were positive reactions in excess of 30% per sample, repeat PCR with lower/higher dilutions was performed.

2.7.2 First Round Amplification Reaction

PCR reaction mix was made for one-100 reactions (94 test + two negative controls). Four extra reactions were made to account for pipetting error- see table 2.3 below.

Table 2.3. Mastermix for the first round PCR

Reagent	Volume(µl/tube)	x100 reactions(µl)
dH_2O	15.3	1,530
5X buffer (supplied)	2	200
$MgS0_4$ (supplied)	0.8	80
dNTPs (10mM mix)	0.4	40
Taq (High Fidelity Platinum)	0.1	10
Primer: OFM19 (20µM)	0.2	20
Primer: VIF1 (20µM)	0.2	20
Final volume	19	1,900

Nineteen µl of the PCR reaction mastermix was added per /well. For the negative control, 1 µl of water was added to 19 µl of the PCR reaction mixture to make up the negative controls for the PCR and 1µl cDNA per dilution per well was added for the test reactions. The plate was split into two sections of 48 wells each to accommodate two different participant samples in a single PCR run. Once the correct cDNA dilution was determined through the dilution, 50 µl of diluted cDNA was added to each half of the master mix and then 20 µl of this reaction mixture was added per well. The plate was placed in a thermal cycler and run with following PCR parameters**:** one cycle of 94°C for 2 mins; 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 4 mins and one cycle at 68 °C for 10-20 mins; and finally on hold at 4°C.

From all positive wells, the amplicons from the first round were stored at -20°C for future envelope cloning once the second round products were resolved, visualized and confirmed using gel electrophoresis.

2.7.3 Second Round (Nested) PCR Reaction

PCR reaction mixture for the second round was prepared for one-100 reactions (94 test + two negative controls) according to the tabulated protocol (table 2.4).

Table 2.4. Mastermix for the second round PCR

Reagent	Volume (µl/tube)	x100 reactions (µl)
dH_2O	15.3	1,530
5X buffer (supplied)	2	200
$MgS0_4$ (supplied)	0.8	80
dNTPs (10mM mix)	0.4	40
Taq (High Fidelity Platinum)	0.1	10
Primer: ENV A (20µM)	0.2	20
Primer: ENV N (20µM)	0.2	20
Final volume	19	1,900

Nineteen µl of the master mix was pipetted into each well of a 96-well plate and 1µl from each of the first round PCR reaction samples was added to the corresponding well of the nested PCR plate. Mixing was achieved by pipetting up and down. The 96 well plate was placed in a thermal cycler and PCR performed with the following cycling parameters: one cycle of 94°C for 2 mins; 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 4 mins and one cycle at 68°C for 10-20 mins; and finally on hold at 4°C.

2.7.4 Analysis of PCR.

Three to 5 µl of products from the second round PCR was run on a 1% agarose gel, and the product size was confirmed by comparing it to the Molecular X marker (Roche Diagnostics, Indianapolis, USA). All positive wells were selected. The amplicons from the second round PCR were then subjected to PCR product clean up using the QiaQuick PCR Purification Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's protocol. PCR products were either quantified on a 1% agarose gel using a low molecular weight marker or on the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Surrey, United Kingdom). Thereafter full-length *env* sequencing was performed.

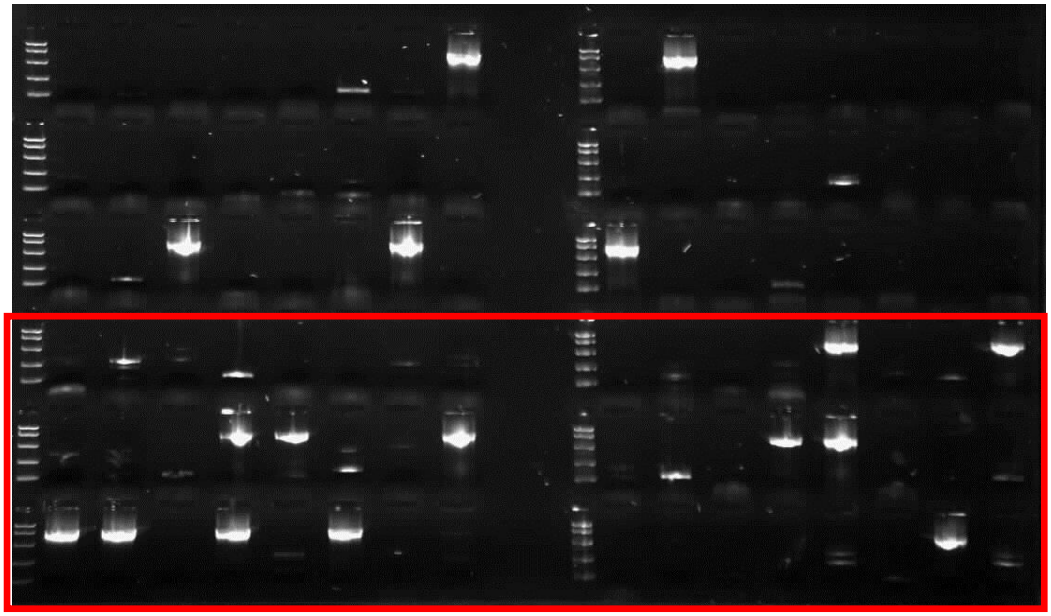

Figure 2.2. Representative confirmatory PCR on a 96 well plate visualized on a 1% gel. The plate is divided into two parts to screen for *env* amplicons in two patients. In the second half of the plate (indicated by a red outline) there is a 25% detection rate for the number of positive wells, which is in keeping with the Poisson's distribution. The amplicons for the first and second round PCR reactions were stored for future use for cloning and sequencing respectively.

2.8 Sequencing Reaction

The ABI 3130xl Sequencer was used to resolve sequences of the envelope amplicons generated. To facilitate high throughput sequencing, 96 well plates were used. A master mix for all the samples to be sequenced was prepared according to the tabulated protocol (table 2.5). This protocol was used for a single reaction for a single primer. Envelope sequencing requires an average of eight primers, four for the forward and four for reverse sequencing in order to generate a full-length 3kb contig. For each 96 well plate 12 full-length *env* amplicons were sequenced (purification of the sequencing products were performed on the same day as the sequencing reaction).

Table 2.5. Mastermix for sequencing reactions

Reagent	Volume (µl/reaction)
BigDye Terminator Ready Reaction Mix (Applied Biosystems Version 3.1 cycle sequencing kit, Catalogue No.	0.4
5X Sequencing Buffer	2.0
Primer (pmol)	3.2
Template DNA	(20ng*)
De-ionised Water	Up to 10µl
Final volume	10

The above reaction mixture was mixed well and spun down. The mixture was then aliquotted into tubes or a plate depending on the number of sequencing reactions. The diluted template (20 ng of DNA in total) was then added to the reaction. The plate was spun down briefly. The PCR plate was placed in a thermo-cycler and the following cycling conditions were used: 1 min at 96°C, 25 cycles of 96°C - 10 sec; 50°C for 5 sec; 60°C for 4 min, and finally 4°C on hold.

Note: 35 cycles was used if the cleaned PCR product had a low concentration or for achieving higher signal intensities. The products were stored at 4°C, and protected from light. Primers used for sequencing reactions were as follows as depicted in table 2.6.

Table 2.6. List of primers used to sequence HIV-1 subtype C Env gp160

Primer name	**HXB2 Position**	**Primer Sequence**
ED31	6818→6845	5’ CTCAGCCATTACACAGGCCTGTCCAAAG
SQ13F(2)C	7672→7701	5’TATATAAATATAAAGTGGTAGAAATTAAGC
SQ14FC	7925→7944	5’ ACTCACGGTCTGGGGCATTA
EF00	6204→6228	5’AAA GAG CAG AAG ACA GTG GCA ATG A
ENV N	9171←9145	5’ CTGCCAATCAGGGAAGTAGCCTTGTGT
SQ3R(2)C	8651←8680	5’ GCTATGGTATCAAGCAGACTAATAGCACTC
SQ5.5RC	7979←8004	5’ CTAGGAGCTGTTGATCCTTTAGGTAT
SQ6RC(2)	6839←6864	5’ GAATTGGGTCAAAAGAGACCTTTGGA

→ Denotes a forward primer. ← Denotes a reverse primer

2.9 Purification of Sequencing Products

2.9.1 Plate Cleanup

This protocol assumed a 10µl cycle-sequencing reaction, and all sequencing clean-up was done on a 96 well-plate. One µl of 125mM EDTA pH 8.0 was added to each well add and mixed. A solution of 3M NaOAc pH 5.2 was made up with 100% ethanol (kept at -20°C) according to a 1:25 ratio respectively and 26 µl of this solution was added per well, and mixed and vortexed briefly. A sealing tape was placed on the plate making sure each well is sealed and protected from cross-contamination during the vortex steps. The plate was centrifuged at 3,000 x g for 20 mins. The plate was carefully inverted onto paper towel and centrifuged at 150 x g for 5 mins to dry. Immediately after, 35 µl of 70% cold ethanol (freshly made up and kept at 4°C) was added to each well. The plate was centrifuged at 3,000 x g for 5 mins. The plate was carefully inverted onto paper towel and centrifuged at 150 x g for 1 min to dry. The samples were dried in a thermo-cycler at 50°C for 4 mins. The samples were then re-suspended in 10 µl formamide and vortexed thoroughly for 15 seconds and denatured in a thermo-cycler (95°C for 3 mins, and then 4°C for a minimum of 3 mins). The plates were then placed in a 3130 XL Sequencer for sequences to be detected.

2.9.2 Sequencing Analysis of gp160

The full-length envelopes were sequenced in the forward and reverse directions using the ABI Prism Big Dye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) utilizing primers spanning the entire envelope and approximately 300 bp apart (as described in detail above). Sequences were then resolved on the ABI 3130 XL genetic analyzer. Contigs were assembled and edited using the Sequencher v 4.8 software (Genecodes, Ann Arbor, MI). The sequences were aligned using Clustal W (Thompson et

al., 1994) and manually edited in the Genetic Data Environment (GDE 2.2). For phylogenetic analysis, subtype reference strains were obtained from the Los Alamos HIV sequence database http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html). Phylogenetic trees were generated in PAUP*4.0b10 using the TVM I + G model of substitution as determined by MODELTEST 3.7 (Posada and Crandall, 1998). Trees were rooted with a homologous region of Group O reference (O.CM.96). Maximum likelihood (ML) trees of sequences from individual patients were also drawn using the appropriate evolutionary model (as determined by MODELTEST 3.7) and rooted with the "Best-fit root" as determined by Path-O-Gen v1.2 (Rambaut, 2008). All trees were bootstrapped with 1,000 sampling replicates. Trees were viewed with FigTree v1.1.2 (Rambaut, 2008). The approximate time of HIV-1 infection was estimated using BEAST (Bayesian Evolutionary Analysis Sampling Trees) version 1.4.8 (http://beast.bio.ed.ac.uk) in order to predict approximate time of infection prior to study enrollment (Drummond and Rambaut, 2007). BEAUTi was used to generate the .xml file to generate the BEAST file. The GTR substitution model with estimated base frequencies and a site heterogeneity model of gamma + invariant sites were used. A relaxed, uncorrelated lognormal molecular clock model was chosen. The MCMC (Monte Carlo Markov Chain) length of chain was set at 30,000,000 to give an effective sample size (ESS) > 170. The number and location of putative N-linked glycosylation sites (PNGs) was estimated using N-GlycoSite (http://www.hiv.lanl.gov/content/hiv-db/GLYCOSITE/glycosite.html) from the Los Alamos National Laboratory database. Sequence diversity was calculated using the Maximum Composite Likelihood option in Mega 4.0 (Tamura et al., 2007). Characteristic differences between progressors and slow progressors including corresponding study entry and exit time-points were identified using VESPA (Viral Epidemiology Signature Pattern Analysis) (Korber and Myers, 1992). Nucleotide substitution rates were calculated using baseml from the PAML software package (Yang, 1997). Sites under positive selection were

identified using the SLAC option in HyPhy (Pond et al., 2005) and CODEML as implemented in the PAML software package.

Positively selected sites and signature mutations were mapped onto the X-ray structure of a clade C HIV-1 gp120 (3LQA.pdb) (Diskin et al.) using the BIOPREDICTA module in the VLifeMDS software package (VLife Science Technologies, 2007). Gp41 was modeled in SWISS-MODEL (Schwede et al., 2003) using 1ENV.pdb (Weissenhorn et al., 1997) as a template. Structures were rendered and annotated in PyMol (DeLano, 2006).

2.9.4 Genebank Accession Numbers

Sequences have been assigned the following GenBank accession numbers: GU216702-GU216737 and GU216739-GU216847.

2.10 Directional Cloning

In order to clone the envelope generated from PCR though SGA, the first round envelope products were subjected to a second round PCR amplification using a specific primer with an ATGG overhang (Env A directional primer). The protocol below was used in order to generate the PCR product to be used in the subsequent cloning step. The Phusion High Fidelity kit (New England Biolabs, Ipswich, USA) was used as stated below to generate the second round PCR product to be used in downstream cytomegalovirus-driven pcDNA 3.1/V5-His TOPO directional cloning experiments. The PCR products were subsequently subjected to agarose gel visualization after PCR clean up using the QiaQuick PCR clean up kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's protocol. The PCR

products were then quantified for DNA using the 2000 Spectrophotometer (Thermo Scientific, Surrey, United Kingdom).

Table 2.7. Mastermix for the second round PCR

Reagent	Volume (µl/reaction)
H2O	37
5x Phusion Buffer	10
dNTP (10mM)	1
EnvAdir (20 pmol/µl)	1
EnvM (20 pmol/µl)	1
TaqPol	0.5
Final volume	50.5

One µl of the first round SGA PCR product was added.

PCR conditions for downstream cloning were as follow: one cycle of 94°C for 5 mins; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 10 mins and on hold at 4°C.

2.10.1 Directional Cloning using pcDNA3.1 Expression Vector

PCR fragments, generated using Env 1A and Env M primers, were cloned into the pCDNA 3.1/V5-His TOPO vector (Invitrogen, Carlsbad, USA) into XL Gold Ultra-competent cells (Stratagene, USA) and bacterial colonies were screened by PCR for insertion and correct orientation using T7 (supplied in the pcDNA 3.1/V5-His TOPO expression kit) and Env M primers through colony PCR.

2.10.2 Cloning Procedure

Sterile tubes were chilled on ice in order to be adequately chilled for downstream cloning.

The cloning reaction mastermix consisted of the following as depicted in table 2.8.

Table 2.8. Mastermix for ligation of PCR product into the pcDNA 3.1/V5-His TOPO vector

Reagent	Volume (µl/reaction)
Sterile Water	3
Salt	1
pcDNA3.1/V5-His TOPO Vector	1
DNA (10 ng/µl)	1
Final Volume	5

This reaction was incubated for 30 mins at RT. 10 mins into the reaction above, the XL Gold - Ultracompetent cells were thawed on ice for 10 mins, thereafter 4 µl of beta-mercaptoethanol was added per vial of XL Gold - Ultracompetent cells (150 µl). Tubes were placed on ice for 10 mins. 50 µl of XL Gold - Ultracompetent cells was aliquotted to each chilled sterile tube and to the cells, 3 µl of the cloning reaction above was added to each tube and left to incubate on ice for 30 mins. Cells were then heat shocked for 30 seconds at 42°C in a water-bath. The tubes containing the cells were then placed on ice for 2 mins and to each tube of cells, 200 µl of SOC medium (from the pcDNA 3.1 kit) was added. The tubes were then shaken for 1 hr at 220 rpm at 37°C in a shaking incubator. Fifty-100 µl of each reaction mixture was then plated out onto LB agar plates containing ampicillin and grown overnight at 37°C in an incubator for at least 18 hrs. Plates were removed the next day and placed in the fridge to prevent further colony growth. Colonies were then picked and added to a 96-well plate containing 100 µl of LB media per well and shaken at 220 rpm for approximately 3-4 hrs at 37°C and screening of the insert was confirmed through colony PCR using the PCR procedure below.

Nine µl of the PCR mastermix (see table 2.9) was added to each well on PCR plate and 1 µl of each inoculated mini-culture was added to the respective wells for the colony PCR. PCR

cycling conditions were as follow: one cycle of 94°C; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 4 mins, one cycle at 68°C for 20 mins, and finally on hold at 4°C.

Table 2.9. Mastermix for Colony PCR

Reagent	Volume (µl/reaction)
Sterile Water	7.6
10X Buffer	1
Magnesium chloride	0.7
dNTP (10mM)	0.2
Primer: T7 (20 pmol/µl)	0.2
Primer: EnvM (20 pmol/µl)	0.2
TaqPol	0.05
Final volume	9.95

All PCR products were visualized on a 1% agarose gel and those positive for the 3.1 kilobase inserts were then selected and inoculated overnight into 3 ml of LB medium containing ampicillin (220 rpm at 37°C), and thereafter grown in 100 ml of LB media in order to amplify the amount of plasmid DNA using the same conditions in the shaking incubator. The Qiagen Maxi-Prep Kit (Qiagen, Dusseldorf, Germany) was used according to the manufacturer's protocol in order to extract the plasmid DNA. The plasmid DNA was then subjected to restriction enzyme digest analysis in order to confirm the presence of the 3.1 kilobase Env insert as depicted in Table 2.10.

Table 2.10. Restriction Enzyme Digest using BamH1 and Xba1

Reagent	Volume (µl/reaction)
Sterile Water	11.6
Xba1 (New England Biolabs, Ipswich, USA)	0.7
BamH1 (New England Biolabs, Ipswich, USA)	0.7
10x Bovine Serum Albumin	2.0
New England Buffer 2 (New England Biolabs, Ipswich, USA)	2.0
Plasmid DNA (100 ng)	3
Final Volume	20

2.11 Functional Assays

2.11.1 Pseudovirus generation, Pseudovirus Titre and Neutralization assays

The production of molecularly cloned pseudoviruses in 293T cells by co-transfection with an *env*-expressing plasmid plus a backbone plasmid lacking *env* is necessary to carry out such assays. Co-transfection generates pseudoviruses that are able to infect cells but, due to the absence of a complete genome, are unable to produce infectious progeny virions. This single round of infection is readily detectable in genetically engineered cell lines that contain a Tat-responsive reporter gene, such as luciferase.

The TZM-bl cell line is a HeLa cell clone that is used in functional assays. This cell line was engineered to express CD4, CCR5 and CXCR4 and contains integrated reporter genes for firefly luciferase and E. coli β-galactosidase under control of an HIV-1 long terminal repeat (LTR) (Wei et al., 2002). TZM-bl cells therefore permit sensitive and accurate measurements of infection. The cells are highly permissive to infection by most strains of HIV-1, SIV and SHIV, including primary HIV-1 isolates and molecularly cloned pseudoviruses. DEAE dextran is used in the medium during neutralization assays to enhance infectivity. Luciferase activity is quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum.

2.11.2 Experimental Overview

The schematic below (figure 2.3) illustrates the chronology of the experimental methods used during the course of this study to achieve the study objectives.

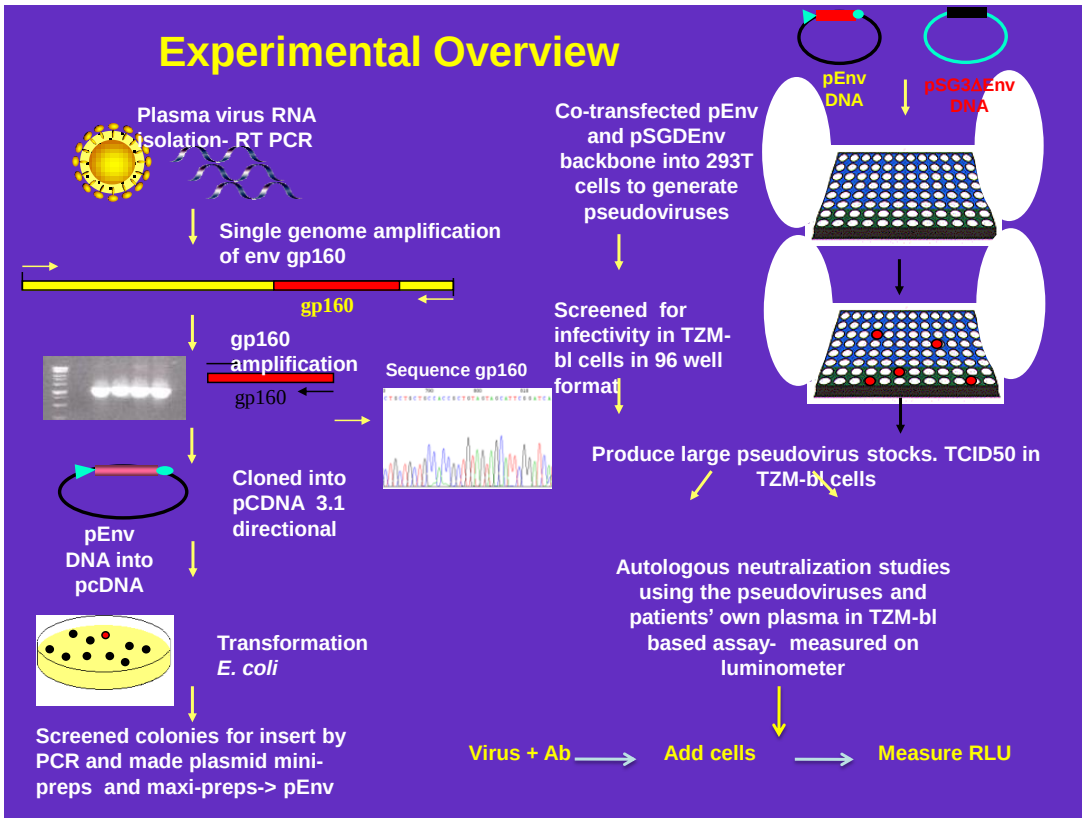

Figure 2.3. The experimental overview for the project spanning the initial virus isolation, the single genome sequencing analysis and subsequent directional cloning to the phenotypic assays for the measurement of neutralizing antibody responses using Env-pseudotyped viruses in a TZM-bl-based assay.

2.11.3 Pseudovirus Generation

In order to carry out neutralization assays, pseudoviruses were generated through co-transfection experiments. The Env-pseudotyped virus stocks were generated by co-transfecting 1,000 ng of the *env* encoding plasmid DNA with 1,500 ng of the HIV-1 genomic vector SG3Δ *env* into an 80% confluent monolayer of 293T cells in a T-25 culture flask in the presence of 7.5 µl of Fugene 6 Transfection Reagent (Roche Diagnostics, Indianapolis,

USA). The media was replaced 6–8 hrs after transfection; 48 hrs later, culture supernatant containing the pseudoviruses was harvested, and stored at -80°C. The tissue culture infectious doses ($TCID_{50}$) were quantified by infecting TZM-bl cells with serial four-fold dilutions of the supernatant in the presence of DEAE dextran- see below for detailed methodology.

2.11.4 Determining Viral Titer on TZM-bl Cells

TZM-bl cells were seeded into 24 well plates 24 hrs before viral titration. Each well contained 50,000 cells in 400 ul of 10% Fetal Bovine Serum (FBS- Gibco, Catalogue No. 10106-169) in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen, Carlsbad, USA) and 1% Gentamicin (Sigma-Aldrich, St Louis, USA). Seeded cells were left for 24 hrs in a 5% CO_2 incubator at 37°C. 1% FBS in DMEM was used to dilute DEAE-Dextran (Sigma-Aldrich, St Louis, USA) to make an 80 µg/ml solution and to dilute the virus stocks. Virus stocks were serially diluted in microtitre tubes according to the following algorithm.

a. Well A: 8 µl virus + 232 µl 1% FBS in DMEM
b. Well B: 40 µl from Well A + 200 µl 1% FBS in DMEM
c. Well C: 40 µl from Well B + 200 µl 1% FBS in DMEM
d. Well D: 40 µl from Well C + 200 µl 1% FBS in DMEM
e. Well E: 40 µl from Well D + 200 µl 1% FBS in DMEM
f. Well F: 40 µl from Well E + 200 µl 1% FBS in DMEM

TZM-bl 24-well plates were 30-40% confluent when viewed under the microscope. Media was removed from the seeded TZM-bl 24-well plates using the vacuum aspirator. DEAE-Dextran diluted (80 µg/ml) in 1% FBS in DMEM was added to each well first (150 µl) and

then 150 µl virus dilutions to the appropriate wells. Plates were briefly shaken back and forth and placed in 37°C incubator for 1-2 hrs. After 1-2 hr incubation, 0.5 ml 10% FBS in DMEM was added to each well. Plates were then incubated for 48 hrs in 37°C incubator. After 48 hrs, cells were viewed and found to be 100% confluent and thereafter stained using X-gal (see appendix for B-galactosidase staining) staining solution.

2.11.5 Staining Procedure

Media was removed from each well. To each well 400 µl/well of fixing solution was added and was incubated for 5 mins at RT. Fixing solution was then removed, and the plate was washed three times with PBS. Thereafter, 400 µl/well of staining solution was added per well and incubated at 37°C for 1-2 hrs. Contents of plate were discarded and the plate was washed twice with PBS. Infection of the TZM-bl cells was then scored according to the algorithm below in table 2.11. To score for infection, each well of the 24-well plate was divided into four quadrants. All blue cells were counted within view, once in each of the four quadrants of a well. Infectious Units per µl were computed as follows: [(# blue cells/4) x 67] / (µl virus added) = IU/µl. The average of each well should be similar for an accurate titration.

Table 2.11. Titration algorithm used for serial dilution of pseudoviruses

	A	B	C	D	E	F
Volume of virus (µl) added to the wells of a 24-well plate row	5	1	0.2	0.04	0.008	0.0016

Pseudovirus stocks with titers less than 40 IU/µl were discarded and the remaining stock tubes with corresponding titer and stored at -80°C.

2.12 Neutralization Assays for Autologous Antibody Assays

2.12.1 Day 1- Seeding TZM-Bl Cells into Flat-Bottomed 96-Well Plates

Cells were seeded into 96 well-flat bottomed plates. Each well contained 100 µl of cells with an average count of 8,000 cells/100 µl of 10% FBS-DMEM and the plates were incubated at 37°C for 24 hrs.

2.12.2 Day 2- Infecting TZM-Bl Cells with Pseudoviruses

Cells were 30-40% confluent at the time of infection. Pseudoviruses and test plasmas were removed from the -80°C freezer simultaneously, and thawed out at RT. Any plasma should have been previously heat-inactivated by the following protocol:

A volume of 0.5 ml of plasma was inactivated in a water bath at 56°C for 30 mins. The plasma was centrifuged at 3,000 x g for 5 mins at RT. The plasma was then transferred to another tube, without disturbing the pellet, and ~0.4 ml of plasma supernatant was aliquotted per cryogenic vial.

2.12.3 Plasma Preparation

The final, total volume of plasma reagent was determined using the following equation: number of (#) pseudoviruses x 120 µl x 1.4 = volume of plasma needed. Plasma in the first tube constituted 10% of the total volume. Serial dilutions were made by multi-channeling (150 µl serial transfers) down five tubes of 6% DMEM to achieve a final dilution of 1:100, 000. No plasma was added to the last tube which contained 6% DMEM only in this step and was later mixed with pseudovirus to create the virus only control. A control row of six tubes was made that contained a 1:1 ratio of 6% DMEM to a solution of DEAE Dextran (80 µg/ml)

in 1% DMEM only. This was used to determine what the average baseline luciferase ("background") reading is, which will, in analysis, be subtracted from the other assay wells, this tube represents the negative control.

Figure 2.4 below illustrates the schema used for autologous neutralizing antibody assays using the study entry and study exit plasma nAb samples to test for neutralization to the study entry and exit Envs. This type of matrix yields both a contemporaneous response and an evolutionary response where escape from neutralization or resistance is often observed.

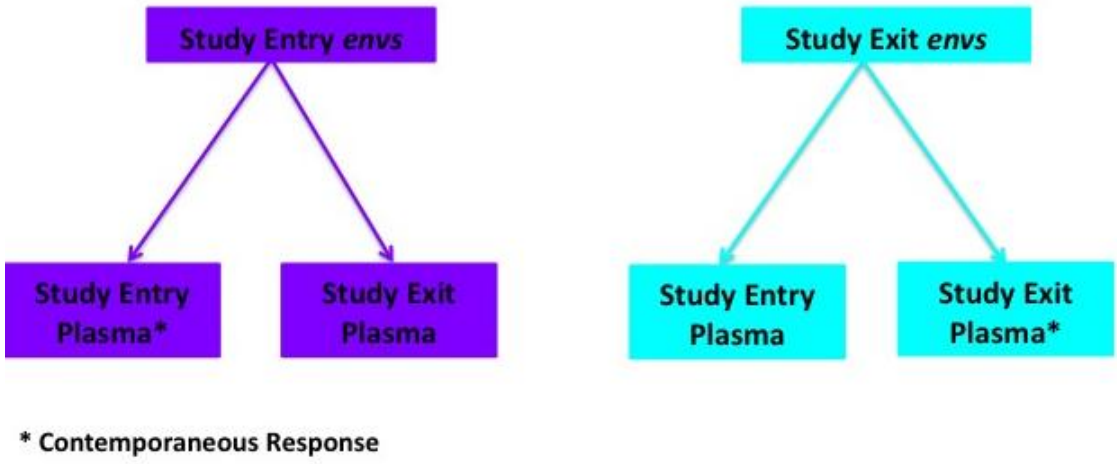

Figure 2.4. Schematic illustrating the autologous neutralizing antibody assays in all participants. The study entry Envs were tested for neutralization by nAbs from the study entry and study exit plasma samples. Likewise the study exit Envs were tested for neutralization by nAbs from the study entry and study exit plasma samples.

2.12.4 Pseudovirus Preparation

A volume of 952.32 µl 1% DMEM was pipetted into the first microtiter tube for each pseudovirus test. The appropriate amount of virus was added to achieve the desired 2000IU/100 µl. (The volume of the tube was equivalent to 952.32 µl). The first tube only contained 6.7 µl of 10mg/ml DEAE-dextran and was mixed well. Thereafter 140 µl of the

pseudovirus preparation was aliquotted to each of five microtiter tubes. A volume of 120 µl of the plasma reagent and then 120 µl pseudovirus was added to the wells of a round-bottomed 96-well plate and incubated at 37°C for 1 hr as illustrated in figure 2.5A.

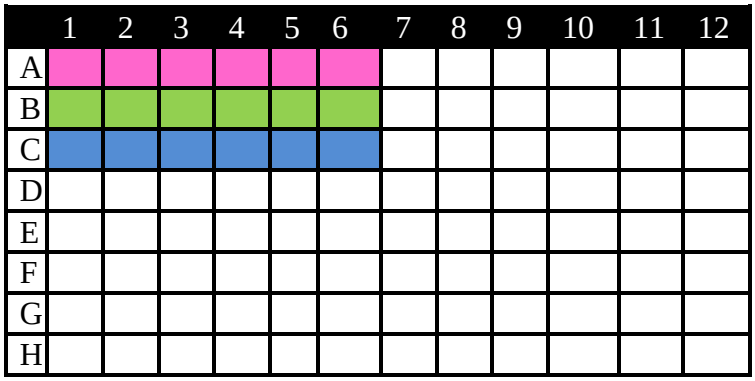

Figure 2.5A. 96-well round-bottomed plate illustrating the plasma and virus incubation for three different samples

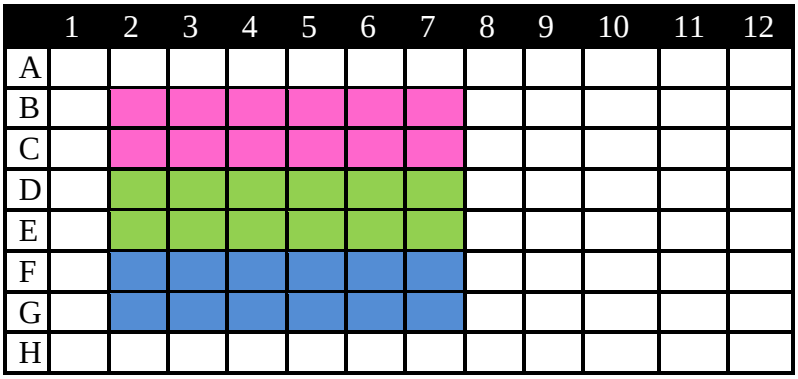

Figure 2.5B. TZM-bl –seeded 96-well flat-bottomed plate illustrating the plasma and virus incubation with pre-seeded TZM-bl cells for three different samples.

The media (~100 µl) only from the wells of TZM-bl cells that were to be infected were aspirated off. The media from outside rows or columns were not removed in order to prevent evaporation. The middle section of the plate was only used for infection (See schematic - figure 2.5B).

Thereafter, 100µl of pre-incubated pseudovirus/plasma was added in duplicate to all intended cells and was incubated for 48 hrs at 37°C.

2.12.5 Day 4- Terminating Assay And Reading Luciferase On The Luminometer

After 48 hrs, the media or supernatant from the infected wells were aspirated, one plate at a time. Lysis buffer (75 µl) was added to each well. Each plate was wrapped with tape to prevent the lid from slipping off during the freeze/thaw process. Plates were frozen at -80°C for 30 min (until opaque) and then shaken at RT for 30 mins (until clear). This freeze/thaw cycle was repeated for a total of two freeze/thaw cycles. Luciferase reagent was reconstituted according to the manufacturer's protocol and kept in the dark until use. A volume of 20µl of lysed cells was added to each of the 96 wells on a white luminometer 96-well plate. To this 100µl/well of luciferase assay reagent was added, and measured on the luminometer. Data was exported to Microsoft Excel and was then analyzed.

2.13 Neutralizing Antibody Assay Protocol for Heterologous Responses

2.13.1 Envelope Clones

Envelope genes used in this study were either cloned as previously described or obtained from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP). Briefly, the single genome amplification derived *env* amplicons were directionally T/A cloned into the CMV-driven expression plasmid pcDNA3.1-V5 HisTOPO-TA and screened for biological function as pseudoviruses following co-transfection with an *env*-deficient subtype B proviral plasmid (SG3ΔEnv) into 293T cells as described previously (Derdeyn et al., 2004). A total of 20 standard reference envelope clones were used to test for heterologous neutralization.

These included eight subtype C, seven subtype B and five subtype A envelope pseudoviruses as depicted in table 2.12. Tier 1, Tier 2 and Tier 3 viruses were stratified previously based on clustering analysis of sensitivity patterns- with Tier 1 being the most sensitive, Tier 2 displaying moderate to low sensitivity and Tier 3 displaying the lowest sensitivity to neutralization (Seaman et al., 2010). The ConC plasmid carrying a consensus of all the HIV-1 subtype C sequences from the Los Alamos database by 2001 (Kothe et al., 2006) and the envelope plasmids containing single point mutations (as described in Gray et al. (2011), were obtained from Lynn Morris. Table 2.12. depicts the envelope clones used, the subtype of the clone, the tiered categorization and the references for the viral isolates.

Table 2.12. HIV-1 Env pseudovirus panel of subtype C, B and A reference strains

HIV-1 Isolate	Subtype C, B or A	Tiered Category	Reference
MW965.25	C	1	NIH ARRRP
ZM197 M.PB7	C	1	(Li et al., 2006b)
ConC	C	2	(Kothe et al., 2006)
DU156.12	C	2	(Li et al., 2006b)
DU172.17	C	2	(Li et al., 2006b)
ZM214 M.PL15	C	2	(Li et al., 2006b)
CAP45.G3	C	2	(Li et al., 2006b)
CAP239.G3	C	2	(Gray et al., 2007)
SF162.LS	B	1	(Stamatatos and Cheng-Mayer, 1998)
6535.3	B	1	(Li et al., 2005)
AC10.0.29	B	2	(Li et al., 2005)
QHO692.42	B	2	(Li et al., 2005)
WITO 4160.33	B	2	(Li et al., 2005)
TRO.11	B	2	(Li et al., 2005)
PVO.4	B	3	(Li et al., 2005)
Q23ENV17	A	2	(Blish et al., 2007)
Q842ENVd12	A	2	(Blish et al., 2007)
Q168ENVa2	A	2	(Blish et al., 2007)
Q461ENVe2	A	2	(Blish et al., 2007)
Q769ENVd22	A	2	(Blish et al., 2007)

Using the format of a 96-well flat-bottom culture plate as illustrated in Figure 2.6; 150 µl of complete growth medium (GM- see Appendix on the constituents of growth medium) was

added in all wells of column one (cell control). For columns two to twelve 100 µl was added in all wells (column two - virus control). Depending on dilution of test serum or plasma sample (see standard dilution algorithm- below in Tables 2.13 and 2.14): an additional amount of growth medium (GM) was added to all wells of columns three to twelve, row-H so that for example if your starting dilution of plasma was 1:45, the volume of test plasma added was 5 µl and the corresponding total amount of GM was 45µl.

This format was designed to assay five samples in duplicate at each serum dilution (Figure 2.6. Template A). Adjustments may be made to test a larger number of samples per plate (Ten samples, Figure 2.6. Template B).

A positive control with a known neutralization titer against the target virus was included on at least one plate in series each time assays were performed. Also, at least one negative control sample was used. The required number of vials of virus was thawed by placing them in an ambient temperature water bath. When the viruses were completely thawed, the virus was diluted in GM to achieve a concentration of 4,000 $TCID_{50}$/ml.

To each well in columns three to four, row H only, 5 µl of test plasma sample was added. Serial dilutions of the test plasma was done by pipetting 50 µl of this dilution to row G, and this process was repeated until eight serial dilutions were done to achieve a final dilution of 1:98415. To all wells in columns two to twelve, rows A through H, 50 µl of cell-free virus was dispensed. and mixed by pipette action after each transfer. Pipette tips were rinsed in a reagent reservoir containing sterile PBS between each transfer to avoid carry-over. The plate was covered and incubated for 1 hr, at 37°C.

A suspension of TZM-bl cells at a density of 10,000 cells/ml in GM containing DEAE dextran was prepared. Thereafter, 100 µl of cell suspension was dispensed (10,000 cells per well) to each well in columns one to eleven, rows A though H. Rinse pipette tips in a reagent

reservoir containing sterile PBS between each transfer to avoid carry-over. Plates were covered and incubated for 48 hrs, at 37°C.

From each well, 150 µl of culture medium was removed and 100 µl of Bright Glo™ Reagent was then dispensed to each well and incubated at RT for 2 mins to allow complete cell lysis and was mixed by pipetting action (two strokes) and 150 µl of this mixture was then transferred to a corresponding 96-well black plate. The plate was read immediately in a luminometer.

Percent neutralization was determined by calculating the difference in average relative luminescence units (RLU) between test wells (cells + serum sample + virus) and cell control wells (cells only, column one), and dividing this result by the difference in average RLU between virus control (cell + virus, column two) and cell control wells (column one), subtracting from one and multiplying by 100.

nAb IC_{50} or nAb ID_{50} was defined as the neutralizing antibody titers that were expressed as the reciprocal of the serum dilution required to inhibit 50% virus inhibition (or reduce the RLU by 50%). Each experiment was performed independently at least twice with duplicate wells.

2.13.2 Analyzing Results

Criteria defining the set limits of the pseudovirion assay included: Cell control (CC) to be greater than 100 and less than 1,000 RLU (>100 CC < 1,000 RLU). Virus control (VC) was ten times greater than the cell control (CC). If these criteria are not met, the assay was regarded as a failure.

Template A

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8
B	CC	VC	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7
C	CC	VC	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6
D	CC	VC	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
			Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	

Template B

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
B	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
C	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
D	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
			Sample 1&2		Sample 3&4		Sample 5&6		Sample 7&8		Sample 9&10	

Figure 2.6. Template for measuring the titer of nAbs. Template A is for five samples per plate. Template B is for ten samples per plate. CC, cell control wells (cells only); VC, virus control wells (virus and cells but no serum) (adapted from Montefiori, 2004).

Standard Dilution Algorithm

Table 2.13. Standard dilution algorithm for two-fold dilutions

Desired Start Dilution	GM Volume (µl)	Sample Volume (µl)
1:5	40	60
1:10	70	30
1:15	80	20
1:20	85	15
1:25	90	12
1:30	90	10
1:50	95	6

Growth medium (100 µl) was added to all wells of columns three to eleven. Then an extra amount of growth medium listed above was added, and then the desired sample volume was added to the first three wells. Thereafter two-fold dilutions were performed (i.e., serial transfers of 100 µl).

Table 2.14. Standard dilution algorithm for three-fold dilutions

Desired Start Dilution	GM Volume (µl)	Sample Volume (µl)
1:5	5	45
1:8	25	28
1:10	30	22
1:15	35	15
1:20	40	11
1:24	50	10
1:45	45	5

Growth medium (100 µl) was added to all wells of columns three to eleven. Then an extra amount of growth medium listed above was added, and then the desired sample volume was added to the first three wells. Thereafter three-fold dilutions were performed (i.e., serial transfers of 100 µl) (adapted from Montefiori, 2004).

2.14 HIV-1 Antigen Specific Non-Neutralizing Antibody Binding Affinities

Total immunoglobulins (IgGs) were isolated from plasma samples of slow progressors and progressor at study entry and study exit time points. Using an ELISA based assay, Fc portion of the IgGs were tested for their ability to bind to various activating and inhibitory (Fcγ) receptors in order to use this as a surrogate marker of potential antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody dependent cell-mediated viral inhibition (ADCVI) activity. The activating receptors assayed for binding affinities were FcγRI, FcγRIIa, FcγRIIIa and the inhibitory receptor was FcγRIIb. In addition, the binding affinities of the IgGs to specific HIV-1 antigens were also determined. The rationale for this substudy was to determine whether there were differences in binding affinities of the IgGs in divergent states of disease progression.

2.14.1 IgG Isolation

2.14.1.1 Column Preparation:

Melon Gel (Thermo Scientific, Surrey, United Kingdom) (500 µl) was added onto the Pierce column. The column was spun for 1 min at 5,000 x g and thereafter 300 µl of buffer was added and washed through the column twice. Columns were spun for 30 seconds at 5,000 xg.

2.14.1.2 Plasma IgG Isolation

Test plasma/serum was diluted and 500 µl was added to the column that was capped at the bottom to prevent the eluent from leaking (100 µl of plasma /serum plus 900 µl buffer). The tubes were placed in a hula-mixer and rotated for 5 mins in order to ensure maximum capture of the IgGs. The tubes were then placed in clean eppendorf tubes and spun down for 1 min at 5,000 x g (without the cap) to elute the IgG's. The remaining 500 µl of diluted serum was

added to the same spin column and the cap was replaced onto the column. The tubes were placed in a hula-mixer and again rotated for 5 mins. The tubes were then spun down for 1 min at 5,000 x g (without the cap) to elute the IgGs from the column. IgG concentrations were measured on the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Surrey, United Kingdom) and stored at 4^0C for downstream ELISA assays.

2.15 Fcgamma (Fcγ) ELISA Protocol

2.15.1 Fcγ Receptors

Four different Fc gamma (γ) receptors were used: FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa (R & D Systems, Minneapolis, USA) and the ELISAs for each of the receptors were performed using a standard protocol. FcγR (50 µg) was dissolved in 1 ml PBS for a 50 µg/ml solution.

2.15.2 FcGamma (γ) Receptor ELISA

The diluted Fcγ receptor (FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa) was added to nickel coated plates Ni-NTA HisSorb™ Plates (Qiagen, Dusseldorf, Germany) at a concentration of 5µg/ml using PBS (100 µl/well). One receptor per plate was added and incubated for 1 hr at RT, then overnight at 4°C. The plate was washed three times with PBS-Tween (0.05%) and blocked with 5% PBS-Bovine Serum Albumin (BSA) (Sigma-Aldrich, St Louis, USA), 250 µl/well and was incubated for 1 hr at RT. The plate was washed three times 0.05% PBS-Tween and 100 µl of antibodies were added (the IgG's derived from the test plasma was diluted serially starting at 100ng/µl diluted down to 3.12 ng/µl) to the respective wells and the plate was left to incubate for 1hr at RT. The plate was washed three times 0.05% PBS-Tween. Anti-human IgG Antibody, Fab fragment, peroxidase labeled (100µl per well was

added (KPL Protein Research Products, Maryland, USA). (For the HRP Fab 20 µl was added to 10 mls PBS for a final concentration of 1 mg/ml for one plate). The plate was incubated for 1 hr at RT and covered in tinfoil to protect it from light. The plate was washed three times with 0.05% PBS-Tween. The colour was resolved with o-phenylenediamine (OPD) (Sigma-Aldrich, St Louis, USA) by adding one OPD tablet to 11 mls of StrepHRP substrate, 100 µl/well was added. The reactions were stopped by adding 100 µl/well of 2.5 N sulphuric acid (Sigma-Aldrich, St Louis, USA). The plate was read at 490 nm on an ELISA plate reader.

2.15.3 Gp120, gp41, and p24 ELISAs

ELISA plates were coated with 80µl/well of a 250 ng/ml gp120-(YU2) or gp41 (HXBc2) or p24-(HIV-1/HXBc2) (Immune Technology, New Jersey, USA) and were left to incubate overnight at 4°C. All antigens were diluted with PBS. The plates were washed three times with PBS Tween 0.05%. The plates were blocked with 5% PBS-BSA for 2 hrs at RT (100µl/well). IgGs isolated previously were diluted in PBS and added to respective wells according to a serial dilution algorithm. IgG's isolated previously was added at log serial dilutions to respective wells and left to incubate for 2 hrs at RT at a starting concentration of 1 mg/ml. The plates were washed three times with PBS Tween 0.05% and 100 µl anti-human IgG Antibody, Fab fragment, peroxidase labeled was added to each well (KPL Protein Research Products, Maryland, USA) and left for 1 hr at RT (1:500 diluted-per plate: 20 µl HRP in 10 ml PBS was made up) and cover in tinfoil. The plate was washed three times 0.05% PBS-Tween. One OPD tablet was added to 11 mls of phosphate citrate buffer and to this 4.4 µl hydrogen peroxide (H_2O_2) was added and 50 µl/well of this solution was added to each well to develop the colour. The reaction was stopped by adding 50 µl/well of 2.5 N

H_2SO_4 after 15 min. The plate was read at 490nm on a microplate reader and the data was analyzed using GraphPad Prism 5 software programme.

2.16 Statistical Analyses

Pairwise comparisons of different parameters including genetic diversity, PNGs and length polymorphism, and autologous and heterologous nAb responses between subjects in the two groups were calculated by the Mann-Whitney non-parametric test and correlations performed using Spearman's non-parametric rank test implemented in the GraphPad Prism 5 software programme unless otherwise stated. Correlations were regarded as statistically significant with a p-value ≤ 0.05. All reported p-values are for two-sided tests.

Chapter Three – Results

3.0 HIV-1 Subtype C Envelope Characteristics Associated with Divergent Rates of Chronic Disease Progression

Chapter Three is a reprint of published work: **Derseree Archary**, Michelle L. Gordon, Taryn N. Green, Hoosen M. Coovadia, Philip J.R. Goulder and Thumbi Ndung'u. HIV-1 subtype C envelope characteristics associated with divergent rates of chronic disease progression. *Retrovirology* 2010 Nov 4;7(1):92.

3.1 Abstract

Background: HIV-1 envelope diversity remains a significant challenge for the development of an efficacious vaccine. The evolutionary forces that shape the diversity of envelope are incompletely understood. HIV-1 subtype C envelope in particular shows significant differences and unique characteristics compared to its subtype B counterpart. Here we applied the single genome sequencing strategy of plasma derived virus from a cohort of therapy naïve chronically infected individuals in order to study diversity, divergence patterns and envelope characteristics across the entire HIV-1 subtype C gp160 in four slow progressors and four progressors over a median of 21 months.

Results: Sequence analysis indicated that intra-patient nucleotide diversity within the entire envelope was higher in slow progressors, but did not reach statistical significance (p=0.07). However, intra-patient nucleotide diversity was significantly higher in slow progressors compared to progressors in the C2 (p=0.0006), V3 (p=0.01) and C3 (p=0.005) regions. Increased amino acid length and fewer potential N-linked glycosylation sites (PNGs) were observed in the V1-V4 in slow progressors compared to progressors (p=0.009 and p=0.02 respectively). Similarly, gp41 in the progressors was significantly longer and had fewer

PNGs compared to slow progressors (p=0.02 and p=0.02 respectively). Positive selection hotspots mapped mainly to V1, C3, V4, C4 and gp41 in slow progressors, whereas hotspots mapped mainly to gp41 in progressors. Signature consensus sequence differences between the groups occurred mainly in gp41.

Conclusions: These data suggest that separate regions of envelope are under differential selective forces, and that envelope evolution differs based on disease course. Differences between slow progressors and progressors may reflect differences in immunological pressure and immune evasion mechanisms. These data also indicate that the pattern of envelope evolution is an important correlate of disease progression in chronic HIV-1 subtype C infection.

3.2 Background

The rate of disease progression in HIV-1 infected individuals is determined by a complex interplay of viral characteristics, host genetic factors, immune responses and environmental factors. The high viral replication rate, the lack of proof-reading mechanism by the HIV-1 reverse transcriptase enzyme, and high recombination rate are characteristics that ensure that the virus continuously mutates and evolves, resulting in both HIV-1 diversification and viral escape from host immune responses (Preston et al., 1988, Mansky and Temin, 1995). Viral diversity and the constant generation of new viral quasispecies that may not be recognized or eliminated by the host immune mechanisms, particularly contemporaneous virus-specific cytotoxic CD8 T-cells or nAbs, are major impediments for the development of an efficacious HIV-1 vaccine (Brander et al., 2006, Walker and Burton, 2008).

The HIV-1 envelope (Env) subunits gp120 and gp41 are the only viral proteins that are exposed on the virus surface, and they are under continuous host selective pressure, as they are key determinants of the target host cell range and are important targets of nAbs and CD8 T-cell responses. Specific Env sequence characteristics such as the overall amino acid diversity, the number of putative N-linked glycosylation sites (PNGs), and the length of variable loops have been shown to influence or correlate with antibody neutralization sensitivity, cell tropism, co-receptor utilization and virus transmission (Resch et al., 2001, Wei et al., 2003, Rademeyer et al., 2007). Studies of Env diversity can also provide important clues for selective forces that may significantly influence the rate of disease progression or alternatively identify specific regions of the Env protein that comprise important targets of effective immune pressure which may be important considerations in rational HIV-1 vaccine design.

In HIV-1 subtype B, the relationship between HIV-1 Env diversity and disease progression is complex, as illustrated by a series of studies. In one early study, HIV-1 Env hypervariable region 3 (V3 loop) diversity was shown to increase with time (Nowak et al., 1991). A subsequent study showed that Env hypervariable regions 3 to 5 (V3 to V5) diversity was directly associated with duration of patient survival, positive selection for change, and inversely correlated with the rate of disease progression as measured by the slope of CD4+ T cell loss (Wolinsky et al., 1996). Another study that examined Env C2-V5 sequences in men followed for 6 to 12 years following seroconversion demonstrated a complex pattern of viral diversity characterized by an early phase of linear increases in divergence and diversity, followed by an intermediate phase with increase in divergence but stabilization or decline of diversity, and a final phase showing stabilization or reduction in divergence and continued stability or decline in diversity (Shankarappa et al., 1999). In another study, analysis of C2-V5 Env sequences among typical progressors versus slow progressors showed that the typical progressors exhibited higher diversity, lower intra- and inter-sample divergence, evidence of lower host selective pressure and increases in both synonymous and non-synonymous substitutions over time while only non-synonymous substitutions increased in slow progressors (Bagnarelli et al., 1999).

The aforementioned studies and a comprehensive body of similar studies on HIV-1 diversity, divergence, and host selective forces that may impact on disease progression have been performed on HIV-1 subtype B (Shankarappa et al., 1999, Delwart et al., 2002, Learn et al., 2002, Freel et al., 2003, Ritola et al., 2004, Frost et al., 2005a, Gottlieb et al., 2008, Keele et al., 2008). Furthermore, these studies clearly demonstrate that patterns of Env diversity, divergence, and associated selective pressures identified can differ according to the stage of disease, the sampling methodology, the region of Env analyzed, the founder virus, and the host genetic background.

HIV-1 subtype C is the most rapidly spreading subtype worldwide (Rong et al., 2007b, Coetzer et al., 2007), and an effective global vaccine will have to show efficacy against this subtype. A number of studies have explored Env diversity and diversification within HIV-1 subtype C (Tscherning et al., 1998, Ping et al., 1999) but data on this subtype remain relatively limited, despite accumulating evidence that this subtype may differ significantly from HIV-1 subtype B in certain biological properties mediated by the *env* gene (Tscherning et al., 1998, Ping et al., 1999, Ball et al., 2003, Abraha et al., 2009, Rong et al., 2009). In particular, possible differences in Env diversity, divergence, and selective pressures between HIV-1 subtype C-infected individuals with divergent rates of disease progression remain understudied.

In this study, we used single genome amplification and sequencing to explore the evolution of the Env gp160 protein. Specifically, we investigated differences in diversity and divergence in 4 slow progressors and 4 progressors of black African descent infected with HIV-1 subtype C. Further, we investigated differences in Env features such as the extent of putative N-linked glycosylation, lengths of the variable and constant regions of gp160, and positive selection in slow-progressors and progressors in order to assess the correlation of these variables with rates of disease progression.

3.3 Materials and Methods

3.3.1 Participants

Participant samples were retrospectively identified from the Sinikithemba cohort, which is a prospective natural history study of HIV-1 infected individuals based at McCord Hospital, Durban, South Africa as previously reported (Kiepiela et al., 2004). Ethics approval was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee and all participants gave written informed consent to participate in the study. CD4 T-cell counts were performed at three month intervals whereas viral loads were done at six month intervals.

For this substudy, CD4 T-cell count over two years was chosen as the primary determinant of disease progression for stratification into slow progressor and progressor categories. Both slow progressors and progressors were selected on the basis of a CD4 T-cell counts >500 cells/μl at study entry time point. However, at study exit, slow progressors maintained a CD4 count above 500 cells/μl or a viral load less than 10,000 viral RNA copies/ml. In contrast, progressors declined in CD4 T-cell counts to below 500 cells/μl and had a viral load above 10,000 copies/ml. The overall median time was 21 months between study entry and study exit sampling. All individuals were antiretroviral therapy naive before and during the window of evaluation. When the virological and immunological data became available beyond the study window (follow-up of an average of 39.8 months for slow progressors and 36.8 months for progressors, we analyzed these parameters relative to the study entry criteria and they remain statistically different for the progressors only ($p=0.03$ for both CD4 T-cell count and viral load).

3.3.2 Sample Collection, CD4 T-Cell Counts and Plasma Viral Load

Blood was drawn from each subject into EDTA tubes and plasma was separated by centrifugation and stored at −80°C until use. Viral load was measured using the Amplicor Version 1.5 assay (Roche Diagnostics, Indianapolis, USA). CD4+ T-cell counts were enumerated by Trucount technology on a four colour FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

3.4.3 cDNA Synthesis and Single Genome Amplification

HIV-1 RNA extraction, cDNA synthesis, and single genome amplification were performed as previously reported with some modifications (Salazar-Gonzalez et al., 2008) (see detailed methodology in Chapter Two). Briefly, primers were designed for the efficient amplification of HIV-1 subtype C envelope through nested PCR. For the first round PCR, the external primers used were VIF1: 5'– GGGTTTATTACAGGGACAGCAGAG-3' (HXB2 positions 4900-4923) and OFM19: 5'– GCACTCAAGGCAAGCTTTATTGAGGCTTA-3' (HXB2 positions 9604-9632). Primers for the second round PCR reaction were ENV A: 5'-GCTTAGGCATCTCCTATGGCAGGAAGAA-3' (HXB2 positions 5954-5982) and ENV N: 5'– CTGCCAATCAGGGAAGTAGCCTTGTGT-3' (HXB2 positions 9145-9171) (Salazar-Gonzalez et al., 2008). Cycling conditions for first round PCR were as follows: 94°C for 4 min, 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 68°C 4 min, and final extension of 68°C for 20 min followed by hold at 4°C. Second round PCR conditions were as follows: 94°C for 2 min, 45 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 4 min; final extension at 68°C for 20 min and 4°C hold. PCR products were visualized on a 1% agarose gel and amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Dusseldorf, Germany).

3.3.4 Sequencing Analysis of Gp160

The full-length envelopes were sequenced in the forward and reverse directions using the ABI Prism Big Dye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA)- see detailed methodology in Chapter Two.

3.3.5 Statistical Analyses

Pairwise comparisons of different parameters including genetic diversity, PNGs, and length polymorphism between subjects in the two groups were calculated by the Mann-Whitney non-parametric test using the GraphPad Prism 5 software programme unless otherwise stated. Correlations were regarded as statistically significant with a p-value <0.05. All reported p-values are for two-sided tests.

3.4.6 Genebank Accession Numbers

Sequences have been assigned the following GenBank accession numbers: GU216702-GU216737 and GU216739-GU216847.

3.4 Results

3.4.1 Study Participant Characteristics

There were eight participants in this study, seven female and one male. Table 3.1 below depicts the demographic profile of the participants for this retrospective study. All participants were of African descent. All participants were antiretroviral treatment naïve for the period of evaluation that was a median of 21 months. There were seven females and one

male in the study. The participants were stratified into two groups, slow progressors and proressors based on their immunological (CD4 T-cell count) and virological (viral loads) over a median period of 21 months and beyond the window of evaluation. Based on sample availability, the average period of follow-up between study entry and study exit samples for slow progressors and progressors were 21 and 18 months respectively. CD4 T-cells were measured three-monthly and viral loads six-monthly. The average age of the participants was 34 years old (range: 22-59 years). The average age for the slow progessors was 38 years (range 30-59 years) and for the progressors was 31 years (range: 22-40 years).

At study entry, both progressors and slow progressors did not differ in their CD4 T-cell counts (medians of 621 cells/µl versus 571 cells/µl (p=0.39) as shown in figure 3.1. However, at study exit the median CD4 T-cell count of slow progressors was 506 cells/µl, which is not significantly different from the CD4 count at study entry (p=0.7), while the progressors' median CD4 T-cell count had significantly declined to 283 cells/µl, (p=0.03).

Table 3.1. Patient demographics and virological and immunological characteristics

Patient Identification	**Sex**	**Age at Baseline**	**Viral Load at study entry (copies/ml)**	**Viral Load at study exit (copies/ml)**	**CD4 count at study entry (cells/ul)**	**CD4 level at study exit (cells/ul)**
SK035 [SP]	F	31	5800	2950	680	**322**
SK036 [SP]	M	32	5100	10600	936	**575**
SK169 [SP]	F	30	2210	2440	561	**437**
SK312 [SP]	F	59	3460	3630	545	**881**
SK010 [P]	F	31	6480	345000	649	**268**
SK200 [P]	F	40	14360	24900	595	**416**
SK221 [P]	F	30	9740	23700	503	**297**
SK233 [P]	F	22	11800	18900	547	**218**

[SP]= Slow progressor [P]= Progressor

Slow progressors also had no significant difference for viral load (p=1.0) between study entry and exit time-points, whereas progressor participants had significantly lower viral load (p=0.03) at study entry compared to exit time-point. In addition, CD4 T-cell (figure 3.1) and viral load were statistically different for progressors only at the latest available time-point compared to study entry (p=0.03 for both parameters). When slow progressors were compared to progressors, the analysis yielded significant differences when the CD4 T-cell counts at study exit and last available time-points - as shown in figure 3.1 (p=0.04 and p=0.02 respectively). Likewise viral load between the groups was significantly different for study exit and the latest available time-point (p=0.03 and p=0.02) respectively.

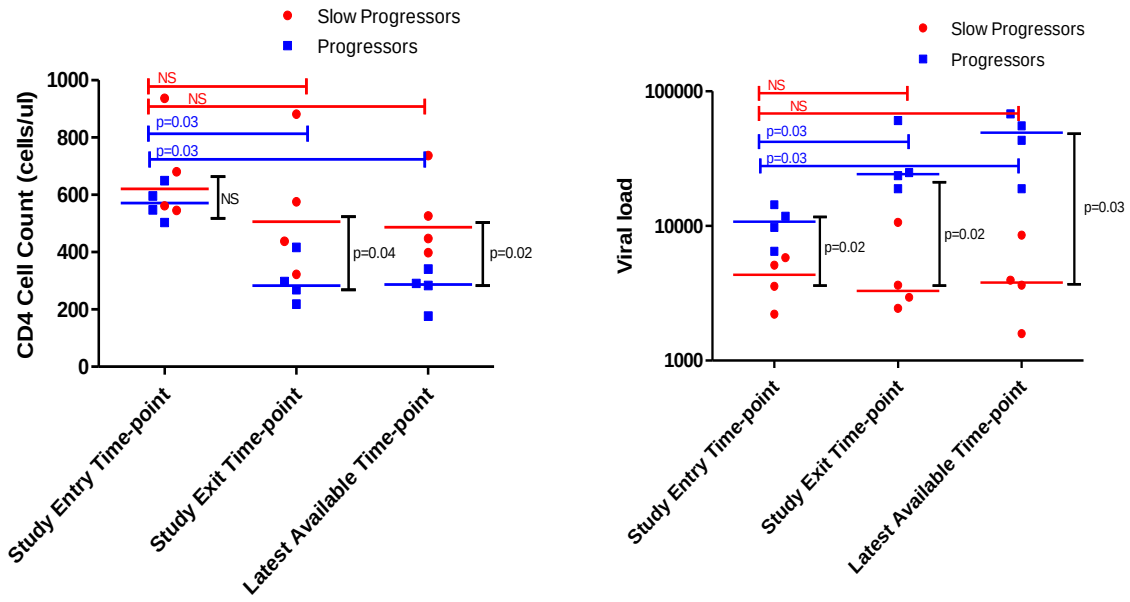

Figure 3.1. CD4 T-cell counts and viral loads of study entry, exit and latest available time point data for slow progressors and progressors. The red circles depict the data points for the slow-progressors. The blue squares depict data points for the progressors. Red bars and blue bars represent the p-values for the slow progressors and progressors respectively. Black bars represent p-values for inter-group comparison for the different time-points. NS = not significant. All comparisons between the study entry exit and latest available time-point parameters were performed using the Mann-Whitney unpaired t test, and p-values are shown. Differences were regarded as statistically significant with a p-value <0.05.

Furthermore, we used BEAST to estimate the approximate time of infection in both groups of participants. Slow progressors were estimated to be infected for a mean period of 8.2 years (range 4.75-15 years) compared with 2 years (range 0.75-3.75 years) for progressors.

Next, single genome sequence analysis was undertaken to delineate the differences in the Env genotype between the two groups. Phylogenetic analysis is shown in the figure 3.2.

3.4.2 Phylogenetic Relationships

To analyze phylogenetic relationships and changes in envelope sequences in slow progressors and progressors over a median period of 21 months, a mean of nine single genome full-length gp160 amplicons per participant per timepoint (range four to eleven amplicons) for the study entry and exit time-point were analyzed, for a total of 146 sequences. One of the slow progressors (SK312) had a few putative functional Env amplicons that were included in the final analysis when compared to the other study participants. This was due to a low number of SGA-derived clones that was limited by the low viral load and plasma sample availability. All participants' consensus sequences bootstrapped confidently with subtype C reference strains, as determined by a Maximum Likelihood tree for each patient at each time point (figure 3.2A). As expected, consensus sequences from the study entry and study exit for each patient formed monophyletic groups.

Overall, there were no distinguishing phylogenetic patterns noted between sequences from the slow progressors and progressors (figure 3.2A). Slow progressors showed a more diverse pattern characterized by either separate (sub)clusters at study entry and exit (figure 3.2B - SK035) or intermingling of sequences from early and exit time points (figure 3.2E - SK312). Additionally, phylogenetic clusters at study exit typically showed similar (figure 3.2C -

SK036) or longer branch length (figure 3.2D, example subject - SK169), compared with that of the study entry sequences. However, individual participant sequence trees for the progressors tended to show segregation between entry and exit time-point sequences (Figures 3.2F-3.2I).

3.4.3 Intra-patient Diversity Analysis

Intra-patient diversity, defined as the mean pair-wise nucleotide distance, was calculated by measuring distances between all sequences from a single individual at a single time-point, and is shown alongside the phylogenetic trees (figures 3.2B - 3.2I). Mean overall intra-patient diversity was 2.75% for the four slow progressors and 2.21% for the four progressors (p=0.07). The mean baseline intra-patient nucleotide diversity for the slow progressors was 2.63% (range 1.8-3.3%) and 1.42% (range 1.0-2.0%) for the progressors, but this did not reach statistical significance (p= 0.08). Study exit time point mean intra-patient diversity was 2.88% (range 1.9-4.2%) and 3.0% (range 1.0-7.4%) for slow progressors and progressors, respectively, which was not a significant difference (p-value = 0.56). Collectively, these data show that in this cohort, slow progressors trended to higher intra-patient sequence diversity compared to progressors although the differences did not reach statistical significance.

3.4.4 Nucleotide Substitution Rates in Study Entry and Exit in Slow Progressors and Progressors

To examine the evolution of the envelope gene over the study period, we calculated the rate of nucleotide divergence for each patient's *env* sequences. On average the nucleotide substitution rate was higher in the progressors (1.2×10^{-2} nucleotide substitutions/site/year;

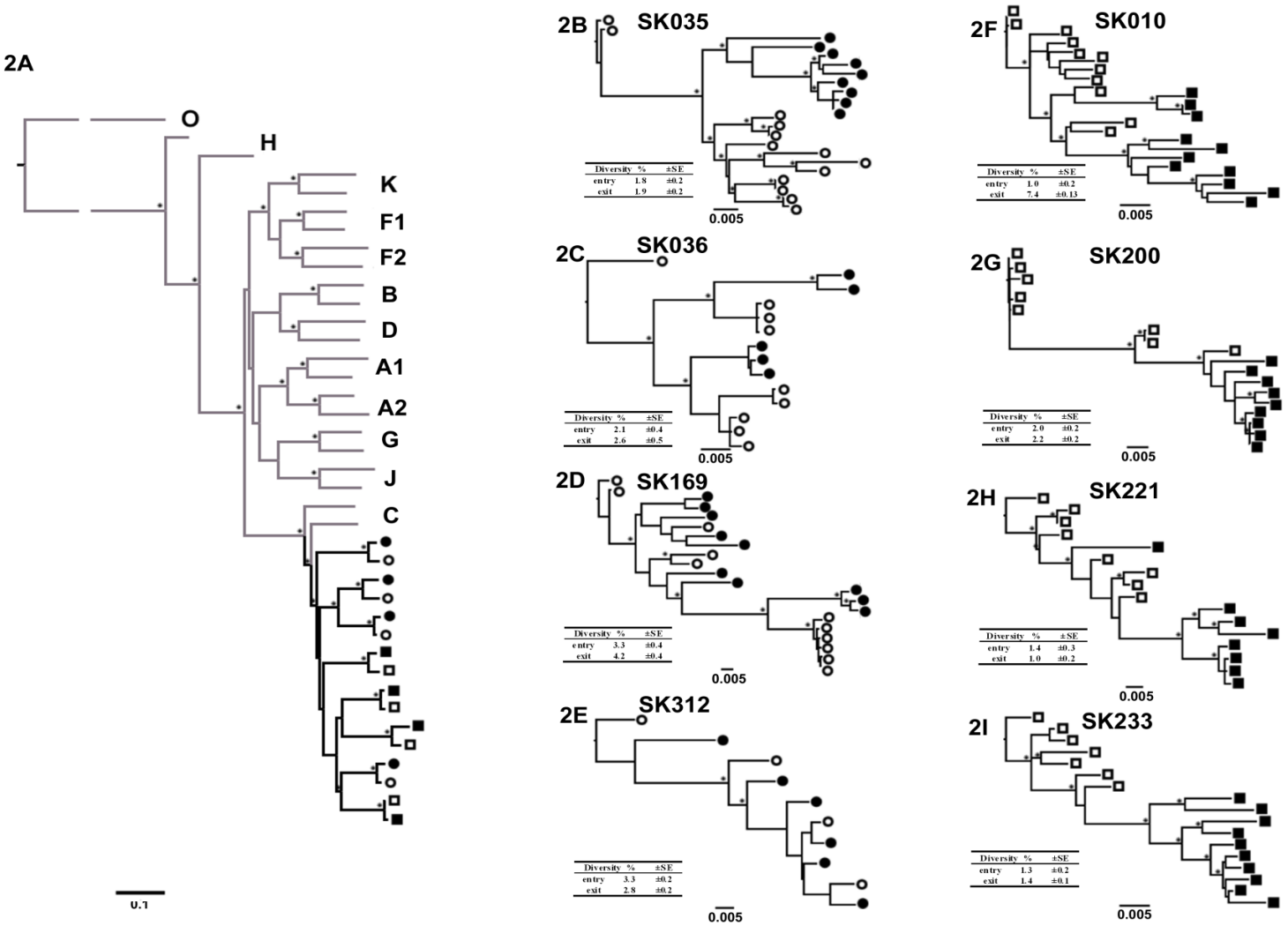

Figure 3.2. Maximum Likelihood trees of SGA-derived full-length *env* sequences from Progressors and Slow progressors. Figure 3.2A Subtype tree of consensus sequences for slow progressors entry (●) and exit (O) and progressors entry (■) and exit (□) time-points. Subtype reference strains were obtained from the Los Alamos database (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html). The tree was rooted with Group O as the outgroup. Figures 3.2B to 3.2E represent maximum likelihood trees for the slow progressor sequences and Figures 3.2F to 3.2I represent trees for the progressor sequences. All trees were drawn in Paup* using the appropriate substitution model. Bootstrap support from 1000 bootstrap resamplings is indicated by •. Only values >70% are shown. The scale bar is shown at the bottom of figure 3.2A is 0.1 and for figures 3.2B-3.2I the scale bar is

range 6-17 $x10^{-3}$), compared to the slow progressors ($3x10^{-3}$ nucleotide substitutions/site/year; range 0.1-7 $x10^{-3}$), but did not differ significantly (p=0.12). The nucleotide substitution rate appeared to follow the viral load pattern, such that there was a positive but non-significant linear correlation between divergence (nucleotide substitution rate) and the $\log_{10}$ viral load (p=0.12) - data not shown.

3.4.5 Heterogeneity of Diversity in Env in Slow Progressors and Progressors for the Variable and Constant Regions

To assess whether there were overall differences in diversity between regions of *env* at study entry and exit, we analyzed distinct regions of the *env* gene separately and compared diversity scores between the slow progressors and progressors for the five variable loops, three constant regions and gp41 over time as seen in figure 3.3A. Significant diversity differences between slow progressors and progressors were noted for the C2 (p=0.004), V3 (p=0.01) and C3 (p=0.005), with differences remaining significant for C2 and C3 even after applying Bonferroni correction for multiple comparisons (≤0.006). There was no significant difference in overall inter-patient percentage diversity between slow progressors and progressors for V1 (p=0.12), V2 (p=0.09), V4 (p= 0.29), C4 (p=0.13), V5 (p=0.08) and gp41 (p=0.40).

Next, the differences in inter-individual *env* diversity patterns across *env* for study entry and exit time-points were assessed. The results of this analysis are summarized in figure 3.3B for slow progressors and figure 3.3C for progressors. There were no significant differences between the early and exit time-point intra-patient diversity for either of the groups in any of the regions.

3.4.6 Length Polymorphisms and Glycosylation Patterns for the Variable and Constant Regions

Overall length of certain regions and changes in the number of N-linked glycosylation sites (PNGs) in Env have been shown to influence the sensitivity or resistance of the virus to antibody neutralization and may also influence efficiency of interactions with receptors on the cell surface (Wei et al., 2003, Sagar et al., 2006). However, these characteristics have not been comprehensively analyzed for HIV-1 subtype C and most studies have focused on the V3 loop, which is an important but not exclusive determinant of viral tropism and cell entry (Huang et al., 2008). We sought to determine whether Env sequence characteristics are associated with disease progression in HIV-1 subtype C.

Table 3.2 depicts Env region length polymorphisms and numbers of PNGs in slow progressors and progressors over time. Mean V1-V2 length for progressors and slow progressors was 66 amino acids and 69 amino acids respectively (table 3.2) but this difference was not statistically significant ($p=0.32$). Similarly, we observed no differences in C4-V5 amino acid length ($p=0.29$) or PNGs ($p=0.15$), and length polymorphism for C2-V3 showed no significant difference between the groups. However, a significant difference was noted in the overall number of PNGs in C2-V3 between slow progressors and progressors ($p=0.009$), a result that remained significant after Bonferroni test correction ($p<0.01$).

For C3-V4, slow progressors had a significantly higher mean of 85 (range 81-90) compared to 82 (range: 76-88) amino acids in progressors ($p=0.02$), however analysis of PNGs indicated no difference between the groups ($p=0.96$). Interestingly, there was a significant difference overall between the groups in the numbers of PNGs for C3 only in the progressors compared to the slow progressors ($p=0.0006$) (data not shown).

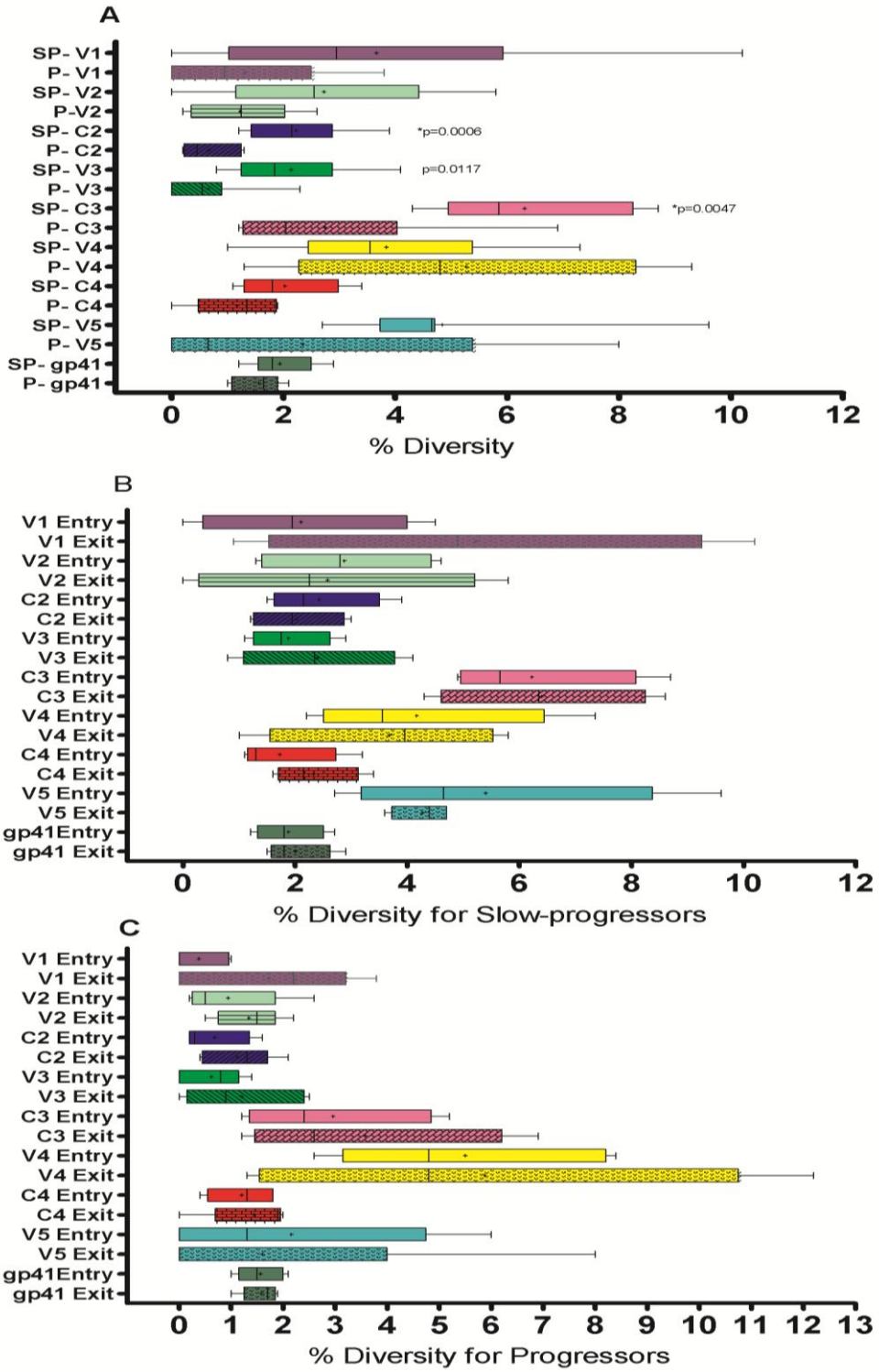

Figure 3.3. Box-and-whisker plots of genetic diversity of the dissected envelope gene for V1, V2, C2, V3, C3, V4, C4 and V5 and gp41 for slow progressors and progressors. The whiskers extend to the upper and lower adjacent values. Comparisons between the groups were done with the Mann Whitney unpaired t test, and p-values are shown. Correlations were regarded as statistically significant with a p-value <0.05 and only significant p-values are shown. p-values depicted with an asterisk (*) indicate the ones corrected for multiple comparisons using the Bonferroni correction of $p \leq 0.006$. Mean diversity value is depicted as (+). Figure 3.3A Diversity of V1, V2, C2, V3, C3, V4, C4, V5 and gp41 in slow progressors (SP) and progressors (P) overall. Figure 3.3B Box and whisker plots of intra-patient diversity analysis for slow progressors for different regions of the *Env* gene for study entry and study exit. Figure 3.3C Box and whisker plots of intra-patient diversity analysis for progressors for different regions of the *Env* gene for study entry and study exit.

Table 3.2. Env sequence characteristics of amino acid length and potential N-linked glycosylation sites for slow progressors and progressors

Patient	***V1V2***		***C2V3***		***C3V4***		***C4V5***		***gp41***	
	mean length (range)	Mean PNGs (range)	mean length (range)	Mean PNGs (range)	mean length (range)	mean PNGs (range)	mean length (range)	mean PNGs (range)	mean length (range)	mean PNGs (range)
Slow progressors										
SK035 entry	69 (62-72)	6 (3-7)	133	8	80 (75-81)	7 (5-8)	53 (52-56)	3 (3-4)	252	5 (3-5)
SK035 exit	69 (59-70)	6 (4-8)	133	8	82 (80-88)	7 (6-8)	53 (52-58)	3 (2-4)	250(243-252)	5 (4-5)
SK036 entry	64 (61-73)	5 (4-6)	133	6 (7-8)	84 (82-84)	8 (8-9)	52	3 (2-3)	243(243)	4 (3-5)
SK036 exit	66 (59-73)	4 (3-6)	133	8 (7-8)	84	8 (7-9)	52	3 (2-3)	243(243)	5 (4-5)
SK169 entry	75 (71-80)	6 (5-7)	133(132-133)	6 (6-8)	85 (84-88)	7 (6-8)	54 (52-55)	3 (2-4)	245(241-245)	3 (3-4)
SK169 exit	76 (71-77)	7 (6-7)	133	6 (6-8)	86 (84-95)	7 (4-10)	54 (51-55)	3 (2-4)	245(245)	3 (3-4)
SK312 entry	66 (60-69)	5(3-5)	133	6	90 (85-97)	9 (5-11)	51 (50-54)	3 (2-4)	239(233-252)	3
SK312 exit	67 (67-69)	5	133	6	90 (84-97)	8 (4-10)	51 (50-55)	3 (1-4)	239(236-252)	3
Mean (range) over time	69 (64-75)	6 (4-7)	133	7 (6-8)	85 (81-90)	8 (7-9)	53 (51-54)	3	245(239-252)	4 (3-5)
Progressors										
SK010 entry	65	6	133	8	79 (77-82)	8 (7-9)	52 (52-53)	3	252	3
SK010 exit	65 (65-66)	6	133	8	78 (75-79)	7 (5-8)	52 (50-54)	3	252	3
SK200 entry	66 (64-78)	6 (6-7)	133	8	76 (75-76)	6 (6-7)	52	2 (2-3)	252	3 (2-3)
SK200 exit	73 (71-73)	6 (6-8)	133	8	76 (75-76)	7	52	3	252	3 (2-3)
SK221 entry	72 (55-74)	7 (3-8)	133	9	77 (73-82)	7 (7-8)	51	3 (3-4)	252	2
SK221 exit	71 (63-76)	5 (4-5)	133	9	85 (74-90)	8 (6-9)	51	3 (3-4)	246(245-252)	2
SK233 entry	58	4	133	8	84 (84)	9 (8-9)	52 (50-51)	3	245	3 (3-4)
SK233 exit	59 (59-63)	5 (5-6)	133	8 (7-8)	84 (84)	9 (8-9)	53 (52-53)	3 (2-4)	245	3 (3-4)
Mean (range) over time	66 (59-72)	6 (4-7)	133	8 (8-9)	82 (76-88)	8 (7-9)	52 (51-53)	3 (2-4)	250(245-252)	3 (2-3)
p-value	***p= 0.32***	***p=0.78***	***NS***	****p=0.009***	***p=0.02***	*p=0.96*	***p=0.29***	***p=0.15***	***p= 0.02***	***p= 0.02***

p-value was calculated using the two-tailed Mann-Whitney non-parametric test overall between the slow progressors and progressors. Where only the mean is reflected it is because it is equivalent to the range. * represents the p-value that remained significant after Bonferroni adjustment for multiple comparisons ($p<0.01$), NS represents a non-significant p-value. Potential N- linked glycosylation = PNGs.[#] Data for V1-V4 length is as follows: slow progressors had a mean of 286 amino acids (range 282-294) versus progressors' 281 amino acids (range 276-292; p=0.009). [#] Data for V1-V4 PNGs is as follows: slow progressors had a mean of 20 PNGs (range 19-21) versus a mean of 22 PNGs (range 20-23) in progressors (p=0.02).

V1-V4 length overall was significantly different, with slow progressors displaying longer V1-V4 length of 286 amino acids (range 282-294) compared to progressors' 281 (range 276-292; p=0.009). In contrast, we found that the numbers of PNGs for V1-V4 overall was significantly higher with a mean of 22, (range 20-23) in progressors compared to a mean of 20 (range 19-21) in slow progressors (p=0.02). Gp41 length was significantly higher in progressors (range 245-252) compared to slow progressors (range 239-252; p=0.02) (table 3.2). However, the number of PNGs in gp41 in slow progressors (range 3-5) was statistically different from those of progressors (range 2-4 PNGs; p=0.02).

3.4.7 Positive and Negative Selection Pressure

The dN/dS (ω) ratio reflects non-synonymous (dN) substitutions to synonymous (dS) substitutions per codon site, with a value of >1 at any site indicating positive selection pressure (Yang et al., 2003). The ω values for the whole of gp160, as well as the variable and constant regions within envelope, were calculated using the M1a and M2a models implemented in CODEML. The settings for the M1a (neutral) model were: model = 0, NSsites = 1, and for the M2a (selection) model were: model = 0, NSsites = 2. A Likelihood Ratio Test (2ΔlnL) was performed between the likelihood scores of the M1a (null) vs. M2a (alternative) models. A χ^2 test was performed using two degrees of freedom (Yang, 1997). For V1, the M2a (selection) model was supported only in the slow progressors (p<0.005). For V2 and V3, the null hypothesis (M1a) could not be rejected for both slow and typical progressors (p=0.25), while the M2a model was supported for all remaining envelope regions (p<0.005) for both groups.

Analysis of the entire Env gp160 in the two groups using CODEML and the SLAC option in HyPhy identified 9 common sites under positive selection in slow progressors and 5 sites in progressors. In slow progressors (Figures 3.4A and 3.B), these were at codons 87, 138 and 140 (V1), 336 and 340 (C3), 396 and 410 (V4), 460 (V5) and 832 (gp41). Most of the sites under positive selection in slow progressors were either adjacent to a putative N-linked glycosylation site (codons 87, 138, 336 and 410) or were located at N-linked glycosylation sites (codons 140, 340, 396 and 460). Interestingly, positions 336 and 340 are within the α-2-helix (HXB2 position 335-352); it has been previously reported that changes within this region may confer autologous antibody neutralization resistance (Rong et al., 2007b).

For progressors (Figures 3.4C and 3.4D), 4 of 5 positively selected sites were located in gp41 (codons 607, 612, 641 and 821), while the remaining site, codon 350, was located in the α-2-helix of C3 immediately downstream of V3. Two of the sites under positive selection in the progressors were either adjacent to, (codon 612) or located at a putative N-linked glycosylation site (codon 641).

One additional site identified using CODEML, codon 671, is located at a linear epitope NWFNIT, which is within the membrane proximal external region (MPER) of gp41, an epitope that is well recognized by a broadly neutralizing antibody (4E10) (Zwick et al., 2005).

Negative selection or purifying selection is the selective removal of alleles that are deleterious and can result in stabilizing selection through this purging process (Loewe, 2008). Negatively selected sites were also assessed using HyPhy, these sites provide information about amino acids that cannot undergo mutation or should not undergo mutation, as changes

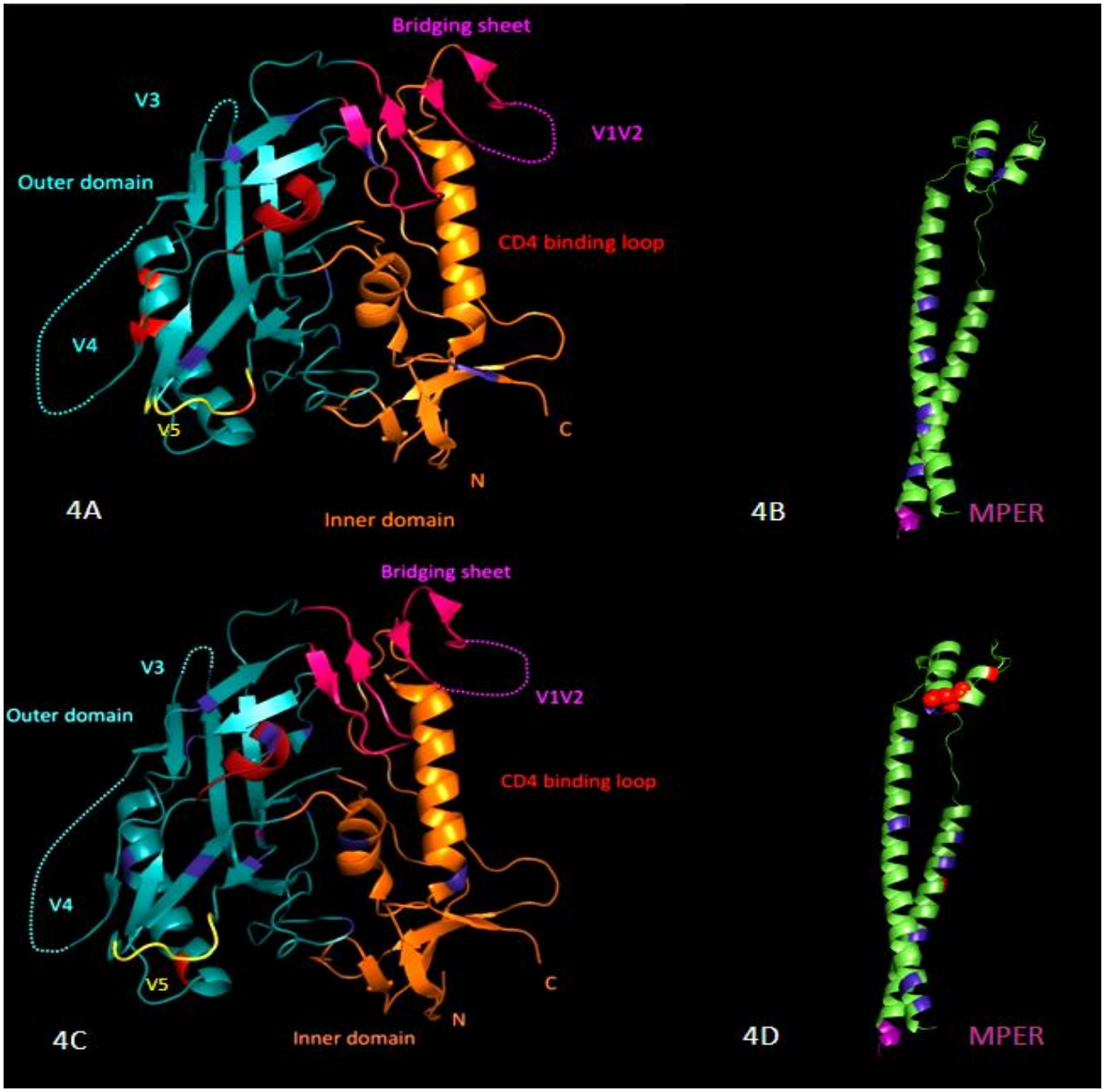

Figure 3.4. Three dimensional structural illustrations of positions associated with positive negative and neutral selection. Locations were mapped onto a model of gp120 based on the X-ray structure of the gp120 core in complex with sCD4 and 21c Fab (3LQA.pdb) for slow progressors - Figure 3.4A and for progressors - Figure 3.4C. V1-V2, V3 and V4 loops were drawn onto the core for completeness. In the orientation shown, the cellular and viral membranes would be located above and below the protein respectively. Figure 3.4B and 3.4D represent ribbon structures of gp41 for slow progressors and progressors with the MPER region highlighted. Cartoon diagrams showing locations under positive selection, as determined by dN/dS ratios for subtype C sequences. Red indicates strong positive selection (dN/dS >4) as shown above in HXB2 positions 87, 336, 340, 396, 410 and 460 for slow progressors (Figure 3.4A) and in progressors at positions 350 (Figure 3.4C) and 607, 612 and 641 in Figure 3.4D. Blue indicates strongly negatively selected positions (<-3). Purple and purple arrows denote changes in putative functional sites as shown in Figures 3.4B, 3.4C and 3.4D. Spheres indicate signature sequence differences. It should be noted that the gp120 core crystal structures which were modeled on the 3LQA.PDB structure, include amino acid residues from HXB2 position 86-491. The gp41 structure based on 1ENV.pdb includes amino acid residues from HXB2 position 541-662. Therefore all the positively and negatively selected sites are not indicated on the gp120 and

may be deleterious to overall function of the protein and therefore to the virus. There were 49 and 51 negatively selected sites in slow-progressors and progressors respectively as depicted in tables 3.3 and 3.4, and all sites were significantly negatively selected for ($p < 0.05$).

The distribution of sites under negative selection was widespread along Env gp160 in both slow progressors and progressors (see tables 3.3 and 3.4 respectively). Percentage (%) of negatively selected sites was calculated by counting the numbers of negatively selected sites within a particular region of Env (e.g. V2) and dividing it by the total number of negatively selected sites spanning the entire Env gp160. Slow-progressors had negative selection in most regions of Env except for C3. 12.2% of the negatively selected sites were found in the C1 (6/49), 2% in V1 (1/49), 8.6% in V2 (4/49), 14.2% in C2 (7/49), 4% in V3 (2/49), 6% in C4 (3/49), 2% in V5 (1/49), 4% in C5 (2/49) and a majority of 46.9 % in gp41 (23/49). A long interval of sites from codons 211-282 was found in C2. 17.4% (4/23) negatively selected sites in gp41 were in the leucine zipper region.

Progressors had negative selection in all regions of the Env gp160 except for V1. 11.7% of the negatively selected sites were found in the C1 (6/51), 5.8% in V2 (3/51), 7.8% in C2 (4/51), 1.9% in V3 (1/51), 7.8% in C3 (4/51), 1.9% in V4 (1/51), 3.9% in C4 (2/51), 1.9% in V5 (1/51), 7.8% in C5 (4/51) and 49% in gp41 (25/51). 20% (5/25) of the negatively selected sites in gp41 were in the leucine zipper region of gp41.

In gp41 there was a long interval of negatively selected sites spanning codons 501-813 (23 sites) in slow progressors and codons 544-853 (25 sites) in progressors. Interestingly, amino acids common to both groups were only in the region proximal to or in the leucine zipper area, these were at positions 787R, 794K and 813S. The preservation of conserved amino acids in the leucine zipper region is crucial to HIV-1 Env oligomerization. These results

could be biased because we did not account for phylogenetic relatedness and there were too few participants to perform multivariate analysis. Although the p-values represented in the tables are highly statistically significant, when we do adjust for multiple comparisons using the Bonferroni correction, none of the p-values were < 0.00005.

3.4.8 Signature Sequence Differences between Slow Progressors and Progressors

To identify key differences between the groups, consensus sequences of slow progressors and progressors study entry and exit were generated in VESPA using an 80% threshold (i.e. sequence differences were in >80% of the sequences). Signature differences were noted at 6 amino acid positions between the progressors and slow progressors consensus sequences. Four of six of these differences occurred in gp41 (codons 607, 727, 770 and 837), and the remaining two were at codons 80 and 133. No signature differences were noted between the entry and exit time points within each group.

Except for an N to S/D mutation in the progressors at codon 80, which resulted in the gain of a casein-kinase-2 (CK2) phosphorylation site at codons 77-80, most of the signature changes were not at putative functional sites. Other changes, although not in the signature, but resulting in a change in putative functional sites in the progressors, are: a V to T mutation at codon 455 resulting in the gain of a myristoylation site at codon 451-456, a Q to K mutation at codon 665 (within the ALDSQWN epitope) resulting in the gain of a tyrosine kinase phosphorylation (TKP) site at codons 665-667, and an N to S mutation at codon 671 resulting in the gain of a CK2 phosphorylation site at codons 671-674 within the NWFDIT epitope. Interestingly, the loss of a putative N-linked glycosylation site in the progressors in the V4 region was compensated for by a gain of an N-linked glycosylation site in the C3 region

(codons 362-365). When these signature patterns were compared with the subtype B reference strain, it was noted that an L to V mutation at codon 800 in the subtype C signature sequences resulted in a loss of a putative leucine zipper (codons 793-814). Whether the gain or loss of putative functional sites influence viral pathogenesis needs to be confirmed with functional assays.

3.4.9 Predicted Coreceptor Usage based on V3 Crown Sequence Motifs in Slow Progressors and Progressors

Based on sequence analysis of the V3 sequences, predicted co-receptor usage of the viruses was done using the Web Position Specific Scoring Matrix (WebPSSM-http://indra.mullins.microbiol.washington.edu/webpssm). This programme takes into account the sentinel amino acid positions 11 and 25 of the 35-36 amino acid motif of V3 that determine whether a virus uses either the CCR5 or CXCR4 chemokine receptors or both in order to infect a target cell. A total of 147 sequences were analyzed, 78 for progressors and 69 for slow-progressors. All the progressors and two slow-progressors had CCR5 using viruses. The remaining two slow-progressors SK035 and SK169 had viruses in the study entry time-point that were predicted to be CXCR4 or CCR5 using, or both and the percentage of viruses in these participants were 15% (2/13) and 50% (6/12) for SK035 and SK169 respectively. Additionally, all these V3 sequences were 35 amino acids in length whereas the CCR5 only using viruses ranged between 35 and 36 amino acids in length. Interestingly, these viruses were only evident at the study entry time-point and had disappeared at the study exit time-point over an average period of 23 months for these participants.

Table 3.3. 49 Negatively selected sites spanning Env gp160 for slow progressors using HyPhy

HXB2 position and wild type amino acid	dN/dS Ratio	p- value	Position in Env	HXB2 position and wild type amino acid	dN/dS Ratio	p-value	Position in Env
38V	-4.00	0.012	C1	**488V**	-3.00	0.037	C5
54C	-3.29	0.048	C1	**501A**	-3.00	0.037	Gp41
60A	-3.00	0.037	C1	**510K**	-7.30	0.0009	Gp41
N80S	-3.29	0.04	C1	**529T**	-3.00	0.037	Gp41
86L	-6.54	0.0017	C1	**540Q**	-7.30	0.0009	Gp41
121K	-4.23	0.016	C1	**553N**	-9.87	0.00007	Gp41
131C	-3.29	0.048	V1	**554N**	-8.23	0.0003	Gp41
159F	-3.29	0.041	V2	**563Q**	-4.16	0.027	Gp41
176F	-3.29	0.041	V2	**569T**	-5.00	0.004	Gp41
191Y	-4.93	0.012	V2	**597G**	-4.00	0.012	Gp41
N195S	-3.29	0.41	V2	**A607N/D/T**	-3.50	0.045	Gp41
E211D	-6.59	**0.002**	C2	**H643Y**	-4.94	0.013	Gp41
212P	-3.00	0.037	C2	**657E**	-4.40	0.013	Gp41
222G	-4.00	0.012	C2	**679L**	-3.20	0.041	Gp41
243S	-4.94	**0.008**	C2	**685F**	-6.59	0.002	Gp41
260L	-3.73	0.023	C2	**694G**	-4.00	0.012	Gp41
263G	-3.00	0.037	C2	**747R**	-4.40	0.013	Gp41
282K	-5.91	**0.003**	C2	**759D**	-9.88	0.00007	Gp41
304R	-3.68	0.024	V3	**765L**	-6.00	0.0013	Gp41
327R	-6.69	0.0009	V3	**787R**	-7.77	0.0017	Gp41-proximal to the leucine zipper region
418C	-3.29	0.048	C4	**794K**	-6.94	0.001	Gp41 Leucine zipper
421K	-4.44	0.014	C4	**806E**	-8.84	0.0002	Gp41 Leucine zipper
425N	-8.23	0.0003	C4	**808K**	-5.12	0.029	Gp41 Leucine zipper
466E	-5.73	0.004	V5	**813S**	-7.39	0.005	Gp41 Leucine zipper
487K	-4.44	0.014	C5				

Table 3.4. 51 Negatively selected sites spanning Env gp160 in progressors using HyPh

HXB2 position and wild type amino acid	dN/dS Ratio	p-value	Position in Env	HXB2 position and wild type amino acid	dN/dS Ratio	p-value	Position in Env
44V	-5.00	**0.004**	C1	**544L**	-3.84	0.021	Gp41
70A	-4.00	0.012	C1	**547G**	-4.00	0.012	Gp41
74C	-3.26	0.05	C1	**569T**	-4.00	0.012	Gp41
99D	-5.68	0.018	C1	**581L**	-4.02	0.018	Gp41
117K	-4.22	0.016	C1	**604C**	-3.26	0.048	Gp41
128S	-3.5	0.045	C1	**626T**	-12.74	0.025	Gp41
153E	-3.99	0.043	V2	**634E**	-4.37	0.014	Gp41
171K	-4.37	0.041	V2	**638Y**	-8.16	**0.0007**	Gp41
S190E	-3.99	0.04	V2	**647E**	-4.35	0.014	Gp41
239C	-6.53	**0.002**	C2	**649S**	-4.00	0.018	Gp41
262N	-3.26	0.041	C2	**S668N**	-6.52	**0.0012**	Gp41
264S	-3.26	0.041	C2	**685F**	-3.26	0.041	Gp41
V275E	-5.84	**0.003**	C2	**692L**	-3.73	0.027	Gp41
314G	-3.00	0.042	V3 Crown motif	**765L**	-3.00	0.037	Gp41
339N	-3.26	**0.04**	C3	**780R**	-8.40	0.0002	Gp41
P369L	-3.26	0.04	C3	**787R**	-4.83	0.028	Gp41
375S	-4.30	0.031	C3	**788R**	-3.54	0.024	Gp41
377N	-3.26	0.04	C3	**794K**	-6.92	0.0011	Gp41 Leucine zipper
386N	-4.89	**0.009**	V4	**798N**	-6.10	0.007	Gp41 Leucine zipper
418C	-4.89	**0.009**	C4	**806E**	-5.53	0.004	Gp41 Leucine zipper
454L	-3.74	0.03	C4	**811A**	-4.00	0.012	Gp41 Leucine zipper
468F	-6.52	**0.0017**	V5	**813S**	-7.32	0.005	Gp41 Leucine zipper
477D	-3.26	0.04	C5	**840I**	-3.56	0.022	Gp41
489V	-3.00	0.04	C5	**842H**	-4.89	0.009	Gp41
499T	-5.00	**0.004**	C5	**R853A**	-8.50	0.0004	Gp41
L518V	-6.00	**0.001**	C5				

3.5 Discussion

In this study we aimed to identify *env* sequence characteristics that may distinguish progressors from slow progressors in a chronically HIV-1 infected anti-retroviral naïve subtype C-infected cohort. We used a single genome amplification approach in order to accurately and comprehensively represent the diversity of viral quasi-species. Several indicators of evolutionary forces were used to elucidate putative differences between the groups including heterogeneity of envelope sequence diversity, Env length polymorphisms, numbers of PNGs, positive selection, and signature sequence characteristics.

Our study suggests that regions of Env are shaped by different evolutionary forces that may in turn leave viral sequence footprints that may distinguish slow progressors from progressors in chronic HIV-1 subtype C infection. It has previously been shown that in subtype B infection there may be Env region-specific differences in evolutionary forces between those with high versus low viral loads (Wolinsky et al., 1996). Our study demonstrated a non-significant trend towards increased intra-patient diversity in slow progressors, a finding consistent with other studies on HIV-1 disease progression (McNearney et al., 1992, Markham et al., 1998, Mani et al., 2002). We therefore could not prove our hypothesis that reduced viral diversification correlated with lack of or slower disease progression. In contrast, a study of primary HIV-1 subtype C infection has found that increased envelope diversity is inversely correlated with CD4 T-cell counts and is associated with rapid disease progression (Gray et al., 2007). Together, these results may imply that evolutionary forces that drive HIV-1 subtype C diversification differ according to the phase of infection. On close examination of the envelope regions we found that diversity in C2, V3 and C3 was higher in slow progressors compared to progressors suggesting co-evolution of these regions. These findings are consistent with findings from other studies (Menzo et al., 1998, Gaschen

et al., 2002). From a functionality standpoint it appears that, because the V3 loop is very important for viral entry, increased diversity in this region is a correlate of viral attenuation (Abraha et al., 2009).

Length polymorphisms in the constant and variable envelope regions may also contribute to structural diversity in terms of glycan packing and protein folding of the virion structure. An unusual finding was that the longer V1-V4 in slow progressors had fewer PNGs whereas the longer gp41 domain contained fewer PNGs in progressors. Several studies have shown the association between neutralization sensitivity and shorter V1-V4 length (Derdeyn et al., 2004, Rong et al., 2007a). In contrast, other studies have shown longer V1-V4 with extensive glycosylation mask neutralizing antibody sensitive epitopes in subtype C (Rademeyer et al., 2007); however, in subtype B no such association was found (Liu et al., 2008b). Our observations may imply that longer length regions may be masking neutralization sensitive epitopes as suggested by Gray et al. (Gray et al., 2007). Additionally in progressors, a loss of a glycan in V4 was compensated for by a gain in a PNG within C3, implying a shifting glycan shield as suggested previously (Wei et al., 2003).

High dN/dS ratios indicative of strong diversifying selection due to humoral immune pressure (Yang et al., 2003), occurred mainly within gp41 in progressors, while slow progressors had a number of regions targeted. This suggests that the nature of antibody targets may differ between the groups. Interestingly, both groups had positive selection in the α-2-helix within C3. It has been suggested that, because the V4 loop is shorter in subtype C than in subtype B, the α-2 helix is more exposed and more antigenic (Gaschen et al., 2002, Gnanakaran et al., 2007, Lynch et al., 2009). Interestingly, position 607 of gp41 was positively selected in progressors and was also a signature sequence difference between progressors and slow-progressors, indicating that there may be putative humoral immune

pressure driving escape at that position. Additionally, gp41 in progressors showed differences at two putative antibody sites. Firstly, ELDKWAS was recognized by nmAb-2F5, where DKW are the sentinel amino acids that determine sensitivity to 2F5 (Zwick et al., 2005). This appears in the majority of the slow progressors's sequences; however, it is substituted by DSW in all the progressors indicating a loss of a putative antibody recognition site. In addition there is a sequence change from Q at position 665 to K, making the overall progressor sequence ALDSWKN. Secondly, an N to S change at codon 671, which is within a linear epitope- **N**WFNIT- that is recognized by nmAb 4E10, may result in a loss of this recognition site. In addition, this codon was positively selected for in the progressors. The effect of the loss of these putative recognition sites during chronic disease progression is unknown. In addition, the negatively selected sites along the entire gp160 in both slow progressors and progressors may imply that there are some sentinel amino acids that need to be preserved in order to maintain the overall integrity of the Env. Identification of negatively selected sites may be tool we can use to hone in on crucial or functionally important sites in HIV-1 Env and may be included in future immunogen design to cripple the virus where it cannot afford mutations**.** Together these results may imply that the virus uses multiple strategies to evade the immune system, including increased V1-V4 amino acid length, increased numbers of PNGs, and specific mutations resulting in the virus gaining selective advantages. Essentially, the cat and mouse game that persists during chronic infection as a result of the dichotomy between antigenic stimulation and immunological response, which impacts and influences viral characteristics, needs further investigation.

The limitations of the study are that firstly, we do not know the exact time of infection for these subjects. Therefore stratification of study subjects as progressors or slow progressors relied on short-term (24 months) follow-up immunological data, which may be an

unrepresentative snap-shot of the entire natural history of disease progression for these participants. However, this concern was somewhat allayed by bioinformatic analysis of the study sequences that showed that consistent with the stratification, progressors in this cohort were more likely to have been infected for shorter period of time than slow progressors. Second, the sample size of the study cohort was relatively small, which may have limited our statistical power to identify differences. Third, we had a limited number of SGA-generated amplicons for one of the study participants in particular, due to their low viral load and sample volume limitation. In addition, many more *env* amplicons were generated than were included in the final analyses as some of the amplicons had sequences with stop codons. Fourth, although the slow progressors and progressors differed in markers of disease progression at study exit, more stringent selection criteria could potentially identify additional significant differences. Overall, therefore, the findings reported here will require duplication in larger cohorts with longer periods of follow-up and more significant differences in immunological and virological outcomes.

3.6 Conclusions

The dynamics of HIV-1 *env* evolution between chronic slow progressors and progressors are distinct. Single genome sequence analysis of circulating viruses in slow progressors and progressors indicate that diversity, *env* length polymorphisms, sites under positive selection pressure, and PNGs consistently map to specific regions in slow progressors or progressors. Varied diversity across the *env* genome, the relationship between amino acid length, number of PNGs or sites under positive selection may provide further insight to the intrinsic differences between the viruses from both groups and the influence of the host's selective pressures which may be used to inform more effective vaccine design.

Competing interests

The author(s) of this paper declare that they have no competing interests.

Authors' contributions

DA and TN conceived the experiments. DA, MG and TG carried out the experiments. DA, MG and TN wrote the paper and all authors including HMC and PJRG helped interpret the data and reviewed the manuscript.

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Chapter Four - Results

Deciphering Neutralizing and Non-Neutralizing Antibody Responses in HIV-1 Subtype C Chronically Infected Patients: Association with Divergent Rates of Disease Progression.

4.1 Abstract

Background: Neutralizing antibodies (nAbs) play a critical role in the protection against many viral infections, however their role in HIV/AIDS disease progression remains controversial. Similarly, the role that non-neutralizing antibodies may play in HIV/AIDS disease attenuation is unclear. In this study we aimed to identify HIV-1 subtype C (HIV-1C) infected individuals with broad and potent nAbs, and the epitope targets of these nAbs that could assist in rational vaccine design. In addition, we also undertook to assess the binding affinities of several HIV-1 antigen-specific IgGs and Fcγ receptors as surrogate indicators of antibody-dependent cell-mediated cytoxicity (ADCC) to investigate possible differences associated with divergent states of HIV-1 disease progression.

Results: Autologous nAb (AnAb) IC_{50} titers were significantly different in progressors when the study entry viruses were challenged with study exit plasma nAbs (p=0.003) compared to the contemporaneous response. There was a positive correlation between amino acid length and nAb IC_{50} titer in C3-V5 (p=0.03) and V1-V5 (p=0.04) in slow progressors, however in the progressors there was a negative correlation in V1-V2 (p=0.04) and nAb IC_{50} titer. Neutralization breadth and titer to subtype B reference strains however, was significantly higher in progressors compared to slow progressors (both p<0.03) with increasing nAb breadth from study entry to study exit in progressors. There were no significant differences in breadth of responses between the groups for either subtype A or C. Average nAb IC IC_{50} titers to subtype B tier 2 viruses were significantly higher in progressors compared to slow

progressors (p=0.005). Fine mapping of two progressors nAbs indicated target epitopes in V2 and V3. There were no differences between the groups in the binding affinities of the IgGs to the HIV-1-specific antigens or to the FcγRs. However, there was a significant correlation between CD4 T-cell count and declining binding affinities of FcγRIIa in slow progressors (p=0.005).

Conclusions: Differences between slow progressors and progressors nAb profiles may reflect differences in immunological pressure and immune evasion mechanisms. The potencies of autologous nAbs are influenced by amino acid length and numbers of PNGs in particular Env regions in chronic progressive HIV-1 infection. Neutralization breadth is a correlate of chronic disease progression. Progressors had cross-reactive neutralizing antibodies targeting epitopes in V2 and V3 and also displayed neutralization breadth against subtype B viruses and this could have important implications in HIV-1 vaccine development. Overall, the data suggest that neither neutralizing nor non-neutralizing antibodies could be directly associated with disease attenuation in this cohort of chronically HIV-1 infected individuals. However, continuous evolution of nAbs was a potential marker of disease progression.

4.2 Background

In 2009 alone, there were an estimated 2.4 to 2.9 million new HIV-1 infections worldwide with ~70% of these new infections occurring in sub-Saharan Africa (UNAIDS, 2010). This high HIV-1 incidence makes the development of a protective vaccine a global public health priority. Such a vaccine will likely need to elicit antiviral antibodies, similar to the successful vaccines against other viral infections such as hepatitis B, measles, mumps and polio that are thought to mediate their effects primarily through antibody mechanisms (Plotkin, 2008). However, despite intense efforts in the study of HIV-1 envelope structure and immunogen design, the development of an efficacious vaccine able to induce broadly effective antibody responses remains elusive.

During natural HIV-1 infection, only a subset of individuals, about <30%, develop broad, cross-neutralizing antibodies after many years (Li et al., 2009, Sather et al., 2009, Simek et al., 2009, Stamatatos et al., 2009, Gray et al., 2011a). Such individuals have been an important source of new neutralizing monoclonal antibodies (nmAbs) against the HIV-1 envelope. For example, Walker and colleagues reported the isolation of nmAbs that target the variable loops, termed PG9 and PG16 from a subtype A-infected individual (Walker et al., 2010). In addition, VRC01, an anti-CD4 binding site nmAb is able to neutralize more than 90% of primary isolates and more recently, the PGT series of nmAbs with PGT128 which targets glycans, is able to neutralize diverse viruses at much lower concentrations than other nmAbs (Wu et al., 2010, Pejchal et al., 2011). Collectively, this work shows that in some cases natural infection is able to induce potent anti-HIV-1 antibodies with studies in non-B subtype infected populations providing putative targets to include in globally relevant HIV-1 vaccine design. Given that HIV-1 subtype C is the predominant circulating and most

rapidly spreading subtype worldwide (Esparza, 2005, Coetzer et al., 2007, Hemelaar et al., 2011), screening, characterizing and understanding the types of nAbs produced by HIV-1 subtype C-infected individuals; and defining the potencies and breadth of these nAbs will contribute to the design of the next generation of envelope immunogens.

While only some people develop cross-neutralizing antibodies, autologous nAbs (AnAbs) appear in almost all HIV-1-infected individuals usually within the first year. A number of studies have shown contemporaneous viruses are less sensitive to AnAbs than earlier autologous viruses suggesting that viral evolution and escape occurs rapidly and this remains a significant obstacle to HIV-1 vaccine development (Delwart et al., 1997, Wei et al., 2003, Richman et al., 2003, Rong et al., 2009, Moore et al., 2009). Several mechanisms of viral escape have been documented; these include insertions and deletions of amino acids, amino acid substitutions and shifting the position of N-linked glycans in Env (Frost et al., 2005b, Rong et al., 2009, Moore et al., 2009, Lynch et al., 2011). Further understanding of HIV-1 antibody escape patterns and mechanisms may improve the design of immunogens.

Although the major focus of the HIV-1 vaccine field is the development of immunogens able to induce broadly neutralizing antibodies, the role of non-neutralizing antibodies is now being considered as potentially important. Non-neutralizing antibodies are defined as those which do not show classical neutralizing activity, rather, they inhibit virus replication through an Fc (fragment crystallizable) receptor- (FcR)-dependent mechanism (Peressin et al., 2011). A recent study showed that antibody-dependent cell-mediated cytotoxicity (ADCC) activity was significantly higher in elite controllers compared to chronic progressors (Lambotte et al., 2009). These data on HIV-1 are consistent with earlier studies suggesting a role for non-neutralizing antibodies in viral control and disease attenuation (Baum et al., 1996, Alsmadi et al., 1997). Fc receptors (FcRs) are part of the immunoglobulin (Ig) superfamily and bind to

the Fc regions of antibodies (Nimmerjahn and Ravetch, 2007). There are five different isoforms of FcγRs with either an activating or inhibitory function with relative strengths of IgG binding affinities. FcγRI (high affinity), FcγRIIa and FcγRIIIa (medium-low affinity)- all with activating functions; and the inbibitory FcγRIIb (medium-low affinity) (Siberil et al., 2007, Forthal and Moog, 2009). The Fc portions of antibodies form a bridge between the cell bearing the target antigens and the effector cell. The effector cell bearing the FcR is then able to mediate virus killing through ADCC or through antibody-dependant cell-mediated viral inhibition (ADCVI). Following the recent RV144 HIV-1 vaccine trial (Rerks-Ngarm et al., 2009), there has been speculation that non-neutralizing antibodies may have contributed to the 31% vaccine efficacy (Tomaras and Haynes, 2009). Recently, Burton and colleagues (2011) demonstrated the lack of efficacy of a weakly neutralizing antibody (b6) compared to a non-neutralizing antibody (F240) against vaginal SHIV challenge (Burton et al., 2011). The role of ADCC/ADVCI in HIV-1 acquisition and/or control is poorly understood and no studies have reported on the characterization of these antibodies in HIV-1 subtype C infections.

In this study, we used standard high throughput neutralization assays to evaluate the potency and breadth of neutralizing activity in patient plasma and map potential Env targets associated with nAb breadth. We profiled autologous and cross-reactive (against a standard panel of 20 subtype A, B and C viruses) nAb titers in subtype C-infected slow progressors and progressors. Envelope genotypic characteristics (amino acid length and numbers of potential N-linked glycosylation sites (PNGs) correlating with autologous nAb titers were assessed. Binding affinities of non-neutralizing antibodies to FcRs were quantified and used as a surrogate for ADCC/ADCVI activity. A correlation between nAbs and non-neutralizing

antibodies and markers of disease progression (namely CD4 T-cell counts and viral loads) was investigated.

4.3 Materials and Methods

4.3.1 Participants

Participant samples were retrospectively identified from the Sinikithemba cohort, which is a prospective natural history study of HIV-1 infected individuals based at McCord Hospital, Durban, South Africa as previously reported (Kiepiela et al., 2004). Ethics approval was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee and all participants gave written informed consent to participate in the study.

4.3.2 Sample Collection, CD4 T-Cell Counts and Plasma Viral Load

CD4 counts were performed at three-month intervals whereas viral loads were done at six-month intervals. Blood was drawn from each subject into EDTA tubes and plasma was separated by centrifugation and stored at $-80^{\circ}C$ until use. Viral load was measured using the Amplicor Version 1.5 assay (Roche Diagnostics, Indianapolis, USA). CD4+ T-cell counts were enumerated by Trucount technology on a four-colour FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

4.3.3 Envelope Clones

Envelope genes used in this study were either cloned as previously described or obtained from the NIH AIDS Research and Reference Reagent Program (NIH ARRRP). Briefly, the

single genome amplification derived *env* amplicons were directionally T/A cloned into the CMV-driven expression plasmid pcDNA3.1-V5 HisTOPO-TA and screened for biological function as pseudoviruses following co-transfection with an *env*-deficient subtype B proviral plasmid (SG3Δ*env*) into 293T cells as described previously (Derdeyn et al., 2004) (see detailed protocol in Chapter Two). A total of 20 standard reference envelope clones were used in the heterologous neutralization assays. These included eight subtype C, seven subtype B and five subtype A envelope pseudoviruses as depicted in table 2.12 (see chapter two). Tier 1 and Tier 2 viruses were stratified previously based on a clustering analysis of sensitivity patterns- with Tier 1 being the most sensitive, Tier 2 displaying moderate to low sensitivity and Tier 3 displaying the lowest sensitivity to neutralization (Seaman et al., 2010). The ConC plasmid carrying a consensus of all the HIV-1 subtype C sequences from the Los Alamos database by 2001 (Kothe et al., 2006) and the envelope plasmids containing single point mutations (as described in Gray et al. (2011), were obtained from Lynn Morris.

4.3.4 Neutralization Assays

Patient plasma samples were evaluated for neutralizing antibody activity against virions pseudotyped with autologous patient-derived viral Envs using a single reporter assay as described previously (Wei et al., 2003, Derdeyn et al., 2004, Li et al., 2005, Li et al., 2006b). Figure 2.4. (see Chapter Two) illustrates the schema used for autologous nAb assays where the study entry and exits Envs were tested for neutralization using the study entry and study exit plasma nAb samples. This type of matrix yields both a contemporaneous response and an evolutionary response where escape from neutralization or resistance is often observed. Neutralization was measured as a reduction in luciferase gene expression after a single round of infection of JC53bl-13 cells, also known as TZM-bl cells (NIH AIDS Research and

Reference Reagent Program). Two thousand infectious units of each pseudovirus was combined with five-fold dilutions of heat-inactivated participant plasma and incubated for 1 hr at 37°C. Subsequently, the virus and antibody reaction was added to the plated TZM-bl cells, and left to incubate at 37°C for 48 hrs. The cells were lysed and the luciferase activity was determined using a luminometer. The background luminescence of the uninfected wells was subtracted from the test wells. The percentage of infectivity was calculated by dividing the number of luciferase units at each plasma dilution by the value in the well containing no test plasma. The dilution that determined 50% inhibitory activity against the virus, known as the nAb IC_{50} titer (IC_{50} titer) was determined on the Microsoft Excel. Each experiment was performed in duplicate and independently at least twice for replicability. The nAb IC_{50} titer was defined as: the reciprocal plasma dilution causing a 50% reduction of relative light units (IC_{50}). Heterologous neutralization assays were done as previously reported by Montefiori, et al. (2004). A detailed description of these methods is in Chapter Two.

4.3.5 IgG Isolation

Detailed explanations of IgG isolation and Fcγ receptor binding assays are provided in Chapter Two. Briefly, the IgGs were isolated according to the manufacturer's instructions. 500 µl of a 1:10 dilution of plasma to buffer was added to a prepared Melon Gel column (Thermo Scientific, Surrey, United Kingdom) (100 µl of plasma /serum plus 900 µl buffer). The tubes were rotated for 5 mins in order to ensure maximum capture of the IgGs and were then spun down for 1 min at 5,000 x g to elute the IgGs. IgG concentrations were measured on the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Surrey, United Kingdom) and stored at 4°C for downstream ELISA assays

4.3.6 Fcgamma (Fcγ) ELISA Protocol

4.3.6.1 Binding Affinity for Fcγ Receptors

Binding affinities of IgGs for the various activating and inhibitory receptors were done in order to see if there may be a difference between the IgGs of the slow progressors and progressors in their ability to bind to different activating (FcγRI, FcγRIIa and FcγRIIIa) or the inhibitory receptor (FcγRIIb). Four different Fc gamma (γ) receptors were used: FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa (R & D Systems, Minneapolis, USA) and the ELISAs for each of the receptors were performed using a standard protocol as seen in Chapter Two.

4.3.6.2 Gp120, gp41, and p24 ELISAs

ELISA plates were coated with 80 µl/well of a 250 ng/ml gp120 (Immune Technology, New Jersey, USA) or gp41 (HXBc2) (Immune Technology, New Jersey, USA) or p24 **(**Immune Technology, p24 (HIV-1/HXBc2 (Immune Technology, New Jersey, USA) and then incubated O/N at 4°C. ELISAs for each of the antigens were performed using a standard protocol as detailed in Chapter Two**.**

4.4 Statistical Analyses

Pair-wise comparisons of different parameters including nAb IC_{50} titers for both the autologous and heterologous responses, and affinity binding of the different Fcγ receptors and intergroup and intra-group comparisons were performed using the Mann-Whitney non-parametric test using the two-tailed test and Spearman's non-parametric rank test was

performed using the GraphPad Prism 5 software programme unless otherwise stated. Correlations were regarded as statistically significant with a p-value ≤ 0.05. All reported p-values are for two-sided tests.

4.5 Results

In chapters two and three of this thesis we described the participants' stratification into slow progressors and progressors , based mainly on CD4 T-cell counts (see chapter three, figure 3.1) (Archary et al., 2010). Briefly, slow progressors maintained their CD4 T-cell levels over the window of evaluation with no differences between their study entry and study exit levels. However, the progressors had experienced a significant decline in CD4 T-cell counts at study exit compared to the study entry CD4 T-cell counts (p=0.03). Similarly, when viral loads were compared between the study entry and study exit time-points, only the progressors had increased viral loads over time (p=0.03). In addition, a generalized estimating equation (GEE) model, adjusting for repeated measures of CD4 T-cells to assess the overall mean square root CD4 count across all time points was used and it was found that there was 19.16 (SE=0.3073) cells/µl in the progressors and 23.96 (SE= 0.4400) cells/µl in the slow progressors and these are significantly different (p<0.0001) between the groups. Furthermore, the overall mean log viral load across all time points is 4.56 (SE=0.0796) log copies/ml in the progressors and 3.65 (SE=0.0766) log copies/ml in the slow progressors and these are significantly different (p<0.0001). In addition, when the median values for both CD4 T-cells and viral loads were compared between the slow progressors and progressors for study entry, study exit and the last available time-points, the differences remained statistically significant except for the study entry time-points for CD4 only (p=0.39)- see tables 4.1 and 4.2.

Table 4.1. CD4 T- cell count with inter-quartile ranges (IQR) of study entry, exit and latest available time-point

Visit	Slow progressor n	Median CD4 (IQR)	Progressor n	Median CD4 (IQR)	p-value*
Study entry	4	621 (553 – 808)	4	571 (525 – 622)	0.39
Study exit	4	506 (380 – 728)	4	283 (243 – 357)	0.043
Latest available time-point	4	486 (423 – 631)	4	287 (230 – 315)	0.021

Table 4.2. Viral loads with inter-quartile ranges (IQR) study entry, exit and latest available time-point

Visit	Slow progressor n	Median VL (IQR)	Progressor n	Median VL (IQR)	p-value*
Study entry	4	4 280 (2 835 – 5 450)	4	10 770 (8 110 – 13 080)	0.021
Study exit	4	3 290 (2 695 – 7 115)	4	24 300 (21 300 – 42 800)	0.021
Latest available time-point	4	3 950 (1 580 – 8 527)	4	49 350 (18900– 67 900)	0.029

4.5.1 Autologous and Heterologous Responses

4.5.1.1 Autologous Antibody Responses

To better understand the autologous neutralization of the Env glycoprotein during chronic infection we tested the participants' AnAbs against their autologous Envs using the functional pseudovirus-based assay. Each of the participants' study entry and exit viruses were tested against their study entry and study exit plasma samples. In the slow progressors a total of 19 autologous Envs were tested against their study entry plasma and study exit plasma (range: four to six viruses per participant). Figures 4.1 and 4.2 illustrate the patterns of autologous neutralization in both groups, generally in the slow progressors their Envs were less sensitive to neutralization requiring higher levels of nAbs as evidenced by the lower nAb IC_{50} titers than in the progressors. In the progressors a total of 18 autologous Envs were

tested against their study entry plasma and study exit plasma (range: three to six Envs per participant). The figures illustrate the patterns of autologous neutralization (figures 4.1 & 4.2). Whenever the study entry point Env was tested for neutralization to the study exit plasma, it was noticed that this response was always more potent than the contemporaneous response, i.e. when study entry Env was neutralized by the study entry plasma.

The slow progressors displayed a consistent pattern when the autologous viruses were neutralized by the study entry and study exit plasma samples. Although all the viruses were neutralized, they required higher concentration of plasma nAbs (evidenced as lower nAb IC_{50} titers) for neutralization, approximately more than five-fold to achieve 50% neutralization compared to the progressors (see figure 4.1).

In all the progressors there was a distinct neutralization profile when the entry time point (baseline) viruses are neutralized by the study exit plasma; viruses from the entry time-point were more effectively neutralized at a higher nAb IC_{50} titers the study exit plasma compared to the contemporaneous responses. In three out of four progressors (SK010, SK200 and SK221), a ten-fold higher dilution of plasma was required to achieve 50% neutralization compared to the rest of the study cohort. In summary, there were no differences in nAb IC_{50} titers between the groups when the study entry and study exit AnAb responses were compared.

As shown in Figure 4.3A, there was no difference in the nAb titers when the study entry Envs were tested against the study entry or study exit plasmas in the slow progressors (median nAb IC_{50} of 502 versus 627, p=0.08). In contrast, there were significant differences in responses in progressors when study entry Envs (Figure 4.3B) were tested against study exit plasma, compared to the contemporaneous response at study entry (median nAb IC_{50} of 2,172 versus 425, p=0.003) and study exit (median nAb IC_{50} of 2,172 versus 223, p= 0.0002). This result

suggests that a robust nAb response is continuously mounted against the evolving virus during progressive HIV-1C disease. However, there was no difference between the slow progressors and progressors nAb titers of exit plasma against entry Envs (median nAb IC_{50} 502 versus 2,172; p=0.05).

4.5.1.2 Env Autologous Neutralization (nAb IC_{50} Titers) correlates with the Length of The Hypervariable Regions in C3-V5 and V1-V5 in Slow Progressors and in V1-V2 For Progressors

Previous studies have shown an inverse association between neutralizing antibody titers and length of variable loops (V1-V2) and numbers of potential N-linked glycosylation sites (PNGs) (Chackerian et al., 1997, Pinter et al., 2004, Sagar et al., 2006, Gray et al., 2007, Rong et al., 2007a). To better understand the relationship between the genotypic and phenotypic characteristics, the amino acid length of the hypervariable loops of *env*, and PNGs were correlated to autologous neutralization titers as shown below in figure 4.4.

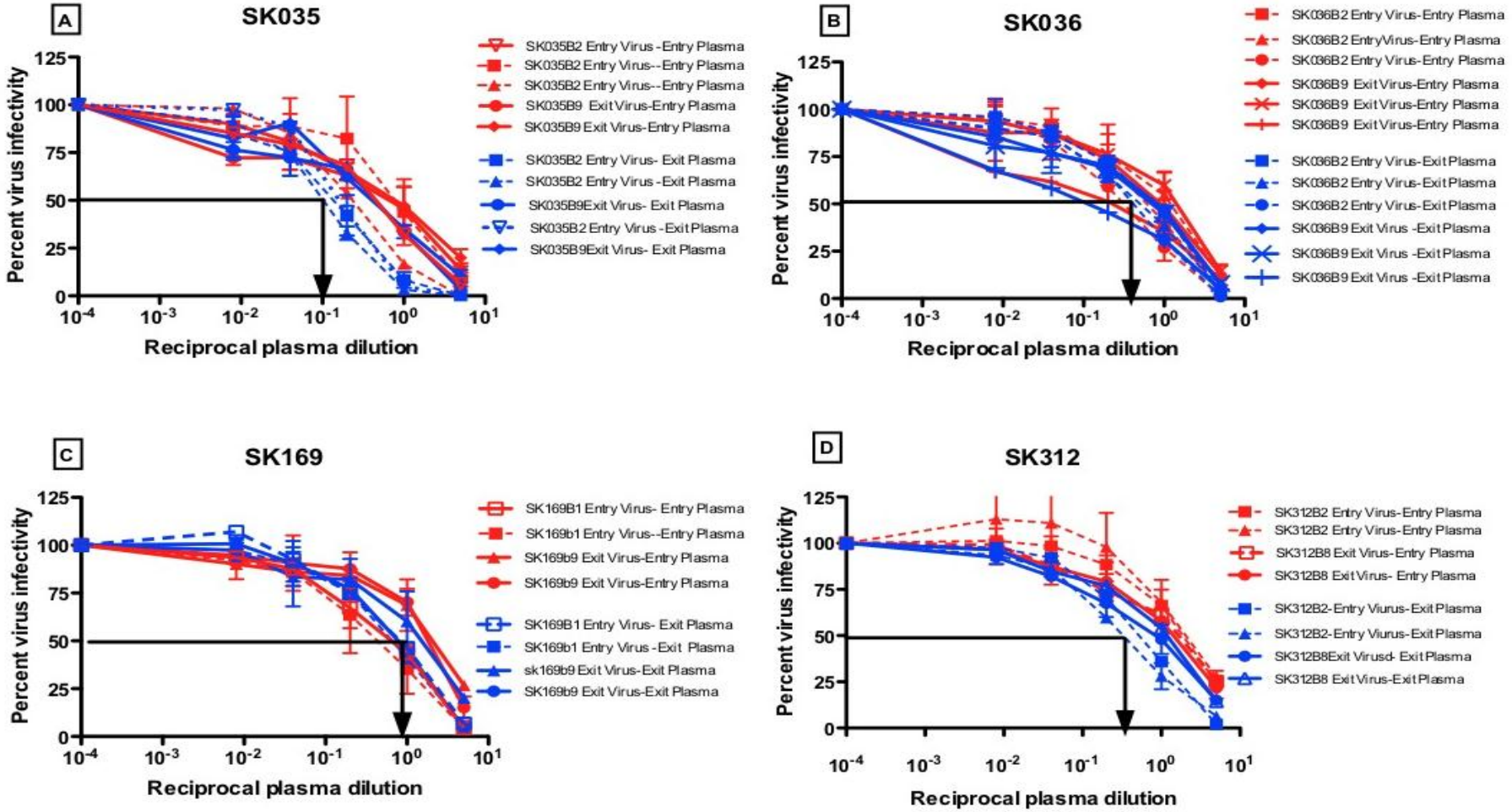

Figure 4.1. Autologous neutralization profiles for individual slow progressors for study entry and study exit autologous Env pseudoviruses. Red dotted lines represent study entry viruses neutralized by the study entry plasma (contemporaneous response), blue dotted lines represent study entry viruses neutralized by the study exit plasma. Solid red lines represent study exit viruses neutralized by the study entry plasma and solid blue lines represent study exit viruses neutralized by the study exit plasma (contemporaneous response). Black arrows indicate the reciprocal plasma dilution effecting 50% viral inhibition when study entry viruses were neutralized by the study exit plasma.

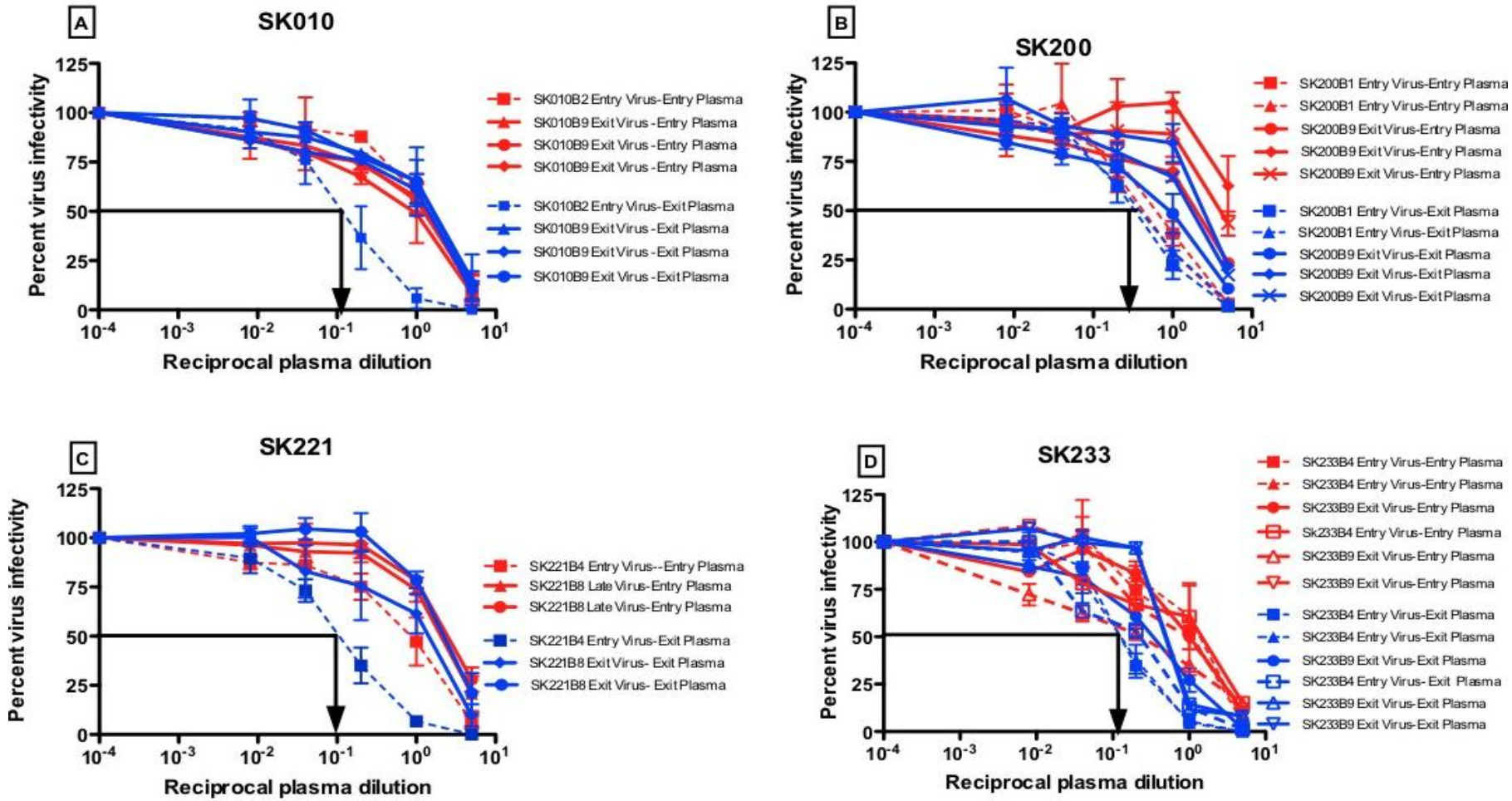

Figure 4.2. Autologous neutralization profiles for individual progressors for study entry and study exit autologous Env pseudoviruses. Red dotted lines represent study entry viruses neutralized by the study entry plasma (contemporaneous response), blue dotted lines represent study entry viruses neutralized by the study exit plasma. Solid red lines represent study exit viruses neutralized by the study entry plasma and solid blue lines represent study exit viruses neutralized by the study exit plasma (contemporaneous response). Black arrows indicate the reciprocal plasma dilution effecting 50% viral inhibition when study entry viruses were neutralized by the study exit plasma.

Overall, there were no significant correlations between the amino acid lengths of V1-V2, C3-V5 or V1-V5 to autologous nAb IC_{50} titers (p=0.16; p=0.4; and p=0.16 respectively) for the entire group (when data for slow progressors and progressors were combined).

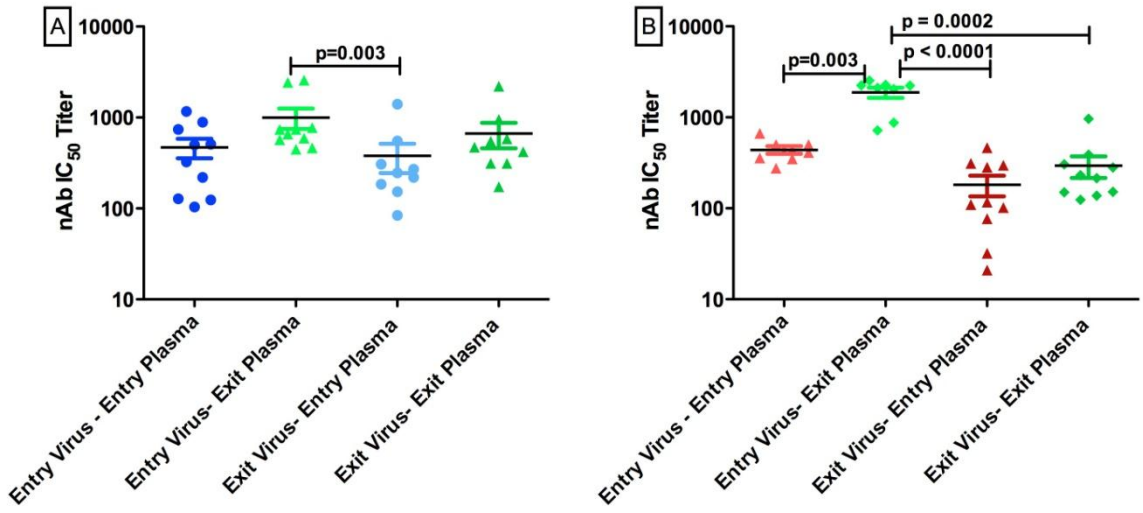

Figure 4.3. Autologous nAb IC_{50} titers in study participant study entry and study exit plasma samples for slow progressors (A) and progressors (B). p-values <0.05 were considered significant. *p*-value was calculated using the two-tailed Mann-Whitney non-parametric test overall. All the p-values (p< 0.0125) remained statistically significant after Bonferroni adjustment for multiple comparisons.

In figure 4.4, the amino acid lengths of V1-V2, C3-V5 and V1-V5 was correlated to nAb IC_{50} titer for the entry virus neutralized by the study exit plasma. The rationale for using this sample for the correlation is based on the progressors maintaining a statistically higher nAb IC_{50} titer compared to other time-points as shown in figure 4.2 above. There were significant positive correlations between amino acid length and nAb IC_{50} titer in C3-V5 (p=0.03) and V1-V5 (p=0.04) in slow progressors, however in the progressors there was a significant negative correlation in V1-V2 (p=0.04) and nAb IC_{50} titers (Figure 4.4, Panel B). The same analysis was extended for the numbers of potential N-linked glycosylation sites (PNGs) in the

various hypervariable loops and nAb IC_{50} titers. Interestingly, the only significant positive correlation was the numbers of PNGs in V1-V2 for the slow progressors only (p=0.03) with nAb IC_{50} titer.

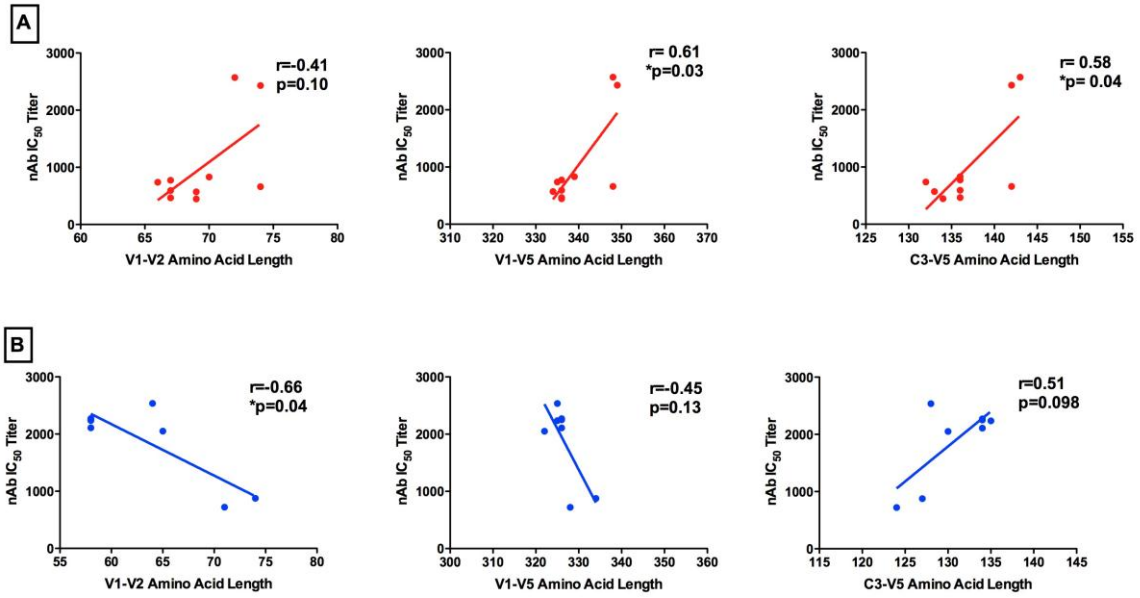

Figure 4.4. Correlation between the length of V1-V2, C3-V5 and V1-V5 of Env and autologous neutralization titer (nAb IC_{50} titer) when study entry viruses were neutralized by the study exit plasma samples in slow progressors (panel A) and progressors (Panel B). The Spearman r coefficient and p-values are shown and the correlation was significant when $p<0.05$.

4.6 Neutralization Assays using Heterologous Viruses

4.6.1 Neutralization Breadth

To better understand and assess the extent of heterologous neutralization in this chronic infection cohort, we investigated plasma neutralization activity over a median period of 21 months between sampling. Figure 4.5 depicts the neutralization profile of heterologous

viruses for the eight study participants at study entry and study exit. All plasma samples were assayed using the TZM-bl neutralization assay against various standard virus reference panels. A total of 20 Envs (figure 4.5) from a standard reference panel as depicted in table 2.12 (see chapter two) above were tested for breadth of neutralization. The virus panels included eight subtype C viruses of which two were from South African isolates identified during the acute phase of infection from the CAPRISA 002 study (Gray et al., 2007), seven subtype B, and five subtype A. Positive neutralization was scored at titers above 1:45. The percentage of neutralization was calculated by the number of viruses neutralized by each sample divided by the total number of viruses tested. Likewise the percentage viruses neutralized per subtype panel was calculated by the number of viruses neutralized by each participant sample divided by the total number of viruses tested in that subtype panel. We discuss the results for this experiment in terms of percentage (%) breadth of neutralization per subtype reference panel, and total % neutralization breadth including all the subtypes C, B and A panels. In addition, potency of neutralization is also discussed in terms of the nAb IC_{50} titer, with a titer of >1,000 as being highly potent.

There was a wide range of variation in neutralization titers with most of the sera from both slow progressors and progressors displaying heterologous activity particularly against the subtype C panel, indicating a very subtype-specific response. MW965.25 (a tier I virus) was potently neutralized by every participant's samples as evidenced by a nAb IC_{50} titer >10,000- figure 4.5. All of the participants neutralized ConC, except for one progressor (SK010). Of particular interest were the neutralization titers for DU156.12, a Tier 2 virus because in both groups there was a consistent number of participants at study entry and study exit that neutralized this virus. Three of the four slow progressors (nAb IC_{50} titer-median 143; range 66-455) and three of the four progressors (nAb IC_{50} titer- median 1332; range 197-9305)

neutralized DU156.12. When the median nAb IC_{50} titers for DU156.12 were compared, progressors had a significantly (p=0.03) higher nAb IC_{50} titer relative to the slow progressors. None of the Tier 2 viruses in either panels of subtypes A or B had similar neutralization profiles when tested against any of the plasma samples.

For the subtype B panel, all study participants potently neutralized SF162 (Tier 1- see figure 4.5) except for SK010. However, it should be noted that although SF162 was potently neutralized by most of the participants' plasma, this was not the case for the rest of the subtype B panel. Within the slow progressor group, only two participants (SK036 and SK312) displayed low nAb IC_{50} titers (nAb IC_{50} titer- median 83; range 77-162) to 6535.3 and in the progressors, three out of four (SK200, SK221 and SK233) had neutralization activity to this virus, although the nAb IC_{50} titers were not different (nAb IC_{50} titer- median 222; range 52-391) to those of the slow progressors. Although none of the nAb IC_{50} titers exceeded 1,000, progressors- SK200, SK221 and SK233 did neutralize between five and six out of the seven viruses in the subtype B panel. QHO692.42 was the only subtype B virus not neutralized by SK200 (progressor) plasma.

Progressors showed a significantly higher median of 57% neutralization breadth (range 14-86%) for the subtype B panel compared to slow progressors 21% (range 14-28%, p=0.03). There was no difference in % neutralization between the groups for either subtype A (median- 50% in both slow progressors and progressors) or subtype C (median 63%

		Subtype C Viruses								Subtype B viruses								Subtype A Viruses						
Participant ID	MW965.25	ZM197M.PB7	ConC	DU156.12	DU172.17	ZM214M.PL15	CAP45.G3	CAP239.G3	% Neutralized	SF162.LS	6535.3	QHO692.42	AC10.0.029	WITO4160.33	TRO.11	PVO.4	% Neutralized	Q23.17	Q842.d12	Q168.a2	Q461.e2	Q769.d22	% Neutralized	Total % Viruses Neutralized
SK035B2*	64815	45	227	45	451	289	45	45	50	26474	45	45	45	45	45	45	14	45	45	45	45	45	0	25
SK035B9*	59544	54	331	45	839	512	45	45	63	52005	45	45	45	45	45	45	14	45	45	45	45	45	0	30
SK036B2*	70370	45	160	66	59	45	49	68	75	20861	88	45	45	45	45	45	28	94	149	106	59	110	100	65
SK036B9*	167454	45	111	111	71	45	45	57	63	57314	162	45	45	45	45	45	28	76	167	96	45	110	80	55
SK169B1*	19573	45	1070	281	60	45	45	45	50	1307	45	45	45	45	45	45	14	591	45	45	45	45	20	30
SK169B9*	26925	121	1383	455	146	45	147	882	88	2592	45	45	45	45	45	45	14	1447	45	45	45	109	40	50
SK312B2*	37815	170	222	120	45	45	172	45	63	392	78	45	45	45	45	45	28	234	254	60	45	45	60	50
SK312B8*	63666	114	253	174	45	45	134	160	75	550	77	45	45	45	45	45	28	208	223	66	45	45	60	55
SK010B2#	21551	45	45	45	45	45	161	45	25	42500	45	45	45	45	45	45	14	45	45	45	45	45	0	15
SK010B9#	43309	45	45	45	45	45	45	45	13	32462	45	45	45	45	45	45	14	45	45	45	45	45	0	10
SK200B1#	24592	971	741	9305	101	935	5326	155	100	10945	121	45	45	45	46	45	43	1316	45	54	45	45	40	65
SK200B9#	29147	4725	847	8643	92	1534	2185	632	100	18709	231	45	56	83	73	156	86	1829	234	118	45	45	60	85
SK233B4#	10523	45	63	231	48	45	200	45	63	4976	52	45	49	95	45	45	57	312	45	45	45	45	20	50
SK233B9#	31354	89	773	197	170	45	878	57	88	17454	212	45	301	762	66	45	71	1678	104	113	45	93	80	80
SK221B4#	41559	48	823	1940	45	45	163	45	63	7615	248	45	79	45	111	45	57	294	101	120	45	45	60	60
SK221B8#	30264	218	375	724	62	45	750	194	88	3380	391	45	45	153	116	51	71	155	77	117	45	45	60	75

>1000
>100 and <1000
>45 and <100
45

B2= Study Entry Timepoint
B9 = Study Exit Timepoint
* Slow Progressor
# Progressor

Figure 4.5. Heterologous responses of participant plasma neutralization at study entry and study exit to 20 Env pseudoviruses from a standard reference panel including subtypes C, B and A of tier 1, tier 2 and tier 3 categories over a median period of 21 months. The neutralization titer is shown as reciprocal plasma dilution required to inhibit 50% of virus infection when the virus is neutralized by the participant's plasma. The highest titer (>1000) is shown in red, and the lowest in light orange, yellow depicts a titer of <1:45 that is below detection as shown above.

in both slow progressors and progressors). Overall, the progressors had higher % neutralization against all Envs (median 62% vs. 50%, p=0.4) but this difference was not significant.

The slow progressors displayed greater neutralization breadth against the subtype A panel compared to the progressors, with SK036 having a 100% neutralization at study entry and 80% at study exit as shown in figure 4.5. One progressor, SK233 had evolved neutralization breadth over time for the subtype A panel, with 20% at study entry and 80% at study exit with modest increases of nAb IC_{50}. However, the progressors did display more neutralization breadth against the subtype B panel compared to the slow progressors.

Interestingly, the total percentage neutralization in three of the four progressors increased over a median of 21 months (SK200, SK221 and SK233). Similarly, three out of four slow progressors (SK035, SK036 and SK312)) had an increase in the total percentage of viruses neutralized over time. Generally in both progressors and slow progressors there was no nAb IC_{50} titer to any of the tier 2 subtype A or B viruses that was greater than 1000. Three out of the four slow progressors also had an evolution of neutralization breadth from study entry to study exit to the subtype C panel of viruses (50 to 63% for SK035, 50 to 88% for SK169 and 63 to 75% for SK312) with the remaining participant SK036, having a loss of neutralization breadth at study exit (75 to 63%).

There was a substantial heterogeneity of neutralization breadth, particularly in the progressors. We discuss a progressor, SK200 in detail, as this participant displayed the most neutralization breadth and also had potent nAb IC_{50} titers (>1,000- see figure 4.5) to five of the eight subtype C viruses tested including three of five tier 2 subtype C viruses. Progressor SK200 had an evolving breadth at study exit, this participant's plasma neutralized 100%, 86% and 60% of subtypes C, B and A respectively. SK200 plasma neutralized a total of 65%

at study entry and 85% at study exit of all the reference viruses tested that indicated a higher breadth of neutralization over time. In particular, SK200 displayed 100% neutralization breadth to the subtype C viruses and for progressors- SK221, and SK233 there was an evolving neutralization breadth to the subtype C panel from study entry to study exit (63 to 88% for SK221 and SK233), whereas SK010 lost neutralization for CAP45.G3 over time, which also translated into loss of breadth. Tier 2 viruses -CAP45.G3 and CAP239.G3 were neutralized by SK200 study entry and exit plasmas, and CAP45.G3 in particular, was neutralized at nAb IC_{50} titers of >2,000. SK221 and SK233 both neutralized CAP45G3 more potently at study exit, being about > four-fold increase in nAb IC_{50} titers than at study entry with none of the nAb IC_{50} titers greater than 1,000. In addition, these two participants neutralized CAP239.G3 at study exit only. Another observation was that the neutralization titers (nAb IC_{50} titers) were generally higher in the progressors (nAb IC_{50} titer- median 475; range 161-5326) compared to the slow progressors (nAb IC_{50} titer- median 56; range 49-172), particularly against the tier 2 subtype C CAP45.G3. For the slow progressors, none of the nAb IC_{50} titers against the tier 2 Subtype C viruses were greater than 1,000.

It is noteworthy that SK200 also displayed potent nAb IC_{50} titers >1000 to a subtype A tier 1 virus- Q23ENV17 compared to the other progressors. One slow progressor however, SK169 also had an approximate three-fold increase in nAb IC_{50} titer at study exit to Q23ENV17 (nAb IC_{50} titer of 591 to 1,447).

We extended the analysis to each of the subtype virus panels and compared the average nAb IC_{50} titers of the progressors to the slow progressors. There was a significantly higher average nAb IC_{50} titer to the Tier 2 viruses in the subtype B virus panel only in progressors (average nAb IC_{50} titer- 77) when compared to slow progressors (nAb IC_{50} titer- 45; $p=0.005$), and this remained significant when corrected for multiple comparisons ($p<0.008$).

None of the other comparisons either inclusive or exclusive of the Tier 1 viruses for either subtypes A or C were statistically different.

4.6.2 Correlation of Neutralization Breadth with CD4 T-Cell Counts and Viral Loads

There are a battery of studies that showed an inverse relationship between neutralization breadth and CD4 T-cells and a positive correlation with viral loads (Piantadosi et al., 2009, Euler et al., 2010, Sather et al., 2009, Gray et al., 2011a). To better understand the relationship between neutralization and clinical disease markers in this chronic cohort, we correlated the participants' CD4 T-cell counts and viral loads over time to total percentage (%) of viruses neutralized (neutralization breadth).

As shown in figures 4.6A and 4.6B, in slow progressors and in figures 4.6C and 4.6D in progressors there were no significant correlations between CD4 T-cell count or viral loads and total % of viruses neutralized by each individual.

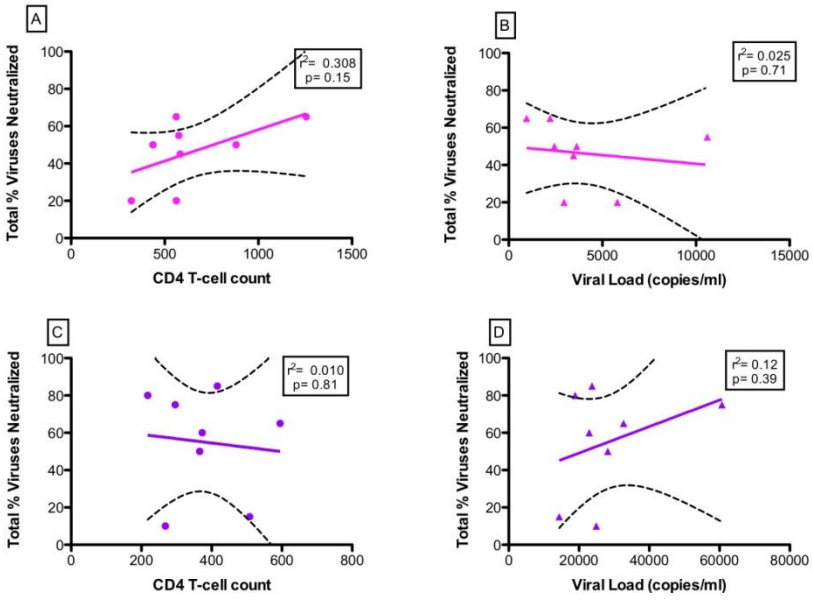

Figure 4.6. Correlation of total percentage of viruses neutralized (neutralization breadth) with CD4 T-cell counts and viral loads in slow progressors (A & B) and progressors (C & D). r^2 values and p-values are shown.

4.6.3 Mapping of Epitopes Targeted by Cross-Neutralizing Antibodies

The targets of broadly neutralizing antibodies are subjects of much interest in the vaccine field. A recent study by Walker and colleagues (2010) elucidated a number of key sites targeted by individuals with neutralization breadth. The sites included N160, I165 in V2 and N332 in V3 (Walker et al., 2010). Therefore, participants in our study that displayed the highest breadth of neutralization against different subtypes (e.g. A, B and C) - SK200, SK221 and SK233, were then further investigated using their study exit plasma samples only (study entry plasma samples were no longer available). Using CAP45.G3, ConC, Du156.12, TRO.11 and Q23.17 with single-point mutations in specific regions e.g. position N160A, K169E, I165A and N332A that map to V2, V3 and C3 respectively, we further defined putative targets of the progressors' nAbs. A decrease of >three-fold for the neutralization titers was indicative of neutralization resistance (Gray et al., 2011a).

As depicted in Table 4.3, 5.6-fold and 4-fold drops in neutralization titers (compared to the wild type neutralization titers) were seen when participants' SK221 and SK233 study exit plasma samples neutralized CAP45 K169E respectively. These observations suggest that the broadly neutralizing antibodies in these two participants most likely target the V2 region and that a charge change from a lysine (K) to glutamic acid (E) induced neutralization resistance. In addition SK221 plasma also showed a 4.2 fold drop in neutralization titers to TRO.11 N332A indicating that this asparagine (N), or perhaps the N-linked glycosylation at this position, was essential for the antibody activity. Overall, the variability in the fold-drop in neutralization titers for the K169E mutations in CAP45 and ConC, and for the N332A mutation in Tro.11 may be reflective of structural and conformational differences of the viruses studied. Together, these results suggest that SK221 most likely has cross nAbs that target the V2 and C3 regions on Env however we could not establish whether these nAbs

evolved as the study entry sample was not tested due to limited availability of plasma to perform such experiments. Intriguingly, SK200, the participant that displayed the most neutralization breadth, also neutralized the mutated Env indicating that this particular participant may have antibodies directed to other regions of Env and this participant's nAbs therefore warrants further investigation.

Table 4.3. Effects of single point mutations on neutralization sensitivity and summary of antibody specificities.

Plasma sample identity	Fold effect of mutation[a]									Antibody specificity conferring breadth
	ConC N160A	ConC I165A	ConC K169E	CAP45 N160A	CAP45 I165A	CAP45.G3 K169E	Du156.12 N332A	Q23.17 N332A	Tro.11 N332A	
SK200 study exit	1.1	0.5	0.7	1.0	0.6	1.9	1.2	1.5	0	Unknown
SK221 study exit	0.4	0.3	1.1	2.0	0.4	**5.6**	1.4	0.6	**4.2**	Quaternary, PG9/PG16 like, N332
SK233 study exit	0.1	0	0.6	0.8	0.9	**4**	0.7	1.2	0	Quaternary, PG9/PG16 like

[a] Calculated as wild type IC_{50}/mutant IC_{50} for the plasma. Changes in titer of >3-fold are shown in bold.

4.7 HIV-1-Specific IgG Binding Affinities for Gp120, Gp41 and p24 between Slow Progressors and Progressors

We isolated total immunoglobulins (IgGs) in both groups of participants at study entry and study exit time points and studied the binding of IgGs to HIV-1-specific antigens and to Fcγ receptors. We then quantified the 50% effective concentration (EC_{50}) of each batch of total IgGs needed to bind HIV-1-specific gp120, gp41 and p24 antibodies and various Fcγ receptors using an ELISA-based assay. EC_{50} is a concentration in µg/ml- it corresponds to the concentration of antibody needed to achieve half of the maximal binding to an Fcγ receptor for example. The EC_{50} binding titer of the IgGs was used as a surrogate indicator of

how strongly or poorly the IgGs' Fc portion may bind to the Fcγ receptor to recruit effector cells like macrophages, dendritic cells etc to initiate either ADCVI or ADCC activity. Essentially, the higher the EC_{50} the more antibody is needed for antiviral activity like ADCC or ADCVI to occur, the lower the affinity of antibody binding to the respective target cells bearing the antigens e.g. p24, gp120 or gp41 or to the Fcγ receptors to antibodies to initiate effector functions like ADCVI. The median EC_{50} for gp120 IgGs (EC_{50} 141.5 μg/ml) and gp41 IgGs (EC_{50} 135.8 μg/ml) trended toward being higher at study entry in the progressors (EC_{50} range 130.2-153 μg/ml for gp120 and range 71.6-143.5 μg/ml for gp41) only, but this result was not statistically significant either when compared to the slow progressors study entry and exit EC_{50}levels or to the progressors study exit values (figures 4.7A and 4.7C). Over the study period, there was no difference between the slow progressors and progressors median EC_{50} for both gp120 or gp41 ($p=0.50$ and $p=0.33$ respectively).

For p24 IgG, the slow progressors showed a median EC_{50} increase from study entry (134.92 μg/ml) to study exit (150.17μg/ml- figure 4.7E) the same trend was seen in the progressors (142.17 μg/ml at study entry and 166.35μg/ml at study exit). The median EC_{50} binding titers for p24 IgGs were also not different between the groups (136.6 μg/ml for slow progressors and 166.3 μg/ml for progressors; $p= 0.88$). There was also no difference in the median EC_{50} values between the groups over time with the progressors displaying an overall higher median EC_{50} binding titers for all three antigen-specific IgGs. In addition, there was no significant correlation between the levels of CD4 T-cells or viral loads with the EC_{50} of gp120, gp41 or p24 (see figures 4.8A to 4.8D) over the study period.

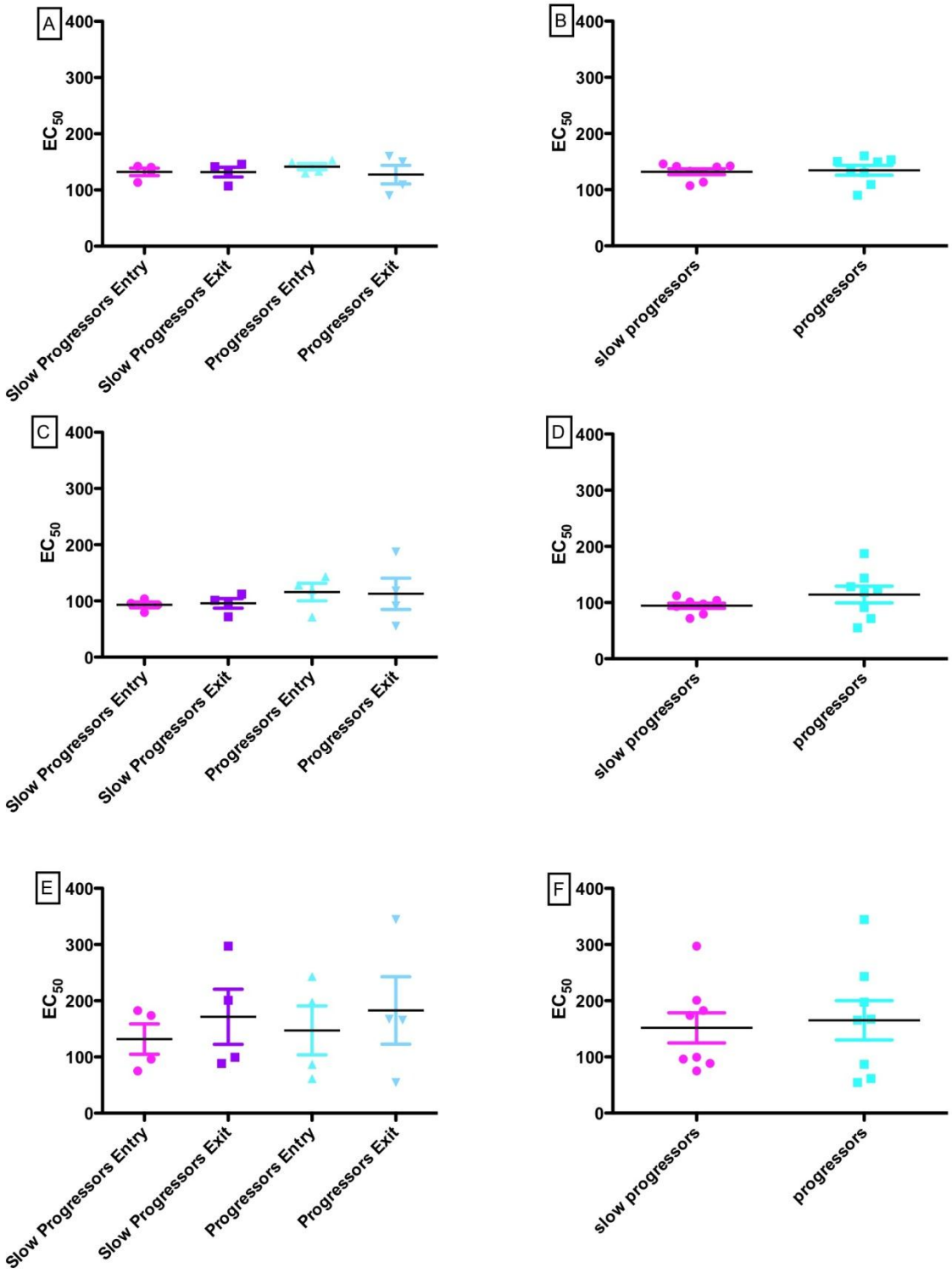

Figure 4.7. EC_{50} binding titers of gp120, gp41 and p24-specific IgGs in slow progressors and progressors stratified into study entry and study exit values (A, C and E), and also over time with combined study entry and study exit values for slow progressors and progressors (B, D and F). There were no statistical differences between the slow progressors and progressors for any of the EC_{50} binding titers of the various IgGs.

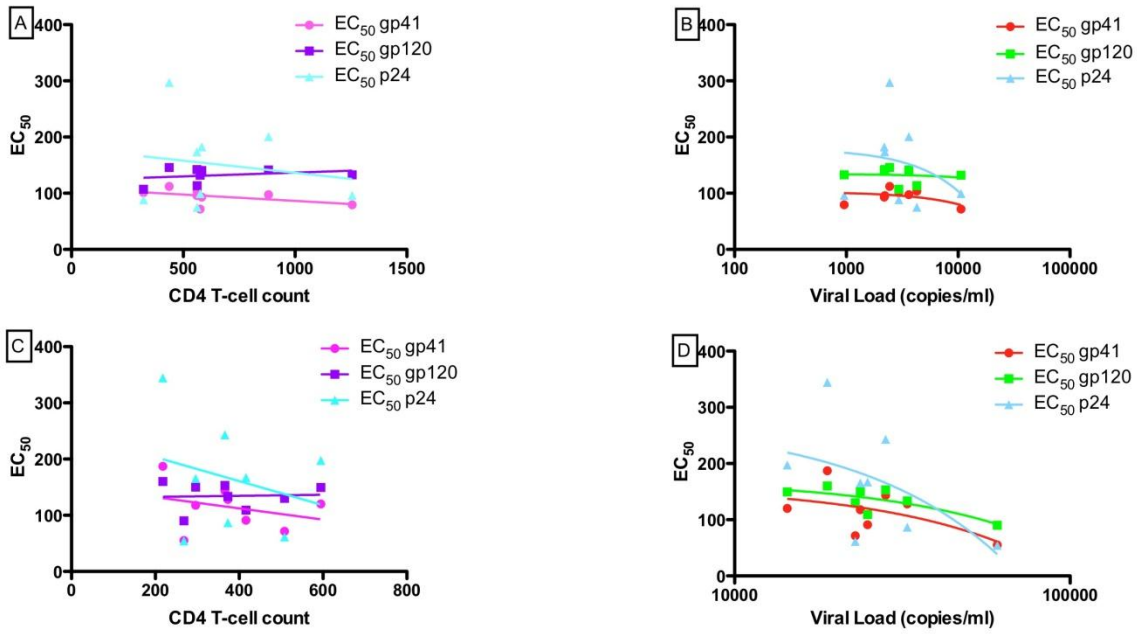

Figure 4.8. Correlation of IgG EC_{50} binding titers for gp120, gp41 and p24-specific antibodies versus CD4 T-cell counts and viral loads in slow progressors (A & B) and progressors (C & D). None of the correlations were statistically significant.

4.8 IgG Binding Titers for FcγR Ia/IIa/IIb And IIIa between Slow Progressors and Progressors as a Surrogate Marker For Binding Affinity to Initiate ADCC or ADCVI

To determine if the binding affinities of the IgGs with various activating and inhibitory Fcγ receptors were different between the slow progressors and progressors, we measured and compared the EC_{50} binding titers at study entry and study exit time-points, and overall between the two groups. When the EC_{50} of the IgGs for the various Fcγ receptors were measured, there were no differences either for the intra-group comparison for study entry versus study exit or between the slow progressors and progressors over time (figures 4.9A – 4.9G). The slow progressors IgGs consistently showed a higher median EC_{50} for FcγRI, IIa, IIb and IIIa at study entry (FcγRI 7.58 μg/ml; FcγRIIa 10.73 μg/ml; FcγRIIb 22.55 μg/ml and FcγRIIIa 27.24μg/ml) and a consistent relative decline at study exit (FcγRI 6.71; FcγRIIa

8.53 μg/ml; FcγRIIb 18.75 μg/ml and FcγRIIIa 27.22 μg/ml). Similarly, the median IgG EC_{50} for progressors for FcγRI (7.80μg/ml), FcγRIIa (8.42μg/ml) and FcγRIIb (20.92μg/ml) and FcγRIIIa (26.23μg/ml) was higher at study entry compared to study exit (FcγRI 6.89 μg/ml; FcγRIIb 20.46 μg/ml and FcγRIIIa 25.21 μg/ml) except for FcγRIIa (9.16μg/ml). Overall, there were no differences in the median IgG EC_{50} binding titers between the slow progressors and progressors over time for any of the Fcγ receptors (p=0.65 for FcγR1; p=0.72 for FcγRIIa and FcγRIIb; p=0.89 for FcγRIIIa).

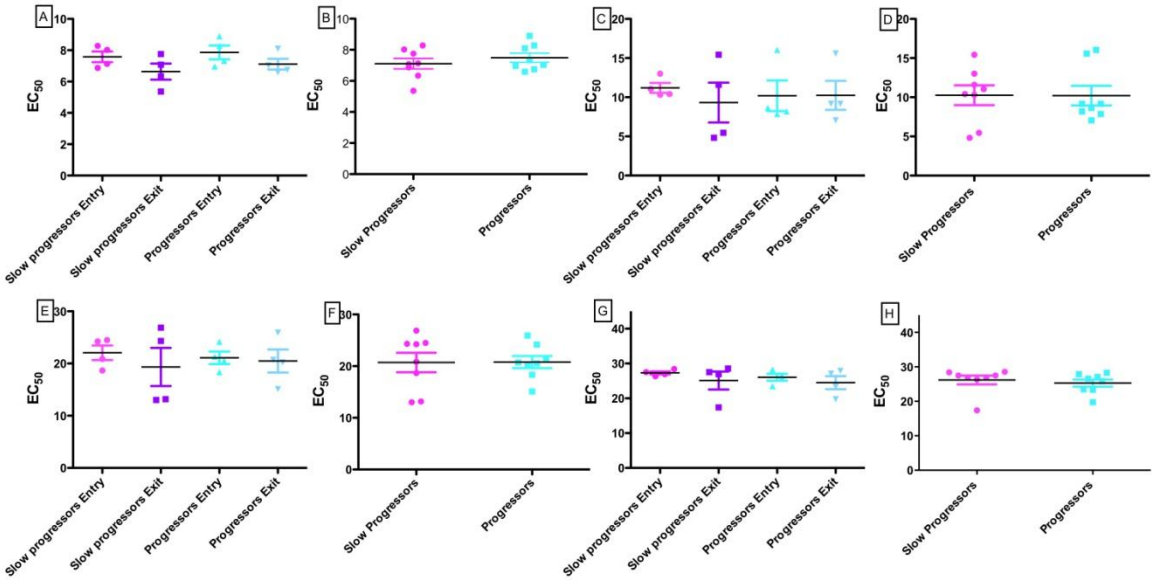

Figure 4.9. EC_{50} binding titers of IgGs for FcγRI, FcγRIIa, FcγRIIb and FcγRIIIa in slow progressors and progressors stratified into study entry and study exit values (A, C, E, and G), and also over time with combined study entry and study exit values for slow progressors and progressors (B, D, F and H). None of the intra- or inter-group comparisons were statistically different i.e. $p<0.05$.

In addition, the median IgG EC_{50} ratios of the stimulatory (FcγRI, FcγRIIa, and FcγRIIIa) to the inhibitory receptors (FcγRIIb) over time in both groups (figure 4.10A, 4.10B) were also similar with the slow progressors having a higher ratio for FcγRIIIa/ FcγRIIb (figure 4.10C)

compared to progressors, but this difference was not statistically different (p=0.80). There were no significant differences between slow progressors and progressors for FcγRI/ FcγRIIb (p=0.96) or for FcγRIIa/ FcγRIIb (p=0.80).

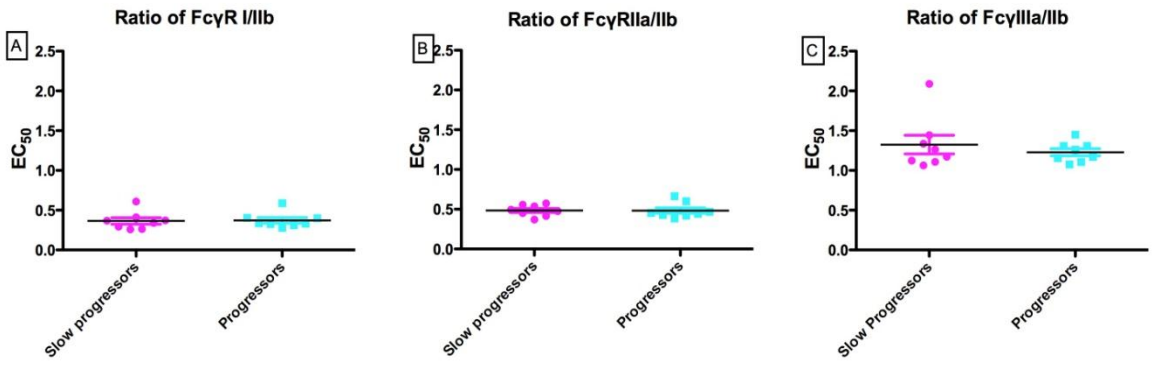

Figure 4.10. Ratios of IgG EC_{50} for FcγRI, FcγRIIa, and FcγRIIIa to FcγRIIb i.e. the activating versus inhibitory receptors in slow progressors and progressors (A, B, C), over time with combined study entry and study exit values. There were no differences between the groups over time.

4.8.1 FcγIIa Receptor Levels Correlated With CD4 T-Cell Counts In Slow Progressors

Next, the IgG EC_{50} of Fcγ receptors I, IIa, IIb and IIIa were correlated to CD4 T-cell count and viral loads for the slow progressors and progressors. There was a positive correlation only in the slow progressors between the levels of CD4 T-cell count and IgG EC_{50} for FcγRIIa with r^2 value=0.75 and p=0.005 (figure 4.11A), this value remained statistically different after adjusting for multiple comparisons using the Bonferroni adjustment (p=0.0125). There were no other significant correlations between viral loads or CD4 T-cells with the EC_{50} binding titers of the various receptors in the slow progressors or progressors.

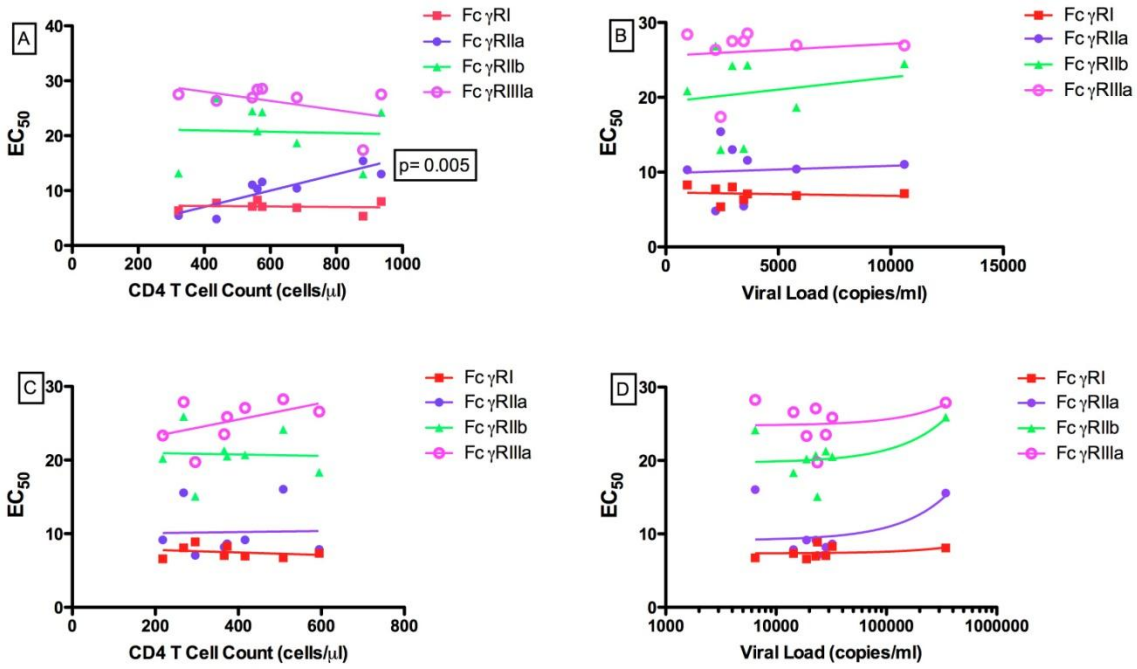

Figure 4.11. Correlation of CD4 T-cell counts and viral loads with IgG Effective Concentration at 50% (EC_{50}) for activating (FcγRI, FcγRIIa and FcγRIIIa) and inhibitory (FcγRIIb) receptors in slow progressors (A and B) and progressors (C and D). Only p-values <0.05 are shown.

4.9 Discussion

The role that nAbs and non-neutralizing antibodies play in disease progression particularly in HIV-1 infection, is poorly understood and has been the subject of much debate. We therefore undertook this study in this HIV-1 subtype C chronically infected cohort in order to establish whether neutralizing and non-neutralizing antibodies may be able to attenuate disease progression, and whether certain genotypic *env* characteristics influence autologous neutralizing antibody responses.

Certain envelope characteristics such as the amino acid length of certain hypervariable regions, PNGs, and mutations of sentinel amino acids in antibody target epitopes have an effect on neutralization sensitivity/resistance. We also investigated what the putative

differences were between the neutralization sensitivities relative to the *env* genotypic characteristics in different states of disease progression. In addition, the binding affinities of HIV-1-specific IgGs and the affinities of the IgGs to various Fcγ receptors (both activating and inhibitory) were used as a surrogate indication of recruitment of effector functions of cells of the innate immune system e.g. macrophages or natural killer cells to initiate ADCC or ADCVI and these were correlated to markers of disease progression like CD4 T-cell count and viral loads. Several studies have shown that the binding interaction and the strength of the binding between the Fc and the FcγR directly affects the potency of the effector functions of FcγR–bearing cells, and by altering the antibody properties through deglycosylation and site directed mutagenesis one can manipulate the strength of binding affinity (Shields et al., 2001). This may in turn affect the downstream ADCC/ADCVI activity of the effector cells. Based on these studies we therefore exploited an "in house" binding affinity ELISA

One of the more controversial issues regarding nAbs, is the extent to which they are protective or not during different stages of HIV-1 disease progression. Using a single-cycle of infection assay with recombinant pseudoviruses to measure nAbs against autologous and heterologous viruses, we made a number of observations for the role of nAbs in slow progressors and progressors during the chronic HIV-1 subtype C infection state. Firstly, although high level neutralizing titers to contemporaneous autologous virus were not observed in most of the participants, our findings of higher autologous responses over time in the progressors (when the study entry viruses were neutralized the study exit plasma) with higher viral loads, argues against the protective role of nAbs in chronic infection (Deeks et al., 2006). Secondly, the autologous viruses in all the participants could not completely escape AnAbs, which attests to the constraints on Env's mutability to escape the humoral immune system during the chronic infection stage. Indeed, we could not prove the

hypothesis that slow progressors have a more potent nAb response that protects them against disease progression as there was no difference in nAb titers when compared to progressors. Thirdly, the study entry viruses were more effectively neutralized by the study exit nAbs in all participants compared to the contemporaneous response, suggesting that viral evolution was present in both the slow progressors and progressors in a chronic stage of HIV-1 disease. Fourthly, it was found that the longer the amino acid lengths for regions V1-V5 and C3-V5 of Env were directly correlated with autologous nAb IC_{50} titers in the slow progressors. Interestingly, in contrast, there was an inverse correlation for V1-V2 length and nAb IC_{50} titers in the progressors. This observation suggests that increased length in these regions may be leading to neutralization resistance. Lastly, the correlation between the numbers of PNGs and autologous nAb IC_{50} titer in slow progressors, suggests that in a progressive disease state, the PNGs may be critical for neutralization by nAbs, increasing the potencies of nAbs or that there is a point beyond which the "evolving" or "shifting" glycan shield cannot escape nAbs.

Much of the nAb responses against heterologous viruses appears to be very type-specific, even more so for the slow progressors than the progressors. The progressors had evolved breadth of neutralization indicating that breadth and increasing breadth over time is a marker of disease progression in chronic subtype C infection (Euler et al., 2010). These observations are in keeping with other studies indicating that antigenic stimulation through higher viral load burden may be responsible for dictating the breadth of heterologous antibody responses (Mellors et al., 1997, Goujard et al., 2006, Fraser et al., 2007, Piantadosi et al., 2009, Sather et al., 2009, Doria-Rose et al., 2010). These findings are however in stark contrast to a recent study done in HIV-1 subtype B-infected patients with undetectable viraemia on ARV treatment, where there was broadly neutralizing antibody activity despite undetectable viral loads (Medina-Ramirez et al., 2011). However, the significant neutralization breadth against

the subtype B panel of viruses in chronic infection may have important implications for HIV-1 vaccine design in that a wider range of subtype B viruses were neutralized by plasma from subtype C infections. Further studies will be needed to confirm these results and to investigate underlying mechanisms.

We did not see any significant association between neutralization breadth and CD4 T-cell count or viral loads, a larger sample size may have established statistically significant correlations. Gray and colleagues (2011) found a significant correlation between the CD4 T-cell count and viral loads with neutralization breadth at six months post-infection only and not at later time points (Gray et al., 2011a). and these results may have important implications for HIV-1 vaccine design, the HIV-1 immunogens may have to be given over long periods of time in order to allow for antibody maturation and the development of broadly neutralizing antibodies through affinity maturation (Medina-Ramirez et al., 2011).

Development of neutralization breadth was not associated with slower disease progression (Euler et al., 2010). Slow progressors showed a lower neutralization breadth overall and maintained their CD4 T-cell counts suggesting that lack of neutralization breadth is associated with slower disease progression. For the progressors who did display cross-neutralizing antibody responses, two participants, SK221 and SK233 had autologous antibodies that recognized the lysine (K) in position 169 in V2, indicating possible quaternary epitopes that are targeted by PG9/PG16-like antibodies (Gray et al., 2011a). In addition SK221 also had antibodies that targeted the N332 glycan in C3 and suggests that SK221 targets epitopes in both V2 and C3 and that the asparagine in that position is essential for neutralization activity. However, we could not establish whether such antibodies were present at the start of the study or whether they evolved over time, as plasma sample availability was a limitation. Likewise we could not test for autologous neutralization against

autologous mutated Env due to lack of plasma sample availability to perform these assays. Despite these limitations and based on our data we propose that in subtype C infection, the V1, V2 and C3 regions are the immunodominant regions commonly targeted by AnAbs (Moore et al., 2008, Moore et al., 2009, Rong et al., 2009, Lynch et al., 2011) particularly during the early stage of infection (Gray et al., 2011a). Here we show that the immunodominance of these regions persist into chronic infection although we cannot delineate whether such antibodies evolved. It has been shown that there is a limited number of antibody specificities that dictate the breadth of neutralization (Walker et al., 2010) and that type specific antibodies sometimes develop into antibodies with cross neutralizing specificities (Gray et al., 2011a).

The decline of p24 antibody titers has been found in disease progression (Lange et al., 1986, Allain et al., 1987, Forster et al., 1987, Binley et al., 1997). In addition, here we show that in HIV-1 subtype C infection that the binding affinity of p24-specific IgGs declines over time to these antigens as demonstrated in both slow progressors and progressors. Chargelegue and colleagues (1995) found that low p24 IgG affinity correlated to HIV-1 disease progression (Chargelegue et al., 1995). In addition, the trend of declining affinities of gp120, gp41 and p24 antibodies in the progressors may be linked to disease progression as they may lose the ability to recruit the Fc-mediated functions. It is plausible that declining binding affinity of FcγRIIa in the slow progressors, heralds dysfunction in the expression of these receptors on the cells of the innate immune system (Dugast et al., 2011). Dugast and colleagues purport that HIV-infection is associated with a number of changes in FcR expression on phagocytic cells that are associated with changes in their ability to respond to antibody-opsonized targets, leading to a failure in viral clearance in different stages of infection (Dugast et al., 2011). Other factors that have been shown to affect the binding affinity of the non-

neutralizing antibodies include polymorphisms in the FcγRIIa (Forthal and Moog, 2009), and the extent of glycosylation of the antibodies (Forthal et al., 2010).

One of the strengths of our study is that we used single genome RNA plasma-derived *env*s to generate our recombinant viruses, by performing limited dilution assays we therefore would have sampled both the major and minor circulating *env*s and therefore have neutralization data based on these representative strains. Single genome amplification also allowed us to study real-life representative Env strains in patients, and thereby avoid possible artifactual PCR generated recombinants. However, one of the limitations of the study is that we only tested a limited number each of the viruses derived from the SGA amplicons, and therefore may have biased the results of the study. Another limitation is that we do not know the exact time of infection for these subjects. Therefore stratification of study subjects as progressors or slow progressors relied on short-term (21 months) follow-up immunological data, which may be an unrepresentative snap-shot of the entire natural history of disease progression for these participants. However, this concern was somewhat allayed by bioinformatic analysis of the study sequences that showed that consistent with the stratification, progressors in this cohort were more likely to have been infected for shorter period of time than slow progressors. Another limitation is that the sample size was too small to make significant conclusions regarding the significance of breadth of neutralization and autologous responses in these participants, however, this study does corroborate the trends of increasing neutralization breadth with disease progression.

4.10 Conclusions

NAbs and non-neutralizing antibodies do not protect against disease progression during the chronic stage of HIV-1 subtype C infection in this cohort. Breadth of neutralization correlates to disease progression in chronic infection. Breadth of nAb responses was higher in progressors which may indicate that *env* sequence footprints found in chronically infected progressors may be used in effective HIV-1 immunogen design for candidate vaccines. Target vaccine immunogens may have to be given over long periods of time to elicit the production of broad cross neutralizing antibodies with high binding affinity. Progressors had cross-reactive neutralizing antibodies targeting epitopes in V2 and V3 indicating that nAb breadth may be dictated by a limited number of target Env epitopes. The potencies of autologous nAbs are influenced by amino acid length of V1-V5 and C3-V5 and numbers of PNGs in V1-V2 in chronic progressive HIV-1 infection. The decreased binding affinities of IgGs to HIV-1-specific antigens and to FcγRIIa are associated with chronic HIV-1 disease progression.

Chapter Five- Discussion and Conclusions

5.1 Discussion

HIV-1 subtype C is the most rapidly spreading and globally dominant subtype. However, much of the vaccine research endeavor and immunogen design to date has focused on subtype B, largely due to the ease of availability of this subtype in the high income countries of North America and Europe where most basic studies on HIV-1 immunogen design have been done. Therefore there is urgency, despite the challenges of developing an HIV-1 vaccine, to elucidate the correlates of immune protection for HIV-1 subtype C in order to develop a globally relevant vaccine.

In this descriptive study, we aimed to identify *env* sequence characteristics that may distinguish progressors from slow progressors in a chronically HIV-1 subtype C infected antiretroviral naïve cohort. We used a single genome amplification approach in order to accurately and comprehensively represent the diversity of viral quasi-species. Several indicators of evolutionary forces were used to elucidate putative differences between the groups including heterogeneity of envelope sequence diversity, Env length polymorphisms, and numbers of PNGs as well as features such as positive selection, negative selection and signature sequence characteristics.

In addition, we also investigated putative differences in autologous antibody responses of slow progressors and progressors and did comparisons of titers of autologous (AnAb) responses relative to the *env* genotypic characteristics. Also, the breadth of the heterologous nAbs responses using a standard reference panel of subtypes A, B and C viruses were also determined in the divergent states of disease progression. Mapping of the putative AnAbs epitopes in progressors with neutralization breadth were also investigated through the

mutations of certain specific sentinel amino acids in putative antibody target epitopes of Env that have an effect on the neutralization sensitivity/resistance.

Furthermore, to define the role that non-neutralizing antibodies may have in HIV-1 disease attenuation, the binding affinities of HIV-1-specific IgGs to gp120, gp41 and p24; and the affinities of the IgGs to various Fcγ receptors (both activating- FcγRI/IIa/IIIa and inhibitory FcγRIIb) were assessed. These binding affinities were used as a surrogate indication of recruitment of effector functions of cells of the innate immune system that bear these specific receptors e.g. macrophages or natural killer cells to initiate ADCC or ADCVI and these were correlated to markers of disease progression like CD4 T-cell count and viral loads.

This study extends our knowledge of the interplay between both the *env* sequence and bioinformatic analysis of the *env* gene, and relates this information to nAbs, and examines whether non-neutralizing antibodies are different in divergent rates of chronic HIV-1 subtype C disease. In addition, the role of both neutralizing and non-neutralizing antibodies in HIV-1 subtype C disease progression also needs to be further defined as much of the knowledge thus far is based on subtype B infection. The correlates of immune protection and disease attenuation in subtype C infection remain understudied and poorly understood. We therefore undertook this study in order to fill in some of the gaps in knowledge regarding the evolution of *env*, and the roles of neutralizing and non-neutralizing antibodies in divergent rates of HIV-1 subtype C disease progression.

5.2 Envelope Evolution and Diversity

The full extent of the genotypic and structural nature, and the plasticity of the Env glycoprotein needs to be further understood in the context of chronic HIV-1 subtype C

disease progression in order to develop effective immunogens for an HIV-1 vaccine. Our study suggests that regions of *env* are under differential evolutionary pressures in slow progressors and progressors. Slow disease progression was associated with increased intra-patient diversity that is consistent with other studies on disease progression (McNearney et al., 1992, Markham et al., 1998, Mani et al., 2002). This finding contradicts our initial hypothesis that there is reduced viral diversification in slow disease progression. Perhaps this is reflective of the heterogeneity of diversity one sees in chronic HIV-1 disease compared to the primary stage where increased diversity was associated with rapid disease progression (Gray et al., 2007). Specific regions of Env including C2, V3 and C3 in slow progressors showed increased diversity suggesting that co-evolution of amino acids due to the close proximity of these regions is likely (Menzo et al., 1998, Gaschen et al., 2002). From a functionality standpoint it appears that, because the V3 loop is critical for viral entry, increased diversity in this region could be a correlate of viral attenuation (Abraha et al., 2009). Interestingly, we found that viruses in the slow progressors used both CCR5 and CXCR4 coreceptors equally and this could explain the higher diversity in this group.

Our study contradicts other studies that have found direct associations between amino acid lengths of the V1-V4 Env regions and the numbers of PNGs (Derdeyn et al., 2004, Rong et al., 2007b). These studies also illustrate the direct relationship between neutralization sensitivity and shorter V1-V4 regions (Derdeyn et al., 2004, Rong et al., 2007a). Interestingly, when we examined genotypic characteristics in relation to the phenotypic antibody responses, in progressors there was negative correlation between the nAb IC_{50} titers and the amino acid length in V1-V2, implying that increased length of certain regions of envelope may be a mechanism to evade nAb recognition. We found that longer lengths for V1-V4 loops with fewer PNGs had positive association with neutralization titers that is

discussed later on in this chapter. We propose that length polymorphisms in the constant and variable envelope regions may lead to varied protein folding of Env that ultimately affects the conformation and adds to structural diversity of Env. Additionally, when we examined the sequence footprints in progressors, a loss of a glycan in V4 was compensated for by a gain in a PNG within C3, implying a shifting glycan shield as suggested previously (Wei et al., 2003), which may be crucial for immune evasion.

High dN/dS ratios indicative of strong diversifying selection due to humoral immune pressure (Yang et al., 2003), occurred mainly within gp41 in progressors. Slow progressors on the other hand, had a number of regions targeted positively selected for along the Env gp160. This suggests that the nature of antibody targets may differ between the groups that may be due to the intrinsic sequence differences between the Envs. Interestingly, the α-2-helix within C3 of both groups experienced positive selection pressure and this suggests that C3 is under diversifying selection pressure (Gaschen et al., 2002) and a likely neutralization target in subtype C viruses (Gnanakaran et al., 2007, Rong et al., 2007b). Position 607 of gp41 was positively selected for in progressors and was also a signature sequence difference between progressors and slow-progressors, indicating that there may be putative humoral immune pressure driving escape at that position. Furthermore, mutations occurred at two putative antibody sites in gp41. These antibody sites were- ELDKWAS which was recognized by nmAb- 2F5, where DKW are the sentinel amino acids that determine sensitivity to 2F5 (Zwick et al., 2005) and a linear epitope- **N**WFNIT- that is recognized by nmAb 4E10. Mutations may confer possible loss of a putative antibody recognition sites which may then affect both autologous and heterologous virus neutralization. The effect of the loss of putative antibody recognition sites during chronic disease progression is unknown. In addition, the negatively selected sites along the entire gp160 in both slow progressors and

progressors may imply that there are some sentinel amino acids that need to be preserved in order to maintain the overall integrity of the Env. Negatively selected sites may be tool that we can use to hone in on crucial or functionally important sites in HIV-1 *env* and may be included in future immunogen design as a way to identify functionally important parts of the virus that would make attractive immunological targets. Together these results imply that the virus may gain selective advantages by exploiting multiple strategies to evade the immune system, including amino acid length, increased numbers of PNGs, and specific mutations. This study highlights the epic battle between virus and selective forces that impact on the virus and ultimately, clinical disease outcome. Further studies will be required to better understand these forces in chronic HIV-1 infection.

5.3 Neutralizing and Non-Neutralizing Antibodies

NAbs and non-neutralizing antibodies may play a role in HIV disease progression but these parameters have rarely been investigated concurrently. We therefore undertook this study in individuals chronically infected with HIV-1 subtype C, the most abundant subtype worldwide, in order to establish whether neutralizing and non-neutralizing antibody patterns differ in individuals with divergent rates of disease progression, and to determine whether certain genotypic *env* characteristics are associated with autologous or heterologous nAb responses. Our data indicated that nAbs did not appear to protect against disease progression, rather greater neutralization breadth against subtype B Envs and increasing autologous nAb titer were associated with disease progression. NAb IC_{50} titers were correlated with *env* genotypic characteristics, including increased amino acid length and numbers of PNGs in hypervariable regions of gp120.

In this study, nAb potency or breadth did not predict disease progression rate in individuals with chronic HIV-1 subtype C infection. However, a number of interesting findings were apparent. Firstly, high-level neutralizing titers to contemporaneous autologous virus were not observed in most of the participants. Instead, we observed significantly higher autologous responses over time in the progressors (when the study entry viruses were tested against the study exit plasma) compared to contemporaneous responses, which suggests that nAbs are continuously evolving in these subjects. These findings also argue that an increasing nAb titer per se is not effective at attenuating disease progression but rather is a marker of disease progression. Secondly, autologous nAb IC_{50} titers correlated with longer amino acid length for V1-V5 and C3-V5 of *env* in slow progressors. In contrast, the inverse correlation of nAb IC_{50} titer with V1-V2 length was found in the progressors. Indeed evidence for V2 dependent epitopes was observed in progressors SK221 and SK233. This data suggest that different regions of Env may be targeted by nAb in the progressors versus the slow progressors. Alternatively, there may be other intrinsic genetic differences of *env* between slow progressors and progressors that dictate neutralization potency and breadth that need to be further defined. Lastly, the correlation between autologous nAb IC_{50} titer and number of PNGs in V1V2 in slow progressors, suggests these glycans may be targeted by slow progressors but not in progressors.

The selective increase in neutralization breadth over time in progressors suggests that this parameter could be a marker of disease progression in chronic subtype C infection (Euler et al., 2010). Gray and colleagues (2011) found significant correlations between CD4 T-cell count and viral load with neutralization breadth at six months post-infection only and not at later time points (Gray et al., 2011a). These observations suggest that higher antigenic stimulation may dictate the breadth of antibody responses to heterologous viruses (Mellors et

al., 1997, Goujard et al., 2006, Fraser et al., 2007, Piantadosi et al., 2009, Sather et al., 2009, Doria-Rose et al., 2010). In contrast, a study reported broad cross-neutralizing antibodies in individuals on antiretroviral treatment (ART) with undetectable viraemia (Medina-Ramirez et al., 2011). The authors hypothesized that the lack of antigenic stimulation resulting in undetectable viraemia through effective ART may have compensated for an improved B cell function, resulting in broadly neutralizing antibodies. Although increased neutralization breadth in both study groups suggests that nAbs may not be protective against disease progression, they may be effective against super-infection as has been suggested by some studies (Smith et al, 2006 and Deeks et al, 2006) but not others (Blish et al., 2008). Together, these studies have implications for HIV vaccine design, as vaccine immunogens may need to be given over long periods of time to stimulate the B cell response, and to facilitate affinity maturation which appears to be necessary for antibodies to acquire cross-neutralizing activity (Pancera et al., 2010; Scheid et al., 2009). For the progressors who displayed potent cross neutralizing antibodies responses, their nAbs likely targeted quaternary V2 epitopes similar to PG9/PG16-like antibodies (Moore et al., 2011, Gray et al., 2011a). In addition, nAbs that targeted the N332 glycan in C3 suggest that the asparagine in that position is essential for neutralization activity. However, we could not establish whether these cross neutralizing antibodies had been there from study entry or had evolved. Due to the lack of plasma sample availability, we could not test for this. In subtype C infection, the V1-V2 and C3 regions are the immunodominant regions commonly targeted by AnAbs particularly during the early stage of infection (Moore et al., 2008, Moore et al., 2009, Rong et al., 2009, Lynch et al., 2011). Our results do indicate the V2 and C3 regions are immunodominant and the focus of the nAb response resulting in broadly cross-neutralizing antibodies even during chronic progressive HIV-1 disease.

A decline of p24 antibody titers as disease progresses has previously been reported (Lange et al., 1986, Allain et al., 1987, Forster et al., 1987, Binley et al., 1997), and it is therefore not surprising that the p24-specific IgGs may lose their affinity over time as demonstrated in both groups. A study found that low p24 IgG affinity correlated with HIV-1 disease progression (Chargelegue et al., 1995). It is plausible that in the slow progressors, a decrease in the binding affinity of activating FcγRIIa, heralds dysfunction in the expression of these receptors on the cells of the innate immune system despite a relatively unchanged CD4 T-cell count over time (Dugast et al., 2011). We found no differences in the binding affinities of the IgGs to the various FcγRs in divergent states of disease progression suggesting that non-neutralizing antibodies may have a limited or no protective effect during the chronic infection stage. Indeed it was found that non-neutralizing antibodies have no or a limited ability to protect against SHIV infection in mucosally challenged animals (Burton et al., 2011).

The limitations of the study are that firstly, we do not know the exact time of infection for these subjects. Therefore stratification of study subjects as progressors or slow progressors relied on a snapshot of the entire natural history of disease progression for these participants. However, this concern was somewhat allayed by bioinformatic analysis of the study sequences that showed that consistent with the stratification, progressors in this cohort were more likely to have been infected for shorter period of time than slow progressors. Second, the sample size of the study cohort was relatively small, which may have limited our statistical power to identify differences. Third, we had a limited number of SGA-generated amplicons for one of the study participants in particular, due to their low viral load and sample volume limitation. In addition, many more *env* amplicons were generated than were included in the final analyses as some of the amplicons had sequences with stop codons.

Fourth, we used binding affinity assays as a surrogate indication of non-neutralizing antibody activity, which may not always be reflective of ADCC/ADCVI activity *in vivo*.

The low sample size in each group was a limitation of this study, which led to poor statistical power. This study was a descriptive pilot analysis, based on strict criteria on participants having a similar CD4 T-cell count at study entry, patients remaining ARV naïve for the duration of the study and sample availability. Based on results from Mani et al. (2002) and Bagnarelli et al. (1999), we expected a mean intra-patient diversity of 2% and 4%, respectively; assuming 80% power, a Type I error of 5% and a standard deviation of 1%, we would require a sample size of at least 6 participants in each group and therefore our study was underpowered. For the autologous and heterologous neutralization, we wanted to assess the evolution of these responses in both groups of participants. Overall, therefore, the findings reported here will require duplication in larger cohorts with longer periods of follow-up and more significant differences in immunological and virological outcomes.

Despite these caveats, one of the strengths of the study is that we used single genome RNA plasma-derived *envs* to generate our recombinant viruses, by performing limited dilution assays we therefore would have sampled the both major and minor circulating *envs* and therefore present the autologous neutralization and genetic data based on these representative strains. Another strength of this study is that the groups were initially stratified according to CD4 T-cell counts and viral loads as progressors and slow progressors over the initial period of observation (of 24 months) in the absence of long-term follow-up data that only became available later on in the study. These groups were shown to be indeed different at study exit and beyond the study period, and therefore we were able to tease out putative viral sequence differences in divergent states of disease progression.

5.4 Conclusions

The dynamics of HIV-1 *env* evolution between chronic slow progressors and progressors are distinct. Single genome sequence analysis of circulating viruses diversity, envelope length polymorphisms, sites under positive and negative selection pressure, and PNGs consistently map to specific regions in slow progressors versus progressors. Our findings suggest that an ongoing de novo nAb response does not directly protect against disease progression during chronic HIV-1 subtype C infection. Our results are consistent with data from non-human primate studies showing that high titer nAb titers are not found in sooty mangabeys (Li et al., 2010) suggesting that autologous nAb are not part of the protection against disease progression in HIV and SIV infections. Furthermore, overall nAb breadth increased over time in most subjects, regardless of their disease status. There was evidence that progressors may target different regions of Env than slow progressors, which could have influenced the ability of the former to neutralize heterologous subtype B Envs. The mechanism by which nAb breadth increased in these subjects is of interest. Thus, it will be important to determine which Env epitopes in chronically infected progressors elicit broadly neutralizing antibodies and whether these provide any clinical benefit to the patient.

5.5 Future Directions

Going forward, the evolution of the autologous and heterologous responses in larger HIV-1 subtype C acute infection cohorts should be explored. This type of study would be valuable for the characterization of the antibodies and the definition of the target immunogens. In so doing we may be able to characterize putative subtype C immunogens to include in a globally

relevant HIV-1 vaccine that may induce potent and broadly neutralizing antibodies. In addition, using the Luminex platform to screen for large numbers of HIV-1 antigen specific IgGs, will facilitate the development of algorithms for predicting HIV-1 disease progression based on the immune correlates that prevail. For example the contribution that V1-V2 IgG binding antibodies play in disease attenuation can also be explored. In addition, we can explore the contribution of ADCC and ADCVI in controlling or attenuating HIV-1 disease progression.

Chapter Six- References

6.0 References

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Chapter Seven- Appendix

7.0 Appendix

Luria Bertani Medium

20 LB tablets (Sigma-Aldrich, St Louis, USA)

1 litre of distilled water

Autoclave for 20 mins at 121 kPa, allow to cool

LB Agar

20 LB tablets (Sigma-Aldrich, St Louis, USA)

10 g of Agar (Sigma-Aldrich, St Louis, USA)

1 litre of distilled water

Autoclave for 20 mins at 121 kPa, allow to cool.

1ml of Ampicillin (Invitrogen, Carlsbad, USA) to the LB- Agar was added when it had reached 65°C.

The LB-Agar was poured into sterile petri dishes and left to set before storing at 4°C. Petri dishes were wrapped in foil to prevent the degeneration of the ampicillin. Plates were used within 1 month.

X-Gal staining for TZM-bl Virus Titration

Fixing Solution (can be kept at 4°C)

500mls PBS 7.4 pH no Ca++ or Mg++

4mls Gluteraldehyde (Sigma-Aldrich, St Louis, USA)

11mls Formaldehyde (Sigma-Aldrich, St Louis, USA)

Staining Solution

7.4 PH PBS NO Ca++, Mg++

40mg/ml X-Gal Stock

1M MgCl2

0.2M Potassium Ferricyanide

0.2M Potassium Ferrocyanide

* 0.4ml/well of staining solution is needed per 24 well plate.

For 10ml of staining buffer (which will stain 1 24 well plate) required the following:

100µl X-Gal (40mg/ml stock)

40µl 1m mgcl2

9.5ml 7.4 ph PBS which contained no Calcium (Ca++) or Magnesium (Mg++)

200µl 0.2m Potassium Ferricyanide

200µl 0.2m Potassium Ferrocyanide

Growth Medium

500 ml of DMEM (Invitrogen, Carlsbad, USA)

50ml Fetal Bovine Serum (FBS)- (Invitrogen, Carlsbad, USA)

12.5ml (25mM) HEPES (Invitrogen, Carlsbad, USA)

2.5 ml Gentamicin (Sigma-Aldrich, St Louis, USA)

Remove 65ml of DMEM and then add all of the above components of the media and store at 40°C.

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01 July 2011

Mrs D Archary
Paediatrics and Child Health
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Mrs Archary

PROTOCOL: Neutralizing Antibody responses and Viral Evolution in a Longitudinal Cohort of HIV Subtype C infected Antiretroviral-naïve individuals. Mrs D Archary, Paediatrics and Child Health. Ref: E055/06.

PROTOCOL AMENDMENT RATIFICATION

Further to our letter to you dated 09 May 2011 this letter serves to notify you that at a full sitting of the Biomedical Research Ethics Committee Meeting held on **14 June 2011**, the Committee RATIFIED the sub-committee's decision to approve Protocol Amendment.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

Nelson R Mandela
School of Medicine

UNIVERSITY OF
KWAZULU-NATAL

12 April 2007

Professor T Ndung'u
HIV Medicine
DDMRI
Nelson R Mandela School of Medicine

Dear Professor Ndung'u

PROTOCOL: Neutralizing antibody responses and viral evolution in a longitudinal cohort of HIV subtype C infected antiretroviral-naïve individuals
D Archary Paediatrics Student number: 893281029 PHD H055/06

The Postgraduate Education Committee ratified the abovementioned study on 10 April 2007

Please note:

- the Postgraduate Education Committee must review any changes made to this study.
- the study may not begin without the approval of the Ethics Committee.
- the study may not begin without the approval of the Bioethics Research Ethics Committee. Please submit the copies to us to forward to the Ethics Committee. (The Ethics application form is available on the LAN – **(V:) /User/Staff/General/Ethics**

Many thanks
Yours sincerely

PROFESSOR P MOODLEY
Chair : Postgraduate Education Committee

c.c Derseree Archary

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02 November 2006

Mrs D Archary
Paediatrics and Child Health
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Mrs Archary

PROTOCOL: Neutralizing Antibody responses and Viral Evolution in a Longitudinal Cohort of HIV Subtype C infected Antiretroviral-naïve individuals. Mrs D Archary, Paediatrics and Child Health. Ref: E055/06

Further to our letter to you dated 14 July 2006, I wish to notify you formally that the above study has been approved. I apologise for the lateness of this notification.

The Biomedical Research Ethics Committee considered the abovementioned application and the protocol was approved at its meeting held on 16 May 2006 pending appropriate responses to queries raised. These conditions have now been met and the study is given full ethics approval and may begin as at 30 July 2006.

This approval is valid for one year from 30 July 2006. To ensure continuous approval, an application for recertification should be submitted a couple of months before the expiry date. In addition, when consent is a requirement, the consent process will need to be repeated annually.

I take this opportunity to wish you everything of the best with your study. Please send the Biomedical Research Ethics Committee a copy of your report once completed.

Yours sincerely

DR J MOODLEY
Chair: Biomedical Research Ethics Committee

Doctoral Student's contribution to journal article.

Student name: **Derseree Archary** Student No: **893281029**

Title of the article: HIV-1 subtype C envelope characteristics associated with divergent rates of chronic disease progression.

Authors (sequence as in the article): Derseree Archary, Michelle L. Gordon, Taryn N. Green, Hoosen M. Coovadia, Philip J.R. Goulder and Thumbi Ndung'u.

Journal (with year, volume and pages)*: Retrovirology* 2010 Nov 4;7(1):92

Please state your contribution to the article under the following headings:

(Describe your involvement. A simple yes or no is not acceptable.)

1. **Formulation of the hypothesis**

The original study was designed and proposed by Derseree Archary and Prof Thumbi Ndung'u. Together we proposed and developed an outline of a PhD study. I then put together a proposal including literature review, specific aims and objectives of the study.

2. **Study design**

I was involved in the proposal design to address the PhD study objectives.

3. **Work involved in the study**

I carried out all of the experiments and the analysis of the data. The experiments included RT-PCR, PCR, single genome sequencing, bioinformatic analysis and modeling of the sequences.

4**. Data analysis**

I analyzed data generated from the single genome sequencing and bioinformatic analysis as presented in this article.

5. **Write-up**

I wrote the first draft of this article and this was later reviewed and approved by coauthors before submission.

I declare this to be a true reflection of my contributions to this journal article.

Signature: _____ Date:_____