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Founder Virus Envelope Glycoproteins as Novel Oligomeric HIV-1 Vaccine Immunogens

Mark Killick



A thesis submitted to Faculty of Health Sciences,

University of the Witwatersrand,

in fulfillment of the requirements for the degree of

Doctor of Philosophy

Johannesburg, March 2014

“It always seems impossible until it’s done.”

Nelson Rolihlahla Mandela

(18 July 1918 – 5 December 2013)

Declaration

I, Mark Andrew Killick declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____

Mark Killick

_____day of_____2014

Publications and presentations arising from this study

Publications:

1. **M. Killick**, A. Capovilla and M.A. Papathanasopoulos. Generation and Characterization of an HIV-1 subtype C transmitted and early founder virus consensus sequence. AIDS Research and Human Retroviruses. 2014. Volume 30, Number 00.
2. **M. A. Killick**, N. Cerutti, A. Capovilla and M.A. Papathanasopoulos. Env-2dCD4^{S60C} complexes act as super immunogens and elicit high titre, broadly neutralizing antibodies against clinically relevant HIV-1. *In preparation.*

Conference presentations:

1. M. L. Grant, **M. A. Killick**, N. Cerutti, A. Capovilla and M. A. Papathansopoulos. Comparative antigenicity and immunogenicity of Indian and South African HIV-1 subtype C native and CD4-liganded envelope glycoproteins. AIDS Vaccine conference 2013, Barcelona, Spain.
2. **M. A. Killick**, A. Capovilla and M. A. Papathanasopoulos. Immunogenicity of native and CD4 liganded monomeric and trimeric envelope glycoproteins based on HIV-1 Subtype C consensus Founder virus sequences. October 2012, 4th Cross-faculty postgraduate symposium, WITS University, JHB, SA. Oral presentation.
3. **M. A. Killick**, A. Capovilla and M. A. Papathanasopoulos. Immunogenicity of native and CD4 liganded monomeric and trimeric envelope glycoproteins based on HIV-1 Subtype C consensus Founder virus sequences. 9-12 September 2012, AIDS Vaccine 2012, Boston, MA USA. Late breaker poster.
4. **M. A. Killick**, N. Moolla, A. Capovilla and M. A. Papathanasopoulos. Novel Covalently Engineered HIV-1 Env-CD4 Mimetic Vaccine Immunogens Increase the Magnitude of Neutralizing Antibody Responses. 22 September 2010, Faculty of Health Sciences, Research Day and Postgraduate Expo., University of the Witwatersrand, Johannesburg, South Africa.

Abstract

The ability to induce a broadly neutralizing antibody (bNAb) response following vaccination is regarded as a crucial aspect in developing an effective vaccine targeting the human immunodeficiency virus type 1 (HIV-1). The bNAbs target the HIV-1 envelope glycoprotein (Env) which is exposed on the surface of the virion, thereby preventing cell entry. Previous work in our laboratory focused on the generation of a 2dCD4^{S60C} molecule (a variant of the CD4 primary Env receptor) with higher affinity for HIV-1 Env through targeted disulphide exchange. This study reports on the design and construction of an HIV-1 subtype C founder virus consensus Env immunogen derived from newly transmitted/founder virus sequences, and the ability of the purified recombinant Env proteins (2dCD4^{S60C}-liganded and unliganded) to induce a broadly neutralizing antibody response in small animals. A total of 1894 founder sequences from 80 HIV-1 subtype C infected patients were available and downloaded from the databases. A consensus sequence was generated for each of the patients, and this alignment was subsequently used to generate a founder virus consensus env sequence. The env sequence was used to create codon-optimized constructs encoding monomeric (gp120$_{FVC}$m), dimeric (gp120$_{FVC}$GCN4d) and trimeric (gp140$_{FVC}$GCN4t(+) and gp140$_{FVC}$GCN4t(-) founder virus conformations cloned into the pcDNA3.1(-) mammalian expression vector. All four Env constructs were successfully expressed in HEK293T mammalian cell culture. The 2dCD4^{S60C} was expressed in *E. coli* BL21 (DE3) and purified by nickel affinity chromatography. Large scale expression and purification of the gp120, gp120GCN4 and gp140GCN4 +/- in the unliganded or 2dCD4^{S60C} liganded state were purified by lectin affinity chromatography, followed by conformation and complex purification using size exclusion chromatography. Immunogens/immune complexes were evaluated by ELISA, SDS-PAGE, native PAGE and surface plasmon resonance, and confirmed they were functional and conformationally intact. Immunogenicity of each conformation alone or complexed to 2dCD4^{S60C} was evaluated in rabbits. Breadth and potency of the rabbit sera was tested against 12 pseudoviruses (Tiers 1-3), derived from HIV-1 subtype B and C Env, using the PhenoSense Neutralizing antibody assay (Monogram Bioscience, Inc.). Minimal neutralizing breadth was obtained from animals immunized exclusively with Env conformations. However, animals that received the Env/2dCD4^{S60C} complex showed extensive neutralizing capacity against all 12 viruses tested, including the tier 2 and 3 virus strains. End-point ELISA titer results revealed that the rabbits that were immunized with Env/2dCD4^{S60C} produced both Env and 2dCD4^{S60C} specific titers, but those directed towards 2dCD4 were on average 10x lower than the 2dCD4^{S60C} control group. This implies a proportion of the NAb activity is directed towards conserved epitopes exposed on

the Env/2dCD4^{S60C} immunogens. Overall, these results show that the use of founder Env/2dCD4^{S60C} complexes as vaccine immunogens dramatically improves the antibody neutralization breadth and magnitude as compared to founder Env or 2dCD4^{S60C} alone. This level of broad neutralization has not been previously reported in the literature, and these results provide encouraging data to inform us of the best envelope vaccine immunogen to include in a preventative vaccine.

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List of abbreviations

µ	micro
293-F	FreeStlye™ 293-F suspension cell line
Ad5	adenovirus 5
ADCC	antibody-dependent cell-mediated cytotoxicity
ADCVI	antibody-dependent cell-mediate viral inhibition
AIDS	acquired immunodeficiency syndrome
Anc	ancestral
ART/ARV	anti-retroviral therapy
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
bNAbs	broadly neutralizing antibody/ies
BSA	bovine serum albumin
CCR5	cellular chemokine co-receptor type 5
$CD4^+$ T cells	human CD4 positive T lymphocytes
CD4bs	CD4 binding site
CD4i	CD4 induced conformation/epitope
cDNA	complementary DNA
CDRH3	third complimentarity determining region of the heavy chain variable regions of human antibody
CHO	Chinese hamster ovary
CRFs	circulating recombinant forms
CTLs	cytotoxic T-lymphocytes
CXCR4	cellular chemokine co-receptor type 4
DEAE Dextran	diethylaminoethyl-dextran hydrochloride
DMEM	Dulbecco's modified Eagles Medium
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
E. coli	*Escherichia coli*

EDC	1-thyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Env	HIV-1 Envelope glycoprotein
env	HIV-1 envelope gene
FCS	fetal calf serum
FDA	U.S. Food and Drug Association
FLSC	flexible linker sequence
FPLC	fast protein liquid chromatography
GALT	gut-asssociated lymphoid tissues
G. nivalis	*Galanthus nivalis*
GSH	reduced glutathione
GSSG	oxidized glutathione
HAART	highly active anti-retroviral therapy
HEK	human embryonic kidney
HIV-1	human immunodefieciency virus , type 1
HIS	polyhistidine
HLA	human leukocyte antigen
HR1	heptad repeat region 1
HR2	heptad repeat region 2
HRP	horse-radish peroxidase
i.d.	internal diameter
IC_{50}	50% inhibition
IPTG	isopropyl 1-thio-β-D-galactopyranoside
k_a	association constant
k_d	dissociation constant
K_D	equilibrium dissociation constant

kDa	kiloDaltons
LB	Luria-Bertani
LTR	long terminal repeat
MAb	monoclonal antibody
MHC	major histocompatibility complex
MMP	methyl α-D-mannopyranoside
MPER	membrane proximal external region
mRNA	messenger RNA
Mw	molecular weight
MWCO	molecular weight cut-off
NAb	neutralizing antibody
NaCl	sodium chloride
NHP	non-human primate/s
NHS	N-hydroxysuccinimide
p.i.	post infection
PBS	phosphate buffered saline
PBS-T	Dulbecco's PBS containing Tween 20
PBS-TE	Dulbecco's PBS containing EDTA and Tween-20
PMTCT	prevention of mother to child tranmission
PNGs	potential N-linked glycosylation sites
PrEP	pre-exposure prophylaxis
R_{max}	maximum theoretical response of the analyte for given ligand level
RU	response units
sCD4	soluble, 4 domain CD4
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
Se^{75}	radio-labelled selenium
SEC-FPLC	size exclusion chromatography-fast protein liquid chromatography

SFMII	serum free media II
SGA	single-genome amplification
SHIV	SIV and HIV chimera virus, containing the Env portion from HIV
SIV	simian immunodeficiency virus
SPR	surface plasmon resonance
STIs	sexually transmitted infections
$TCID_{50}$	median tissue culture infectious dosage
TCLA	T cell line adapted
Tris	hydroxymethyl aminomethane
T-TBS	Tris-buffered saline containing Tween-20
WHO	World Health Organisation
α	Alpha or Anti (in the context of antibody)
α-HIS	anti-HIS probe
β	Beta

Chapter 1: Introduction

1.1. The HIV-1 pandemic and the need for a vaccine

Since the discovery of the Human Immunodeficiency Virus-type 1 (HIV-1) in the early 1980's and its identification as the causative agent of Acquired Immunodeficiency Syndrome (AIDS) in humans [1, 2], it is estimated that by the end of 2012 there were approximately 35.5 million people living with HIV globally [3]. Improved access to life-saving antiretroviral therapy (ART) has witnessed a 31% decrease in the number of AIDS related deaths since the 2005 peak (2.1 to 2.6 million in 2005, down to 1.6 million AIDS deaths in 2012) [3]. In-roads into the prevention of new HIV-1 infections have also been made as indicated by a 33% decrease in the number of new infections per annum since 2001 (3.4 million in 2001, down to 2.3 million in 2012) [3]. Despite these global improvements, sub-Saharan Africa continues to bear a disproportionate burden of the pandemic, accounting for an estimated 70% (25 million; Figure 1.1) of the global HIV infected population and 70% of the total new HIV infections (1.6 million) worldwide. By the end of 2012, South Africa had over 6.1 million individuals living with HIV-1, with a prevalence of 17.9% [3].

Access to ART is far from universal, and estimates (based on the 2013 World Health Organization (WHO) guidelines which recommend ART initiation in patients with CD4 T cell counts <500 cells/ml) show that currently only 34% of people eligible to receive ART therapy are currently accessing treatment in the low and middle income countries [3]. The increasing imbalance between treatment coverage and the number of new infections annually foretells of an impending future global health crisis with regard to the treatment of HIV-1 infection. Thus, it is of paramount importance that there is continued investment in current prevention strategies and the development of novel methods to combat new HIV-1 infections. Most importantly, the development of an effective prophylactic vaccine that prevents transmission within the general population remains our best opportunity at bringing an end to this pandemic, particularly in resource-limited settings that are the most severely affected.

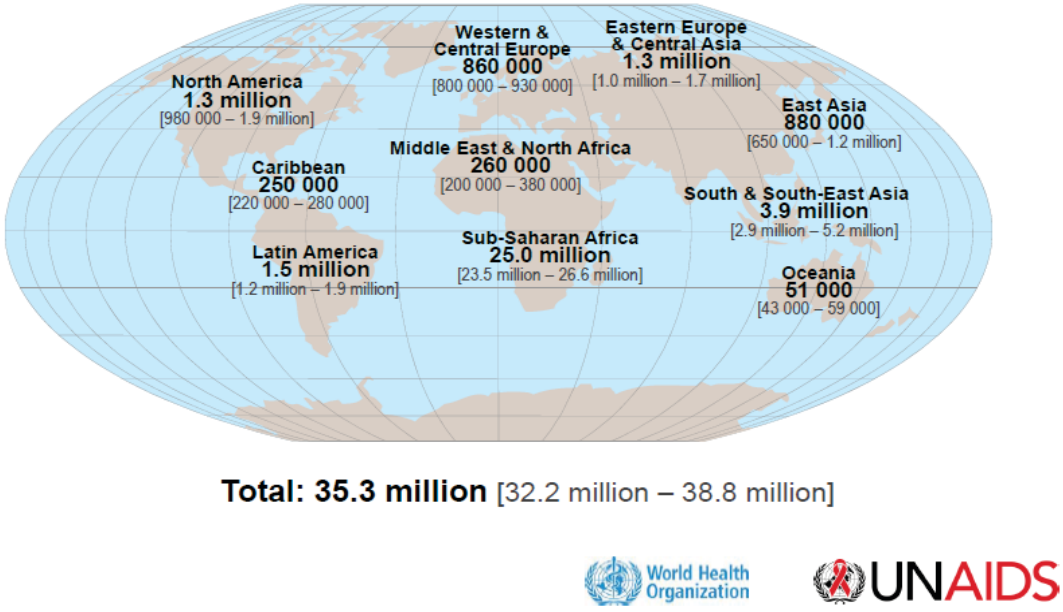


Figure 1.1: Schematic representation of the world map showing the total number of adults and children estimated to be living with HIV infection by the end of 2012. Image was downloaded from [4].

1.2. HIV-1 infection prevention strategies

Current HIV-1 infection prevention strategies employ a multifaceted approach that includes both social and pharmaceutical or biomedical interventions. Social interventions include, but are not limited to, the identification of high-risk individuals within the population, education programs aimed at reducing HIV-1 acquisition through initiating safer sex behavioral changes, promoting condom usage, prevention of gender based violence and needle exchange programs in areas where there is a high incidence among injecting drug users [5, 6]. Proven biomedical interventions include voluntary medical male circumcision which has been shown to reduce the risk of HIV transmission from females to males by as much as 60% [7-9], pre-exposure prophylaxis (PrEP) which was shown to be efficacious in reducing risk of HIV acquisition by 40% in cohorts of men who have sex with men [10], 49% among injecting drug users [11], and up to 54% in heterosexual transmission amongst high-adherence females using a tenofovir-containing vaginal gel [12]. Furthermore, evidence to support the use of ARV therapies as treatment in prevention strategies (reviewed in [13]) comes from highly effective programs in preventing mother to child transmission (PMTCT), as well as clinical trials of serodiscordant couples (HPTN052) which showed a reduction in transmission of the virus by up to 96% [14]. While these current HIV-1 infection prevention methods will continue to play a critical role in the fight against HIV and AIDS, the development of a safe and effective HIV-1 vaccine would provide a much needed addition to the HIV prevention armory.

1.3. Structural features and the life cycle of HIV-1

1.3.1. Structure of the HIV-1 virion

HIV-1 is a lentivirus that belongs to the family of *Retroviridae.* Its genome consists of two positive, single-stranded RNA molecules, which code for nine genes (*gag, pol, env, tat, rev, nef, vif, vpr* and *vpu;* approximately 9.7 kilobases) [6, 15, 16]. The single stranded RNA molecules are enclosed, along with the enzymes required for viral replication and processing, reverse transcriptase, protease and integrase, within a conical capsid comprised of the viral protein p24 (Figure 1.2) . The p24 is surrounded by the matrix p17 protein. The envelope surface of the HIV-1 virion is acquired from the human cell membrane as the virus particle buds from an infected cell [15, 17]. Embedded within the host derived envelope is the virus trimeric envelope glycoprotein spike (Env), comprising a heterodimer of surface gp120 and transmembrane gp41 viral proteins [17]. The HIV-1 Env plays a pivotal role in the viral life cycle, and is responsible for viral transmission, host cell entry, tropism, influences replication kinetics and is the target of neutralizing antibodies (NAb) [18].

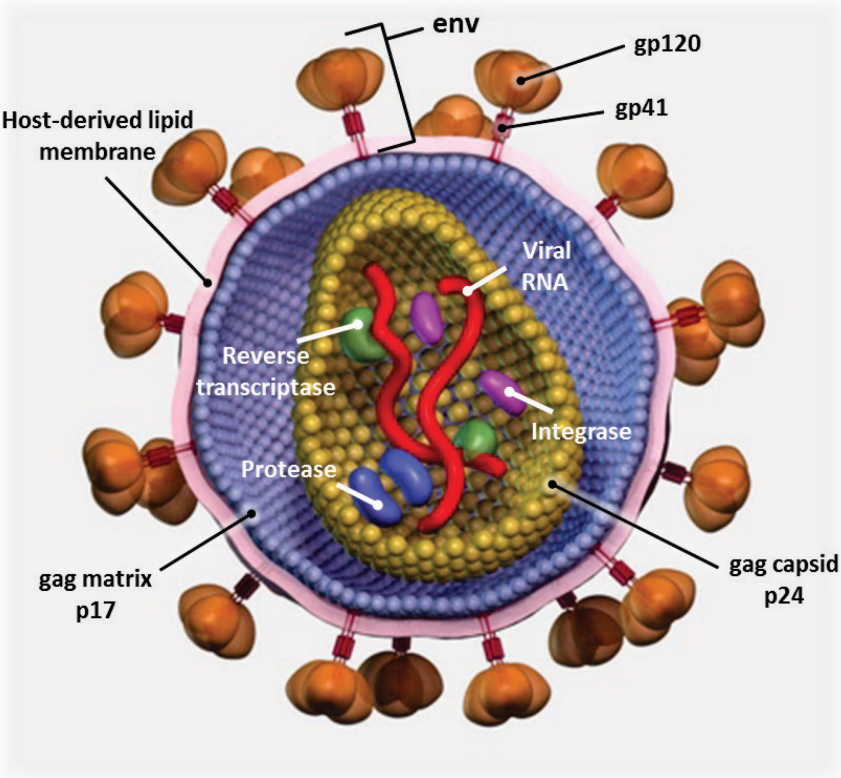


Figure 1.2: A model representation of the structural organization of the HIV-1 virion and its associated proteins. Image downloaded from [19].

1.3.2. The HIV-1 life cycle

HIV-1 predominantly infects T lymphocytes and/or primary monocytes/macrophages that express the CD4 glycoprotein, which results in the progressive loss of CD4 positive ($CD4^+$) T lymphocytes, ultimately leading to AIDS. An in-depth understanding of the HIV-1 life cycle (Figure 1.3) is paramount to designing and developing preventative approaches such as vaccines.

Viral entry is initiated with the binding of the viral gp120 component of Env to the primary CD4 on a $CD4^+$ T lymphocyte, followed by the sequential binding to a chemokine co-receptor, either CCR5 or CXCR4 [20, 21]. Once bound to the co-receptor, the gp120 may dissociate from the gp41 component of Env which is stably anchored/inserted within the viral membrane, triggering the gp41 fusion peptide and formation of the six helix bundle/hairpin structure, resulting in fusion of the viral and host membranes [18, 22]. The p24 is inserted into the host cytoplasm where it disintegrates (uncoats) and releases the viral RNA and other viral enzymes required for viral replication. Reverse transcriptase then transcribes the viral single stranded RNA molecules into double stranded complementary DNA molecules (cDNA). The cDNA aggregates with viral integrase, Vpr and other host proteins to form a preintegration complex, which moves into the nucleus, and facilitates the irreversible integration of the viral cDNA into the host chromosome [23, 24]. The proviral DNA is subsequently transcribed, and singly, multiple spliced and full length mRNA is exported to the cytoplasm where at least 16 viral proteins are translated [25, 26]. The Gag-Pol polypeptides and full length Env (gp160) are then intracellularly cleaved into functional shorter proteins by the viral protease and host proteases (such as furin), respectively [17, 27, 28]. Some proteins undergo post-translational modifications such as glycosylation and myristolation. The structural and enzymatic proteins then assemble at the inner surface of the host cell membrane in cholesterol rich lipid rafts and bud out of the cell as immature HIV virions which have the viral Env incorporated within host membrane lipids and glycoproteins [26]. This allows HIV-1 to remain similar in phenotype to the host cell, with the exception of the protruding Env, thus contributing to the viral strategy of immune avoidance. After budding, the viral protease completes the cleavage of the Gag-Pol polypeptide to form a fully infectious virion capable of subsequent rounds of infection. The viral replication cycle is extremely successful, and takes approximately 2.5 days [16]. Moreover, the reverse transcriptase enzyme is highly error prone and introduces mistakes with each replication cycle (estimated misincorporation rate of $3x10^{-5}$ per base per replication cycle)[29-31]. This results in a swarm of viral quasispecies circulating within an infected individual. A consequence of this high genetic diversity and viral evolution is ongoing immune escape, which has implications for the development of effective vaccines [32].

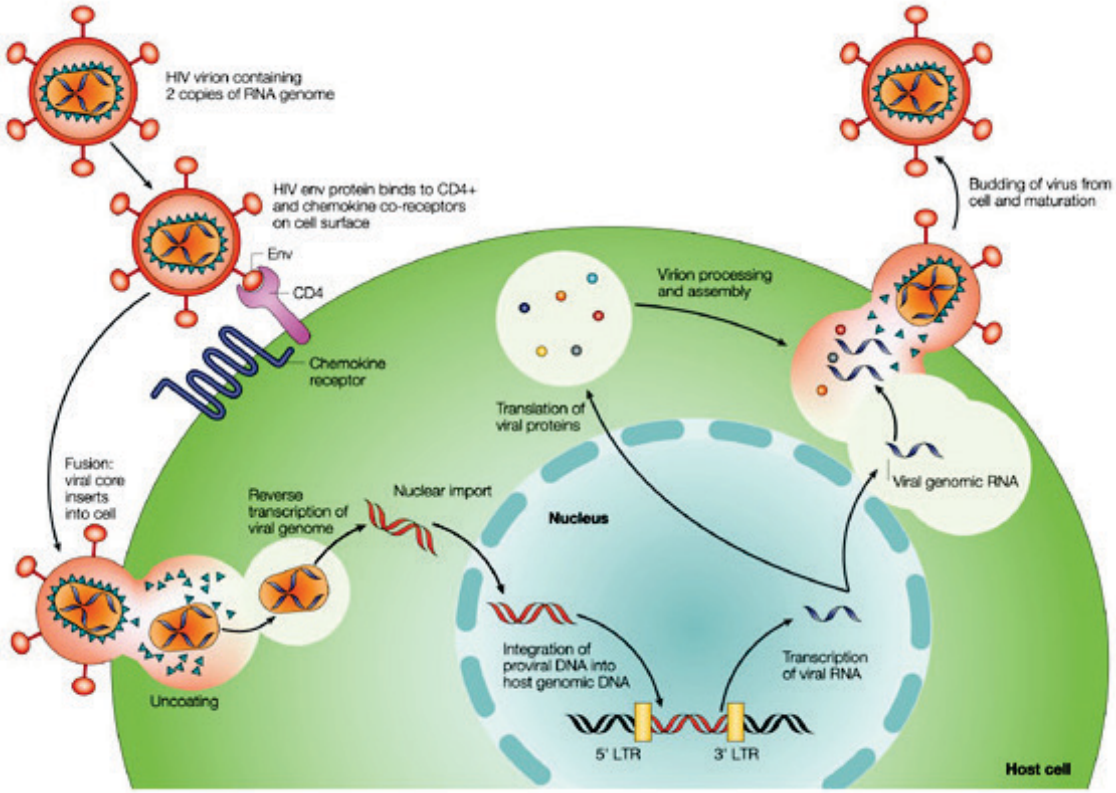


Figure 1.3: Schematic representation of the HIV-1 life cycle within a CD4⁺ T lymphocyte. Image obtained from [33].

The viral Env is visible on the surface of the virus, and because it is responsible for viral entry, it is an ideal target for vaccine elicited NAbs, which if present, could provide sterilizing immunity [34-36]. By contrast, all the HIV-1 proteins in infected cells are processed and presented on the surface of major histocompatibility complex (MHC) class I and II, and would be targets of HIV-1 specific CD4⁺ T cell and CD8⁺ cytotoxic T lymphocyte (CTL) responses [37, 38]. Since HIV-1 irreversibly integrates into the host genome, and CD8⁺ CTL responses emerge only after infection has occurred, it is unlikely they can prevent infection, but they could alter the course of disease progression to AIDS [39].

1.4. Sexual transmission and HIV-1 disease progression to AIDS

Although largely an inefficient process, sexual transmission (heterosexual and homosexual) of HIV-1 predominates as the mode of transmission amongst humans and is dependent on a number of interlinking factors [6, 40, 41]. Transmission inefficiency is characterized by low transmission rates observed per coital act (1 in 100 to 1 in 1,000) amongst discordant heterosexual couples, although during the acute phase of infection transmission rates can

increase to 1 in 30 [42]. Furthermore, despite being challenged by a myriad of HIV-1 quasispecies circulating within the donor, in the overwhelming majority of cases productive infection arises as a result of single transmitted viruses, termed the founder virus/population [43-52]. These studies have all evaluated the diversity of *env* sequences in newly infected individuals in South Africa, Zambia, Malawi, Rwanda, Uganda and USA.

Detailed research into the early transmission events have for practical and ethical reasons only been carried out using the simian immunodeficiency virus (SIV) and rhesus macaque non-human primate (NHP) models [53, 54]. Nonetheless these studies provide valuable insights and parallels to the critical early events of HIV-1 transmission [55, 56]. The first stage in HIV-1 transmission requires the virus to cross the mucosal barrier in order to gain access to viable target cell populations located just below the epithelial layer or deeper submucosa [57, 58]. In the context of heterosexual transmission, the mucosal epithelial barrier not only provides a passive physical barrier that prevents against invasion, but the epithelial cells lining the endocervix and upper female reproductive tract mediate the innate responses of the host immune system [59, 60]. Penetration of the mucosal barrier most commonly occurs at sites of enhanced accessibility, perhaps as a result of microtrauma during sexual intercourse, where there is thinning of the barrier due to genital ulcers, or naturally favored target sites of the endocervix and transformation zone between the endo- and ectocervix regions, which are protected by only a single layer of columnar epithelial cells [46, 54, 59, 61, 62].

Access to susceptible cells initiates small foci of productively infected cells where local expansion of the founder virus population occurs, before dissemination to the secondary lymphoid organs [63]. Upon reaching the draining lymph nodes, viral replication increases exponentially due to the increased availability and close proximity to target cell populations. This coincides with peak viral loads in peripheral blood and other tissues in approximately the second week following transmission (Figure 1.4), before declining to establish a viral set point. It is also during this period that rampant viral replication in secondary lymphoid tissues inflicts its most severe damage on the host immune system, culminating in massive $CD4^+$ T lymphocyte destruction within the gut-associated lymphoid tissues (GALT) and the establishment of latent viral reservoirs [64-67]. The high plasma viral loads associated with the acute or primary phase of HIV-1 infection have been associated with at least 50% of new transmissions [42, 68].

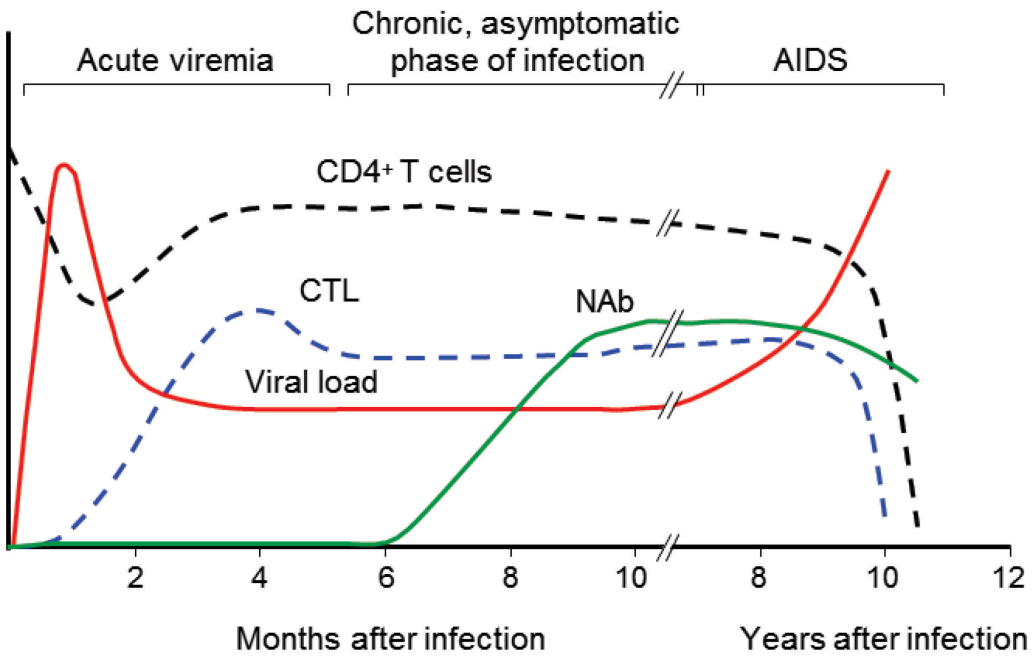


Figure 1.4: Schematic representation of the three typical stages of HIV-1 disease progression to AIDS in an infected individual over time. Viral loads (red), measured as RNA copies/ml, peak shortly after infection during the acute/primary phase, and the $CD4^+$ T cell (black) numbers (cells/µl) rapidly decline. After several weeks viral load decreases to a set point, and $CD4^+$ T cell counts improve. The emergence of patient CTL and NAb immune responses are shown in blue and green, respectively. The chronic/asymptomatic phase of infection can persist for several years with steadily increasing viral load and declining $CD4^+$ T cell count, until an individual becomes symptomatic with AIDS defining opportunistic infections (adapted from [69]).

The decline in the plasma viral load and establishment of a viral set point correlates with deployment of the host adaptive immune response, specifically $CD8^+$ CTL responses [70]. Establishment of the viral set point marks the start of the clinical latency or asymptomatic phase, which coincides with a recovery in the number of $CD4^+$ T lymphocytes. Viral load set point is thought to be an important indicator of disease progression, with lower viral set points associated with slower disease progression to AIDS [71-73]. In general, binding antibodies appear within a few weeks to months post infection, and the individual then seroconverts and enters the asymptomatic chronic phase of infection [74]. In some individuals, antibodies capable of neutralizing the autologous virus emerge within months to years [75-77]. However, the presence of binding and neutralizing antibodies is not associated with control of infection. If left untreated, $CD4^+$ T lymphocytes numbers will progressively decrease over time, until there is generalized immune dysfunction and dysregulation [78]. It is during this advanced symptomatic phase of the infection, that the infected individual is susceptible to a range of opportunistic infections (such as tuberculosis, pneumonia and certain malignancies) resulting increased morbidity and mortality. If highly active anti-retroviral therapy (HAART) is not initiated within

these patients, death rapidly follows within two years of an AIDS diagnosis. A typical progressor may develop AIDS within 8 to 10 years post infection [78].

Once infection and a latent reservoir have been established, the currently available antiretroviral drugs used in HAART regimens cannot eradicate HIV-1 [64]. Hence, the ideal vaccine should prevent HIV-1 transmission. Of particular interest, research into the early events of HIV-1 transmission and establishment of productive infection has identified "the window of opportunity" which is defined as the period in which a potentially successful HIV-1 vaccine must target the genetically restricted founder virus population in order to exert its protective effect [79, 80]. This is a finite period which extends from the time of transmission until the establishment of the latent pool of $CD4^+$ T cells, and subsequent diversification of the viral quasispecies as a result of the error prone reverse transcriptase and immune pressure.

1.5. HIV-1 natural infection: lessons for vaccine design

Since the discovery of HIV-1 as the etiological agent of AIDS, numerous attempts to generate an effective vaccine have thus far proven unsuccessful [81]. These failures within the field are due to a unique set of challenges presented by the virus and its interaction with host target cells including the enormous sequence diversity of circulating HIV-1 strains worldwide, the preferential targeting of $CD4^+$ T lymphocytes of the host immune system, the establishment of latent viral reservoirs, multiple mechanisms of immune evasion such as concealment of critical/neutralizing epitopes on the surface of the virus, specific down regulation of MHC class I molecules and the high degree of human genetic diversity coupled with viral diversity that thwart targeted $CD8^+$ CTL responses and mediate escape from NAbs (reviewed in [34, 36, 82-86]). These evasion mechanisms allow HIV-1 to persist within the host, avoiding eradication while retaining the ability to re-infect target immune cells. HIV-1 replication continues despite intense and sustained immune responses by both humoral and cellular mediated arms of the immune system. Moreover, the existence of "HIV-1 superinfection" coupled with the inability of viral immune clearance suggests that the presence of replicating virus (constant antigenic challenge) does not generate an adequate protective immune response during the natural course of infection – a fact that is discouraging for classical vaccine design [87]. Hence, the precise immune correlates of protection required to prevent HIV-1 acquisition in humans remains uncertain.

Research investigating the development of immune responses during the natural course of HIV-1 infection and disease progression to AIDS in humans has provided valuable insights into the development of immunogens and vaccine strategies. However, the lack of an appropriate animal model of protection that directly mimics HIV-1 infection in humans has significantly hindered advancement in the vaccine field. Currently, SIV or chimeric SHIV (SIV containing HIV Env) infection of NHP is used as the preclinical model in vaccine testing, prior to entering human clinical trials (reviewed in [88, 89]). Traditionally, the HIV vaccine field has been divided into evaluating the CTL mediated responses and antibody mediated responses. More recently, the role of innate immunity in HIV-1 transmission and disease progression has come under the spotlight.

1.5.1. $CD8^+$ CTL directed cellular immune responses to HIV-1

An HIV-1 directed CD8+ CTL response is a major driving force in the evolution of HIV-1, both at a population level and within the individual [90, 91]. The continuous mutation requirement and rapid evolution within immunodominant epitopes of the virus to avoid the host $CD8^+$ CTL responses is testament to the validity of this response [92, 93]. Additional evidence to support the significance of eliciting HIV-1 specific $CD8^+$ CTL responses through vaccination stems from the significant role that $CD8^+$ CTL responses play in controlling viremia, particularly during the acute phase of infection [38, 70]. The initial reduction in plasma viral load has been temporally associated with the appearance of virus specific $CD8^+$ CTL responses, which precedes the appearance of HIV-1 directed NAb activity (Figure 1.4) [70].

During the chronic phase of HIV-1 infection, higher levels of HIV-1 specific $CD8^+$ CTL responses have been reported in HIV-1 infected individuals clinically classified as long-term nonprogressors [94]. Furthermore, favorable clinical outcomes and slower disease progression to AIDS has been linked to individuals with favorable human leukocyte antigen (HLA)-class I alleles, such as HLA-B27 and HLA-B57 [95-97]. $CD8^+$ T cell depletion studies in NHP models, with a concomitant rise in SIV viremia further promotes the significant role for $CD8^+$ T cells in controlling HIV replication [98-100]. However, what has become evident from human vaccine trials is that $CD8^+$ T cell control of viremia in vaccinated NHP who are subsequently challenged with SIV/SHIV is not an adequate model [101]. This was most evident in the Merck STEP trial (discussed below), where challenge studies demonstrated that adenovirus 5 (Ad5) prototype vaccines led to $CD8^+$ CTL control of viremia in NHP but not in humans [101, 102].

1.5.2. B cell and antibody directed responses to HIV-1

A major gap in the HIV-1 vaccine development field is the failure to generate a vaccine immunogen that elicits effective antibodies that are protective and/or neutralizing. B cell responses are present throughout the natural course of HIV-1 infection and can be detected as early as 8-14 days following plasma viral load detection [74]. Unfortunately these early antibody responses are only binding antibodies directed towards all the HIV-1 proteins, and do not significantly impact on acute-phase viremia [74]. In the case of NAbs which target critical epitopes on the Env and prevent viral entry into the host cell, they require several months to years to mature. These antibody responses are generally directed towards the immunodominant variable regions of HIV-1 Env and thus display a very narrow neutralizing breadth usually confined to the infecting autologous virus [103-106]. These initial neutralizing antibody responses are frequently evaded through the introduction of neutralization escape mutations in Env [107, 108]. It is estimated that approximately 10-30% of HIV-1 infected individuals will develop a moderate to broadly NAb response, with 1% developing unusually broad and potent NAb activity capable of neutralizing a range of HIV-1 subtypes [75-77]. Interestingly, the broadly NAb (bNAb) responses in these patients are generally as a result of antibodies targeting a limited number of specificities [109].

Most currently available efficacious vaccines correlate with the induction of specific antibodies [110, 111]. In order to be effective, antibodies must be present at the site of infection or replication and the induction of B cell memory is critical in developing the prolonged protection associated with vaccination. The traditional view of antibody mediated protection against viral infection is that it is mediated through the binding and sequestering of free virus, thereby preventing infection. However, induced antibody responses may also exert effector functions such as antibody dependent cell mediated cytotoxicity (ADCC) or antibody-dependent cell-mediated viral inhibition (ADCVI) type responses that may functional in virological control. In the context of HIV-1 infection, passive immunization studies in NHP models have provided proof-of-concept that pre-existing NAb responses can prevent HIV-1 infection [112-118]. Unfortunately, such bNAb responses have not been effectively developed following immunization in NHP and humans.

1.5.3. HIV-1 human efficacy vaccine trials

Since 1988, a total of 218 clinical trials have been conducted in humans, most of which were early phase I safety/immunogenicity trials [81]. The vaccine candidates include immunogens

capable of inducing $CD8^+$ CTL responses, such as replication-competent or incompetent viral vectors (adenovirus, pox virus) containing HIV-1 gene inserts and naked DNA plasmids encoding various HIV-1 genes, immunogens capable of inducing NAbs, such as soluble HIV-1 protein/peptide subunits, or a combination of the two approaches. Heterologous prime boost immunization regimens have been employed in an attempt to enhance the breadth and potency of the elicited host immune response (CTL and antibody responses). Of the clinical trials conducted to date, only five progressed through to phase III efficacy studies (Reviewed by [81]).

The first phase III clinical trial aimed to elicit a NAb response following immunization, and was carried out using a recombinant expressed gp120 subunit vaccine evaluated in North America (VAX004) and Thailand (VAX003). These trials were carried out using bivalent preparations of gp120 (AIDSVAX B/B in North America and AIDSVAX B/E in Thailand) derived from CCR5- and CXCR4-utilizing viruses. Combined, approximately 8,000 volunteers took part in the efficacy trials, and in 2003 the released vaccine results showed that these vaccines failed in preventing HIV-1 acquisition [119-121].

In 2004-2007, the efficacy of a $CD8^+$ CTL based HIV-1 vaccine was evaluated in humans, using the Merck, replication-defective Ad5 vector [122]. This recombinant Ad5 vector expressed HIV-1 subtype B derived *gag*, *pol* and *nef* genes, and was evaluated in two major clinical trial studies, the STEP and Phambili trials. Unfortunately in 2007, both studies were stopped prematurely as an interim review revealed inefficacy and safety concerns with regard to increased risk of HIV-1 acquisition in the vaccine receipt arms of these studies [102].

By contrast, results of the RV144 announced in 2009 provided a much need boost to the HIV-1 vaccine field. This vaccine trial assessed the prime-boost combination of a CTL and antibody based vaccine approach using the ALVAC (Canarypox vector expressing HIV-1 *gag*, *pol* and *nef* genes) and AIDSVAX B/E (recombinant expressed gp120 subunit vaccine). When administered independently these vaccine approaches did not confer a protective effect, however in combination as a prime/boost strategy this vaccine trial resulted in a modest 31.2% efficacy in preventing HIV-1 infection [123]. Subsequent analysis of serum samples from the RV144 trial participants established an inverse correlation between the presence of gp120 (V1/V2 loop specific) binding antibodies and the risk of acquiring HIV-1 [124]. The lack of NAbs associated with protection from infection in this trial may hint towards other antibody effector functions such as ADCC and ADVCI. The RV144 vaccine trial result reinforced the idea that a safe and effective HIV-1 vaccine will most likely require the co-stimulation of innate, cellular and humoral arms of the immune system.

More recently, the HVTN505 [125] vaccine trial that used a DNA/Ad5 (DNA encoding *gag*, *pol*, and *nef* from HIV-1 subtype B and *env* from subtypes A, B, C, and Ad5 encoding *gag* and *pol* from HIV-1 subtype B and *env* from subtypes A, B, and C) prime boost regimen was stopped prematurely (2013) after interim analysis revealed that vaccination did not correlate with the prevention of HIV-1 infection, nor was there a reduction in viral load set points in the volunteers that received the vaccine and subsequently became infected [81].

Although there is currently no direct evidence that the presence of NAb will prevent HIV-1 transmission in humans, several studies show that the passive transfer of broadly NAb affords sterilizing protection in NHP models of HIV infection [112-117, 126]. Thus, it is now widely accepted that an effective HIV vaccine should elicit broad and potent anti-HIV NAb responses.

1.6. HIV-1 Env as a target of bNAb

The HIV-1 Env is expressed as a gp160 precursor polypeptide which undergoes extensive asparagine-linked glycosylation and cleavage by host proteases (furin) in the Golgi apparatus, resulting in the surface exposed gp120 and transmembrane gp41 subunits [17, 27]. These subunits assemble via non-covalent and hydrophobic interactions to form gp120/gp41 heterodimers that cooperate with additional gp120/gp41 heterodimers via their gp41 transmembrane regions, to form a trimeric quaternary complex (Env spike) on the surface of the infected cells [17, 18]. These Env spikes are incorporated on the surface of the HIV-1 virus particles as it buds from the infected $CD4^+$ T cell.

Amino acid sequence analysis of gp120 has identified five regions that show a high degree of conservation (designated as constant regions (C1 to C5)) interspaced by sections that display significant variability (designated variable regions/loops (V1 to V5)) [127]. The first four variable regions of gp120 protrude from the surface as loop structures that are stabilized via intramolecular disulphide bonds at their bases [18, 128]. The conserved regions (C1 to C5) comprise the core structure of gp120 (5 α-helices and 25 β-strands), folded into two major domains (inner and outer domains) and conjoined by an antiparallel, four-β-stranded (β2, β3, β20 and β21) bridging sheet [128, 129]. The core structure of gp120 contains the primary CD4 and co-receptor binding sites, which are both comprised of discontinuous amino acids sequences and therefore conformationally dependent. The primary receptor CD4, binds a surface depression or “pocket” on the gp120 molecule formed at the interface between the inner, outer domains and the bridging sheet, known as the CD4 binding site (CD4bs) (Figure 1.5). Contact residues between CD4 and gp120 are limited to residues 25 to 64 on domain 1 of

CD4, yet on gp120 these contacts are distributed over 6 segments including leucine at position 125 (all numbering according to HXBc2), loop D of the constant region 2 (C2) (residues 276-283), the CD4 binding loop (β15-α3 excursion) in the C3 region (residues 362-374), the β20-β21 hairpin (residues 425-433), and the V5/β24 region (residues 455-462 and 469-476) (Figure 1.5) [128, 129]. The chemokine co-receptor binding site (CCR5 or CXCR4) on gp120 is induced following engagement of the primary receptor CD4 and thus referred to the CD4-induced (CD4i) conformation [128, 129]. The exact residues that comprise the co-receptor and CD4i binding sites are unknown, but they are highly conserved and include contact sites in the bridging sheet and the base of the V3 loop [128-132]. The V3 in particular is known to be an important determinant in co-receptor usage and viral tropism, and displays features such as a characteristic tetrapeptide crown motif, inclusion of particular amino acids at positions 11 and 25, and overall positive charge, which impact on whether CCR5 or CXCR4 is used for viral entry [133].

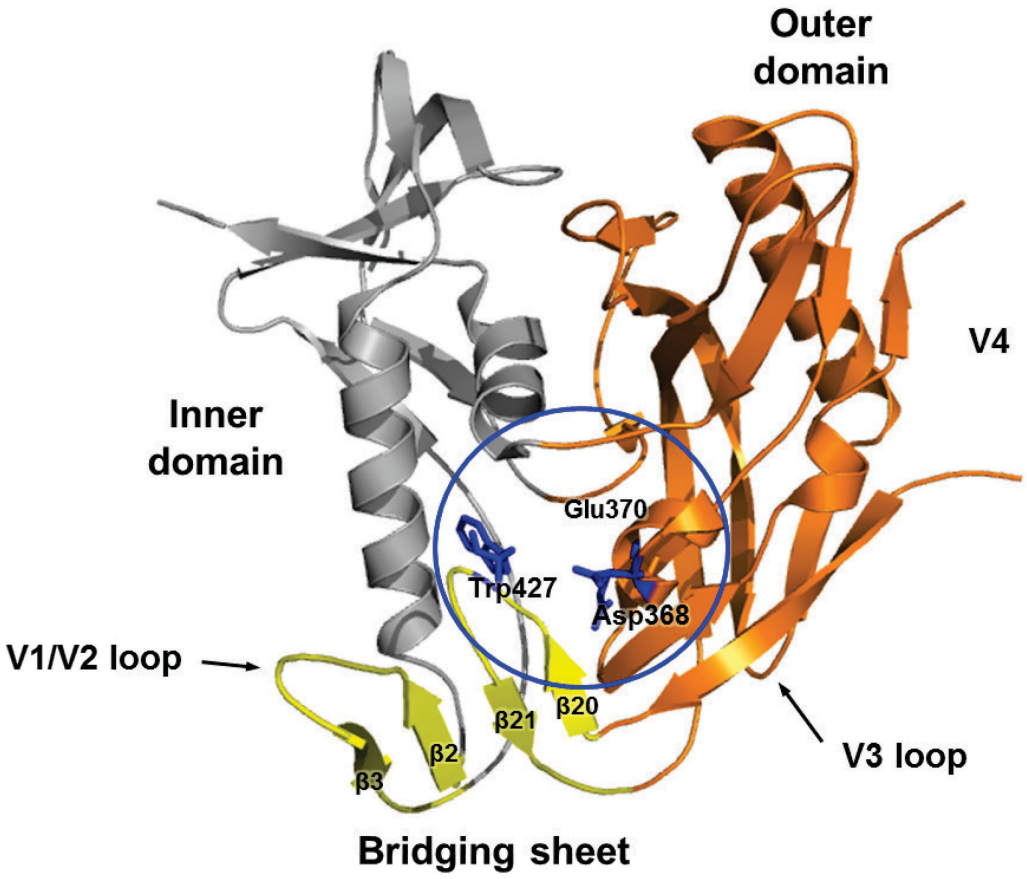


Figure 1.5: Ribbon diagram representation of the deglycosylated gp120 core monomer showing the inner domain (grey), outer domain (orange) and bridging sheet (β3, β2, β21, β20) (yellow). Position of the CD4 binding site is indicated (blue circle) with crucial residues Asp368, Glu370 and Trp427 that make contact with CD4, depicted in stick representation (blue). Positions of the truncated V1/V2 and V3 are shown. (Protein Data Bank 1G9M [128]). Image adapted from [84, 128].

The gp41 transmembrane Env subunit is sub-divided into three domains, the extracellular ectodomain, the membrane-spanning or transmembrane region and the intracellular endodomain [134]. The ectodomain of gp41 contains the hydrophobic fusion peptide sequence (FLGFLG) crucial for mediating membrane fusion as well two leucine-zipper like heptad repeat regions (HR1 and HR2) positioned at the N- and C- termini of the ectodomain, respectively. The quaternary structure and trimerization of HIV-1 Env is mediated by the HR1 region in the ectodomain, which has been shown to form a trimeric coiled-coil [135, 136]. The HR2 helices interact with the outside of the HR1 central trimeric coiled-coil in an antiparallel fashion to form a six-stranded helical bundle that represents the post-fusion conformation of gp41 [136].

1.6.1. HIV-1 Env mediates viral entry

Entry of HIV-1 into target host $CD4^+$ T cells has been described as a complex, multiconformational process that is initiated by the engagement of the HIV-1 gp120 with the surface expressed cellular CD4 receptor [128]. The projected critical contact residues Phe 43 and Arg 59 on CD4 enable access to the recessed CD4bs on gp120, forming multiple contacts with residues Asp368, Glu370 and Trp120 of gp120 (Figure 1.5) [128, 129]. CD4 binding induces significant conformational changes in gp120, triggering rearrangements in the inner, outer and bridging sheet domains. These rearrangements are accompanied by repositioning of the V1/V2 and V3 loop structures, resulting in exposure of the co-receptor binding site and epitopes comprising the CD4i conformation [128, 132, 137]. Chemokine co-receptor binding is thought to trigger further rearrangements in the gp120 molecule that exposes the gp41 domain, initiating the viral and host cell membrane fusion process [138]. The precise mechanism of viral and host cell membrane fusion is unclear as structural intermediates present following the induction of the co-receptor binding site and establishment of the post-fusion, six-helix bundle of gp41 have not yet been elucidated. However it is postulated that co-receptor binding induced conformational change extends the N-terminal fusion peptide of gp41 into the host cell membrane, establishing an inter-membrane anchor between the viral and host cell membranes bilayers. The ectodomain of gp41 folds back on itself through the interaction of the HR1 and HR2 regions, establishing the post-fusion stabilized, antiparallel six helical bundle conformation which draws the two distal membrane anchor points into close proximity allowing for membrane fusion and release of the viral p24 capsid into the host cell cytoplasm [136].

Strategies employed to target HIV-1 Env mediated entry (reviewed in [139, 140]) include the use of non-peptide, co-receptor antagonists (for example, Maraviroc), thereby preventing engagement of the co-receptor on the surface of the host cell, as well as fusion inhibitor peptides (for example, Enfuvirtide/T-20) which binds the gp41 HR1 region and prevents formation of the six helix bundle. Antibodies targeting crucial epitopes on the surface exposed gp120 (e.g. CD4bs) or the gp41 ectodomains or structural intermediates, such as the co-receptor and CD4i binding sites have been shown to prevent HIV infection *in vitro* and *in vivo* (reviewed in [141, 142]). The ability to elicit these types of antibodies is expected to form a crucial component of an effective HIV-1 preventative vaccine.

Overall, the HIV-1 Env has a complex tertiary/quaternary trimeric structure which exhibits conformational flexibility. Thus, different transient epitopes are exposed in the unliganded versus the CD4- and co-receptor liganded Env. Furthermore, the protein is extensively glycosylated, and as much as 50% of the molecular mass is attributed to glycans [18]. These factors all contribute towards making the Env less immunogenic, and HIV-1 able to avoid immune clearance.

1.7. bNAbs identify crucial HIV-1 Env vulnerabilities

In spite of the extensive sequence diversity of HIV-1 that exists globally, the identification and characterization of antibodies that target highly conserved epitopes on Env affords these antibodies extraordinary neutralization breadth capable of exerting inter-subtype neutralizing activity (reviewed in[141, 142]). Although originally thought to occur rarely during the natural course of infection, recent advances in technology which allow for high throughput screening of large cohorts, the development of novel techniques such as single B cell amplification and sophisticated B cell sorting using uniquely generated gp120 capture molecules has facilitated the discovery of a new generation of bNAbs that show enhanced neutralizing breadth and potency (reviewed in [141-144]). Characterization of the epitopes targeted by these bNAbs have so far identified four vulnerable antigenic sites on the HIV-1 Env spike (three sites in gp120 and one site in gp41) that provide valuable insights into HIV-1 Env immunogen design (Figure 1.6).

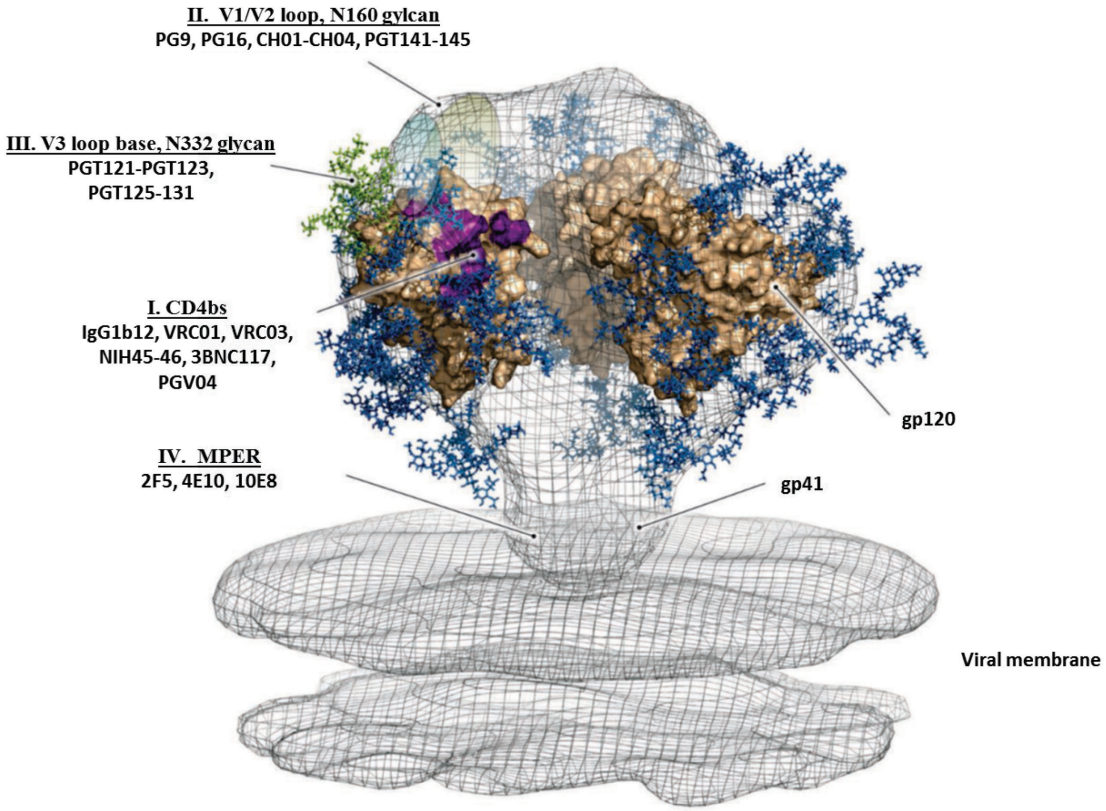


Figure 1.6: Broadly neutralizing antibodies (bNAbs) identify crucial neutralizing epitopes on the surface of the HIV-1 envelope spike. The trimeric envelope is comprised of three heterodimers of gp120 (surface domain) and gp41 (transmembrane domain). This model was generated by combining cryoelectron tomographic [145], crystallographic [146] and computational analysis. The molecular surface of the gp120 core structure (tan) fitted in the electron density map (grey mesh) of the spike. The approximate positions of the V1/V2 and V3 loops are shown (green and blue ovals, respectively), the CD4 footprint on gp120 is highlighted in purple and glycans are shown in blue and green. Major sites targeted by bNAbs are indicated (I-IV). Image obtained from [82].

1.7.1. The CD4 binding site

Accessibility to the CD4bs on gp120 is restricted since it is located in a recessed pocket surrounded by glycans and variable loop regions. Nonetheless antibodies directed towards this highly conserved region on gp120 display exceptional neutralization breadth and potency. The monoclonal antibody IgG1b12 was the first of this antibody class to be discovered, it was derived from phage display and was generated through the random pairing of light and heavy antibody chains [147]. The new generation of bNAbs targeting the CD4bs include VRC01 and its somatic derivatives VRC03, VRC06 [148] and NIH45-46 [149], PGV04 [150] and 3BNC117 [149]. These antibodies neutralize the virus directly by competing for the CD4bs on gp120. These antibodies show similar levels of breadth, neutralizing approximately 75% of pseudoviruses (177-180 pseudoviruses) when tested at a concentration of <1.0 μg/ml [151].

1.7.2. The V1/V2 variable loop

The V1/V2 variable loop region on gp120 is most frequently associated with the shielding of highly conserved neutralizing epitopes, evasion of neutralizing antibody responses through sequence changes and insertion or deletions in this region [51, 106, 107, 152-154]. However the glycan at asparagine residue 160 (N160) occurs within a highly conserved sub-domain within the V1/V2 loops structure and is targeted by bNAbs PG9, PG16 [155, 156], PGT141-145 [157] and CH01-CH04 [158]. PG9 and PG16 antibodies display extended anionic charged, third complementarity determining region of the heavy chain variable regions (CDRH3) that penetrate the glycan shield to interact with the cationically charged, β-strand C loop of the V1/V2 loop [155]. Furthermore these antibodies show preferential binding to the native Env spike expressed on the surface of transfected cell lines, suggesting quaternary stabilization of the targeted epitope, or the involvement of multiple V1/V2 loop domains [156]. PG9 and PG16 have been shown to neutralize 65 and 59% of 177 pseudoviruses tested at a concentration of <1.0 μg/ml, respectively [151].

1.7.3. The glycan-dependent V3 variable loop

Similar to the glycan-dependent neutralizing epitope located in the V1/V2 variable loop structure, the N-linked glycan positioned at the base of the V3 variable loop (N332) forms a critical component of the epitope targeted by bNAbs PGT121-PGT123 and PGT125-PGT131 [157]. The crystal structure of PGT128 reveals that this antibody also targets a proteoglycan epitope on gp120, recognizing two conserved glycans at positions N301 and N332, and a short β-strand at the carboxyl terminus of the V3 loop which it makes contact with through an extended CDRH3 region [159]. The most potent of these antibodies PGT121 and PGT128 were shown to neutralize approximately 60% of 162 pseudoviruses tested at a concentration of <1.0 μg/ml [157].

1.7.4. Membrane proximal external region (MPER) of gp41

The MPER region of gp41 is a highly conserved region located at the N-terminus transmembrane spanning domain. The originally described bNAbs targeting this region, 2F5 and 4E10, interact with linear epitopes within the MPER. These antibodies have also been shown to be polyreactive, displaying activity towards the membrane phospholipid cardiolipin, which due to the close proximity of the MPER region to the viral/host membrane, is thought to

increase the affinity of these antibodies towards HIV-1 [160, 161]. However the latest bNAb targeting the MPER region, 10E8 displays exceptional breadth, neutralizing 72% of 180 pseudoviruses tested at <1.0 μg/ml [151]. Although recognizing residues that overlap with the 4E10 bNAb epitope, 10E8 appears to differ in its recognition, targeting a structural element within the MPER and also lacks the polyreactivity associated with other MPER targeting bNAbs [151].

1.8. Development of an HIV-1 vaccine that exploits Env vulnerabilities

The identification of these bNAbs and their constituent neutralizing epitopes has revived humoral-focused HIV-1 vaccine strategies. Indeed, passive transfer studies carried out using bNAbs alone, or in combination, have validated the role of NAbs in providing protection against virus infection using the SHIV-rhesus macaque NHP challenge model [112-118]. More recently, the improved neutralization potency of these new generation bNAbs has been shown to confer protection in NHP at relatively low serum levels, believed to be achievable by vaccination [116, 117]. Structural and sequence analysis have revealed unusual characteristics associated with these bNAbs which include displaying a high degree of somatic hypermutation, long variable CDRH3 regions and polyreactivity with self-antigens. Somatic hypermutation is the natural process of affinity maturation that takes place in the germinal centers of secondary lymphoid tissues and improves antibody binding affinity to its cognitive antigen. The extensive somatic hypermutation displayed in these bNAbs suggests multiple rounds of affinity maturation are required to develop such responses *in vivo* [35, 141, 144]. Furthermore it has been noted that the B cell lineages of these bNAbs display limited binding to currently available Env immunogens and raises concerns with regard to the initial engagement of the germline precursors during vaccination [162-164].

Extended CDRH3 regions are not a prerequisite to determining neutralizing breadth or potency. However, in certain instances these extended CDRH3 regions allow these bNAbs to access sterically restricted or inaccessible epitopes on the HIV-1 Env spike. These include the complex proteoglycan epitopes located in the V1/V2 loop dependent on the glycan at position 160, the N332 glycan dependent epitope at the base of the V3 loop, as well as the restricted access site of the MPER region in gp41. For the bNAbs targeting the complex glycan dependent epitopes in the V1/V2 and V3 loops structures, the extended CDRH3 region allows these antibodies to penetrate the glycan shield gaining access to the relevant epitopes in the protein structures below [155, 159].

It is estimated that 70% of all B cells producing antibodies specific to HIV-1 Env are polyreactive [165, 166]. The increased polyreactivity of HIV-1 bNAbs is thought to be compensatory in response to the relatively low numbers (approximately 15) of functional HIV-1 Env trimers incorporated on the surface of the virion [167]. Polyreactivity improves the affinity of these bNAbs by increasing the heterotypic valency (the ability to bind the target epitope with one Fab arm and the nearby polyreactive low-affinity ligand with the other) and thereby increase the avidity effects [160, 166]. Although polyreactivity is a product of natural somatic hypermutation, it may be selected against during B cell development resulting in clonal deletion [144].

These unusual characteristics displayed by HIV-1 bNAbs have raised concerns to the possibility of eliciting such responses by traditional vaccination approaches. However the existence of these bNAbs and relatively high prevalence (10-30%) of HIV-1 infected patients able to develope a moderate to broad NAb response suggests that given the appropriate vaccine immunogen/regime such a response can be achieved by immunization.

1.9. Challenges of HIV-1 Env-based subunit immunogens

Development of an effective HIV-1 preventative vaccine will most likely require the combined immunological response of both the innate and adaptive (cellular and B cell) arms of the immune system. In particular, NAb responses directed towards the Env spike on the surface of HIV-1 virion will be critical in a preventative vaccine. Harnessing the protective effect of these NAbs will require sufficient quantities of vaccine-elicited antibodies to be present at the time of a transmission event, that exhibit adequate breadth to handle the myriad of circulating strains within the population and can prevent establishment of the founder virus population and/or restrict dissemination to the secondary lymphoid tissues [54, 86]. The window of opportunity in which NAbs must exert their protective effect is very narrow, as demonstrated in studies evaluating protective-passive transfer of bNAbs which showed the ability to confer protection at six hours but not at twenty-four hours post inoculation in the NHP macaque model [168]. Nonetheless, these passive immunization/transfer experiments validate the protective effect of NAbs preventing SHIV acquisition [112-118].

To date, Env based subunit vaccine strategies have failed to induce bNAb responses in various animal models and humans. Although the majority of these bNAb epitopes are present and exposed on these Env-based immunogens, they appear to be poorly immunogenic in the context of vaccination [120, 169]. This finding along with the identification of sophisticated immune

evasion and escape mechanisms on gp120 highlights the challenges associated with eliciting bNAbs responses using Env-based immunogens [85, 170]. These challenges include the extraordinary genetic diversity of HIV-1 strain circulating globally, the extensive N-linked glycosylation of the gp120 surface, transient exposure of vulnerable NAb epitopes, as well as conformational masking or occlusion of critical neutralizing epitopes by the quaternary Env spike conformation and immunodominant variable loop structures [105, 107]. The following sections will discuss the relevant challenges to this thesis and the proposed strategies aimed at addressing them.

1.9.1. HIV-1 sequence diversity

HIV-1 group M is responsible for approximately 95% of the HIV-1 infections worldwide and is subdivided into 9 different clades or subtypes A-D, F-H, J and K with 61 identified recombinant forms (CRFs) [171, 172]. The HIV-1 error-prone reverse transcriptase, high replication rate, the ability to undergo major genetic recombination and insertion and deletion events during viral replication all contribute to the global diversity observed [173]. Immunological pressure exerted by the host immune system is also an important determinant in driving HIV-1 viral evolution [90, 91, 174]. Although fairly homogeneous to begin with, HIV-1 rapidly evolves under the selective pressure of the immune system that may result in up to 10% of nucleotides being altered in *env* [107, 108, 175, 176]. Differences of up to 30% in amino acid sequences in the HIV-1 Env glycoprotein have been observed between the group M subtypes, while strains within the same subtype can differ by up to 20% [32, 177]. With such global sequence diversity and evolution worldwide, selection and design considerations concerning the appropriate Env sequence needs to be evaluated more closely.

Two general approaches have been proposed by Gaschen *et al*.,[32] in terms of selection of vaccine strains that will contend with the high levels of HIV sequence variation. The first approach is one of biological consideration, where the vaccine strain is matched to the predominant or geographic circulating isolate in the proposed region where the vaccine is needed. Additional biological considerations may include co-receptor usage, naturally occurring antibody resistance/susceptibility or the preferential use of isolates from recent sero-converters. The second approach utilizes bioinformatic tools that aim to minimize the genetic variation by generating consensus, center-of-tree or the prediction of ancestral sequences [32]. These approaches are referred to as “centralized genes” and are proposed to offer benefit to HIV-1 vaccine design and are referred to as centralized immunogens [32, 178-180].

1.9.1.1. Centralized immunogens

The majority of HIV-1 Env based immunogens used in preclinical and clinical settings were derived from contemporary viruses that were selected based on availability or geographical considerations that attempted to match the circulating strain within a target population [32]. The disadvantage of this approach is that the high levels of HIV-1 genetic diversity translate into diverse antigenicity between the vaccine strain and the current circulating strains a population is exposed to [178]. The simplest method of minimizing genetic diversity is to generate a consensus sequence, where the most common amino acid at each position in an alignment is included based on a cut-off frequency or threshold (e.g. ≥50% frequency). Alternatively, centralized sequences can be reconstructed from a phylogenetic tree to a particular central point where the evolutionary distance is minimized as is the case for center-of-tree, or to the most recent common ancestor sequence (Anc). The advantage of these approaches is that they take into account the evolutionary history of the samples which may impact biological interactions such as co-varying sites [181]. A concern in the development of centralized immunogens, particularly consensus and center-of-tree sequences, is that the generation of these sequences is subjected to sampling bias which may result in the linking of unnatural combination of polymorphisms, detrimentally affecting the native, quaternary conformation of these proteins or the display of particular neutralizing epitopes [182]. The sample sets used to infer these sequences should therefore ideally factor in the phylogenetic distribution and the relative frequencies of the circulating strains [178].

The generation of centralized sequences of the HIV-1 Env glycoprotein has proven to be challenging. The hypervariable domains (V1 to V5) contain insertions and deletions that do not evolve according to substitution models currently assumed for deriving maximum likelihood trees (i.e. single point mutation) [32, 183]. Instead, these hypervariable regions are aligned by anchoring common N-linked glycosylation sites and incorporating only minimal shared spanning elements [184]. It has been suggested that the "artificial" nature of these sequences may be to the detriment of correctly folded proteins, potentially affecting elicitation of antibodies by immunization and therefore may be better suited to the development of CTL immunogens [32]. Nonetheless several groups have reported the development of centralized Env immunogens that are functional and antigenically correct [182-187].

1.9.1.2. Restriction of HIV-1 genetic diversity following sexual transmission

As mentioned previously, research into early transmission and acute HIV-1 infection has revealed a severe genetic bottleneck in the transmission from donor to recipient, so much so,

that in the majority of cases (65-90%) productive infection arises as a result of a single viral variant, termed the founder virus [43-47, 49, 50]. Importantly, this restriction in genetic diversity occurs in the recipient, rather than the transmitting partner thereby representing the viral strain that managed to overcome the challenges associated with mucosal transmission to establish productive infection. Analyses of these founder virus populations may uncover certain genotypic or phenotypic characteristics that can be exploited by an appropriate vaccine-elicited response. While there is currently no clear association between the genotype of these founder virus populations and transmission efficiency, sequence analysis has shown that these transmitted variants are predominately CCR5 utilizing viruses [47, 52] and contain shorter, more compact variable loop structures and fewer potential N-linked glycosylation sites (PNGs) in the gp120 as compared to more contemporary viruses present during chronic infection [44, 48, 50]. Indeed it has been observed that the expansion of the V1/V2 loop structures and an increase in the number of PNGs or a shift in their position occurs naturally over the course of infection in response to immunological pressure exerted by the host [51, 153, 154]. The accumulation of Env modifications over the course of infection may negatively affect the viral entry efficiency of cells with low CD4 receptor surface expression, reducing the transmission fitness of these viruses [48, 51]. The more compact structure of these founder virus variants are thought to be more fit in a transmission setting due to greater exposure of the CD4bs, which is associated with an increased sensitivity to NAbs [44, 50]. Founder virus populations also have increased affinity for the α4β7 integrin which is proposed to lead to the enhancement in the transmission fitness of these variants, through improved cell-to-cell transmission or specific infection of lymphocytes that home to GALT [188, 189].

1.10. Structural improvement of Env-based immunogens

Numerous strategies have been employed over the last 30 years in making Env-based vaccine immunogens which are antigenically correct, including gp120 monomers, resurfaced stabilized gp120 cores, gp120 outer domain, gp140 trimers, and scaffolds displaying particular NAb epitopes (reviewed in [159, 190]). Overall, these strategies have been evaluated in small animal as well as NHP models, and have been shown to elicit antibodies that display some autologous NAb activity, but none have successfully generated bNAbs. Further structural improvement of Env-based immunogens encompasses a broad category of novel approaches aimed at improving/facilitating the elicitation of bNAbs following immunization.

The failure of monomeric gp120 to protect humans from HIV-1 acquisition in clinical trials (VAX003 and VAX004) has been attributed to structural differences between the monomeric

Env and the native trimeric Env spike on primary HIV-1 isolates. Monomeric Env conformations are now known to elicit non-neutralizing antibodies to epitopes that are usually buried or non-exposed on the native HIV-1 Env spike [191]. A number of novel methodologies are currently being explored to improve the exposure of bNAbs epitopes of Env. These include modifications to the monomeric form aimed at increasing the exposure of neutralizing epitopes through the deletion of one or more variable loop domains [192-195], or the removal of N-linked glycans [196, 197]. While these approaches have increased the exposure of neutralizing sensitive epitopes on gp120, as demonstrated through the increased antibody neutralizing sensitivity of pseudoviruses, these Env immunogens have failed to induce bNAbs following immunization. Alternatively, introducing additional PNGs (hyperglycosylation) or specific point mutations into monomeric Env conformations with the aim of reducing the exposure of non-neutralizing epitopes and refocusing the immune response to critical neutralizing epitopes has been attempted [198]. Additionally, the use of gp120 subdomains (outer subdomain which contains the CD4bs), structural analogs and epitope "transplantation" on unrelated molecular scaffolds (for example the CD4bs – β15 loop and 2F5 constrained epitopes located in the MPER region of gp41) have been attempted to display singular bNAb epitopes in isolation [199]. Methodologies aimed at mimicking the native Env trimeric spike by producing soluble, stable trimers of Env and the stabilization of intermediary epitopes during the binding process are discussed in greater detail below.

1.10.1. Strategies aimed at producing soluble, stable HIV-1 Env trimers

The purpose of producing stabilized trimeric Env immunogens not only stems from intended mimicry of the native Env spike, but antigenically this quaternary structure restricts recognition by non-neutralizing antibodies [200] and is preferentially targeted by bNAbs such as PG9, PG16 and PGT145 [155, 156, 201]. Furthermore the relatively high prevalence (21%) of PG9/PG16-like antibodies identified in patients that develop broad and potent NAbs warrants pursuit of vaccine strategies aimed at eliciting these type of responses [109].

On the native trimeric Env spike, the HR1 domain of gp41 is thought to stabilize the trimeric quaternary conformation by forming a leucine-zipper like coiled-coil with the two other HR1 domains [136]. However, the inherent instability of the non-covalent gp120/gp41 interaction frequently results in gp120 "shedding" during purification procedures and has hampered the development of stable trimeric Env immunogens [202-204]. Recombinantly expressed Env trimers most often use the soluble gp140 polypeptide which contains the gp120 and gp41 ectodomain (includes the gp41 extracellular portion but lacks the cytoplasmic and

transmembrane domains). The two most common approaches used to stabilize the gp120/gp41 interaction include (i) mutation of the furin proteolytic cleavage site which eliminates cleavage of gp120 and gp41, or (ii) cross-linking the cleaved gp120 and gp41 subunits by introducing appropriately positioned cysteine residues (HXBc2 numbering 501 of gp120 and 605 of gp41, respectively) in each subunit that allows for the formation of an intermolecular stabilizing disulphide bond (designated SOS) [205]. These SOS gp140 trimers are further stabilized by additional isoleucine to proline substitution at position 559 (HXBc2 numbering) (termed SOS-IP) that strengthens the trimerization of the gp41 ectodomains [206] and truncation of the carboxyl-terminal hydrophobic residues in the MPER region which improves trimer homogeneity [207]. The recent success of generating stable, soluble SOS-IP trimers have allowed crystallization of the trimeric Env for the first time [200]. Cryo electron microscopy and X-ray crystallization confirm that the SOS-IP trimer resembles that found on the surface of the virion [200, 208]. However, when immunized into NHPs the SOS-IP trimer generated autologous NAb, but was unable to elicit bNAbs [209].

Although the elimination of the gp120/gp41 cleavage site is non-native by virtue of its obligatory requirement in mediating viral infection of target cells [210], the role that furin-cleavage has on Env antigenicity and subsequent immunogenicity remains unclear. While efficient cleavage has been shown to affect the display of neutralizing epitopes [211], particularly benefiting the antigenicity of gp41 [212], uncleaved gp160 on the expressed cell surface has shown comparable binding of PG9 and PG16 antibodies to cleaved Env [156].

Uncleaved gp140 can be further stabilized by the introduction of heterologous trimerization motifs to the carboxyl terminus. The two most commonly used trimerization motifs include the GCN4 leucine zipper peptide ([213-215] and the carboxyl terminal region of bacteriophage T4 fibritin [216]. Other less frequently used trimerization motifs include chicken cartilage matrix protein [217] and catalytic chain of aspartate transcarbamoylase [218]. The GCN4 as well as other coiled-coil proteins are characterized by a seven-residue repeat with incorporation of hydrophobic (H) residues corresponding to the first and fourth positions of the repeat region, and polar (P) residues elsewhere (H·P·P·H·P·P·P) [213]. Manipulation of amino acids at position 1 or 4 in the heptad repeat allows for selective formation of dimeric, trimeric or tetrameric conformations. Alternating isoleucine and leucine at positions 1 and 4 of the heptad repeat preferentially selects for the formation of the dimeric conformation similar to the native the heptad repeat selects for a parallel, trimeric conformation and is incorporated as the heterologous carboxyl terminal trimerization motif in gp140-GCN4 constructs [213-215].

Interestingly, comparative immunization studies of matched monomeric and trimeric Env conformations have shown marginal improvements in antibody breadth and neutralizing titer [219-222]. Overall, none of the abovementioned approaches have managed to elicit bNAbs that show activity against tier 2 pseudoviruses (reviewed in [223]. Presumably these trimeric Env conformations still do not completely mimic the native Env spike and adequately display relevant NAb epitopes in the context of vaccination.

1.10.2. Stabilization of the CD4-bound conformation

By virtue of their neutralizing breadth, bNAbs target conserved regions of the HIV-1 Env spike [224]. While certain conserved epitopes are constitutively exposed, others appear to be occluded by the variable loop structures and only transiently exposed during binding and entry processes [225, 226]. Unbound Env is presumably in a closed conformation, and relatively inaccessible to bNAbs. The CD4-bound conformation of gp120 induces significant conformational changes in the core domains (inner, outer and bridging sheet), reorienting the surface variable loop structures and the co-receptor binding site, and exposing residues preferentially recognized by CD4i antibodies [128, 132]. The CD4i epitope is antigenically highly conserved amongst the lentivirus family (HIV-1, HIV-2 and SIV), despite only sharing approximately 50% amino acid sequence similarity [130]. This antigenic conservation of the co-receptor binding site is illustrated by the ability of CD4i MAbs to neutralize both HIV-2 and SIV strains following priming with soluble CD4 [130]. Stabilized complexes of gp120-CD4 were shown to elicit NAbs in macaques [227], also showing accelerated clearance of plasma viremia and an absence of long term tissue viremia following heterologous challenge with $SHIV_{SF162P3}$ [228].

Due to the transient nature of the CD4i epitope, a number of strategies aimed at stabilizing the CD4-bound conformation gp120 thereby limiting the conformational flexibility between the inner and outer domains [146] and stabilizing the CD4i epitope are being explored as vaccine immunogens. These include chemical cross-linking of gp120 and CD4 molecules [227, 229]; expression of a CD4, gp120 single chain polypeptide separated by a flexible linker sequence (FLSC) [229, 230]; the generation of CD4 mimetic peptides (CD4M33, M9 and M64U1-SH) [231-233]; CD4bs directed antibodies that induce the CD4i epitopes (such as the monoclonal antibody A32) [234]; introduction of structure-guided amino acid substitutions that stabilize the CD4-bound conformation of gp120 such as the “cavity-filling” mutations (S375W and T257S) [235]; the introduction of cysteine pairs in gp120 that allow for intramolecular

disulphide bond formation [236, 237]; as well as Env sequences derived from CD4-independent viruses that naturally display enhanced exposure of the co-receptor binding site [238].

1.11. Justification for the study

Overall, despite recent advances in the design of HIV-1 Env-based vaccine immunogens, such immunogens have not yet elicited broadly cross-reactive NAb against circulating primary HIV. This is now attributed to the fact that current Env-based vaccine immunogens and/or immunization regimens are unlikely to drive the evolution and high levels of antibody affinity maturation (up to appromiately 30% from unmutated common ancestor antibody) needed to get the required protective NAb responses in humans [162, 239]. There is a lack of fundamental knowledge on the way B cells recognize such conserved neutralization epitopes on Env immunogens and how they subsequently respond to that recognition. It has become increasingly evident that improving the immunogenicity of such epitopes, and ensuring appropriate B cell recognition is crucial for the elicitation of broad anti-HIV NAb responses. Strategies to program/educate the B cell response by sequential exposure to native HIV-1 Env quasispecies isolated from individuals with a broadened NAb response or prolonged antigenic stimulation may drive Env-specific antibody maturation both by presenting new epitopes in escape variants and by focusing the response on conserved epitopes, such as conformation-dependent regions of Env involved in CD4 and chemokine co-receptor binding [162, 164, 239].

There is thus renewed interest in the development of alternative Env-based subunit immunogens that may better overcome current roadblocks and chart a clear path towards the development of a safe and effective HIV-1 vaccine. One such immunogen concept is the use of Env and human CD4 in configurations that replicates the conserved Env-CD4 complex and exposes CD4i epitopes, a key transition state that all HIV Env's must realize during infection. Similar to the strategy used to stabilize the SOS-IP gp140 trimeric Env conformations, our group and others have engineered intermolecular disulphide bonds that stabilizes CD4 binding or M64U1-SH CD4 mimetic binding to gp120 [231, 240]. We have previously shown that the introduction of a structurally conserved, serine to cysteine substitution at position 60 in two domain CD4 ($2dCD4^{S60C}$) allows for intermolecular targeting of the disulphide-bridge between cysteine residues 126 and 196, positioned at the base of the V1/V2 loop structure of gp120 [240]. The resultant gp120-$2dCD4^{S60C}$ interaction is thus further stabilized by the newly-formed intermolecular disulphide-bridge between cysteine residues 60 on CD4 and 126 on gp120 (Figure 1.7). One potential mechanism of stabilizing an Env dimer which was investigated in this study was the introduction of a selenocysteine residue on the Env carboxy-terminus. The

rationale behind this approach is based on the highly nucleophilic properties of selenocysteine (reviewed in [241]), which we reasoned would facilitate highly stable intermolecular diselenide bond formation between the two gp120 monomers.

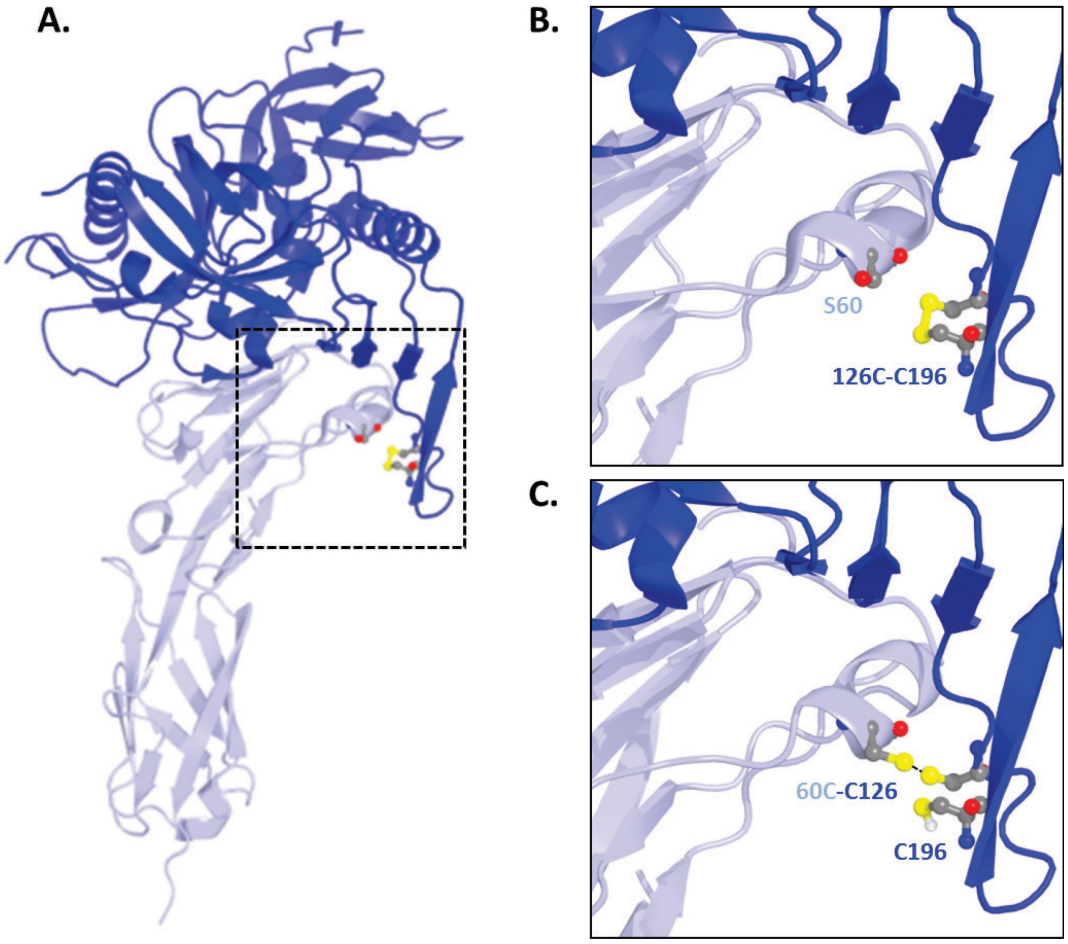


Figure 1.7: Ribbon diagram showing the molecular structure of the gp120-CD4 receptor complex (Protein Data Bank: 1G9M). A. gp120 (dark blue), CD4 (grey) with the Ser60 on the CD4 domain one represented and the disulphide bond between 126Cys -Cys196 on gp120 represented in ball-and-stick format. **B.** Enlarged graphic showing the proximal positioning Ser60 on CD4 to the 126Cys -Cys196 disulphide located at the base of the V1/V2 loop of gp120. **C.** Introduction of a cysteine residue at position 60 in CD4 predicts the formation of an intermolecular CD4 60Cys -Cys126 gp120 disulphide bond. Image adapted from [240].

HIV-1 subtype C viruses account for over 48% of the total new infections worldwide and predominate in those countries most adversely affected by the global pandemic, particularly South Africa (14). The development of an effective HIV-1 vaccine will require answering two key questions. Firstly, what are the relevant properties of HIV-1 isolates on which to base Env vaccine immunogen designs? Secondly, what is the best mechanism for eliciting bNAb responses against primary HIV-1 isolates? Vaccine constructs should be representative of the circulating subtypes in each country, and therefore HIV-1 subtype C sequences should be incorporated in vaccines targeting the South African population. The availability of *env* sequences from

transmitted founder viruses from South Africa [43] was exploited in this study to infer a consensus founder virus Env sequence that encodes more relevant sequences for vaccine design than viruses that are the basis for current vaccines. In addition, this study focused on the production of novel functional HIV-1 subtype C gp120 monomers, gp120 dimers and gp140 trimers/oligomers that better expose neutralizing epitopes in the unliganded or 2dCD4^{S60C}-liganded conformation, as compared to the matched gp120 monomer, with the aim of improving the immunogenicity of these glycoproteins. An understanding of the structure and systematic immunogenicity of these novel dimeric and trimeric molecules should assist development of their potential as immunogens for the induction of broadly NAbs in future South African vaccine recipients.

Thus, the overall aim of the study was to generate an HIV-1 subtype C founder virus Env consensus sequence and evaluate the immunogenicity of monomeric gp120, dimeric gp120 and oligomeric gp140 consensus founder virus Envs in the 2dCD4^{S60C}-liganded and unliganded conformations.

This was achieved by the following objectives:

1. To identify, select and design a consensus founder virus subtype C sequence and to produce codon optimized gene constructs of consensus founder virus monomeric gp120, dimeric gp120 and trimeric gp140.

2. To express, purify and characterize recombinant gp120 monomer, gp120 dimer and gp140 trimer/oligomer.

3. To create and characterize stable complexes of Env and 2dCD4^{S60C} (CD4-liganded conformation).

4. To evaluate the immunogenicity of 2dCD4^{S60C}-liganded and unliganded Envs in rabbits, and determine the NAb titers.

Chapter 2: Materials and Methods

2.1. Design and generation of HIV-1 envelope glycoprotein (*env*) constructs

2.1.1. Selection and design of HIV-1 subtype C founder virus *env* consensus sequences

Sequence data published previously using single genome amplification of HIV-1 env sequences from patients acutely infected with HIV-1 subtype C was used to generate an HIV-1 subtype C founder virus Env sequence. The patient cohort comprised 81 individuals from South Africa (40) [43], Malawi (29) [43] and Zambia (12) [49] all acutely infected with HIV-1 subtype C, as classified according to the Fiebig stage classification system [242], following heterosexual transmission (Table 2.1). Twenty four of the individuals were in Fiebig stage I/II (5-10 days post infection (p.i.), 20 patients were in stage III/IV (13-19 days p.i.) and 36 patients were identified to be in the latter stages of acute infection, Fiebig stages V/VI (88 days onwards p.i.) (Table 2.1). Overall, in 70% (n=62) of the patients identified in these cohorts, productive infection occurred as a result of a single transmitted viral variant. For the remaining 30% (n-=19) of patients, productive infection was as a result of simultaneous transmission of multiple variants (mean 2.4, range 2 to 5).

Table 2.1: Selected sequence information for the generation of an HIV-1 subtype C founder virus *envelope* consensus sequence.

Geographical Location	Total number of sequences analyzed	Number of infected patients arising from:		Fiebig stage		Ref.
		Single variant	Multiple variants			
South Africa	823	33	7	I/II III/IV V/VI	8 9 23	[43]
Malawi	652	21	8	I/II III/IV V/VI	14 10 5	[43]
Zambia	351	8	4	I/II III/IV V/VI	2 1 9	[49]
Total	1894	62	19	I/II III/IV V/VI	24 20 36	

Of the published 1927 *env* sequences isolated from these 81 patients, 1894 sequences were available for downloading as FASTA files from the Los Alamos National Laboratory HIV sequence database [172] and these were analysed further. An average of 22 *env* sequences, with a range of 15-42, were obtained for each patient from 40 South African and 29 Malawian patients (accession numbers: FJ443128-FJ444632 [43]). From 11 Zambian patients, an average of 28 *env* sequences, with a range of 24 to 49, were obtained for each patient (accession numbers: EU166413-EU166473, EU166482-EU166517 and EU166544-EU166919 [49]). Sequence analysis of *env* isolated from patient ZM231F revealed an outlier from the established group M of HIV-1, and this sequence was therefore excluded from further analysis [49].

The individual *env* sequences were aligned for each patient using Clustal X 2.0.11 [243]. Consensus *env* sequences were manually generated for each quasispecies/variant present in each patient, and subsequently translated in Genedoc to obtain the amino acid sequence (Env). Consensus Envs from all 80 patients were aligned in Clustal X 2.0.11 [243], and viewed in GeneDoc [244] to generate a geographical Env consensus sequence from each country manually. The ProSite search function available in GeneDoc was used to identify PNGs, known bNAb epitopes/binding residues and other relevant functional sites.

The South African, Malawian and Zambian consensus Env sequences were then analyzed manually to generate a final HIV-1 subtype C founder virus Env consensus sequence, based on the information generated above. This is subsequently termed the HIV-1 subtype C founder virus *env*. Phylogenetic and molecular evolutionary analysis was achieved using a multiple alignment (Clustal X 2.0.11) of the newly generated gp160 with references from HIV-1 subtypes A to K, CRF01_AE, CRF02_AG, Consensus C and Ancestral C Env sequences, and generating a neighbor joining tree using MEGA 4 [245].

2.1.2. HIV-1 subtype C founder virus *env* construct design

The HIV-1 subtype C founder virus *env* sequence was used to design five constructs to effect the expression of monomeric gp120 ($gp120_{FVC}m$), dimeric gp120 ($gp120_{FVC}GCN4d$), selenocysteine incorporating dimeric gp120 ($gp120_{FVC}Ud$), and trimeric gp140 with and without an intact cleavage site ($gp140_{FVC}GCN4t(+)$ and $gp140_{FVC}GCN4t(-)$, respectively).

The $gp120_{FVC}m$ monomer, $gp120_{FVC}GCN4d$ dimer and $gp120_{FVC}Ud$ dimer constructs all contained the founder virus gp120 envelope sequence. The $gp120_{FVC}GCN4d$ dimer has a dimer-forming, leucine zipper sequence (amino acid sequence: NH_3-GMKQLEDKIEELLSKIYHLENEIARL KKLIGEV-COOH) denoted GCN4d, fused to the carboxyl-terminal end of the glycoprotein [213].

The $gp120_{FVC}$Ud dimer has an in-frame TGA codon followed by CGG (Arg) codon, a TAA stop codon and the first 1509 nucleotide sequence derived from 3'UTR of thioredoxin reductase 1 (*Homo sapiens*) which contains the selenocysteine insertion sequence (SECIS) element (5'ATTTGGCAGGGCATCGAAGGGATGCATCCATGAAGTCACCAGTCTCAAGCCCATGTGGTAGGCGGT GATGGAACAACTGTCAAATCAGTTTTAGCA-3'). For the full TR1, 3'UTR sequence used in this construct refer to (Appendix A).

The $gp140_{FVC}$GCN4t(+) and $gp140_{FVC}$GCN4t(-) trimeric constructs were generated as previously described [214, 215]. These constructs contain the founder virus gp120 envelope sequence and ectodomain of gp41 (designated gp140) with a trimeric GCN4 (GCN4t), leucine zipper sequence (amino acid sequence: NH_3-GMKQIEDKIEEILSKIYHIENEIARIKKLIGEV-COOH) fused to the carboxyl terminal of the ectodomain of gp41 [213-215]. The positive (+) sign refers to the presence of an intact furin-cleavage amino acid sequence (REKR) while the minus sign (-) indicates disruption of this site by introducing arginine to serine mutation at the corresponding positions (SEKS).

The native N-terminal, Env leader peptide sequence was replaced with heterologous human CD5 antigen leader peptide (NH_3-MGMGSLQPLATLYLLGMLVASVLA-COOH), which has been shown to facilitate optimal expression and secretion of Env proteins [246, 247].

The amino acid sequences were provided to GeneArt (Regensburg, Germany), who codon optimized the nucleotide sequence for human/mammalian expression. DNA cassettes encoding the open reading frame of the five codon optimized HIV-1 founder virus *env* were synthesized and sub-cloned by GeneArt into the pcDNA™3.1 (Invitrogen, Life Technologies, Carlsbad, CA, USA) mammalian expression vector using the *Eco*RI and *Bam*HI restriction sites.

2.2. Transformation of constructs into *Escherichia coli* DH5α and large scale recombinant plasmid isolations

2.2.1. *Escherichia (E.) coli* DH5α transformation

The five lyophilized pcDNA™3.1-containing founder virus *env* constructs ($gp120_{FVC}$m monomer, $gp120_{FVC}$GCN4d dimer, $gp120_{FVC}$Ud dimer, $gp140_{FVC}$GCN4t(+) and $gp140_{FVC}$ GCN4t(-)) were re-suspended at a final concentration of 0.1 µg/ml in sterile distilled water. Plasmid stocks of pET15b-2dCD4^{S60C} and pCI-neo-$gp120_{BaL}$, as described in section 2.3.1. were previously cloned in our laboratory and were used for subsequent transformation experiments. Two hundred nanograms of the five founder virus pcDNA3.1 constructs, pET15b-2dCD4^{S60C} and pCI-neo-

$gp120_{BaL}$ were used to transform chemically competent *E. coli,* DH5α cells (Invitrogen, Life Technologies, Carlsbad, CA, USA). Plasmids were incubated with the chemically competent bacterial cells on ice for 20 minutes (mins), heat-shocked at 42°C for 90 seconds and returned immediately to ice for a further 5 mins. Transformed bacterial cells were cultured on selective Luria-Bertani (LB) agar plates (35 g/l LB Agar (Sigma-Aldrich, St. Louis, MO, USA) dissolved in distilled water and sterilized by autoclaving) supplemented with ampicillin (Roche, Mannheim, Germany) at a final concentration of 100 µg/ml (1000 × ampicillin stock solution (100 mg/ml) made up in 70% [v/v] ethanol (Merck-Millipore, Darmstadt, Germany). Plates were incubated overnight at 37°C.

2.2.2. Plasmid isolations

Recombinant plasmid DNA was purified from transformed *E. coli,* (DH5α) using the Midi plasmid purification kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Briefly, a single, transformed bacterial colony was selected and used to inoculate a sterile Erlenmeyer flask containing 150 ml of LB media (10 g/l tryptone (Merck, Darmstadt, Germany); 5 g/l yeast extract (Merck-Millipore, Darmstadt, Germany) and 10 g/l sodium chloride (NaCl) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 µg/ml ampicillin. Flasks were incubated overnight at 37°C in a high-rotation, shaking incubator. Bacterial cells were harvested by centrifugation at 6,000 × g for 15 mins at 4°C. The supernatant was discarded and the bacterial pellet re-suspended in 5 ml of P1 buffer. Bacterial cells were lysed under alkaline conditions using 5 ml of P2 lysis buffer. Bacterial chromosomal DNA was precipitated by the addition of 5 ml of chilled P3 buffer and removed by passing the lysate through the Qiafilter cartridge. The clarified lysate containing the recombinant plasmid was passed over a QBT-equilibrated, anion-exchange chromatography Qiagen-tip. The lysate was allowed to enter the Qiagen-tip by gravitational flow, followed by two, 10 ml washing steps with QC buffer, to remove RNA and protein contaminants. Bound-recombinant plasmid was eluted from the Qiagen-tip in 5 ml QF elution buffer. Plasmid DNA was precipitated by the addition of 0.7 volumes of isopropanol (Merck-Millipore, Darmstadt, Germany) (3.5 ml) and centrifuged at 15,000 × g for 30 mins at 4°C. Residual isopropanol was removed by washing with ethanol (70% [v/v]), followed by air-drying of the plasmid DNA pellet. The DNA pellet was gently re-suspended in sterile distilled water to a final concentration of 0.5 mg/ml, as determined by UV absorption at A_{280nm} using the NanoDrop ND 1000 spectrophotometer (ThermoFischer Scientific, Waltham, MA, USA). Plasmids were stored in aliquots at -20°C.

2.3. Expression, purification and characterization of recombinant monomeric, dimeric and trimeric envelope glycoproteins (Env) and Env-2dCD4^{S60C} complexes

2.3.1. Reagents used

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (contributor in parenthesis): HIV-1 gp120$_{BaL}$ (Dr. Marvin Reitz) recombinant protein was used as a positive control in Western blotting and functional ELISA-binding assays. HIV-1 Monoclonal antibodies (IgG1b12) (Dr. Dennis Burton and Carlos Barbas), 17b (Dr James E. Robinson) and recombinant soluble Human CD4 (sCD4) (Progenics Pharmaceuticals, Inc. Tarrytown, NY, USA) were used in functional ELISA binding assays.

The HEK293T cell line was available in our laboratory and used for recombinant expression of Env glycoproteins and the generation of HIV-1 pseudoviruses (refer to section 2.9.3.1.).

The pET15b-2dCD4^{S60C} expression cassette was available in our laboratory and has been described previously [240]. Briefly, the first 2 amino-terminal domains of human CD4 (2dCD4 residues 1 – 183, wild type sequence) were synthesized by GeneArt (Regensburg, Germany). The 2dCD4 sequence was codon optimized for *E. coli* expression, with a single Ser/Cys substitution introduced at position 60 (S60C) and a 6 x polyhistidine (HIS) encoding sequence introduced in frame at the 3'-terminus. This was used for adequate recombinant expression of 2dCD4^{S60C} in *E. coli* BL21 (DE3). Purified recombinant wild type 2dCD4 (2dCD4^{WT}) protein was available in our laboratory for use as a positive control.

The gp120$_{BaL}$ Env glycoprotein was expressed for use as a positive control. The expression cassette (pCI-neo-gp120$_{BaL}$) was available in our laboratory and was generated as follows: The gp120$_{BaL}$ envelope amino acid sequence is identical to that available from the NIH AIDS Research and Reference Reagent Program (Catalogue number: 4961), as contributed by Dr. Marvin Reitz. The gp120$_{BaL}$ monomer construct was synthesized by GeneArt (Regensburg, Germany), codon-optimized for mammalian cell expression and a 6 x HIS encoding sequence was introduced on the 3'-terminus. Restriction endonuclease sites *Xho*I and *Eco*RI flanked the 5' and 3' ends of the coding sequence, respectively and were used to sub-clone the gp120$_{BaL}$ coding sequence into the mammalian expression vector pCI-neo (Promega, Madison, WI, USA).

2.4. Envelope glycoprotein mammalian expression and purification

2.4.1. General mammalian cell culture protocol

All mammalian tissue culture experiments and maintenance of cultures, unless otherwise stated, were carried out at 37°C using a humidified, water jacketed incubator (ThermoFischer Scientific, Waltham, MA, USA) in an atmosphere supplemented with 5% CO_2 (Medical CO_2, Afrox, Selby, South Africa).

The HEK293T and TZM-bl adherent cell lines were maintained in Dulbecco's modified Eagles Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) (Gibco, Life Technologies, Carlsbad, CA, USA), 2 mM GlutaMax (Gibco, Life Technologies, Carlsbad, CA, USA), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA). Cells were sub-cultured twice a week using a 1 × trypsin/EDTA solution (0.25% [w/v] trypsin and 0.02% [w/v] ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, MO, USA)) and re-seeded at a ratio of 1/5 – 1/10.

The 293 suspension cell line (FreeStyle™ 293-F cells) (Invitrogen, Life Technologies, Carlsbad, CA, USA) was maintained in FreeStyle™ 293 Expression Medium (Gibco, Life Technologies, Carlsbad, CA, USA). Adherent 293-F cells were mechanically dislodged from the tissue culture plate surface by pipetting action during subculturing procedures and re-seeded at a ratio 1/10 in an appropriate volume of FreeStyle™ 293 Expression Medium.

Cells were enumerated manually by staining the cell suspension at a 1:5 ratio with 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA). Viable cells were then enumerated by microscopic examination of the stained cell suspension on a Neubauer haemocytometer (Roth, Karlsruhe, Germany).

For prolonged storage of cell lines, cell suspensions were prepared at 1-2 × 10^6 cells/ml concentration in freezing mix (HEK293T/TZM-bl cell lines – 90% [v/v] FCS and 10% [v/v] dimethyl sulphoxide (DMSO) (Fluka Chemika, Buchs, Switzerland); 293-F cell line – 90% [v/v] Freestyle™ 293 expression medium and 10% [v/v] DMSO), aliquots were placed in cryovials (Nunc, Roskilde, Denmark) and cooled at a rate of -1°C/minute using a Mr Frosty™ freezing container (ThermoFischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The working cell line was generally discarded passage 30 and replaced by rapidly thawing frozen cell line stocks and diluting them into an appropriate volume of growth media.

2.4.2. Transient transfections

Initial expression and characterization of the founder virus Env and $gp120_{BaL}$ glycoproteins were carried out by transient transfection of HEK293T cell lines. Transfections were carried out in T25 tissue culture flasks (Nunc, Roskilde, Denmark) using Polyfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 24 hours prior to transfection, 1.2×10^6 cells were seeded into a T25 flask. Four micrograms of purified plasmid DNA was combined with 40 µl Polyfect in a final volume (500 µl) of serum free media II (SFMII) (Life Technologies, Carlsbad, CA, USA). The transfection complexes were mixed thoroughly and incubated for 5 mins at room temperature, before gently added to the cells. Eight to twelve hours later the media was replaced with 10 mls SFMII media and incubated for a further 48 hours. Supernatants containing the Env glycoproteins were then harvested, and clarified by centrifugation at 1,000 × g for 10 mins and stored at -20˚C until analysis. Expression was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as described in section 2.7.2.

2.4.3. Generation of stable cell lines expressing each construct

Large scale expression of the founder virus Env and $gp120_{BaL}$ glycoproteins was achieved through generating stably expressing 293-F suspension cell lines. Transfections were carried out as described above. Subsequently, stably expressing cell lines were selected using a final concentration of 500 µg/ml G418 sulphate (Gibco, Life Technologies, Carlsbad, CA, USA) with media changes every 4-5 days as required. Further selection of the stable cell lines was achieved through limiting dilution. Recombinant protein expression was confirmed by SDS-PAGE and Western blot analysis as described in section 2.7.2. The stably expressing cell lines were transferred to 1,700 cm^2 expanded surface roller flasks (Corning, NY, USA), and maintained in 150 ml of FreeStyle™ 293 Expression medium, supplemented with 250 µg/ml of G418 sulphate. Cultures were maintained for two weeks and supernatants were harvested daily, centrifuged at 1,000 × g for 10 mins, filtered through a 0.45 µm PALL Supor, low-protein binding filter syringe (PALL Corporation, Port Washington, NY, USA) and stored at -20˚C until lectin-affinity purification.

2.5. Founder virus Env and $gp120_{BaL}$ glycoprotein purification

2.5.1. Lectin-affinity chromatography

Envelope glycoproteins were purified by an optimized lectin-affinity chromatography procedure. Separate lectin bound resin was used for each Env glycoprotein purified. Four milliliters of *Galanthus (G.) nivalis* lectin cross-linked to 4% beaded agarose (Sigma-Aldrich, St. Louis, MO, USA) was incubated with stirring, overnight at 4˚C in approximately 500 ml of harvested culture supernatant containing the expressed protein. The resin was collected and packed into a luer-lock, non-jacketed liquid chromatography column (Dimensions: internal diameter (i.d.) 2.5 × 10 cm) (Sigma-Aldrich, St. Louis, MO, USA). All subsequent washing and elution procedures were carried out using the Biologic LP Chromatography System (BioRad, Hercules, CA, USA). All buffers were maintained on ice. The packed resin bead was washed with 20 column bed volumes (80 mls) of 0.65 M NaCl, in Dulbecco's 1× phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) followed by 20 column bed volumes of 1.0 M NaCl, in Dulbecco's PBS, followed by a final 20 column bed volumes of Dulbecco's PBS. Bound Env glycoproteins were eluted with 7.5 bed volumes of Dulbecco's PBS containing 1.0 M methyl α-D-mannopyranoside (MMP) (Sigma-Aldrich, St. Louis, MO, USA). Eluted Env was filter-sterilized using 0.45 µm PALL Supor, low-protein binding filters and concentrated to approximately 1 ml in volume using an Amicon® Ultra Centrifugation Filter Unit (Millipore, Billerica, MA, USA) with a 50 kDa molecular weight cut-off. Purified and concentrated Env was snap-frozen in liquid nitrogen and stored at -80˚C. The optimized protocol allowed for the routine purification of 2 to 3 mg of each Env from 1 liter of culture supernatant. The purity of the recombinant protein was confirmed by SDS-PAGE and Western blot analysis as described in Section 2.7.2.

2.5.2. Size exclusion chromatography (SEC-FPLC)

Monomeric, dimeric and trimeric conformations of the founder virus Env/$gp120_{BaL}$ glycoproteins as well as each conformation in complex with $2dCD4^{S60C}$ were further purified by size exclusion chromatography. A size exclusion column (Dimensions: i.d. 2.5 × 50 cm) was packed according to the manufacturer's instructions with Superdex 200 prep-grade (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using the ÄKTA FPLC platform (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The Superdex 200 column was cleaned between the various Env glycoproteins using the cleaning-in-place protocol recommended by the manufacturer and re-equilibrated with 3 column volumes of sterile, 0.45 µm filtered Dulbecco's PBS. Elution profiles were monitored using UV absorbance at 280 nm. Founder virus Env glycoproteins

samples were centrifuged at 16,000 × g for 10 mins so as to pellet particulate matter, and adjusted to a final volume containing 1% sucrose (Sigma-Aldrich, St. Louis, MO, USA) (10% sucrose stock made up in Dulbecco's PBS and filtered through a 0.2 μm syringe filter). Recombinant Env glycoproteins were loaded onto the column manually in 500 μl volume batches and resolved at flow rate of 1 ml/min. Three milliliter fractions were collected and relevant fractions were pooled and concentrated using an Amicon® Ultra Centrifugation Filter Unit with a 50 kDa molecular weight cut-off. Finally, protein concentration of the concentrated fractions was determined using the BCA™ Protein Assay Kit (Pierce, ThermoFischer, Rockford, IL, USA) (refer to section 2.7.1. Env glycoproteins stocks were concentrated to 1 mg/ml, snap-frozen in liquid nitrogen and stored at -80˚C.

2.6. 2dCD4^{S60C} expression, purification and refolding

Recombinant 2dCD4^{S60C} was expressed and purified as previously described [240]. Briefly, pET15b-2dCD4^{S60C} constructs were used to transform *E. coli* BL21 (DE3). The 2dCD4^{S60C} protein was expressed in standard LB medium using an isopropyl 1-thio-β-D-galactopyranoside (IPTG)-free system. Cultures were grown at 37˚C at high rotation until mid-log phase and transferred to a 20°C incubator for 20 hours on high rotation. The 2dCD4^{S60C} proteins were purified from inclusion bodies using a denaturing purification protocol, followed by protein re-folding in glutathione-containing buffers. Bacterial cells were harvested by centrifugation, 6,000 × g for 30 mins at 4°C, and re-suspended in 25 ml/l of culture in Dulbecco's PBS containing 0.5 mg/ml chicken egg lysozyme (53,000 units/mg) (Sigma-Aldrich, St. Louis, MO, USA). Cell suspensions were incubated at 4°C for 1 hour on gentle rotation. Bacterial cells were lysed with 3 freeze/thaw cycles in liquid nitrogen, followed by 3 × 1 min cycles at 80% of sonication (Sonopuls, Bandelin, Berlin, Germany). The cell lysate was pelleted at 20,000 × g at 4°C for 45 mins, the supernatant was discarded and pellet re-suspended thoroughly in 25 ml of solubilization buffer (Dulbecco's PBS, pH 7.4 containing 8 M urea (Sigma-Aldrich, St. Louis, MO, USA), 50 mM glycine (Sigma-Aldrich, St. Louis, MO, USA), 20 mM imidazole (Merck-Millipore, Darmstadt, Germany), 0.5 M NaCl and 2 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). The supernatant was harvested by centrifugation at 20,000 × g for 45 mins at 4°C and filtered through a 0.45 μm PALL Supor, low-protein binding syringe filter.

The recombinant 2dCD4^{S60C} was purified by Ni^{2+} affinity chromatography. One milliliter of Ni^{2+} charged, packed resin was added to the homogenate and incubated overnight at 4°C with stirring. The resin was collected using a luer-lock, non-jacketed liquid chromatography column (Dimensions: i.d. 2.5 × 10 cm). Washing procedures were carried out manually, using 10 bed

volumes of wash buffer 1 (Dulbecco's PBS containing 8 M urea, 50 mM glycine, 20 mM imidazole, 0.5 M NaCl, and 2 mM β-mercaptoethanol), followed by 10 bed volumes of wash buffer 2 (Dulbecco's PBS containing 8 M urea, 50 mM glycine, 50 mM imidazole, 0.5 M NaCl and 2 mM β-mercaptoethanol). Bound protein was eluted from the resin in 2 × 10 ml elution buffer (Dulbecco's PBS containing 8 M urea, 50 mM glycine, 500 mM imidazole, 0.5 M NaCl, and 2 mM β-mercaptoethanol) for 5 mins at room temperature. Eluted 2dCD4^{S60C} was refolded by dialysis (SnakeSkin® dialysis tubing, 10 k MWCO, 35 mm dry i.d. (ThermoFischer, Rockford, IL, USA)) overnight at 4˚C in folding buffer 1 (50 mM glycine, 10% sucrose, 1 mM EDTA (BDH, Merck-Millipore, Darmstadt, Germany), 1 mM reduced glutathione (GSH) (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM oxidized glutathione (GSSG) (Sigma-Aldrich, St. Louis, MO, USA), 4 M urea (pH 9.6) (Sigma-Aldrich, St. Louis, MO, USA), followed by overnight dialysis at 4°C in folding buffer 2 (50 mM sodium carbonate (BDH, Merck-Millipore, Darmstadt, Germany), 10% sucrose, 1 mM EDTA, 0.1 mM GSH and 0.01 mM GSSG (pH 9.6)). The purified 2dCD4^{S60C} was then dialyzed 3 times against 20 × volumes of Dulbecco's PBS at 4°C for at least 2 hours each cycle. Precipitate was removed by centrifugation at 3,200 × g for 10 mins at 4°C followed by filtration through a 0.2 µm PALL Supor, low-protein binding syringe filter. Proteins samples were concentrated using an Amicon® Ultra Centrifugation Filter Unit with a 10 kDa molecular weight cut-off, aliquoted, snap-frozen in liquid nitrogen and stored at -80°C. The purity of the recombinant protein was confirmed by SDS-PAGE and Western blot analysis as described in Section 2.7.2.

2.7. Protein biochemical characterization

2.7.1. Total protein concentration determination

Total protein concentration of each purified recombinant Env and 2dCD4^{S60C} was determined using the BCA™ Protein Determination Kit (Pierce, ThermoFischer, Rockford, IL, USA) according to the manufacturer's microplate protocol. Briefly, a two-fold dilution series of the bovine serum albumin (BSA) standards ranging in concentration from 500.00 – 7.81 µg/ml were prepared using the 2 mg/ml BSA ampule supplied by the manufacturer. Standards were diluted in sterile Dulbecco's PBS and stored at 4°C for up to two months. Unknown samples were generally diluted 5 or 10 times in sterile Dulbecco's PBS. The prepared standards and unknown samples were assayed in duplicate. Twenty-five microliters of each sample was transferred to a flat bottomed, 96-well, polysorb Immuno plate (Nunc, Roskilde, Denmark). Two-hundred microliters of the BCA working reagent was added to each sample and incubated at 37°C on an orbital shaker (THERMOstar Microplate incubator, BMG Labtech, Ortenberg,

Germany) at 300 rpm for 1 hour. Following the incubation, plates were allowed to cool to room temperature before the absorbance at 570 nm was determined using the BioRad Model 680 microplate reader (BioRad, Hercules, CA, USA). Protein concentration of unknown samples was determined using a standard curve, which was generated by linear regression analysis using Microsoft Excel version 14.0.6129.5000 (Microsoft Corporation, Redmond, WA, USA) of the prepared standards of known concentration and the corresponding A_{570nm} values. Protein concentrations of unknown samples were determined by interpolating the A_{570nm} against the derived standard curve.

2.7.2. Characterization of recombinant proteins by SDS-PAGE

Initial verification of the expression and purification steps of the five HIV-1 founder virus envelope glycoproteins and $gp120_{BaL}$ was evaluated by SDS-PAGE followed by Western blotting or Coomassie blue staining. Mammalian tissue culture supernatants containing transiently or stably expressed envelope glycoproteins were prepared in 2 × Laemmli sample buffer (0.125 M Tris-HCl (Tris - (hydroxymethyl) aminomethane) (Sigma-Aldrich, St. Louis, MO, USA), 4% [w/v] sodium dodecyl sulphate (SDS) (Sigma-Aldrich, St. Louis, MO, USA), 20% [v/v] glycerol (Merck-Millipore, Darmstadt, Germany), 0.2 M dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA) and 0.02% bromophenol blue (Saarchem, Merck-Millipore, Darmstadt, Germany), pH 6.8.) boiled for 5 mins and resolved on a 10% SDS-PAGE gel (separating gel - 10% acrylamide (Sigma-Aldrich, St. Louis, MO, USA), 0.9% NN-methylenebisacrylamide (Sigma-Aldrich, St. Louis, MO, USA), 0.1% SDS (Sigma-Aldrich, St. Louis, MO, USA), 0.375 M Tris-HCl, pH 8.8; stacking gel – 4% acrylamide, 0.36% NN-methylenebisacrylamide, 0.1% SDS, 0.125 M Tris-HCl, pH 6.8). For Western blot analysis, PAGE gels were removed from the gel casting apparatus and equilibrated for approximately 10 mins in transfer buffer (20% [v/v] methanol (Merck-Millipore, Darmstadt, Germany), 0.025 M Tris, 0.192 M glycine and 0.1% SDS) at room temperature. Resolved bands were transferred to nitrocellulose membrane support (Hybond-C extra, Amersham, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 400 mA for 1 hour using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Hercules, CA, USA). Nitrocellulose membranes were blocked overnight in Tris-buffered saline containing Tween-20 (T-TBS) (0.25 mM Tris-HCl, 17 mM NaCl, 3 mM potassium chloride (KCl) (Sigma-Aldrich, St. Louis, MO, USA) pH 7.4 and 0.1% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA)) and 1% lyophilized BSA (Sigma-Aldrich, St. Louis, MO, USA). Founder virus Env glycoproteins were detected using a 1:2,000 dilution of HIV-1 infected patient serum (presumably infected with HIV-1 subtype C and obtained from the South African National Blood Services (SANBS), Constantia Kloof, SA) in T-TBS. Non-specific

antibodies were removed by 5 × 5 min washes, on an orbital shaker, with an appropriate volume of T-TBS. Bound antibodies were detected using a 1:25,000 dilution of goat anti-human IgG (Fc specific) alkaline phosphatase conjugate secondary antibody (Sigma-Aldrich, St. Louis, MO, USA). Bands were visualized on the nitrocellulose membrane by a precipitation reaction using SIGMFAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (Sigma-Aldrich, St. Louis, MO, USA) prepared according to the manufacturer's instructions. The reaction was allowed to proceed until clear visualization of bands (approximately 10-20 mins at room temperature) and terminated by extensive rinsing-off of the remaining BCIP/NBT substrate with water.

The incorporation of a carboxyl-terminal 6 x HIS tag on the expressed 2dCD4^{S60C} and gp120$_{BaL}$ Env glycoprotein allowed for the expression and purification of these proteins to be monitored using a 1:1,000 dilution of the Penta-HIS, Horse-Radish peroxidase (HRP) conjugated antibody (Qiagen, Hilden, Germany). The remaining anti-HIS antibody was rinsed off using the above Western blot washing protocol, before bound antibody was detected by conventional chemiluminscence reaction procedures using the SuperSignal West Pico Chemiluminescent Substrate (ThermoFischer Scientific, Rockford, IL, USA) and visualized using the ChemiDOC™ - XRS imager and Quantity One® analysis software (BioRad, Hercules, CA, USA).

2.7.3. Characterization of recombinant proteins by native PAGE

Analysis of the conformation states of the Env glycoproteins following size exclusion chromatography was evaluated using the NativePAGE™ Novex® 4-16% Bis-Tris Gel System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, a total of 5 µg of each purified Env glycoprotein conformation was prepared in deionized water containing the NativePAGE™ 4 × sample buffer (Invitrogen, Carlsbad, CA, USA). Samples were resolved using the Dark Blue Cathode buffer protocol (NativePAGE™ Running Buffer (20×), NativePAGE™ Cathode buffer additive, containing 0.4% Coomassie® G-250 (20×) made up in deionized water). The Anode buffer was prepared using the NativePAGE™ Running Buffer (20×). Electrophoresis was performed using the XCell *SureLock*™ Mini-Cell chamber at a constant voltage of 150 V for 110 mins. Protein sizes were estimated using the NativeMark™ Unstained Protein Standard (Invitrogen, Carlsbad, CA, USA) and individual protein preparations available for non-denaturing analysis (Kit for Molecular Weights 14,000-500,000 non-denaturing, Sigma-Aldrich, St. Louis, MO, USA).

Envelope-liganded 2dCD4^{S60C} complexes were evaluated under reducing conditions using the NuPAGE® Novex® 4-12% Bis-Tris Gel system (Invitrogen, Life Technologies, Carlsbad, CA, USA). A total of 10 µg of each protein complex was prepared in 3 × sample buffer (2:1[v/v] NuPAGE® LDS sample buffer (4×) (Invitrogen, Life Technologies, Carlsbad, CA, USA)) and 500 mM DTT, boiled for 5 mins before loading onto the gel. Electrophoresis was performed using the XCell *SureLock*™ Mini-Cell chamber at a constant voltage of 200 V for 40-50 mins.

Coomassie staining was used to visualize proteins following SDS-PAGE and native PAGE analysis. Gels were removed from the casting apparatus and placed in coomassie staining solution (50% [v/v] methanol, 10% [v/v] acetic acid (Merck-Millipore, Darmstadt, Germany), 0.025% [w/v] Coomassie R250 (VWR, Radnor, PA, USA) overnight, followed by de-staining (5% [v/v] ethanol, 7% [v/v] acetic acid).

2.7.4. Characterization of recombinant protein by ELISA

The functional integrity and conformation of the purified recombinant Env glycoproteins were evaluated by ELISA. Medium binding, 96-well ELISA plates (Greiner Bio-One, Frickenhausen, Germany) were coated overnight at 4°C with 100 µl of the monoclonal anti-gp120 antibody D7324 (1 µg/ml, Aalto Bio Reagents Ltd., Dublin, Ireland). Plates were then blocked with 250 µl per well with assay buffer (Dulbecco's PBS containing 0.05% Tween 20 and 1% [w/v] lyophilized BSA) for 1 hour at room temperature. A five-fold dilution series of each Env glycoprotein was prepared in Dulbecco's PBS (concentration ranges for various founder virus Env conformations were 2,000 – 16 ng/ml and for gp120$_{BaL}$ 1,000 – 8 ng/ml). Each Env dilution was incubated with an equal volume of 2 µg/ml of soluble CD4 (sCD4) (Progenics Pharmaceuticals Inc., Tarrytown, NY, USA) at room temperature for 1 hour. The remainder of each Env dilution series was adjusted to the sCD4/Env concentration by performing a further 2 fold dilution, to yield a final testing concentration of each founder virus Env conformation from 1,000 – 8 ng/ml and for gp120$_{BaL}$ 500 – 4 ng/ml.

The assay buffer was removed and plates were washed with PBS-T (Dulbecco's PBS containing 0.05% [v/v] Tween 20), 3 × 300 µl per well using a BioTek, ELx 50 automated plate washer (BioTek, Winooski, VT, USA). One hundred microliters of each dilution of either Env only, Env/sCD4 or Env/2dCD4^{S60C} was placed in the respective wells and incubated at room temperature for 2 hours. Proteins were assayed in duplicate and non-Env containing wells were included as a negative control. Unbound proteins were removed by washing each well 5 times with 300 µl PBS-T. Wells containing Env conformations only, were probed with IgG1b12 or 17b

antibodies, while Env/sCD4 or Env/2dCD4^{S60C} complexes were probed exclusively with 17b. One hundred microliters, of 1:2,000 dilution of IgG1b12 or 17b, prepared in assay buffer, was used to probe the respective wells and incubated for a further 2 hours at room temperature. Each well was washed 5 times with 300 µl PBS-T. Bound antibody was detected using 100 µl of HRP-conjugated monoclonal anti-human Fc antibody (Amersham Biosciences, Buckinghamshire, UK) diluted 1:2,000 in PBS-T for 1 hour at room temperature. Unbound secondary antibody was removed by washing each well 5 times with 300 µl PBS-T. Bound secondary antibody was detected by chromogenic methods using the Sure Blue™ TMB peroxide substrate (KPL, Gaitherberg, MD, USA) and TMB stop solution (KPL, Gaithersburg, MD, USA) as per the manufacturer's instructions. Absorbance values were quantified spectrophotometrically at 450 nm using a BioRad Model 680 microplate reader (BioRad, Hercules, CA, USA).

2.7.5. Characterization of immunogens by surface plasmon resonance (SPR)

The kinetics of the interactions between monomeric, dimeric and trimeric founder virus Env conformations and 2dCD4^{WT} were evaluated using SPR. Experimental procedures were performed on the ProteOn XPR36™ (BioRad, Hercules, CA, USA) platform, using BioRad recommended reagents and carried out at a flow rate of 30 µl/min, unless otherwise stated. Briefly, a GLC (compact capacity amine coupling for protein-protein interactions) sensor chip was pre-conditioned by sequentially passing solutions containing 0.5% SDS, 50 mM sodium hydroxide and 100 mM HCl, over the surface of the chip with a total contact time of 1 min/solution. The surface of the chip was activated by passing a mixture of 0.2 M 1-thyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.05 M N-hydroxysuccinimide (NHS) over the surface for 5 mins. Immediately following surface activation, 2dCD4 was immobilized by injecting a 2 µg/ml solution prepared in 10 mM sodium acetate (pH 5.0) over the surface for 5 mins, or until a minimum of 2,000 response units (RU) were reached. The chip surface was then blocked by performing a 5 min injection of 1 M ethanolamine-HCL (pH 8.0) and equilibrated for 5 mins in PBS-TE buffer (Dulbecco's PBS containing 3 mM EDTA and 0.005% Tween-20) to establish a baseline signal. The surface of the chip was regenerated between each Env conformation by injecting 10 mM glycine N-hydroxysuccinimide (pH 2.5) over the surface for 5 mins. Founder virus Env analytes were prepared in PBS-TE ranging in concentration from 0 – 500 nM. Kinetic binding data was obtained by injecting the analytes over the surface at a flow rate of 20 µl/min using a 20 min (1200 seconds) association and a 20 min (1200 seconds) dissociation phase. Reference flow cells (control) were subtracted from the

reaction data, before the data was fitted to a 1:1 Langmuir binding model. The ratio of rate constants (k_d/k_a) were used to calculate the equilibrium dissociation constant K_D.

2.7.6. Selenocysteine incorporation

HEK293T cells were transiently transfected with either $gp120_{FVC}m$ (control) or $gp120_{FVC}Ud$ constructs as previously described. Culture media was supplemented with 0.5 µM sodium selenite (Sigma-Aldrich, St. Louis, MO, USA) and 6 µCi/ml radiolabeled Se^{75} as selenite (University of Missouri Research Reactor, Columbia, MO, USA). Culture supernatants were harvested 72 hours later, centrifuged at 1,000 × g for 10 mins and concentrated ten-fold using an Amicon® Ultra Centrifugation Filter Unit with molecular weight cut-off of 50 kDa. The cellular fraction was collected, washed in 5 ml Dulbecco's PBS and re-suspended in a final volume of 500 µl Dulbecco's PBS. The concentrated supernatant samples were treated with an increasing amount of DTT from 0 - 100 mM (final concentration) in 2 × Laemmli sample buffer. Samples were boiled for 5 mins and resolved on a 10% SDS-PAGE gel. Expression of the $gp120_{FVC}m$ and $gp120_{FVC}Ud$ proteins was monitored by Western blot, using the protocol described in section 2.7.2. For the autoradiography analysis, SDS-PAGE gels were stained according to the protocol described in section 2.7.2. and dried using the DryEase® Mini-Gel Drying System (Invitrogen, Life Technologies, Carlsbad, CA, USA). Dried gels were placed on filter paper and the corresponding position of the molecular weight was spotted with diluted Se^{75} radioactive label and exposed to Amersham Hyperfilm MP (GE Healthcare, Uppsala, Sweden).

2.7.7. Founder virus envelope immunogen conformation/complex purification

Envelope-liganded $2dCD4^{S60C}$ complexes of each founder virus and $gp120_{BaL}$ conformation were generated using the following protocol. One milligram of purified, native Env glycoprotein was incubated with 1 mg of $2dCD4^{S60C}$ in the presence of a final concentration of 1 mM β-mercaptoethanol at room temperature for 1 hour. A precipitate was noted and samples were centrifuged at 16,000 × g for 10 mins before loading onto the Superdex 200 column. Envelope–liganded $2dCD4^{S60C}$ complexes were loaded and resolved according to the procedure mentioned above (section 2.5.2. and 2.7.2. Elution peaks corresponding to either monomeric, dimeric or trimeric Env were collected, pooled and concentrated using an Amicon® Ultra Centrifugation Filter Unit with a 50 kDa molecular weight cut-off. The protein concentration was determined

using a BCA™ Protein Assay Kit as described in section 2.7.1. Envelope-liganded 2dCD4^{S60C} complexes for immunizations were aliquoted in 120 µg quantities, snap-frozen using liquid nitrogen and stored at -80°C.

2.8. Immunogenicity testing of recombinant monomeric, dimeric and trimeric envelope glycoproteins (Env) and Env-2dCD4^{S60C} complexes

2.8.1. Rabbit immunization schedule

Animals were obtained, housed, and all immunization/bleeds carried out, by the staff of the Central Animal Service located at the University of the Witwatersrand, Health Sciences, Johannesburg, South Africa. Prior to initiation of the study, animal ethics clearance was obtained (University of the Witwatersrand, Animal Ethics Screening Committee, clearance certificate number: 2010/12/03, Appendix B). Immunizations were carried out in New Zealand White Rabbits (*Oryctolagus cuniculus*). Each rabbit received 20 µg of total protein-immunogen formulated in Dulbecco's PBS and Adjuplex (Advanced Bioadjuvants, LLC. Omaha, NE, USA distributed by Sigma-Aldrich, St. Louis, MO, USA) (Final volume 500 µl). Animals were evenly distributed between groups according to age and gender where possible, with 3 to 5 rabbits per group. Immunizations were administered intramuscularly, alternating right and left hind legs at 4 week intervals (week 0, 4, 8 and 12). Baseline serum samples were collected at weeks -2 and 0, with additional serum samples collected every 2 weeks post-immunizations (weeks 2, 6, 10 and 14). Ten milliliters of whole blood was collected by venipuncture of the marginal vein on the ear and collected in serum tubes (BD Vacutainer® red-capped tubes, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Terminal bleeds were carried out at week 14, 2 × 10 ml of whole blood was collected from the ear of each animal in serum tubes. Rabbits were subsequently anesthetized and an additional 5 × 10 ml serum tubes were collected by cardiac puncture. Coagulation was initiated by inversion of tubes several times before separation of the serum by centrifugation at 1,300 × g for 10 mins. Serum samples were aspirated and stored at -80°C until further characterization.

2.9. Neutralizing antibody assays

Terminal rabbit serum samples collected at week 14 and a single pre-bleed serum sample (week 0) from each group were evaluated for neutralization potency and breadth using a commercially available and in-house assay. Rabbit sera were heat inactivated at 56°C for one hour in a water bath before ELISA and neutralizing titers were determined.

2.9.1. Reagents used

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (contributor in parenthesis). Pseudoviruses were produced by transient transfection using the *env*-deficient backbone, pSG3$^{\Delta env}$ (Dr. John C. Kappes and Xiaoyun Wu) backbone and complementing Env(gp160)-expressing plasmids ZM53M.PB12 (Drs E. Hunter and C. Derdeyn) and SF162.LS (obtained from Dr. D. Monefiori, Laboratory for AIDS Vaccine R&D, Duke University Medical Center). The TZM-bl (JC53-bl) reporter cell line (Dr. John C. Kappes, Dr. Xiaoyun Wu and Transzyme Inc.) which contains the β-galactosidase and *Photinus pyralis* (firefly) luciferase reporter genes under the control HIV-1 long terminal repeat (LTR) promoter [248, 249]. This cell line was used to determine pseudovirus infectivity and subsequent rabbit sera neutralization activity in a single round infectivity assay described below in section 2.9.3. The TZM-bl cell line was maintained in complete DMEM and cultured according to the procedure described in section 2.4.1.

2.9.2. PhenoSense neutralizing antibody assay

Serum samples were evaluated using the PhenoSense Neutralizing Antibody Assay (Monogram Biosciences Inc., San Francisco, CA, USA), against a total of 12 pseudotyped viruses (Tier 1 – SF162, BaL and NL43; Tier 2 – 93IN905, 93MW959, 97ZA012, IAVI_C22, JRCSF, MGRM-C-004, MGRM-SC-B-012, MGRM-C-015; Tier 3 – MGRM-SC-B-005, rabbit sera 53 and 68 were additionally tested against HIV-2_{P2} and SIV_{mac316}, human sera Z23 was used as a positive neutralization control and aMLV envelope was used as a specificity control). Fifty-percent inhibitory dilution (IC_{50}; 1/dilution) for neutralizing sera was determined as the reciprocal of the dilution of serum required to achieve 50% inhibition.

2.9.3. In-house neutralizing antibody assay

2.9.3.1. Pseudovirus production

In-house testing of selected sera was achieved using the HIV-1 Env pseudotyped virus neutralizing assay as previously described [250]. Briefly, HIV-1 pseudoviruses were generated by co-transfection of HEK293T with *env*-deficient backbone expression plasmid ($pSG3^{\Delta env}$) and either ZM53M.PB12 or SF162.LS. Transfections were carried out in T25 culture flasks using Polyfect as described above. HEK293T cells were transfected with a total of 8 µg DNA plasmid, using a ratio of 2:1 (Env:Backbone plasmids) 40 µl Polyfect and made up to a final volume of 500 µl in SFMII. After eight to twelve hours the supernatant was discarded and replaced with 5 ml of complete DMEM. Supernatants were harvested 48 hours later, centrifuged at 1,000 × g, adjusted to 20% FCS, and filtered through a 0.2 micron syringe filter. Aliquots were stored at -80°C.

2.9.3.2. Pseudovirus titration and infectivity determination

Aliquots of the HIV-1 pseudoviruses were titrated against the TZM-bl cell line so as to determine the Median Tissue Culture Infectious Dosage ($TCID_{50}$) according to methods described previously [250]. A five-fold dilution series of each pseudovirus stock was prepared, using 96-well tissue culture plate (Nunc, Roskilde, Denmark), in a final volume of 100 µl of complete DMEM. Freshly trypsinized TZM-bl cells were visualized by Trypan Blue (Sigma-Aldrich, St. Louis, MO, USA) staining and enumerated using a haemocytometer as described in section 2.4.1. One hundred microliters of 1×10^5 cells/ml containing diethylaminoethyl-dextran hydrochloride (DEAE Dextran) in complete DMEM were transferred to each well. Pseudovirus stocks were titrated in quadruplicate and the media was supplemented with a final concentration of 20 µg/ml DEAE Dextran (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated for a further 48 hours, before infectivity was determined by means of the tat-inducible luciferase-based reporter assay. Following the 48 hour incubation the cell culture medium was aspirated from each well and cells were lyzed by incubating at room temperature for 5 mins with 100 µl of Glo-Lysis buffer (Promega, Madison, WI, USA). Fifty microliters cell lysate was transferred to a 96-well white solid luminometer plate (Promega, Madison, WI, USA) to which 50 µl Bright-Glo™ Luciferase reagent (Promega, Madison, WI, USA) was added and the luminescence was quantified using the Veritas luminometer (Turner Biosystems, CA, USA). The $TCID_{50}$ for each virus was determined using the Microsoft Excel macro established by the Duke Central Reference Laboratory, available online at the Los Alamos National Laboratory HIV immunology database (http://www.hiv.lanl.gov/).

2.9.3.3. Neutralizing antibody assay

Heat-inactivated rabbit sera were evaluated for neutralizing antibody activity by the reduction in tat-inducible luciferase activity following a single round infectivity assay as described previously [250]. A three-fold dilution series of each rabbit sera was prepared in a 96-well tissue culture plate and incubated with 200 $TCID_{50}$ of HIV-1 pseudovirus in a final volume of 150 µl of complete DMEM for 1 hour at 37°C. Serum samples were assayed in duplicate with matched pre-bleed serum to serve as a baseline, non-virus and virus containing only wells were included as appropriate controls. Subsequently, 10^4 freshly trypsinized TZM-bl cells prepared in a final volume of 100 µl using complete DMEM and supplemented with DEAE Dextran (20 µg/ml final concentration), were added to each well. Plates were returned to the incubator for a further 48 hours before the luminescence was quantified as described above (section 2.9.3.2.). The reciprocal of serum dilution required to achieve 50% inhibition (IC_{50}) relative to the virus control wells, after the control background has been accounted for, was calculated by fitting a variable-slope, non-linear regression curve using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

2.10. Preliminary characterization of antibody responses

2.10.1. End-point antibody titer ELISA

Total Env and/or $2dCD4^{S60C}$ binding antibody titers were determined by ELISA. For Env binding titer determination, medium binding 96-well ELISA plates were coated overnight at 4°C with 50 µl of a 1 µg/ml solution of the matched Env immunogen (either $gp120_{FVC}$m monomer, $gp120_{FVC}$GCN4d dimer, $gp140_{FVC}$GCN4t(+) trimer, $gp140_{FVC}$GCN4t(-) trimer or $gp120_{BaL}$ monomer). For determination of the $2dCD4^{S60C}$ specific binding titer, ELISA plates were coated with 50 µl of 1 µg/ml $2dCD4^{S60C}$ overnight at 4°C. All the subsequent incubation steps were carried out at room temperature with reaction volumes of 50 µl, unless stated otherwise. The following day the coating solution was discarded and plates were blocked for 1 hour with 250 µl of assay buffer (Dulbecco's PBS containing 0.05% Tween 20 and 1% [w/v] lypholized BSA) per well. Blocking solution was removed and plates were washed 3 × 300 µl/well with PBS-T (Dulbecco's PBS containing 0.05% Tween 20) using an automated plate washer. Heat-inactivated rabbit sera were prepared in assay buffer using a two-fold dilution series starting at 1:400 up to 1:1,638,400. Samples were transferred to the plate and incubated for 1 hour. Unbound rabbit antibodies were removed during the PBS-T washing protocol (5 × 300 µl/well). Bound rabbit antibodies were detected using a 1:5,000 dilution of a donkey anti-rabbit – HRP

linked, whole antibody (GE Healthcare UK, Buckinghamshire, UK), in assay buffer and incubated for a further 1 hour. Following a 5 × 300 µl/well wash, bound secondary antibody was detected by chromogenic methods using the Sure Blue™ TMB peroxide substrate and TMB stop solution as per the manufacturer's instructions. Absorbance values were quantified spectrophotometrically at A_{450nm} using a BioRad Model 680 microplate reader (BioRad, Hercules, CA, USA). End-point titers were determined as the reciprocal of the dilution required to obtain an A_{450nm} of 0.5 units. This value represents aminimum point in the linear range of the dilution curve, allowing comparison across all samples.

2.10.2. Specific antibody depletion of rabbit sera

2.10.2.1. Anti-Env specific antibody depletion

Anti-Env specific antibody responses of selected rabbit sera were depleted using $gp140_{FVC}$GCN4t(-) covalently cross-linked tosylactivated paramagnetic beads according to procedures described previously [251]. Protein coupling was performed using the Dynabeads MyOne™ tosylactivated magnetic beads (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 400 µg of $gp140_{FVC}$GCN4t(-) for anti-Env specific depletion (Dyna-gp140), or BSA – 2 mg/ml ampules, available from Pierce, ThermoFischer, Rockford, IL, USA) which served as a non-specific depletion control (Dyna-BSA) (mock depleted) was covalently cross-linked to 20 mg of Dynabeads. Protein coupling was carried out at 37°C in a final volume of 1.25 ml of coating buffer (0.1 M sodium borate buffer, pH 9.5 containing 1 M ammonium sulphate ($(NH_4)_2SO_4$) (Sigma-Aldrich, St. Louis, MO, USA) for 16 hours using a rotary mixer (Intelli-Mixer, ELMI, Aizakraukles, Latvia). The Dynabeads were retained using a magnetic separator while the supernatant was aspirated and discarded. The beads were then blocked overnight at 37°C in 1.25 ml of blocking buffer (Dulbecco's PBS, containing 0.5% BSA and 0.05% Tween-20) using a rotary mixer to maintain the beads in suspension. The following day the blocking buffer was discarded while the beads were retained using the magnetic separator and washed with 3 × 500 µl of washing/storage buffer (Dulbecco's PBS, containing 0.1% BSA and 0.05% Tween-20). Following the final wash, protein-coupled Dynabeads were re-suspended at a final concentration of 0.1 mg/ml in washing/storage buffer and stored at 4°C until use in the depletion experiments. Ten micrograms of the protein-conjugated Dynabeads were used to deplete 500 µl of the diluted rabbit sera.

Selected, heat-inactivated rabbit sera were prepared in complete DMEM, at the same dilution as the same starting concentration of the neutralizing antibody assay (13.72 ×). Ten micrograms of

the protein-conjugated Dynabeads were used to deplete 500 µl of the diluted rabbit sera, by incubating the bead suspension on a rotary mixer for 2 hours at room temperature. Following the 2 hour incubation, the depleted rabbit sera were evaluated for depletion efficacy and specificity by ELISA and impact on neutralizing activity (see section 2.10.2.3.).

2.10.2.2. Anti-CD4 specific antibody depletion

Anti-CD4 specific antibody responses were depleted using 2dCD4^{S60C} coupled via the 6 x HIS tag to magnetic, nickel charged (Ni^{2+}) beads as described below. Thirty microliters of HIS-Mag™ Agarose Beads (Novagen, Merck-Millipore, Darmstadt, Germany) suspension were rinsed with 3 × 500 µl Dulbecco's PBS using a magnetic separator to capture and retain the magnetic agarose beads. Two-domain CD4^{S60C} was linked to the HIS-Mag beads by incubating overnight at 4°C on a rotary mixer in the presence of 100 µg of 2dCD4^{S60C} made up in a final volume of 500 µl of Dulbecco's PBS. Beads incubated with just 500 µl of Dulbelcco's PBS were included as a non-depletion control while also serving as a non-specific depletion control. The supernatant was discarded and HIS-Mag beads were retained using a magnetic separator and washed using 3 × 500 µl of Dulbelcco's PBS to remove any unbound 2dCD4^{S60C}. Beads were then equilibrated and blocked using 500 µl complete DMEM. The HIS-Mag beads were retained using a magnetic separator and re-suspended in a final volume of 100 µl of complete DMEM. Fifty microliters of this final suspension (i.e. 15 µl of the original HIS-Mag suspension) was used to deplete 500 µl of selected diluted rabbit serum samples. Heat-inactivated rabbit serum samples were prepared in complete DMEM and diluted to the same extent as the starting concentration of the neutralizing antibody assay (13.72 ×).

2.10.2.3. Evaluation of anti-CD4 and anti-Env specific antibody depleted sera

Depletion of anti-Env and anti-CD4 specific antibodies responses was monitored by binding ELISA and subsequent impact on neutralizing capacity evaluated by the in-house neutralizing antibody assay (sections 2.10.1. and 2.9.3.3. respectively). Supernatants collected following the 2 hour depletion incubation, anti-Env specific antibody depletion (Dyna-gp140) or anti-CD4 specific antibody depletion (HIS-Mag-2dCD4^{S60C}), or the non-specific depletion control incubations (Dyna-BSA and HIS-Mag, respectively) were diluted further to 1:400 in assay buffer and evaluated for anti-CD4 and anti-Env binding levels. All subsequent steps were carried out as described in the End-point antibody titer ELISA protocol (section 2.10.1.). Antibody binding levels at 1:400 were normalized to the untreated terminal bleed serum samples.

Supernatants from depletion experiments were also evaluated for loss of neutralizing activity against the tier 1, SF162.LS and/or tier 2, ZM53M.PB12 pseudoviruses. One hundred and fifty microliters of the anti-Env or anti-CD4 specific depleted, or non-specific depleted controls were transferred directly into a 96-well tissue culture plate, at the position that corresponds to the highest serum concentration. The subsequent 3-fold dilution series, viral inoculum and incubations were carried out as described previously (section 2.9.3.3.).

Chapter 3: Results

3.1. Construction of the HIV-1 subtype C founder virus consensus Env sequence

An HIV-1 subtype C founder virus Env consensus sequence was successfully generated after detailed analysis of 1894 env sequences, derived from 80 acutely infected patients (Figure 3.1, Appendix C). To derive the final founder virus Env consensus sequence, the consensus Env amino acid sequences of each patient were extensively analyzed and PNGs, known residues/epitopes of bNAbs and other functional sites were identified (Appendix C). The amino acid length of the variable loops and number of PNGs were calculated and are shown in Table 3.1 and Table 3.2 respectively. The founder virus Env consensus sequence generated had shorter V1/V2, V4 and V5 loop sequences (Table 3.1; Figure 3.1), one less PNGs site in the V1/V2 loop structure and an additional PNG included in the C2 region and ectodomain of gp41, as compared to the average number found in early transmitted subtype C viruses (Table 3.2; Figure 3.1). Importantly, the founder virus consensus sequence contained the relevant residues for CD4 binding and includes leucine at position 125 (all numbering according to HXBc2) loop D of the constant region 2 (C2) (residues 276-283), the CD4 binding loop in the C3 region (residues 362-374) and V5/β24 region (residues 458-469) (Figure 3.1, red boxes with critical residues shaded red). Residues within these regions form the epitopes of the CD4bs-directed bNAbs such as IgG1b12, VRC01, NIH45-46, PGV04 and 3BNC117 [147-150, 252]. Glycan-dependent epitopes such as those required for PG9/PG16 (residues N156 and N160) and PGT family (PGT 125-128, 130, 131) of bNAbs (residues N301 and N332) were present (Figure 3.1, green boxes). The linear epitope for α4β7 integrin binding site (LDI/V – residues 179-181) and MPER directed bNAb 4E10 (NWFDIT - residues 671-677) were also present (Figure 3.1, black-background with pink or yellow text, respectively).

Phylogenetic tree analysis revealed that the founder virus Env consensus sequence was most closely related to the inferred ancestral and consensus C sequences available from the Los Alamos database, rather than those from circulating viruses (Figure 3.2).

Table 3.1: Average number of amino acids present in the variable loops of gp120 and the gp41 ectodomain in early transmitted HIV-1 subtype C viruses from each country and the newly inferred founder virus Env consensus sequence.

Geographical Location	Mean number of amino acid residues				
	gp120				ectodomain of gp41
	V1/V2	V3	V4	V5	
South Africa	70.2	32.8	24.8	8.4	170.9
Malawi	72.5	32.9	26.5	7.9	170.9
Zambia	65.9	32.9	27.8	8.3	171.1
HIV-1 subtype C founder virus Env consensus	**55**	**33**	**18**	**6**	**171**

Table 3.2: Average number of potential N-linked glycosylation sites as predicted by ProSite in the conserved (C) and variable (V) regions of gp120 and the gp41 ectodomain of early transmitted HIV-1 subtype C viruses from each country and the newly inferred founder virus Env consensus sequence.

Geographical Location	Mean number N-Linked glycosylation sites									
	gp120									ectodomain of gp41
	C1	V1/ V2	C2	V3	C3	V4	C4	V5	C5	
South Africa	1.0	6.3	6.4	1.0	2.8	3.8	1.7	1.5	0	4.2
Malawi	1.0	6.2	6.3	0.9	2.8	4.0	1.4	1.2	0	3.9
Zambia	1.0	5.7	6.2	0.9	2.9	3.9	1.7	1.4	0	3.8
HIV-1 subtype C founder virus Env consensus	**1**	**5**	**7**	**1**	**3**	**4**	**2**	**1**	**0**	**5**

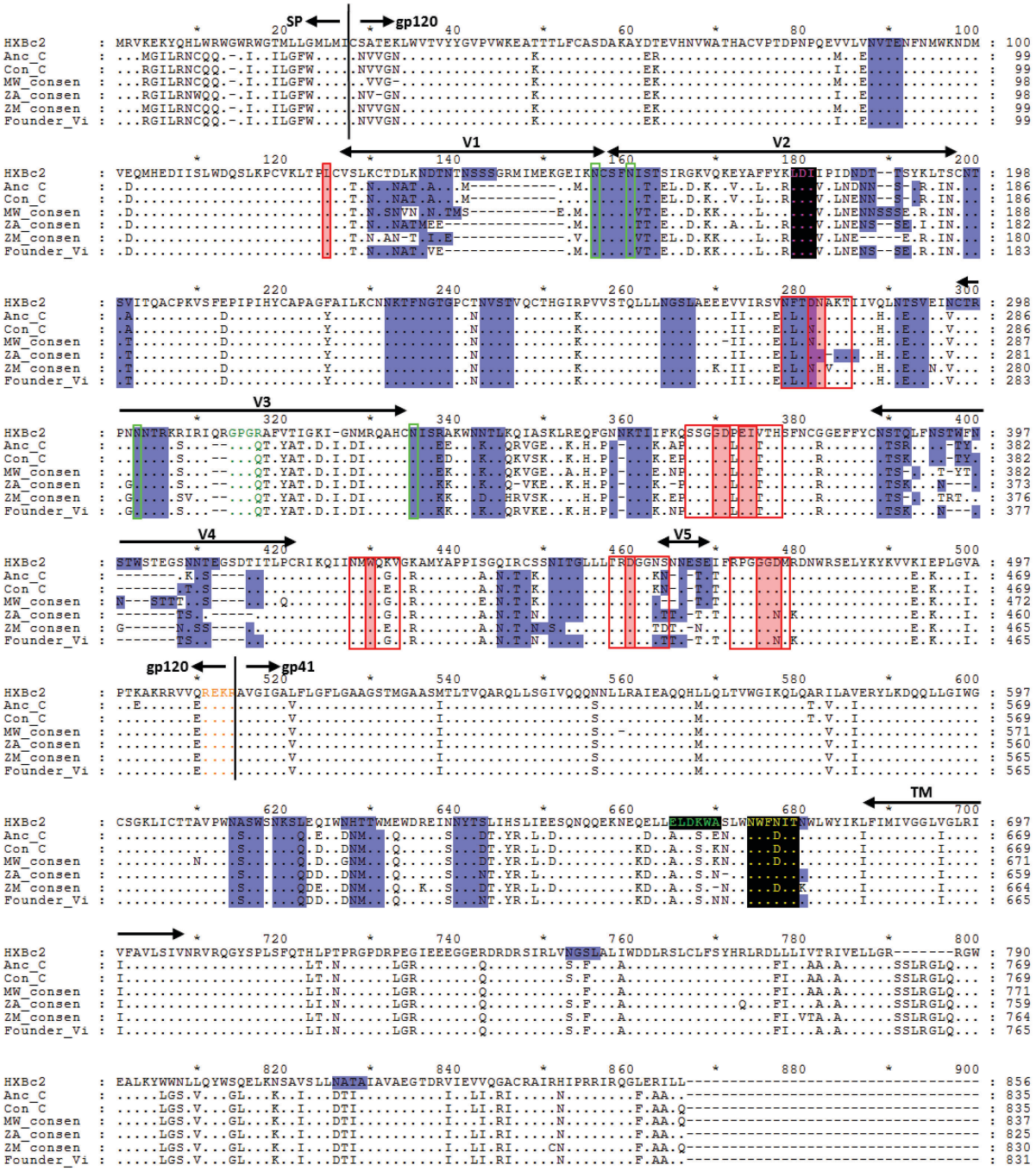


Figure 3.1: Alignment of the envelope glycoprotein (Env) amino acid consensus sequences derived from HIV-1 subtype C acutely infected individuals from Malawi (MW_consen), South Africa (ZA_consen), Zambia (ZM_consen) and used to infer a subtype C founder virus Env consensus (Founder_Vi) sequence. Sequences were aligned to the ancestral (Anc_C), consensus (Con_C) and HXBc2 reference sequences for comparative analysis and orientation purposes. The variable loop structures signal peptide and Env precursor sites are all indicated above the sequence alignment. Potential N-linked glycosylation sites (PNGs) are shaded in blue, α4β7 integrin binding site tri-peptide motif (LDI/V) indicated in pink with a black background, the tetra-peptide crown of the V3 loop structure (GPGQ/R) is shown in green and the linear epitopes of the two bNAbs targeting the gp41 MPER region 2F5 (ELDKWA) and 4E10 (NWFDIT) are shown with a black background and highlighted green and yellow, respectively. Regions boxed in red containing the residues important for CD4 binding and CD4bs-directed bNAbs epitopes, critical residues are shaded. Glycan-dependent epitopes targeted by bNAbs PG9/PG16 and PGT family antibodies are boxed in green. (.) indicates identity to the consensus sequence, (-) indicates insertions/deletions at a particular position.

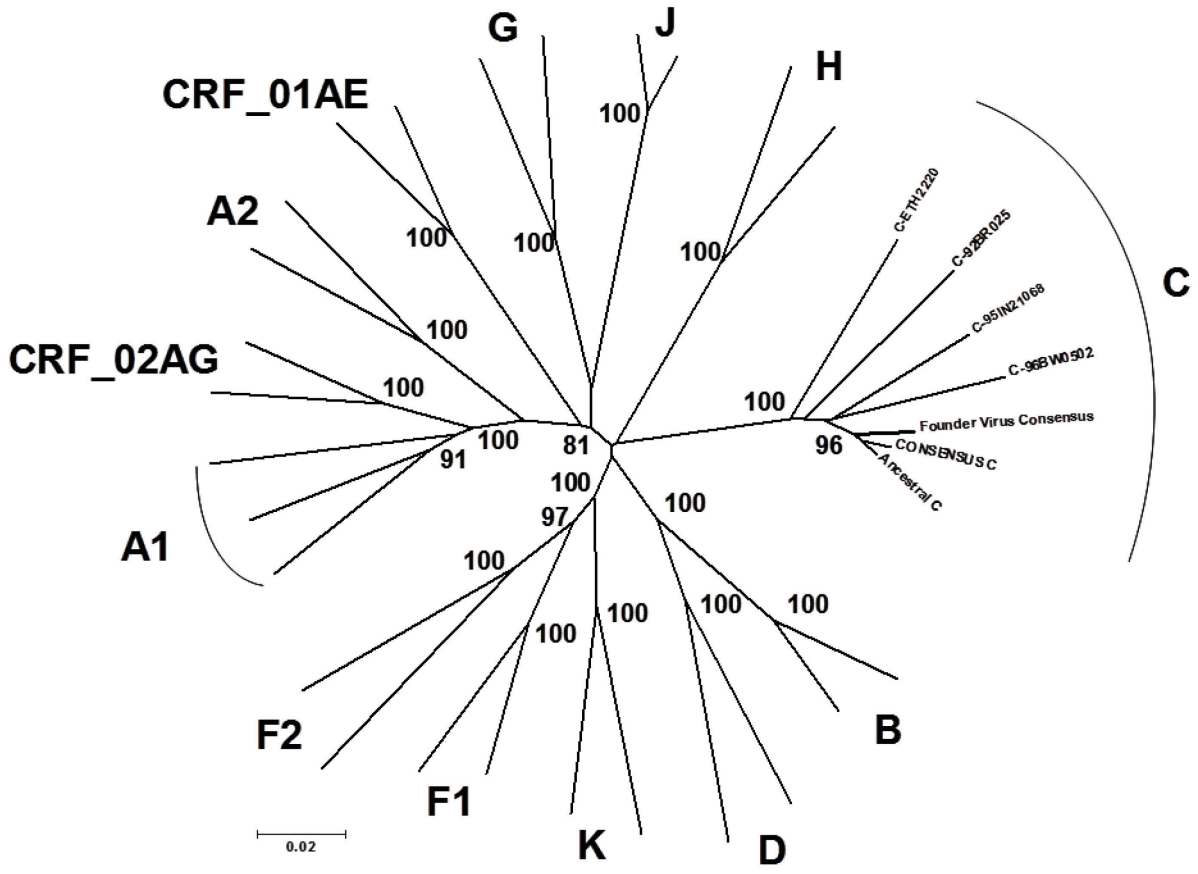


Figure 3.2: Phylogenetic analysis of the generated founder virus consensus Env sequence as compared to the Env from ancestral C, consensus C and HIV-1 subtype reference panel (available from Los Alamos sequence database [172]). The phylogenetic tree was generated using nucleotide sequences and neighbor-joining method with a maximum parsimony bootstrap to estimate the stability of nodes. Bootstrap values of greater than 70% are shown. HIV-1, group M subtypes A-D, F-H, J, K, CRF_01AE and CRF_02AG are indicated.

3.2. Generation of constructs

Constructs encompassing the codon optimized monomeric gp120 (pcDNA-gp120$_{FVC}$m), dimeric gp120 (pcDNA-gp120$_{FVC}$GCN4d and pcDNA-gp120$_{FVC}$Ud) and trimeric gp140 (pcDNA-gp140$_{FVC}$GCN4(+) and pcDNA-gp140$_{FVC}$GCN4(-)) were successfully designed, cloned into the pcDNA3.1 mammalian expression vector and obtained from GeneArt (Figure 3.3). Lyophilized constructs were resuspended in sterile distilled water, successfully transformed into chemically competent *E. coli* DH5α and used to purify the recombinant plasmids on a large scale (results not shown), for mammalian transfection and recombinant protein expression.

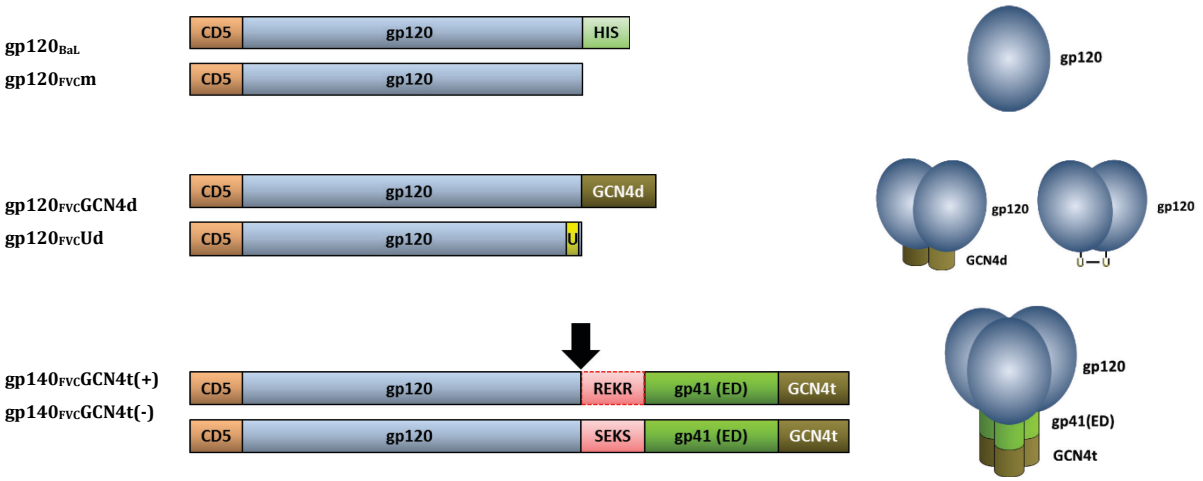


Figure 3.3: Schematic representation of the *env* constructs used in this present study. Monomeric, dimeric and trimeric conformations containing the HIV-1 subtype C founder virus *env* consensus sequence were generated for comparative biochemical characterization and immunogenicity. Monomeric *env* constructs comprised just the gp120 glycoprotein amino acid sequence. Dimeric *env* conformations contained the gp120 glycoprotein with either a heterologous dimerization motif (GCN4d) or selenocysteine (U) incorporated at the C-terminus. The gp140 constructs comprise the gp120 and gp41 ecto domain (gp41(ED)) either with an intact (REKR/(+)) or disrupted (SEKS/(-)) cleavage site and a heterologous trimerization motif (GCN4t) fused to the C-terminus of gp41(ED). The $gp120_{BaL}$ constructed was included as a control and comprised the HIV-1 subtype B $gp120_{BaL}$, with a C-terminal His-tag (HIS). In each construct the *env* leader sequence was replaced with the human CD5 leader sequence (CD5). Proteolytic cleavage sites in the trimeric Env are indicated by the arrow.

3.3. Expression, purification and characterization of recombinant Env glycoproteins

3.3.1. Transient and stable expression of HIV-1 subtype C founder virus Env constructs

Initial transient expression, SDS-PAGE and Western blot analyses of all HIV-1 subtype C founder virus Env constructs in HEK293T cells confirmed that all recombinant proteins were expressed (Figure 3.4). When assessed under reducing and denaturing conditions, monomeric ($gp120_{FVC}$m) and dimeric ($gp120_{FVC}$GCN4d and $gp120_{FVC}$Ud) constructs produced Env that migrated at equivalent levels (approximately 120 kDa) (Figure 3.4, lanes 3-5). The trimeric constructs ($gp140_{FVC}$GCN4t(+) and $gp140_{FVC}$GCN4t(-)) produced Env that migrated to approximately 140 kDa (Figure 3.4, lanes 1 and 2). The $gp140_{FVC}$GCN4t(+) construct which contains an intact proteolytic cleavage site produced an additional, less intense band that resolved to the equivalent position of the gp120 constructs ($gp120_{FVC}$m, $gp120_{FVC}$GCN4d and $gp120_{FVC}$Ud) at approximately 120 kDa (Figure 3.4). Expression of $gp120_{BaL}$ Env was confirmed using an anti-HIS probe (Figure 3.4, lane 6).

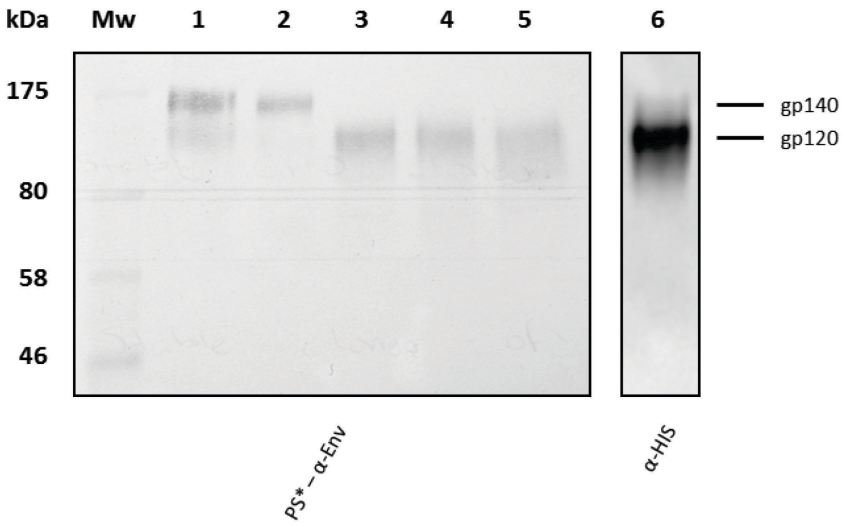


Figure 3.4: Western blot analysis confirming expression of the 5 recombinant founder virus consensus C and gp120$_{BaL}$ Env glycoproteins. Following transient transfection of HEK293T, unpurified cell culture supernatants were analysed by SDS-PAGE (10% gel) and Western blotting. Expression of the founder virus containing constructs (left panel): lane 1, gp140$_{FVC}$GCN4t(+); lane 2, gp140$_{FVC}$GCN4t(-); lane 3, gp120$_{FVC}$GCN4d; lane 4, gp120$_{FVC}$Ud and lane 5, gp120$_{FVC}$m, was confirmed by probing for Env using HIV-1 positive patient sera (PS*) and subsequent detection of bound human antibodies with alkaline phosphatase conjugated, anti-human secondary antibody. Expression of gp120$_{BaL}$ Env (lane 6) was confirmed using a HRP conjugated, anti-HIS probe (α-HIS) (right panel). The molecular weight marker (lane Mw - prestained protein marker, broad range (7-175 kDa) (New England Biolabs, Ipswich, MA, USA) and corresponding molecular weights are shown to the left in kiloDaltons (kDa).

To confirm selenocysteine incorporation in the gp120$_{FVC}$Ud Env, expression was monitored following transient transfection of HEK293T cells with pcDNA-gp120$_{FVC}$Ud or pcDNA-gp120$_{FVC}$m (control) constructs in the presence of radiolabeled selenium (Se^{75}) (Figure 3.5). The formation of a reducing-reagent resistant, di-selenide stabilized Env dimer was evaluated in the presence of increasing DTT concentrations (6.25-100 mM). Western blot analysis of culture supernatants confirmed expression of both Envs that migrated at equivalent levels (~ 120 kDa) under reducing conditions (Figure 3.5A and B, upper panels), and elimination of the reducing agent from sample buffer (0 mM DTT) resulted in the formation of a dimeric species which migrated at approximately 250 kDa for both Envs (Figure 3.5A and B, upper panels lane 7). Autoradiography analysis revealed exclusive incorporation of selenocysteine into the expressed gp120$_{FVC}$Ud Env (Figure 3.5A and B, lower panels). Formation of the dimeric Env conformation was not evident on the autoradiography analysis in the presence of DTT (100 – 6.25 mM) and was present only under non-reducing conditions (0 mM DTT). Additionally, there was non-specific labelling under non-reducing conditions, as evidenced by an approximately 55 kDa band in Figure 3.5, lanes 7. Taken together, these results suggest that despite inclusion of a C-terminal selenocysteine in the gp120$_{FVC}$Ud Env protein, this is not sufficient to form a di-selenide stabilized Env dimer and therefore further work on this construct was halted.

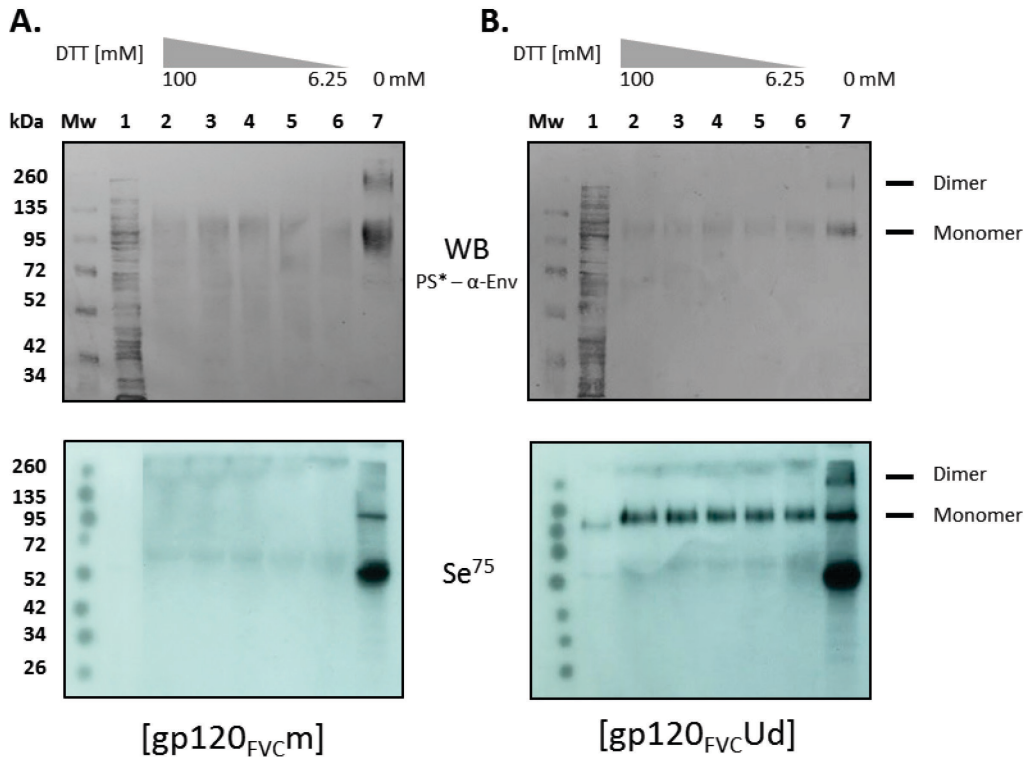


Figure 3.5: Selenocysteine incorporation and dimerization assessment of the gp120$_{FVC}$Ud. HEK293T were transiently transfected with **A.** gp120$_{FVC}$m (control) or **B.** gp120$_{FVC}$Ud constructs and cultured in the presence of radiolabeled selenium (Se^{75}). Expression of gp120$_{FVC}$m and gp120$_{FVC}$Ud Envs was monitored by SDS-PAGE (10%) followed by Western blotting (WB) (upper panels), using a HIV-1 positive patient sera (PS*). Bound antibodies were detected using an alkaline phosphatase conjugated, anti-human secondary antibody. Selenocysteine incorporation was monitored by autoradiography (lower panels). Cell culture supernatants were treated with decreasing concentration of DTT (100 – 6.25 mM) (lanes 2-6) and under non-reducing conditions (0 mM DTT) (lane 7). Lane 1, cellular fraction resolved under reducing conditions (100 mM DTT) was included as a control of endogenously expressed selenocysteine-containing proteins (thioredoxin reductase ~50 kDa). The molecular weight marker (lane Mw – Spectra multicolour broad range protein ladder (Fermentas, Thermo Scientific, Waltham, MA, USA) and corresponding molecular weights are shown on the left in kiloDaltons (kDa). The position of monomeric (~120 kDa) and dimeric (~250 kDa) gp120 Env is indicated on the right.

To obtain sufficient quantities of all four subtype C founder virus Env consensus (gp120$_{FVC}$m, gp120$_{FVC}$GCN4d, gp140$_{FVC}$GCN4t(+) and gp140$_{FVC}$GCN4t(-)) and gp120$_{BaL}$ glycoproteins (collectively referred to as Envs) required for biochemical characterization, formulation of Env/2dCD4^{S60C} complexes and subsequent small animal immunizations, stably expressing 293-F cell lines were successfully generated for each Env construct (results not shown). The expression levels generated by the stable lines was similar to those potentiated by the corresponding transiently transfected cells (Figure 3.4). Stable cells lines were used to collect supernatant containing the relevant Envs for all subsequent work.

3.3.2. Large-scale protein expression and purification of Env glycoproteins

Aliquots of approximately 150 ml of culture supernatant were harvested daily from stably expressing cell lines cultured in the roller flasks, clarified by centrifugation and filtration, and stored at -20°C. Expression was confirmed by SDS-PAGE and Western blot analyses. Collected supernatant aliquots were batched together into 500 ml volumes for the optimized purification protocol.

Recombinant Env glycoproteins were successfully purified to homogeneity using a two stage protein purification protocol. Recombinant Env was initially purified from the filtered culture supernatant by *G. nivalis* lectin-affinity chromatography, followed by oligomeric assessment and conformation purification by size exclusion-fast protein liquid chromatography (SEC-FPLC). Each step in the Env purification procedure was monitored by SDS-PAGE and Western blotting (Figure 3.6). Gel filtration profiles are shown in Figure 3.7.

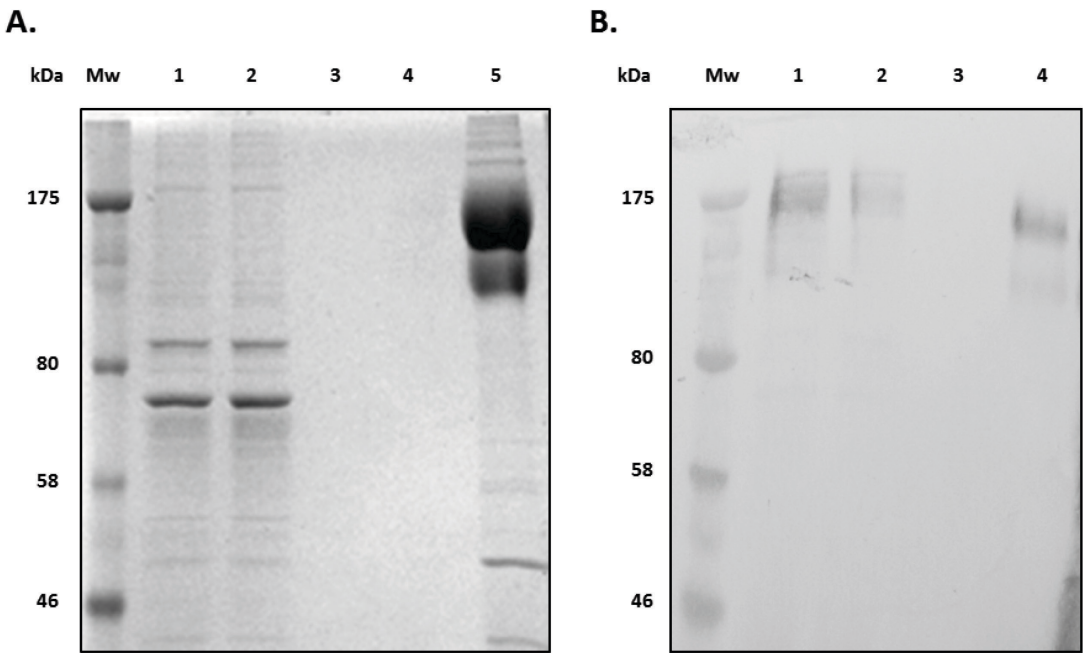


Figure 3.6: SDS-PAGE and Western Blot analysis of *G. nivalis* lectin-affinity purification of Env (gp140$_{FVC}$GCN4t(+)). The 293-F stably expressing gp140$_{FVC}$GCN4t(+) cell line (293-F/gp140$_{FVC}$GNC4t(+)) was cultured in roller flasks. Culture supernatant was clarified by centrifugation and passed through a 0.45 µm filter. Matched samples, collected following the various steps in the lectin purification procedure were analysed by **A.** SDS-PAGE (10%) and **B.** Western blotting, using HIV-1 positive patient sera and an alkaline phosphatase conjugated, anti-human secondary antibody. Lane 1, 293-F/gp140$_{FVC}$GCN4t(+) stable cell line culture supernatant; lane 2, 293-F/gp140$_{FVC}$GCN4t(+) culture supernatant post-binding to *G. nivalis*-linked agarose beads (overnight); lane 3, combined NaCl washes; lane 4, elution of bound Env (gp140$_{FVC}$GCN4t(+)) from *G. nivalis*-linked agarose beads (1M MMP); lane 5, concentrated Env (Centricon, centrifugal concentrator). Molecular weight marker (lane Mw - prestained protein marker, broad range (7-175 kDa) (New England Biolabs, Ipswich, MA, USA)) is shown to the left of the diagram in kiloDaltons (kDa).

The gel filtration profiles represent the heterogeneity of native Env conformations present following lectin-purification. All Envs demonstrated a common elution peak, that corresponded to the void volume of the column (approximately 33.5 ml) and represent conformations of Env that are greater in molecular weight than the exclusion limit of the column (>600 kDa) (Figure 3.7F). The monomeric constructs ($gp120_{FVC}$m and $gp120_{Bal}$) display a predominantly double-peak gel filtration profile that comprises both monomeric and dimeric conformations (Figure 3.7A and B). Monomeric gp120 was shown to have a peak retention volume of approximately 49.5 ml ($gp120_{FVC}$m ~ 49.5 ml and $gp120_{BaL}$ ~ 48.5 ml) while the dimeric conformation was eluted prior to the monomer peak, with a peak retention volume of approximately 43.0 ml. The majority of the $gp120_{FVC}$GCN4d lectin-purified Env was calculated to have peak retention volume of 42 ml, consistent with a dimeric conformation (Figure 3.7C). An additional smaller monomeric peak was eluted at approximately 50 ml and there was evidence of an additional peak (that could not be resolved despite optimization attempts) just prior to the elution of the dimer (35-38 ml). The lectin-purified Env expressed from the trimeric constructs ($gp140_{FVC}$GCN4t(+) and $gp140_{FVC}$GCN4t(-)) displayed similar gel-filtration profiles, characterized by two major peaks that eluted at relatively low volumes (Figure 3.7D and E). A larger proportion of these Envs were eluted in the void volume of column (33.5 ml) than compared to the monomeric and dimeric constructs ($gp120_{BaL}$, $gp120_{FVC}$m and $gp120_{FVC}$GCN4d). The trimeric Env conformation had a peak retention volume of 37.0 ml.

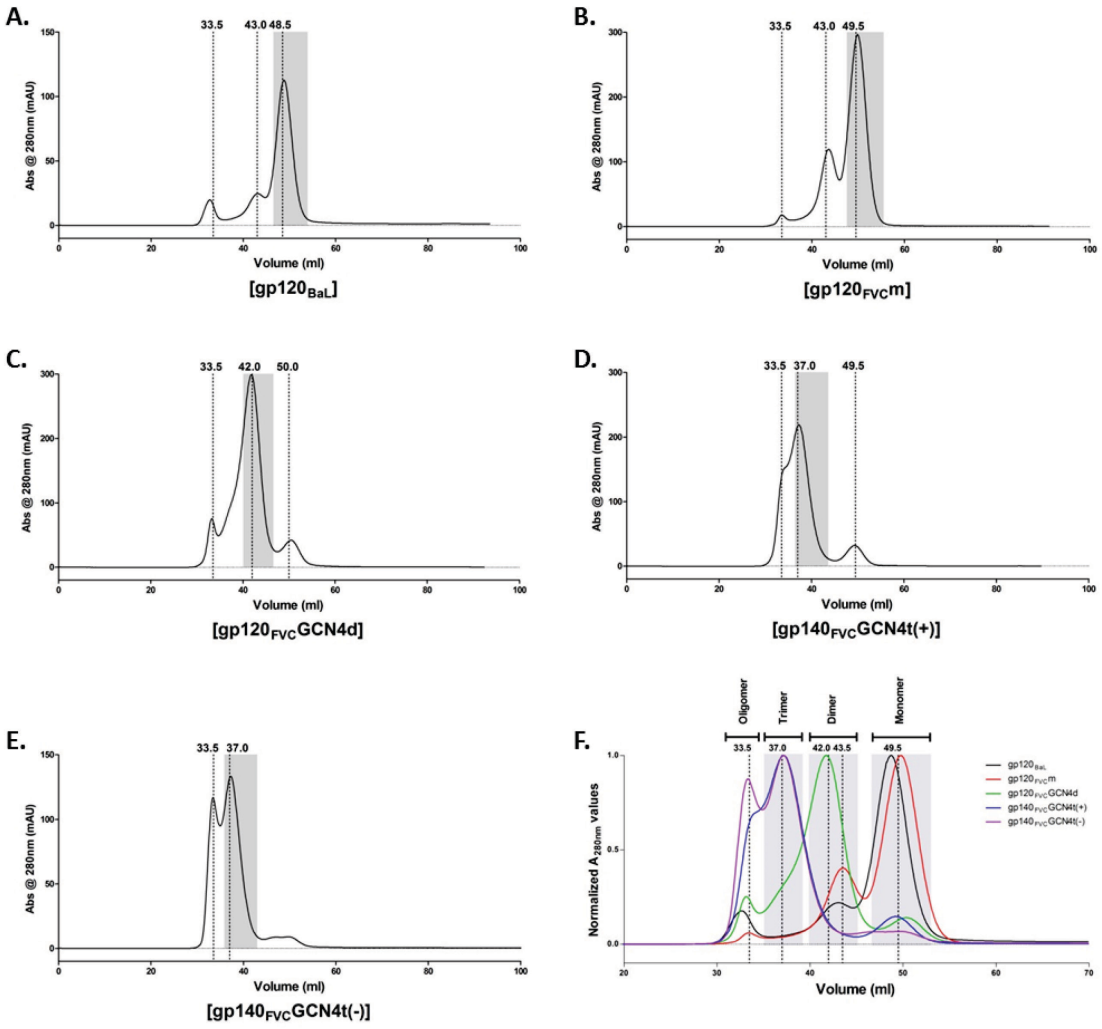


Figure 3.7: Gel filtration profiles of lectin-affinity purified monomeric, dimeric and trimeric Env constructs. Following lectin-affinity purification, Envs were resolved over a Superdex 200 gel filtration column. Gel-filtration profiles (A_{280nm}) for monomeric Env constructs **A.** $gp120_{BaL}$ and **B.** $gp120_{FVC}m$; dimeric Env construct **C.** $gp120_{FVC}GCN4d$; trimeric Env constructs **D.** $gp140_{FVC}GCN4t(+)$ and **E.** $gp140_{FVC}GCN4t(-)$. **F.** Overlay of normalized gel-filtration profiles of each Env showing the retention volumes that correspond to monomeric, dimeric and trimeric conformations. Shaded areas represent collected fractions for each conformation and peak-retention volumes are indicated above the peaks in ml.

Size exclusion-FPLC, in combination with fractionation, allowed for adequate isolation of monomeric, dimeric and trimeric Env conformations (Figure 3.7, shaded areas), although some of the selected fractions may contain minority conformations due to incomplete resolution. Following fractionation, Env samples were re-concentrated and the conformation purity was assessed by blue native PAGE (Figure 3.8) and a second round of SEC-FPLC (described later in Figure 3.11).

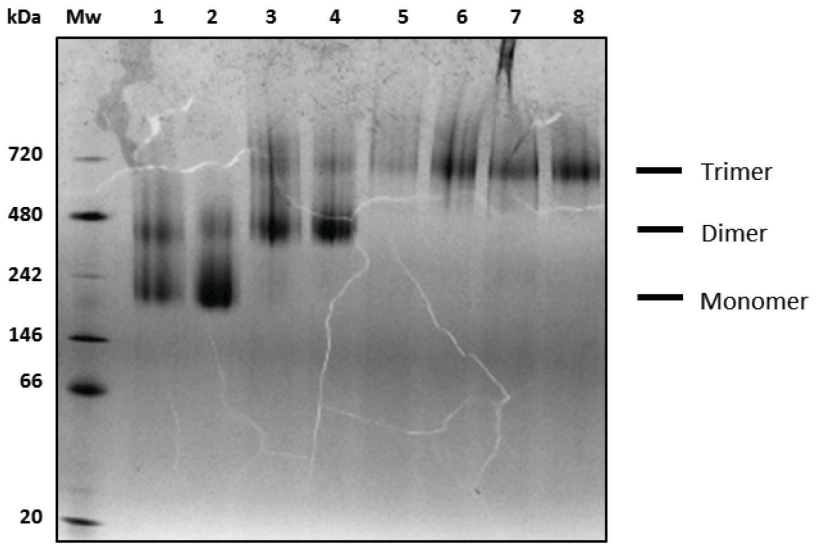


Figure 3.8: Blue native PAGE analysis of pre- and post- SEC-FPLC Env purification. Equivalent microgram amounts (5 µg) of lectin-purified and conformational purified Env were resolved on 4-16% Native PAGE Novex Bis-Tris Gel. Lane 1, lectin-purified gp120$_{FVC}$m; lane 2, gp120$_{FVC}$m SEC-FPLC purified monomer; lane 3, lectin-purified gp120$_{FVC}$GCN4d; lane 4, gp120$_{FVC}$GCN4d SEC-FPLC purified dimer; lane 5, lectin-purified gp140$_{FVC}$GCN4t(+); lane 6, gp140$_{FVC}$GCN4t(+) SEC-FPLC purified trimer; lane 7, lectin-purified gp140$_{FVC}$GCN4t(-); lane 8, gp140$_{FVC}$GCN4t(-) SEC-FPLC purified trimer. Molecular weight (Mw) marker (NativeMark™, Novex, Life Technologies, Carlsbad, CA, USA) with molecular weight in kiloDaltons (kDa) is shown on the left.

The native PAGE analysis confirmed the heterogeneity of native conformations present following lectin-affinity purification of Env which was consistent with the gel-filtration profiles obtained in Figure 3.7. The monomeric conformation migrated at approximately 200 kDa, the dimeric conformation at approximately 450 kDa and the trimeric conformation at approximately 720 kDa (Figure 3.8), in line with what has been shown previously [253]. Again, the monomeric construct (gp120$_{FVC}$m) was found to comprise both monomeric and dimeric conformations, the dimeric construct (gp120$_{FVC}$GCN4d) comprises predominately dimeric conformation and high-order oligomers, equivalent in size to trimers and the trimeric constructs (gp140$_{FVC}$GCN4t(+) and gp140$_{FVC}$GCN4t(-)) comprise trimeric and high-order oligomeric conformations. Following SEC-FPLC, there was noticeable enrichment of the desired conformation for each construct however the minority conformations are still clearly visible in the monomeric and dimeric constructs (Figure 3.8, lanes 2 and 4). The trimeric constructs showed the greatest improvement in conformational homogeneity as depicted by a distinct band at ~720 kDa (Figure 3.8, lane 6 and 8). However, to limit potential complications that may have been introduced as a result of conformational heterogeneity, as far as possible, all subsequent protein characterization, generation of 2dCD^{S60C} complexes and immunizations were carried out using the SEC-FPLC purified conformations of each Env.

3.3.3. Expression, purification and characterization of 2dCD4^{S60C}

Sufficient quantities of 2dCD4^{S60C} were expressed in *E. coli* BL21 (DE3), purified to homogeneity from inclusion bodies under denaturing conditions using Ni^{2+} affinity chromatography and refolded in a controlled oxidative environment (Figure 3.9A). The refolded 2dCD4^{S60C} was shown to bind $gp120_{FVC}$GCN4d in a similar manner to that of commercially available soluble, four-domain CD4 (sCD4) in a functional binding "sandwich ELISA" (Figure 3.9B and C). Since the 17b antibody preferentially binds the CD4i epitope on Env following engagement of the CD4 receptor, results indirectly confirmed the presence of bound CD4 to Env, and consequently the presence of functional 2dCD4^{S60C}.

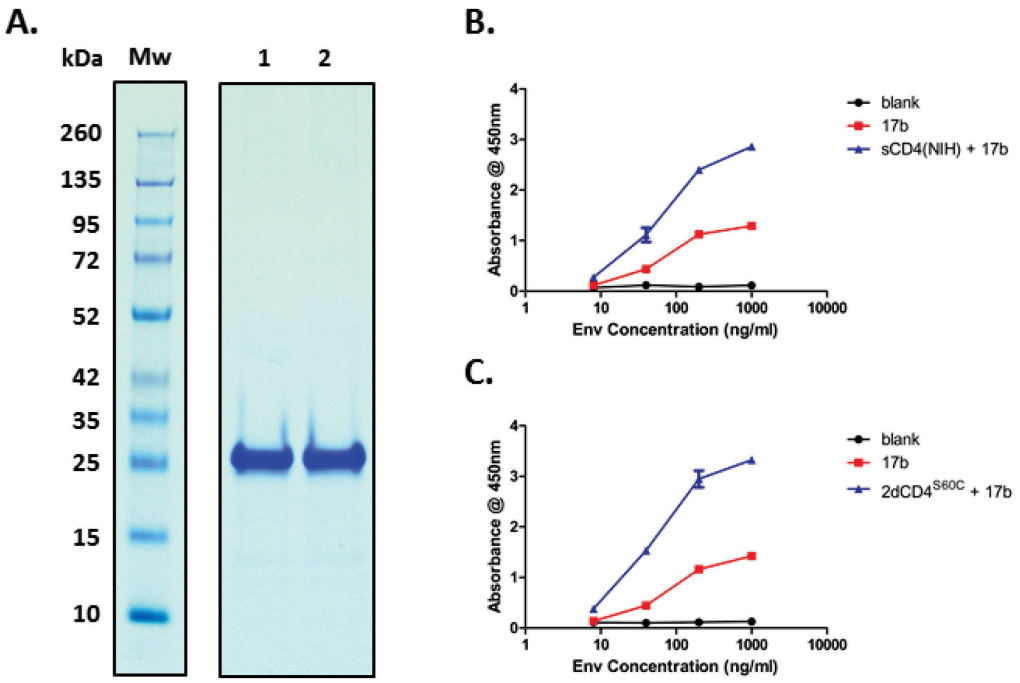


Figure 3.9: Purity and functionality assessment of 2dCD4^{S60C}. A. Ni^{2+} affinity purified and eluted 2dCD4^{S60C} as compared to 2dCD4^{WT}. Lane 1, 2dCD4^{S60C} and lane 2, 2dCD4^{WT} were assessed under reducing SDS-PAGE (NuPAGE, Novex 4-12% Bis-Tris gel) and visualized by Coomassie staining/destaining protocols. Molecular weight (Mw) marker (Spectra multicolour broad range protein ladder (Fermentas, Thermo Scientific, Waltham, MA, USA) with molecular weights shown in kiloDaltons (kDa) on the left. Refolded 2dCD4^{S60C} binding to Env ($gp120_{FVC}$GCN4d) was compared to commercially available soluble CD4 (sCD4(NIH)) (Progenics) using a functional binding ELISA. **B.** sCD4-liganded Env (blue trace), **C.** 2dCD4^{S60C}-liganded Env (blue trace) or Env alone (red traces) were captured with D7324 antibody, probed with a 1:2,000 concentration of 17b antibody and bound 17b antibody detected using an HRP-conjugated, anti-human antibody. Wells containing no Env were used as a control (blank).

3.3.4. Characterization and functional analysis of SEC-FPLC purified Env glycoproteins

Conformational integrity and functionality of SEC-FPLC purified monomeric, dimeric and trimeric founder virus Envs were confirmed by ELISA (Figure 3.10) and SPR, with particular focus on the CD4-binding site on Env and its ability to bind 2dCD4^{S60C} and subsequently induce the native CD4i-17b epitope.

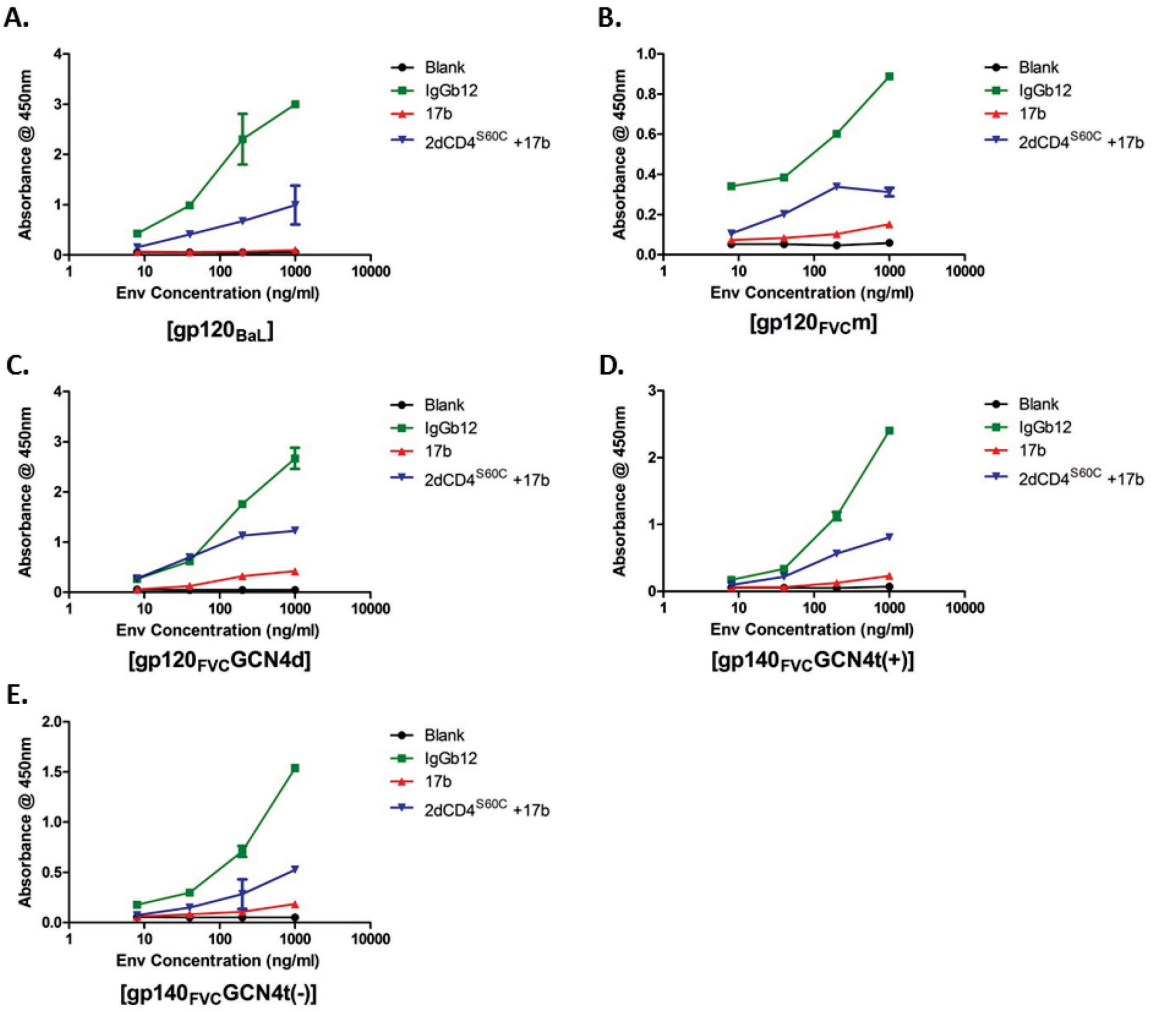


Figure 3.10: Conformational integrity and functionality assessment of SEC-FPLC purified, founder virus constructs. Unliganded or 2dCD4^{S60C}-liganded Env conformations were captured using the D7324 antibody. Unliganded Env conformations were probed with IgG1b12 (green trace) or 17b (red trace), while the 2dCD4^{S60C}-liganded Env conformations were probed exclusively with 17b (blue trace). Wells containing no Env were included as a control (blank – black trace). Bound antibodies were detected using a HRP-conjugated, anti-human IgG antibody and established TMB/stop solution chromogenic methods. **A.** monomeric gp120$_{BaL}$, **B.** monomeric gp120$_{FVC}$m, **C.** dimeric gp120$_{FVC}$GCN4d, **D.** trimeric gp140$_{FVC}$GCN4t(+) and **E.** trimeric gp140$_{FVC}$GCN4t(-).

Results show that IgG1b12 bound to all Env conformations tested, and increased with Env concentration (Figure 3.10, green trace). The 17b monoclonal antibody bound to Env to a lesser extent (Figure 3.10, red trace), but Env binding was greatly enhanced in the presence of 2dCD4^{S60C} (Figure 3.10, blue trace), confirming that the 2dCD4^{S60C} bound Env and induced the CD4i epitope recognised by 17b. It was noted that the OD values for antibody binding with increasing concentrations of gp120$_{BaL}$ and gp120$_{FVC}$m varied, despite repeated testing.

Further binding kinetic characterization of the Env conformations with 2dCD4^{WT} was carried out using SPR, and the association (k_a), dissociation (k_d) and equilibrium (K_D) constants for each Env conformation was determined by fitting a 1:1 Langmuir binding model to the generated sensograms (Table 3.3).

Table 3.3: Calculated binding kinetics of the four founder virus Env conformations interaction with 2dCD4^{WT} as determined by surface plasmon resonance.

Envelope	Conformation	k_a (1/Ms)	k_d (s^{-1})	K_D (nM)	R_{max} (RU)[a]	χ^2 [b]
gp120$_{FVC}$m	monomer	1.62×10^4	3.65×10^{-4}	22.53	100.65	11.54
gp120$_{FVC}$GCN4d	dimer	9.14×10^3	1.68×10^{-4}	18.38	101.17	7.20
gp140$_{FVC}$GCN4t(+)	trimer	4.88×10^3	1.43×10^{-5}	2.93	95.95	5.30
gp140$_{FVC}$GCN4t(-)	trimer	3.09×10^3	1.41×10^{-5}	4.56	72.61	4.00

k_a – Association constant
k_d – Dissociation constant
K_D – Equilibrium constant (k_d/k_a)
R_{max} – Maximum theoretical response assuming all of the ligand is active, ligand is 100% pure and all binding sites are available
χ^2 – Chi-squared is the average of squared residuals and indicates fitting confidence
[a] – For kinetic analysis, R_{max} ≤200 RU is recommended
[b] – Chi-squared values less than 10% of R_{max} are considered acceptable

Overall the two trimeric Envs showed the most favourable CD4-binding kinetics with K_D values of 2.93 and 4.56 nM for the gp140$_{FVC}$GCN4t(+) and gp140$_{FVC}$GCN4t(-) constructs, respectively. The monomeric gp120$_{FVC}$m and dimeric gp120$_{FVC}$GCN4d conformation had an approximate 5 fold reduction in affinity (22.53 and 18.38 nM, respectively) as compared to the trimeric conformations. The monomeric Env conformation showed the most favourable association rates (k_a values; 1.62×10^4 Ms^{-1}) followed closely by the dimeric conformation (9.14×10^3 Ms^{-1}) while the trimeric conformations showed a three-fold reduction in k_a values (Table 3.3). The dissociation rates, k_d values were the inverse of the association rates, with the trimeric Env conformations having the slowest off-rates, followed by the dimer and monomer conformations. Overall, all Env conformations were functional and able to bind CD4, and as expected, the quaternary structure of Env was shown to impact on the binding affinity to CD4.

3.4. Env-based immunogen preparation and characterization

3.4.1. Generation and purification of 2dCD4^{S60C}-liganded Env immunogens

Sufficient quantities of 2dCD4^{S60C}-liganded Env conformations for small animal immunogenicity studies were generated by incubating the relevant SEC-FPLC purified Env conformation with an approximate five-molar excess of 2dCD4^{S60C} (equivalent microgram amounts of Env and 2dCD4^{S60C}) at room temperature under reducing conditions. Unbound 2dCD4^{S60C} was removed by passing the 2dCD4^{S60C}-liganded Env complexes through a second round SEC-FPLC (Figure 3.11). Fractions corresponding to the relevant Env conformation were collected by fractionation and assessed for the presence of bound 2dCD4^{S60C}.

The Env gel-filtration profiles generated from the second round of SEC-FPLC depict a vastly improved homogeneity for each Env conformation, as indicated by a single peak, with retention volumes corresponding to monomeric, dimeric or trimeric for the respective constructs (Figure 3.11A, B, C, D and E). This result is in contrast to the native PAGE analysis obtained following the first round of size exclusion chromatography (Figure 3.8), where the monomeric and dimeric Env conformations (gp120$_{BaL}$, gp120$_{FVC}$m and gp120$_{FVC}$GCN4d) still exhibited minority conformations of dimeric and higher-order oligomers, respectively. The presence of high-order oligomeric conformations in the trimeric constructs (gp140$_{FVC}$GCN4t(+) and gp140$_{FVC}$GCN4t(-)) was still evident as indicated by an unresolved peak that eluted just prior to that of the trimeric conformation at ~37 ml (Figure 3.11D and E). Gel filtration peaks that corresponded to the relevant 2dCD4^{S60C}-liganded Env conformations were collected and pooled (Figure 3.11, shaded areas). Fractionations of the resolved trimeric Env/2dCD4 complexes were used to limit the inclusion of higher-order oligomeric conformations as far possible (Figure 3.11D and E, shaded areas).

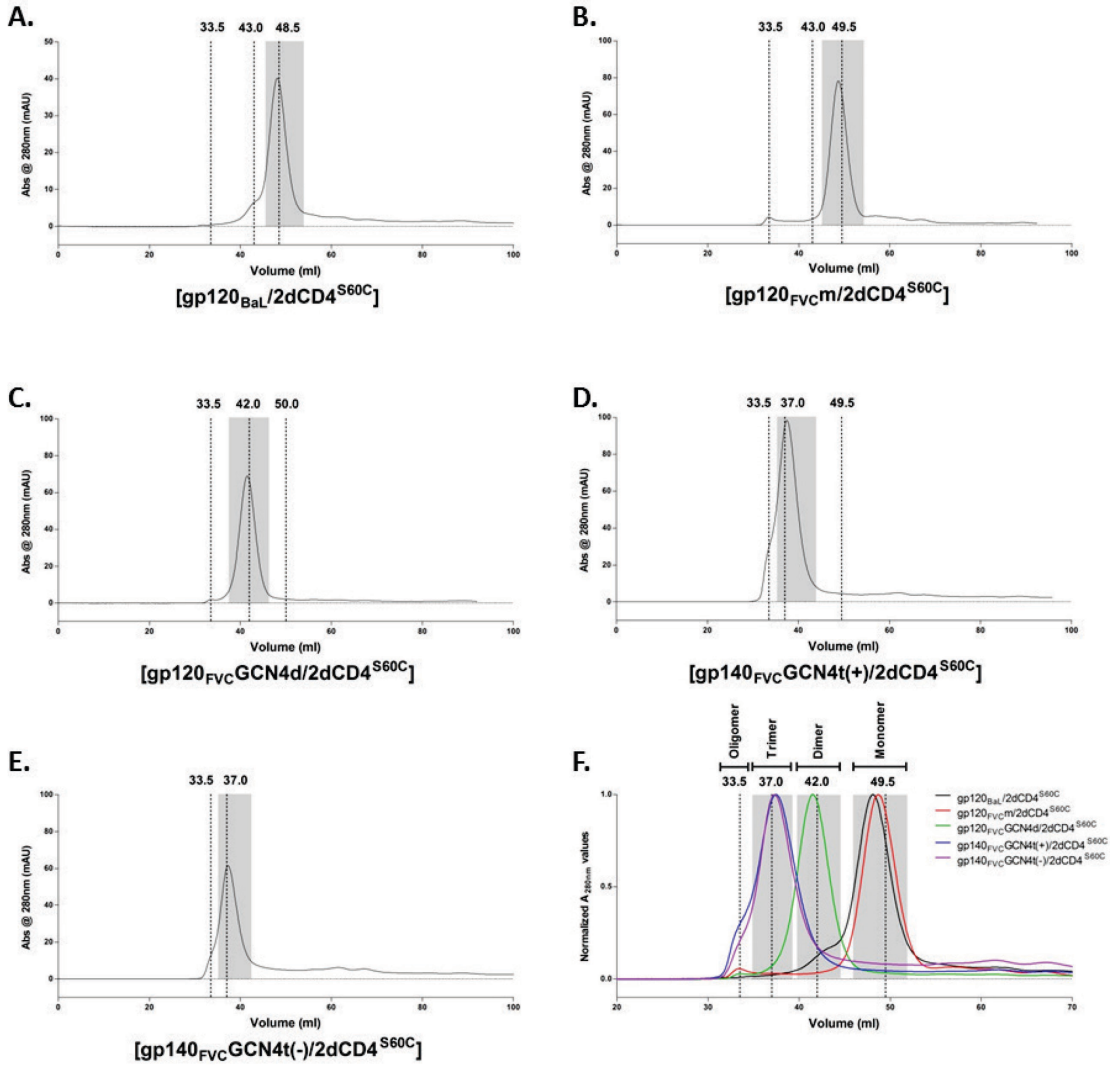


Figure 3.11: Gel filtration profiles of 2dCD4^{S60C}-liganded monomeric, dimeric and trimeric Env complexes. Env/2dCD4^{S60C} complexes were generated by incubating the relevant SEC-FPLC purified Env conformation with excess 2dCD4^{S60C} under reducing conditions at room temperature. Unbound 2dCD4^{S60C} was removed by passing the complexes through a Superdex 200 gel filtration column. Fractions corresponding to the relevant Env conformation were collected and pooled (shaded areas). **A.** gp120$_{BaL}$/2dCD4^{S60C}, **B.** gp120$_{FVC}$m/2dCD4^{S60C}, **C.** gp120$_{FVC}$GCN4d/2dCD4^{S60C}, **D.** gp140$_{FVC}$GCN4t(+)/2dCD4^{S60C}, **E.** gp140$_{FVC}$GCN4t(-)/2dCD4^{S60C} and **F.** Overlay of the normalized gel-filtration profiles showing the retention volumes that correspond to monomeric, dimeric and trimeric Env conformations.

3.4.2. Confirmation and purity assessment of 2dCD4^{S60C}-liganded Env complexes and unliganded Env immunogens

SDS-PAGE confirmed the presence of bound 2dCD4^{S60C} in the 2dCD4^{S60C}-liganded Env complexes and provided an overall purity assessment of the unliganded and 2dCD4^{S60C}-liganded Env conformations that were to be used in subsequent small animal immunizations studies (Figure 3.12). Total protein concentration was determined for each unliganded and 2dCD4^{S60C}-liganded

Env conformation and SDS-PAGE loading amounts standardized to 10 µg. Two micrograms of 2dCD4^{S60C} alone was loaded in order to estimate the quantity of this protein present in the 2dCD4^{S60C}-liganded Env complexes (Figure 3.12, lane 6).

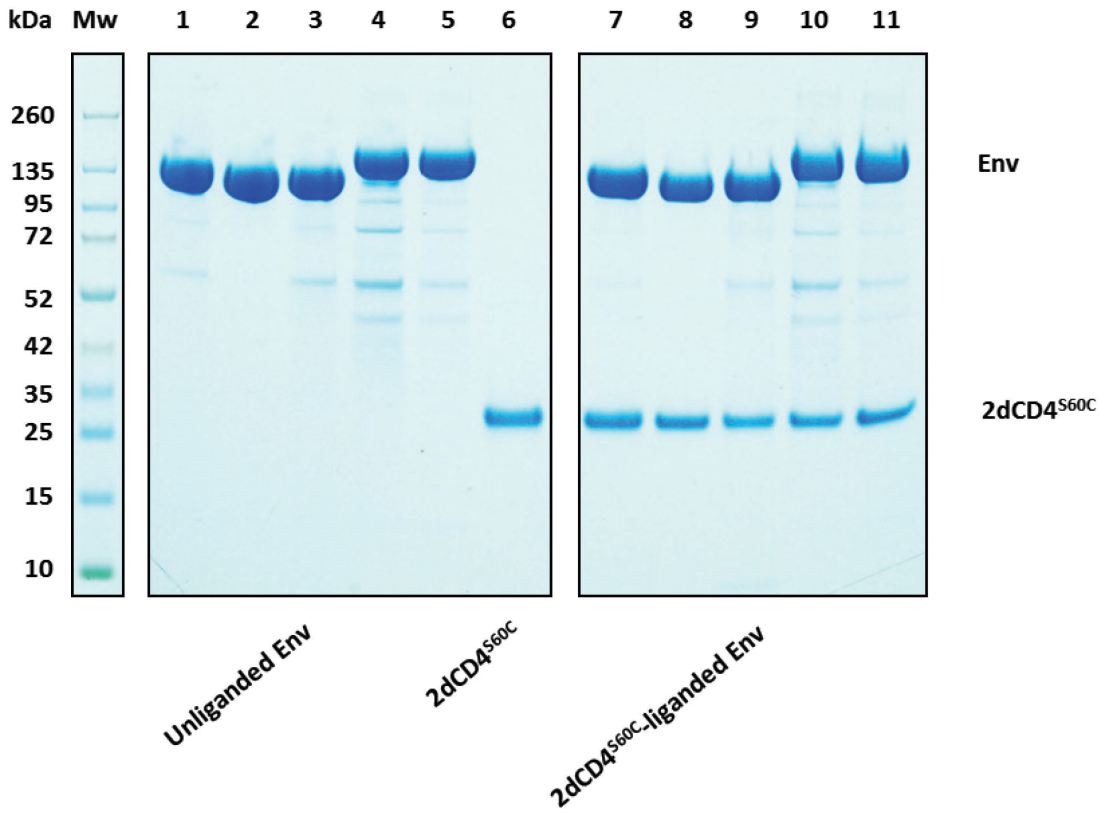


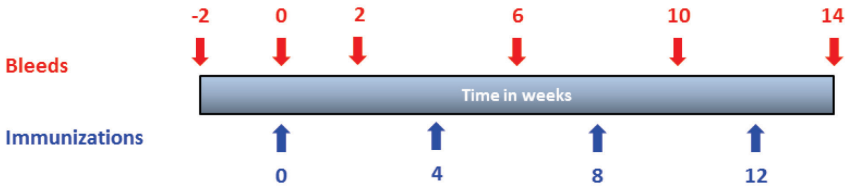
Figure 3.12: SDS-PAGE analysis of unliganded and 2dCD4^{S60C}-liganded Env complexes. Ten micrograms of total protein of unliganded (left panel) or 2dCD4^{S60C}-liganded Env complexes (right panel) were resolved on a NuPAGE, Novex 4-12% Bis-Tris gel, under reducing conditions. Bands were visualized by Coomassie blue staining and destaining protocols. Lane 1, gp120$_{BaL}$; lane 2, gp120$_{FVC}$m; lane 3, gp120$_{FVC}$GCN4d; lane 4, gp140$_{FVC}$GCN4t(+); lane 5, gp140$_{FVC}$GCN4t(-); lane 6, 2dCD4^{S60C} (2 µg); lane 7, gp120$_{BaL}$/2dCD4^{S60C}; lane 8, gp120$_{FVC}$m/2dCD4^{S60C}; lane 9, gp120$_{FVC}$GCN4d/2dCD4^{S60C}; lane 10, gp140$_{FVC}$GCN4t(+)/2dCD4^{S60C} and lane 11, gp140$_{FVC}$GCN4t(-)/2dCD4^{S60C}. Molecular weight (Mw) marker (Spectra multicolour broad range protein ladder (Fermentas, Thermo Scientific, Waltham, MA, USA) with molecular weights shown in kiloDaltons (kDa) is indicated on the left.

The purity of the unliganded and 2dCD4^{S60C}-liganded Env immunogens was estimated to be 90-95% pure by Coomassie staining. Lower molecular weight bands were particularly noticeable in the trimeric Env conformations and remained, even after a second round of SEC-FPLC (Figure 3.12, lanes 4, 5, 10 and 11). The presence of 2dCD4^{S60C} in the relevant Env conformation fractions following the SEC-FLPC isolation of 2dCD4^{S60C}-liganded Env complex confirms the successful generation of these immunogen complexes.

3.5. Immunogenicity testing of unliganded and 2dCD4^{S60C}-liganded Env conformations

The immunogenicity of each Env conformation alone or in complex with 2dCD4^{S60C} was evaluated in New Zealand White Rabbits. A control group that received 2dCD4^{S60C} alone was included. Immunizations were carried out at 4 week intervals, with each rabbit receiving a total of 20 μg of protein immunogen formulated in Adjuplex, per immunization, and baseline reference bleeds were collected prior to the first immunization and 2 weeks post each immunization/boost for a period of four months (Figure 3.13A). All terminal bleed samples (week 14) were evaluated for their ability to neutralize a panel of 12 HIV-1 pseudotyped viruses using the PhenoSense Neutralizing antibody assay (Monogram Biosciences, Inc.) (Figure 3.13B). Neutralization data is shown in Figure 3.13B as the reciprocal of the dilution of serum required to achieve 50% inhibition (IC_{50}) of each pseudotyped virus, and represented as a heat map. The panel of HIV-1 pseudotyped viruses was specifically selected to comprise genetically and geographically diverse *env* sequences derived from subtype B and C viruses with representatives of well characterized neutralization phenotypes (Tiers 1 to 3) [254].

A.



B.

	Immunogen	Tier 1			Tier 2								Tier 3	Control
		SF162	BaL	NL43	93IN905	93MW959	97ZA012	IAVI_C22	JRCSF	MGRM-C-004	MGRM-SC-B-012	MGRM-C-015	MGRM-SC-B-005	aMLV
Envelope only	$gp120_{BaL}$	450	<10	51	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		3870	62	39	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		1203	45	45	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		886	23	199	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		1520	46	251	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	$gp120_{FVCm}$	10876	94	11	<10	<10	<10	<10	<10	<10	<10	12	<10	<10
		20098	59	23	19	<10	<10	<10	<10	<10	19	26	<10	<10
		181	<10	18	10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		571	<10	21	15	<10	<10	<10	<10	<10	<10	10	<10	<10
		56	<10	27	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	$gp120_{FVC}GCN4d$	109	<10	<10	13	<10	<10	<10	<10	<10	<10	<10	<10	<10
		1120	18	41	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		215	<10	12	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		114	<10	33	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		1206	11	20	<10	<10	<10	<10	<10	<10	<10	12	<10	<10
	$gp140_{FVC}GCN4t(+)$	1575	<10	22	21	<10	<10	<10	<10	<10	<10	14	<10	<10
		694	<10	24	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		1117	15	38	<7	<7	<7	<7	<7	<7	<7	<7	<7	<7
		1118	16	39	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
		1119	17	40	<9	<9	<9	<9	<9	<9	<9	<9	<9	<9
	$gp140_{FVC}GCN4t(-)$	1115	13	36	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
		1116	14	37	<6	<6	<6	<6	<6	<6	<6	<6	<6	<6
		408	<10	64	11	<10	<10	11	<10	11	<10	24	<10	<10
		7668	52	103	21	<10	<10	<10	<10	12	17	16	<10	<10
		648	<10	33	13	<10	<10	<10	<10	<10	<10	<10	<10	<10
Envelope/$2dCD4^{S60C}$ complex	$gp120_{BaL}/2dCD4^{S60C}$ complex	1027	394	254	906	900	760	1087	789	1234	1314	1004	956	<10
		842	163	404	331	332	265	418	241	481	524	437	334	<10
		2143	556	331	599	594	508	709	609	852	652	665	744	<10
		406	216	178	261	142	175	312	219	416	297	298	298	<10
		1034	505	256	1291	1442	1141	1700	924	1645	1788	1219	1242	<10
	$gp120_{FVCm}/2dCD4^{S60C}$ complex	288	165	119	251	179	178	232	247	286	204	324	281	<10
		1386	596	325	689	602	559	724	764	1082	934	952	1416	<10
		446	327	182	685	589	518	821	522	844	678	640	628	<10
		350	265	141	728	588	613	828	536	950	768	571	774	<10
		708	242	238	669	408	386	825	446	1226	814	923	849	<10
	$gp120_{FVC}GCN4d/2dCD4^{S60C}$ complex	1929	316	222	1258	845	853	1631	733	1394	1233	914	710	<10
		356	253	129	798	714	703	1446	558	1114	945	1030	707	<10
		1704	618	278	1836	1559	1727	2580	1461	2386	2406	1435	1628	<10
		402	271	184	917	768	848	1264	565	1127	1213	650	638	<10
		759	232	110	710	562	485	825	401	680	771	519	575	<10
	$gp140_{FVC}GCN4t(+)/2dCD4^{S60C}$ complex	1096	431	204	1096	876	856	1268	834	1244	1027	1028	1089	<10
		706	358	177	1240	889	854	1328	734	1415	1154	851	899	<10
		1261	553	611	1537	1165	1195	1636	1178	1838	1916	1799	1169	<10
		688	263	172	696	548	558	755	476	889	724	729	563	<10
		842	499	277	1677	1582	1403	1680	1167	1890	2116	2046	1257	<10
	$gp140_{FVC}GCN4t(-)/2dCD4^{S60C}$ complex	1270	387	197	1041	872	839	1112	765	1295	874	979	847	<10
		2076	1420	588	3727	3908	2822	4353	2950	5290	3610	2734	3129	<10
		3641	1144	480	2507	2343	1763	3102	2169	3086	3226	1658	2231	<10
		738	412	196	1247	856	859	1630	773	1372	1476	1082	925	<10
		1897	796	382	1729	1650	1215	2145	1489	2322	1992	1581	1486	<10
	$2dCD4^{S60C}$	10	<10	<10	42	28	33	90	30	72	55	50	28	<10
		<10	<10	<10	<10	12	11	27	<10	20	17	10	<10	<10
		<10	<10	10	19	20	15	23	12	23	22	17	14	12
	Z23	16778	892	1582	341	308	148	328	306	149	1643	197	<100	<100
		13613	850	1340	419	300	113	382	278	118	1616	157	<100	<100
		14451	631	1086	437	242	129	284	252	114	1561	130	110	<100

IC_{50} value key: >10,000 | 10,000 -1,000 | 1,000 -500 | 500 -100 | 100 -20

Figure 3.13: Immunization schedule and neutralizing antibody profile data of immunized rabbit sera against a panel of 12 HIV-1 pseudovirus isolates. A. Schematic diagram showing the immunization/bleed schedule. Each rabbit received 20 µg of total protein/immunization. **B.** Neutralization data is indicated as the reciprocal of the dilution required to achieve 50% inhibition. Z23 is derived from a human patient sera exhibiting broadly neutralizing activity and used as a positive control (Monogram Biosciences, Inc.). Murine leukaemia virus (aMLV) was included as a specificity control. A reaction is determined positive (shaded) when the IC_{50} value obtained is at least 3 fold higher (95% confidence) than the IC_{50} against the specificity control, aMLV.

The five groups of animals that were immunized with the Env conformations alone exhibited a very narrow neutralizing breadth that was confined to the neutralization sensitive, tier 1 HIV-1 pseudoviruses and were unable to neutralize any tier 2 or 3 pseudoviruses, with the exception of $gp120_{FVC}m$, where one rabbit exhibited very low neutralizing activity against the tier 2 pseudovirus MGRM-C-015 (Figure 3.13B).

In contrast, immunization of rabbits with all the $2dCD4^{S60C}$-liganded Env complexes exhibited significantly enhanced breadth and IC_{50} neutralizing titers across all tiers of pseudoviruses tested (Figure 3.13B). The induction of this broadly neutralizing response appeared to be independent of the Env subtype and conformation, however it was noted that the $2dCD4^{S60C}$-liganded, trimeric Env conformations ($gp140_{FVC}GCN4t(+)/2dCD4^{S60C}$ and $gp140_{FVC}GCN4t(-)/2dCD4^{S60C}$) demonstrated on average, an improved neutralizing titer, particularly against the more candidate vaccine-relevant, neutralization resistant tier 2 and 3 pseudoviruses.

3.6. Characterization of $2dCD4^{S60C}$-liganded Env complex induced neutralizing responses

3.6.1. End-point ELISA titer

Env and CD4 directed immune responses in all rabbit sera were determined by end-point titer ELISA, with the mean titer obtained for each immunized group of rabbits shown in Figure 3.14. Overall, all Env based immunogens, whether unliganded or $2dCD4^{S60C}$-liganded, induced similar anti-Env titers that were not shown to be significantly different, with the exception of the $gp120_{FVC}m/2dCD4^{S60C}$ complex immunogen, which elicited a significantly lower ($p<0.05$) anti-Env titer compared to the matched unliganded Env conformation ($gp120_{FVC}m$) (Figure 3.14A). Immunization with the $2dCD4^{S60C}$-liganded Env complexes induced anti-CD4 responses, however the titer of anti-CD4 directed responses was significantly lower ($p<0.0001$) than those induced by $2dCD4^{S60C}$ immunization alone (Figure 3.14B). The end-point anti-CD4 titers induced by the Env/2dCD4 complexes ranged from five-fold for the $2dCD4^{S60C}$-liganded monomeric Env complexes ($gp120_{BaL}/2dCD4^{S60C}$ and $gp120_{FVC}m/2dCD4^{S60C}$) up to 25–fold lower for the $2dCD4^{S60C}$-liganded dimeric Env ($gp120_{FVC}GCN4d/2dCD4^{S60C}$).

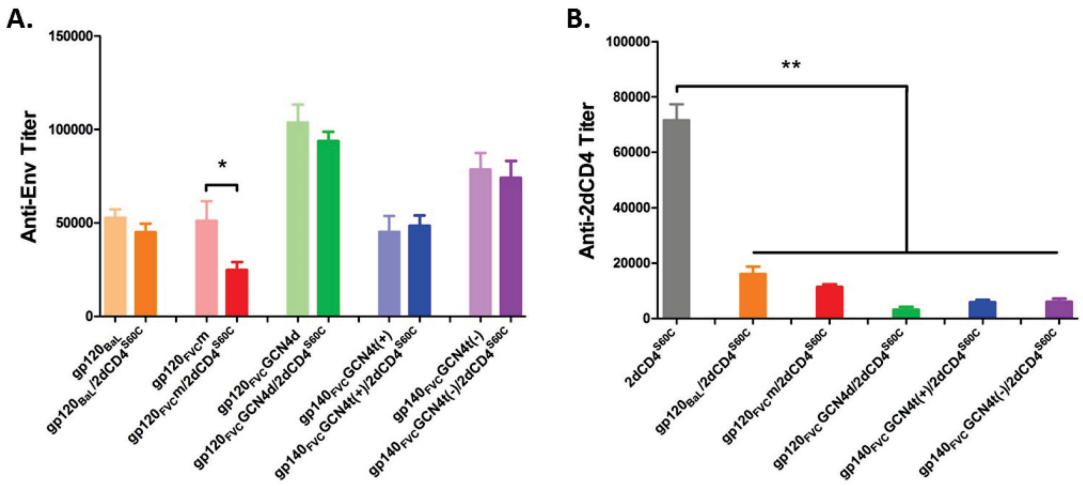


Figure 3.14: Specific end-point ELISA titer of rabbit sera determined against Env and 2dCD4^{S60C}. The immunogen is indicated on the x-axis. **A.** Anti-Env titers were determined by titrating the terminal bleed rabbit sera against the matched Env immunogen. **B.** Anti-CD4 titers were determined by titrating the terminal bleed rabbit sera against 2dCD4^{S60C}. ELISA titers represent the reciprocal of the dilution required to obtain a 0.5 absorbance reading at 450 nm. Statistical difference was calculated using a student's *t*-test. (*) – $p < 0.05$ and (**) – $p < 0.0001$.

Rabbits that received the unliganded Env only conformations and the group of animals that received 2dCD4^{S60C} alone did not show any cross-reactivity to either 2dCD4^{S60C} or any of the Env conformations when assessed by ELISA at 1:100 dilution (data not shown).

3.6.2. Specific antibody depletion of neutralizing antibody responses

Sequential depletion of anti-Env or anti-CD4 specific responses was performed to further characterize the broadly neutralizing response induced by the 2dCD4^{S60C}-liganded Env complexes. The IC_{50} results for the selected rabbit serum samples for antibody depletion experiments are re-shown in Figure 3.15A, and include 2dCD4^{S60C}-liganded Env complex immunized (68 – gp140$_{FVC}$GCN4t(-)/2dCD4^{S60C}, selection based on the highest average NAb titers), and unliganded Env immunized (53 – gp140$_{FVC}$GCN4t(-), matched Env control) rabbits. In addition, these two rabbit sera were resent to Monogram Biosciences and tested against HIV-2$_{P2}$ and SIV$_{mac316}$. Serum 68 showed neutralizing activity against HIV-2$_{P2}$ and SIV$_{mac316}$. However, the IC_{50} values against SIV$_{mac316}$ were >10 fold lower (Figure 3.15A).

A.

Immunogen	Serum ID	Tier 1			Tier 2								Tier 3	HIV-2	SIV	Control
		SF162	BaL	NL43	93IN905	93MW959	97ZA012	IAVI_C22	JRCSF	MGRM-C-004	MGRM-SC-B-012	MGRM-C-015	MGRM-SC-B-005	HIV-2$_{P2}$	SIV$_{mac316}$	aMLV
gp140$_{FVC}$GCN4t(-)	53	7668	52	103	21	<10	<10	<10	<10	12	17	16	<10	11	<10	<10
gp140$_{FVC}$GCN4t(-)/2dCD4^{S60C} complex	68	2076	1420	588	3727	3908	2822	4353	2950	5290	3610	2734	3129	3045	215	<10

B.

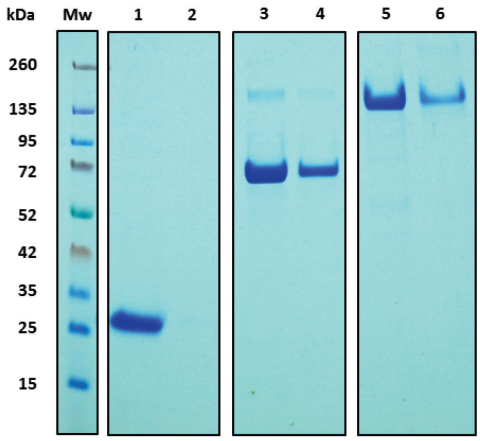


Figure 3.15: Selected rabbit sera for antibody-specific depletion experiments and target protein coupling to magnetic beads. A. Summary of selected rabbit sera for depletion experiments showing the neutralization profiles extracted from Figure 3.13B and additional IC_{50} data against HIV-2$_{P2}$ and SIV$_{mac316}$. Rabbit 53, immunized with unliganded trimeric Env conformation gp140$_{FVC}$GCN4t(-) and rabbit 68, received the 2dCD4^{S60C}-liganded trimeric Env complex gp140$_{FVC}$GCN4t(-)/2dCD4^{S60C}. **B.** SDS-PAGE analysis of target protein coupling to magnetic beads visualized by Coomassie staining/destaining protocols. Band intensities are shown prior to coupling for 2dCD4^{S60C} (lane 1), BSA (lane 3) and gp140$_{FVC}$GCN4t(-) (lane 5) and post coupling for the respective proteins (lanes 2, 4 and 6). 2dCD4^{S60C} was coupled by Ni^{+} to HIS-Mag Agarose beads (Novagen) whereas the BSA and gp140$_{FVC}$GCN4t(+) were covalently coupled to Dyna tosylactivated polystyrene beads (Invitrogen). Molecular weight (Mw) marker (Spectra multicolour broad range protein ladder (Fermentas, Thermo Scientific, Waltham, MA, USA) with molecular weights in kiloDaltons (kDa) is shown on the left.

Envelope-specific depletion of rabbit sera was performed by covalently cross-linking Env or BSA (specificity control) to the surface of paramagnetic, polystyrene beads. Anti-CD4 specific depletions were performed by coupling 2dCD4^{S60C} via the HIS-tag to the surface of Ni^{+} charged, magnetic agarose beads. Target protein coupling to the surface of the HIS-Mag or Dyna magnetic beads was monitored by SDS-PAGE (Figure 3.15B). Coupling efficiencies were estimated to be between 50-80% for the Dyna bead coupling (BSA and gp140$_{FVC}$GCN4t(-)) (Figure 3.15B, lanes 3, 4, 5 and 6) and up to 95% for 2dCD4^{S60C} coupling to HIS-Mag beads (Figure 3.15B, lanes 1 and 2).

Selected rabbit sera were depleted by specific adsorption of antibodies to the proteins immobilized on the surface of the magnetic beads. Antibody adsorption efficacy and specificity was monitored by ELISA at 1:400 dilution and the subsequent depleted-sera were tested for loss of neutralizing activity using the in-house pseudovirus assay (Figure 3.16 and Figure 3.17).

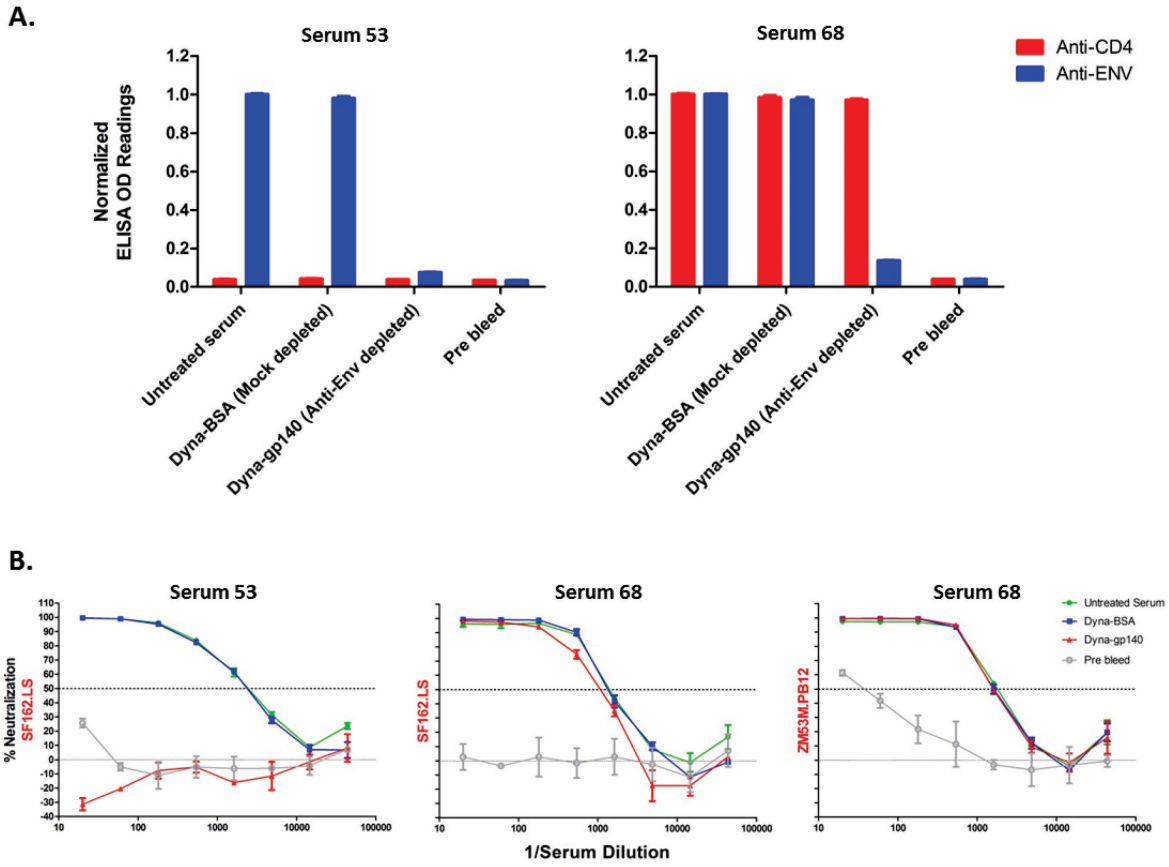


Figure 3.16: Anti-Env specific antibody depletion of selected rabbit sera. Antibody specific depletion experiments were performed on terminal bleed sera samples obtained from selected animals immunized with gp140$_{FVC}$GCN4t(-) alone (serum 53) or with gp140$_{FVC}$GCN4t(-)/2dCD4^{S60C} complex (serum 68), using Env (gp140$_{FVC}$GCN4t(-)) or BSA (control) covalently coupled to tosylactivated magnetic beads, Dyna-gp140 or Dyna-BSA respectively. **A.** Antibody depletion specificity and efficacy was monitored by ELISA. Following antibody adsorption, rabbit sera were diluted 1:400 and the relative binding levels to Env (gp140$_{FVC}$GCN4t(-)) and CD4 (2dCD4^{S60C}) determined. Antibody binding levels were normalized to the untreated serum binding levels. **B.** Neutralization curves of sera 53 and 68, following antibody adsorption against neutralization sensitive subtype B, tier 1 pseudovirus SF162.LS and more resistant subtype C, tier 2 pseudovirus ZM53M.PB12 as compared to untreated serum and pre bleed (week 0) serum samples.

As a control for the Env specific depletion, rabbit 53 serum was selected as it showed high (7,658 IC_{50}) neutralizing titers against the tier 1 pseudovirus SF162 and Env conformation was matched (trimeric - gp140$_{FVC}$GCN4t(-)) to that used to induce the broadly neutralizing response observed in the serum of rabbit 68 (Figure 3.15A). Envelope-directed antibody responses were

specifically and effectively depleted in sera 53 and 68 following incubation with the covalently linked $gp140_{FVC}$GCN4t(-) tosylactivated beads (Dyna-gp140) (Figure 3.16A.). Mock depletions performed using Dyna-BSA did not result in the non-specific depletion of anti-Env or anti-CD4 directed antibodies relative to the untreated sera (Figure 3.16A). Depletion of anti-Env directed response from serum 53 effectively abolished the neutralizing activity to levels that correspond to the pre-bleed serum sample taken at week 0, against the neutralization sensitive, tier 1, subtype B pseudovirus SF162.LS (Figure 3.16B, left panel) and validates the Env-specific depletion using Dyna-gp140 adsorption. However, depletion of anti-Env responses from serum 68 did not affect the neutralizing capacity against the SF162.LS nor the breadth as indicated by the neutralization of the tier 2, subtype C pseudovirus ZM53M.PB12 (Figure 3.16B, middle and right panels respectively) relative to the untreated serum control.

To confirm the contribution of anti-CD4 directed responses in broadly neutralizing activity observed in serum 68, serum samples were specifically depleted of anti-CD4 responses. Mock depletion performed using the uncoupled HIS-Mag beads did not result in the non-specific depletion of anti-Env or anti-CD4 responses (Figure 3.17A). Anti-CD4 responses were specifically and effectively depleted in serum 68 (Figure 3.17A, right panel) following single adsorption incubation with HIS-Mag-2dCD4 beads. Incubations of rabbit sera 53 and 68 with HIS-Mag-2dCD4 did not result in the non-specific depletion of anti-Env responses but was able to specifically and effectively deplete serum 68 of anti-CD4 responses following a single adsorption (Figure 3.17A, left and right panels, respectively). Anti-CD4 specific depletion of serum 68 effectively abolished the neutralizing activity, reducing the IC_{50} against ZM53M.PB12 by approximately 40 fold (from ~1600 to ~40 IC_{50}) (Figure 3.17B, left panel).

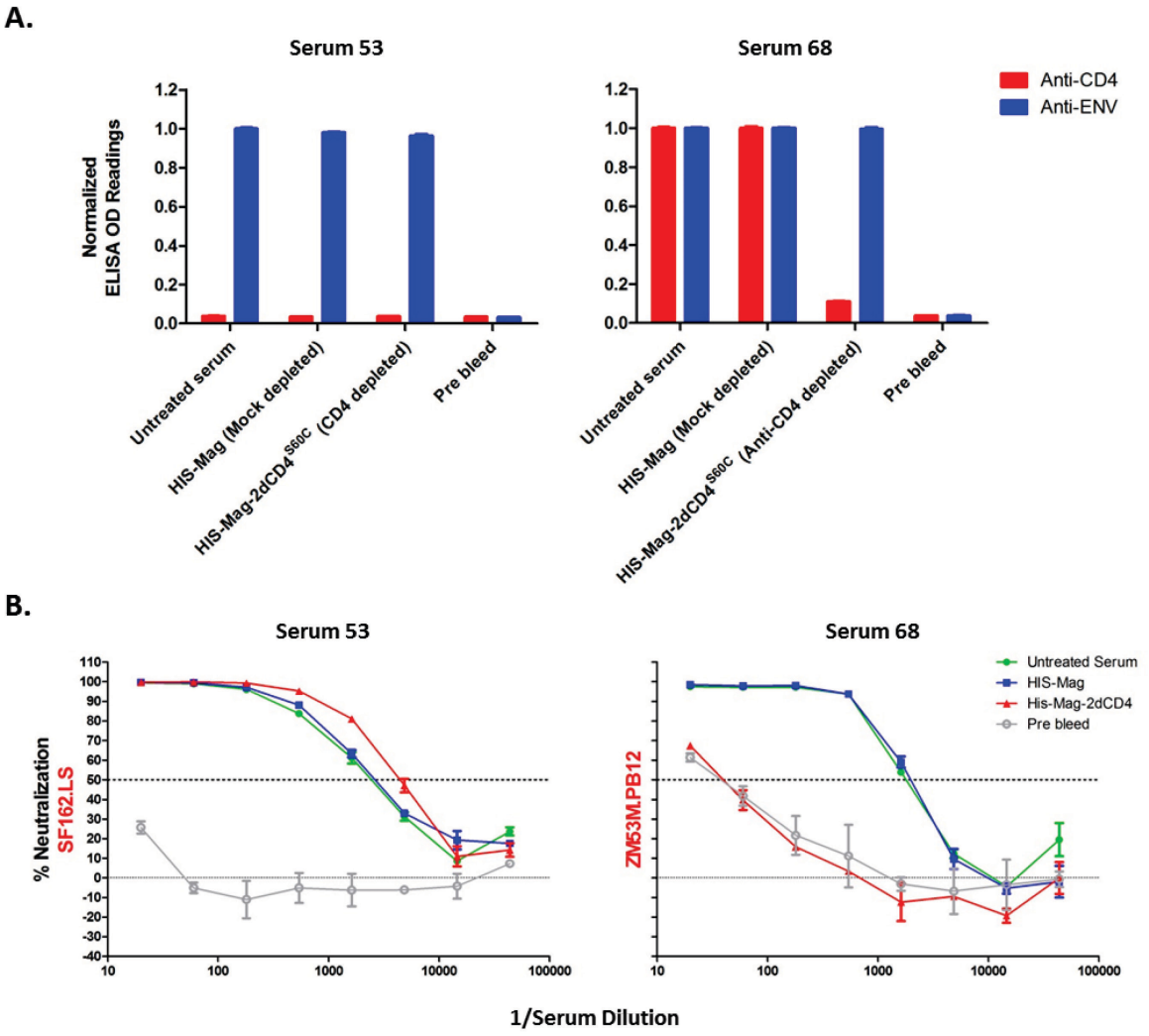


Figure 3.17: Anti-CD4 specific antibody depletion of selected rabbit sera. Antibody specific depletion experiments were performed on terminal bleed sera samples obtained from selected animals immunized with gp140$_{FVC}$GCN4t(-) alone (serum 53) or with gp140$_{FVC}$GCN4t(-)/2dCD4^{S60C} complex, using 2dCD4^{S60C}-coupled or uncoupled (control) HIS-Mag magnetic beads, HIS-Mag-2dCD4^{S60C} and HIS-Mag, respectively. **A.** Antibody depletion specificity and efficacy was monitored by ELISA. Following antibody adsorption, rabbit sera were diluted 1:400 and the relative binding levels to Env (gp140$_{FVC}$GCN4t(-)) and CD4 (2dCD4^{S60C}) determined. Antibody binding levels were normalized to the untreated serum binding levels. **B.** Neutralization curves of sera 53 and 68, following antibody adsorption against a subtype B, tier 1 pseudovirus SF162.LS and a subtype C, tier 2 virus ZM53M.PB12 as compared to untreated serum and pre bleed (week 0) serum samples.

Taken together, these results illustrate the contribution of anti-CD4 specific responses in inducing the neutralization breadth observed in the animals that received Env/2dCD4^{S60C} complexes. The Env-directed antibody response was shown to have little effect on the neutralizing breadth and was confined to the neutralization sensitive tier 1 pseudoviruses.

Chapter 4: Discussion

The ability to induce a bNAb response capable of preventing primary HIV-1 acquisition *in vivo* following immunization remains a crucial obstacle in the development of an effective HIV-1 vaccine. The surface exposed Env glycoprotein of HIV-1 which mediates viral entry is an obvious target of the humoral arm of the immune system. Unfortunately Env based vaccine immunogens have met with limited success largely due to the inability to recapitulate native Env structure and expose the critical, conserved neutralizing epitopes required to induce a broadly neutralizing response capable of neutralizing cross-clade or subtype variants (reviewed in [84, 170, 191, 255]. Moreover, it has become increasingly evident that the appropriate ongoing antigenic stimulation is required for the host to undergo the extensive B cell affinity maturation required to generate a bNAb response (reviewed in [144]). A multitude of strategies have been employed in an attempt to overcome the challenges associated with Env-based immunogens that ultimately induce the desired immunological response capable of protecting from HIV-1 acquisition. This thesis describes the design and production of an HIV-1 subtype C founder virus consensus Env based immunogen and the ability of different conformations thereof, alone or via an intermolecular-disulphide-stabilized 2dCD4^{S60C} immune complex, to induce a broadly neutralizing antibody response in a small animal model.

4.1. Selection and design of the HIV-1 subtype C founder virus Env consensus sequence

Given the vast degree of genetic diversity of HIV-1 strains circulating globally, identification and selection of a single sequence on which to base a vaccine immunogen capable of inducing sufficient cross reactivity to offer benefit globally presents a significant challenge [32, 171]. The development of "centralized immunogens" is gaining popularity as these sequences minimize the genetic diversity between the contemporary circulating viruses and the vaccine sequence while also providing an opportunity to cover more conserved features or epitopes over any single natural isolate [32, 182-185, 256]. During sexual transmission of HIV-1, the donor virus inoculum has to overcome the physical barriers of the mucosal surface, immunological barriers of the innate immune system and gain access to suitable target host cells that will ultimately result in the establishment of a productive infection in the recipient. The inefficiency of this

process is highlighted by a dramatic restriction in genetic diversity from donor to recipient that culminates in 65-90% of new infections arising from a single transmitted variant [43-52]. Despite this inefficiency, sexual transmission predominates as the mode of HIV-1 transmission, with HIV-1 subtype C accounting for over 50% of new heterosexual transmission worldwide [171, 257]. These early events and host/viral interactions represent a finite window of HIV-1 vulnerability which can be exploited by an appropriate vaccine-elicited immune response. Several phenotypic and genotypic characteristics have been associated with founder Env sequences, including the predominant transmission of CCR5 utilizing viruses [47, 52] and shorter, more condensed variable loop structures (V1/V2 and V4) with fewer PNGs [44, 48, 50]. These sequences have also been linked to α4β7-reactivity which may aid in early stages of transmission [188, 189, 258] while enhanced neutralization sensitivity to the donor plasma [44] and CD4bs-directed antibodies IgG1b12 and VRC01 has also been reported [52]. While the precise transmission-benefit provided by the genotype of founder virus Env remains unclear, it has been suggested that the shorter, less glycosylated variable loop structures may promote improved access to the CD4bs on gp120 impacting on the susceptibility of the virus to NAbs [44]. Thus, Env sequence data available from these founder viruses may provide valuable information regarding structural, functional and immunogenic properties crucial for vaccine development.

Here we describe the construction of an HIV-1 subtype C founder virus Env consensus sequence derived from patients acutely infected with HIV-1 subtype C that had acquired the infection via heterosexual transmission [43, 49]. The final HIV-1 subtype C founder viruses Env gp160 consensus sequence was calculated to be 831 amino acids in length (Figure 3.1). The gp120 sequences of the geographical and founder virus consensus sequences were notably shorter than the original patient sequence data sets used to derive the consensus, particularly in the variable loop structures V1/V2, V4 and V5 (Table 3.1). This is attributed to the high degree of amino acid sequence variability in these loop structures, which during the construction of the consensus sequences, alignments were anchored using common PNGs and minimal shared amino acids spanning elements were incorporated [184]. This resulted in a 20-30% reduction in the amino acid length in each of the V1/V2, V4 and V5 loop structures (Table 3.1). While changes in variable loop length are often accompanied by the addition or loss of PNGs [44], it was our intention to conserve the position and number of PNGs in the founder sequence as far as possible. Overall the founder virus consensus Env sequence showed good conservation of the number and PNGs distribution with only minor alterations in numbers occurring in the V1/V2, C2 and ectodomain of gp41 noted as compared to the data set average (Table 3.2).

The identification of bNAbs that target glycan-dependent epitopes in the V1/V2 and the base of the V3 loops structures [156-158] combined with the recent immune analysis of the RV144 vaccine trial, which found that the presence of V1/V2 binding antibodies correlated inversely with HIV-1 infection risk [123, 124, 259], prompted us to examine these domains in greater detail with regard to immunogen design. The bNAbs, PG9 and PG16 target a proteoglycan epitope that comprises asparagine linked glycans at position 160 and 156 (N173 in some strains) and the cationic C loop of the V1/V2 loop [155]. Similar binding properties and neutralization profiles have been described for bNAbs CH01-04 [158], PGT141-145 [157] which suggests targeting similar or overlapping epitopes to that of PG9 and PG16. Removal of the glycan at position N160 by mutation of the PNGs motif or through de-glycosylation treatment abrogates the neutralizing effect of these antibodies, highlighting the crucial role of this glycan in the formation of the epitope [155-158]. Likewise, bNAbs PGT121-123 and PGT125-135 also target a proteoglycan epitope, where the glycan at position N332 is indispensable to the formation of the bNAb epitope [157, 159]. Pertinent to the design of the founder virus consensus Env sequence it was noted that despite the conservation of the PNGs at positions 160 and 332 in chronic viruses, these sites have been reported to be significantly reduced in subtype C transmitted/founder viruses sequences [260]. The founder virus Env consensus sequence contains both PNGs at position 160 and 332, as these were highly conserved within our data set (Appendix C). Elimination of these specific PNGs correlates with resistance to the bNAbs PGT128, PG9, PG16 and PGT145 [260]. This raises a concern with regard to the inclusion of these specific PNGs in Env based immunogens, as antibody responses elicited towards these glycans may prove to be ineffective against certain founder viruses that lack the epitope. Interestingly, Moore and colleagues described host-immunological driven evolution of bNAbs epitopes, whereby glycosylation of N332 arises in response to early immunological pressure resulting in the formation of a conserved, broadly neutralizing epitope [260]. The inclusion of PNGs at positions N160 and N332 is further supported by longitudinal antibody response studies that have shown that bNAb responses targeting these glycans occur more frequently in HIV-1 infected patients than those targeting the other well described bNAb sites such as the CD4bs or MPER region [109, 261, 262]. Moreover, Walker *et al.,* [201] reported that in a SHIV-infected macaque where the N332 was present in the transmitted strain, the macaque subsequently developed a N332-dependent bNAb response. Thus, the inclusion of these PNGs at position N160 and N332 in Env-based immunogens requires further investigation.

The V1/V2 loop also contains the α4β7 integrin binding motif LDV/I which allows gp120 to bind and signal through $\alpha4\beta7^{+}/CD4^{+}$ T cells [263, 264]. Although the interaction of gp120 with the α4β7 integrin is not required for viral entry, a subset of T cells that express high levels of

α4β7 integrin (α4β7$^+$/CCR5$^+$/CD4$^+$ T cells) on their surface are prone to highly productive HIV-1 infection [188]. Furthermore these α4β7$^+$/CCR5$^+$/CD4$^+$ T cells have been shown to be present at the major sites involved with early HIV replication and include the cervicovaginal and rectal mucosal sites as well as the gut-associated lymphoid tissues (GALT) [188]. Nawaz and colleagues, have reported that founder viruses may have improved affinity for the α4β7 integrin than compared to more contemporaneous strains due to differential PNGs on Env [189]. Targeting of α4β7$^+$/CCR5$^+$/CD4$^+$ T cells may provide enhanced transmission efficiency, as the α4β7 integrin mediates trafficking of CD4$^+$ T cells to the GALT thereby providing an ideal environment in which to establish infection [188, 265]. Anti-α4β7 integrin directed antibodies have been shown to reduce viral load during acute SIV infection in macaques [266], yet were unsuccessful in preventing the spread of infection in an *in vitro* assay [258]. Thus the relevance of inclusion of the α4β7 integrin binding motif as a vaccine target requires further elucidation. Nonetheless the α4β7 integrin binding motif was included in the founder Env consensus sequence due to the high conservation rate present in the sample set.

Analysis of the bNAb epitopes in the MPER region of gp41 revealed single amino acid substitutions in the 4E10 and 10E8 epitopes, located at positions N674D [267] and R683K [151], respectively. These have been reported to be well tolerated in binding and neutralization assays with the respective antibodies and have not been shown to convey resistance [151, 267]. Moreover, a crucial amino acid substitution was found to occur at position 665 (K665S) which correlates with resistance to the bNAb 2F5 and has been reported previously for HIV-1 subtype C sequences [267, 268].

The discontinuous amino acids sequences, as determined by structural and mutagenesis analysis, indicate that residues located in loop D of the C2 region, the CD4 binding loop in the C3 region, the bridging sheet (β20/21 hairpin) and β23 in the C4 region and β24-α5 connection in the C5 region together make up the conformational CD4bs on gp120 (Figure 3.1) [128, 269-271]. As expected, the CD4 molecule contact residues are shared or overlap with those that comprise the epitopes targeted by CD4bs-directed bNAbs, including IgG1b12, VRC01, VRC03, VRC06, NIH45-46, PGV04 and 3BNC117 on gp120 [147-150, 252, 269]. The discontinuous nature of the CD4bs presents a challenge when analyzing Env amino acid sequences for CD4 binding functionality or CD4bs-directed bNAbs epitopes, as alanine-substitution analysis has exposed unrelated contact sites that influence neutralization sensitivity to these CD4bs ligands, presumably as a result of alteration in the quarternary structure of the surface Env spike [269]. Thus the impact of these non-contact mutations, whether naturally occurring or induced, can only be determined empirically. The founder virus Env consensus sequence showed a high degree of conservation of the CD4bs contact residues, with only a single substitution, of a

aspartic acid (D, acidic polar, negatively charged) to asparagine (N, polar, neutral charge) at position 474, occurring in one of the critical contact residues. Interestingly this was due to the high degree of conservation of asparagine at the position in the data set (Appendix C). Based on subsequent experiments, the D474N substitution does not have any detectable impact on the ability of Env to bind CD4 (Figure 3.10 and Table 3.3).

Overall, the HIV-1 subtype C founder virus Env consensus sequence clustered more closely with the inferred ancestral C and consensus C sequence showing a high degree of conservation with these two sequences. In addition, the inferred founder virus Env consensus sequence incorporates characteristics of transmitted and early founder viruses and was used in the subsequent development of an Env immunogen for novel vaccine design.

4.2. HIV-1 subtype C founder virus Env consensus constructs

4.2.1. Monomeric, dimeric and trimeric constructs of the HIV-1 subtype C founder virus Env consensus sequence

The native Env spike on the surface of HIV-1 is comprised of a trimer of non-covalently bound heterodimeric subunits of the surface gp120 and transmembrane gp41 domains [17, 18]. However, monomeric forms of gp120 Env have traditionally been employed as subunit vaccine candidates, predominantly due to their ease of manufacture [119]. The inability of monomeric gp120 Env to protect from HIV-1 acquisition in human vaccine trials has been attributed to the elicitation of non-neutralizing antibody responses in the host that are directed to epitopes usually buried or non-exposed on the native HIV-1 Env spike of primary isolates [120, 169, 272]. In addition, the bNAbs PG9 and PG16 have been shown to preferentially recognize their epitopes in the context of the quaternary, trimeric Env structure and suggests a potential benefit trimeric Env conformations have over monomeric immunogens for eliciting these type of responses [156]. Preliminary comparative studies between matched trimeric and monomeric Env conformations suggest an improved antibody neutralizing breadth and titer following immunization with trimeric conformations [219-222]. Overall, the development of trimeric Env-based subunit immunogens is aimed at replicating the native Env spike, however it presents its own unique set of challenges.

The development of trimeric Env-based immunogens has primarily focused on using the soluble gp140 ectodomain of Env, since expressed recombinant gp160 remains membrane bound [17, 156]. The gp140 polypeptide contains the gp120 and the ectodomain of gp41 and therefore

includes the bNAbs epitopes contained within the MPER (2F5, 4E10 and 10E8) while eliminating any solubility issues that may result from inclusion of the transmembrane region [216, 222, 273, 274]. The inherent instability of the gp120/gp41 non-covalent interaction has been addressed by generating cleavage-defective Envs (designated gp140 (-)) through mutation of the furin cleavage site that negates the processing of the gp140 polypeptide [275, 276]. Alternatively, processed (furin cleavage site intact) gp140 Env has been stabilized through the induction of a intermolecular disulphide bond between the gp120 and gp41 subunits (designated SOS gp140) [205]. Further stabilizing amino acid substitutions of isoleucine to proline at position 559 and others in the N-terminal heptad region of gp41 have resulted in fully cleaved trimers that show favourable antigenic properties [206, 277]. It has been suggested that the cleaved and uncleaved forms of HIV-1 Env may differ in their antigenicity, particularly in the gp41 component as demonstrated by the preferential binding of non-neutralizing anti-gp41 MAbs to uncleaved gp140SOS-IP trimers [212].

The addition of heterologous trimerization motifs fused to the carboxyl terminus of Env (gp140), have been used in an attempt to stabilize the formation of Env trimers. Most commonly used trimerization motifs include the GCN4 transcription factor (GCN4)[213], the C-terminal region of bacteriophage T4 fibritin [216], trimerization motif of chicken cartilage matrix protein [217] and the catalytic chain of aspartate transcarbamoylase [218]. The trimerization of Env utilizing the GCN4 motif has been well described in the literature [214, 215, 218, 221, 253, 278, 279] and therefore was selected as a means for generating trimeric, consensus founder virus Env.

In addition to comparative biochemical, antigenicity and immunogencity analysis between monomeric ($gp120_{FVC}m$) and GCN4, stabilized trimeric Env forms ($gp140_{FVC}GCN4t$ (+)/(-)), we also examined a dimeric Env conformation in this regard. Recombinant expression of Env (gp120/gp140) is notoriously heterogeneous, comprising monomeric, dimeric, trimer/oligomeric and even higher order aggregates [214, 274, 278, 280-282]. The native gp120 dimeric conformations that form have been shown to be as a result of intermolecular disulphide linkages between two monomeric units [281, 283] and show reduced recognition by CD4i antibodies and reduced binding to CCR5 following CD4 priming, as compared to the monomeric form. This suggests that these dimeric conformations represent aberrant forms that may not be functional and are undesirable in certain forms of analysis. Taking the abovementioned into consideration, we explored the possible role of GCN4 stabilized ($gp120_{FVC}GCN4d$) or novel, carboxyl terminal, di-selenide stabilized ($gp120_{FVC}Ud$) dimeric conformations from a production/expression and biochemical/immunogenicity aspect.

4.2.2. Expression, purification and characterization of founder virus Env consensus constructs

The inferred HIV-1 subtype C founder virus *env* consensus sequence was successfully utilized to encode functional, gp120 monomeric, gp120 dimeric and gp140 trimeric Env glycoproteins. Evaluation of the transiently expressed founder virus consensus Envs produced recombinant proteins with the expected molecular weight ranges for gp120 and gp140 Env proteins (Figure 3.4). Inefficient full proteolytic cleavage of the expressed $gp140_{FVC}$GCN4t(+) Env was noted, as only a fraction of this secreted protein was found to exist in its cleaved gp120 and gp41 ectodomain subunits (Figure 3.4, lane 1). This finding is consistent with those of Binley *et al.*, [205], Zhang *et al.*, [284], Kothe *et al.*, [184] and Kothe *et al.*, [185] who suggested that incomplete processing of Env arises due to saturation of the furin proteases or potential differences in proteolytic Env processing routes between infected and transfected cells which may be exacerbated during recombinant expression of soluble Env. To address the furin cleavage inefficiency the authors suggested co-transfecting with a furin protease-encoded plasmid, which has been shown to reduce Env expression [205] or through the enhancement of the furin recognition cleavage site, by replacing the native REKR sequence with six consecutive arginine residues (RRRRRR) [205, 212]. Since efficient cleavage impacts on the quaternary structure of Env [200, 207, 211, 285], future work could investigate the differences between constructs containing an REKR and RRRRRR cleavage site.

We first set out to examine whether the engineered carboxyl terminal, selenocysteine residue in the expressed $gp120_{FVC}$Ud Env construct would be sufficient to generate di-selenide stabilized gp120 dimeric conformations. We noted that expression of soluble $gp120_{FVC}$m naturally dimerizes and that the formation of this dimeric conformation is sensitive to reducing reagents, consistent with the finding of Finzi *et al.,* [281] and Center *et al.,* [283]. In addition, previous work conducted in our laboratory showed that synthetically generated peptide sequences containing selenocysteine residues form intermolecular di-selenide stabilized bonds that are highly resistant to reducing reagents (unpublished data). Thus, to establish whether the carboxyl terminal, selenocysteine residue was sufficient to generate a di-selenide stabilized gp120 dimeric conformation, we comparatively assessed the dimer formation of the expressed $gp120_{FVC}$m and $gp120_{FVC}$Ud Envs under increasing DTT concentrations (Figure 3.5). As the $gp120_{FVC}$m and $gp120_{FVC}$Ud Envs differ in length on the carboxyl terminus by only two amino acids (selenocysteine and arginine, respectively), it provides a suitable control for selective selenocysteine incorporation and natural dimerization of gp120 Env that occurs in the absence of any dimerization motifs. Expression of $gp120_{FVC}$m and $gp120_{FVC}$Ud expressed Envs was monitored by Western blotting. Under reducing conditions (100 – 6.25 mM DTT) both Envs

resolved as homogeneous monomers. However, elimination of the reducing reagent (0 mM DTT) resulted in the appearance of a dimeric Env conformation in both Envs. Autoradiography analysis of SDS-PAGE gels confirmed exclusive incorporation of Se^{75} radioisotope in the $gp120_{FVC}$Ud expressed Env but not in the $gp120_{FVC}$m. Thus, confirming the successful read-through and recoding of the UGA codon that resulted in the insertion of a selenocysteine at this position (Figure 3.5A and B, lower panels). We also examined the cellular fraction of each of the $gp120_{FVC}$m and $gp120_{FVC}$Ud transfected HEK293T cell lines for the presence of the Se^{75}-labelled, endogenously expressed selenocysteine-containing protein thioredoxin reductase. This was present and resolved at ~50 kDa (Figure 3.5A and B, lower panels, lane 1). More importantly, formation of the $gp120_{FVC}$Ud, dimeric Env conformation was not evident on the autoradiography analysis in the presence of DTT (100 – 6.25 mM). This confirmed that the successful inclusion of a C-terminal selenocysteine residue, is insufficient for formation of a di-selenide stabilized Env dimer. A further interesting observation to note was that under non-reducing conditions (DTT – 0 mM) the autoradiography analysis clearly detected two bands in the supernatant from the HEK293T/$gp120_{FVC}$m Env (~120 and ~ 50 kDa) and a third band was detected in the HEK293T/$gp120_{FVC}$Ud supernatant corresponding in size to a dimeric Env (~250 kDa). The presence of these additional bands was unexpected, but given that they were only present under non-reducing conditions suggests a non-specific labelling of expressed Env with the Se^{75} radioisotope.

For large-scale Env expression (mg amounts) required for downstream biochemical functionality, antigenicity and immunogenicity testing, we established stably expressing 293-F cell lines (non-adherent subclone of the HEK293T cell line). This cell line was selected due to its high-transfection efficiency and its adaptation to serum free media facilitates less stringent protein purification methods that would otherwise be required to remove contaminating serum proteins. The Chinese hamster ovary (CHO) cell line is FDA approved for the recombinant expression of proteins destined for clinical usage in humans including HIV-1 Env [119]. There is growing appreciation within the HIV-1 vaccine field of the importance of glycosylation and glycosylation patterns of the host cell system from which the proteins are derived. Raska *et al.*, [286] suggest that the commonly used mammalian expression systems (HEK293T/CHO) for antigen production do not exhibit the same N-glycosylation patterns as compared to the natural, T cell line host of HIV-1. This differential in N-glycan composition has been shown to affect recognition of Env by HIV-1 infected patient sera and may also influence antigenicity or elicitation of the glycan-dependent 2G12, PG9, PG16, CH01-04 and the PGT family of bNAbs [155-159]. While the significance of the glycosylation patterns falls beyond the scope of this current study, this does present necessary future avenues of exploration and optimization.

Using a one-step *G. nivalis* lectin-affinity purification protocol we were able to achieve approximately 90% purity of the expressed founder virus Envs (Figure 3.6). Conformational heterogeneity of the expressed founder virus Envs as determined by SEC-FPLC and native PAGE was consistent with previously reported data that shows the expression of soluble HIV-1 Env is comprised of a heterogeneous mixture of monomeric, dimeric, trimeric as well as high order oligomeric conformations [214, 218, 235, 253, 274]. The expressed trimeric founder virus Envs ($gp140_{FVC}$GCN4t(+) and $gp140_{FVC}$GCN4t(-)) were particularly susceptible to the formation of higher-order aggregates, presumably comprising dimers-of-trimers or tetrameric conformations of these Envs (Figure 3.7F). Interestingly, the gel filtration profile of $gp140_{FVC}$GCN4t(+) exhibited an additional distinct peak that corresponded to the equivalent position of the eluted monomeric gp120 conformation (~50 mls) (Figure 3.7D and F). This peak was not as prominent in the $gp140_{FVC}$GCN4t(-) expressed Env and therefore most likely represents the cleaved monomeric gp120 Env that has disassociated from the trimeric/higher order oligomeric conformations.

The gel filtration profiles also provided valuable insights into the construct design and conformational stability of each expressed Env protein. The peak magnitude and the area under the curve provide an overall estimate of the conformational heterogeneity present in each Env protein. We were encouraged by the observation that following lectin-purification, the majority conformations corresponded to the respective construct design e.g. monomeric constructs ($gp120_{BaL}$ and $gp120_{FVC}$m) predominately comprised monomers, etc. (Figure 3.7). The SEC-FPLC purified Env conformations were highly stable. Comparative analysis of retention volumes that correspond to the different conformations, between the first (conformational purification of Env) (Figure 3.7) and second round (removal of unbound $2dCD4^{S60C}$ from immune complexes) (Figure 3.11) of SEC-FPLC produced almost identical values. Furthermore, the gel filtration profiles for each Env conformation were homogeneous in nature, as indicated by a single peak (Figure 3.11), and did not show any evidence of further disassociation/association into a heterogeneous mixture of conformations. It should be noted that there was a discrepancy between the native PAGE data and the gel filtration profiles generated from the second round of SEC-FPLC (Figure 3.8 and Figure 3.11, respectively). The native PAGE analysis following the first round of SEC-FPLC revealed the continued presence of conformations, particularly in the $gp120_{FVC}$m and $gp120_{FVC}$GCN4d Envs (Figure 3.8). However only single peaks were seen in the second round of SEC-FPLC (Figure 3.11). To the best of our knowledge, there is no evidence in the existing literature to explain and support these findings, since most researchers only publish native PAGE gels of trimeric Env conformations or core gp120 monomers [253, 278]. Future

work can evaluate the integrity of these Env conformations and the methodologies in more detail.

4.2.3. HIV-1 subtype C founder virus consensus Envs are functionally intact

Having expressed and purified relatively homogenous monomeric, dimeric and trimeric conformations of the HIV-1 subtype C founder virus consensus Envs, we then proceeded to confirm the biophysical functionality of each conformation by ELISA and SPR analysis. A concern with the development of centralized immunogens, such as consensus sequences, is that generation of these sequences is subjected to sample biasing that may result in the unnatural combination of polymorphisms, detrimentally affecting the native, quaternary conformations of these proteins or the display of particular neutralizing epitopes [182]. Such circumstances were illustrated by the findings of Lian *et al.,* [186] who reported generating a Botswana derived, subtype C consensus sequence derived from published sequences, that did not result in the expression of functional Env protein capable of binding CD4 and facilitating fusion. Our analysis of the CD4bs contact residues of the inferred founder virus Env consensus sequence, revealed a charge-altering, sterically-conserved substitution (D474N) that was present at a critical contact site between gp120-CD4 (Figure 3.1). Validating the interaction between the founder virus consensus Envs and CD4 was not only important to establish the functionality of this inferred sequence, but was also critical for the downstream generation and immunogenicity evaluation of Env/2dCD4^{S60C} complexes. The CD4bs for each founder virus Env conformation was successfully shown to bind the IgG1b12 and induce the 17b epitope following priming with 2CD4^{S60C} (Figure 3.10). The IgG1b12 targets an epitope that overlaps the CD4 binding site. Due to the discontinuous nature of this epitope, binding of IgG1b12 confirms the native conformation of the CD4bs and that the Env core has folded correctly [147]. The 17b antibody preferentially recognizes a CD4 induced epitope that overlaps with the co-receptor binding site on gp120 Env. Recognition of the monomeric, dimeric and trimeric conformations of the founder virus consensus Envs by 17b following priming by CD4 (2dCD4^{S60C}), not only confirms the native functioning of these proteins through their ability to bind CD4 but also illustrates that each Env conformation was able to proceed with the significant conformational changes required to induced the co-receptor binding site [128, 132]. These Env conformations are therefore able to present native conformations required during the HIV-1 entry process and the intermediary-states targeted by immune responses. It is likely that the dimer described here folds correctly, and is functional since it dimerizes as a result of the GCN4 dimerization motif, rather than the aberrant intermolecular disulphide bonds [281, 283]. The high affinity of the

gp120-CD4 interaction for each founder virus consensus Env conformation was confirmed in the low nano molar range (2.93-22 nM) consistent with other reports [253, 284, 287]. Interestingly, we observed that the quaternary structure or conformation influenced the affinity of the Envs for CD4. This observation is not all that unexpected if one considers the differential in CD4-affinity reported between primary and laboratory adapted isolates, yet their respective monomeric gp120 Env subunits have comparable affinities [288, 289]. We propose a similar logical progression of reasoning for the observed decrease in the on-rates (k_a) as the Env conformation gains complexity. The monomeric Env ($gp120_{FVC}m$) conformation is more likely to be less rigid than the trimeric conformations ($gp140_{FVC}GCN4t(+)$ and $gp140_{FVC}GCN4t(-)$), thus promoting easier access to the CD4bs as indicated by a more favourable on-rate (k_a). Interestingly, the trimeric Env conformations showed a slower (10-fold) off-rate *(*k_d) value as compared to the monomeric and dimeric conformations, possibly as a result of avidity effects. These trimeric Env conformations, in theory, possess three functional CD4bs and may be able to engage with more surface-immobilized CD4 at a single time point, thereby further stabilizing the complex interaction and decreasing the off-rate. This hypothesis is supported by the findings of Zhang *et al.,* [287] who showed that the HIV-1 ADA gp140 trimer was able to bind more than one CD4 molecule. In addition Pancera *et al.,* [278] who performed microcalorimetric experiments using a YU2gp120 GCN4 stabilized trimer demonstrated that these trimers could on average bind two CD4 molecules. However, this is contradicted by the findings of Kovacs *et al.,* [220] who calculated a 1:1 stoichiometry of CD4 binding to trimeric Env, using two different gp140 stabilized trimers, derived from different subtypes (subtype A – 92UG037.8 and subtype C – CZA97012). These authors proposed that the structural rearrangements induced in the gp120 as result of the CD4-bound conformation, causes an asymmetry in the trimeric structure, possibly obstructing access to the remaining two CD4bs. Pancera *et al.,* [278] and Srivastava *et al.,* [290] demonstrated that deletion of the V1/V2 loop structures in gp120 or gp140 stabilized trimers resulted in the viability of all three CD4bs. Combined, the data suggests that the stoichiometry of the CD4-trimeric Env interaction may be related to the Env sequence, or that modifications to the variable loop structures, such as length shortening as is the case with our derived HIV-1 subtype C founder Env consensus sequence, may result in improved accessibility of all three CD4bs of the trimer. While we did not calculate the specific stoichiometry of CD4 binding to the different founder virus conformations, Figure 3.12 indicates remarkably similar amounts of $2dCD4^{S60C}$ were found to have bound the different conformations, supporting the theory that all three CD4bs site are available and functional on the founder virus consensus Env trimers.

The dimeric Env conformation was interesting in that it displayed a hybrid of monomeric and trimeric conformational CD4-binding characteristics. The dimer displayed an improved association rate as compared to the trimeric conformations, yet not as favorable as the monomeric conformation. A similar pattern emerged in the dissociation rates, with the dimeric conformation displaying a slower dissociation rate than the monomer although still 10-fold faster than the trimeric conformations. While the dimeric Env conformation does not necessarily represent the native, trimeric Env spike, this result does underline the influence that quaternary structure of Env has on CD4-binding.

Although outside the scope of this study, the abovementioned findings can be strengthened by the future evaluation of the Env binding kinetics of a panel of bNAbs such as PG9, PG16, PGT family, VRC01, 4E10 and 10E08.

4.3. Immunogenicity of HIV-1 subtype C founder virus consensus Env based immunogens

As previously stated and reiterated here, critical to the development of an effective HIV-1 vaccine is the induction of a bNAb response following immunization. We are exploring a multi-angled approach in the development of improved Env based immunogens. We have described the rationale behind developing a subtype C founder virus Env based immunogen, as well as comparative analysis between different Env conformations. Lastly, we explored the potential immunogenicity benefit of these various Env conformations in complex with 2dCD4^{S60C}. The intention of developing such Env-based complex immunogens is to stabilize intermediary epitopes during the binding or fusion process, or reveal cryptic epitopes that are otherwise unexposed or occluded on the functional trimeric Env spike. Entry of HIV-1 requires the binding of both CD4 and chemokine co-receptors. Binding of CD4 to gp120 induces significant conformational changes in the outer and inner domains as well as the bridging sheet of gp120 [128, 132, 137]. This is accompanied by repositioning of V1/V2 and V3 loops structures, exposing the co-receptor binding site and the epitopes targeted by CD4i antibodies [128, 129]. The CD4i epitope presents an attractive vaccine target as it has been shown to exhibit an extraordinary degree of antigenic conservation across the lentiviruses (HIV-1, HIV-2 and SIV). Regardless of infecting HIV-1 subtype (subtypes A-F and H, including recombinant forms CRF01_AE, CRF02_AG and CRF11_cpx) the co-receptor binding site is frequently targeted by the host immune system during the natural course of disease progression [103, 130], eliciting high titers of anti-CD4i antibodies capable of neutralizing highly divergent HIV-2 following priming

with CD4 [130]. Previous immunization studies conducted, utilizing gp120-CD4 complexes, reported the ability to elicit neutralizing antibodies against primary isolates and correlated with accelerated clearance and long-term absence of tissue viremia in a SHIV macaque challenge model [228]. Thus the CD4i epitopes present an attractive target for vaccine/immunogen development. Efforts to exploit this epitope on gp120 include; the chemical cross-linking of gp120 and CD4 proteins [227, 228]; expression of a CD4, gp120 single chain polypeptide separated by flexible linker sequence (FLSC) [228, 230]; stabilization of the CD4-bound conformation by site-directed mutagenesis [237, 291]; the use of CD4-mimetics (CD4M33, M9, M64U1-SH) or antibodies (A32) to stabilize the CD4i conformation of gp120 [231-234, 292]; as well as Env sequences derived from CD4-independent viruses that naturally display enhanced exposure of the co-receptor binding site [238]. Our group developed a novel 2dCD4 containing a serine to cysteine substitution at position 60 ($2dCD4^{S60C}$) that allows for targeted intermolecular disulphide exchange to occur between the cysteine 60 of $2dCD4^{S60C}$ and the cysteine 126, positioned at the base of V1/V2 loop structure on gp120, thereby stabilizing this interaction [240].

We assessed the immunogenicity of the unliganded HIV-1 subtype C founder virus consensus Env conformations and $2dCD4^{S60C}$-liganded conformations in rabbits. A number of factors influence the ability to compare outcomes from the various immunization studies conducted using Env glycoproteins of HIV-1 accurately. Firstly, there is no standardized animal model. While non-human primates are considered to be the gold standard and provide an *in vivo* challenge model, they are prohibitively expensive and therefore the immunogenicity of the majority of Env-based immunogens have been investigated in the smaller animals models such as mice, guinea pigs, rabbits, goats and even llamas [185, 236, 256, 293-297]. Secondly, the immunization protocols vary greatly, from the concentration of protein immunogen used, number of immunizations conducted, immunization regimen, to priming with DNA or viral vectors as well as the various adjuvants used, all of which are aimed at strengthening the directed immune response. Lastly, although much effort has been made to characterize the viruses and standardize methodologies and assays used to monitor the neutralizing antibody responses [250, 254, 268], this does present an area where inter-laboratory variation may result in the under or over estimation of potential neutralizing effects. To avoid the potential of inter-laboratory variation, assessment of our terminal rabbit sera was carried out by Monogram Biosciences Inc. using their PhenoSense assay.

4.3.1. Antibody neutralization titers induced by unliganded Env conformations

Single-round *in vitro* infectivity assays provide a reproducible, quantitative measure for evaluating magnitude and breadth vaccine sera. Standardized HIV-1 Env-pseudotyped virus reference panels have been developed to represent genetically and geographically diverse subsets of viruses [250, 268]. These Env-pseudotyped viruses have been further categorized into tiers, according to neutralization sensitivity. Antibody-mediated neutralization sensitivity increases from tier 1 to tier 3, with the majority of primary circulating viral isolates sensitivity represented by tier 2 pseudoviruses [254]. This tiered categorization allows for increased stringency testing of potency and breadth on NAbs, while providing a platform for comparative analyses of different vaccine candidates [254]. Consistent with previous immunization studies carried out using Env glycoproteins, despite the elicitation of high levels of Env binding antibodies (Figure 3.14A), the HIV-1 subtype C founder virus consensus Env conformations exhibited a very narrow antibody neutralization breadth that was confined to tier 1 neutralization-sensitive pseudoviruses (Figure 3.13B). These findings have not only been reported for derivatives of so-called centralized immunogens but also for HIV-1 Env immunogens derived from TCLA and contemporary primary viral isolates [182, 184-186, 253, 298]. In addition, comparative immunization studies conducted previously have suggested improved breadth and neutralization titers following immunization with trimeric Envs compared to the matched monomeric Env conformations [219-222]. Due to the narrow neutralizing breadth obtained, induced by the un-liganded Env immunizations in this present study, comparative neutralizing titers assessment is limited to the tier 1 pseudoviruses (SF162, BaL and NL43). There was no definitive improvement in neutralizing breadth of the trimeric founder virus Env conformations over either the monomeric or dimeric versions, as the rabbit sera showed activity against SF162 and NL43. One could argue that the trimeric Env conformations were more consistent in eliciting a neutralizing antibody response against NL43 as all rabbits in that particular group responded (5/5), whereas only 3/5 rabbits showed neutralizing antibody responses in the monomeric and dimeric groups. However, the low-level neutralization titers obtained against NL43 does not inspire much confidence in this correlation and would require additional testing of these rabbit sera against other susceptible viral strains (tier 1) to better evaluate the neutralizing antibody breadth. Indeed, our control $gp120_{BaL}$m Env immunization group exhibited the broadest response. This was not unexpected as the immunogen ($gp120_{BaL}$) was tested against its autologous pseudovirus (BaL) while the other two tier 1 pseudoviruses are also derived from subtype B viral isolates. Thus subtype B immunogens are more likely to elicit antibodies that target closely related subtype B pseudoviruses than the

founder virus subtype C consensus Env immunogen. Comparative assessment of neutralization potency (IC_{50}) between groups that received the different conformations revealed that the trimeric Envs $gp140_{FVC}$GCN4t(+) and $gp140_{FVC}$GCN4t(-) displayed an average IC_{50} neutralization titer against SF162 of 1125 and 2191, respectively. The dimeric $gp120_{FVC}$GCN4d Env conformation produced an average IC_{50} neutralization titer of 553. Two rabbits in the founder virus monomeric group ($gp120_{FVC}$m) exhibited significantly higher titers against the SF162 pseudovirus (>10 000 IC_{50}), which resulted in an average IC_{50} neutralization titer of 6356. Exclusion of these outliers resulted in greatly reduced average IC_{50} titer of 269 for the remaining 3 animals in the group (Figure 3.13B). More importantly, despite the induction of high neutralizing antibody titers against SF162 by these two outliers in the $gp120_{FVC}$m group, this did not correlate with an increase in neutralization breadth against tier 2 viruses, although low-levels of neutralizing titers were noted against BaL. In summary, these findings again highlight the limitation of HIV-1 Env based immunogens. While many of these Env-based immunogens are designed to better expose conserved bNAb epitopes, it is unfortunately not possible to accurately predict the immunogenic properties based on their antigenicity [299].

4.3.2. Breadth and potency of neutralizing response elicited by 2dCD4^{S60C}-liganded Env complexes

Undoubtedly the most striking result of this thesis was the extraordinarily broad and potent neutralizing response that was consistently induced following immunization of rabbits with 2dCD4^{S60C}-liganded Env complexes (Figure 3.13B). To the best of our knowledge, such a broad and potent neutralizing response using an Env-based immunogen has not been reported in the literature to date. Upon closer inspection of the neutralizing antibody data (Figure 3.13B) a number of critical observations were noted. These include the fact that every single rabbit that received the 2dCD4^{S60C}-liganded Env complex, regardless of Env subtype (subtype B – gp120BaL vs subtype C – founder virus consensus) or conformation (monomer, dimer or trimer), developed the equivalent neutralization breadth. It was noted however that the trimeric Env conformations on average, elicited a higher potency in neutralizing titer than compared to the monomeric and dimeric conformations (Figure 3.13B). Secondly, the neutralization titers obtained against the tier 2 and 3 pseudoviruses were higher than those obtained against the tier 1 viruses. Typically, high neutralization titers are associated with the relatively neutralization sensitive tier 1 pseudoviruses, and these IC_{50} neutralizing titers are reduced when challenged against the more resistant tier 2 and tier 3 pseudoviruses, as illustrated by the positive control sera (Z23). These data, in conjunction with findings published by other groups [227, 233, 294]

conducting similar immunizations studies using Env/CD4 complexes, prompted us to suspect that the unusual neutralizing breadth that we observed may be attributed to anti-CD4 activity.

Further analysis of rabbit sera 68 (immunized with $gp140_{FVC}$GCN4t(-)/2dCD4^{S60C}) confirmed the contribution of anti-CD4 responses with regard to its broadly neutralizing antibody activity. Sera 68 displayed neutralizing antibody activity against the evolutionary divergent HIV-2_{P2} and SIV_{mac316} viral strains whereas rabbit sera 53, that received the unliganded $gp140_{FVC}$GCN4t(-) Env only, did not (Figure 3.15A). Decker *et al.*, [130] reported the extraordinary degree of antigenic conservation of co-receptor binding of lentiviral lineages, as demonstrated by the ability of CD4i-directed antibody responses in neutralizing HIV-1, HIV-2 and SIV following priming with CD4. Our data suggests two possible scenarios. Firstly, if a CD4i-directed response was responsible for the neutralization breadth, then antibodies within the sera have either managed to overcome the high entropic barrier that prevents antibody access to the co-receptor binding site on the native trimer [128, 132, 300-302] or have managed to access the exposed co-receptor binding site following CD4 engagement amidst the sterically-restricted, viral-host cell synapse [225, 303]. The second scenario, suggests that neutralization of these viral strains occurs prior to the formation of the co-receptor and CD4i epitopes, through the direct targeting and blocking of the host CD4 cell receptor. This was indeed confirmed to be the case, as depletion of anti-CD4 specific responses from serum 68 abrogated the neutralization affect against the tier 2 pseudovirus ZM53M.PB12 (Figure 3.17), whereas specific depletion of anti-Env responses did not (Figure 3.16). Even though we did not analyze the rabbit sera for the induction of CD4i-directed response, which are presumably present, the fact that the neutralization response could be exclusively abolished following depletion with 2dCD4^{S60C}-coupled beads negates the role CD4i responses in the neutralization breadth within our study, or at least with regards to rabbit sera 68. A further possibility is that the $gp140_{FVC}$GCN4t(-)/2dCD^{S60C} complexes elicit a bivalent antibody, capable of binding epitopes on both Env and CD4 which were depleted in the abovementioned experiments.

Varadarajan *et al.,* [233] reported that immunization of guinea pigs using single-chain gp120-$CD4_{D12}$ (gp120(JRFL)2dCD4 expressed as a single polypeptide chain, separated by a flexible 20 amino acid linker sequence) elicited a broadly neutralizing response that was shown to be as a result of high anti-CD4 titers. As these authors did not include a CD4-only control immunization group and given our shared concern with regard to the immunogenicity studies, the variability in ELISA reagents and protocols, we are cautious to draw any definitive conclusions from comparative ELISA titer data. Our data demonstrate that elicitation of high anti-CD4 titers does not correlate with improved neutralizing breadth. Rabbits that were exclusively immunized with 2dCD4^{S60C}, did not develop as potent a bNAb response despite displaying significantly

higher anti-CD4 titers as compared to those that received the Env/2dCD4^{S60C} complexes that did develop bNAbs (Figure 3.13B and Figure 3.14B). This is further supported by the observation that the dimeric and trimeric Env/2dCD4^{S60C} complexes showed lower anti-CD4 binding than what was obtained for the monomeric Env/2dCD4^{S60C} complex and yet displayed more potent neutralizing antibody titers (Figure 3.13B and Figure 3.14B). These findings are supported by those of Celada *et al*., [304] whereby the authors immunized mice using an unpurified, complex mixture of CD4-IgG (domains 1 and 2, spliced to CH2 and CH3 domain of human IgG) and gp120. These authors reported that mice receiving the CD4-gp120 showed superior syncytia-blocking properties and markedly lower anti-CD4 titers when compared to the group that was immunized with CD4 alone [304]. In summary these findings indicate that CD4 binding titer does not necessarily correspond with the neutralizing breadth or the potency of the response and that the anti-CD4 response elicited by these Env/2dCD4^{S60C} complexes are highly efficacious in blocking CD4 mediated viral entry in the assay used.

The differences in anti-CD4 antibody titer between the 2dCD4^{S60C} only group and the Env/2dCD4^{S60C} complex groups could simply be a result of differences in 2dCD4^{S60C} amounts present in the immunogen formulation. Considering the immunizations were carried out using 20 μg of total protein per immunization, and assuming every Env CD4bs is functional and occupied by CD4, its estimated that each rabbit received approximately 7 fold less 2dCD4^{S60C} per immunization in the groups that were immunized with the Env/2dCD4^{S60C} complex versus the rabbits that were exclusively immunized with 2dCD4^{S60C} (control group). Celada *et al*., [304] suggested that binding of the larger gp120 protein to its relatively small CD4 ligand may occlude the CD4 epitopes and prevent the interaction with B cell surface immunoglobulins, a term these authors referred to as “blanketing.” While this hypothesis may not necessarily correlate with the differences in CD4-binding titers observed between the different Env conformations used to generate the Env/2dCD4^{S60C} complex, it may offer some insight into the difference in neutralizing antibody potency developed. Structural studies of core gp120 in complex with 2dCD4 [128, 129] indicate that even in the absence of N-linked glycans and shortened variable loop structures, the gp120 molecule engulfs a large portion of domain 1 on CD4, limiting the exposure of this domain compared to that of domain 2. Thus, we hypothesize that exposure of CD4 domain 1 epitopes is limited as compared to domain 2 in respect of a formed gp120-CD4 complex. However, in the monomeric conformation a large portion of the gp120 molecule would still be exposed, including the inner domain which is thought to be occluded on the functional trimeric Env spike. In contrast, the proposed orientation of a trimeric Env/2dCD4 purified complex would resemble a central Env trimeric core structure with bound 2dCD4 molecules radiating outwards like spokes on a wheel [145]. This central Env axis would reduce the surface

exposure of gp120 epitopes present in the complex and may promote immunological-focusing on the exposed CD4 molecules, most likely on the C-terminal end of domain 1 or the complete domain 2. While there is no doubt that the trimeric Env/2dCD4^{S60C} complexes elicit anti-Env titers in rabbits (Figure 3.14A), the trimeric Env conformations may focus the rabbit humoral response to the exposed CD4 portion of the complex, thereby inducing higher specificity antibodies which translate into improved neutralizing potency in virological assays.

Fouts *et al.,* [227] also demonstrated that chemically cross-linked complexes comprising gp120 or gp140 and sCD4 (soluble, 4 domain CD4) elicited an antibody response that was capable of neutralizing a wide range of primary viral isolates following immunization of rhesus macaques. In addition, these chemically cross-linked Env/CD4 complexes were shown to induce an anti-CD4 response, which showed strong immunoreactivity with the third and fourth domains of sCD4 [227]. Interestingly, these authors also report that the anti-Env/CD4 complex sera showed a reduced neutralizing effect of TCLA viral strains (tier 1) than compared to primary viral isolates [227]. Our complexes were generated using 2dCD4, therefore the subsequent anti-CD4 response induced can only be directed towards the first or second domains of CD4. Interestingly, our data also exhibited this inverse relationship between neutralization titer and pseudovirus tier categorization as referred to by Fouts *et al.*, [227]. The tiered categorization of pseudoviruses provides a measure of neutralizing sensitivity to HIV-1 directed humoral responses [254]. The highly sensitive tier 1 viruses arose from the frequent passaging of HIV-1 isolates through T cell lines (T cell line adapted) that ultimately resulted in high sensitivity to antibody-mediated neutralization [305]. The majority of circulating, contemporary primary viral isolates fall in the tier 2 category and exhibit reduced neutralizing sensitivity to HIV-1 positive patient plasma pools. The difference in neutralization sensitivity displayed between the different tiers is thought to be a result of increased exposure of neutralizing epitopes on Env. The increase in neutralizing epitope exposure correlates with an increase exposure of motifs required for binding and fusion (CD4 and co-receptor binding sites) [272, 306]. Thus the more flexible and open conformation of Env associated with tier 1 viruses translates into a more CD4-independent phenotype than compared to tier 2 or 3 category viruses. In other words, engagement of the CD4 receptor is far more obligatory for the tier 2 and 3 category viruses in order to mediate the subsequent conformational changes in Env essential for co-receptor binding, membrane fusion and virus entry. Thus the requirement for CD4-mediated viral entry may differ amongst the different tiered pseudoviruses, resulting in the observed difference in neutralizing potency of the CD4-directed response.

4.4. Future vaccine potential of Env/2dCD4^{S60C} complexes

It is becoming increasingly evident that despite recent advances in the design and development of Env-based vaccine immunogens, these immunogens have failed to elicit bNAb responses required to protect against circulating primary viral isolates. Although bNAb epitopes are present and exposed on such immunogens, they appear to be poorly immunogenic in the context of vaccination [120, 169]. This has been attributed to the fact that current Env-based immunogens are unable to stimulate the clonal lineages of many of these bNAbs and that current immunization regimes are unable to drive the affinity maturation required to develop the neutralizing breadth [162-164]. Thus, there has been renewed interest in the development of alternative Env-based subunits immunogens that may be better over the current roadblocks and chart a clear path to the development of a safe and effective HIV-1 vaccine. The strategy described in this thesis presents an alternative method for eliciting potent broadly neutralizing antibody titers against HIV-1 requiring a relatively modest immunization regime.

While the immediate response to a vaccine-induced anti-CD4 response would naturally raise safety concerns of potential autoimmune reactions, we explored the potential of such a strategy using information gathered from the literature. Other groups involved with the development of Env/CD4 based immunogens and exposure of the CD4i epitopes have expressed concern with regard to eliciting autoimmune responses. The generation of CD4 mimetics may present an alternative option to stabilizing the gp120/CD4 conformation without inducing significant CD4 responses [231, 232]. However, the extraordinary neutralization breadth and potency observed in the sera of our immunized rabbits compelled us to examine whether such a response could be replicated in a NHP or human model. Firstly, we considered the possibility of inducing a CD4-directed response in these model systems. In the early 1990's, Watanabe *et al.,* [307-309] explored the potential of sCD4 as an immunogen in NHP models. The authors reported the successful elicitation of anti-CD4 responses following immunization of rhesus macaques with a rhesus derived CD4 immunogen. Additionally, sera obtain from these animals was shown to inhibit SIV_{mac} infection of rhesus monkey PBMC's *in vitro*. These results indicate that a "self-recognizing" antibody response can be elicited by immunization in such models without any apparent reported deleterious effects.

Secondly, we considered the potential safety concerns of a CD4-directed immune response. A CD4-directed antibody may bind to the surface CD4 receptor, disrupting the normal MHC signaling, or these bound antibodies may elicit antibody-dependent cell mediated cytotoxicity (ADCC) type responses that could potentially lead to the lethal targeting of these immune cells and overall immune dysfunction. Despite these concerns, a humanized anti-CD4 (domain 2)

specific antibody (ibalizumab) has shown excellent anti-HIV-1 activity and safety data in phase 1a, 1b, 2a and 2b human clinical trial studies [310-312]. Ibalizumab blocks HIV-1 entry by targeting human CD4 and has shown extensive neutralization breadth and potency, neutralizing 92% of pseudoviruses tested and requiring significantly lower antibody concentrations than other bNAbs PG9 or VRC01 [163, 164, 313]. The impact of ibalizumab binding on MHC class II binding is currently under investigation, however structural studies have shown that this antibody interacts opposite to the site of gp120 and MHC class II binding [314, 315]. Taking all the above into consideration, these data suggest that elicitation of the "right type" of anti-CD4 directed antibody response (such as ibalizumab-type antibodies) can be accomplished in the NHP and human models, offering significant protection from HIV-1 acquisition. Thus, the Env/2dCD4^{S60C} complex immunogens described here represent a suitable candidate for future exploration.

4.5. Concluding remarks and future directions

This thesis assessed the ability of HIV-1 subtype C founder virus consensus Env glycoproteins (monomeric, dimeric and trimeric conformations) alone or bound to 2dCD4^{S60C}, to elicit a broadly neutralizing antibody response against HIV-1 within a small animal model. While the founder virus Env only conformations did not elicit a broadly neutralizing response, the biochemical data presented here, indicate that each Env conformation was functional and antigenically correct. Important future research on the founder virus consensus Envs and conformations thereof will include assessing different host-cell derived glycosylation patterns and the potential effects on binding kinetics to currently available bNAbs (PG9, PG16, PGT family, VRC01, etc). Most relevant to the current research thrust in the HIV-1 vaccine field is to establish whether this founder virus consensus Env is able to bind the germ line or unmutated common ancestor of these bNAbs, as this may provide a starting point for modern vaccine strategies aimed at driving antibody affinity-maturation.

The Env/2dCD4^{S60C} complexes did indeed elicit a potent and broadly neutralizing response in rabbits. Principally our future work will focus on developing monoclonal antibodies that will map these responses and accurately determine epitopes that constitute this neutralizing activity. In addition, these mapping experiments may uncover novel drug targets on CD4 that can be exploited by pharmaceutical interventions. Central to developing these Env/2dCD^{S60C} complexes as a vaccine strategy will be to replicate our findings in the more appropriate NHP model. Additionally, repeating our experiments with more frequent immunizations in NHP, to mimic the constant antigenic challenge faced during natural HIV-1 infection may elucidate

whether the appropriate affinity maturation to generate broadly neutralizing antibodies can be achieved. The NHP model affords the opportunity for viral challenge and will be crucial in determining the potential of an anti-CD4 directed response as a preventative HIV-1 vaccine strategy.

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Appendices

Appendix A

5’GCCCCAGTGTGGATGCTGTTGCCAAGACTGCAAACCACTGGCTCGTTTCCGTGCCCAAATCCAAGGC
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CTCACCATTATTTGTCATGGAGGCAGACATACACCAGAAATGGGGGAGAAACAGTACATATCTTTCTG
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ATGCATCTGCCTGGCATTTAGGCAGCAGAGCCCCTGACCGTCCCCCACAGGGCTCTGCCTCACGTCCTC
ATCTCATTTGGC 3’

Figure A-1: The first 1509 nucleotide sequence derived from 3’UTR of thioredoxin reductase 1 (TrxR1) (*Homo sapiens*) used to incorporate selenocysteine at the penultimate amino acid position in the gp120$_{FVC}$Ud construct. The sequence was obtained from SelenoDB [316]. The selenocysteine insertion sequence (SECIS) element is shown in red.

Appendix B

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2010/12/03

APPLICANT: Dr MA Papathanasopoulos

SCHOOL: Molecular Medicine
DEPARTMENT:
LOCATION:

PROJECT TITLE: **Immunisation of the New Zealand white rabbit with novel HIV-1 envelop glycoprotein immune complexes and the evaluation of anti HIV humoral immune responses**

Number and Species

30 M, Rabbits

Approval was given for the use of animals for the project described above at an AESC meeting held on 23.02.2010. This approval remains valid until 23.02.2012

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

The application was approved subject to details being supplied for all persons involved in the study, specifically those for the person involved with sample collection and animal monitoring..

Signed: _____ (Chairperson, AESC) Date: 09/03/2010

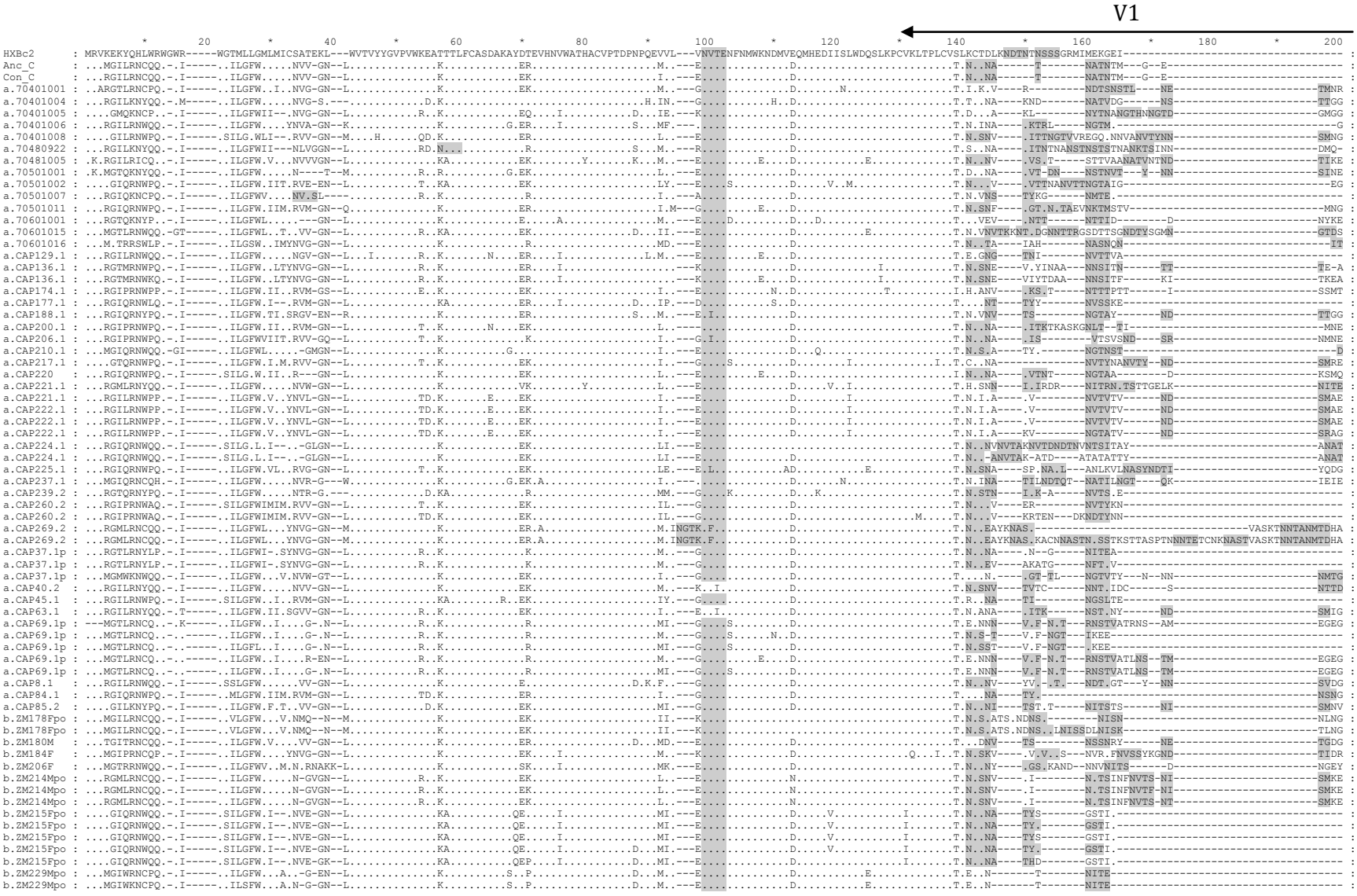
I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

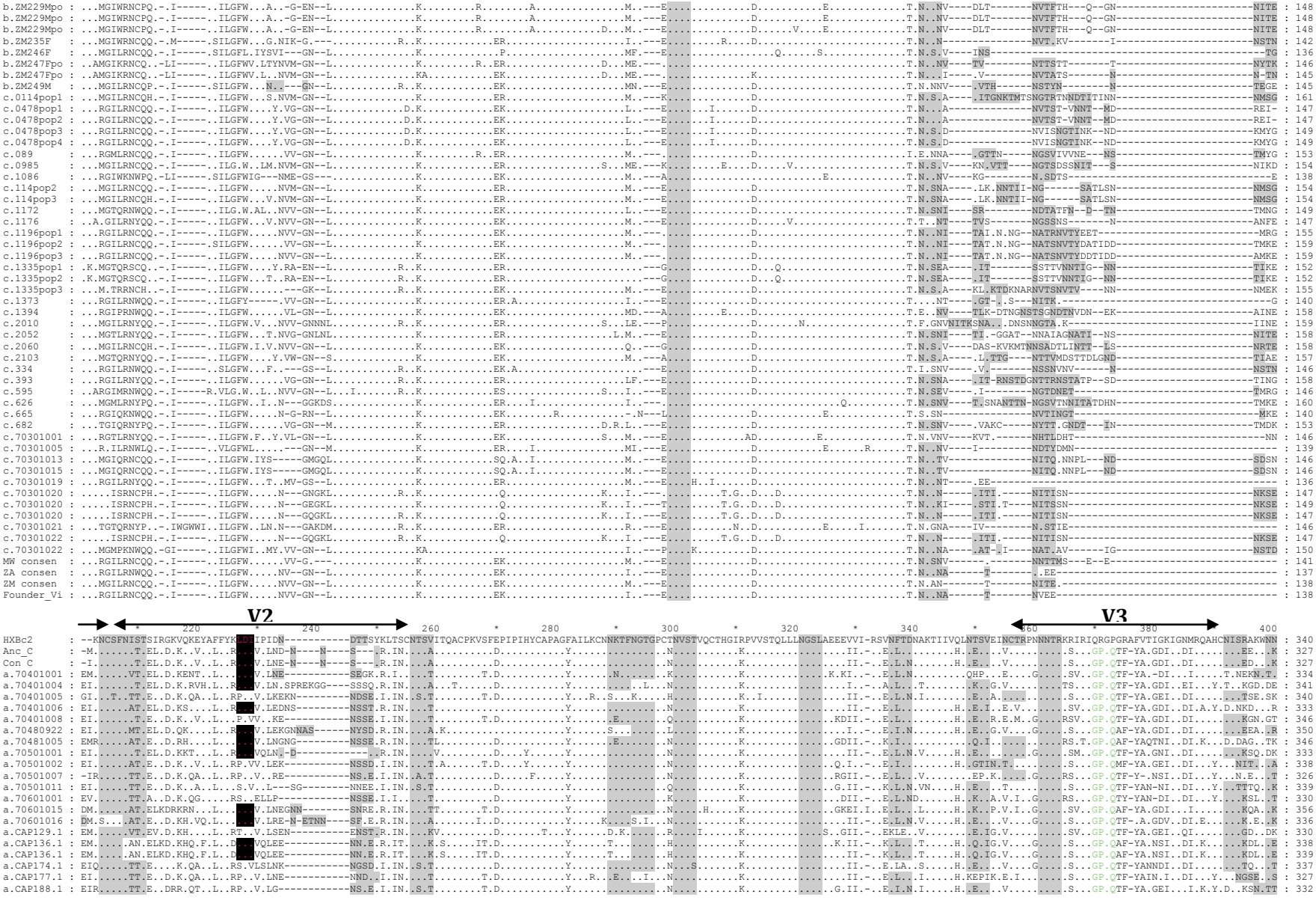
Signed: _____ (Registered Veterinarian) Date: 09/03/2010

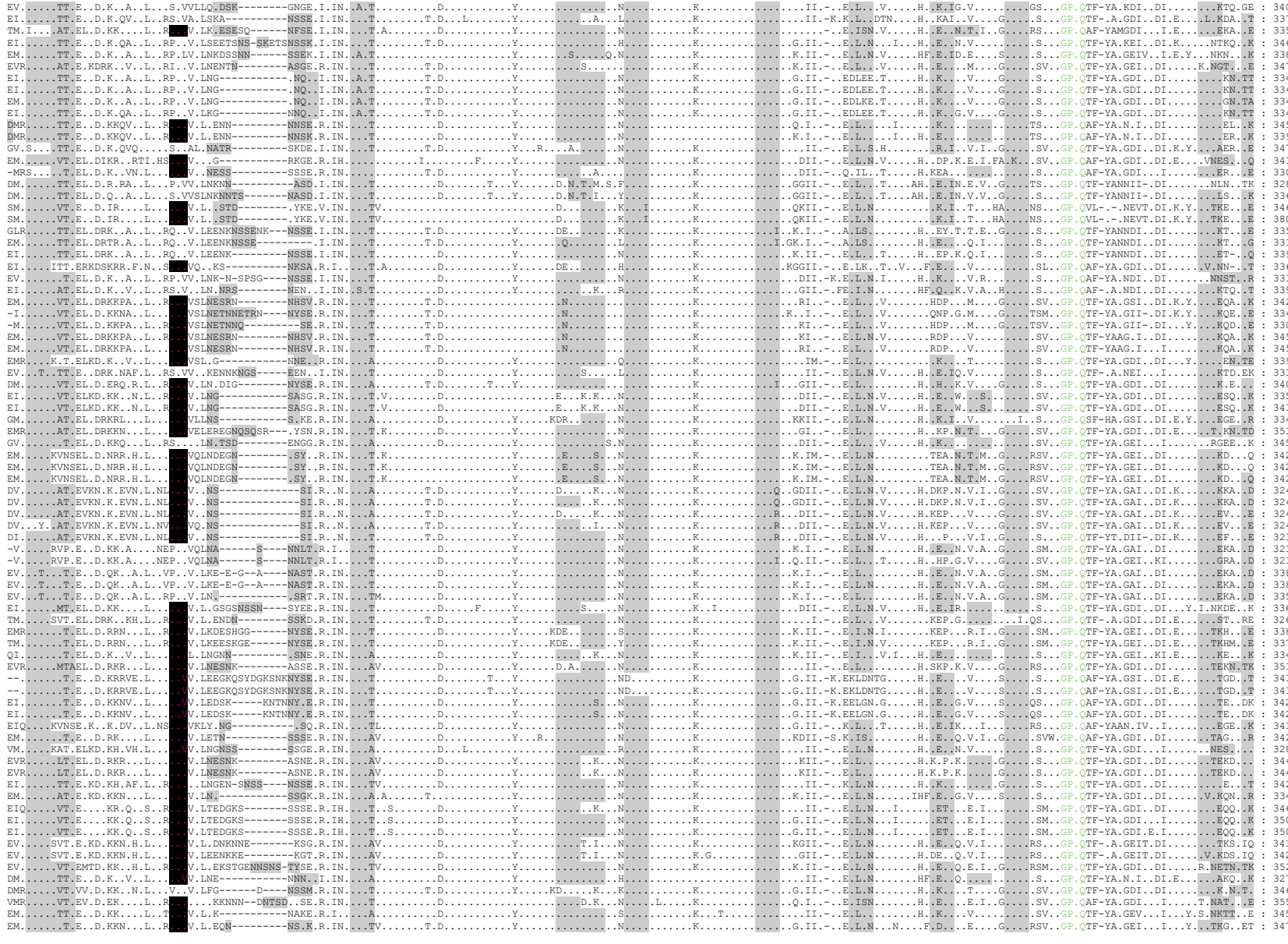
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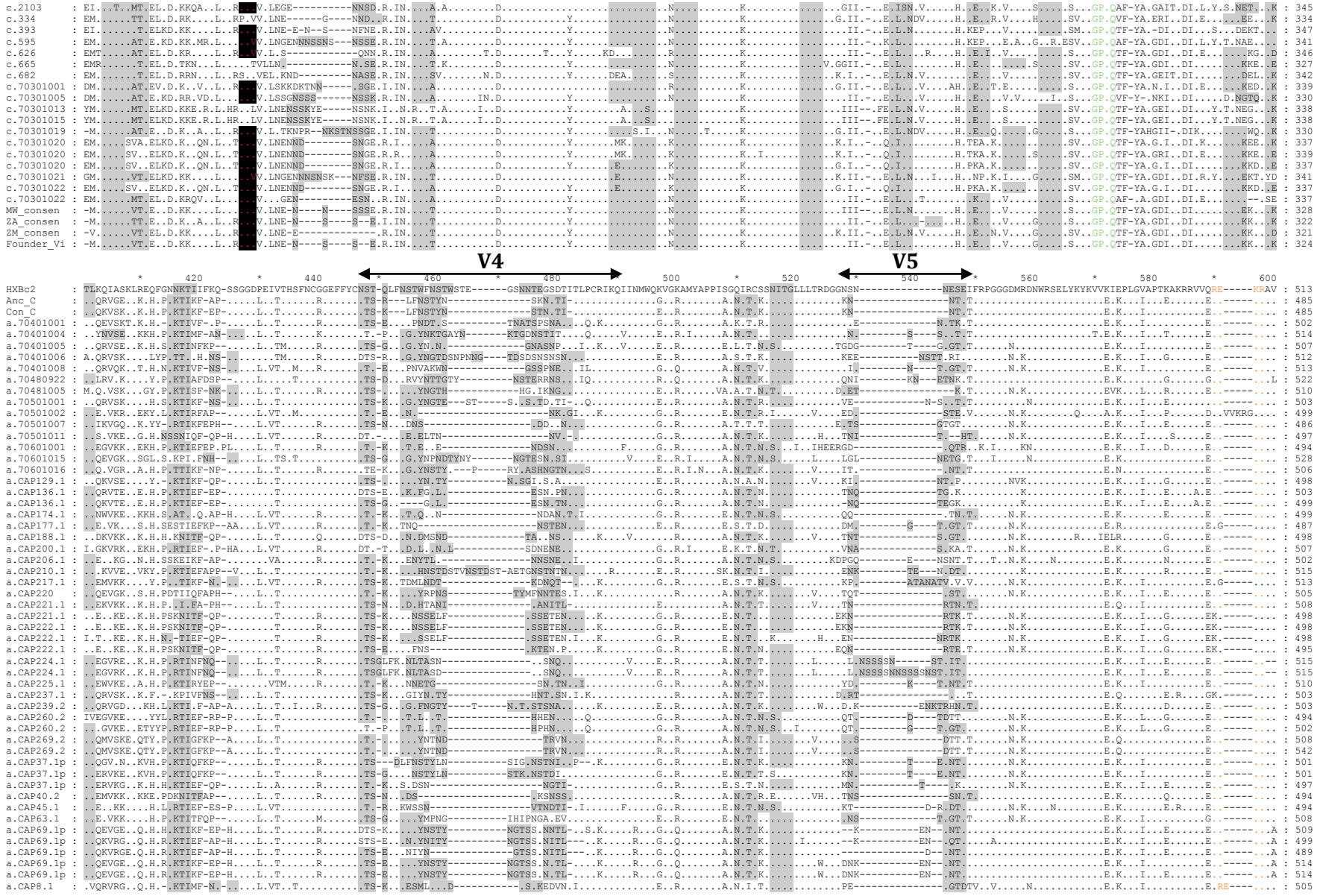
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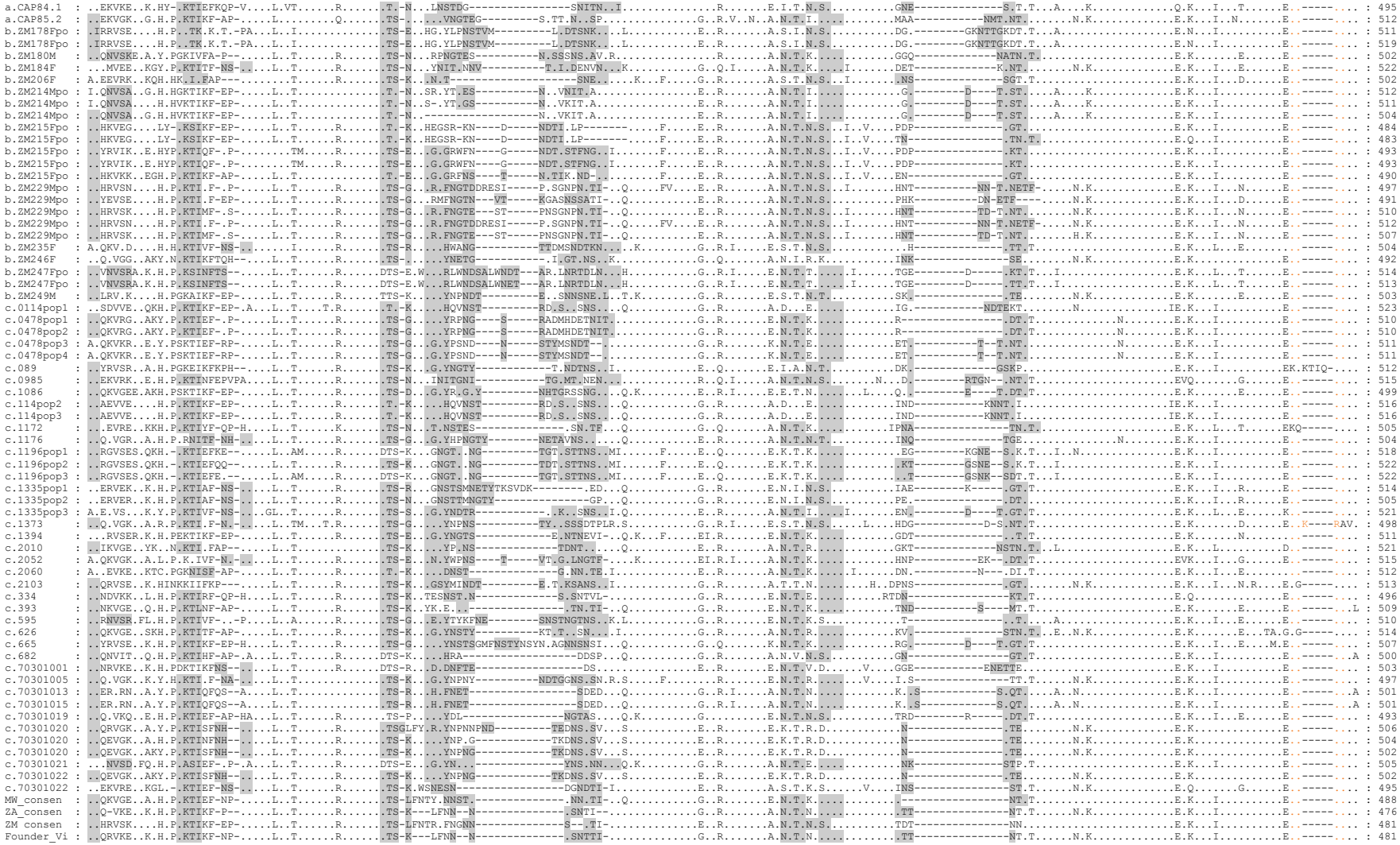
Appendix C

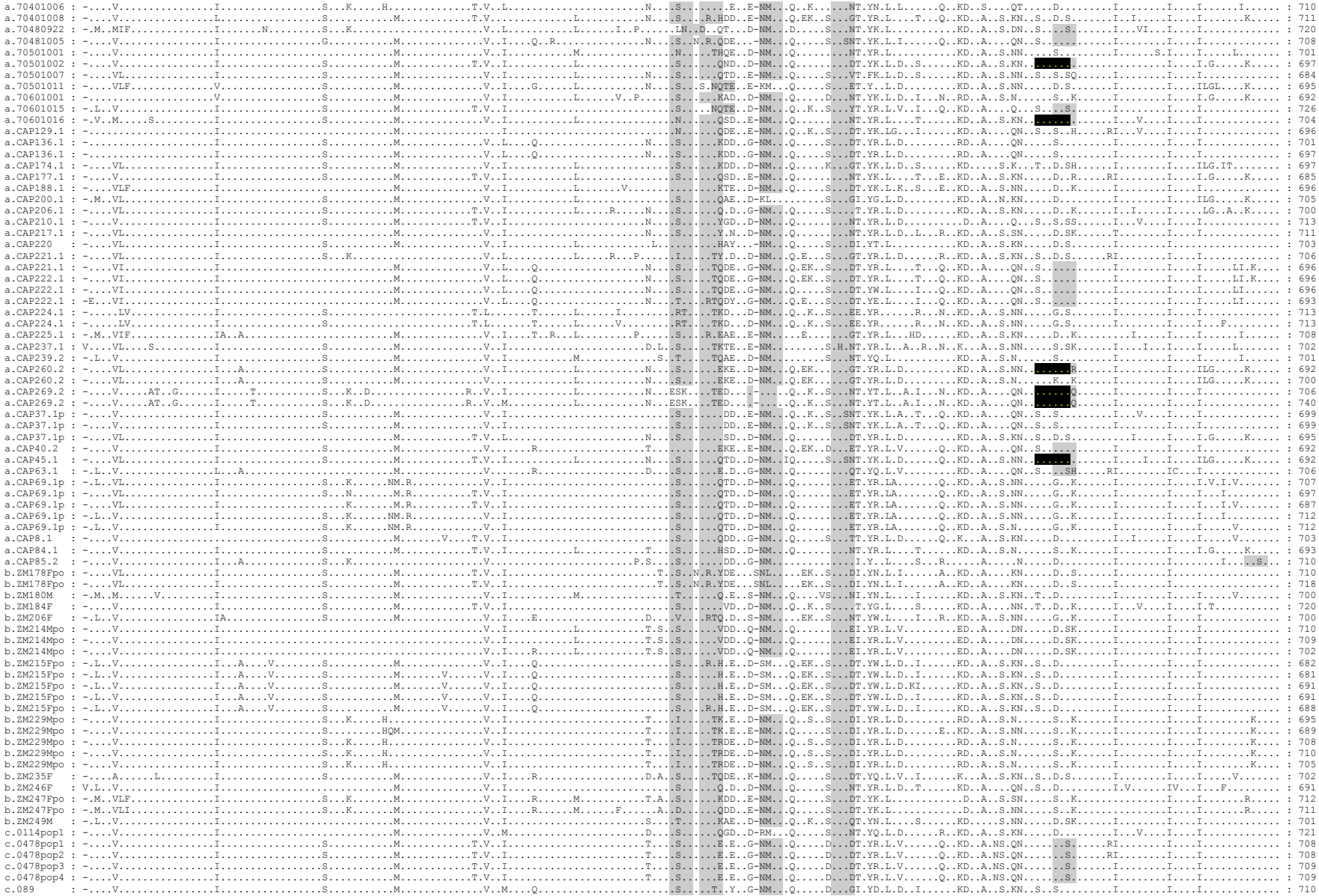


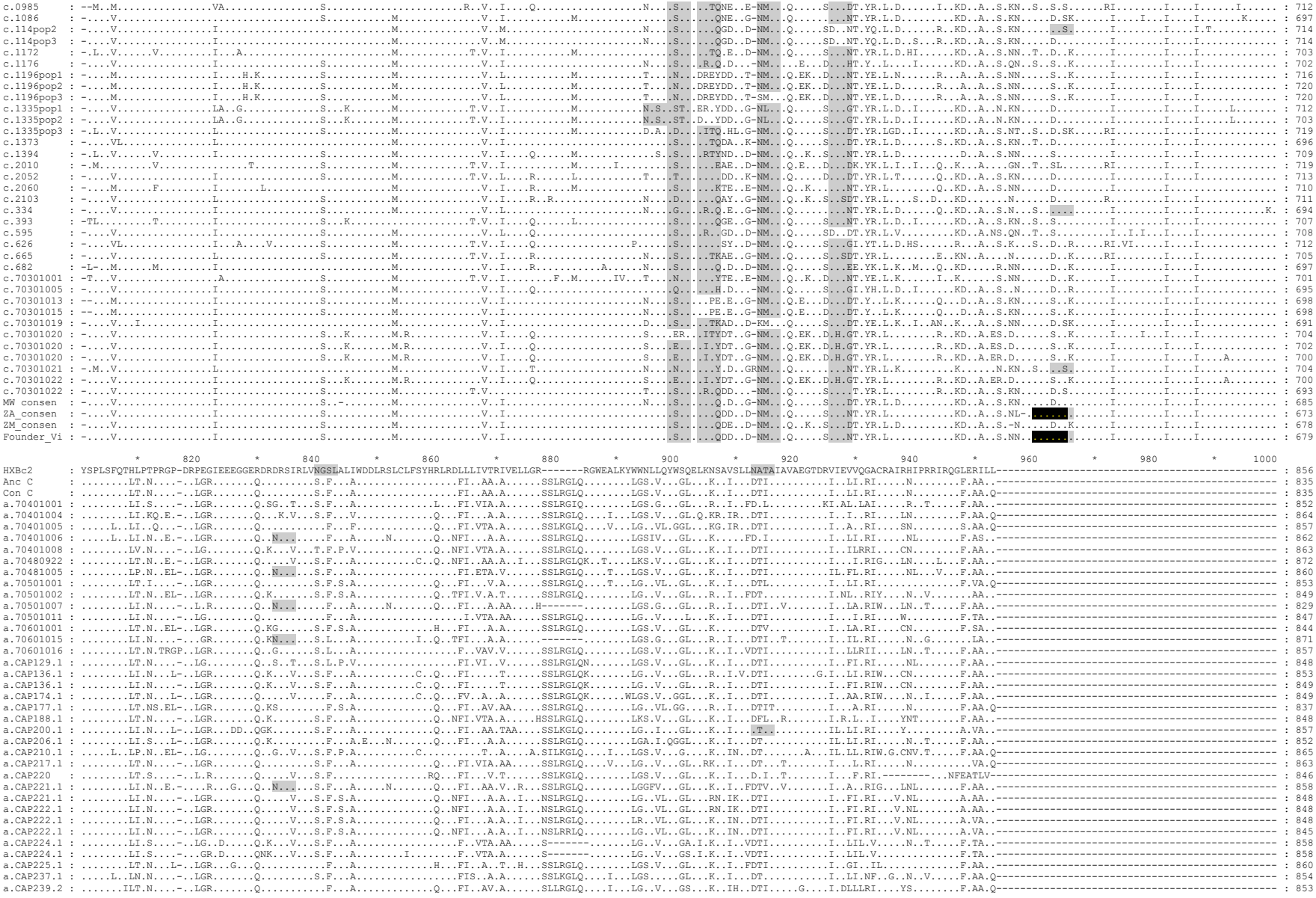

V2
V3

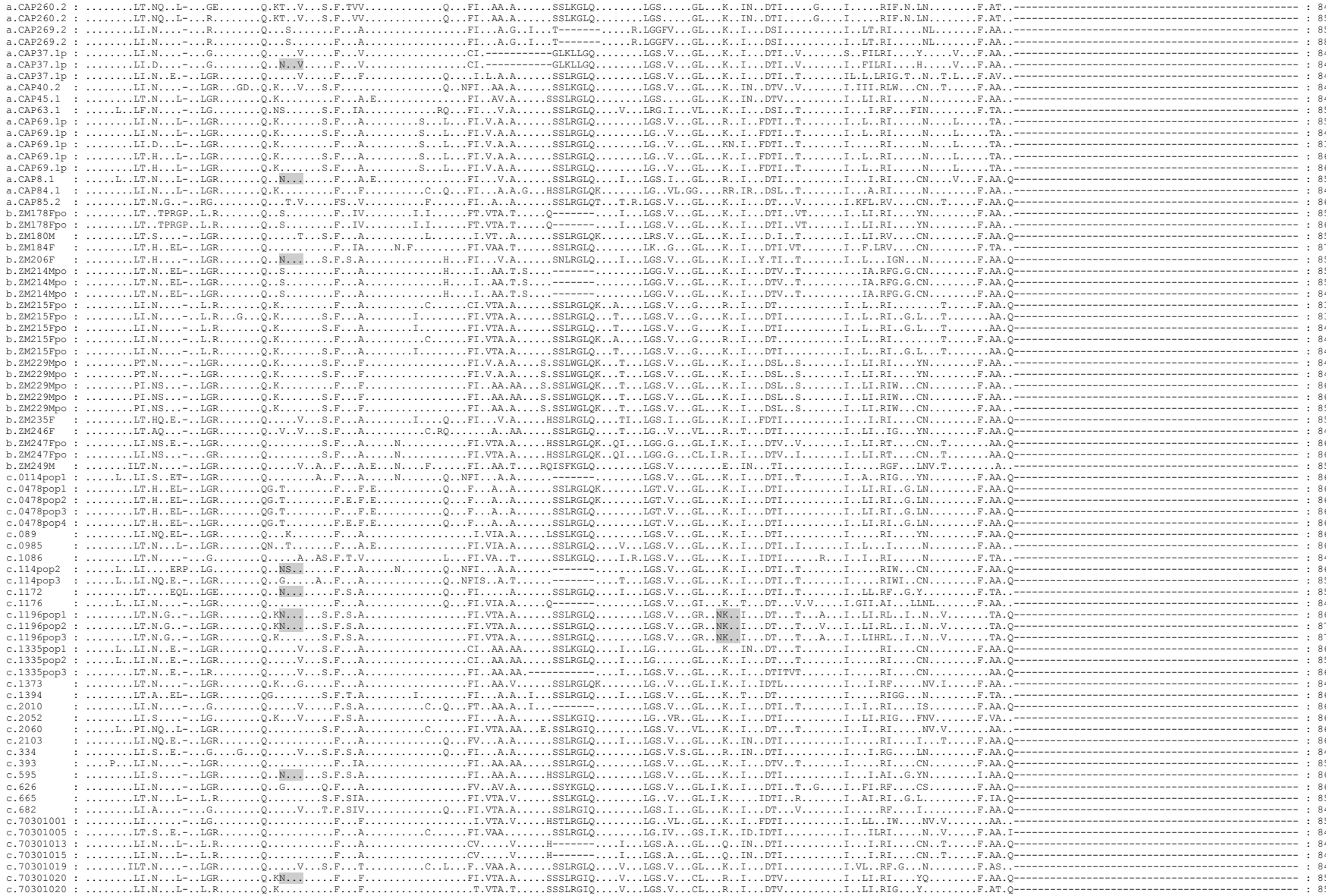



V4
V5









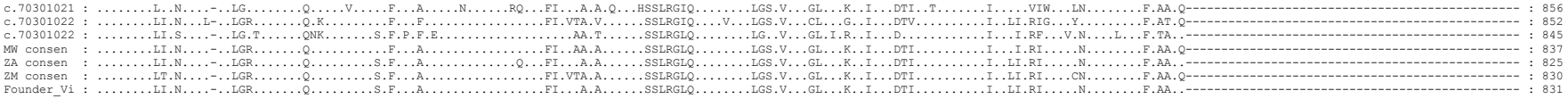


Figure C-1: Alignment of the env glycoprotein (Env) amino acid sequences derived from 80 HIV-1 subtype C acutely infected individuals from Malawi, South Africa and Zambia. Geographical consensus sequences are shown for each country, Malawi (MW_consen), South Africa (ZA_consen), Zambia (ZM_consen) and used to infer a subtype C founder virus Env consensus (Founder_Vi) sequence. Sequences were aligned to the ancestral (Anc_C), consensus (Con_C) and HXBc2 reference sequences for comparative and orientation purposes. Refer to Figure 3.1 for the description of the relevant shaded/highlighted functional sites.

Appendix D



R14/49 Mr Mark Killick

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130386

NAME: (Principal Investigator)	Mr Mark Killick
DEPARTMENT:	Molecular Medicine & Haematology Medical School
PROJECT TITLE:	founder Virus Envelope Glycoproteins as Novel Oligomeric HIV-1 Vaccine Immunogens
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof M Papathanasopoulos
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 08/04/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES