HIV-1 Subtype B and C Envelope glycoprotein based immunogens as preventative vaccines

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DECLARATION

I, Michelle Lilly Grant declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

Michelle Grant

_____day of _____2017 in _____

To my husband James,

and my children: Kate, Christopher and Nina,

you make every day beautiful.

"As many times as [HIV] changes its clothes, it's still wearing the same socks, and now our job is to make sure we get the body to really hate those socks"

~ Seth Berkley ~

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Publications:

Killick, M.A., Grant, M.L., Cerutti, N.M., Capovilla, A., Papathanasopoulos, M.A., 2015. Env-2dCD4(S60C) complexes act as super immunogens and elicit potent, broadly neutralizing antibodies against clinically relevant human immunodeficiency virus type 1 (HIV-1). *Vaccine* 33, 6298-6306.

Conference presentations:

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.

ABSTRACT

The ability to induce a potent and broadly neutralizing antibody (bNAb) response following vaccination is critical in developing an effective HIV-1 vaccine. To date, no HIV-1 envelope glycoprotein (Env) immunogens have elicited bNAbs in preclinical or human clinical trials. This study compared the antigenicity and immunogenicity of a panel of HIV-1 subtype B and C Env-based immunogens in various immunization regimens in a small animal model. The Env-based immunogens used included matched monomeric (gp120) and trimeric (gp140GCN4(+)) conformations of 6 subtype C Env's (IN26191, IN25710, IN25925, ZACAP45, ZACAP210 and ZA706010164; all designed during the course of this study), and subtype B stabilized Env derivatives Cyc4OD gp120 cyclic permutant, gp140 cyclic permutant h-CMP V1cyc 144-142, OD_{EC}COBPICS gp120 fragment, VRC01 engrafted scaffold peptides 1WR2 and 1ORC, and DNA (Wt-JRFL-Env, JRFL Env-570D and JRLF Env-SEKS; all obtained from collaborators).

The six HIV-1 subtype C Env sequences (IN26191, IN25710, IN25925, ZACAP45, ZACAP210 and ZA706010164) were selected, matched gp120 and gp140GCN4(+) constructs were designed, codon optimized and cloned into a mammalian expression vector. All 12 *env* constructs were expressed in HEK293T or 293FS cell lines, and the Env purified by lectin affinity chromatography, followed by size exclusion chromatography. Additionally, two domain soluble CD4 (2dCD4) wildtype, folding defective and an S60C mutant were expressed in a bacterial system and purified by nickel affinity chromatography. 2dCD4^{S60C}-liganded Env was prepared, and purified further, as required. The antigenicity of all 12 Env's was evaluated against 2dCD4 and a panel of bNAbs using surface plasmon resonance (SPR). The immunogenicity of two of these subtype C Env variants (liganded and unliganded to 2dCD4^{S60C}) were subsequently compared to that of the subtype B immunogens in rabbits, using various prime-boost regimens. Rabbit sera were subsequently tested for anti-Env binding antibody titres by enzyme linked immunosorbent assay (ELISA), and neutralization by an *in*

vitro phenotypic neutralization assay against a panel of HIV-1 pseudoviruses.

All 12 recombinant Envs were successfully expressed and purified to homogeneity. Binding of all the gp120/gp140GCN4(+) Env variants to 2dCD4 variants (Wt and S60C) confirmed that they were all functional and conformationally intact with an accessible CD4 binding site (CD4bs). Binding to a panel of CD4bs directed bNAbs (IgG1b12, VRC01, HJ16, VRC-CH31, NIH45-46^{G54W}, VRC03) revealed that overall, the trimeric gp140GCN4(+) variants showed higher binding affinities to these bNAbs compared to the matched monomeric gp120, attributed to their resemblance to the native trimer on the viral surface. With the exception of VRC03 and IgG1b12, the Indian Env variants bound with an approximately 10-fold higher affinity compared to the South African Env's. Overall, nine different immunization regimens were performed. Immunization of rabbits induced high titres of antibodies (Abs) for all the immunogens tested, as determined by ELISA, however, minimal neutralization breadth (against Tier 1 pseudoviruses) was obtained for the Env-only variants for subtype B and C immunogens. Of these, the VRC01 engrafted scaffold peptide (1ORC) showed improved neutralization of the Tier 1 pseudovirus SF162 compared to the other Env only based immunogens. The only promising neutralization results were obtained from rabbits immunized with the Env/2dCD4^{S60C} liganded immunogens that potently neutralized both subtype B and C, Tier 1, 2 and 3 pseudoviruses. This response was improved for the trimeric Env/2dCD4^{S60C} complexes compared to the monomeric ones and was consistently elicited regardless of the Env sequence used. The neutralization response is likely either due to Abs targeting one or more epitopes on 2dCD4 or Env or both. Therefore, the use of CD4 liganded Env immunogens in vaccine design should be investigated further as they provide a promising "Ibalizumab-like" neutralization response. Overall, based on emerging evidence on how the bNAb responses evolve in HIV-1 infected individuals, the findings in this study are promising and lay the

groundwork for further testing of these HIV-1 Env based immunogens in various combinations using sequential prime-boost strategies to optimally drive affinity maturation of the appropriate B cell lineages.

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ABBREVIATIONS

Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immune deficiency syndrome
ART	antiretroviral therapy
ARV	antiretroviral
BCR	B cell receptor
BGH	bovine growth hormone
bNAbs	broadly neutralizing antibody/ies
BSA	Bovine Serum Albumin
CAS	Central Animal Service (University of the Witwatersrand, Johannesburg, South Africa)
CD4+ T cell	human CD4 positive T lymphocytes
CD4bs	CD4 binding site
CD4i	CD4 induced binding site
cDNA	complementary deoxyribose nucleic acid
CDR	complementarity determining region
CDR H3	third complementarity determining region of the heavy chain variable regions of human antibody
CDRH2	heavy chain complementarity-determining region 2
Complete DMEM	Dulbecco's modified Eagles Medium supplemented with 10% fetal calf serum and 2 mM GlutaMax
CT	cytoplasmic tail
CTL's	cytotoxic T lymphocytes
DEAE Dextran	diethylaminoethyl-dextran hydrochloride
DMEM	Dulbecco's modified Eagles Medium
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
env	HIV-1 envelope glycoprotein gene
Env	HIV-1 envelope glycoprotein

FCS	Fetal calf serum
FPLC	fast protein liquid chromatography
HAART	highly active antiretroviral therapy
HEK	human embryonic kidney
HIV-1	human immunodeficiency virus, type 1
HIV-2	human immunodeficiency virus, type 2
ID_{50}	The reciprocal of the dilution of serum required to achieve 50% (1/dilution) inhibition of viral infection
IN	integrase
ITC	isothermal titration calorimetry
kDa	kilo Daltons
LB	Luria Bertani
LTNP	long term non-progressor
mABs	monoclonal antibody/ies
MAC	membrane attack complex
MBL	mannose binding lectin
MMP	methyl α-D-mannopyranoside
MPER	membrane-proximal external region of gp41
mRNA	messenger RNA
NAbs	neutralizing antibody/ies
NaCl	sodium chloride
Nef	negative factor
NHP	non-human primate
NK cells	natural killer cells
PBS	phosphate buffered saline (Dulbecco's)
PEP	post-exposure prophylaxis
PIC	preintegration complex
PNG	potential N-glycosylation
PNLG	potential N-linked glycosylation site
PR	protease
PrEP	pre-exposure prophylaxis
Rev	regulator of expression of virion proteins

RT	reverse transcriptase
RU	response units
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SIV	Simian immunodeficiency virus
SPR	Surface Plasmon Resonance
Tat	transactivator of transcription
$TCID_{50}$	50% Tissue Culture Infectious Dose
Th1	T helper 1 cells
TM	transmembrane
TMB	3,3',5,5'-tetramethylbenzidine
T-TBS	Tris-buffered saline containing Tween-20
UCA	Unmutated common ancestor

AMINO ACID ABBREVIATIONS

Amino acid	3-letter abbreviation	1-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter 1 : Introduction

1.1 The HIV-1 pandemic continues to be a major health challenge

By the end of 2015, approximately 36.7 million people were living with Human Immunodeficiency Virus-type 1, (HIV-1) globally and approximately 2.1 million of these individuals became newly infected during 2015. Thus HIV/AIDS (acquired immune deficiency syndrome) remains one of the world's major health challenges UNAIDS (2016). Of these newly acquired HIV-1 infections, sub-Saharan Africa remains the most heavily affected region, accounting for almost 70 % of new HIV-1 infections (World Health Organization, 2016).

Antiretroviral therapy (ART) has had a positive effect on the lives of HIV-1 infected people and has a host of benefits including: suppression of viral replication in HIV-1 infected individuals to such an extent that they are able to maintain a healthy immune system; prevention (reduced risk of 95 %) of sexual transmission of the virus to a non-infected partner if taken by the HIV-1 positive partner; prevention of HIV-1 acquisition by an HIV-1 negative person who uses ART as pre-exposure prophylaxis (PrEP); use of ART as post-exposure prophylaxis (PEP) to prevent HIV-1 acquisition in cases of accidental exposure; and prevention of mother-to-child transmission if ART is used during pregnancy (Sued et al., 2016; World Health Organization, 2016).

HIV-1 prevention strategies such as condom use, voluntary medical male circumcision, the antiretroviral (ARV) drug based prevention strategies mentioned above, and the use of sterile needle exchange programmes for injecting drug users have been widely publicised in education programmes (Sued et al., 2016). The implementation of these prevention and treatment strategies has had some success, as indicated by the drop in global HIV-1 infections by 35 %, and the decrease in AIDS-related deaths by 28 % since 2000. Although ART has played a major role in the decline on the morbidity

and mortality associated with HIV-1 infection, it is not curative and as at June 2016, only half of people living with HIV-1 were receiving ART worldwide. Access to treatment by individuals in low- and middle-income countries represent the majority of the treatment gap and this issue remains high on the agenda towards obtaining the goal of ending the AIDS epidemic by 2030 (UNAIDS, 2016). As a step towards achieving this goal, the UNAIDS “90-90-90” programme set 2020 as the target year to achieve the following goals (i) successfully diagnose 90 % of people living with HIV, (ii) administer antiretroviral treatment to 90 % of the HIV-1 infected individuals diagnosed, (iii) attain viral suppression in 90 % of the treated individuals (Corey and Gray, 2017).

Despite the positive statistics mentioned above, 1.1 million people died of AIDS-related illnesses globally in 2015 (UNAIDS, 2016). Successfully diagnosing 90 % of HIV-1 infected individuals, especially during their high-risk transmission period of infection, in a country such as South Africa that has a high burden of disease is proving to be logistically difficult (Corey and Gray, 2017). Preventing HIV-1 infection is key in the fight against the HIV/AIDS pandemic, and historically viruses have been most effectively controlled by vaccination (Agwale et al., 2011). A prophylactic HIV-1 vaccine would have a major impact on the transmission rates of the virus and help control the HIV/AIDS pandemic (Esparza, 2013). Corey and Gray (2017) suggest that an HIV-1 vaccine with a partial efficacy of 50 % could avert over 17 million new infections globally. Thus, a vaccine in combination with the 90-90-90 programme could substantially reduce HIV-1 incidence. However, despite intensive research on vaccine development over the past 30 years, an HIV-1 prophylactic vaccine remains elusive (Esparza, 2013).

1.2 HIV-1 biology and life cycle

1.2.1 HIV-1 Structure

HIV-1 is a lentivirus belonging to the family *Retroviridae*. It is enveloped by a lipid bilayer derived from the host cell which contains host cell proteins

such as major histocompatibility antigens, actin and ubiquitin (Figure 1.1). The inner surface of the host lipid membrane is lined with viral matrix p17 protein. A conical capsid core at the centre of the virus, composed of p24 viral protein, contains two copies of positive, single-stranded RNA molecules stabilized by the nucleocapsid protein, as well as three virally encoded enzymes ((protease (PR), reverse transcriptase (RT) and integrase (IN)). The RNA contains approximately 9,700 basepairs and encodes for nine genes (*gag, pol, env, tat, rev, nef, vif, vpr* and *vpu*) (Frankel and Young, 1998; Goto et al., 1998; Turner and Summers, 1999).

Envelope glycoprotein (Env) viral spikes are projected randomly on the surface of the host derived lipid membrane and these are composed of three non-covalently associated exterior envelope glycoproteins of approximately 120 kDa in size (gp120), which are each non-covalently associated with a transmembrane envelope glycoprotein of approximately 41 kDa in size (gp41), which anchors them into the membrane (Figure 1.1).

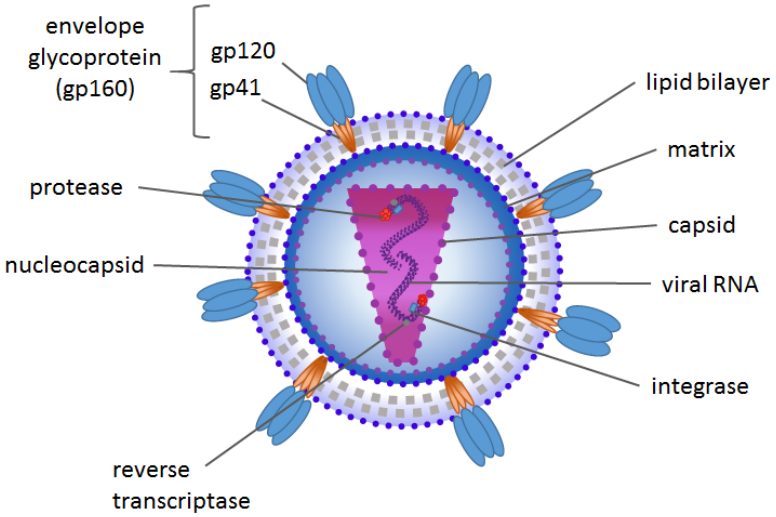


Figure 1.1: Schematic representation of the HIV-1 virion. Virus envelope glycoproteins (gp120) exposed on the outside of the virus are anchored to the virus by a transmembrane protein gp41. A conical capsid core at the centre of the virus contains two identical copies of RNA and three virally encoded enzymes (protease, reverse transcriptase and integrase) (Adapted from University of London, 2016).

The full length Env is approximately 160 kDa in size (gp160) and is expressed as a gp160 precursor that is subsequently cleaved into the functional gp120 and gp140 units (Benjelloun et al., 2012; Berger et al., 1999; Goto et al., 1998; Kwong et al., 1998; Turner and Summers, 1999).

The molecular structure of gp120 was originally elucidated using X-ray crystallography by using a deglycosylated gp120 in complex with a two-domain fragment of CD4 (Kwong et al., 1998). The gp120 core consists of an inner and outer domain and a ß-sheet (Figure 1.2). The inner domain faces the trimer axis and gp41 and comprises a two-helix, two-strand bundle and a five-stranded ß-sandwich. The V1V2 stem emanates from the distal end of this inner domain. The outer domain is mostly exposed on the trimer surface and is a stacked double barrel that lies approximately parallel to the inner domain and comprises a six-stranded ß-sheet. The variable loops V4 and V5 are on the proximal end of the outer domain and the base of the V3 loop is on the distal end. The ß-sheet is an antiparallel four-stranded bridging sheet linking the inner and outer domain (Kwong et al., 1998; Wyatt and Sodroski, 1998). A depression at the interface of the outer domain with the inner domain and the bridging sheet forms the CD4 binding site (CD4bs). The CD4 binding residues on gp120 are distributed over six segments and binding of these two molecules induces a conformational change in gp120 that exposes conserved regions of gp120 (Kwong et al., 1998).

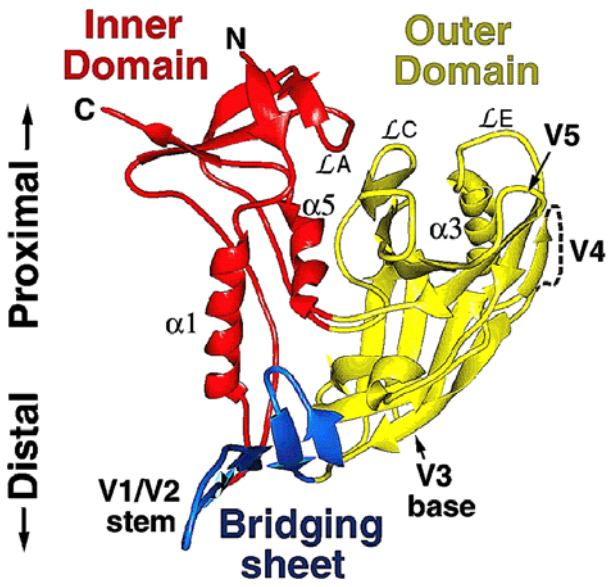


Figure 1.2: Ribbon diagram representation of a deglycosylated gp120 core from the perspective of the CD4 glycoprotein. The inner domain is shown in red, the outer domain is shown in yellow and the bridging sheet is shown in blue (Wyatt et al., 1998a).

Understanding the structure of Env at a molecular level assists in vaccine design since gp120 mediates attachment of the virus to the host cell and is an essential step in the life cycle of HIV-1. Additionally, the Env is the only "foreign" antigen exposed on the surface of the virion, and is thus an antibody target.

1.2.2 HIV-1 Life Cycle

The HIV-1 virion targets susceptible host cells, primarily CD4 positive ($CD4^+$) cells such as T lymphocytes, dendritic cells and macrophages (Figure 1.3) and infection with HIV-1 results in the gradual destruction of $CD4^+$ cells ultimately leading to immune deficiency and AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1984).

As mentioned above, attachment of the virus to the host cell is mediated by gp120 which binds to the host's CD4 surface receptor. This gp120-CD4 interaction stabilizes the bridging sheet between the inner and outer domains of gp120, exposing the co-receptor binding site. The two major co-

receptors that facilitate virus entry into the target cells are CCR5 or CXCR4, with CCR5 being the most common. The co-receptor phenotype has been found to be determined by the overall charge of the V3 loop, where neutral and/or acidic amino acids at positions 11 and 25 in the V3 loop favour CCR5 tropism, whereas basic amino acids in these positions favour CXCR4 tropism (Engelman and Cherepanov, 2012; Feng et al., 1996; Klasse, 2012; Kwong et al., 1998; Willey et al., 1988; Xu et al., 2007; Yao et al., 2015).

Once the co-receptor has been engaged, gp120 dissociates from gp41, is shed into the peripheral blood and serves as an immune decoy (Moore et al., 1990; Moore et al., 2006). The gp41 subunit mediates fusion between the viral and cellular membranes by forming a six-helix hairpin structure (resulting from the rearrangement of the trimerized amino- and carboxyl-terminal heptad repeat sequences) which juxtaposes the two membranes for fusion (reviewed in Engelman and Cherepanov, 2012). The membrane fusion results in the delivery of the viral core capsid into the cytoplasm where replication of the virus using the machinery of the infected cell takes place (Coetzer et al., 2006; Freed, 1998; reviewed in Sued et al., 2016).

HIV-1 infectivity of host cells has been found to be enhanced by complement protein, which is present in human sera and attaches to the surface of microorganisms in order for phagocytic cells to bind to the pathogen for phagocytosis. HIV-1 infection utilizes this process to increase binding of HIV-1 to target cells such as dendritic cells, thereby facilitating HIV-1 interaction and infection of these cells. HIV-1 infected cells escape complement-mediated neutralization by utilizing the complement's self-regulation system and incorporates complement regulating membrane proteins into its envelope which down regulate complement activation (Bajtay et al., 2004; Yu et al., 2010).

After membrane fusion, the HIV-1 virion p24 capsid is released into the cytoplasm of the host cell and uncoated to release viral RNA and enzymes

RT, IN and PR (Sierra et al., 2005). The viral enzyme RT transcribes this single stranded viral RNA into double stranded complementary DNA (cDNA) molecules. This step lacks a proof-reading function making it highly error prone and results in mutations and extensive diversity of viral quasispecies. The viral cDNA is transported into the nucleus as a preintegration complex (PIC), which includes the cDNA, IN and a range of viral and host proteins, and moves into the nucleus of the host cell where the viral enzyme IN incorporates the cDNA into the host's genome. The integrated proviral DNA becomes the blueprint for producing messenger RNA (mRNA). Initially mRNA transcripts are synthesized and transported out of the nucleus into the cytoplasm where they are translated into the regulatory viral proteins Rev (regulator of expression of virion proteins), Tat (transactivator of transcription) and Nef (negative factor). Once the levels of Rev in the cytoplasm are sufficiently high, it is imported back into the nucleus where it binds to full-length unspliced HIV mRNA forming oligomers and mediates their export out the nucleus for translation of the viral proteins, therefore Rev acts as a critical shuttle for viral mRNA between the nucleus and cytoplasm (Frankel and Young, 1998; Goto et al., 1998; Pollard and Malim, 1998).

The Env precursor (gp160) is synthesized in the endoplasmic reticulum where it forms a trimeric glycoprotein complex that is heavily glycosylated. It is then post-translationally modified in the endoplasmic reticulum and Golgi apparatus and cleaved by a furin-like protease to form the non-covalently associated gp120-gp41 trimeric glycoprotein complex (Klasse, 2012; Willey et al., 1988). These meta-stable trimers are transported to the host cell membrane and incorporated into the virus when the viral Gag protein drives the assembly of the virions before they bud from the infected cell. A small minority of the gp160 precursor is cleaved intracellularly to produce gp120 and gp41. The remainder is transported to the lysosomes and degraded (Willey et al., 1988). The immature virions bud out of the cell by folding of the lipid bilayer to form pockets which are pinched off and released from the cells. HIV-1 PR cleaves the Gag-Pol polypeptide to

construct the enzymes and structural proteins (matrix, capsid, nucleocapsid and p6), which rearrange during maturation to form infectious virions (Sued et al., 2016; Zheng et al., 2005). The infectious virions are therefore encased by the host cell's lipid bilayer with the viral trimeric Env's protruding from this membrane minimizing recognition by the immune system (Checkley et al., 2011).

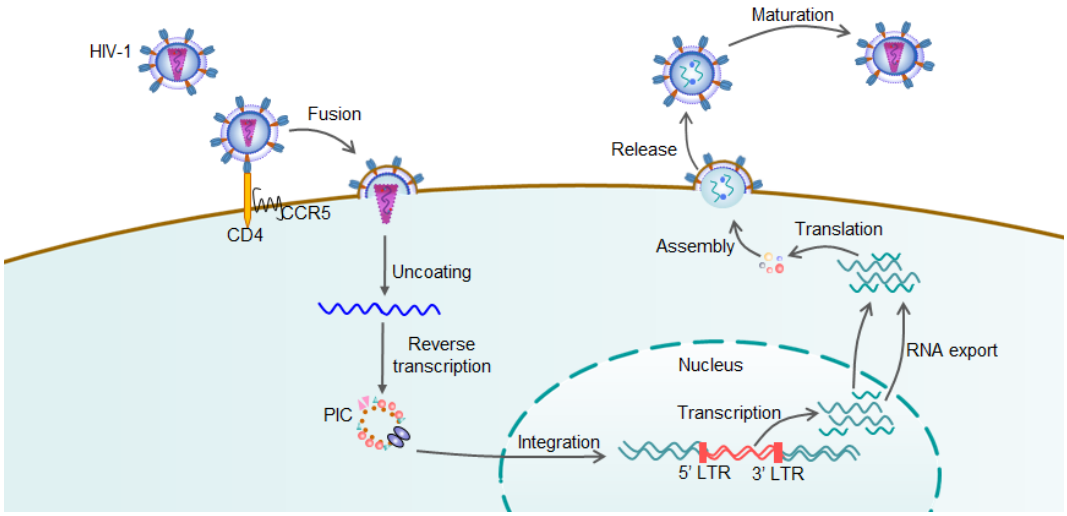


Figure 1.3: Schematic diagram of the life cycle of HIV-1. The virus binds to the CD4 receptor and fuses with the host's cell membrane, releasing the capsid into the cell. The capsid is uncoated and releases viral RNA into the cytoplasm. Reverse transcription converts the RNA into cDNA to form the pre-integration complex which is translocated into the nucleus. This cDNA is incorporated into the host's chromosome by integrase. Messenger RNA is synthesised and exported back into the cytoplasm of the host cell where it is translated into viral proteins and translocated to the cell surface where they are assembled into immature virus particles. These immature virions mature and bud out of the cell to form infectious virions (adapted from Barre-Sinoussi et al., 2013).

Since Env mediates viral entry into host cells, this critical role has made it an attractive target for vaccine design, aimed at eliciting a broadly neutralizing antibody (bNAb) response (Benjelloun et al., 2012). Therefore knowledge of the structure and functionality of Env is key to designing an immunogen capable of eliciting the appropriate immune response to provide sterilizing immunity to the host and this is expanded on in section 1.3 below.

1.2.3 Consequences of the viral replication cycle:

1.2.3.1 Diversity and evolution

The continual generation of new HIV-1 variants is a consequence of the highly error-prone RT enzyme resulting in an extensive genetic diversity of HIV-1 and recombination of the resulting quasispecies in an infected individual. This coupled with the extremely efficient replication cycle where approximately 10^9 virions can be generated in an infected individual daily, assists in the rapid evolution of the virus (Rambaut et al., 2004; Robertson et al., 1995; Santoro and Perno, 2013).

The diversity of HIV is demonstrated by the vast number of groups and subtypes. HIV can be divided into two types: HIV-1 and HIV-2. HIV-1 is most closely related to the simian immunodeficiency virus (SIV) isolated from chimpanzees and each group (see below) is thought to have been transmitted during a single chimpanzee-to-human incident and diverged in humans into the diverse virus it is today (http://www.hiv.lanl.gov, 2012). HIV-2 is closely related to SIV isolated from sooty mangabeys, suggesting it was transmitted from this host (Reeves and Doms, 2002). HIV-2 is largely confined to West Africa and is less easily transmitted from person to person (Reeves and Doms, 2002).

HIV-1 is the predominant virus worldwide which undergoes rapid viral evolution and recombination during replication resulting in an extraordinary viral diversity, with HIV-1 being further classified into four groups, namely HIV-1 M (Main group), HIV-1 O (Outlier Group), HIV-1 N (Non M, Non O or New Group) (http://www.hiv.lanl.gov, 2012) and HIV-1 P (a group of HIV-1 thought to have been transmitted by the gorilla host) (Plantier et al., 2009). Group M is the predominant circulating group worldwide and has been further classified into a number of phylogenetically associated subtypes or clades namely A1, A2, A3, A4, B, C, D, F1, F2, G, H, J and K. This continual diversification of HIV-1 results in mutational escape of the virus from the immune system as well as ARV drug treatment, and is therefore one of the

major obstacles for the development of an effective vaccine (Taylor et al., 2008). To overcome this problem, researchers are currently focusing on the selection of subtype-specific immunogens that are specific to the geographical area where the vaccine is intended to be used. Vaccines will also need to be continually updated as viral epitopes evolve (Agwale et al., 2011). The high genetic variability consequently has proven to be a major hurdle in vaccine development (Esparza, 2013).

1.2.3.2 ARV drug escape

ARV drug therapy using highly active antiretroviral therapy (HAART) in which a combination of drugs are used to act against several steps of the HIV life cycle, has not completely rid the virus from HIV-1 infected patients due to the ability of the virus to establish latent reservoirs, and continual evolution of the virus to escape pressure from both the ARV drugs and immune system (detailed below in Section 1.3) (Rambaut et al., 2004).

Four classes of antiretrovirals have been developed based on their molecular mechanism against various steps in the HIV-1 life cycle namely RT inhibitors (nucleoside/tide-analog RT inhibitors (NNRTIs), non–nucleoside RT inhibitors (NNRTIs)), IN inhibitors, PR inhibitors (PIs), and entry inhibitors (fusion inhibitors and coreceptor antagonists) (Arts and Hazuda, 2012).

Despite several steps in the life cycle of HIV-1 being targeted at once using HAART, HIV-1 continues to evolve escape mutations. The rapid replication cycle of HIV-1 means that virus producing cells have a half-life of approximately two days (Murray et al., 2011; Zhou et al., 2005). This coupled with the high rate of mutation attributed to the error prone nature of RT means that HIV-1 variants resistant to antiretroviral agents can replace wild-type strains in plasma within 2 – 4 weeks. In addition, genetic recombination of two single drug resistant strains leading to multiple drug

resistant strains rapidly drives the evolution of drug resistance in HIV-1 (Santoro and Perno, 2013).

Thus the latent reservoirs which are established within the first couple of weeks post transmission, and the ongoing evolution of the virus has hindered efforts for the development of a sterilizing cure and rather a 'functional' cure in which viral replication and remission of HIV-1 symptoms are controlled, is the current standard of care (Liu et al., 2015a; Rambaut et al., 2004). Research efforts focussing on eliminating the reservoir of HIV-1 using techniques such as "shock and kill" therapy to reactivate dormant virus with gene and cell therapy strategies could provide a 'sterilizing' cure. This could negate the need for the life-long treatment with ARVs and subsequent development of ARV resistant strains (Leong et al., 2017; Schwartz et al., 2017).

1.3 Natural disease progression to AIDS and immunological response to HIV infection

As occurs in all viral infections, HIV infection first activates the innate immune system, which is followed by the development of the B and T cell responses of the adaptive immune system (Chang and Altfeld, 2010).

The natural progression of HIV-1 in an average untreated individual is shown in Figure 1.4. In the first weeks after infection (acute phase) the virus replicates rapidly by targeting $CD4^+$ T lymphocyte cells ($CD4^+$ T cells) which results in a severe depletion of the $CD4^+$ T cell population (Tomaras et al., 2008). The likelihood of HIV-1 transmission from an HIV-1 infected individual to another person is highest during this stage of infection (Tomaras et al., 2008). During the acute phase, a latent reservoir is established and occurs within the first two to three weeks post infection (Chun et al., 1999). This occurs when HIV infects $CD4^+$ T cells that are naturally reverting into a resting memory state (Eisele and Siliciano, 2012). Latency is established in these $CD4^+$ T cells because they are less

permissive to HIV-1 gene expression, resulting in $CD4^+$ T cells with integrated provirus rather than rapid death of the cell as is normally the case (Eisele and Siliciano, 2012). These resting $CD4^+$ T cells have been found to contain persistent replication-competent HIV even in patients on suppressive HAART (Chun et al., 1997b). HIV reservoirs have been found in various tissues including the brain (Churchill and Nath, 2013; Pierson et al., 2000), male urogenital tract (Pierson et al., 2000) and in gut-associated lymphoid tissue (Chun et al., 2008).

Anti HIV-1 CD8+ cytotoxic T lymphocyte (CTL) cells are produced and this response results in the rapid decline of plasma viral RNA to a viral set point, the near-recovery of $CD4^+$ T cells and is the start of the chronic/asymptomatic phase of infection characterized by sustained immune activation and viral replication (Borrow et al., 1994; Koup et al., 1994; Maartens et al., 2014; Moir et al., 2011). This initial control of the HIV viral load by anti HIV-specific $CD8^+$ CTLs is short-lived as the virus quickly evolves escape mutations (Betts et al., 2006). Viral replication continues and HIV-specific $CD8^+$ T cells continue to proliferate during the chronic phase of HIV infection. The highly error prone step in which RT transcribes the RNA to DNA continually results in a diverse quasispecies of the virus (Betts et al., 2006). Without treatment, the chronic stage of infection can last several years depending on the infected individual's viral load set point and immune response. The onset of AIDS occurs when the $CD4^+$ T cells are depleted to such low levels that they can no longer fight off opportunistic infections (Maartens et al., 2014; Okoye and Picker, 2013). Within two years of an AIDS diagnosis, and in the absence of HAART, the individual will die from opportunistic infections.

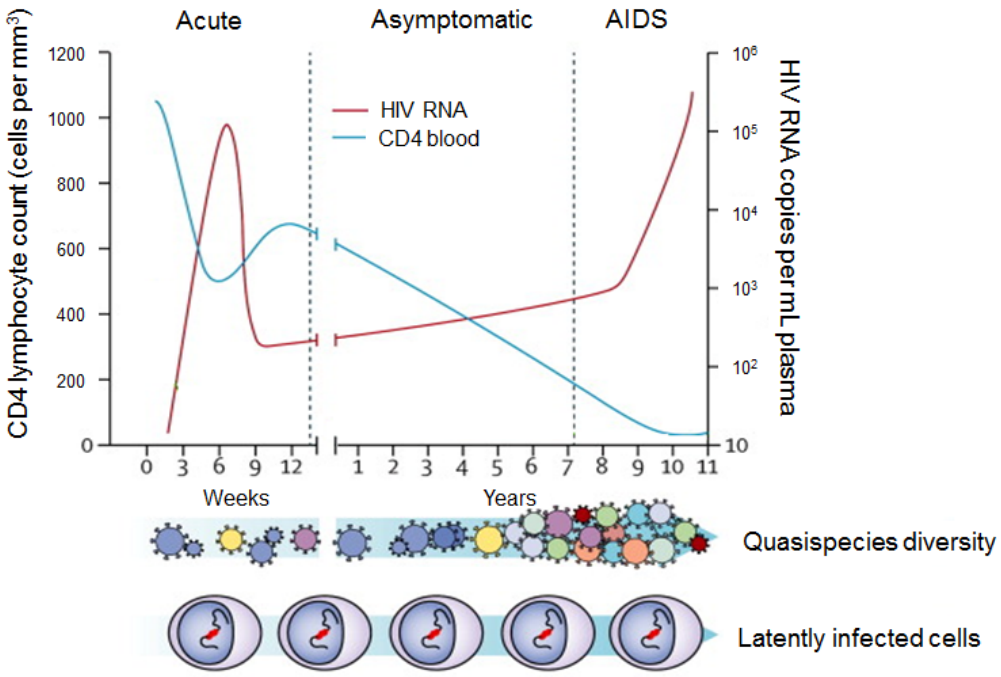


Figure 1.4: Natural progression of HIV-1 infection in an average untreated individual. The HIV viral load (red line) increases to high levels a few weeks after infection, and in a very short period establishes latently infected cells. The HIV RNA levels decrease as the host immune system responds to infection, then slowly increases again. The high mutation rate of HIV-1 results in a highly diverse quasispecies as HIV infection progresses. The CD4+ T cells (blue line) become depleted to a point where they can no longer fight opportunistic infections, signifying the onset of AIDS (adapted from Maartens et al., 2014).

1.3.1 Innate immune response to HIV-1 infection

Early after infection, the dendritic cells, natural killer cells and macrophages of the innate immune system respond with a “cytokine storm” inflammatory response to the virus (Stacey et al., 2009).

The complement system is activated as one of the first-line defenders against HIV-1 by: a) binding of complement protein C3 present in human sera to the virus; b) binding of mannose binding lectin (MBL) present in human sera to carbohydrates on the surface of the virus; or c) binding of HIV-1 specific antibodies (Ab) to HIV-1 which is initiated by binding of the Ab’s Fc tail to the complement protein C1. Once the complement system

has been activated, C3-convertase cleaves and activates C3 into C3a and C3b which leads to a cascade of reactions resulting in inflammation, recruitment of phagocytic cells, the formation of the membrane attack complex (MAC) which forms lytic pores and ultimately lysis of the virus. In addition, the complement system links the innate and adaptive immune response by enhancing and regulating humoral immunity. HIV-1 escapes complement-mediated neutralization by utilizing the complement's self-regulation system by incorporating complement regulating membrane proteins in the membrane as it buds from the infected cell, which down regulate complement activation (reviewed in Ballegaard et al., 2014; Lund et al., 1995; and Yu et al., 2010).

1.3.2 Adaptive immune response to HIV-1 infection

1.3.2.1 CD8+ Cytotoxic T-lymphocyte response

The adaptive immune system responds with a CD8+ CTL response as early as three weeks post infection in which $CD8^+$ T-lymphocytes kill infected cells expressing viral antigens (Borrow et al., 1994; Koup et al., 1994; Streeck et al., 2009). This CTL response is co-ordinated by $CD4^+$ T cells by priming the $CD8^+$ T-cell responses, maintaining the $CD8^+$ T-cell memory and maturing $CD8^+$ T-cell function. HIV-1 attacks these $CD4^+$ T-cells early in infection resulting in a drastic decline in their numbers thereby limiting this immune response (reviewed in McMichael and Rowland-Jones, 2001).

1.3.2.2 CD4+ T cell response

In addition to mediating the CD8+ CTL and B cell responses of the immune system, the CD4+ T cells produce a host of cytokines, specifically IFNγ, TNFα and IL-2 that stimulate macrophages, natural killer cells and the differentiation of T helper (Th1) cells that assist in fighting viral infection. However, HIV-1 infection disrupts the optimal balance of costimulatory signals required for optimal immune function. T cells have been found to express higher levels of inhibitory receptors designed to reduce harmful inflammatory responses, contributing to the loss of CD4+ T cell function

(Kassu et al., 2010; Ostrowski et al., 2006). The loss of immunological control is therefore a result of CD4 dysfunction and loss of CD4+ T cells.

This loss of CD4+ T cells has been associated with the presence of bNAbs. It's hypothesized that the loss of CD4+ T cells and resulting higher viremia contributes to bNAb development due to increased exposure to the virus (Doria-Rose et al., 2010; Euler et al., 2010; Sather et al., 2009). Interestingly, this correlation seems to be true only for adults, whereas in children antibody-mediated anti-HIV-1 neutralization correlates with a lower CD4+ T cell population (Agthe et al., 2014).

1.3.2.3 Humoral immune response

The humoral arm of the immune response is activated approximately one week after infection when the host's virus-specific B-cells recognize an antigen of HIV-1 (Li et al., 2007). The initial response of the B cells is to the gp41 region of Env, and results in non-neutralizing antibodies (Tomaras et al., 2008). These Abs bind to linear epitopes of Env, but are unable to bind to an epitope on the correctly folded native functional Env trimer spike, resulting in the inability to neutralize the virus and have little control of viraemia (Tomaras et al., 2008). Having said this, non-neutralizing Abs do display some neutralizing properties to Env because the Env spikes decay over time, resulting in epitopes being revealed that these Abs can recognise (Burton and Mascola, 2015).

A second Ab response approximately 14 days later results in the production of gp120 specific antibodies which have limited neutralizing breadth, and again, the virus acquires escape mutations. This second Ab response encompasses neutralizing antibodies that commonly target regions with high sequence variation such as the variable loop regions. However, these neutralizing Abs are strain specific and emerge early in infection before changes in the variable epitopes lead to Ab escape by the virus (Tomaras

et al., 2008). The B cell response is coordinated by the CD4+ T cells, and again, their decline directly impacts the Ab response (Oxenius et al., 1998).

Some HIV-1 infected individuals develop bNAbs which can neutralize a broad range of HIV-1 strains and typically develop years after infection (Mikell et al., 2011) and these bNAbs evolve with the virus to target conserved regions of Env (Burton and Mascola, 2015; Li et al., 2007; Mikell et al., 2011).

It has been determined that bNAbs mature from an unmutated common ancestor (UCA) B-cell receptor post binding to the transmitted founder virus early after infection. BNAb development occurs as a result of complex or prolonged maturation pathways, which is driven by Env evolution. Understanding the developmental pathways of these Abs has important implications for vaccine development since a successful immunogen or immunization protocol would likely need to reproduce such a maturation pathway. First, an Env immunogen would need to target UCAs to trigger the appropriate Ab lineage, and second, this would need to be followed by vaccination with Env immunogens that stimulate the somatic mutation pathways required for the development of breadth and potency against the virus (Doria-Rose et al., 2014; Liao et al., 2013b).

BNAbs target several conserved sites on the highly diverse HIV-1 envelope spike. The glycoprotein gp120 consists of five variable regions (V1 – V5) interspersed with five conserved regions (C1 – C5) (Figure 1.5), and these regions are extensively glycosylated (Kwong et al., 1998).

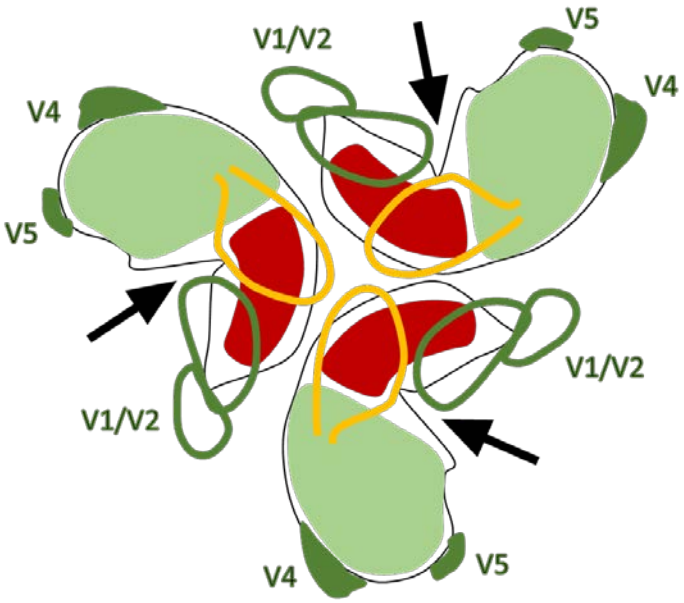


Figure 1.5: Generalized schematic representation of the arrangement of HIV-1 gp120 glycoproteins as a native trimer, viewed from the perspective of the target cell membrane. The areas shaded in dark green represent the variable loops as indicated (V1/V2, V4 and V5). The yellow loops represent the V3 region. The black arrows indicate CD4 binding pockets and the areas shaded in red represent the conserved chemokine-receptor-binding region (adapted from Wyatt et al. (1998b)).

Further CD4 induced (CD4i) conserved regions are presented during the entry process when gp120 binds to CD4, resulting in conformational changes of gp120 that orients and exposes the co-receptor binding site (Kwong et al., 1998). During this process the V2 loop which partially occludes the V3 loop and CD4i epitope moves and this stabilizes the bridging sheet on which these epitopes are situated. The V3 region changes to expose altered V3 epitopes, which together with the CD4i epitope make up the co-receptor binding site (Kwong et al., 1998). This is followed by fusion between the viral and cellular membranes mediated by gp41 (reviewed in Engelman and Cherepanov, 2012).

1.4 Using bNAbs to identify vulnerable epitopes on Env

Each of the epitopes targeted by bNAbs provides a conserved region that can block HIV-1 infection. BNAbs isolated from B cells of HIV-1 infected patients has facilitated the identification and analysis of vulnerable epitopes

on Env which in turn can aid the template-based design of vaccine immunogens, or reverse vaccinology (Corti et al., 2010). Some important bNAbs are discussed below.

1.4.1 Antibodies targeting the variable V1/V2 region of gp120

Despite the high diversity and heavy glycosylation of this region, a number of bNAbs have been isolated that are directed to this region including PG9, PG16, CH01-CH04, PGT141-145 and PGDM1400. These bNAbs preferentially bind to a quaternary epitope that spans V1/V2 on gp120 and they generally share dependence upon the N-linked glycan at position 160 in the V1/V2 region (McLellan et al., 2011; Sok et al., 2014b).

1.4.2 Antibodies targeting the variable V3 loop and associated glycans

BNAbs have been identified that target an epitope in the V3 loop in which the N-linked glycan at position 332 is directly involved. These bNAbs include PGT121-123, 125-126, 130 and 131 (Walker et al., 2011). PGT127 and PGT128 share a similar mode of epitope recognition in that they rely on two glycans for binding, one at position 332 and the other at 301, as well as the C-terminal end of the V3 loop binding epitope. The two glycans N332 and N301 have been found to be conserved in 93% and 73% of HIV isolates respectively, which explains the broad neutralization profiles of these antibodies (Pejchal et al., 2011). However, the neutralization potency of these bNAbs is not solely restricted to these two glycans, as described by Sok et al. (2014a). Their research described these bNAbs as “promiscuous” since many of them were still able to neutralize variants in which the glycan at N332 was shifted to N334, as well as variants in which the glycan was missing and suggests that these bNAbs have the ability to use other glycan sites in proximity (N136/N137 or N295) to the N332 glycan.

An unusual bNAb 2G12, targets a host cell-derived glycan on the gp120 outer domain and blocks membrane fusion as a consequence of steric

hindrance. 2G12 differs to the bNAbs described above because it's binding to the conserved N-linked glycans is dependent on N332. In addition, it is less potent and broad in neutralization compared to the above bNAbs (Doores et al., 2010; Li et al., 2007; Murin et al., 2014).

1.4.3 Antibodies targeting the CD4 binding site

The CD4bs is a functionally conserved site on gp120 and antibodies that target this region are often induced in an HIV-1 infected person within the first four to 16 weeks post infection. However, it takes years to develop potent antibodies with the affinity or serum concentration required for neutralization of the virus (Lynch et al., 2012; Mascola and Haynes, 2013). The CD4bs is recessed in a depression of gp120 hidden by glycosylation and variable loops and binding of gp120 with CD4 relies on electrostatic potential at the contact surfaces between the two molecules (Kwong et al., 1998).

The bNAb IgG1b12, originally generated from the lymphocyte of an asymptomatic person who had been HIV-positive for 6 years, has a protruding finger-like third complementarity determining region of the heavy chain variable regions (CDRH3) that is able to penetrate the CD4bs on Env (Burton et al., 1991; Saphire et al., 2001). IgG1b12 recognizes a discontinuous epitope that overlaps the CD4 binding region and has been reported to neutralize 30 – 40 % of primary isolates of subtype B and C viruses by inhibiting its entry into the cell (Burton et al., 1994; Georgiev et al., 2013). However IgG1b12 is far less potent than the more recently isolated bNAbs that target the CD4bs as discussed below (Bures et al., 2002; McInerney et al., 1997; Zwick et al., 2003).

The somatically related bNAbs VRC01, VRC02 and VRC03 were isolated from a subtype B HIV-1 infected individual classified as a long term non-progressor (LTNP) since this patient had been infected with the virus for more than 15 years and still maintained a $CD4^+$ T-cell count of over 500

cells/µl plasma despite being treatment naïve (Wu et al., 2010). The crystal structure of VRC01 in complex with gp120 has shown that VRC01 partially mimics the interaction of CD4 with gp120 by targeting a highly conserved region of the CD4bs on gp120 and enables it to achieve potent neutralization of over 90 % of circulating HIV-1 isolates. The neutralization of VRC03, a somatic variant of VRC01, is less broad than that of VRC01. VRC03 is able to neutralize 50 % of circulating HIV-1 isolates (Wu et al., 2010; Wu et al., 2011). This group of antibodies is able to access a highly conserved region of the CD4bs without the Env conformational change that occurs after gp120 binding, giving them a unique property distinct from other CD4bs antibodies (Georgiev et al., 2013; Li et al., 2011; Zhou et al., 2010).

HJ16 was isolated from an individual who had been infected for 12 years, had a $CD4^+$ T-cell count of 274 cells/µl and was on HAART (Corti et al., 2010). HJ16 is a glycan-dependant CD4bs specific antibody that binds to an epitope that partially overlaps the IgGb12 epitope and is dependent on the interaction with the glycan at position 276 (Balla-Jhagjhoorsingh et al., 2013).

The somatically related bNAbs VRC-CH30, VRC-CH31, VRC-CH32, VRC-CH33 and VRC-CH34 were isolated from an individual chronically infected with subtype A of HIV-1 (Wu et al., 2011). These antibodies have been classed as VRC01-like antibodies since they bind to the same CD4bs epitope as VRC01. VRC-CH31 is able to neutralize 75% – 95% of HIV-1 viruses of multiple subtypes (Bonsignori et al., 2012; Wu et al., 2011).

NIH45-46^{G54W} is a CD4 directed bNAb originally isolated from the same patient as VRC01. A single amino acid substitution of glycine to tryptophan in the second heavy chain complementarity-determining region (CDRH2) of this antibody increased the breadth and potency of this antibody by increasing the surface contact with the gp120 bridging sheet (Diskin et al., 2011).

The CD4bs bNAb 3BNC117 was isolated from a patient classified as an elite controller. It has been shown to prevent and control HIV-1 infection in animal models and is currently showing promise as treatment in early phase clinical trials in humans (Scheid et al., 2016; Schoofs et al., 2016).

1.4.4 Antibodies targeting the gp41 membrane proximal membrane (MPER) region

The initial B-cell response is to the gp41 region of Env and is mostly non-neutralizing but it is thought that the function of these early Abs is to activate the complement system (Tomaras et al., 2008). BNAbs that target this region have been isolated though, such as 2F5 which has been found to neutralize HIV-1 subtype B but not those of subtype C, 4E10 which is directed against the C terminus of the gp41 ectodomain (Binley et al., 2004) and 10E8 which binds to highly conserved residues within MPER and is distinct from the other gp41 bNAbs in that it does not bind lipids and is not autoreactive (Huang et al., 2014). The gp41 region has been found to be poorly immunogenic and therefore research efforts shifted their focus to the other conserved regions of Env (Binley et al., 2008; Corti et al., 2010). However, recent findings suggest the gp41 region may be more immunogenic than previously thought. Falkowska et al. (2014) isolated the bNAbs PGT151-152, which specifically recognise a glycan-dependent epitope on gp41 of a cleaved Env trimer. In addition, Williams et al. (2017) reported the isolation of a DH511 bNAb lineage directed to the distal portion of the gp41 region.

1.5 Challenges of HIV-1 vaccine development

The development of a preventative HIV-1 vaccine has proven challenging. Challenges include extensive viral diversity, establishment of latent viral reservoirs early after infection, multiple strategies for evading immune responses, the immune correlates of protection are unclear, there is no small animal model that can be used to study the virus and to date the

immunogens tested have not successfully elicited broadly reactive neutralizing antibodies (bNAbs) (Barouch, 2008; Chun et al., 1997a).

In addition, HIV-1 harbours a number of structural immune evasion strategies including low density surface expression of Env, antigenic variability, glycan shielding, transient epitope exposure, conformational masking, steric occlusion and the expression of non-functional Env (Figure 1.6).

The exposed surfaces of Env are expressed at low densities on the virus surface and are heavily glycosylated with an enormous diversity of N-linked oligosaccharides which shield it from neutralizing antibodies (NAbs). Accessibility of conserved epitopes is available only transiently prior to co-receptor binding and membrane fusion, allowing HIV-1 to escape host immune humoral responses. In addition, antibodies are prevented from binding to these conserved epitopes due to steric restriction during gp120 receptor binding and membrane fusion (Burton et al., 2012; Douek et al., 2006; Mizuochi et al., 1988; Mouquet, 2014; Zanetti et al., 2006).

After co-receptor binding, gp120 is shed into the peripheral blood, and serves as an immune decoy. Non-functional Env such as non-cleaved precursors or decaying gp120 monomers are expressed on the virus surface which results in the generation of non-neutralizing antibodies (Burton et al., 2012; Douek et al., 2006; Mizuochi et al., 1988; Mouquet, 2014; Zanetti et al., 2006).

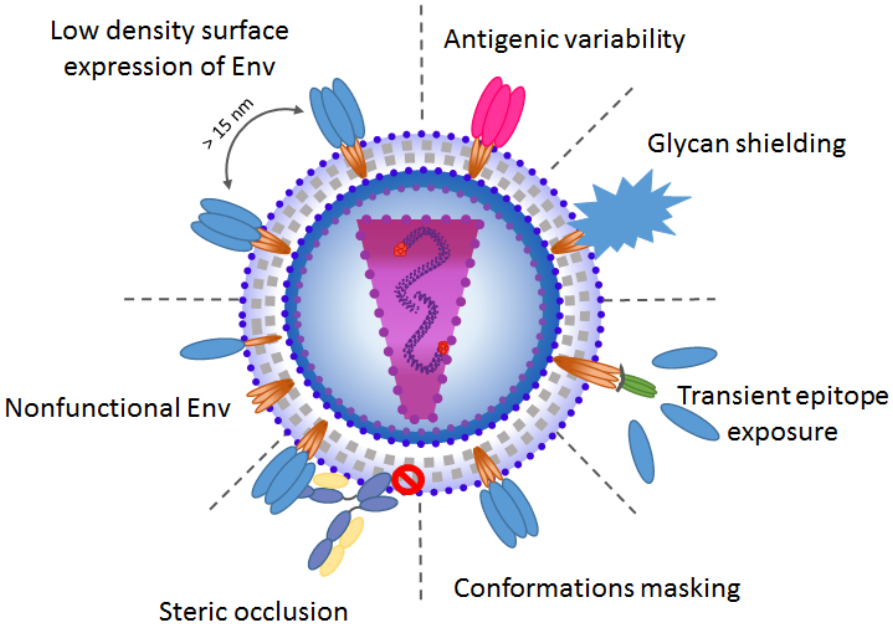


Figure 1.6: Structural immune evasion strategies of HIV-1. Low density expression of Env on the virus surface; heavy glycan shielding of conserved epitopes; non-functional Env including non-cleaved precursors or decaying gp120 monomers on the virus surface; conformational changes during CD4 binding resulting in conformational masking of conserved epitopes; brief epitope exposure during CD4 binding; steric restriction of binding by antibodies to conserved epitopes during gp120 receptor binding and membrane fusion (adapted from Mouquet, 2014).

One of the major challenges in HIV-1 vaccine design is the lack of an animal model for the development and preclinical testing of effective vaccines. This is because HIV-1 is highly specific to humans and traditional animal models fail to provide a suitable environment for HIV-1 replication because they lack the cellular proteins that support it (Hatziioannou and Evans, 2012). Animal models have been developed to study HIV-1 infection such as the humanised mouse, in which genetically immunocompromised mice engrafted with human tissues have enabled the study of HIV-1 infection as well as virus-specific humoral and cellular immune responses (Brainard et al., 2009). However, humanised mice have a non-functional immune system requiring them to live in an artificially sterile environment with specialised housing and handling procedures, and since only parts of the human immune system can be generated in these mice, the effects of HIV-1

infection are limited. An important limitation of the humanized mouse model is its size limitation for the study of latent reservoirs of HIV-1. Large amounts of cells are required for this which necessitates the use of a large number of animals (Policicchio et al., 2016). In addition, humanised mice cannot be bred, requiring them to be generated *de novo* for each experiment, which is costly (Hatziioannou and Evans, 2012).

Overall, non-human primates (NHP) are the closest animal model to humans. HIV-1 does not replicate in NHP's and if it does, such as in some chimpanzees, it is non-pathogenic (Ambrose et al., 2007). By using the NHP equivalent of HIV in macaques, namely SIV, and modifying SIV to represent the challenges of HIV, a useful tool in HIV research has been developed (Hatziioannou and Evans, 2012). However, HIV and SIV are still different viruses, and although some of the immune responses to infection have been elucidated in NHP models, the biological properties of SIV and HIV and those of their respective hosts differ, often making the results incomparable to human HIV-1 biology. The recent work by Li et al. (2016) however, reported that substituting residue 375 of the chimeric SIV-HIV's with that of the naturally occurring SIV, enhanced binding to rhesus macaque CD4. This allows for improved viral entry and replication of SHIV in the rhesus macaque model, and ultimately may provide an improved animal model for HIV pathogenesis and vaccine research (Hatziioannou and Evans, 2012).

While the NHP and humanized mouse models present challenges for studying the complete pathogenesis of HIV-1, combinations thereof provide important insights into the study of specific aspects of the viral life cycle, and critically the development and evaluation of prophylactic vaccines, cure strategies and novel ARVs.

The lack of a suitable animal model has further hindered the elucidation of the immune correlates of protection against HIV. In order to design an effective immunogen, the correlates of protection need to be elucidated,

therefore the only approach available is to test promising vaccine candidates that have undergone preclinical trials in animal models, in human efficacy trials (Esparza, 2015).

1.6 HIV-1 vaccine efficacy trials

Historically vaccines have proven to be the most cost effective way to save lives against disease with very little knowledge of the immunological mechanisms they involve to induce protective immunity (Pulendran and Ahmed, 2011). Unlike other viruses such as smallpox, natural infection with HIV-1 does not result in sterilizing immunity, therefore understanding the immunological factors required to control HIV-1 infection have proven difficult.

HIV-1 vaccines can be been grouped into three groups: (1) vaccines that induce NAbs to confer protection against viral entry using Env based immunogens; (2) vaccines that induce $CD8^+$ T-cell responses using live recombinant viral vectors and DNA vaccines; (3) vaccines that induce multiple immune responses using prime-boost combinations (Esparza, 2015).

The development of a vaccine undergoes three phases of clinical trials. In the first phase, the vaccine is given to a small group of healthy individuals for safety assessment. The second phase can be performed in two stages where the vaccine is given to a large group of approximately 100 individuals from the vaccine target population to assess safety, dosage and immunogenicity (Phase 2a) as well as proof-of-concept (Phase 2b). In the third phase, the vaccine is administered to thousands of individuals of the vaccine target population to test the safety and efficacy of the vaccine (CDC, 2016).

To date, 284 HIV-1 vaccine candidates have been tested in Phase I clinical trials worldwide. Of those, 59 have advanced to Phase 2 trials but only six

Phase 2b or Phase 3 clinical efficacy trials have been completed namely VAX 003, VAX 004, Step, HVTN (HIV Vaccine trials network) 503 (Phambali), RV144 and HVTN 505 (Buchbinder et al., 2008; Flynn et al., 2005; Gray et al., 2011; Hammer et al., 2013; IAVI, 2017; Pitisuttithum et al., 2006; Vanichseni et al., 2004). The VAX 003 and VAX004 trials used a recombinant expressed gp120 subunit vaccine containing a bivalent preparation from subtypes B and E (VAX003) and two gp120s from subtype B (VAX004). These trials aimed to induce an HIV-1 Env-specific antibody response in trial participants, however they were found to be ineffective (Excler and Michael, 2016; Sheets et al., 2016; Shin, 2016).

The HVTN 502 (STEP) and HVTN 503 (Phambili) trials aimed to induce HIV-1 specific $CD8^+$ CTL cell responses in trial participants by using a trivalent replication-incompetent Merck Adenovirus type 5-vectored vaccine (Ad5) vector expressing the *gag*, *pol* and *nef* genes derived from HIV-1 subtype B. These trials showed no vaccine efficacy and in addition, had to be terminated early due to safety concerns as the trial participants showed an increased trend towards infectiousness to HIV-1 (Excler and Michael, 2016; Sheets et al., 2016; Shin, 2016).

The RV144 and HVTN505 trials focussed on using a prime-boost approach to elicit both arms of the immune response, the HIV-1 antibody response as well as a specific T-cell response. In the RV144 trial, the vaccine consisted of a canarypox vector prime expressing the HIV-1 subtype B *gag and protease* genes followed by a boost with a recombinant gp120 subunit vaccine derived from subtypes B and E. This approach conferred a modest 31.2 % (95% CI, 1.1 to 52.1; P=0.04) efficacy with post-hoc analyses indicating a vaccine efficacy of 60 % one year after initial vaccination (95 % CI 22-80 %) which declined quickly. To date this is the only HIV-1 vaccine trial showing a degree of efficacy (Excler and Michael, 2016; Robb et al., 2012; Sheets et al., 2016; Shin, 2016). Analysis of the Ab responses to this vaccine revealed they were mainly directed against a conserved region of

the V1/V2 loop of Env and were correlated inversely with HIV-1 risk of infection whereas plasma IgA Abs directed to Env correlated directly with HIV-1 infection, possibly mitigating the effect of the protective Abs. The lack of NAbs turned the field's attention to the possible protective role of non-neutralizing Abs in antibody-dependent cell-mediated cytotoxicity (ADCC) in protective immunity to HIV-1 (Esparza, 2013; Haynes et al., 2012; Karasavvas et al., 2012; Wren and Kent, 2011).

The HVTN 505 trial tested a DNA vaccine prime consisting of six DNA plasmids expressing HIV-1 subtype B *gag*, *pol, nef and env* genes as well as two Env proteins from subtype A and C followed by a boost using Ad5-vectors expressing Env from subtypes A, B and C as well as a Gag-Pol fusion derived from subtype B. This study too was halted due to safety concerns and this trial showed no efficacy (Excler and Michael, 2016; Sheets et al., 2016; Shin, 2016).

The HVTN 702 Phase 2b/3 efficacy trial is currently ongoing in southern Africa (AVAC: AIDS Vaccine Advocacy Coalition; Global Advocacy for HIV Prevention, 2016). The trial aims to confirm and extend the RV144 trial's partial protection, and will test a recombinant canarypox vector prime (ALVAC-HIV vCP2438) expressing a subtype C HIV-1 Env linked to subtype B gp41, and subtype B *gag* and *protease* genes. The boost immunogen is a bivalent gp120 subunit vaccine, both gp120's derived from subtype C isolates (Andersen-Nissen, 2015; Zolla-Pazner et al., 2016).

The result from the efficacy studies have not led to an HIV vaccine but lessons learnt from the trials are valuable in driving the design of future vaccines (Sheets et al., 2016).

1.7 Structure-based reverse vaccinology

It has been found that approximately 15–30 % of HIV-1 infected humans develop bNAbs against the virus, and some of these bNAbs are able to

neutralize 80-90% of circulating HIV-1 viruses (Moore et al., 2015). As discussed above, these bNAbs are important in defining conserved epitopes on Env and which can be used for the structure-based reverse vaccinology to develop HIV-1 vaccines (Van Regenmortel, 2016).

In structure-based reverse vaccinology, the crystal structure of Env in complex with a bNAb is used to identify the epitope recognized by that bNAb, and this epitope is reconstructed as an immunogen with the hope that it will elicit a bNAb response with the same neutralizing capacity as the bNAb originally isolated (Van Regenmortel, 2016). The ability of these designed immunogens to elicit an immune response in animal models is tested, leading to the development of a vaccine antigen (Burton, 2010). Understanding the structural and biological information with regards to bNAb interactions with gp120 can be used in this way to design immunogens (Nabel et al., 2011).

If the bNAbs that develop in patients' years after infection could be induced prior to infection, they may be able to prevent the establishment of latent infections and provide protective immunity. Studies of the passive administration of NAbs in NHP's have provided proof-of-concept that NAbs directed to Env can protect against HIV-1 infection (Halper-Stromberg and Nussenzweig, 2016; Hessell et al., 2009a; Hessell et al., 2009b; Hessell et al., 2010; Mascola et al., 2000; Shibata et al., 1999; Tomaras et al., 2008).

1.8 Env-based immunogens for vaccine design

As discussed above, the high host cell specificity of HIV-1 is as a result of Env which mediates viral entry by binding to the host's CD4 receptor. The conserved regions of Env provide important target sites that are vulnerable to neutralization by bNAbs (Halper-Stromberg and Nussenzweig, 2016; Tomaras et al., 2008).

Of particular interest is the CD4bs since binding to CD4 is vital for viral entry. The contact residues on CD4 that have been found to be crucial for gp120 binding are phenylalanine-43 (Phe43) on the opening of the deep cavity and arginine-59 (Arg59) which forms a salt bridge with amino acid residue aspartic acid-368 (Asp368) on gp120. Other important contact residues on gp120 that bind to CD4 are tryptophan (Trp112), valine (Val255), threonine-257 (Thr257), glutamic acid (Glu370) and isoleucine (Ile371) (Kwong et al., 1998; Wyatt and Sodroski, 1998). The conformational change resulting from gp120-CD4 binding triggers high-affinity binding to CD4 and the structural rearrangement required to expose the co-receptor binding surface and at the same time exposes glycan-free conserved regions on the viral spike (Mascola and Haynes, 2013).

The lability of the Env trimer, a result of the noncovalent bond between gp120 and gp41, has made it extremely difficult to present a functional Env immunogen to the immune system. It is thought that interdomain flexibility disrupts the CD4bs epitopes. In addition, the variable loops and glycosylation of Env mask the CD4bs. Overall, protection from HIV-1 infection by Abs elicited against Env are limited (Wyatt and Sodroski, 1998).

The immunogenic requirements for bNAb development are poorly understood since it is thought that during natural infection bNAbs only develop in the chronic stage of infection, a result of years of co-evolution between the virus and maturing bNAb lineages (Doria-Rose et al., 2014; Liao et al., 2013b). Somatic hypermutation that leads to the development of bNAb's appears to be driven by the co-evolution of antibody and virus as described by Doria-Rose et al. (2014) who carried out a longitudinal study of NAbs that developed in a CAPRISA-donor (CAP256) that was super-infected at week 15. The unmutated ancestor of the V1V2-directed NAbs developed between 30-38 weeks post infection shortly after virus diversification of the super infecting virus. Further improvement of neutralization breadth occurred at 59 weeks post infection, again after virus

diversification. This relatively rapid maturation of V1V2 directed NAbs demonstrates that the initial engagement of an appropriate B-cell receptor with the relevant attributes (in this case protruding CDR H3s) along with limited somatic hypermutation can result in an Ab with the breadth and potency required to neutralize the virus. This implies that the development of an antigen capable of engaging the appropriate naïve B-cell receptor in combination with sequential immunogens to drive affinity maturation of the Ab are required for an effective vaccine (Doria-Rose et al., 2014).

As mentioned previously, the HIV-1 Env glycoprotein (gp120 and gp41) is the only viral protein to protrude through the host-derived surface of the virion. It is essential for viral entry and is the target of bNAbs, therefore it has been the subject of intensive immunogen design for an HIV-1 vaccine (Burton et al., 2012; Nabel et al., 2011).

The HIV vaccine field has attempted numerous approaches to develop immunogens by stabilizing Env in a native conformation while at the same time exposing conserved regions to the humoral arm of the immune response in the hope of eliciting bNAbs. Although great advances have been made over time in this area, the neutralizing responses elicited in small animals, NHP or humans have been autologous in nature, showing only strain specific neutralization activity (Sanders et al., 2015).

Initial studies using gp120 as an immunogen were disappointing because they resulted in antibodies being elicited to linear epitopes of gp120 and not against the tertiary or quaternary epitopes on the correctly folded monomer when they are part of the native trimer (Schultz and Bradac, 2001; VanCott et al., 1995a). Attempts to modify gp120 to enhance stability and present only important epitopes to the immune system have had limited success and include fusing gp120 to a hepatitis B surface carrier protein (Berkower et al., 2004), designing peptides that bind to important epitopes on gp120 (Mester et al., 2009), engineering gp120 molecules with extra N-linked

glycans to mask decoy epitopes and minimize the conformational flexibility of gp120 (Pantophlet et al., 2003), removal of glycans such as the CD4bs proximal N197 glycan from a stabilised trimer to expose conserved epitopes and increase binding to CD4bs antibodies, and removal of variable loops V1 and V2 to expose the CD4bs epitope (Cao et al., 1997; Liang et al., 2016).

Slightly improved neutralizing antibody responses directed to various epitopes on gp120 and gp41 have been obtained by using soluble gp140 trimers. These are generated by excluding the hydrophobic membrane-spanning domain of gp160. However Env is extremely labile and removing the transmembrane region results in the disintegration of the gp120 and gp41 subunits (Earl et al., 1994; Sanders et al., 2015).

Over time, this has led to various approaches to stabilise gp140 to mimic the native Env trimer and includes mutations to eliminate the cleavage site between gp120 and gp41, covalent cross-linking of the gp160 subunits by introducing cysteines to form a disulphide bond and/or the addition of a trimer-stabilizing motif to extend the N-terminus or C-terminus of gp41 (Sanders et al., 2013; Yang et al., 2000). Env has been stabilized in a trimeric conformation by introducing several trimerization motifs such as the GCN4 leucine zipper peptide derived from a yeast transcriptional activator (Harbury et al., 1993); h-CMP, derived from the C-terminus of human cartilage matrix protein (Saha et al., 2012); a trimerization domain derived from the C-terminus of bacteriophage T4 fibritin (T4F) (Yang et al., 2002); the catalytic chain of aspartate transcarbomoylase (ATC) (Chen et al., 2004); and a trimerization domain derived from chicken cartilage matrix (CART) (Selvarajah et al., 2008).

Of particular interest are coiled coil proteins such as GCN4 and h-CMP. The GCN4 motif has a characteristic seven-residue repeat containing hydrophobic residues at the first and fourth positions of each repeat with the

remainder residues being polar. Buried residues in the GCN4 leucine zipper direct dimer formation and manipulation of the heptad repeat residues can direct the conformation of the protein as monomer, dimer or trimer (Harbury et al., 1993; O'Shea et al., 1989). When trimers stabilized with trimerization motifs have been used as vaccine immunogens, they have shown marginal improvement in bNAb breadth over monomeric gp120 (Du et al., 2009).

The h-CMP trimerization motif contains three subunits linked by disulphide bonds and forms a homotrimer. The C-terminal domain of CMP serves as the trimerization site, and two cysteine residues preceding the C-terminal domain stabilize the complex (Beck et al., 1996; Jenkins et al., 1990). This trimerization motif has been used to trimerize monomeric cyclic permutants which bound bNAbs IgG1b12, PG9, PG16 and PGT145 with slightly higher affinities compared to monomeric gp120 (Saha et al., 2012)

Proteolytic cleavage of gp160 into gp120 and gp41 has since been shown to be essential for the native conformation of gp140 (gp120 with the gp41 ectodomain) and the elicitation of bNAbs (Ringe et al., 2013; Yasmeen et al., 2014). A properly folded and proteolytically cleaved trimer denoted the SOSIP trimer has been generated by introducing an intermolecular disulfide bond (SOS) between gp120 and the gp41 ectodomain as well as an amino acid substitution from isoleucine to proline at residue 559 (IP) on the N-terminal heptad repeat region of gp41 to strengthen the interactions between the gp41 subunits. These modifications have resulted in consistently tri-lobed propeller-shaped trimers. Unfortunately the Abs elicited using this immunogen lack the breadth of activity required for neutralization of heterologous primary isolates of the virus (Beddows et al., 2007; Sanders et al., 2013; Sanders et al., 2015; Sanders et al., 2002).

Another approach to elicit bNAbs has been to cross-link gp120 or gp140 with CD4 to stabilize Env in a conformation that exposes the conserved CD4i epitopes to the immune system and this method has been shown to

elicit polyclonal antibody responses in animal models (Devico et al., 1996; Fouts et al., 2002). It has been found that although immunization using this approach in guinea pigs resulted in neutralizing activity, subsequent analysis revealed the response was directed to CD4 and not an epitope on Env (Varadarajan et al., 2005). Rhesus macaques primed with HIV_{BaL} gp120 linked to rhesus macaque 2dCD4, followed by boosts with rhFLSC protein elicited neutralizing antibodies, however protection following rectal challenge with $SHIV_{Sf162P4}$ was not attained (Thomas et al., 2014). Recently, our laboratory published findings of Env/2dCD4 liganded complexes, in which a soluble CD4 protein containing the first two domains of CD4 ($2dCD4^{S60C}$, serine at position 60 is substituted with cysteine) was used to generate the complexes (Killick et al., 2015). The S60C mutation allows for the Env/$2dCD4^{S60C}$ complex to be stabilized through an inter-molecular disulphide formation, and has previously been shown in our laboratory to have a higher binding affinity to gp120 than wild-type 2dCD4 (Cerutti et al., 2010). Although preliminary results indicate that the Env/2dCD4 induced response is directed to CD4, ongoing work is currently attempting to map the epitopes responsible for the neutralization of the virus. The use of recombinant soluble CD4 to bind to cell-free virus has been tested in clinical trials as an inhibitor of HIV-1 and shown to be safe. Research groups are still trying various modifications of CD4 to improve its efficacy (Chen et al., 2014; Kahn et al., 1990; Schacker et al., 1995).

The stabilization of gp120 and exposure of CD4i epitopes has also been performed by generating cyclic permutants of gp120 by covalently linking the N- and C-termini of gp120 with an amino acid linker and introducing new termini into one of the variable loops by complete or partial deletion of that loop. In addition, a trimerization motif added to the new N-termini results in a native-like trimer that presents loop-derived neutralization epitopes (Saha et al., 2012).

An alternative approach to stabilizing gp120 in a native trimer conformation while exposing the CD4bs, is to introduce a mutation to destabilize the post fusion conformation of gp41. This prevents the post fusion six-helix bundle formation after the co-receptor has been engaged by gp120, effectively halting Env in a stable CD4bs exposed conformation (Kesavardhana and Varadarajan, 2014).

Additionally, techniques to design a peptide that mimics a pre-determined epitope recognized by bNAbs on Env has been described. The target epitope is grafted onto a scaffold protein to replicate the antigenic surface recognized by the bNAb of interest. This type of antigen is designed *in silico* by searching the Protein Data Bank for a suitable peptide with backbone similarity to segments of the Env-antibody epitope. The scaffold peptide selected is then modified to enhance epitope exposure but at the same time minimize non-epitope interactions with the antibody. The antibody binding epitope on Env is then transplanted into the acceptor scaffold resulting in a mimicry of the target epitope in its recognized conformation (Burton, 2010; Ofek et al., 2010; Zhou et al., 2014).

A further strategy is to use plasmid DNA encoding fully cleaved trimers as an antigen. The plasmid DNA contains the gene of interest as well as promoter/enhancer and transcript polyadenylation/termination sequences to allow expression of the gene in mammalian cells. NHP's vaccinated with plasmid DNA can elicit both the cellular and humoral arms of the immune system. Such DNA vaccines are usually boosted with an antigenic protein similar to the protein produced after expression from the DNA plasmid (Chakrabarti et al., 2013; Shiver et al., 1996).

1.9 Designing immunogens to elicit the desired immune response

The results from the HIV-1 vaccine trials, especially the RV144 trial, suggest that eliciting both arms of the adaptive immune response, one in which Abs are generated to inactivate the virus thereby aborting productive infection in

the host cell and the other in which HIV-1 specific $CD8^+$ T cell responses are elicited to control breakthrough infections, is the best approach (Picker et al., 2012).

1.9.1 How can you elicit bNAbs?

The ideal HIV-1 vaccine should be able to induce bNAbs that can neutralize a diverse range of HIV-1 subtypes (Moore et al., 2015) to prevent viral entry into cells as well as stop replication once cells have been infected.

The elicitation of a NAb response requires that: an immunogen binds to the appropriate naïve B-cell receptor to induce antibodies; autoreactive B cells are deleted by clonal deletion; and the immunization process drives affinity maturation in which somatic mutations result in high-affinity antibodies specific to its cognate antigen (Kwong et al., 2011). In the case of anti-HIV bNAbs, they are highly somatically mutated and the mutations typically occur in the complementarity determining region (CDR) loops and take years to develop (Klein et al., 2013).

By using the structure-based design of immunogens, specific epitopes can be presented to the immune system. However, this does not guide the affinity maturation process. A way to overcome this is could be to prime the immune response with a restricted epitope to stimulate the correct naïve B cell receptor (BCR) followed by boosts using the native form of Env (gp120 or gp140) to drive the affinity maturation process (Mascola and Haynes, 2013).

1.9.2 The bNAb problem

The anti-HIV-1 bNAbs isolated to date have all been found to target specific regions of Env (Halper-Stromberg and Nussenzweig, 2016; Tomaras et al., 2008). However native Env has proven to be poorly immunogenic mainly due to its conformational flexibility and its vast genetic diversity as well as the fact that extensive somatic hypermutations that evolve over time are

required (Kwong et al., 1998; Liao et al., 2013b; Rambaut et al., 2004; Robertson et al., 1995; Santoro and Perno, 2013; Wyatt and Sodroski, 1998).

The structural improvements discussed in section 1.7 attempt to overcome the flexibility by stabilizing Env, subunits of Env or important epitopes of Env that will focus the immune response and assist in affinity maturation of the humoral immune response. In addition, by focussing the immune response to conserved regions of Env, it is hoped to overcome the genetic diversity of Env at least within one subtype. The ability of various Env-based immunogens to elicit bNAbs in a small animal model is thus the focus of this thesis.

1.10 Justification

People infected with HIV in India and South Africa are predominantly infected with HIV-1 subtype C. Currently, approximately 7 million (6.7-7.4 million) and 2.1 million (1.7-2.6 million) people are HIV-1 infected in South Africa and India, respectively. In addition, South Africa and India had a combined new infection rate of approximately 466 000 in 2015, accounting for 1300 new HIV-1 subtype C infections daily (UNAIDS, 2017). Overall, South Africa and India account for 25% of the global burden of HIV-1 infection, thus targeting HIV-1 subtype C to prevent new infections would have a significant global impact on the disease. In this case, a vaccine that could target all HIV-1 subtype C infections would be required. A smaller proportion of HIV-infected individuals are infected with subtype B (approximately 11 %). However this subtype has a greater geographic distribution than subtype C (Hemelaar, 2012), and is the most widely studied. Our collaborators focused their research efforts on providing proof-of-concept of Env-based immunogens based on the subtype B isolate JRFL. We therefore included immunogenicity testing of these subtype B stabilized Env derivatives, as comparators in our study.

The use of antibody pools or plasma samples in resistance studies has found greater neutralization when assayed against a matched subtype-specific virus isolate compared to a non-matched isolate (Brown et al., 2008; Jacob et al., 2012; Seaman et al., 2010). This warrants the development of a subtype-specific vaccine.

In addition, our laboratory has recently shown potent and bNAb responses in rabbits immunized with an Env immunogen stabilized by the first 2 domains of CD4 (2dCD4) (Killick et al., 2015). This has raised an interest in utilizing the CD4 and CD4i epitopes as a target for the development of bNAbs. Further understanding of the antigenicity and immunogenicity of immunogens exposing this region may elucidate the biochemistry of a vulnerable site conserved amongst all HIV-1 subtype C Env that could be a target for a geographically specific vaccine.

Thus, the overall aim of this study was to evaluate and compare the antigenicity and immunogenicity of South African and Indian gp120/gp140GCN4(+) Env variants (unliganded and liganded with 2dCD4) and to compare their immunogenicity to gp120/gp140 Env derivative immunogens (proteins, peptides and DNA constructs).

The specific objectives of this study were:

a) To express, purify and biochemically characterize recombinant subtype C gp120/gp140GCN4(+) Env variants (2dCD4-liganded and 2dCD4-unliganded) of Indian and South African origin.
b) To select one representative from each region and evaluate the immunogenicity of a recombinant gp120/p140GCN4(+) Env variant (2dCD4-liganded and 2dCD4-unliganded) in rabbits, and determine the breadth and titres of NAb's elicited.
c) To evaluate the immunogenicity of structurally improved subtype B Env based immunogens using gp120/gp140 Env derivative

immunogens (proteins, peptides and DNA constructs) and determine the breadth and titres of NAbs elicited in rabbits.

d) To compare the immunogenicity of the vaccine immunogens tested in b and c.

Chapter 2 : Materials & Methods

2.1 Env selection and generation of recombinant gp120 and gp140 constructs

2.1.2 Selection of *env* and generation of recombinant gp120 and gp140 constructs

Full length HIV-1 *env* oligonucleotide sequences from patients infected with HIV-1 subtype C transmitted/founder viruses, originating from India (Kulkarni et al., 2009) and South Africa (Abrahams et al., 2009) were obtained and downloaded from the Los Alamos database (Unknown, 2016). These gp160 sequences were aligned in Clustal X (Larkin, 2007), extensively analysed and representative sequences were selected. A consensus sequence was designed for the South African patients selected, namely CAP210 (CAP210.1.00T and P), CAP45 (CAP45.1.05A and 1.05B) and 706010164 (706010164_A to 706010164_G) (Abrahams et al., 2009) (hereinafter designated ZACAP210, ZACAP45 and ZA706010164 to indicate South African origin). Sequences selected from Indian patients were 26191 (26191-2 clone 48), 25925 (25925-2 clone 22) and 25710 (25710-2 clone 43) (Kulkarni et al., 2009) (hereinafter designated IN26191, IN25925 and IN25710 to indicate Indian origin) (Table 2.1). These patients all acquired their HIV-1 infection through heterosexual contact. The three Indian sequences were selected based on resistance and sensitivity to neutralization to IgG1b12. Virus from patient IN26191 was susceptible to neutralization with IgG1b12, whereas IN25925 and IN25710 were resistant (Kulkarni et al., 2009).

These 6 *env* sequences formed the basis for the design of monomeric gp120 and matched gp140GCN4(+) constructs. Sequences were codon optimized for mammalian expression as either monomeric gp120, or as trimeric gp140GCN4(+) containing the gp120 envelope sequence, the gp41 ectodomain, with an intact cleavage site between the gp120 and gp41 ectodomains, plus the addition of a 32 amino acid form of the GCN4

transcription factor (Harbury et al., 1993) fused to the carboxyl terminal of the gp41 ectodomain that allows for trimerization of the recombinantly expressed Env. A heterologous human CD5 antigen leader peptide sequence (NH3-MGMGSLQPLATLYLLGMLVASVLA-COOH) was used to replace the native N-terminal Env leader peptide to increase Env protein expression in mammalian cell culture (Aruffo et al., 1990; Haas et al., 1996; Killick et al., 2015).

Table 2.1: Clinical and demographic information of the Indian and South African patients (infected via heterosexual transmission) from which HIV-1 subtype C envelope glycoprotein (Env) sequences were selected for design of monomeric and trimeric Env constructs.

Participant ID	Gender	Age	Geographical location	Fiebig stage[1]	Sampling date	Viral load (RNA copies/ml)	Nucleotide Accession numbers
ZACAP45	Female	41	South Africa	I/II	04/2005	12,500	FJ443292-297
							FJ443149-158
ZACAP210	Female	43	South Africa	I/II	05/2005	468,000	FJ443186-195
							FJ443509-510
							FJ443307-315
ZA706010164	Male	27	South Africa	IV	08/2007	23,600	FJ444058-076
IN25925	Male	NA[2]	India	I – V	09/1999	616,531	EF117273
IN26191	Male	NA	India	I – V	05/2000	5,346,070	EF117274
IN25710	Male	NA	India	I – V	03/1999	3,523	EF117271

1. Fiebig laboratory stages of HIV infection (days post infection): Stage I (5 – 10 days); Stage II (7 – 13 days); Stage III (10 – 17 days); Stage IV (15 – 22 days); Stage V (47 – 129 days); Stage VI (open-ended) (Fiebig et al., 2003).
2. NA, not available

The codon optimized nucleotide sequences were synthesized and sub-cloned into the pcDNA3.1 mammalian expression vector by GeneArt (Regensburg, Germany). The pcDNA™3.1 vector expresses the HIV-1 Env using the human cytomegalovirus (CMV) enhancer-promoter with a bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence for enhanced mRNA stability. It contains the ampicillin resistance

gene and pUC origin for selection and maintenance in *Escherichia (E.) coli* (Invitrogen, Life Technologies, Carlsbad, CA, USA).

2.2 Expression and purification of the recombinant Env and recombinant 2dCD4 proteins

The six gp120 and six matched gp140GCN4(+) constructs were purified and used for large scale expression of the recombinant Envs in mammalian cells, followed by purification.

2.2.1 Transformation of recombinant Env plasmid DNA into *E. coli* DH5α cells

The lyophilized pcDNA™3.1-containing *env* constructs from GeneArt were diluted with 50 µl sterile water to a final concentration of 0.1 µg/µl. Chemically competent *E. coli* DH5α (Invitrogen, Life Technologies, Carlsbad, CA, USA) cells were incubated with 0.2 µg of DNA on ice for 20 minutes. The cells were then heat shocked at 42 °C for 1 minute and immediately returned to ice for 2 minutes, before adding 100 µl sterile Luria Bertani (LB) broth (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)). Transformed *E. coli* DH5α were cultured on selective LB agar plates (35 g/l LB Agar (Sigma-Aldrich, St. Louis, MO, USA) containing ampicillin (Roche, Mannheim, Germany) at a final concentration of 100 µg/ml (1000 x ampicillin stock solution (100 mg/ml) made up in 70% [v/v] ethanol (Merck-Millipore, Darmstadt, Germany) after incubation overnight at 37 °C.

2.2.2 Recombinant Env plasmid isolations

Recombinant plasmids were purified from the transformed *E.coli* DH5α using the Maxi plasmid purification kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Briefly, a single colony was selected from the selective ampicillin-containing LB plate and was used to inoculate a 5 ml starter culture of LB medium containing ampicillin (100 µg/ml). The culture

was incubated for approximately 8 hours at 37 °C with vigorous shaking in a high-rotation, shaking incubator. The starter culture was then diluted (1:1000) in a sterile, one liter, Erlenmeyer flask containing 250 ml ampicillin-supplemented (100 µg/ml) LB broth and incubated at 37 °C for a further 12 – 16 hours with vigorous shaking in a high-rotation, shaking incubator.

Bacterial cells were harvested by centrifugation at 6,000 x g for 15 minutes at 4 °C and re-suspended in 10 ml buffer P1 containing RNase A. The cells were lysed in an alkaline lysis buffer by adding 10 ml buffer P2 (NaOH-SDS) and incubating at room temperature for 15 minutes. Ten milliliters of chilled buffer P3 (acidic potassium acetate) was added to the lysate to neutralize it and incubated for 20 minutes to allow for precipitation of the potassium dodecyl sulphate. The bacterial chromosomal DNA, cellular debris and proteins, which become trapped in the salt-detergent complexes, were removed from the plasmid DNA by centrifugation at 16,000 x g for 10 minutes. The plasmid DNA, which remains in the supernatant, was loaded onto a pre-equilibrated, anion-exchange chromatography Qiagen-tip and was bound to the resin by gravity flow. Any remaining contaminants were removed by washing twice with 30 ml buffer QC (a medium-salt buffer). The plasmid DNA was eluted from the resin with 15 ml buffer QF (high salt buffer). The plasmid DNA was precipitated by centrifugation with 0.7 volumes of isopropanol (Merck-Millipore, Darmstadt, Germany) in approximately 7.0 ml volumes, at 9,200 x g for 30 minutes at 4 °C. Any residual salt was removed from the pellet by washing with 70 % ethanol [v/v], which was then removed by centrifugation at 9,200 x g for 10 min in a microcentrifuge and discarding the supernatant. After air-drying the DNA pellet, the purified plasmid DNA was re-suspended in sterile distilled water. The yield of the plasmid DNA was determined by UV absorption at A_{280nm} using a Nanodrop ND 1000 spectrophotometer (ThermoFischer Scientific, Waltham, MA, USA) and stored in 500 µg/ml aliquots at -20 °C.

2.2.3 Expression of recombinant monomeric and trimeric Env

2.2.3.1 General mammalian cell culture

Mammalian cells used included the HEK293T cell line (available in our laboratory) which was used for the transient expression of the recombinant Env glycoproteins and the generation of HIV-1 pseudoviruses, the 293F suspension cell line (Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) which was used for generating stable cell lines expressing Env and the TZM-bl (JC53-bl) reporter cell line (Dr John C. Kappes, Dr Xiaoyun Wu and Transzyme Inc.), (obtained from the NIH AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH, contributor in parenthesis) which were used to determine the $TCID_{50}$s of pseudoviruses and for pseudovirion neutralization assays. TZM-bl is a HeLa cell clone engineered to express CD4 and CCR5, making it highly sensitive to most strains of HIV infection. It also contains separate integrated copies of the *Photinus pyralis* (firefly) luciferase and *E. coli* ß-galactosidase genes under control of the HIV-1 long terminal repeat (LTR) promoter (Platt et al., 1998; Wei et al., 2002).

Unless otherwise stated, all mammalian cell culture was carried out at 37°C in a humidified, water jacketed incubator (ThermoFischer Scientific, Waltham, MA, USA) and the atmospheric CO_2 was maintained at 5 % (Medical CO_2, Afrox, Selby, South Africa).

HEK293T mammalian cells used to transiently express recombinant Env and TZM-bl cells used for pseudovirion assays were maintained in Dulbecco's modified Eagles Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) (Gibco™, Thermo Fisher Scientific Inc, Waltham, MA) and 2 mM GlutaMax (Gibco™, Thermo Fisher Scientific Inc, Waltham, MA), referred to as complete DMEM. Cryopreserved cells were thawed rapidly at 37 °C and seeded in a T25 tissue culture flask (Nunc, Roskilde, Denmark) and made up to a final volume of 10 ml of supplemented DMEM medium as described above. Cells

were examined for general cell health and microbial contamination by microscopic observation and passaged twice a week. Passaging the cells was done by removing the spent media, followed by washing the cells with 1 x Dulbecco's Phosphate Buffered Saline (PBS, without calcium and magnesium) to remove spent media and fetal calf serum. This was followed by treatment with trypsin/EDTA solution (0.25% [w/v] trypsin and 0.02% [w/v] ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, MO, USA)) and incubated at 37 °C for a maximum of five minutes to release the cells from the vessel surface. Complete DMEM was used to mechanically wash any remaining cells off the vessel surface. One-tenth of the cells were re-seeded in the vessel and the remaining cells discarded and replaced with fresh complete DMEM media. Cells were enumerated by staining with 0.4% Trypan Blue solution (Sigma-Aldrich, St. Louis, MO, USA) (1:5 ratio) to determine viability and were manually counted by microscopic examination using a Neubauer haemocytometer (Roth, Karlsruhe, Germany). HEK293T and TZM-bl cells were sub-cultured up to passage 30 and then discarded.

FreeStyle™ 293-F cells (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) were used to generate stable cell lines for large scale expression of the recombinant Env proteins. These cells have been adapted for suspension culture and were maintained in FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). Cryopreserved cells were thawed rapidly at 37 °C, seeded in a T25 tissue culture flask (Nunc, Roskilde, Denmark) and made up to a final volume of 10 ml in FreeStyle™ 293 Expression Medium (Gibco, Life Technologies, Carlsbad, CA, USA). Cells were examined for general cell health and microbial contamination by microscopic observation and passaged twice a week. Sub-culturing of the cells was done by re-suspending the cells in their spent media by pipetting action, and one-tenth of the cells volume was re-seeded in the vessel and fresh media was used to replace the discarded media. Cells were enumerated by staining with 0.4% Trypan Blue solution (Sigma-Aldrich, St. Louis, MO, USA) (1:5 ratio) to determine viability and were manually

counted by microscopic examination using a Neubauer haemocytometer (Roth, Karlsruhe, Germany). FreeStyle™ 293-F cells were sub-cultured to passage 30 and then discarded.

Cryogenic preservation of cells was performed for long-term storage of HEK293T cells, TZMbl cells and 293F stable cell lines by preparing a sub-cultured cell suspension concentration of 1 to 2 x 10^6 cells/ml in fresh medium used for the particular cell type, and freezing in a final volume of 10% [v/v] dimethyl sulphoxide (DMSO) (Fluka Chemika, Buchs, Switzerland). The cells were stored 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.

2.2.3.2 Expression of the recombinant Env

Each of the 12 recombinant Env proteins (six gp120 and six matched gp140GCN4(+)) were expressed in HEK293T mammalian cells by transient transfection using Polyfect® Transfection Reagent (Qiagen), as per the manufacturer's instructions. Optimal transfection conditions were determined. Briefly, 24 hours prior to transfection, T175 flasks were seeded with 8.0 – 8.5 x 10^6 cells in complete DMEM. Polyfect (125 µl) was added to 25 µg plasmid DNA in a total volume of 500 µl OptiMEM media (Gibco™, Thermo Fisher Scientific Inc, Waltham, MA). Samples were mixed and incubated for 5 minutes to allow complex formation, after which they were added to the cells. Approximately 8 hours post transfection, the media was replaced with serum free media (SFM II, Gibco™, Thermo Fisher Scientific Inc, Waltham, MA), supplemented with a final concentration of 2 mM GlutaMax (Gibco™, Thermo Fisher Scientific Inc, Waltham, MA) that was warmed to 37°C prior to use. The spent media was collected daily for approximately seven days and the supernatant was harvested by centrifugation at 1,000 x g for 5 minutes, filtered (0.45 µm Low protein

binding, Acrodisc® Syringe Filters with Supor® Membrane, PALL Corporation) and stored at -20°C until purification.

In addition, stable cell lines using the 293F suspension cells (Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) were generated for these recombinant proteins in order to express them on a large scale. Each recombinant plasmid was used to express the recombinant proteins in FreeStyle™ 293-FS cells (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). Polyfect® Transfection Reagent (Qiagen) was used to transfect the cells as per the manufacturer's instructions. Briefly, 24 hours prior to transfection, T25 flasks were seeded with 1.2 x 10^6 cells in FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). Polyfect (25 µl) was added to four micrograms of plasmid DNA in a total volume of 500 µl Serum Free Media (SFMII) (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). Samples were incubated for 5 minutes to allow complex formation, after which they were added to the cells. Approximately 8 hours post transfection, the media was replaced with FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). A final concentration of 500 µg/ml Geneticin® selective antibiotic (G418 Sulphate, Gibco™, Thermo Fisher Scientific Inc, Waltham, MA) was used to select stable cells expressing the recombinant proteins. Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; see section 2.5.2) and Western blot (see section 2.5.3) analysis was performed to confirm expression of the recombinant proteins. For large scale expression of Env, stably transfected cell lines were transferred to roller flasks. Approximately 6.0 x 10^6 stably transfected cells were seeded in a 1,700 cm^2 Roller Bottle (Corning® CellBIND® Cell Culture Surface) in a final volume of 100 ml FreeStyle™ 293 Expression Medium supplemented with 250 µg/ml G418 Sulphate. The spent media was collected every second day for approximately one month and the supernatant was harvested by centrifugation at 1,000 x g for 5 minutes, filtered (0.45 µm sterile, low protein

binding, Acrodisc® Syringe Filters with Supor® Membrane, PALL Life Sciences) and stored at -20°C until purification.

2.3 Purification of Env

2.3.1 Primary purification of Env by lectin-affinity chromatography

The recombinant protein was pre-purified from the filtered culture media using lectin-affinity chromatography. A volume of up to 1000 ml of pooled media for each recombinant protein was incubated separately with 4 ml of lectin-agarose conjugated resin from *Galanthus nivalis* (snowdrop) (Sigma; St Louis, MO) overnight with continuous stirring at 4°C. The slurry was loaded onto a luer-lock, non-jacketed liquid chromatography column (Dimensions: internal diameter (i.d.) 2.5 × 10 cm) (Sigma-Aldrich, St. Louis, MO, USA) to collect the recombinant Env-bound resin. The flow through was retained and re-incubated with the resin overnight for a second round of purification to ensure complete capture of expressed Env. All buffers used for washing and elution were maintained on ice. The resin was washed manually with 50 column bed volumes (200 ml) of 0.65 M NaCl in Dulbecco's 1 x PBS (Sigma-Aldrich, St. Louis, MO, USA), followed by 50 column bed volumes (200 ml) of 1 M NaCl in Dulbecco's 1 x PBS. A final wash step was performed using 25 column bed volumes (100 ml) of Dulbecco's 1 x PBS. The column was inverted in-between each wash step to re-suspend the resin. The recombinant Env proteins were eluted from the resin, using 6.25 column bed volumes (25 ml) of Dulbecco's 1 x PBS containing 1.0 M methyl α-D-mannopyranoside (MMP) (Sigma-Aldrich, St. Louis, MO, USA). The eluted recombinant Env protein was concentrated using an Amicon® Ultra Centrifugation Filter Unit (Millipore, Billerica, MA, USA) with a 50 kDa molecular weight cut-off to approximately 1 ml in volume and washed five times (40 ml per wash) with Dulbecco's 1 x PBS. The partially purified recombinant Env was snap-frozen in liquid nitrogen and stored at -80˚C for further purification by Size Exclusion chromatography (SEC).

2.3.2 Secondary purification of Env by SEC

To separate the monomers and trimers from any aggregates from the lectin-purified recombinant Env eluate, they were further purified using SEC with the ÄKTA FPLC (fast protein liquid chromatography) platform (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The lectin purified Env was thawed on ice and centrifugation was performed (16,000 x g for 10 minutes) to remove any particulate matter. Initially, protein purification was carried out using a size exclusion column (Dimensions: i.d. 2.5 × 50 cm) that was packed according to the manufacturer's instructions with Superdex 200 prep-grade resin (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Following further optimization, a pre-packed column (HiLoad 16/600 Superdex 200 prep grade, GE Healthcare Life Sciences) was obtained and used in all subsequent experiments. Env was loaded onto the column and resolved at 1 ml/min in sterile, filtered (0.45 µm) Dulbecco's 1 x PBS. Fractions were collected to obtain the monomeric gp120 and trimeric gp140GCN4(+) forms of the recombinant Env proteins (Guo et al., 2013). The column was cleaned in-between each different Env purification using the cleaning protocol recommended by the manufacturer. Briefly, the column was cleaned with one-half column volume of 0.5 M NaOH (Sigma-Aldrich, St. Louis, MO, US) at a flow rate of 1.5 ml/min to remove non-specifically bound proteins from the resin, and equilibrated with two to three column volumes of sterile, filtered (0.45 µm) Dulbecco's 1 x PBS at a flow rate of 1.5 ml/min until the UV baseline stabilised. The purity of the purified protein was assessed by SDS-PAGE (section 2.5.2) and quantified using a BCA™ Protein Determination Kit as described in section 2.5.1.

2.4 Expression and purification of recombinant 2dCD4$^{Wt/S60C/FD}$ variants

A soluble CD4 protein containing the first two domains of CD4 (2dCD4), in which the serine at position 60 is substituted with cysteine (S60C) was used to generate gp120/2dCD4^{S60C} and gp140/2dCD4^{S60C} complexes. The S60C mutation allows for the complex to be stabilized through an inter-molecular

disulphide formation, and it has previously been shown in our laboratory to have a higher binding affinity to gp120 than wild-type 2dCD4 (Cerutti et al., 2010). A wild-type 2dCD4 ($2dCD4^{Wt}$) and folding-defective 2dCD4 ($2dCD4^{FD}$) were used as controls. The recombinant pET15-2dCD4 (Wt/S60C/FD) vectors (available in our laboratory) were expressed in *E. coli* BL21 (DE3) using a "leaky", IPTG-free (isopropyl-beta-D-thiogalactopyranoside) induction protocol (Cerutti et al., 2010). Freshly transformed bacteria were cultured in standard LB broth medium (10 g/l tryptone (Merck, Darmstadt, Germany); 5 g/l yeast extract (Merck-Millipore, Darmstadt, Germany) and 5 g/l sodium chloride (NaCl) (Sigma-Aldrich, St. Louis, MO, USA)) containing 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) at 37 °C with vigorous shaking until the absorbance at A600 nm was between 0.6 and 0.8, cultures were then transferred to a 20°C incubator for a further 20 hours to allow for protein expression. The recombinant $2dCD4^{S60C}$, $2dCD4^{Wt}$ and $2dCD4^{FD}$ were purified using a denaturing purification protocol and refolded in glutathione-containing buffers as described previously (Cerutti et al., 2010). Briefly, cells were collected by centrifugation (6,000 x g for 30 minutes, 4°C) and re-suspended in a minimum of 25 ml sterile Dulbecco's 1 x PBS per litre of culture. The cells were lysed by incubating with lysozyme (0.5 mg/ml chicken egg lysozyme (53,000 units/mg) (Sigma-Aldrich, St. Louis, MO, USA) on ice for 1 hour with gentle shaking, followed by three freeze/thaw cycles using liquid nitrogen and three x 1 minute sonication cycles at 1.9 kilojoules/min sonication on ice (Sonopuls, Bandelin, Berlin, Germany). The inclusion bodies containing the expressed 2dCD4 protein were pelleted by centrifugation (20,000 x g for 45 minutes, 4°C) and resuspended thoroughly using a tissue homogenizer (Omni International, GA USA) in 25 ml solubilisation buffer ((Dulbecco's 1 x PBS (pH 7.4) containing 8 M urea (Sigma-Aldrich, St. Louis, MO, USA), 50 mM glycine (Sigma-Aldrich, St. Louis, MO, USA), 2 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 20 mM

imidazole (Merck-Millipore, Darmstadt, Germany) and 0.5 M NaCl (Sigma-Aldrich, St. Louis, MO, USA)). After a 30 minute incubation period at room temperature with gentle shaking to allow unfolding of the 2dCD4, the remaining cellular debris was removed by a second round of centrifugation (20,000 x g for 30 minutes, 4°C). The supernatant containing the solubilized 2dCD4 proteins were filtered (0.45 µm Low protein binding, Acrodisc® Syringe Filters with Supor® Membrane, PALL Corporation) before purification by immobilized metal ion affinity chromatography.

Standard Ni^{2+}-affinity chromatography was used to purify the recombinant 2dCD4s. The supernatant was incubated with 1 ml of Ni^{2+} charged resin overnight at 4°C with stirring. The slurry was loaded onto a luer-lock, non-jacketed liquid chromatography column (Dimensions: internal diameter (i.d.) 2.5 × 10 cm) (Sigma-Aldrich, St. Louis, MO, USA) to collect the recombinant 2dCD4-bound resin and washed manually with 250 column bed volumes (250 ml) of wash buffer 1 ((PBS (pH 7.4) containing 8 M urea, 50 mM glycine, 2 mM β-mercaptoethanol, 20 mM imidazole and 0.5 M NaCl)). This was followed by 250 column bed volumes (250 ml) of wash buffer 2 ((PBS (pH 7.4) containing 8 M urea, 50 mM glycine, 2 mM β-mercaptoethanol, 50 mM imidazole and 0.5 M NaCl)). The recombinant 2dCD4 protein was eluted from the resin in 2 x 10 ml elution buffer ((PBS (pH 7.4) containing 8 M urea, 50 mM glycine, 2 mM β-mercaptoethanol, 500 mM imidazole and 0.5 M NaCl)) with a five minute incubation period at room temperature for each elution.

The eluted recombinant 2dCD4 was refolded overnight at 4°C by dialysis using SnakeSkin® dialysis tubing, 10 kDa MWCO, 35 mm dry i.d. (ThermoFischer, Rockford, IL, USA) against 1 litre of folding buffer A (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) and 4 M urea (Sigma-Aldrich, St. Louis, MO, USA), adjusted to pH 9.6

with NaOH). The following day folding buffer A was replaced with folding buffer B (50 mM sodium carbonate (pH 9.6) (BDH, Merck-Millipore, Darmstadt, Germany), 10% sucrose, 1 mM EDTA, 0.1 mM GSH and 0.01 mM GSSG) for a second round of dialysis overnight at 4°C. The final dialysis step was performed against 1 litre volumes of Dulbecco's 1 x PBS (pH 7.4) three times for a minimum of 2 hours for the first two dialysis steps and overnight for the final dialysis step. All buffers were cooled to 4°C before use. The purified recombinant 2dCD4s were recovered and any precipitate was removed by centrifugation (3,200 x g for 10 minutes, 4°C) and filtration (0.45 µm Low protein binding, Acrodisc® Syringe Filters with Supor® Membrane, PALL Corporation). The recombinant 2dCD4s were concentrated using a centrifugation filter device (Amicon® Ultra Centrifugation Filter Unit with a 10 kDa molecular weight cut-off), snap frozen and stored at -80°C. The purity of the each recombinant 2dCD4 was assessed by SDS-PAGE as described in section 2.5.2 and quantified using a BCA™ Protein Determination Kit as described in section 2.5.1.

2.5 Protein biochemical characterisation

2.5.1 Total protein concentration determination

The purified protein was quantified using a BCA™ Protein Determination Kit (Pierce, ThermoFischer, Rockford, IL, USA), as per the manufacturer's instructions. Briefly, a set of protein standards was prepared by serially diluting the 2 mg/ml Albumin Standard (containing bovine serum albumin, supplied with the kit) two-fold in Dulbecco's 1 x PBS to obtain a range of eight concentrations from 500 µg/ml to 7.8125 µg/ml, and a 0 µg/ml blank standard. Twenty-five microliters of each standard and the protein to be assayed was transferred into a flat bottomed, 96-well, polysorb Immuno plate (Nunc, Roskilde, Denmark) well in duplicate. The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B, and 200 µl of this working reagent was added to each well that contained standard or protein sample. The microplate was incubated at 56 °C on an orbital shaker (THERMOstar Microplate incubator, BMG Labtech,

Ortenberg, Germany) at 300 rpm for 30 minutes. The microplate was allowed to cool to room temperature and absorbance was determined at 570 nm using a microplate reader (BioRad Model 680, BioRad, Hercules, CA, USA). A standard curve was prepared using the A_{570nm} readings obtained and the protein concentration of the unknown samples was determined by interpolating the A_{570nm} against the standard curve.

2.5.2 SDS-PAGE

The expression of the recombinant Env by transiently transfected cells and stably transfected cells lines was confirmed by SDS-PAGE followed by Western blot analysis (2.5.3 below). Mammalian cell culture supernatants containing the Env were mixed with 2 x Laemmli sample buffer (0.125 M Tris-HCl (Tris - (hydroxymethyl) aminomethane) (Sigma-Aldrich, St. Louis, MO, USA), 4% [w/v] 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.) and boiled for 5 minutes in a water bath. The samples were briefly centrifuged and loaded onto a 10 % SDS-PAGE gel (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 separation. Gel electrophoresis was performed in tank buffer (0.025 M Tris-HCl (Tris - (hydroxymethyl) aminomethane) (Sigma-Aldrich, St. Louis, MO, USA), 0.192 M glycine (Sigma-Aldrich, St. Louis, MO, USA), 0.1 % SDS, pH 8.3) at 10 mA until the samples had migrated through the stacking gel, after which the current was increased to 20 mA.

2.5.3 Western blot

Clarified serum from an HIV-1 infected patient (obtained from the SANBS, presumably infected with HIV-1 Subtype C) was used as the primary antibody in the relevant Western blots.

Once resolved, the SDS-PAGE gel was equilibrated 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 for approximately 10 minutes. The samples were transferred from the SDS-PAGE gel onto a nitrocellulose membrane (Hybond-C extra, Amersham, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 400 mA for 1 hour-20 minutes, using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Hercules, CA, USA). The nitrocellulose membrane was incubated overnight at 4°C 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 5 % fat free milk powder to block non-specific binding sites. The membrane was probed with 1:2,000 dilution of serum from an HIV-1 infected patient in T-TBS. The membrane was washed 5 times, each wash being five minutes long, in T-TBS on an orbital shaker to remove non-specific antibodies. A 1:25,000 dilution of secondary antibody (goat anti-human IgG (Fc specific) alkaline phosphatase conjugated antibody (Sigma-Aldrich, St. Louis, MO, USA) was used to detect bound antibodies. After washing (5 x five minute washes in T-TBS) to remove unbound probes, the membrane was incubated in 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, for approximately 10-20 minutes in the dark, at room temperature, until a precipitation reaction resulted in clear visualization of bands. The remaining BCIP/NBT substrate was rinsed off with water to terminate the reaction.

2.5.4 Surface Plasmon Resonance (SPR)

2.5.4.1 Reagents used for SPR

The panel of CD4bs directed mAbs used for Surface Plasmon Resonance (SPR) were obtained from the NIH AIDS Research and Reference Reagents Program (contributor in parenthesis). HIV-1 monoclonal antibodies used include IgG1b12 (Dr. Dennis Burton and Carlos Barbas (Barbas et al., 1992; Burton et al., 1991; Roben et al., 1994)), VRC01 (Xueling Wu, Zhi-Yong Yang, Yuxing Li, Gary Nabel, John Mascola (Wu et al., 2010)), HJ16 (Dr. Antonio Lanzavecchia (Corti et al., 2010; Pietzsch et al., 2010)), VRC-CH31 (Duke Human Vaccine Institute, Duke University Medical Center (Bonsignori et al., 2012)) NIH45-46^{G54W} IgG (Pamela Bjorkman (Diskin et al., 2011)), and VRC03 (Xueling Wu, Zhi-Yong Yang, Yuxing Li, Gary Nabel, John Mascola (Wu et al., 2010)). In addition, the quaternary-structure-specific antibody directed to the V1V2 region of Env, PGT145 was kindly obtained from Prof Raghavan Varadarajan, (Indian Institute of Science, Bangalore, India). All reagents used for SPR were ProteON™ reagents supplied by BioRad, with the exception of the PBS running buffer.

All 12 purified recombinant Envs were biochemically characterized to confirm they were functional (binding to CD4) and conformationally intact (binding to a conformational dependent monoclonal antibody (MAb) and CD4bs directed mAbs) by SPR. The antibody and CD4 interaction studies of the recombinant Env proteins were investigated on a ProteOn XPR36 protein interaction array system (Bio-Rad Laboratories, Hercules, CA, USA) at 25 °C in PBS-T running buffer (1 x PBS (274 mM NaCl; 54 mM KCl; 20.29 mM Na_2HPO_4; 3.53 mM KH_2PO_4) autoclaved and filtered (0.45 µm) and supplemented with 0.05 % Tween 20).

2.5.4.2 Env/2dCD4$^{Wt/S60C}$ interactions

The ProteON™ instrument was used to determine binding kinetics of the 2dCD4-Env interactions. A ProteOn™ GLM Sensor Chip was activated by injecting a freshly prepared 1:1 solution of NHS (N-hydroxysuccinimide) and EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). 2dCD4^{Wt} and 2dCD4^{S60C} were covalently captured onto separate flow cells on the chip at a concentration of 0.02 mg/ml in 10 mM sodium acetate buffer pH 4.5 at a flow rate of 30 µl/min for 300 s to a final response units (RU) of 2000. One Molar ethanolamine chloride was injected at a flow rate of 30 µl/ml for 300 s to deactivate any residual active sites. The six gp120 (IN25925, IN26191, IN25710, ZACAP45, ZACAP210 and ZA706010164) as well as six gp140GCN4(+) (IN25925, IN26191, IN25710, ZACAP45, ZACAP210 and ZA706010164) were passed over the ligands at a flow rate of 30 µl/min for 300 s with a dissociation time of 600 s. Binding kinetics was calculated for the different Env's by injecting a serial dilution of five concentrations over the ligands. The concentrations used were such that the highest Env concentration resulted in a RU of approximately 200 and varied between the different Env's in a range of 30 nM to 3000 nM. The chip was regenerated after each Env injection using 4mM $MgCl_2$ at a flow rate of 100 µl/min for 18 s.

2.5.4.3 Antibody-Env interactions

Binding kinetics of the recombinant Env to CD4bs monoclonal antibodies and the quaternary-structure-specific antibody PGT145 was performed using the ProteON™ XPR36 platform. A ProteOn™ GLC Sensor Chip, (Bio-Rad Laboratories, Inc) was activated by injecting a freshly prepared 1:1 solution of NHS (N-hydroxysuccinimide) and EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). Approximately 2000 response units (RU) of each antibody (IgG1b12, VRC01, HJ16, VRC-CH31, NIH45-46^{G54W}, VRC03 and PGT145) was covalently captured onto separate flow cells on the chip at a concentration of 0.02 mg/ml in 10 mM sodium acetate buffer pH 4.5 and a flow rate of 30 µl/min for 300s. One Molar ethanolamine

chloride was injected at a flow rate of 30 µl/ml for 300 s to deactivate any residual active sites. The six gp120 (ZACAP45, ZACAP210, ZA706010164, IN26191 and IN25925 and IN25710) as well as six gp140GCN4(+) (ZACAP45, ZACAP210, ZA706010164, IN25925 and IN25710) were passed over the bound antibodies at a flow rate of 30 µl/min for 300 s with a dissociation time of 600s. The chip was regenerated in between Env batches using 4 mM $MgCl_2$ at a flow rate of 100 µl/min for 18 s. The concentrations of each Env passed over the coupled antibodies were initially started at 500 nM and adjusted according to binding. If binding of the recombinant Env glycoprotein and antibody was observed, and a sufficient quantity of Env was available, a set of 5 serially diluted concentrations of the Env glycoprotein was injected over the antibodies to calculate binding kinetics. The concentrations used were such that the highest Env concentration resulted in a RU of approximately 200 and varied between the different Env's in a range of 0.9 nM to 1500 nM. In some cases (gp120 IN25710) the flow rate was adjusted to 65 µl/min for 160s with a dissociation time of 600s.

2.5.4.4 SPR data analysis

The binding kinetic data obtained from SPR experiments was fitted to a 1:1 Langmuir model using the ProteOn Manager software, version 3.1.0.6 (Bio-Rad Laboratories Inc., CA, USA).

2.6 Generation of Env/2dCD4 complexes

Two protocols were followed for the generation of the Env/2dCD4 complexes, one in which ß-mercaptoethanol was used to generate the complex and one without ß-mercaptoethanol. The two protocols were followed in order to determine whether the presence or absence of a low concentration of reducing agent made a difference in the formation of the inter-molecular disulphide bridge.

The Env/2dCD4 complexes were generated by incubating 1 mg of Env glycoprotein with 1 mg of 2dCD4 with a final concentration of 1 mM ß-mercaptoethanol for one hour at room temperature. For complexes generated without ß-mercaptoethanol, the same protocol was followed but the ß-mercaptoethanol was omitted. Any precipitate formed was removed by centrifugation (16,000 x g for 10 minutes, 4°C). Any unbound 2dCD4 was removed from the complex using Size Exclusion Chromatography (as described in section 2.3.2). Briefly, the Env/2dCD4 complex was loaded onto a pre-packed column (HiLoad 16/600 Superdex 200 prep grade, GE Healthcare Life Sciences) and resolved at 1 ml/min in sterile, filtered (0.45 µm) Dulbecco's 1 x PBS. Peak fractions corresponding to the Env used in the complex were collected and concentrated using a centrifugation filter device (Amicon® Ultra Centrifugation Filter Unit with a 50 kDa molecular weight cut-off). The purified Env/2dCD4 complex was snap frozen and stored at -80°C. The purity of the complexed protein was assessed by SDS-PAGE (see section 2.5.2) and quantified using a BCA protein assay (Pierce, ThermoFischer, Rockford, IL, USA), as per the manufacturer's instructions (section 2.5.1).

2.7 Immunogenicity study of recombinant monomeric, trimeric envelope glycoproteins (Env) and Env/2dCD4 complexes

Following biochemical characterization, gp120/gp140GCN4(+)$_{ZACAP45}$ and gp120/gp140GCN4(+)$_{IN25925}$ were expressed and purified on a large scale required for immunizations.

The following stabilized gp120/gp140 derivative immunogens (proteins, peptides and DNA constructs) were received from our collaborators ((Professor Virander Chauhan, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India; and Prof Raghavan Varadarajan, Indian Institute of Science, Bangalore, India)), as part of the South African Department of Science and Technology/TIA India-SA bilateral on HIV Vaccine Development:

i. Cyc4OD gp120, a cyclic permutant based on HIV-1 subtype B JRFL, in which the N- and C- termini were joined with a GSAG linker and the V4 loop was removed to create new N and C termini.
ii. $gp120^{JRFL}$ cyclic permutant protein (h-CMP V1cyc 144-142), a cyclic permutant based on HIV-1 subtype B JRFL, in which the native N and C-termini of gp120 were joined with a linker, and the V1 loop was removed to create new N and C termini at residues 144 and 142, respectively. The h-CMP trimerization domain was used to extend the termini and stabilize gp120 in a trimeric conformation.
iii. OD_{EC}COBPICS gp120, an outer domain fragment based on a consensus sequence of subtype B viruses.
iv. DNA immunogen consisting of wild type gp120 based on HIV-1 subtype B, JRFL (Wt-JRFL-Env DNA)
v. DNA immunogen consisting of a trimer based on HIV-1 subtype B JRFL, stabilized by a V570D mutation in the N-heptad repeat region of gp41 (JRFL Env-570D DNA)
vi. DNA immunogen consisting of an uncleaved trimer based on HIV-1 subtype B JRFL (JRLF Env-SEKS DNA).
vii. 1WR2, a monomeric scaffold peptide from *Pyrococcus horikoshii* with the VRC01 epitopes engrafted on it.
viii. 1ORC, a monomeric scaffold peptide of the Cro protein from bacteriophage lambda with the VRC01 epitopes engrafted on it.

The $gp140^{FV}$ Env glycoprotein ($gp140^{FV}$GCN4t(+)) has been described previously and was available in our laboratory (Killick et al., 2015).

All immunogens were formulated with Adjuplex™ (Advanced BioAdjuvants, Omaha, Nebraska, USA) from a concentrated starting solution according to manufacturer's instructions. Adjuplex™ adjuvant is a nanoliposomal mixture of carbomer homopolymer and highly purified natural phospholipids.

2.7.1 Rabbit Immunizations

New Zealand White Rabbits (*Oryctolagus cuniculus*) ranging from 3 to 12 months of age (1.7 – 4.1 kg) were provided by the Central Animal Service (CAS), University of the Witwatersrand, Johannesburg, South Africa. The rabbits were housed individually at CAS, given food and water *ad libitum* and all immunization procedures and care of the rabbits were carried out by the CAS staff. Rabbits were allowed to acclimatise to their experimental housing environment for a minimum of one week prior to immunizations.

Animal ethics clearance certificates (2010/12/03/A; 2010/12/03C and 2015/51/B) (Appendix A) were obtained for the immunizations by the Animal Ethics Screening Committee, University of the Witwatersrand), and all animal work was conducted in strict accordance with University and National/International Recommendations.

Three to five rabbits were assigned to each group and groups were matched for gender, age and weight where possible. Rabbits were immunized intramuscularly in their hindquarters, (alternating between the left and right hindquarter for each immunization) per immunogen as indicated in Table 2.2 and 2.3 below. Blood was drawn from the rabbit's marginal ear vein by venipuncture prior to immunization as a pre-bleed negative control, and post-immunization bleeds were collected at 4 week intervals, starting at week 2 post immunization, so that each bleed was performed 2 weeks after an immunization. Approximately 10 ml of whole blood was drawn from each rabbit per bleed and collected in a serum tube (BD Vacutainer® red-capped tubes, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Terminal bleeds were carried out at week 14 for experiment 1; week 22 for groups 1-4 in experiment 2; and week 30 for groups 5 – 8 in experiment 2 (see Table 2.2 and 2.3). Four to five x 10 ml tubes of whole blood were collected per rabbit, followed immediately by euthanasia with 1 ml/kg sodium pentobarbitone (supplied by CAS), administered intravenously. Serum tubes were inverted several times immediately post collection to

activate clotting, and the clot was pelleted by centrifugation (1,000 x g, for 10 minutes). The serum was collected and stored at -80 °C until further characterisation.

Table 2.2: Immunization schedule for the gp120/gp140GCN4(+) Env variants to compare Env's of South African and Indian origin. Each rabbit was administered with a dose of 20 µg of the immunogen per immunization.

Immunogen	No. of rabbits	Immunization (weeks)	Serum sample collection (weeks)
$2dCD4^{Wt}$	5	0, 4, 8, 12	0, 2, 6, 10, 14
$2dCD4^{S60C}$	5	0, 4, 8, 12	0, 2, 6, 10, 14
$2dCD4^{FD}$	5	0, 4, 8, 12	0, 2, 6, 10, 14
$gp120_{IN25925}$	5	0, 4, 8, 12	0, 2, 6, 10, 14
$gp120_{IN25925}/2dCD4^{S60C}$	5	0, 4, 8, 12	0, 2, 6, 10, 14
$gp120_{IN25925}/2dCD4^{S60C}$ (No β-mercaptoethanol)	5	0, 4, 8, 12	0, 2, 6, 10, 14
$gp140_{IN25925}GCN4(+)$	3	0, 4, 8, 12	0, 2, 6, 10, 14
$gp140_{IN25925}GCN4(+)/2dCD4^{S60C}$	3	0, 4, 8, 12	0, 2, 6, 10, 14
$gp120_{ZACAP45}$	3	0, 4, 8, 12	0, 2, 6, 10, 14
$gp120_{ZACAP45}/2dCD4^{S60C}$	3	0, 4, 8, 12	0, 2, 6, 10, 14
$gp140_{ZACAP45}GCN4(+)$	3	0, 4, 8, 12	0, 2, 6, 10, 14
$gp140_{ZACAP45}GCN4(+)/ 2dCD4^{S60C}$	3	0, 4, 8, 12	0, 2, 6, 10, 14

Table 2.3: Immunization schedule for the gp120/gp140 Env derivative immunogens (proteins, peptides and DNA constructs).

	Immunogen (number of doses)		No. of rabbits	Immunization (weeks)	Dose	Serum sample collection (weeks)
1	Prime	CycV4OD protein (2)	3	0, 4,	20 µg	-1, 2, 6, 10, 14, 18, 22
	Boost	$gp120^{JRFL}$ cyclic permutant protein (2)		12, 20	20 µg	
2	Prime	ODECCOBPICS-C protein (2)	4	0, 4	20 µg	-1, 2, 6, 10, 14, 18, 22
	Boost	$gp120^{JRFL}$ cyclic permutant protein (2)		12, 20	20 µg	
3	Prime	1WR2 peptide (3)	3	0, 4, 8	20 µg	-1, 2, 6, 10, 14, 18, 22
	Boost	ZA $gp140^{FV}$ protein (2)		12, 20	20 µg	
4	Prime	1ORC peptide (3)	4	0, 4, 8	20 µg	-1, 2, 6, 10, 14, 18, 22
	Boost	ZA $gp140^{FV}$ protein (2)		12, 20	20 µg	
5	Prime	Wt-JRFL Env DNA (3)	4	0, 4, 8	2 mg	-1, 2, 6, 10, 14, 18, 22, 26, 30
	Boost	$gp120^{JRFL}$ cyclic permutant protein (2)		20, 28	45 µg	
6	Prime	JRFL Env-V570D DNA (3)	4	0, 4, 8	2 mg	-1, 2, 6, 10, 14, 18, 22, 26, 30
	Boost	$gp120^{JRFL}$ cyclic permutant protein (2)		20, 28	45 µg	
7	Prime	JRFL Env-SEKS DNA (3)	3	0, 4, 8	2 mg	-1, 2, 6, 10, 14, 18, 22, 26, 30
	Boost	$gp120^{JRFL}$ cyclic permutant protein (2)		20, 28	45 µg	
8	Prime	JRFL Env-V570D DNA (3)	4	0, 4, 8	2 mg	-1, 2, 6, 10, 14, 18, 22, 26, 30
	Boost	ZA $gp140^{FV}$ protein (2)		20, 28	45 µg	

2.7.2 Immunogenicity testing of rabbit sera

2.7.2.1 End-point antibody titer Enzyme Linked Immunosorbent Assays (ELISA)

Binding antibody titres were determined for the rabbit serum by ELISA. Terminal bleed and prebleed rabbit sera were heat inactivated at 56 °C for one hour. One hundred microlitres of 1 µg/ml matched Env immunogen or 2dCD4^{S60C} was coated onto medium binding 96-well plates (Greiner Bio-One, Frickenhausen, Germany) overnight at 4°C. The coating solution was aspirated from the wells the following day and the plate was blocked for 1 hour at room temperature with 250 µl of Dulbecco's 1 x PBS containing 0.05% Tween 20 and 1% [w/v] lyophilized bovine serum albumen (BSA; Sigma-Aldrich, St. Louis, MO, USA). The plates were washed three times with 300 µl wash buffer (Dulbecco's 1 x PBS containing 0.05% Tween 20). Heat-inactivated rabbit sera (50 µl) was prepared in assay buffer (Dulbecco's 1 x PBS containing 0.05% Tween 20 and 1% [w/v] lyophilized BSA) using a two-fold serial dilution starting at 1:400 up to 1:1,638,400. In cases where the titres were very high, dilutions were first prepared to obtain a dilution of 1:2,560,000, and further diluted using a two-fold dilution series up to 1:327,680,000. The samples were added to the wells in triplicate and incubated at room temperature for 1 hour. The plates were washed five times with 300 µl wash buffer to remove unbound antibodies. Anti-rabbit IgG, Horseradish peroxidase linked whole antibody (from donkey) (AEC Amersham) was added to each well (50 µl, 1:5000 dilution) and incubated for 45 minutes at room temperature. After five plate washes with wash buffer, bound antibodies were detected using TMB peroxide substrate (100 µl per well for 20 minutes at room temperature) (1-Step™ Ultra TMB-ELISA Substrate Solution, ThermoFisher Scientific) and the reaction was stopped using 1M H_2SO_4 (Sigma-Aldrich, St. Louis, MO, USA).

2.7.2.2 Neutralizing antibody assays

Neutralisation breadth and potency of the antibodies raised in rabbits was evaluated using neutralising antibody assays.

2.7.2.2.1 PhenoSense™ neutralising antibody assay

Neutralizing antibody responses in rabbit sera of groups immunized with gp120/gp140GCN4(+) Env variants $gp120_{IN25925}$, $gp120_{IN25925}$/$2dCD4^{S60C}$, $gp120_{IN25925}$/$2dCD4^{S60C}$ (No β-mercaptoethanol) and $2dCD4^{Wt}$, $2dCD4^{S60C}$, $2dCD4^{FD}$ control groups (Table 2.2) were measured using the PhenoSense Neutralising Antibody Assay at Monogram Biosciences Inc. (San Francisco, CA, USA) (Li et al., 2005). All terminal bleed rabbit sera and one prebleed serum sample per group were heat inactivated at 56 °C for one hour and sent to Monogram Biosciences Inc. for evaluation against a panel of 12 HIV-1 pseudovirus isolates (Tier 1: SF162, BaL, 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). The reciprocal of the dilution of serum required to achieve 50% (ID_{50}; 1/dilution) inhibition of viral infection was used to assess neutralisation activity.

2.7.2.2.2 In-house HIV Neutralizing Antibody Assay

Reagents used for pseudovirion assays

The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID (contributor in parentheses): TZM-bl (JC53-bl) reporter cell line (Drs. John C. Kappes, Xiaoyun Wu and Transzyme Inc.). Reagents for pseudovirus production pSG3Δenv (Dr. John C. Kappes and Xiaoyun Wu) and ZM53M.PB12 (Drs E. Hunter and C. Derdeyn). The gp160-expressing plasmid SF162.LS was kindly provided by Dr. D. Montefiori (Duke University Medical Center, North Carolina, USA).

The four gp160 expressing plasmids for the generation of pseudoviruses included HIV-1 subtype B BaL and SF162.LS (obtained from Dr. D. Montefiori, Laboratory for AIDS Vaccine R&D, Duke University Medical Center) and the HIV-1 subtype C ZM53M.PB12 (Drs E. Hunter and C. Derdeyn) and ZACAP210 (Drs. L. Morris, K. Mlisana and D. Montefiori) which were obtained from the NIH Research and Reference Reagents Program (catalogue numbers 11313 and 11317, respectively).

Pseudovirus production

Recombinant HIV-1 pseudoviruses capable of a single round of infection were generated by co-transfecting HEK293T cells with pSG3Δenv (an *env*-deficient backbone expression plasmid, which contains a defective *vpu* gene) and the five gp160-expressing plasmids being tested as described (Li et al., 2005). Briefly, a total of 5 µg DNA at a ratio of 1:2 µg equivalents (Env : Backbone) was incubated for 10 minutes at room temperature with FuGENE® HD Transfection Reagent (Promega Corporation, Madison, WI 53711 USA) at a ratio of 1:6 (total plasmid DNA : FuGENE) in a final volume of 500 µl Opti-MEM®/Reduced Serum Media (Gibco™, Thermo Fisher Scientific Inc, Waltham, MA). This was added dropwise to 60-65 % confluent HEK293T cells in a T25 tissue culture flask (Nunc, Roskilde, Denmark) and incubated as described in 2.3.4 above. The supernatant was replaced approximately 12 hours post-transfection with 7 ml of complete DMEM. Pseudovirus-containing supernatants were harvested 48 and 72 hours post transfection, and FCS (Gibco™, Thermo Fisher Scientific Inc, Waltham, MA) was added to a final concentration of 20% before filtering through a 0.45 µm syringe filter and storing in 1 ml aliquots at - 80°C.

A single thawed aliquot from each pseudovirus batch was titred to determine the 50% Tissue Culture Infectious Dose ($TCID_{50}$) in TZM-bl cells (Li et al., 2005). The pseudovirus was serially diluted five-fold in a 96-well tissue culture plate (Nunc, Roskilde, Denmark) in a final volume of 100 µl complete DMEM across 11 columns of the 96 well plate in quadruplicate. The wells

of the 12th column contained cell-only controls. TZM-bl cells were trypsinized and enumerated as described in 2.3.4 above. Ten thousand cells in 100 µl complete DMEM, supplemented with 40 µg/ml diethylaminoethyl-dextran hydrochloride (DEAE Dextran, Sigma-Aldrich, St. Louis, MO, USA) to give a final DEAE Dextran concentration of 20 µg/ml, were added to each well.

After 48 hours of incubation the cell culture media was aspirated from each well and 80 µl Glo-Lysis buffer (Promega, Madison, WI, USA) was added to the cells and incubated for five minutes at room temperature to allow cell lysis. Fifty microliters of cell lysate was transferred to a 96-well white solid luminometer plate (Promega, Madison, WI, USA) and 50 µl Bright-Glo™ Luciferase reagent (Promega, Madison, WI, USA) was added. Luminescence was measured immediately using a GloMax® Explorer luminometer (Promega, Madison, WI, USA) and the $TCID_{50}$ was calculated using a Microsoft Excel macro available online at the Los Alamos National Laboratory HIV immunology database (http://www.hiv.lanl.gov/).

Neutralising antibody assay

In-house neutralizing antibody responses of week 0 (prebleed) and week 14 (terminal bleed) in rabbit sera of groups immunized with gp120/gp140GCN4(+) Env variants $gp140GCN4(+)_{IN25925}$, $gp140GCN4(+)_{IN25925}/2dCD4^{S60C}$,$gp120_{ZACAP45}$, $gp120_{ZACAP45}/2dCD4^{S60C}$, $gp140GCN4(+)_{ZACAP45}$, $gp140GCN4(+)_{ZACAP45}/2dCD4^{S60C}$ (Table 2.2) were evaluated in-house using a luciferase-based assay in TZM-bl cells (Li et al., 2005). The same assay was used to evaluate neutralizing antibody responses of week 0 (prebleed) and week 10 (post prime) and terminal bleed rabbit sera of all the groups immunized with the stabilized gp120/gp140 Env derivatives (Table 2.3). The assay was carried out as described in (Li et al., 2005). Briefly, the rabbit sera from time points mentioned above and matched prebleed sera (included as baseline control), were heat inactivated at 56 °C for one hour in a water bath. Rabbit sera was

serially diluted three-fold (range 1:20 to 1:43,740), in duplicate, in a 96-well tissue culture plate (Nunc, Roskilde, Denmark). Two hundred $TCID_{50}$ of each HIV-1 pseudovirus in a final volume of 50 µl complete DMEM supplemented with penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) was added to the appropriate wells and the plate was incubated at 37°C, 5% CO_2 for 1 hour. TZM-bl cells were trypsinised and enumerated as described in 2.3.4 and 10^4 cells in 100 µl complete DMEM, supplemented with 50 µg/ml DEAE Dextran (Sigma-Aldrich, St. Louis, MO, USA) to give a final concentration of 20 µg/ml, were added to each well. One column of 8 wells contained cells only (cell control) and another column contained virus and cells (virus control). Plates were incubated for 48 hours and luminescence was measured as described above, and the reciprocal of serum dilution required to achieve 50% inhibition (ID_{50}) after normalizing against the cell only and virus only control, 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).

Chapter 3 : Results

3.1 Env selection and generation of recombinant gp120 and gp140 constructs

To characterize and compare HIV-1 subtype C Env immunogens from South Africa and India, twelve envelope glycoprotein constructs were designed after extensive bioinformatic analysis of Env sequences derived from South African and Indian HIV-1 subtype C infected patients. This led to the selection of sequences from six patients, three from India and three from South Africa (including matched gp120 and gp140GCN4(+)) and formed the basis of our immunogen design.

The sequences were synthesized and successfully cloned by GeneArt into the pcDNA3.1 mammalian expression vector, and included the codon optimised monomeric gp120 for each sequence (pcDNA-gp120$_{ZACAP45}$, pcDNA-gp120$_{ZACAP210}$, pcDNA-gp120$_{ZA706010164}$, pcDNA-gp120$_{IN26191}$, pcDNA-gp120$_{IN25925}$, pcDNA-gp120$_{IN25710}$) and trimeric gp140 with an intact cleavage site for each sequence (pcDNA-gp140GCN4(+)$_{ZACAP45}$, pcDNA-gp140GCN4(+)$_{ZACAP210}$, pcDNA-gp140GCN4(+)$_{ZA706010164}$, pcDNA-gp140GCN4(+)$_{IN26191}$, pcDNA-gp140GCN4(+)$_{IN25925}$, pcDNA-gp140GCN4(+)$_{IN25710.}$ A representation of the gp120 and gp140GCN4(+) derived constructs are depicted in Figure 3.1.

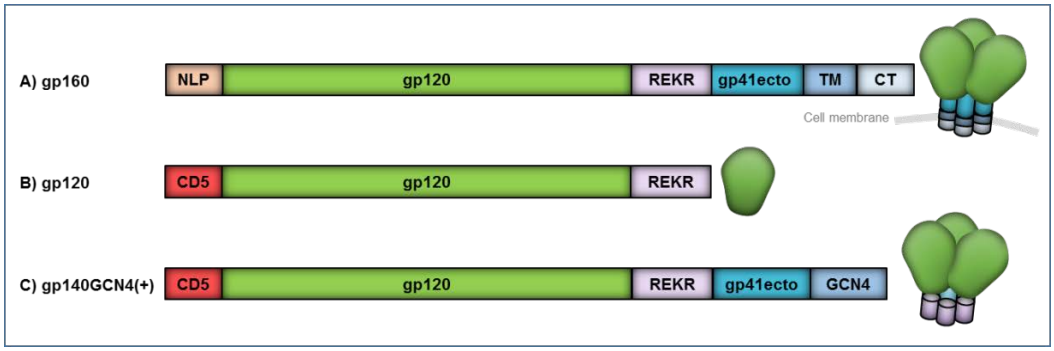


Figure 3.1: Schematic representation of the native HIV-1 envelope glycoprotein and the derived Env constructs used in this study. A) Full length native gp160 precursor. The gp160 comprises three sets of a gp120 monomer, REKR cleavage site, gp41 ecto-domain (gp41ecto), the transmembrane (TM) and the cytoplasmic tail (CT) with the native leader peptide sequence (NLP). B) Representation of the monomeric gp120 with a REKR cleavage site and CD5 leader sequence. C) Representation of the trimeric gp140GCN4(+), which includes three sets of the gp120 monomer, REKR cleavage site and the gp41 ecto-domain, with a trimeric GCN4 leucine zipper sequence and an intact furin cleavage amino acid sequence (GCN4(+)) to form the trimer.

Differences in amino acids and position of potential N-linked glycosylation sites (PNLGs) result in differences in the size and structure of the variable loops of Env, directly influencing epitope exposure to mAbs (Huang et al., 2012; Kumar et al., 2011; Patil et al., 2014). Therefore the amino acid sequences of the six selected Env sequences (a gp120 and matched gp140GCN4(+) of each Env) were examined. The sequences were aligned to HXBc2 for conventional numbering and orientation purposes, the CD5 leader sequence is highlighted in red, the GCN4 motif is highlighted in grey, the cleavage site (REKR) is highlighted in green, gp120 contacts for CD4 are highlighted in yellow (Foley et al., 2015), signature amino acids for Indian isolates are highlighted in purple, and PNLGs as predicted by the N-Glycosylate tool are highlighted in blue (Figure 3.2).

The gp120 sequences ranged from 488 to 510 amino acids, and the gp140GCN4(+) sequences ranged from 694 to 716 amino acids. The PNLG sites were predicted using the Los Alamos HIV databases tool N-GlycoSite (https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html)

where an oligosaccharide chain may be attached to asparagine (N) occurring in the tripeptide sequence N-X-S or N-X-T, where X can be any amino acid except Proline (Zhang et al., 2004). The number of PNLGs ranged between 19 and 29 for the monomeric Env's which is within the expected range for gp120s (Yao et al., 2015) and 23 to 33 for the trimeric Env's. Nine conserved PNLG sites occur in the sequences according to the N-GlycoSite tool, in the conserved and variable regions of Env at amino acid positions N88, N202 (HXBc2 position 197), N267 (HXBc2 position 262), N281 (HXBc2 position 276), N306 (HXBc2 position 301), N345 (HXBc2 position 339), N362 (HXBc2 position 356), N392 (HXBc2 position 386) and N454 (HXBc2 position 442).

No major deviations were found between the lengths of the variable loop regions of the Env variants, except the V2 loop in which Env variant IN26191 was found to have a shorter V2 loop of 40 amino acids compared to 45 amino acids in the other Env variants. All the variable loop lengths were within the expected range according to the results published by Yao et al. (2015).

Sequence analysis of Indian derived HIV-1 subtype C Env revealed three conserved amino acids at positions 346E (HXBc2 position 340) (glutamic acid), 357A (HXBc2 position 350) (alanine) and 441E (HXBc2 position 429) (glutamic acid), which are highlighted in purple in Figure 3.2 (Shankarappa et al., 2001). Only Env IN25710 contains all three amino acids at the corresponding positions.

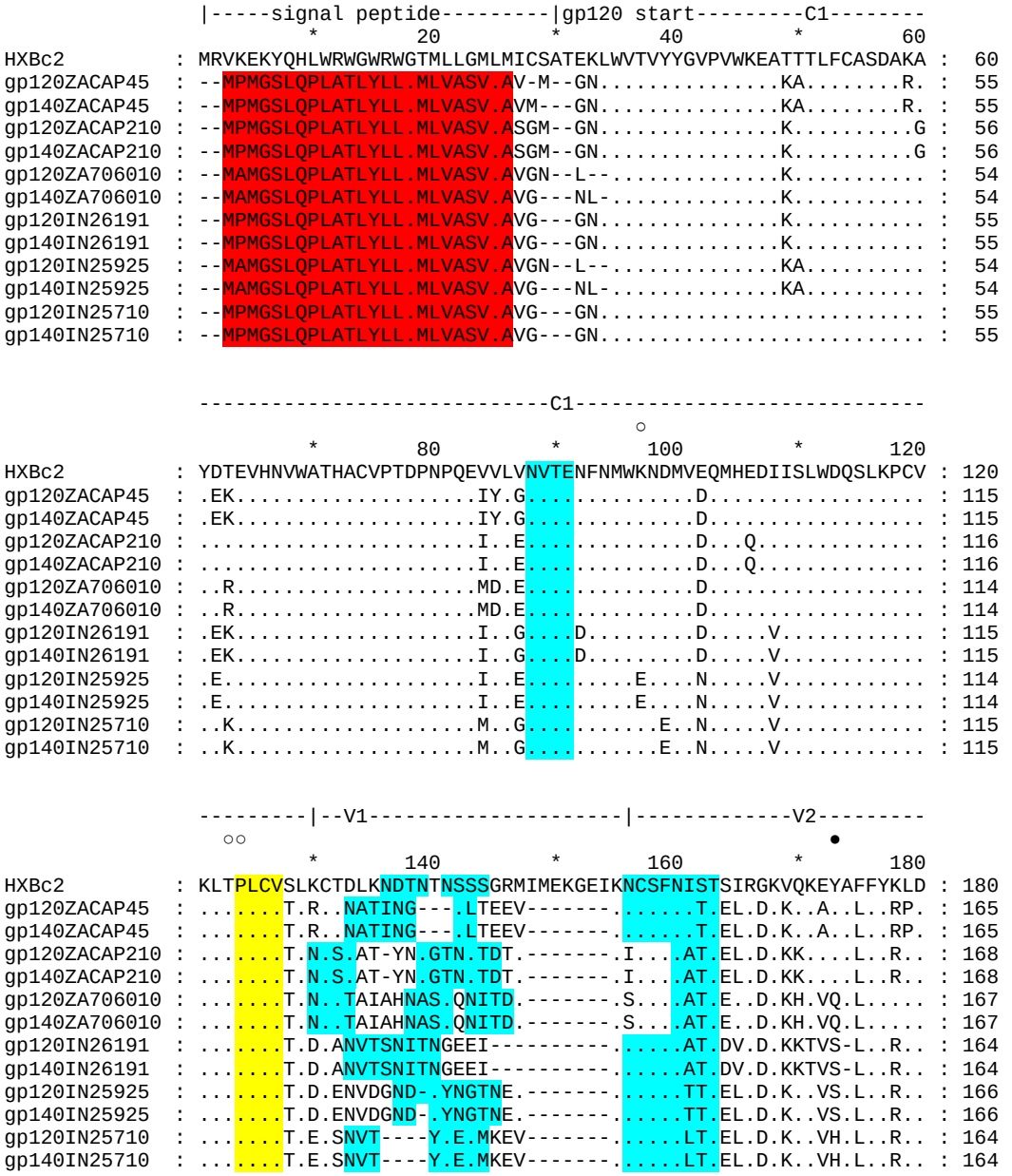
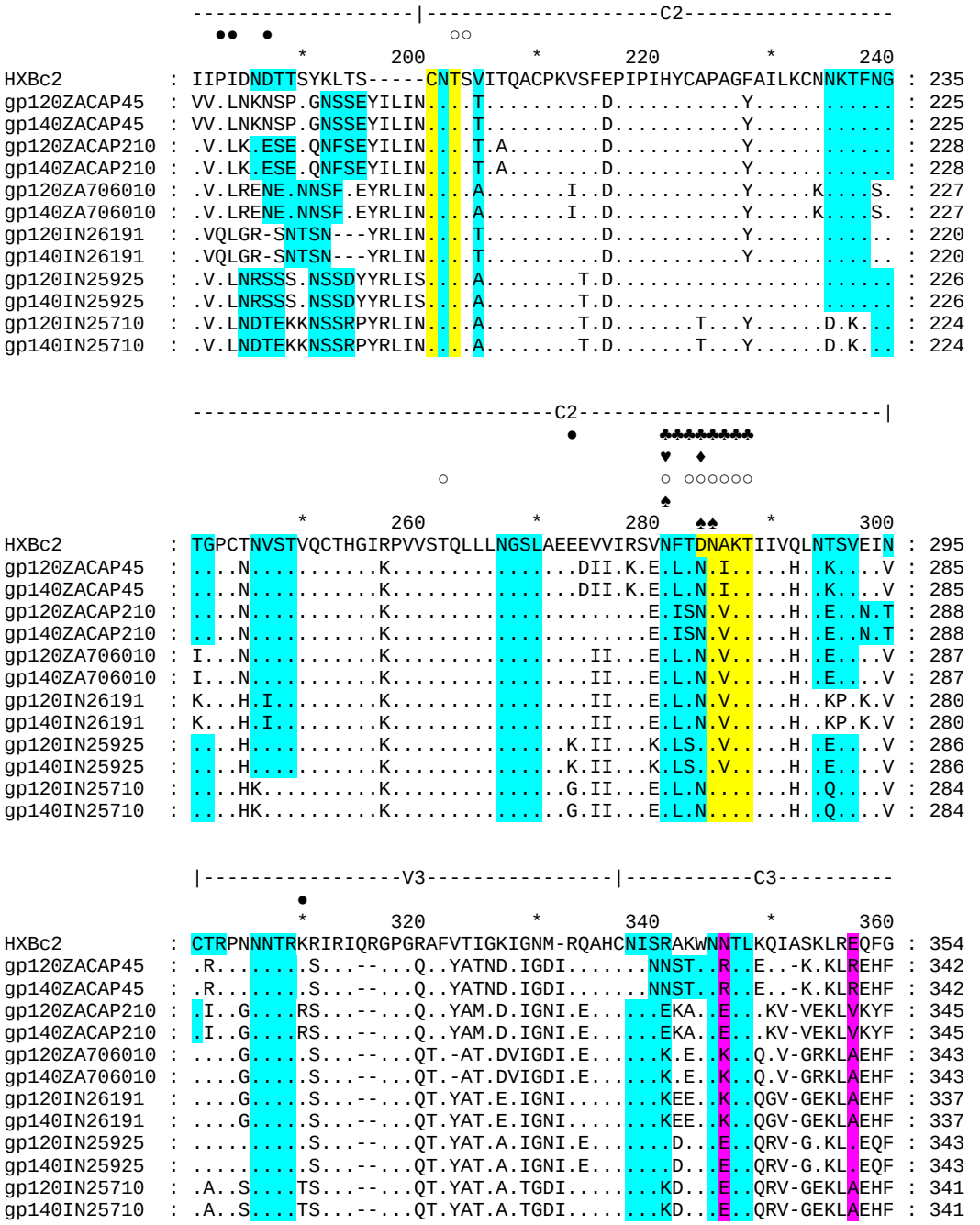
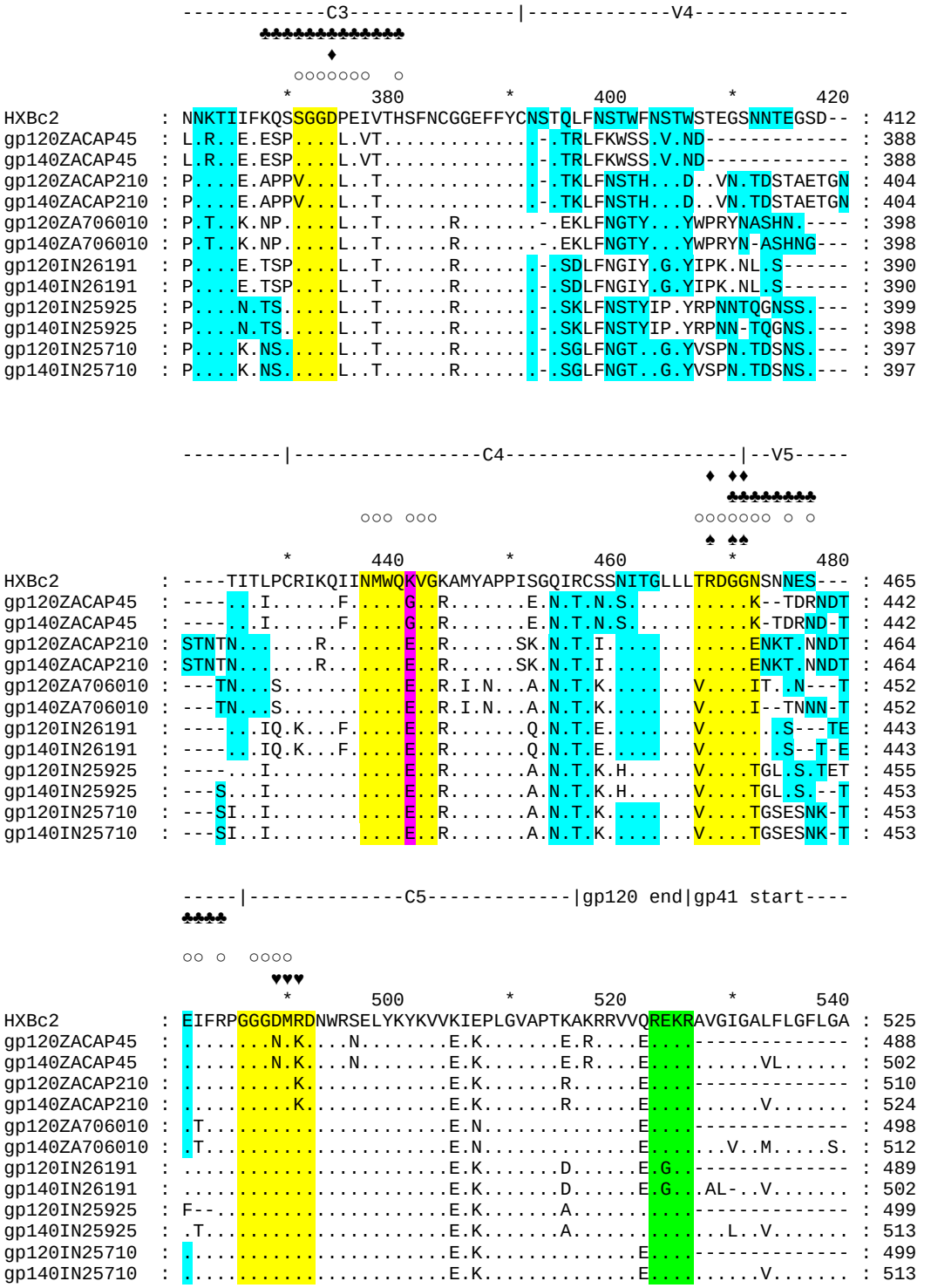


Figure 3.2: Amino acid sequence alignment of the HIV-1 envelope glycoprotein (Env) sequences (gp120 and gp140GCN4(+)) derived from the selected Indian and South African patients. The CD5 leader sequence is highlighted in red, the GCN4 motif is highlighted in grey, cleavage site REKR is highlighted in green, gp120 contacts for CD4 are highlighted in yellow, the relative position of the signature amino acids for the Indian isolates are highlighted in purple, and PNLGs are highlighted in blue. Contact residues important for selected bNAb targeting the CD4bs are indicated by symbols as follows: open circles for VRC03, club suit for important resides shared by VRC01 and IgG1b12, closed circles for IgG1b12, heart for HJ16, diamond for NIH45-46^{G54W} and spade suit sign for VRC-CH31.



Key: Residues on gp120/gp140GCN4(+) important for binding to the following bNAbs:
○ VRC03; ♣ VRC01 and IgG1b12; ● IgG1b12; ♥ HJ16; ♦ NIH45-46^{G54W}; ♠ VRC-CH31.

Figure 3.2: Continued



Key: Residues on gp120/gp140GCN4(+) important for binding to the following bNAbs: ○ VRC03; ♣ VRC01 and IgG1b12; ● IgG1b12; ♥ HJ16; ♦ NIH45-46^{G54W}; ♠ VRC-CH31.

Figure 3.2: Continued

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                  ------------------------------------------------------------
                           *       560         *       580         *       600
HXBc2          : AGSTMGAASMTLTVQARQLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVER : 585
gp120ZACAP45   : ------------------------------------------------------------ :   -
gp140ZACAP45   : .........I.................S...........M............T.V..I.. : 562
gp120ZACAP210  : ------------------------------------------------------------ :   -
gp140ZACAP210  : .........I.................S...........M............T.V..I.. : 584
gp120ZA706010  : ------------------------------------------------------------ :   -
gp140ZA706010  : .........I.................S...........M.............V..I.. : 572
gp120IN26191   : ------------------------------------------------------------ :   -
gp140IN26191   : .........I.................S........................T.V..I.. : 562
gp120IN25925   : ------------------------------------------------------------ :   -
gp140IN25925   : .........I.................S...........M............T.V..I.. : 573
gp120IN25710   : ------------------------------------------------------------ :   -
gp140IN25710   : .........I.................S........................T.V..I.. : 573


                  ------------------------------------------------------------
                           *       620         *       640         *       660
HXBc2          : YLKDQQLLGIWGCSGKLICTTAVPWNASWSNKSLEQIWNHTTWMEWDREINNYTSLIHSL : 645
gp120ZACAP45   : ------------------------------------------------------------ :   -
gp140ZACAP45   : .........L...........N...S.....QTD..DNM..IQ.....S..SNT.YK. : 622
gp120ZACAP210  : ------------------------------------------------------------ :   -
gp140ZACAP210  : ......................N...S.....YGD..DNM..Q.......NT.YR. : 644
gp120ZA706010  : ------------------------------------------------------------ :   -
gp140ZA706010  : .........L..............N.....QSD..ENM..Q.......NT.YR. : 632
gp120IN26191   : ------------------------------------------------------------ :   -
gp140IN26191   : ........................I.....YHE..DNM..Q..K..S...DQ.YR. : 622
gp120IN25925   : ------------------------------------------------------------ :   -
gp140IN25925   : ........................T...R.QAD..GNM..Q.....S...NT.FR. : 633
gp120IN25710   : ------------------------------------------------------------ :   -
gp140IN25710   : ........................Y...R.QDD..DNM..Q..K..S...NT.YK. : 633


                  -------------------------------------|transmembrane domain|
                           *       680         *       700         *       720
HXBc2          : IEESQNQQEKNEQELLELDKWASLWNWFNITNWLWYIKLFIMIVGGLVGLRIVFAVLSIV : 705
gp120ZACAP45   : ------------------------------------------------------------ :   -
gp140ZACAP45   : L.D......Q..KD..A..S.NN.....................I.MKQIEDKIEEILSKIYHIEN : 682
gp120ZACAP210  : ------------------------------------------------------------ :   -
gp140ZACAP210  : L.D..........D..A....Q...S..S.SS......I.MKQIEDKIEEILSKIYHIEN : 704
gp120ZA706010  : ------------------------------------------------------------ :   -
gp140ZA706010  : L....T......KD..A..S.KN.....................IGMKQIEDKIEEILSKIYHIEN : 692
gp120IN26191   : ------------------------------------------------------------ :   -
gp140IN26191   : L.V......S..KD..A.NS.N......D.........I.MKQIEDKIEEILSKIYHIEN : 682
gp120IN25925   : ------------------------------------------------------------ :   -
gp140IN25925   : L.D..I...S..KD..A..S.KN..S..D.........IGMKQIEDKIEEILSKIYHIEN : 693
gp120IN25710   : ------------------------------------------------------------ :   -
gp140IN25710   : L.D..I......KD..A..S.EN.....................I.MKQIEDKIEEILSKIYHIEN : 693


                  cytoplasmic tail start----------------------------------------
                           *       740         *       760         *       780
HXBc2          : NRVRQGYSPLSFQTHLPTPRGPDRPEGIEEEGGERDRDRSIRLVNGSLALIWDDLRSLCL : 765
gp120ZACAP45   : ------------------------------------------------------------ :   -
gp140ZACAP45   : EIA.IKKLIGEV---------------------------------------------------- : 694
gp120ZACAP210  : ------------------------------------------------------------ :   -
gp140ZACAP210  : EIA.IKKLIGEV---------------------------------------------------- : 716
gp120ZA706010  : ------------------------------------------------------------ :   -
gp140ZA706010  : EIA.IKKLIGEV---------------------------------------------------- : 704
gp120IN26191   : ------------------------------------------------------------ :   -
gp140IN26191   : EIA.IKKLIGEV---------------------------------------------------- : 694
gp120IN25925   : ------------------------------------------------------------ :   -
gp140IN25925   : EIA.IKKLIGEV---------------------------------------------------- : 705
gp120IN25710   : ------------------------------------------------------------ :   -
gp140IN25710   : EIA.IKKLIGEV---------------------------------------------------- : 705
```

Figure 3.2: Continued

```
                  ------------------------------------------------------------
                           *       800         *       820         *       840
HXBc2          : FSYHRLRDLLLIVTRIVELLGRRGWEALKYWWNLLQYWSQELKNSAVSLLNATAIAVAEG : 825
gp120ZACAP45   : ------------------------------------------------------------ :   -
gp140ZACAP45   : ------------------------------------------------------------ :   -
gp120ZACAP210  : ------------------------------------------------------------ :   -
gp140ZACAP210  : ------------------------------------------------------------ :   -
gp120ZA706010  : ------------------------------------------------------------ :   -
gp140ZA706010  : ------------------------------------------------------------ :   -
gp120IN26191   : ------------------------------------------------------------ :   -
gp140IN26191   : ------------------------------------------------------------ :   -
gp120IN25925   : ------------------------------------------------------------ :   -
gp140IN25925   : ------------------------------------------------------------ :   -
gp120IN25710   : ------------------------------------------------------------ :   -
gp140IN25710   : ------------------------------------------------------------ :   -


                  ----------cytoplasmic tail end|
                           *       860         *
HXBc2          : TDRVIEVVQGACRAIRHIPRRIRQGLERILL---- : 856
gp120ZACAP45   : ------------------------------------ :   -
gp140ZACAP45   : ------------------------------------ :   -
gp120ZACAP210  : ------------------------------------ :   -
gp140ZACAP210  : ------------------------------------ :   -
gp120ZA706010  : ------------------------------------ :   -
gp140ZA706010  : ------------------------------------ :   -
gp120IN26191   : ------------------------------------ :   -
gp140IN26191   : ------------------------------------ :   -
gp120IN25925   : ------------------------------------ :   -
gp140IN25925   : ------------------------------------ :   -
gp120IN25710   : ------------------------------------ :   -
gp140IN25710   : ------------------------------------ :   -
```

Figure 3.2: Continued

3.1.1 Analysis of amino acid residues important for CD4 binding site directed bNAbs

The CD4bs epitope is highly conserved due to its critical function in viral entry, thus making it an attractive target for vaccine elicited antibody responses (Li et al., 2007; Wyatt and Sodroski, 1998). One of the most characterized CD4bs bNAbs, IgG1b12, is capable of neutralizing 30 – 40 % of primary isolates of subtype B and C viruses (Bures et al., 2002). Interestingly, HIV-1 subtype C viruses of Indian origin have been found to display increased levels of IgG1b12 resistance (Kulkarni et al., 2009; Sneha et al., 2015). To this end, we decided to examine the amino acid differences of each gp120/gp140GCN4(+) Env variants in respect of their CD4bs epitopes.

Recently isolated bNAbs targeting the CD4bs have shown increased potency and breadth, and retain neutralizing activity against IgG1b12 resistant viruses (Li et al., 2007). We propose that including an Env immunogen that is resistant to neutralization by IgG1b12 could potentially reveal further characteristics of the CD4bs epitope that may be applicable to vaccine design and preferentially select for the more potent VRC01-type Abs.

Here we analysed the amino acid residues important for binding to six CD4bs-directed bNAbs; IgG1b12, VRC01, HJ16, VRC-CH31, NIH45-46^{G54W} and VRC03 (Figure 3.2) (Wu et al., 2011). Critical amino acid residues for bNAb binding are indicated as follows: open circles for VRC03, club suit symbol for important resides shared by VRC01 and IgG1b12, closed circles for IgG1b12, heart for HJ16, diamond for NIH45-46^{G54W} and spade suit symbol for VRC-CH31.

Several amino acid differences were noted amongst the Env variants. Env ZACAP45 has a glycine (G) at position 441 (HXBc2 position 429) whereas the other Env's have a negatively charged glutamic acid (E). This affects the charge of the protein in that region. Env IN26191 is lacking the N294 N-linked glycan, which is present in the other Env variants and Env's ZACAP45 and IN26191 lack an N-linked glycan at position 185-190.

The following amino acid mutations, known to confer resistance to IgG1b12 according to a study published by Gnanakaran et al. (2010), were noted in the sequences: serine or histidine at position 173 in all the Env variants of Indian origin; glycine (G) at amino acid position 185 in Env gp120/gp140GCN4(+)$_{IN26191}$; and lysine (K) at amino acid position 273 (HXBc2 position 268) in Env gp120/gp140GCN4(+)$_{IN25925}$.

Amino acid mutations reported to confer resistance to bNAb HJ16 binding (Balla-Jhagjhoorsingh et al., 2013) are present in Env ZACAP45 (D489N, HXBc2 position 474) and Env's ZACAP45 and ZACAP210 R491K (HXBc2 position 476), respectively.

Guided by the bioinformatic analyses we sought to obtain definitive bNAb biochemical binding data, as slight variations in amino acid sequence, including non-contact residues, may influence conformational epitopes of the CD4bs (Li et al., 2011). This was performed by expressing and purifying each gp120/gp140GCN4(+) Env variant, followed by antigenicity analysis with the six CD4 directed bNAbs mentioned above.

3.2 Expression and purification of the recombinant gp120/gp140GCN4(+) Env variants and 2dCD4 proteins

3.2.1 Expression of recombinant gp120/gp140GCN4(+) Env variants

Transient recombinant expression of HIV-1 Env protein was optimized in HEK293T cells. Expression was confirmed by reducing-SDS-PAGE followed by Western blotting (Figure 3.3). As expected, the gp120 constructs expressed Env that migrated to approximately 120 kDa, and the gp140GCN4(+) constructs expressed Env which migrated to approximately 140 kDa (Figure 3.3). Two bands were observed for some of the gp140GCN4(+) Envs, one at 140 kDa representing the uncleaved protein (encompassing both gp120 and gp41), and cleaved gp120 portion (120 kDa). A high molecular weight band, resistant to reducing agent (β-mercaptoethanol), was observed at approximately 260 kDa for Env gp140GCN4(+)$_{IN26191}$ and was suspected to be higher order oligomers of this Env.

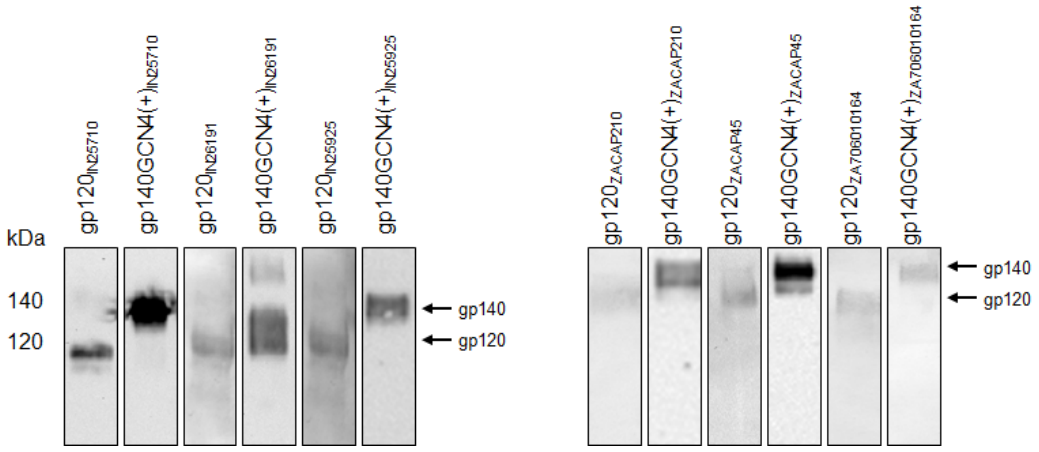

Figure 3.3: Western blot analysis of transient HIV-1 envelope glycoprotein (Env) expression in HEK293T cells with Indian and South African Env constructs. Cell culture supernatants were analysed for expression of each Env by SDS-PAGE (10 % gel) followed by Western blotting by probing with HIV-1 positive patient sera and detection with a secondary antibody (goat anti-human IgG (Fc specific) alkaline phosphatase conjugated antibody. The molecular weight sizes are indicated on the left in kiloDaltons (kDa). Arrows on the right indicate the relative positions of gp120 and gp140.

Subsequently, stable cell lines (293F) expressing each of the 12 Envs were established, and used for large-scale expression of recombinant Env (results not shown). Cell culture supernatant from the transient expression experiments and Env expressing stable cell lines was collected, filtered and stored at -20°C for subsequent use in the purification protocols.

3.2.2 Purification of gp120/gp140GCN4(+) Env variants

3.2.2.1 Primary purification of Env variants by lectin-affinity chromatography

Env was purified from the stored cell culture supernatants using *Galanthus nivalis* lectin-affinity chromatography, and SDS-PAGE (10 %) under reducing conditions was used to assess the purity and confirm elution of the recombinant proteins corresponding to expected sizes (Figure 3.4). Following lectin affinity chromatography, protein yields of the different Env's ranged from 0.5 to 10 mg per litre of culture supernatant as determined by colorimetric BCA total protein quantification. Varying degrees of furin-

cleavage efficiency were observed for the purified gp140GCN4(+) Envs as determined by SDS-PAGE analysis (Figure 3.4). In addition, high molecular weight bands at approximately 260 kDa were observed for some of the Env's (gp140GCN4(+)$_{IN26191}$, gp120$_{ZACAP45}$, gp140GCN4(+)$_{ZACAP45}$, gp1401GCN4(+)$_{ZA06010164}$) and was attributed to higher order oligomers of the Env.

It is well recognised that mammalian expression and purification of Env results in various species and/or higher order oligomers, and that lectin affinity purification does not differentiate the Env conformations of interest. Thus, the presence of these mixed Env species warranted further purification since monomeric and trimeric conformations of each Env variant were required for characterization. Samples were aliquoted, snap frozen, and stored at -80°C until further purification.

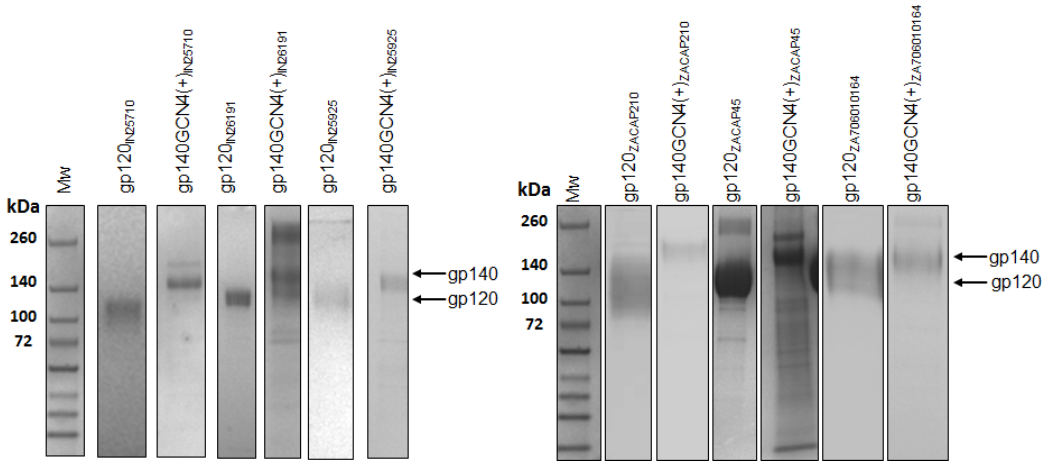

Figure 3.4: Coomassie stained SDS-PAGE (10 %) analysis of the Indian and South African HIV-1 envelope glycoproteins to assess purity after *Galanthus nivalis* lectin-affinity purification. Molecular weight marker (Mw - Spectra™ Multicolor Broad Range Protein Ladder) is shown on the left in kiloDaltons (kDa). Arrows on the right indicate the gp120 and gp140GCN4(+).

3.2.2.2 Secondary purification of Env variants by size exclusion chromatography (SEC)

To further purify the monomeric and trimeric conformations of each Env to homogeneity, eluate from the lectin affinity chromatography was separated using SEC. Examination of the SEC chromatograms of the gp120 glycoproteins (Figure 3.5) revealed that the gp120 glycoproteins consisted of two dominant peaks, corresponding to dimeric and monomeric conformations. Based on the SEC chromatogram a higher proportion of dimer compared to monomer was observed for the majority of the gp120 expressed proteins.

The dimeric gp120 peak retention volumes ranged between 59 – 63 ml for the different Env's, whereas the monomeric gp120 peak retention volumes ranged between 67 – 71 ml. Fractions were collected for the dimeric gp120 as well as the monomeric gp120 (indicated by shaded areas in Figure 3.5) to isolate homogeneous dimers and monomers for each Env.

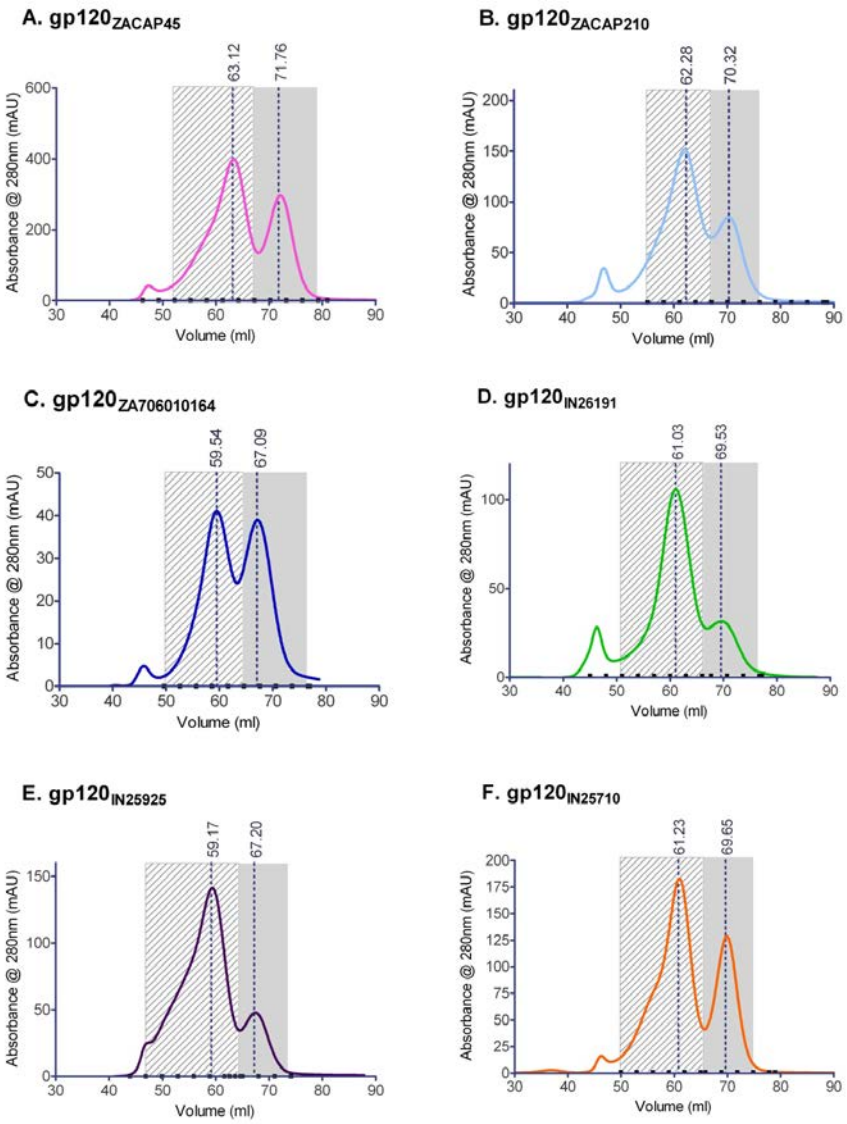

Figure 3.5: Size exclusion chromatograms of the six lectin-affinity purified gp120 HIV-1 envelope glycoproteins (Envs). Peak-retention volumes are indicated above the peaks; areas shaded with diagonal lines represent the dimeric and higher order oligomeric conformations of the Env and the grey-shaded areas represent the fractions collected for the monomeric Env conformation. One representative chromatogram is shown for each Env.

The lectin-purified gp140GNC4(+) Env yielded similar SEC profiles (Figure 3.6) comprising three distinct peaks. The first peak representing an oligomeric form with a peak retention volume between 46 – 49 ml, the second peak representing the trimeric conformation eluted between 46 – 53 ml and a third peak corresponding to the cleavage monomeric gp120 eluted at approximately 67 ml.

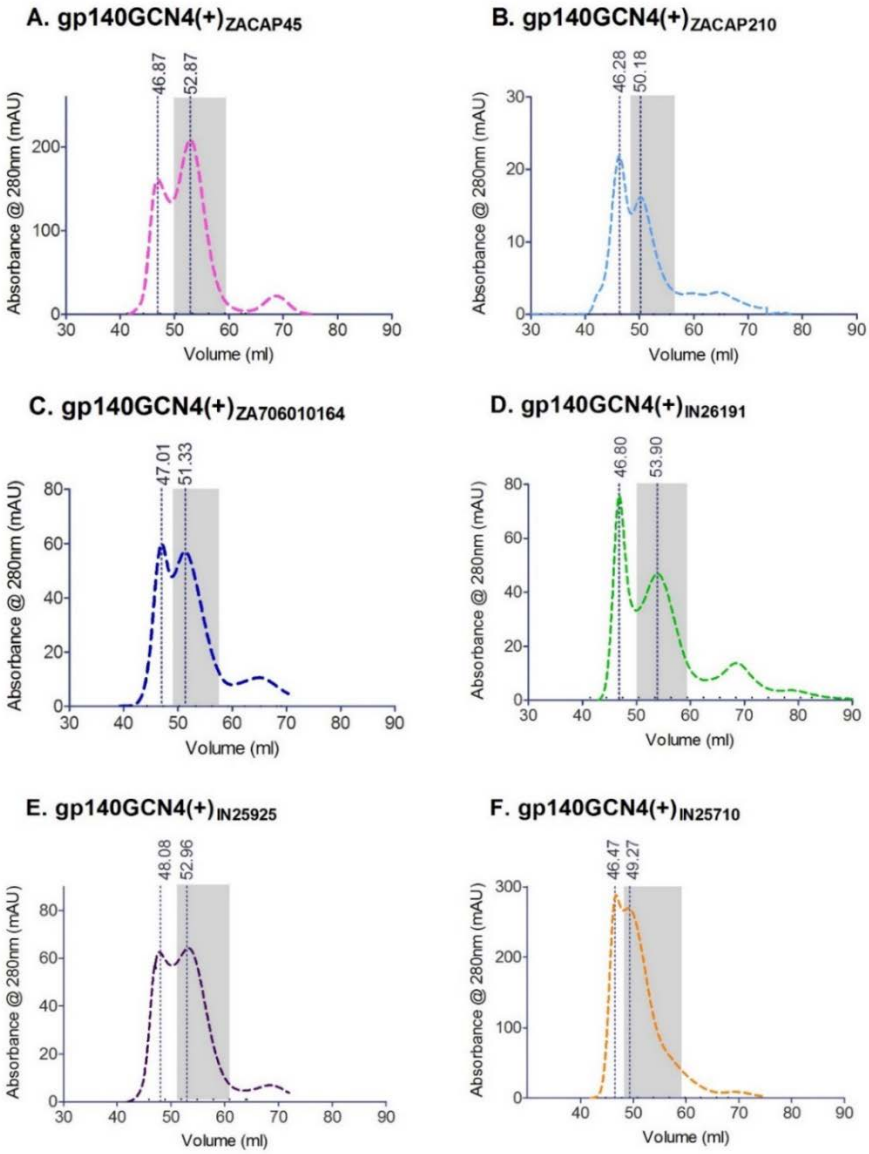

Figure 3.6: Size exclusion chromatograms of the six lectin-affinity purified gp140GCN4(+) HIV-1 envelope glycoproteins (Envs). Peak-retention volumes are indicated above the peaks; shaded areas represent the fractions collected for the gp140GCN4(+) Env conformation. One representative chromatogram is shown for each Env.

An overlay of a normalised SEC chromatograms for each Env (Figure 3.7) illustrates that each Env has a similar SEC profile. The slight differences in peak retention volumes may be attributed to amino acid sequence variation of the Env, as well as differences in glycosylation of each Env, resulting in different molecular weights.

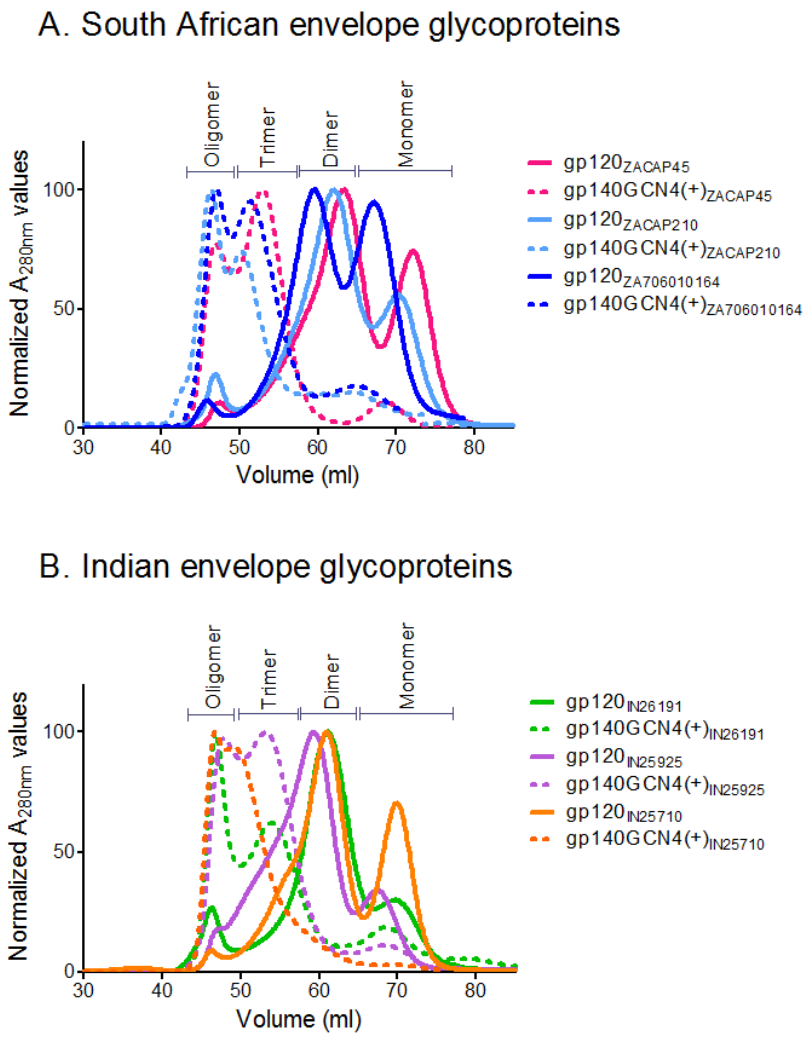

Figure 3.7: Overlay of normalized size exclusion chromatograms for each of the 12 HIV-1 envelope glycoproteins. (A) Env's of South African origin. (B) Env's of Indian origin. Showing a comparison of the profiles for the different conformations (oligomer, trimer (gp140GCN4(+)), dimer, monomer (gp120)). Similar profiles were obtained for each conformation, with slight variations being attributed to amino acid sequence differences, and therefore molecular weight and glycosylation differences.

The relevant trimer, dimer and monomer containing fractions were pooled, concentrated and assessed for purity using Coomassie blue stained SDS-PAGE (4-12 %) under reducing conditions (Figure 3.8). Despite numerous optimization attempts, SEC was unable to remove the contaminating bands observed in the lectin purified glycoproteins seen in Figure 3.7. The amount of recombinant protein was colorimetrically quantified. Fractions were then aliquoted, snap frozen, and stored at -80°C for use in subsequent biochemical characterization, complex formation and/or immunization protocols.

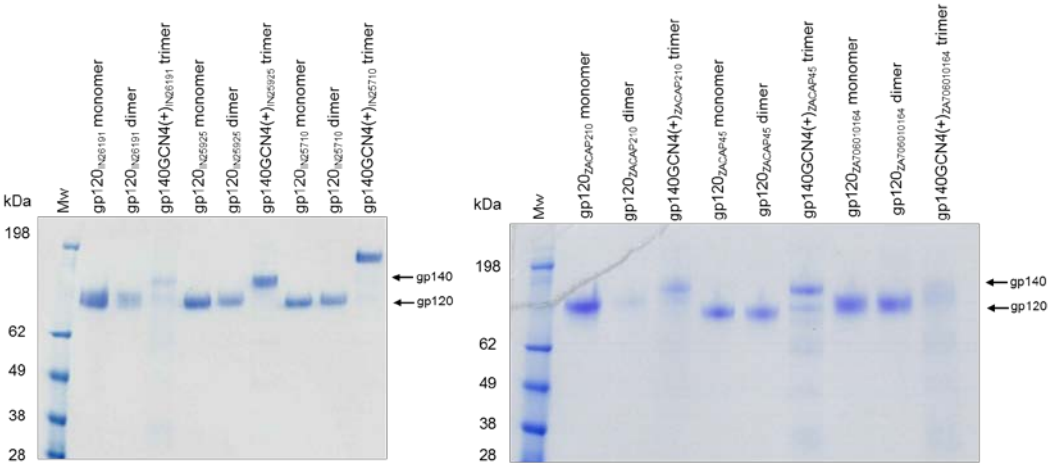

Figure 3.8: Coomassie stained SDS-PAGE (4 – 12 %) analysis of the monomeric and trimeric Indian and South African HIV-1 envelope glycoproteins post SEC. Molecular weight marker (Mw - SeeBlue prestained protein standard) is shown on the left in kiloDaltons (kDa).

Both the monomeric and dimeric species migrated to approximately 120 kDa for all the Env's, which is to be expected under reducing conditions. The gp140GCN4(+) species resolved to approximately 140 kDa for all the constructs, and as described in section 3.2.1 above, in some cases a cleaved gp120 portion was noted, suggesting the presence of a cleaved trimeric conformation. The corresponding partial gp41 component of the cleaved portion was not clearly visible on the gel, perhaps due to insufficient concentration. The gp140GCN4(+)$_{IN25710}$ produced a band which resolved to approximately 200 kDa. This large difference in size of this Env was also noted during SEC, where the gp140GCN4(+) constructs had a peak

retention volume ranging between 50 – 53 ml, however, the gp140GCN4(+)$_{IN25710}$ construct had a peak retention volume of around 46 ml. This may be a result of higher order oligomers or dimers of trimers as noted above.

The final yield of purified gp120 or gp140GCN4(+) recombinant protein obtained from one litre of cell culture supernatant varied between the different Envs and ranged between 0.1 to 2 mg, with higher yields generally obtained for the gp120's compared to the gp140GCN4(+)'s (Table 3.1).

Table 3.1: Average yield of conformation specific purified forms of Env from 1 litre of cell culture supernatant by lectin affinity chromatography followed by further purification using size exclusion chromatography.

Env	Final yield (mg/litre)
gp120$_{ZACAP45}$	2.00
gp140GCN4(+)$_{ZACAP45}$	1.00
gp120$_{ZACAP210}$	2.00
gp140GCN4(+)$_{ZACAP210}$	0.15
gp120$_{ZA706010164}$	1.00
gp140GCN4(+)$_{ZA706010164}$	0.45
gp120$_{IN25925}$	2.00
gp140GCN4(+)$_{IN25925}$	2.00
gp120$_{IN25710}$	0.20
gp140GCN4(+)$_{IN25710}$	0.04
gp120 $_{IN26191}$	0.40
gp140GCN4(+)$_{IN26191}$	0.10

3.2.3 Expression and purification of recombinant 2dCD4$^{Wt/FD/S60C}$

Three recombinant 2dCD4 proteins for Env/2dCD4 complex formation were successfully expressed and purified in *E. coli* BL21 (DE3), namely wild type (Wt), folding-defective (FD) and one in which the serine at position 60 is substituted with cysteine (S60C). These proteins were purified under denaturing conditions using Ni^{2+} affinity chromatography and re-folded using an oxidative post-purification refolding protocol (Cerutti et al., 2010).

The 2dCD4$^{(Wt/FD/S60C)}$ purified proteins were analysed using SDS-PAGE (4-12 %), and as expected all three 2dCD4 proteins resolved to approximately 25 kDa (Figure 3.9). One litre of *E. coli* routinely yielded between 1 – 2 mg of purified recombinant protein for each 2dCD4 variant. Aliquots of each 2dCD4 were aliquoted, snap frozen, and stored at -80°C until used in subsequent biochemical characterization, complex formation or immunization protocols.

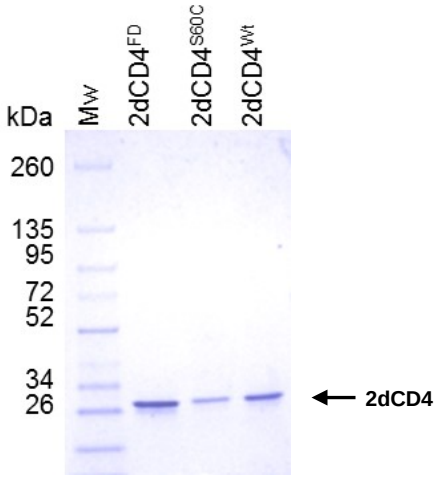

Figure 3.9: Coomassie stained SDS-PAGE analysis of purified 2dCD4 variants. The 2dCD4^{FD} refers to the folding defective control, 2dCD4^{S60C} refers to the 2dCD4 in which the serine at position 60 is substituted with cysteine (S60C) and 2dCD4^{Wt} refers to the wild-type 2dCD4. Molecular weight marker (Mw - Spectra™ Multicolor Broad Range Protein Ladder) is shown on the left in kiloDaltons (kDa). Arrow on the right shows the 2dCD4 variants.

3.3 Antigenicity analysis of the gp120/gp140GCN4(+) Env variants

3.3.1 Surface plasmon resonance interactions of Env gp120/gp140GCN4(+) variants with 2dCD4$^{Wt/S60C}$

We first aimed to assess the structural integrity of the CD4bs of the monomeric and trimeric (gp120/gp140GCN4(+) Env variant) conformations by performing real-time binding experiments to surface immobilised 2dCD4^{Wt} and 2dCD4^{S60C} using SPR. Non-specific analyte interaction with the control surface was too high for kinetics to be determined as shown by the interspot data (interspots > 10% R_{MAX}) (Appendix B1 and B2) (Bio-Rad Laboratories Inc., 2009; Marquart, 2006-2016). However, the sensorgrams showed that all the Env variants were able to bind 2dCD4^{Wt} and 2dCD4^{S60C} to varying degrees (Figures 3.10 and 3.11) which confirmed that the CD4bs epitope on the Env's was conformationally intact and functional.

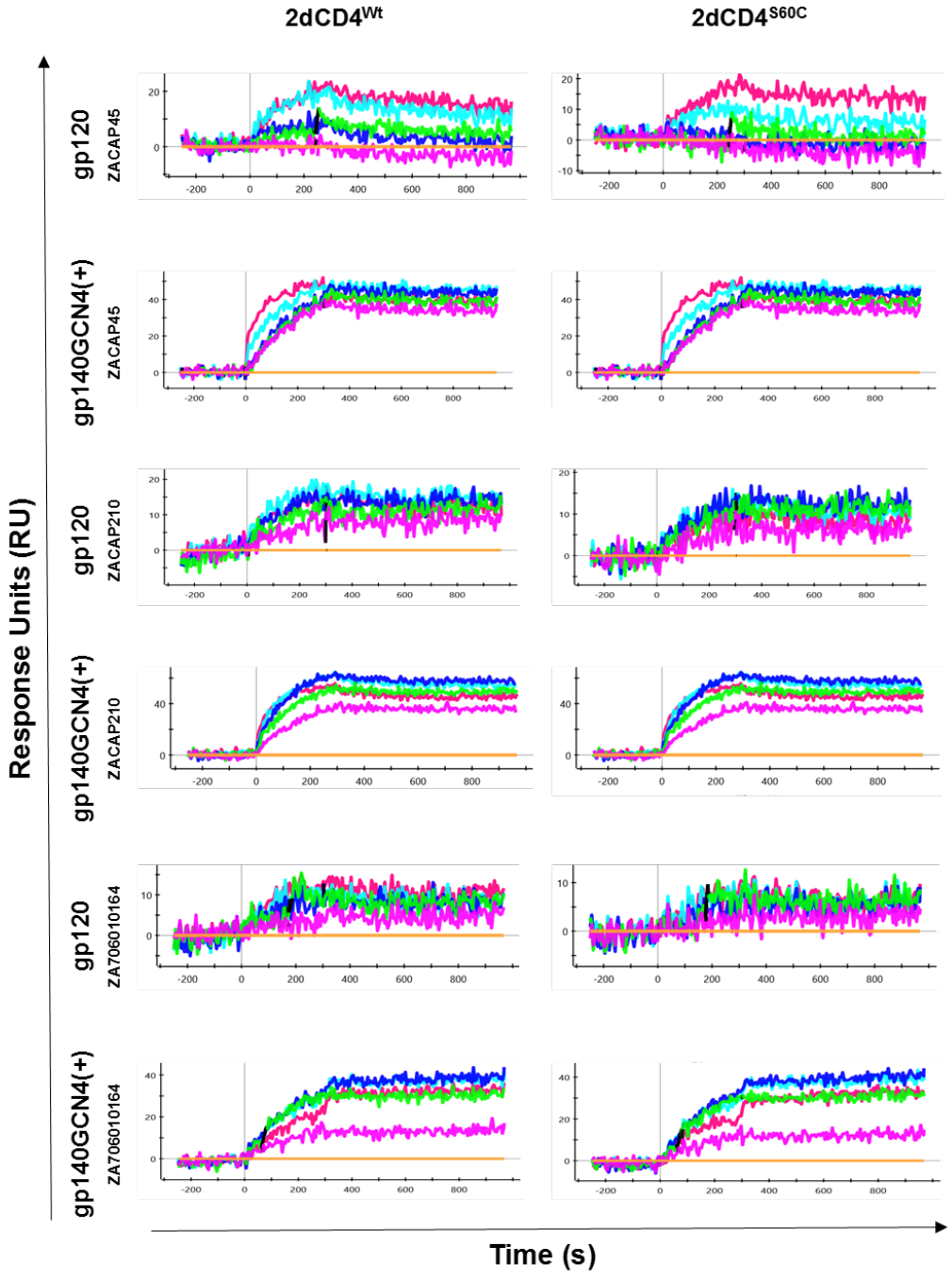

Figure 3.10: SPR sensorgrams showing binding of increasing concentrations of gp120 and gp140GCN4(+) conformations of the HIV-1 envelope glycoproteins of South African origin to 2dCD4^{Wt} and 2dCD4^{S60C}. 2dCD4^{Wt} and 2dCD4^{S60C} were covalently captured onto separate flow cells on the chip at a concentration of 0.02 mg/ml at a flow rate of 30 µl/min for 300 s to a final RU of 2000. The gp120 and gp140GCN4(+) Env's were passed over the ligands at a flow rate of 30 µl/min for 300 s with a dissociation time of 600 s.

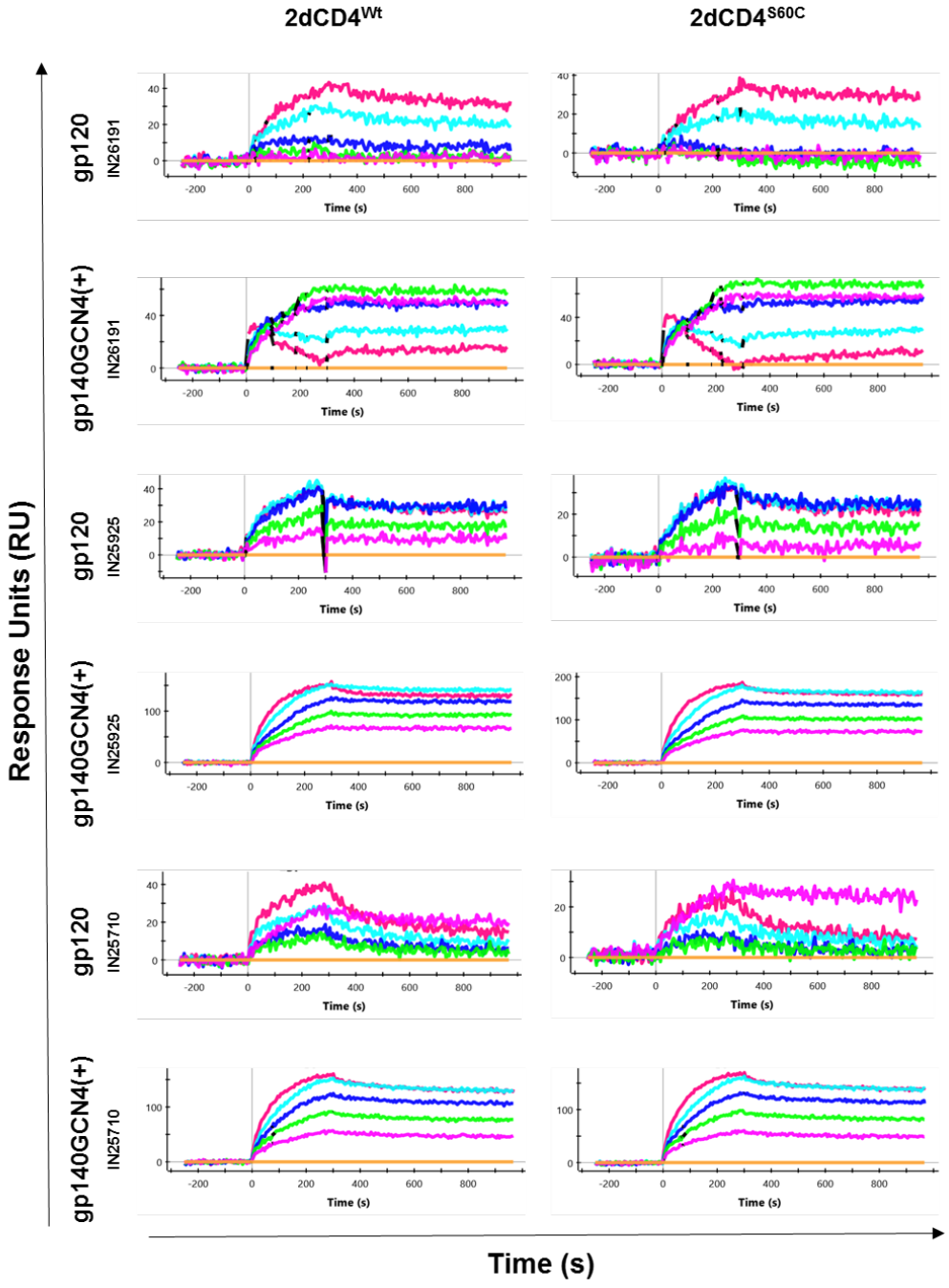

Figure 3.11: SPR sensorgrams showing binding of increasing concentrations of gp120 and gp140GCN4(+) conformations of the HIV-1 envelope glycoproteins of Indian origin to 2dCD4^{Wt} and 2dCD4^{S60C}. 2dCD4^{Wt} and 2dCD4^{S60C} were covalently captured onto separate flow cells on the chip at a concentration of 0.02 mg/ml at a flow rate of 30 µl/min for 300 s to a final RU of 2000. The gp120 and gp140GCN4(+) Env's were passed over the ligands at a flow rate of 30 µl/min for 300 s with a dissociation time of 600 s.

3.3.2 Surface plasmon resonance binding kinetics of the CD4bs directed bNAbs

The antigenicity of the Env variants was evaluated in respect of their CD4bs region using the bNAbs mentioned above (IgG1b12, VRC01, HJ16, VRC-CH31, NIH45-46^{G54W}, VRC03) and trimeric conformation using bNAb PGT145. The low yields obtained for some of the Env variants during mammalian cell culture expression limited the extent of the SPR analysis that could be performed. Initial screening was performed using single-analyte concentration of the Env variants to surface immobilized bNAbs (Table 3.2). Table 3.3 provides an overview of the full kinetic experiments performed.

Table 3.2: Summary of single-analyte concentrations of the gp120/ gp140GCN4(+) Env variants used to screen binding to surface immobilized bNAbs.

Env	IgG1b12	VRC01	HJ16	VRC-CH31	NIH45-46^{G54W}	VRC03	PGT145
$gp120_{ZACAP45}$	✓	✓	✓	✓	✓	✓	ND
$gp140_{ZACAP45}$	✓	✓	✓	✓	✓	✓	ND
$gp120_{ZACAP210}$	✓	✓	✓	✓	✓	✓	✓
$gp140_{ZACAP210}$	✓	✓	✓	✓	✓	✓	ND
$gp120_{ZA706010164}$	✓	✓	✓	✓	✓	✓	ND
$gp140_{ZA706010164}$	✓	✓	✓	✓	✓	✓	ND
$gp120_{IN26191}$	✓	✓	✓	✓	✓	✓	ND
$gp140_{IN26191}$	ND	ND	ND	ND	ND	ND	ND
$gp120_{IN25925}$	✓	✓	✓	✓	✓	✓	✓
$gp140_{IN25925}$	✓	✓	✓	✓	✓	✓	✓
$gp120_{IN25710}$	✓	✓	✓	✓	✓	✓	✓
$gp140_{IN25710}$	✓	✓	✓	✓	✓	✓	✓

✓ = SPR done
ND =no SPR done, therefore binding not determined.

Table 3.3: Summary of SPR experiments completed with a range of concentrations with the aim of obtaining kinetic data of the gp120/ gp140GCN4(+) Env variants binding to surface immobilized CD4 directed bNAbs and 2dCD4 variants.

Env	IgG1b12	VRC01	HJ16	VRC-CH31	NIH45-46^{G54W}	VRC03	PGT145	2dCD4^{Wt}	2dCD4^{S60C}
gp120$_{ZACAP45}$	✓	✓	✓	✓	✓	✓	ND	✓	✓
gp140$_{ZACAP45}$	✓	✓	✓	✓	✓	✓	ND	✓	✓
gp120$_{ZACAP210}$	✓	✓	ND	✓	✓	ND	✓No binding	✓	✓
gp140$_{ZACAP210}$	ND	ND	ND	ND	ND	ND	ND	✓	✓
gp120$_{ZA706010164}$	ND	ND	ND	ND	ND	ND	ND	✓	✓
gp140$_{ZA706010164}$	ND	ND	ND	ND	ND	ND	ND	✓	✓
gp120$_{IN26191}$	ND	ND	ND	ND	ND	ND	ND	✓	✓
gp140$_{IN26191}$	ND	ND	ND	ND	ND	ND	ND	✓	✓
gp120$_{IN25925}$	✓	✓	✓	✓	✓	✓	✓No binding	✓	✓
gp140$_{IN25925}$	✓	✓	✓	✓	✓	✓	✓	✓	✓
gp120$_{IN25710}$	✓	✓	ND	✓	✓	ND	✓No binding	✓	✓
gp140$_{IN25710}$	✓	✓	ND	✓	✓	ND	✓	✓	✓

✓ = Full range of concentrations done and data fitted successfully to1:1 Langmuir model
✓ = Full range of concentrations done but data did not fit the 1:1 Langmuir model
✓= Full range of concentrations done but kinetic data not obtained due to lack of binding or no measurable dissociation.

3.3.2.1 Env gp120/gp140GCN4(+) variant interactions with bNAbs

Having ascertained the structural integrity of the CD4bs of each of the Env variants, we next examined the interaction between the gp120/gp140GCN4(+) and selected CD4bs bNAb's by determining binding affinities using SPR. Single-analyte concentrations of 1000 nM of each of the gp120/gp140GCN4(+) Env variants were used to screen for binding to surface immobilized CD4bs-directed bNAbs (0.02 mg/ml) IgG1b12, VRC01, HJ16, VRC-CH31, NIH45-46^{G54W} and VRC03. Furthermore bNAb PGT145, which recognises a quaternary-structure-specific epitope in the V1V2 region of Env (Yasmeen et al., 2014) was used to assess the trimeric conformation of some the gp140GCN4(+) proteins relative to their gp120 monomers. Sufficient quantities were only available for gp120/gp140GCN4(+)$_{IN25925}$ and gp120/gp140GCN4(+)$_{IN25710}$.

Initial screening confirmed that the CD4bs-directed bNAbs were able to bind with varying degrees to both the monomeric and trimeric Envs (Figure 3.12 and 3.13). Furthermore this experiment confirmed that the antibodies were immoblilized on the chip in a biologically active conformation and provided an indication of the concentration range required to obtain accurate kinetic data.

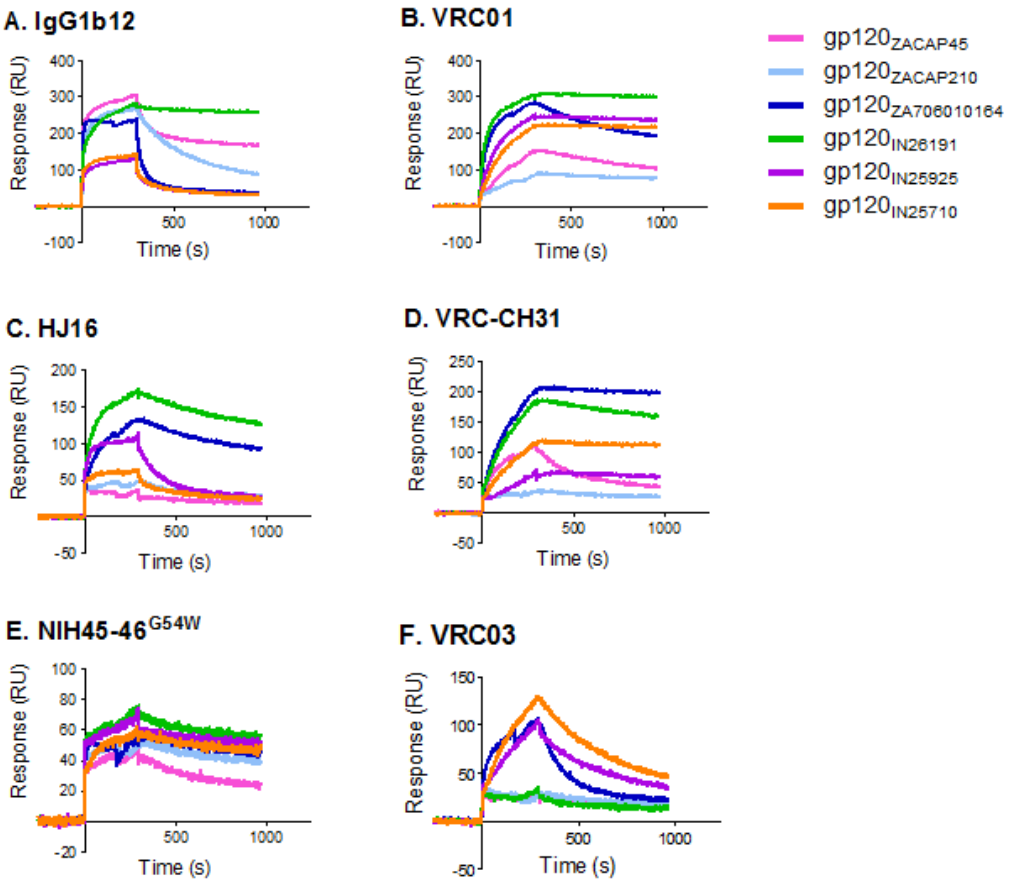

Figure 3.12: SPR sensorgrams of the binding response (RU) of single analyte concentrations (1000 nM) of gp120 Env variants to surface-immobilised monoclonal antibodies (A) IgG1b12, (B) VRC01, (C) HJ16, (D) VRC-CH31, (E) NIH5-46^{G54W} and (F) VRC03 over time (s). Association time was 300 s and dissociation time was 600 s. Monomeric and trimeric (Figure 3.13) proteins were run on the same chip, and PBS-T was included as a non-specificity binding control (Refer to Figure 3.13).

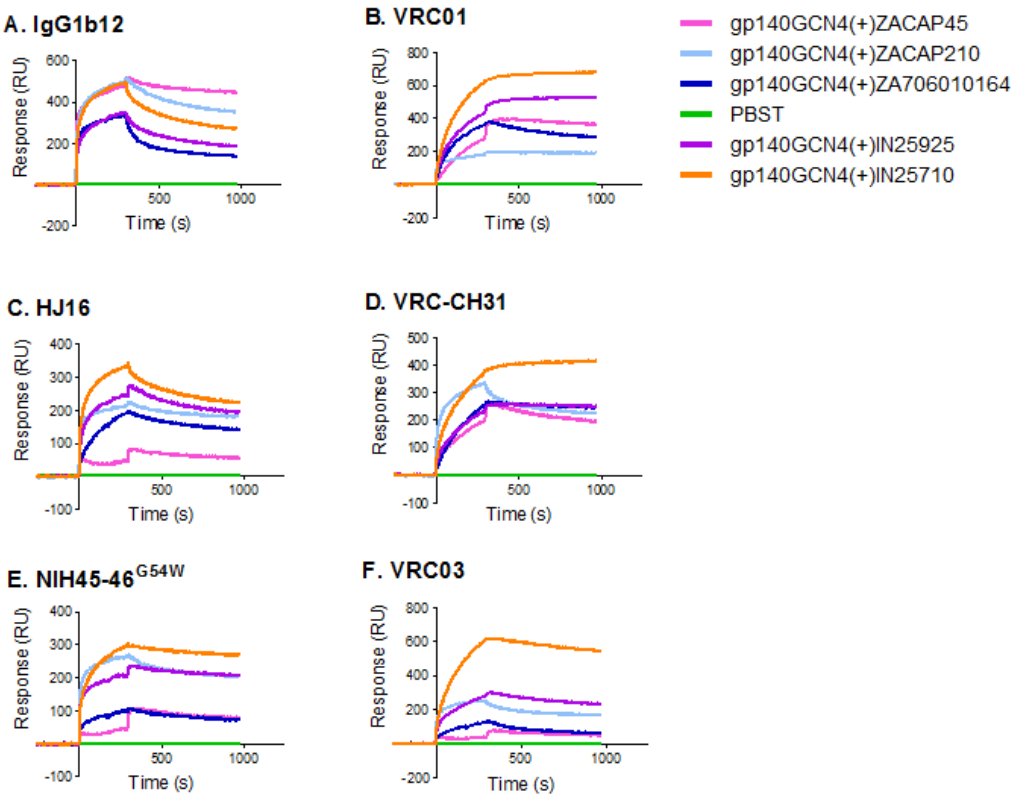

Figure 3.13: SPR sensorgrams of the binding response (RU) of single analyte concentrations (1000 nM) of gp140GCN4(+) Env variants to surface immobilised monoclonal antibodies (A) IgG1b12, (B) VRC01, (C) HJ16, (D) VRC-CH31, (E) NIH5-46^{G54W} and (F) VRC03 over time (s). Association time was 300 s and dissociation time was 600 s. No bNAb binding was performed with Env gp140GCN4(+)$_{IN26191}$ due to insufficient quantities of this Env. Monomeric (Figure 3.12) and trimeric proteins were run on the same chip, and PBS-T was included as a non-specificity binding control.

In order to characterize the gp120/gp140GCN4(+) Env variant interactions with CD4bs bNAbs in more detail, their binding affinities were determined using SPR (fits depicted in Figures 3.14 – 3.20 and kinetics tabulated in Table 3.4 and 3.5). The 1:1 Langmuir binding model was applied to the data to determine their binding kinetics. In some cases mass transfer was observed and binding kinetics were calculated by correcting for this using the ProteON manager software. In cases where mass transfer (Kt) was calculated by the ProteON manager software to be less than 1 x 10^8 (Table 3.4) a 1:1 Langmuir model with correction for mass transfer was applied to these analyses.

As mentioned earlier, the low yields obtained for some of the Env variants during mammalian cell culture expression limited the extent of the SPR analysis that could be performed (refer to table 3.3 for further details).

IgG1b12

The first bNAb evaluated, was the CD4bs directed bNAb IgG1b12. Accurate binding kinetics was only obtained for Env variant gp140GCN4(+)$_{ZACAP45}$ against IgG1b12 with an overall KD binding affinity of 0.424 nM. (Figure 3.14). Although binding was observed for the other Env variants tested, they did not fit the 1:1 Langmuir model (even with factoring in the mass transfer effect), therefore the strength of the interaction could not be accurately determined. Interestingly, Env variants IN25710 and IN25925, reported to be resistant to neutralization by IgG1b12 (Kulkarni et al., 2009) showed binding to this bNAb, which was improved for the trimeric conformations compared to the monomeric ones (Figure 3.14).

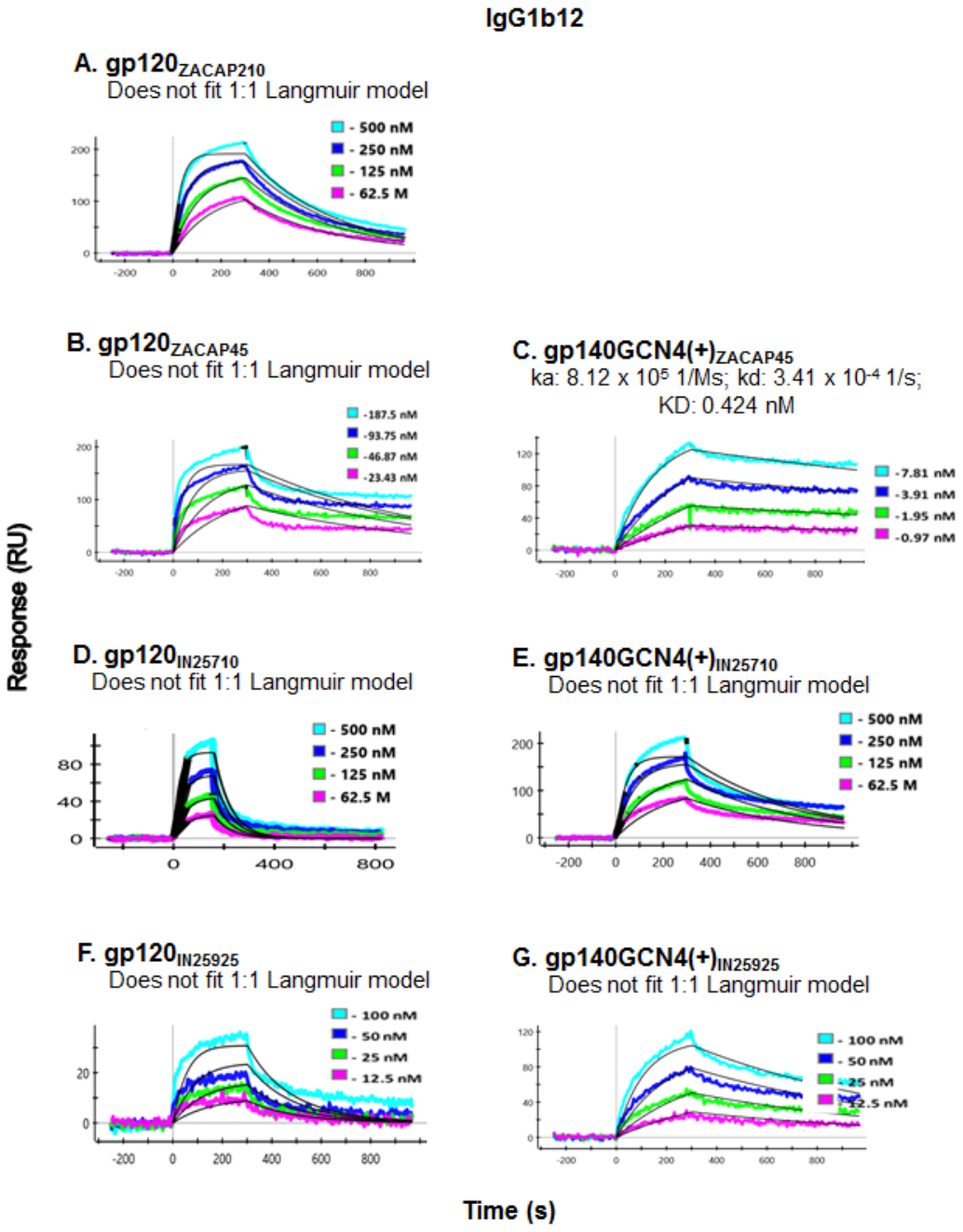

Figure 3.14: SPR sensorgrams of gp120/gp140GCN4(+) Env variant binding to immobilised bNAb IgG1b12 over time (s). Association time was 300 s and dissociation time was 600 s. For Env $gp120_{IN25710}$ association time was 200 s. Concentrations of Env variants used are shown in each sensorgram. Kinetic constants and binding affinities (Table 3.4 and 3.5) were calculated by fitting (black curves) using a 1:1 Langmuir model.

VRC01

Next we evaluated the CD4bs directed bNAb VRC01, which bound strongly to the Env variants derived from Indian patients (Figure 3.15). An approximate ten-fold increase in binding affinity was observed for the ZACAP45 trimeric protein ((gp140GCN4(+)) compared to the monomeric conformation (gp120), (18.7 nM and 139 nM, respectively (Figure 3.15). A similar pattern was observed for the IN25925 trimeric and monomeric Env conformations, however the dissociation rate could not be calculated for the trimer, as insufficient time was allowed for observable decay and thus an overall binding affinity could not be determined (Bio-Rad Laboratories Inc., 2011; Marquart, 2006-2016). The monomeric and trimeric conformations of Env IN25710 showed very little difference in their interaction with VRC01. Again the markedly slow dissociation observed for Env variants gp120/gp140GCN4(+)$_{IN25710}$ and gp140GCN4(+)$_{IN25925}$ could not be accurately quantified.

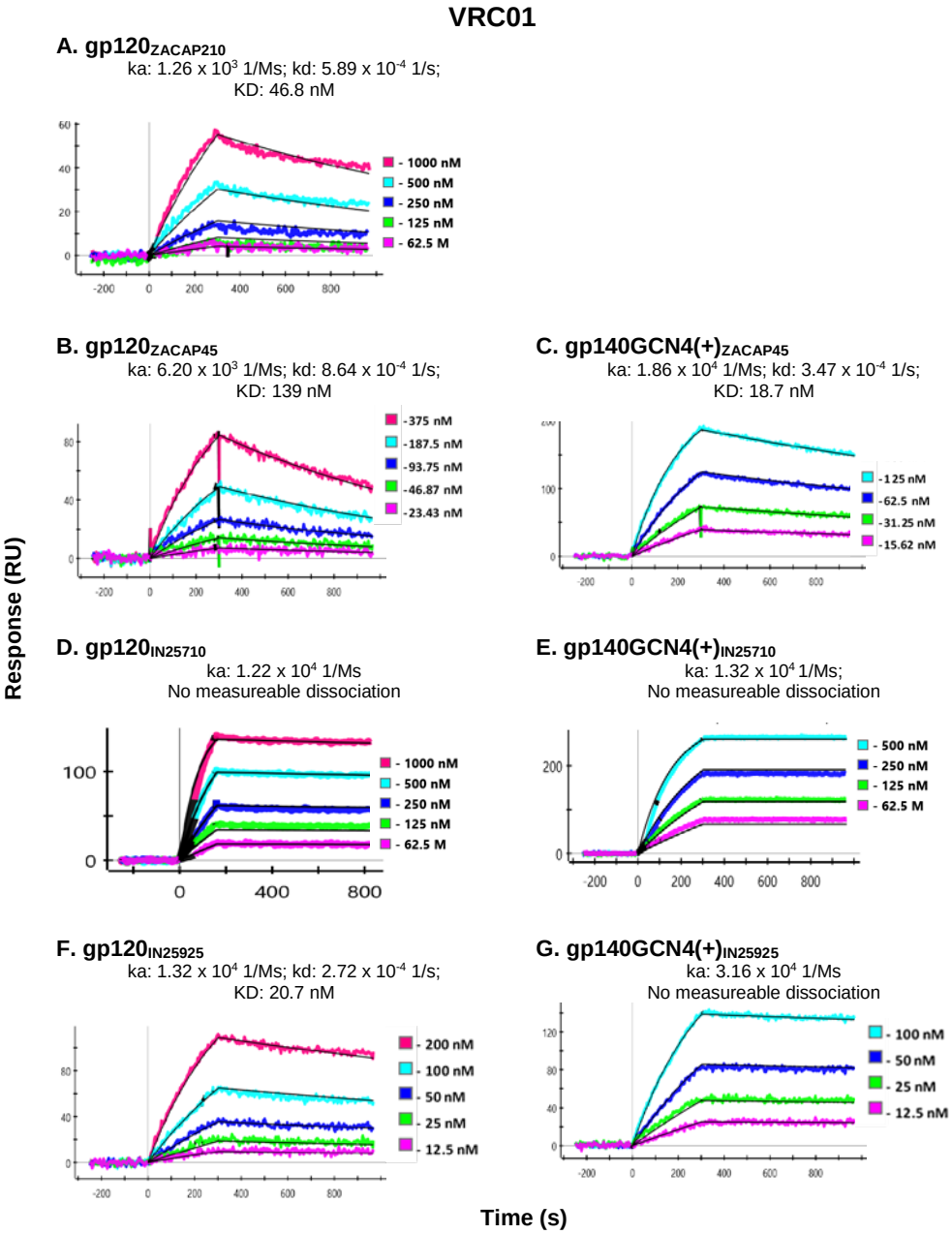

Figure 3.15: SPR sensorgrams of gp120/gp140GCN4(+) Env variant binding to immobilised bNAb VRC01 over time (s). Association time was 300 s and dissociation time was 600 s. For Env gp120$_{IN25710}$ association time was 200 s. Concentrations of Env variants used are shown in each sensorgram. Kinetic constants and binding affinities (Table 3.4 and 3.5) were calculated by fitting (black curves) using a 1:1 Langmuir model.

HJ16

SPR analysis for the interaction between HJ16 was limited to Env variants gp120/gp140GCN4(+)$_{ZACAP45}$ and gp120/gp140GCN4(+)$_{IN25925}$ (Figure 3.16). Negligible binding was observed for both the monomeric and trimeric conformations of Env ZACAP45 with HJ16. However, Env IN25925 was able to bind to HJ16, with improved binding observed for the trimeric conformation with a binding affinity of 21.6 nM compared to 93.2 nM for the monomeric conformation.

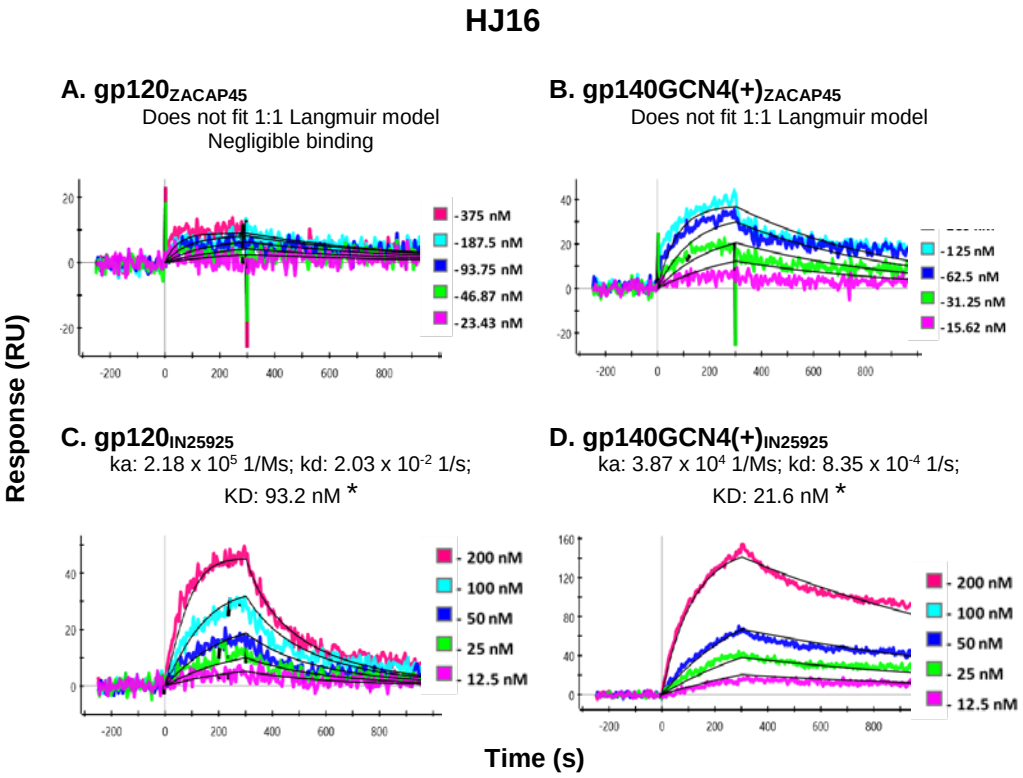

Figure 3.16: SPR sensorgrams of gp120/gp140GCN4(+) Env variant binding to immobilised bNAb HJ16 over time (s). Association time was 300 s and dissociation time was 600 s. Concentrations of Env variants used are shown in each sensorgram. Kinetic constants and binding affinities (Table 3.4 and 3.5) were calculated by fitting (black curves) using a 1:1 Langmuir model and correcting for mass transfer where appropriate (indicated by an asterix *).

VRC-CH31

The interaction of VRC-CH31 was performed with Env variants $gp120_{ZACAP210}$ and $gp120/gp140GCN4(+)_{ZACAP45/IN25710/IN25925}$ (Figure 3.17). VRC-CH31 showed favourable binding to the trimeric conformations of Env's ZACAP45, IN25710 and IN25925 than the corresponding monomers. In addition, negligible binding was observed for the monomer ZACAP210 to this VRC-CH31. A very strong interaction (kd $<10^{-5}$) was observed between VRC-CH31 with Env IN25710, however the dissociation rate could not be measured accurately due to the limited dissociation time of the experiment (Bio-Rad Laboratories Inc., 2011; Marquart, 2006-2016).

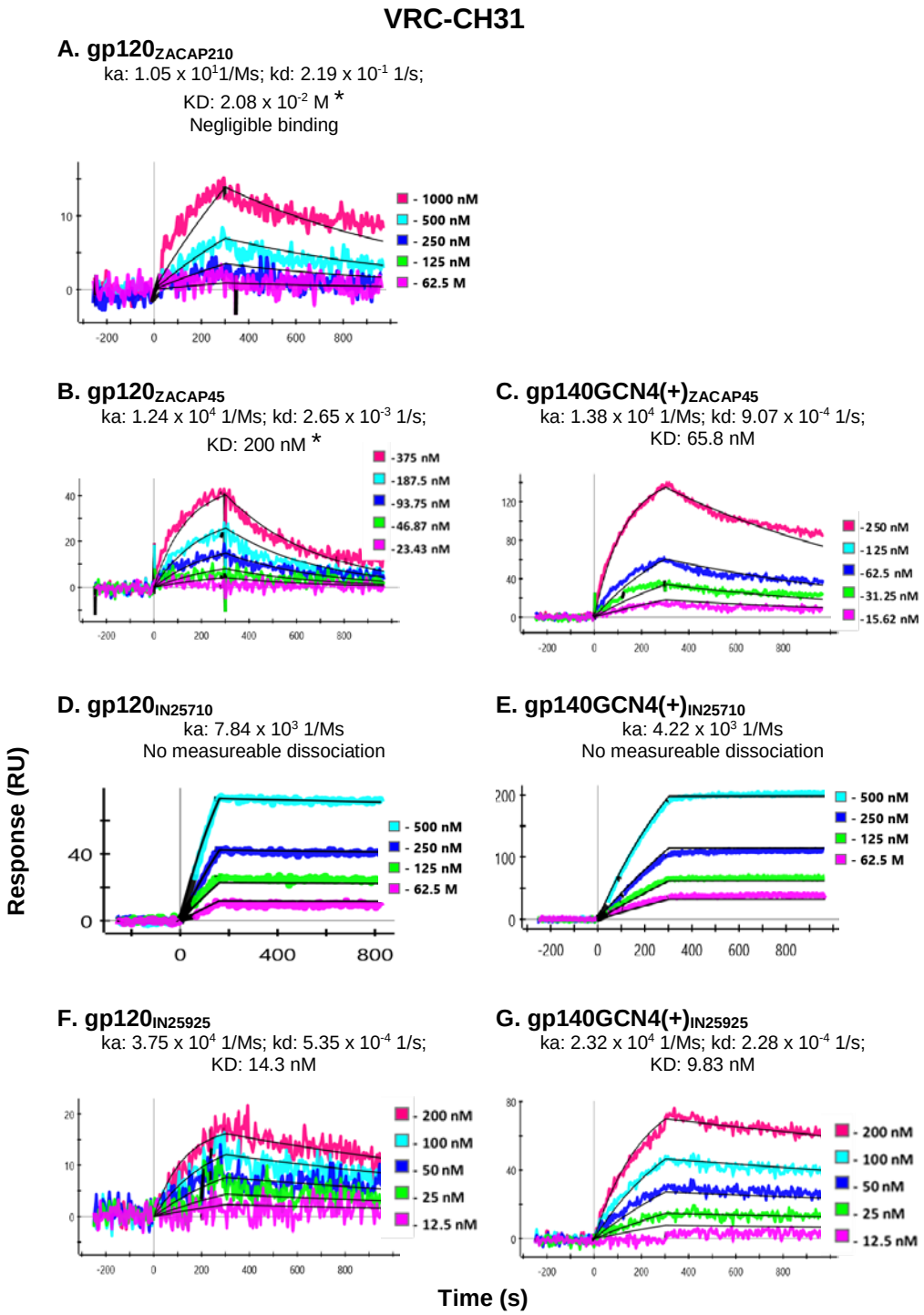

Figure 3.17: SPR sensorgrams of gp120/gp140GCN4(+) Env variant binding to immobilised bNAb VRC-CH31 over time (s). Association time was 300 s and dissociation time was 600 s. For Env gp120$_{IN25710}$ association time was 200 s. Concentrations of Env variants used are shown in each sensorgram. Kinetic constants and binding affinities (Table 3.4 and 3.5) were calculated by fitting (black curves) using a 1:1 Langmuir model and correcting for mass transfer where appropriate (indicated by an asterix *).

NIH45-46^{G54W}

The interaction of NIH45-46^{G54W} was performed with Env variants gp120$_{ZACAP210}$ and gp120/gp140GCN4(+)$_{ZACAP45/IN25710/IN25925}$ (Figure 3.18). Overall, improved binding was observed for the trimeric Env conformations compared to their matched monomeric conformations. In respect of the trimeric conformations, the Indian Env's IN25710 and IN25925 had lower overall binding affinities compared to the South African Env ZACAP45. However, although very little dissociation is seen in the sensorgram with Env variant gp140GCN4(+)$_{IN25710}$, the dissociation rate could not be accurately measured due to limited dissociation time (Bio-Rad Laboratories Inc., 2011; Marquart, 2006-2016).

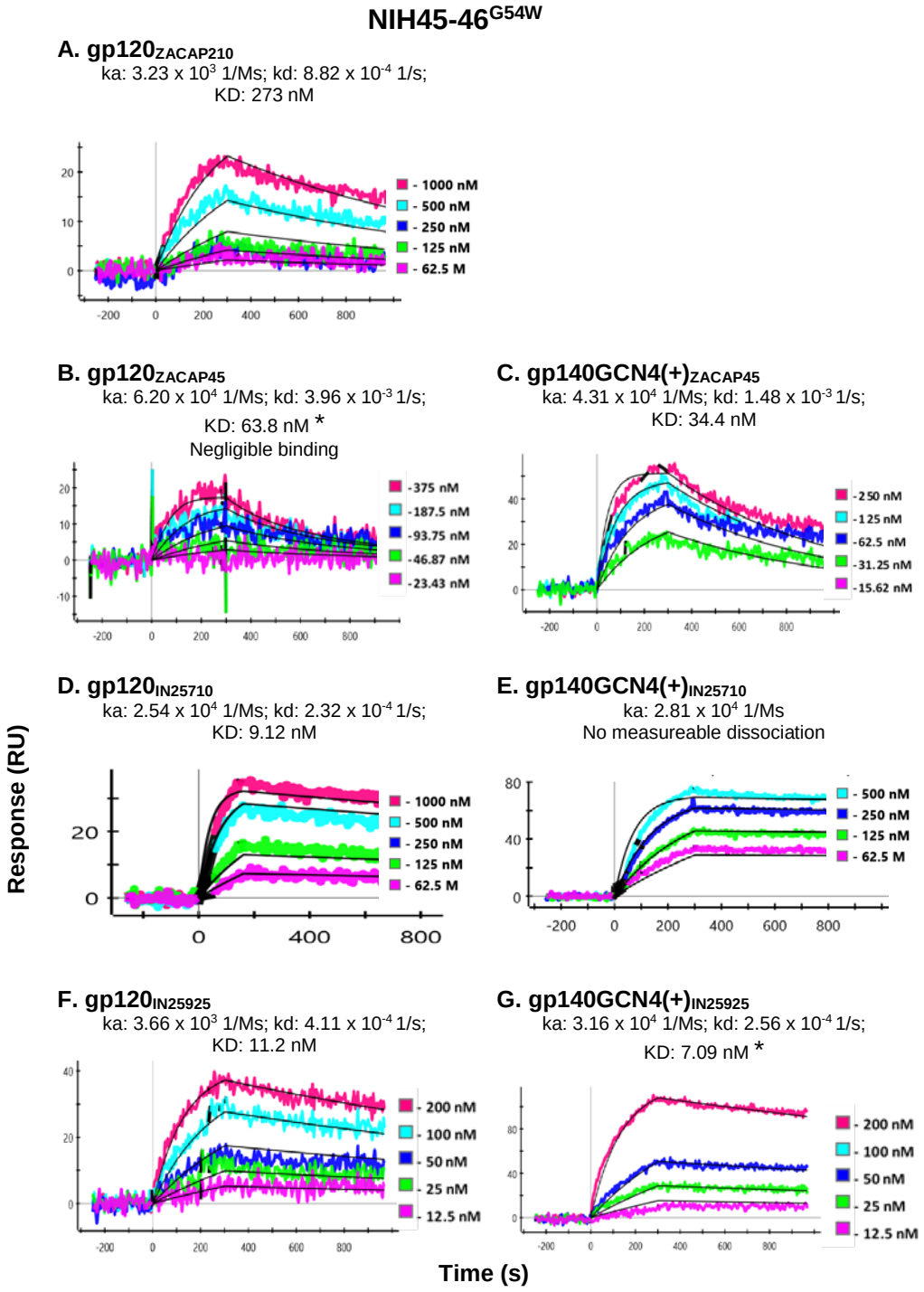

Figure 3.18: SPR sensorgrams of gp120/gp140GCN4(+) Env variant binding to immobilised bNAb NIH45-46^{G54W} over time (s). Association time was 300 s and dissociation time was 600 s. For Env gp120$_{IN25710}$ association time was 200 s. Concentrations of Env variants used are shown in each sensorgram. Kinetic constants and binding affinities (Table 3.4 and 3.5) were calculated by fitting (black curves) using a 1:1 Langmuir model and correcting for mass transfer where appropriate (indicated by an asterix *).

VRC03

The interaction of bNAb VRC03 was only evaluated with Env variants gp120/gp140GCN4(+)$_{ZACAP45}$ and gp120/gp140GCN4(+)$_{IN25925}$ (Figure 3.19) and binding kinetics was only obtained for gp120$_{IN25925}$ since the interaction with the other Env's tested did not fit the 1:1 Langmuir model. In general, binding of VRC03 to these Env variants was poor.

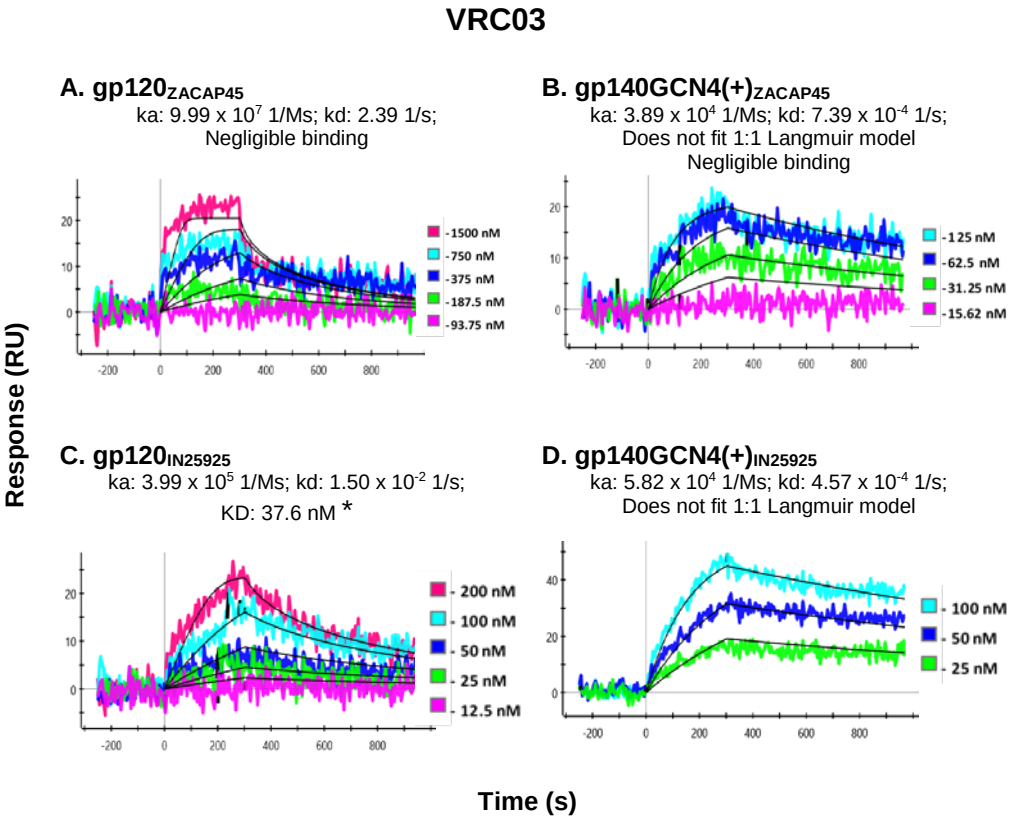

Figure 3.19: SPR sensorgrams of gp120/gp140GCN4(+) Env variant binding to immobilised bNAb VRC03 over time (s). Association time was 300 s and dissociation time was 600 s. Concentrations of Env variants used are shown in each sensorgram. Kinetic constants and binding affinities (Table 3.4 and 3.5) were calculated by fitting (black curves) using a 1:1 Langmuir model and correcting for mass transfer where appropriate (indicated by an asterix *).

PGT145

SPR was performed to evaluate the interaction of the V1V2-directed quaternary-structure-specific bNAb PGT145 with the Env variants. Unfortunately insufficient quantities of the Env variants limited evaluation of this bNAb to gp120/gp140GCN4(+)$_{IN25710/IN25925}$. Initial single analyte screening using a concentration of 1000 nM was performed (data not shown) followed by a range of concentrations to obtain binding kinetics data.

A considerable increase in binding of PGT145 was observed between the trimers of the Env variants evaluated compared to the monomers (Figure 3.20). However, a comparison of the binding kinetics could not be performed due to the interactions not fitting the 1:1 Langmuir model for the trimeric conformations.

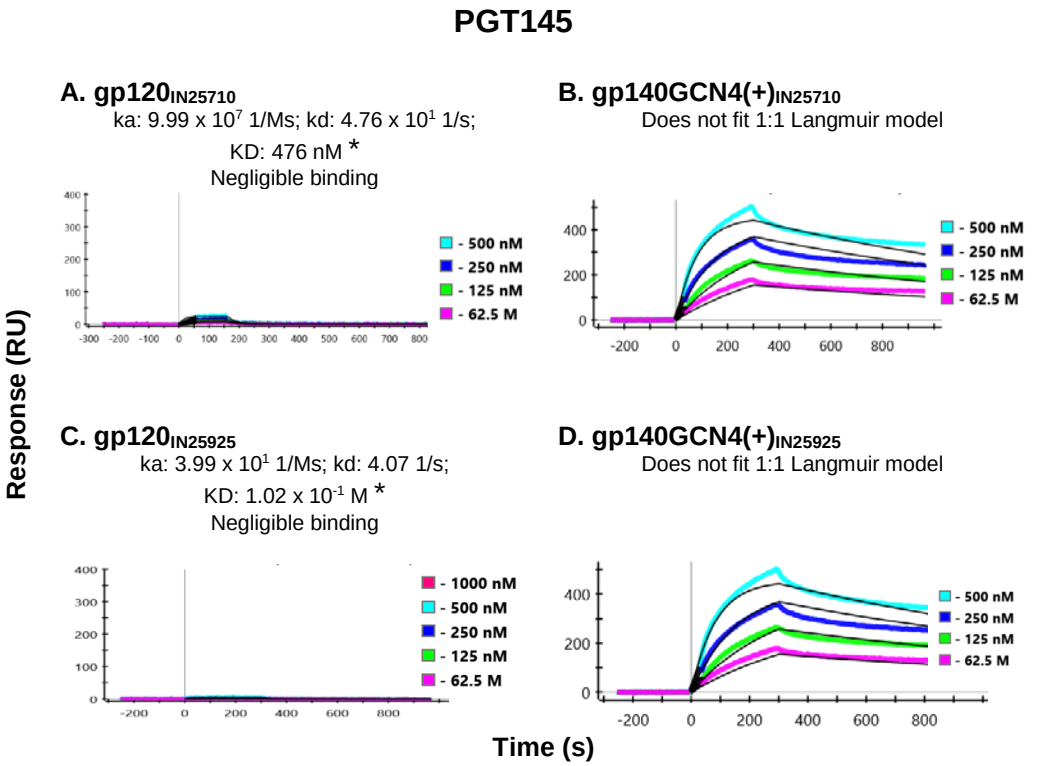

Figure 3.20: SPR sensorgrams of gp120/gp140GCN4(+) Env variant binding to immobilised bNAb PGT145 over time (s). Association time was 300 s and dissociation time was 600 s. For Env gp120IN25710 association time was 200 s. Concentrations of Env variants used are shown in each sensorgram. Kinetic constants and binding affinities (Table 3.4 and 3.5) were calculated by fitting (black curves) using a 1:1 Langmuir model and correcting for mass transfer where appropriate (indicated by an asterix *). Response unit axes (y-axis) were standardized between the monomeric and trimeric conformations of a particular Env variant to highlight the differences between PGT145 binding to a trimer compared to a monomer.

A summary of the binding kinetics obtained for the bNAbs evaluated are shown in Tables 3.4 and 3.5. Overall, the trimeric conformations showed favourable binding to bNAbs IgG1b12, VRC01, VRC-CH31, NIH45-46^{G54W} and PGT145 compared to the monomers. Accurate binding kinetics could not be determined for Env variants gp140GCN4(+)$_{IN25925}$ with VRC01, gp120/gp140GCN4(+)$_{IN25710}$ with VRC01 and VRC-CH31, and gp140GCN4(+)$_{IN25710}$ with NIH45-46^{G54W} due to an insufficient dissociation time to observe decay of the interaction. In addition, kinetics was not obtained for some of the interactions since they did not fit a 1:1 Langmuir

model. Despite the lack of fits, binding was observed and suggests that the epitopes recognized by the CD4bs-directed bNAbs evaluated (IgG1b12, VRC01, HJ16, VRC-CH31, NIH45-46^{G54W} and VRC03) are accessible on the Env variants tested. Overall, this data suggests that the Env variants selected are antigenically correct and suitable for complex formation and immunogenicity testing in a small animal model.

Table 3.4: Binding rate constants for the South African gp120/gp140GCN4(+) Env variants with CD4bs bNAbs and PGT145 as measured by SPR.

Env variant	Antibody	ka (1/Ms)	ka Error (1/Ms)	kd (s^{-1})	kd Error (s^{-1})	KD (nM)	Rmax (RU)	kt (RU/Ms)	Chi^2 (RU)
$gp120_{ZACAP45}$	IgG1b12	Does not fit 1:1 Langmuir model							
	VRC01	6.20E+03	6.18E+01	8.64E-04	3.98E-06	139	187.38	6.33E+18	3.87
	HJ16	Does not fit 1:1 Langmuir model; negligible binding							
	VRC-CH31	1.24E+04	1.32E+03	2.65E-03	2.30E-04	200	68.95	2.28E+06	7.26
	NIH45-46^{G54W}	6.20E+04	4.99E+03	3.96E-03	2.95E-04	Negligible	20.27	9.39E+05	5.05
	VRC03	Does not fit 1:1 Langmuir model							
$gp140GCN4(+)_{ZACAP45}$	IgG1b12	8.12E+05	3.72E+03	3.41E-04	3.77E-06	0.42	151.89	5.13E+21	13.65
	VRC01	1.86E+04	4.98E+01	3.47E-04	1.81E-06	18.7	258.25	1.52E+20	6.35
	HJ16	Does not fit 1:1 Langmuir model							
	VRC-CH31	1.38E+04	7.53E+01	9.07E-04	5.30E-06	65.8	165.94	3.97E+18	16.91
	NIH45-46^{G54W}	4.31E+04	3.23E+02	1.48E-03	7.95E-06	34.4	51.58	1.42E+22	8.45
	VRC03	Does not fit 1:1 Langmuir model							
$gp120_{ZACAP210}$	IgG1b12	Does not fit 1:1 Langmuir model							
	VRC01	1.26E+03	3.54E+01	5.89E-04	6.26E-06	46.8	190.17	2.88E+17	4.51
	VRC-CH31	1.05E+01	1.66E+01	2.19E-01	3.45E-01	Negligible	1.00E+06	5.47E+04	1.95
	NIH45-46^{G54W}	3.23E+03	8.46E+01	8.82E-04	1.38E-05	273	41.67	2.52E+16	3.62

Kd: Dissociation constant
KD: Equilibrium constant (kd/ka)
Rmax: Maximum theoretical response assuming all of the ligand is active, ligand is 100% pure and all binding sites are available
Chi^2: Chi-squared is the average of squared residuals and indicates fitting confidence. Chi^2 values lower than approximately 4 or 5 are seldom achieved due to background noise
Rmax: For kinetic analysis, Rmax ≤200 RU is recommended
Chi^2: chi-squared values less than 10% of Rmax are considered acceptable
Kt: 1:1 Langmuir with mass transfer model applied if kt ≤1.00E+08 RU/Ms
Negligible: binding of Env variant to bNAb was negligible
No measureable dissociation: The dissociation phase was not long enough to observe sufficient decay for kd values ≤ 1.00E-05, therefore kinetics could not be determined.

Table 3.5: Binding rate constants for the Indian gp120/gp140GCN4(+) Env variants with CD4bs bNAbs and PGT145 as measured by SPR.

Env variant	Antibody	ka (1/Ms)	ka Error (1/Ms)	kd (s^{-1})	kd Error (s^{-1})	KD (nM)	Rmax (RU)	kt (RU/Ms)	Chi^2 (RU)
$gp120_{IN25925}$	IgG1b12	Does not fit 1:1 Langmuir model							
	VRC01	1.32E+04	8.67E+01	2.72E-04	2.88E-06	20.7	205.37	4.35E+19	5.02
	HJ16	2.18E+05	3.19E+04	2.03E-02	3.00E-03	93.2	66.22	2.52E+06	6.99
	VRC-CH31	3.75E+04	6.46E+02	5.35E-04	1.59E-05	14.3	18.96	4.37E+19	3.81
	NIH45-46^{G54W}	3.66E+04	3.07E+02	4.11E-04	7.61E-06	11.2	43.50	4.40E+18	4.97
	VRC03	3.99E+05	1.21E+05	1.50E-02	4.52E-03	37.6	27.88	7.45E+05	4.69
	PGT145	3.99E+01	1.42E+04	4.07E+00	1.45E+03	Negligible	1.00E+06	3.67E+05	2.35
$gp140GCN4(+)_{IN25925}$	IgG1b12	Does not fit 1:1 Langmuir model							
	VRC01	3.17E+04	1.39E+02	No measureable dissociation					
	HJ16	3.88E+04	1.89E+02	8.35E-04	5.09E-06	21.6	168.82	5.28E+19	16.40
	VRC-CH31	2.32E+04	1.93E+02	2.28E-04	5.73E-06	9.83	94.98	3.06E+19	9.59
	NIH45-46^{G54W}	3.16E+04	1.40E+03	2.56E-04	5.01E-06	7.09	123.60	6.75E+07	10.09
	VRC03	Does not fit 1:1 Langmuir model							
	PGT145	Does not fit 1:1 Langmuir model							
$gp120_{IN25710}$	IgG1b12	Does not fit 1:1 Langmuir model							
	VRC01	1.22E+04	3.95E+01	No measureable dissociation					
	VRC-CH31	7.84E+03	8.39E+01	No measureable dissociation					
	NIH45-46^{G54W}	2.54E+04	1.83E+02	2.32E-04	7.63E-06	9.12	32.99	1.09E+20	3.50
	PGT145	9.99E+07	1.83E+02	4.76E01	7.63E-06	Negligible	53.26		3.27
$gp140GCN4(+)_{IN25710}$	IgG1b12	Does not fit 1:1 Langmuir model							
	VRC01	1.32E+04	5.60E+01	No measureable dissociation					
	VRC-CH31	4.22E+03	3.97E+01	No measureable dissociation					
	NIH45-46^{G54W}	2.81E+04	1.21E+02	No measureable dissociation					
	PGT145	Does not fit 1:1 Langmuir model							

Kd: Dissociation constant
KD: Equilibrium constant (kd/ka)
Rmax: Maximum theoretical response assuming all of the ligand is active, ligand is 100% pure and all binding sites are available
Chi^2: Chi-squared is the average of squared residuals and indicates fitting confidence. Chi^2 values lower than approximately 4 or 5 are seldom achieved due to background noise
Rmax: For kinetic analysis, Rmax ≤200 RU is recommended
Chi^2: chi-squared values less than 10% of Rmax are considered acceptable
Kt: 1:1 Langmuir with mass transfer model applied if kt ≤1.00E+08 RU/Ms
Negligible: binding of Env variant to bNab was negligible
No measureable dissociation: The dissociation phase was not long enough to observe sufficient decay for kd values ≤ 1.00E-05, therefore kinetics could not be determined.

3.3.3 Selection of gp120/gp140GCN4(+) Envs for immunogenicity studies

In order to further compare the Env variants from South Africa and India, a single matched monomeric (gp120) and trimeric (gp140GCN4(+)) Env from each country was selected for immunogenicity testing in small animal model. The selection of the two Env's was based on extensive amino acid sequence analysis, including the identification of critical amino acid residues that are known to confer resistance to known CD4bs directed bNAbs. In addition, the Env expression yields in mammalian cell culture were taken into consideration.

Env ZACAP45 was selected as the South African representative since it was the only South African Env that did not contain any of the signature amino acids, (346E, 357A and 441E (Figure 3.2) (HXBc2 numbering 340E, 350A and 429E)) as reported by Shankarappa et al. (2001) to be characteristic of Indian Env's. Env ZACAP45 also consistently expressed 2 mg and 1 mg of purified Env per litre of cell culture supernatant for the gp120 and gp140GCN4(+) conformations, respectively.

Envelope sequences gp120/gp140GCN4(+)$_{IN25710}$ and gp120/gp140GCN4(+)$_{IN25925}$ encompass representatives of Indian origin, which are typically resistant to neutralization by bNAb IgG1b12 whereas gp120/gp140GCN4(+)$_{IN26191}$ does not (Kulkarni et al., 2009). However, since the Env produced by the gp140(GCN4(+)$_{IN25710}$ appeared to be an oligomer as observed in SEC (Figure 3.5) and SDS-PAGE analysis (Figure 3.8), it was excluded from selection. In addition, Env gp120/gp140GCN4(+)$_{IN25925}$ consistently expressed 2 mg/litre compared to poor expression of the other Indian Envs. Thus gp120/gp140GCN4(+)$_{IN25925}$ was selected as the Indian Env for immune complex formation and subsequent immunization studies.

3.4 Generation of gp120/gp140GCN4(+) variant Env/2dCD4^{S60C} complexes

The 2dCD4-liganded gp120/gp140GCN4(+) complexes for the immunogenicity studies were generated by incubating equivalent microgram amounts of 2dCD4^{S60C} and Env (equivalent to a five-molar excess of 2dCD4) in the presence or absence of a reducing agent at room temperature for an hour. The reducing reagent (final concentration of 1mM β-mercaptoethanol) was included to help facilitate the intermolecular disulphide exchange between Env variants and 2dCD4^{S60C} (Cerutti et al., 2010), and formation of a disulphide-stabilized Env/2dCD4^{S60C} complex.

After complex formation of the Env/2dCD4^{S60C}, SEC was used to remove any unbound 2dCD4^{S60C} (Figure 3.21). Fractions corresponding to the relevant Env conformation were collected (indicated by the shaded areas in Figure 3.21), concentrated and assessed for the presence of bound 2dCD4^{S60C} using SDS-PAGE (Figure 3.22). SDS-PAGE analysis of Env/2dCD4^{S60C} complexes revealed the presence of Env (120 kDa for gp120; and 140 kDa for gp140GCN4(+)), and 2dCD4^{S60C} at approximately 25 kDa under reducing conditions representing 2dCD4^{S60C} (Figure 3.23). The bands were of similar size and intensity and confirmed the presence of bound 2dCD4 to the Env. The absence of a distinct band at 120 kDa for the monomeric portion of gp140GCN4(+) complex on the SDS-PAGE gel suggests the trimers are predominantly comprised of uncleaved gp140 subunits (Figure 3.22).

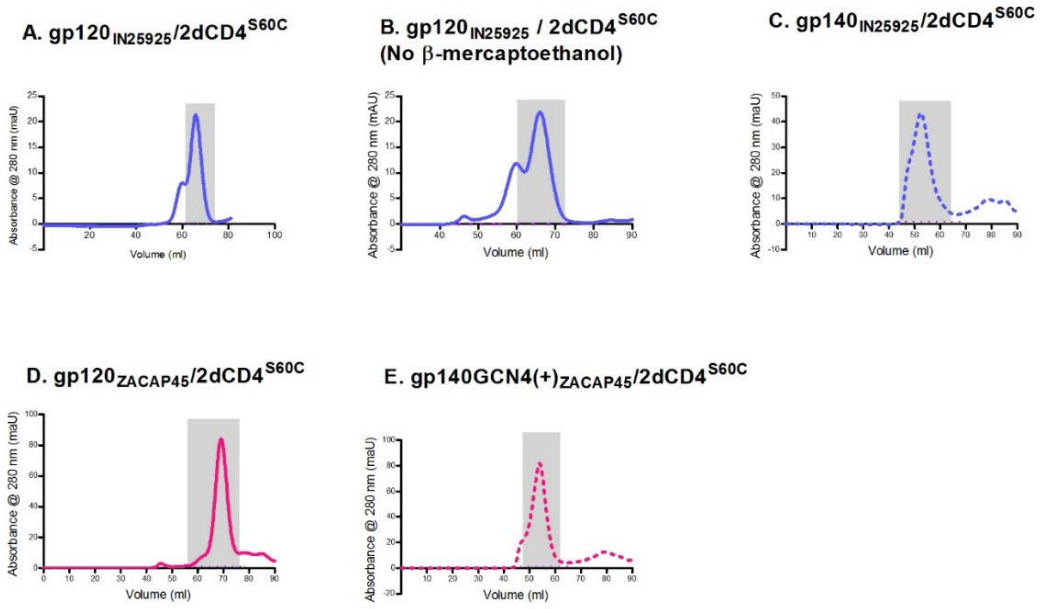

Figure 3.21: Size exclusion chromatograms of envelope glycoproteins complexed with 2dCD4^{S60C}. The Env/2dCD4^{S60C} liganded complexes were generated by incubating the relevant Env with 2dCD4^{S60C} under reducing conditions at room temperature, with the exception of B, which was incubated under non-reducing conditions at room temperature. The complexes were resolved over a Superdex 200 gel filtration column to remove any unbound 2dCD4^{S60C}. The shaded area of the gel filtration profiles (A280nm) represents the fractions collected of the Env/2dCD4^{S60C} liganded complex, corresponding to the relevant Env conformation. A. gp120$_{IN25925}$/2dCD4^{S60C}, B. gp120$_{IN25925}$/2dCD4^{S60C} under non-reducing conditions, C. gp140GCN4(+)$_{IN25925}$/2dCD4^{S60C}, D. gp120$_{ZACAP45}$/2dCD4^{S60C}, E. gp140GCN4(+)$_{IN25925}$/2dCD4^{S60C}.

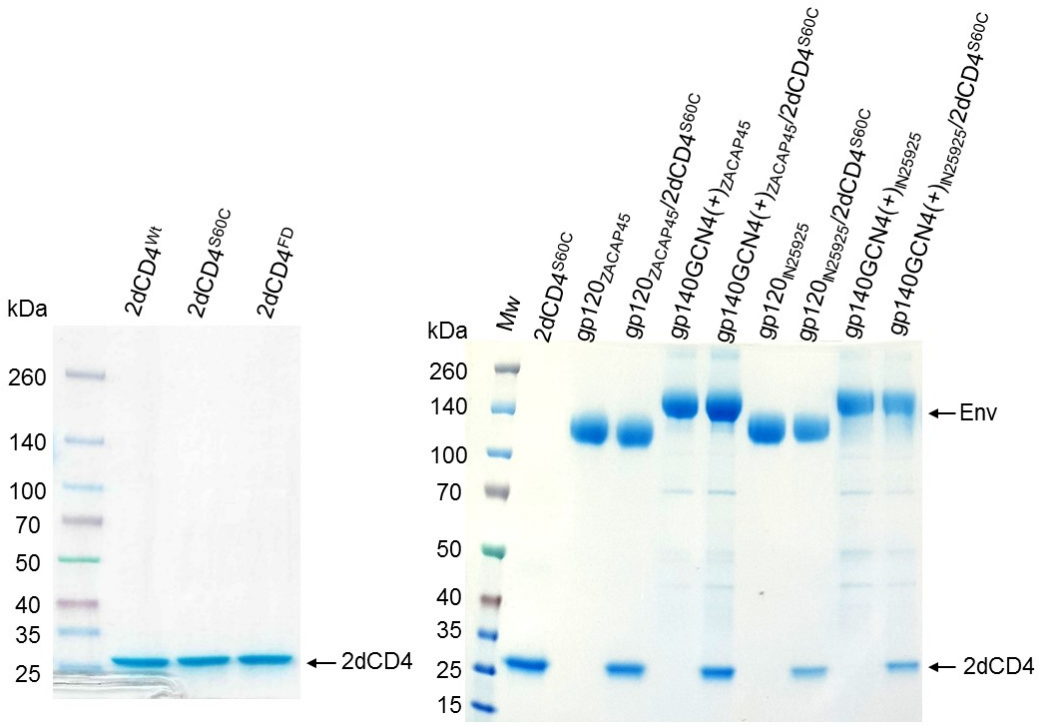

Figure 3.22: Coomassie stained SDS-PAGE analysis of Env/2dCD4^{S60C} complexes used for rabbit immunizations. Complexes were separated on a SDS-PAGE gel under reducing conditions and the presence of 2dCD4^{S60C} in the SEC purified Env/2dCD4 complex confirms the successful formation 2dCD4^{S60C}-liganded complexes.

The use of the reducing reagent during complex formation had no apparent aberrant effect on binding of 2dCD4^{S60C} to Env and complex formation as determined by SDS-PAGE analysis (Figure 3.23).

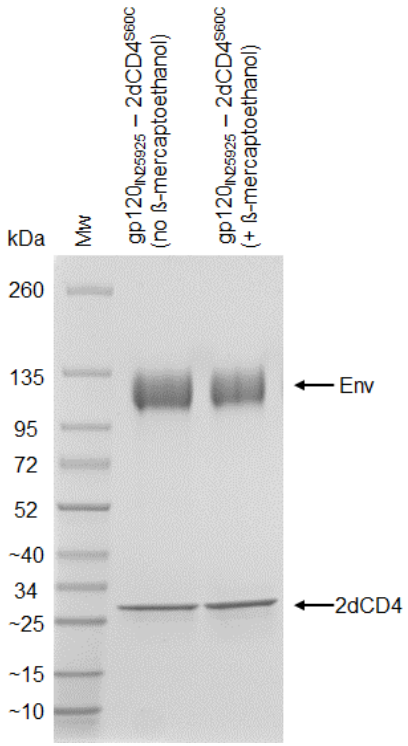

Figure 3.23: Coomassie stained SDS-PAGE analysis of the Env/2dCD4 complexes generated using the two protocols, with and without a reducing agent (ß-mercaptoethanol). The presence or absence of a reducing agent had no effect on the formation of the 2dCD4^{S60C}-liganded Env.

Purified complexes were quantified, aliquoted, snap frozen and stored at -80°C until used in the immunizations.

3.5 Comparative Immunogenicity of recombinant gp120/gp140GCN4(+) Env variants, peptides, DNA vaccines and cyclic permutants

The overall goal of this study was to compare the immunogenicity of two arms of immunogens. The first arm comprised two gp120/gp140 Env variants representative of Env's isolated from patients in South Africa and India. This comparison was made by comparing the use of a monomeric (gp120) and trimeric (gp140GCN4(+)) form of each Env as a subunit vaccine as well as comparing them alone or liganded with 2dCD4 to expose conserved CD4i epitopes. The second arm comprised stabilized gp120/gp140 Env derivatives based on HIV-1 subtype B (peptides, DNA vaccines and cyclic permutants) from our collaborating laboratory in India as part of the South African Department of Science and Technology/TIA India-SA bilateral on HIV Vaccine Development, and these immunogens aimed to present the CD4bs epitopes to the immune system.

To this end, the following were successfully tested for their immunogenicity in New Zealand White rabbits:

Arm 1: subtype C gp120/gp140 Env variants:

- gp120/gp140GCN4(+)$_{ZACAP45}$ (2dCD4^{S60C} – liganded and unliganded)
- gp120/gp140GCN4(+)$_{IN25925}$ (2dCD4^{S60C} – liganded and unliganded)

Arm 2: subtype B stabilized gp120/gp140 Env derivatives

- cyclic permutant Cyc40D JRFL gp120 prime followed by the trimeric hCMP V1cyc 14-142 boost.
- OD$_{EC}$COBPICS gp120 prime followed by the trimeric hCMP JRLF V1cyc 14-142 boost.
- V570D DNA prime followed by the trimeric hCMP JRFL V1 cyclic permutant boost.
- peptide prime 1WR2 followed by gp140^{FV} protein boost.
- peptide prime 1ORC followed by gp140^{FV} protein boosts.

Serum was collected at a relevant time points (Tables 2.2 and 2.3), and tested for the presence of anti-HIV Env antibodies (and anti-CD4, where relevant), as well as neutralizing breadth and potency.

3.5.1 Antibody titres against relevant immunogens in rabbit sera

In order to quantify and compare the antibody response elicited in the first arm immunization study, end-point antibody titers were determined for the terminal bleeds for rabbits immunized with the gp120/gp140GCN4(+)$_{ZACAP45/IN25925}$ Env variants. The sera was titred against matched Env variants for both the 2dCD4^{S60C}-liganded and unliganded groups (Figure 3.24).

Anti-2dCD4 responses elicited by Env/2dCD4^{S60C} immune complexes and 2dCD4$^{S60C/Wt/FD}$ control groups, were determined by titering against immobilized 2dCD4^{S60C}. Rabbits immunized with the 2dCD4^{FD} variant raised the highest anti-2dCD4 antibody titre of 1.77 x 10^7, compared to 2dCD4^{Wt} (7.68 x 10^6) and 2dCD4^{S60C} (1.79 x 10^6) (Figure 3.24 A).

Furthermore, anti-2dCD4 responses were observed in rabbit sera immunized with the gp120/gp140-Env 2dCD40^{S60C}-liganded immunogens (Figure 3.24 A). The anti-2dCD4 titres obtained for the gp120/2dCD4^{S60C}-liganded groups were 1.29 x 10^6 and 1.88 x 10^6 for the monomeric gp120$_{ZACAP45}$/2dCD4^{S60C} and gp120$_{IN25925}$/2dCD4^{S60C} immunogens, respectively, similar to that of the 2dCD4^{S60C} control group. Interestingly, the titres detected in rabbit sera for groups immunized with the trimeric 2dCD4^{S60C}-liganded Env's (gp140GCN4(+)$_{ZACAP45}$/2dCD4^{S60C} and gp140GCN4(+)$_{IN25925}$/2dCD4^{S60C} elicited anti-2dCD4 titres of 1.22 x 10^5 and 2.44 x 10^5 respectively, approximately 10-fold lower than the 2dCD4-liganded monomeric Env's as well as the 2dCD4^{S60C} control group (Figure 3.24)

The matched anti-Env (unliganded) responses that were elicited in rabbits for the South African Env ZACAP45 (6.4 x 10^5 for the monomer and 2.56 x 10^6 for the trimeric conformation) were approximately 10-fold lower than responses detected in the rabbits immunized with the Indian Env IN25925 (4.3 x 10^6 for the monomer and 3.4 x 10^6 for the trimer). In addition, the sera obtained from the rabbit groups immunized with the Env/2dCD4^{S60C} liganded immunogens elicited similar levels of anti-Env titres, except gp140GCN4(+)$_{IN25925}$/2dCD4^{S60C} which showed levels similar to the unliganded Envs (Figure 3.24 B).

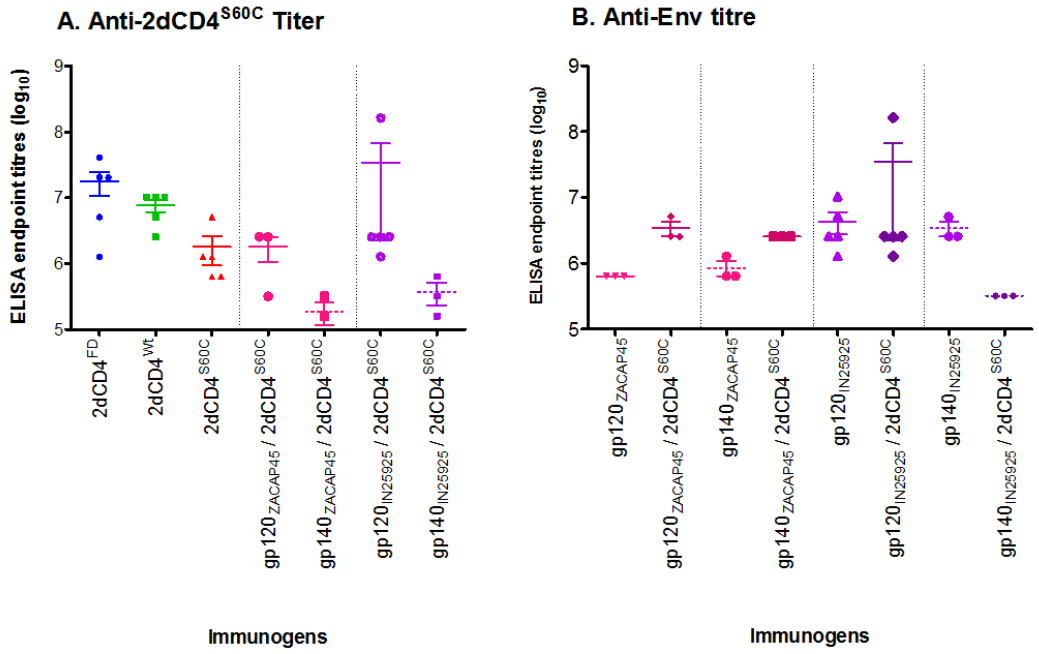

Figure 3.24: End-point ELISA titers of rabbit sera immunized with the first arm of immunogens. ELISA titrations represent the reciprocal of the highest dilution above the cut-off. Cut-off values were calculated using the equation: Cut-off = $\bar{X}$*SDf, where X is the average and SD is the standard deviation for the control serum, and f is SD multiplier corresponding to the 99 % confidence level and number of replicates (Frey et al., 1998). Data points represent end-point titers for each rabbit, the group mean is indicated with a horizontal line and standard errors are indicated with vertical lines. A) Anti-CD4 titers were determined by titrating the terminal bleed sera of immunized rabbits against 2dCD4^{S60C} B) Anti-Env titers were determined by titrating the terminal bleed sera of immunized rabbits against the matched gp120/gp140GCN4(+) Env variant immunogens.

Similarly, the antibody responses were determined in the sera of the rabbits immunized in the second arm immunization study that received the stabilized gp120/gp140 derivative immunogens (proteins and DNA constructs). Mid-point (post-priming and before boosting) serum antibodies were titred against $gp140^{FV}$ and the $gp120^{JRFL}$ cyclic permutant protein. End-point antibody titres of the terminal bleed rabbit sera were titred exclusively against $gp120^{JRFL}$ cyclic permutant protein (Figure 3.25).

The sera obtained from the rabbits immunized with the peptide immunogens were tested for mid-point (post-priming and before boosting) antibodies titres against the matched peptide (1WR2 or 1ORC), while the terminal bleed sera were titred against $gp140^{FV}$ and the $gp120^{JRFL}$ cyclic permutant proteins, respectively (Figure 3.25).

The week 10 (mid-point) anti-$gp140^{FV}$ mean antibody serum titres determined for the rabbits that received the stabilized gp120/gp140 derivative immunogens (Figure 3.25) were higher for the groups 1 and 2 immunized using a protein immunogen as a prime: Cyc4OD (8.25×10^4) and ODECCOBPICS-C (1.6×10^3) compared to the rabbits that were primed with DNA WT-JRFL Env DNA (3.5×10^2) (Group 5); JRFL-Env-V570D (2.5×10^2 and 2.4×10^2 for groups 6 and 8, respectively) and JRFL Env-SEKS DNA (3.3×10^2) (Group 7). The matched anti-peptide titres for the scaffold peptides 1WR2 (Group 3) (1×10^4) and 1ORC (Group 4) (2.24×10^6) were comparable to those obtained from groups primed with protein immunogens when titred against their matched peptides.

Comparing the anti-$gp140^{FV}$ and $gp120^{JRFL}$ titres determined at mid-point (Week 10) we observed a similar titre for group 1 ($gp120^{JRFL}$ Cyc4OD cyclic permutant) when titrering against $gp140^{FV}$ (8.25×10^4) versus gp120 JRFL (1.08×10^5). Whereas, a ten-fold lower titre was obtained for group 2 sera (ODECCOBPICS-C) (1.6×10^3 and 4.3×10^2)) when one compares the titres determined against $gp140^{FV}$ and $gp120^{JRFL}$ respectively (Figure 3.25).

Mid-point titres (week 10) determined against $gp140^{FV}$ and $gp120^{JRFL}$ cyclic permutant for groups that received a DNA prime (group 5 - WT-JRFL Env DNA (group 6 and 8 (JRFL-Env-V570D) and group 7 (JRFL Env-SEKS DNA) showed similar antibody ranges (2.5×10^2 - 1.0×10^3) (Figure 3.25, groups 5-8, mid-point bleeds). Anti-$gp120^{JRFL}$ cyclic permutant titres were not determined for the groups immunized with the peptide immunogens.

Anti-$gp120^{JRFL}$ cyclic permutant titres were determined for all the terminal bleed rabbit sera immunized with the stabilized gp120/gp140 Env derivative immunogens (Second arm immunization study) (Figure 3.25). Animals primed with protein and DNA immunogens showed a marked increase in the antibody titres after the protein boosts compared to the mid-point titres (week 10): approximately five-fold increase for group 1 (Cyc4OD (1.00×10^6), approximately 1×10^4 fold increase for group 2 (ODECCOBPICS-C (5.76×10^6), approximately 4.0×10^3 times increase for group 5 (WT-JRFL Env DNA (3.2×10^6)); 1.2×10^3 times increase for group 6 (JRFL-Env-V570D (1.28×10^6)) and 3.84×10^3 times increase for group 7 (JRFL Env-SEKS DNA (1.28×10^6)). A smaller increase of approximately 400 times was observed for group 8 (JRFL-Env-V570D prime, $gp140^{FV}$ boost). The Ab titres of the terminal bleeds for the animals immunized with the VRC01 epitope engrafted scaffold peptides 1WR2 and 1ORC, followed by $gp140^{FV}$ protein boosts had similar anti-$gp120^{JRFL}$ cyclic permutant titres of 3.2×10^5 and 3.4×10^5, respectively and anti-$gp140^{FV}$ titres of 8.53×10^5 and 2.24×10^6, respectively.

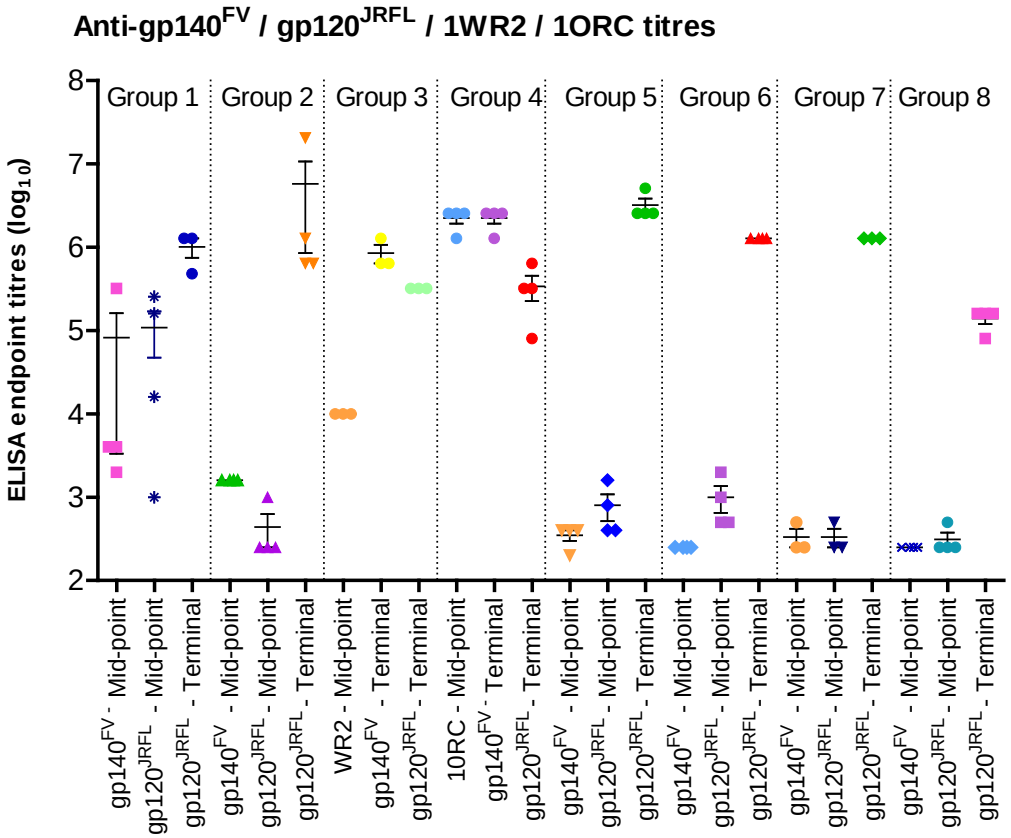

Figure 3.25: End-point ELISA titers of rabbit sera immunized with the second arm of immunogens. ELISA titrations represent the reciprocal of the highest dilution above the cut-off. Cut-off values were calculated using the equation: Cut-off = $\bar{X}$*SDf, where X is the average and SD is the standard deviation for the control serum, and f is SD multiplier corresponding to the 99 % confidence level and number of replicates (Frey et al., 1998). Data points represent end-point titers for each rabbit, the group mean is indicated with a horizontal line and standard errors are indicated with vertical lines.

Immunogens: Group 1: CycV40D gp120 protein prime and gp120^{JRFL} cyclic permutant protein boost; Group 2: ODECCOBPICS-C gp120 prime and gp120^{JRFL} cyclic permutant protein boost; Group 3: 1WR2 peptide prime and gp140^{FV} protein boost; Group 4: 1ORC prime and gp140^{FV} protein boost; Group 5: Wt-JRFL Env DNA prime and gp120^{JRFL} cyclic permutant protein boost; Group 6: JRFL Env-V570D DNA prime and gp120^{JRFL} cyclic permutant protein boost; Group 7: JRFL Env-SEKS DNA prime and gp120^{JRFL} cyclic permutant protein boost, Group 8: JRFL Env-V570D DNA prime and gp140^{FV} protein boost. End-point titers of groups immunized with protein or DNA immunogens (Groups 1, 2, 5, 6, 7, 8) were determined by titrating the mid-point bleed sera (post-priming and before boosting) of immunized rabbits against gp140^{FV} protein and gp120^{JRFL} cyclic permutant protein and the terminal bleed sera against gp120^{JRFL} cyclic permutant protein. End-point titers of groups immunized with peptides (Groups 3, 4) were determined by titrating the mid-point bleed sera (post-priming and before boosting) of immunized rabbits against the matched peptide (1WR2 or 1ORC) and the terminal bleed sera against gp140^{FV} protein and gp120^{JRFL} cyclic permutant protein.

These antibody responses in the rabbit sera illustrate that an immune response was elicited against all immunogens tested. We next sought to further characterize the response by determining whether the antibodies raised in the rabbits were capable of neutralizing a panel of HIV-1 pseudoviruses in an *in vitro* inhibition assay.

3.5.2 Neutralizing antibody assays

The HIV-1 pseudovirus assay was used to evaluate the neutralization capabilities of the antibodies elicited in the rabbits.

The first immunization arm was performed using the Env variant $gp120_{IN25925,}$ liganded and unliganded with $2dCD4^{S60C}$ and included three 2dCD4 variant ($2dCD4^{FD/Wt/S60C}$) control groups, as well as a control in which the reducing agent was omitted during complex formation of the Env/$2dCD4^{S60C}$ immunogen. The terminal rabbit sera obtained from rabbits immunized with these immunogens were sent to Monogram BioSciences Inc. who performed the PhenoSense Neutralizing antibody assay against a panel of 15 HIV-1 pseudotyped viruses (Table 3.6). This panel represented a genetically and geographically diverse range of HIV-1 viruses from different tiers (Tier 1 – 3) and included HIV-2 and SIV (Killick et al., 2015).

In addition, terminal bleed sera obtained from rabbits immunized with the remaining immunogens from the first immunization arm ($gp120/gp140GCN4(+)_{ZACAP45}$ and $gp140GCN4(+)_{IN25925}$, liganded and unliganded with $2dCD4^{S60C}$) were assayed in-house against a smaller panel of four of HIV-1 pseudoviruses from Tier 1 and 2 (Table 3.7).

The neutralization data is presented as a heat map in which the reciprocal of the dilution of serum resulting in 50% inhibition (ID_{50}) of the pseudovirus is shown.

In general, the gp120/gp140GCN4(+) Env variant immunogens exhibited low neutralization breadth, except for the Env/2dCD4^{S60C} liganded immunogens, which consistently resulted in broad neutralization Tier 1 and Tier 2 pseudoviruses irrespective of the origin of the Env sequence used (South African versus Indian).

As expected antibodies raised in the 2dCD4 folding defective (2dCD4^{FD}) control group showed no neutralization (Table 3.6). However three of the five rabbits in the 2dCD4^{Wt} control group raised an Ab response that showed considerable neutralization of the Tier 1, 2 and 3 pseudoviruses (ID_{50} values ranging between 60 - 4381) (Table 3.6). Similarly, three of the five rabbits in the group 2dCD4^{S60C} immunized group also displayed neutralization of Tier 1, 2 and 3 pseudovirus isolates (ID_{50} values ranging between 30 – 3063). Interestingly, the neutralization titres elicited in these 2dCD4 only groups showed lower ID_{50} values against Tier 1 pseudoviruses compared to Tier 2 and 3 (Table 3.6).

The antibodies raised in the rabbits immunized with the Env/2dCD4^{S60C} complex generated in the presence of the reducing agent showed a slightly improved neutralization profile compared to the complex generated without the reducing agent (Table 3.6). This was evident against both Tier 1 and Tier 2 pesudoviruses.

The reduced panel of pseudoviruses analysed in-house corroborated the findings of Monogram Biosciences Inc, with neutralization titres obtained against Tier 1 and 2 pseudoviruses for the 2dCD4^{S60C}-liganded Env complex but not the unliganded Envs (Table 3.7).

Table 3.6: Heat map of neutralizing antibody data of rabbit sera immunized with Env variants (gp120/gp140GCN4(+)$_{IN25925}$) in unliganded and 2dCD4-liganded versions against HIV-1 pseudovirus isolates. Neutralization data is indicated as the reciprocal of the serum 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.

		Tier 1			Tier 2								Tier 3	SIV	HIV-2	Control
		SF162	BaL	NL43	MGRM-SC-B-012	93IN905	MGRM-C-004	MGRM-C-015	IAVI-C-22	93MW 959	97ZA 012	JRCSF	MGRM-SC-B-005	SIVmac 316	P2_HIV-2	aMLV
Monomer only	gp120$_{IN25925}$	12	<10	13	<10	11	<10	<10	<10	<10	<10	<10	<10	<10	20	<10
		11	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		740	<10	<10	<10	16	<10	22	<10	<10	<10	<10	<10	<10	<10	10
		199	<10	10	<10	10	<10	10	<10	<10	<10	<10	<10	<10	<10	<10
		1836	<10	10	<10	19	<10	17	<10	<10	<10	<10	<10	<10	<10	12
	Pooled gp120$_{IN25925}$	466	<10	12	<10	16	<10	22	<10	<10	10	<10	<10	<10	11	12
Monomer / 2dCD4 complex	gp120$_{IN25925}$/2dCD4^{S60C}	183	87	47	346	370	408	309	458	276	275	219	313	23	355	<10
		1780	543	234	2738	2103	2494	1795	3003	896	1558	965	1329	120	1853	<10
		447	510	267	1493	1021	1808	846	1688	851	894	969	977	73	1199	<10
		2217	52	171	75	143	174	293	127	50	82	78	104	13	71	<10
		471	209	131	629	633	714	442	645	427	414	382	524	60	412	<10
	Pooled gp120$_{IN25925}$/2dCD4^{S60C}	892	229	148	897	751	942	749	1008	522	427	418	513	52	782	<10
Monomer / 2dCD4 complex	gp120$_{IN25925}$/2dCD4^{S60C} (No β-mercaptoethanol)	964	104	59	307	447	483	375	510	264	257	251	297	15	241	<10
		2245	242	168	554	524	638	530	596	340	321	288	437	34	426	<10
		367	155	107	341	382	662	306	567	284	288	350	387	34	386	<10
		934	186	97	575	497	688	474	873	356	398	408	434	31	447	<10
		2035	188	147	746	923	732	573	794	262	588	329	348	27	478	<10
	Pooled gp120$_{IN25925}$/2dCD4^{S60C} (No β-mercaptoethanol)	1443	98	101	291	438	458	481	477	237	267	223	254	27	310	<10
2dCD4 controlS	2dCD4^{FD}	<10	<10	<10	13	12	15	<10	19	10	18	15	13	ND	ND	24
		13	<10	14	59	26	65	51	77	36	21	28	43	ND	ND	12
		<10	<10	<10	13	<10	13	<10	12	<10	11	<10	<10	ND	ND	15
		<10	<10	<10	32	18	30	27	41	21	18	16	15	ND	ND	10
		<10	<10	<10	25	16	28	17	34	19	13	18	20	ND	ND	20
		<10	<10	12	<10	<10	10	<10	<10	<10	12	11	11	ND	ND	19
	2dCD4^{Wt}	131	113	70	736	426	677	295	711	407	319	260	476	ND	ND	<10
		<10	<10	<10	23	18	22	13	24	15	17	15	18	ND	ND	22
		137	126	60	644	352	633	438	796	355	388	396	409	ND	ND	<10
		621	660	305	3279	2188	3924	2277	4381	2395	1760	1779	2344	ND	ND	<10
		<10	11	<10	42	23	44	23	81	27	28	24	23	ND	ND	<10
		<10	<10	<10	<10	<10	10	<10	<10	<10	11	<10	13	ND	ND	16
	2dCD4^{S60C}	<10	<10	<10	12	13	14	16	14	<10	10	<10	10	<10	14	<10
		40	33	17	232	147	278	115	380	154	150	99	144	10	170	<10
		16	15	<10	79	46	97	52	118	49	37	25	49	<10	53	<10
		768	788	375	3063	1872	2349	2056	2866	1885	1599	1733	2218	221	2814	<10
		557	483	258	1978	1549	1474	1561	3011	1417	1304	1190	1267	128	2023	<10
	Pooled 2dCD4^{S60C}	281	223	98	1269	896	964	792	1718	787	671	566	667	57	939	<10
Pre-bleed controls		<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	10	<10	<10	13
		<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
		<10	<10	12	<10	<10	10	<10	<10	<10	12	11	11	ND	ND	19
		<10	<10	<10	<10	<10	10	<10	<10	<10	11	<10	13	ND	ND	16
Positive control	Z23	21652	793	1967	932	385	<100	199	223	119	123	169	<100	<100	<100	<100
		14725	844	1548	893	337	<100	169	269	179	144	178	<100	<100	<100	<100
		16246	732	1517	972	264	<100	<100	282	128	<100	239	<100	<100	<100	<100
		17522	756	1889	874	264	<100	195	262	182	<100	192	<100	<100	<100	<100
		17067	607	1868	1002	291	108	146	253	162	109	293	107	<100	<100	<100

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

Table 3.7: Heat map of in-house neutralizing antibody data of rabbit sera immunized with gp120/gp140GCN4(+)$_{ZACAP45}$ immunogens in unliganded and 2dCD4-liganded versions against HIV-1 pseudovirus isolates. Neutralization data is indicated as the reciprocal of the serum dilution required to achieve 50% inhibition.

		Tier 1		Tier 2	
		SF162	BaL	ZM53	CAP210
Monomer only	gp120$_{ZACAP45}$	20	<20	<20	<20
		780	102	<20	<20
		35	<20	<20	23
Monomer / 2dCD4 complex	gp120$_{ZACAP45}$/2dCD4^{S60C}	422	550	114	936
		173	789	478	1975
		409	741	814	1400
Trimer only	gp140$_{ZACAP45}$	101	<20	<20	<20
		97	<20	<20	<20
		160	22	<20	<20
	gp140$_{IN2592S}$	31	<20	<20	<20
		73	<20	<20	<20
		<20	<20	<20	<20
Trimer / 2dCD4 complex	gp140$_{ZACAP45}$/2dCD4^{S60C}	969	1087	480	7401
		1007	1317	1275	4441
		1160	1595	1791	12548
	gp140$_{IN2592S}$/2dCD4^{S60C}	841	1068	671	2450
		976	2113	1908	4887
		750	616	435	1672
Prebleed controls		<20	<20	<20	<20
		ND	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20

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

The neutralization data obtained for the second immunization arm which included the stabilized gp120/gp140 derivatives (peptides, DNA and cyclic permutant immunogens) was performed in-house against a small panel of HIV-1 pseudoviruses (Tier 1 and 2) and included a baseline prebleed reference control. This group of immunogens showed very low neutralization breadth against the viruses tested (Table 3.8). Although neutralization was observed against the Tier 1 neutralization-sensitive pseudovirus SF162, reduced neutralization capacity was observed against BaL and no neutralization was observed against the Tier 2 pseudoviruses tested (ZM53 and CAP210).

Immunization regimes comprising alternate DNA/peptide prime and Env protein boost strategies appeared to enhance the neutralization titres obtained against the SF162 pseudovirus. Overall group 4 (1ORC peptide prime, $gp140^{FV}$ protein boost) immunized rabbit sera yielded the highest average neutralization titre against SF162 (Average ID_{50} 1755.5), followed by group 7 (SEKS DNA prime, $gp120^{JRFL}$ cyclic permutant boost) (average ID_{50} 977) and group 3 (1WR2 peptide prime, $gp140^{FV}$ boost) (average ID_{50} 879).

Comparison of neutralization titres obtained from group 6 and 8, which comprised the same DNA prime immunogen (Wt-JRFL-DNA) but different protein's as a boost ($gp140^{FV}$ versus $gp120^{JRFL}$ cyclic permutant) showed similar, average neutralization titres against SF162 (589.25 and 555 respectively). Interestingly, group 8 (JRFL Env-SEKS DNA prime, $gp120^{JRFL}$ cyclic permutant boost) showed a greater consistency in the levels of elicited titres against SF162 compared to group 6. Furthermore, the DNA prime groups that included the trimeric stabilized DNA cassettes (groups 6 and 7) showed enhanced elicitation of neutralizing antibodies against SF162, compared to the monomeric $gp120^{JRFL}$ cassette primed group (group 5 – Wt-JRFL-DNA).

Table 3.8: Heat map of in-house neutralizing antibody data of rabbit sera immunized with stabilized gp120/gp140 derivatives against selected HIV-1 pseudovirus isolates SF162, BaL, ZM53 and CAP210. Neutralization data is indicated as the reciprocal of the serum dilution required to achieve 50% inhibition.

		Tier 1		Tier 2	
		SF162	BaL	ZM53	CAP210
Group 1	prime - 20ug CycV4OD protein (2) boost - 20ug hCMP V1cyc 144-142 protein (2)	<20	<20	<20	<20
		153	<20	<20	<20
		116	<20	<20	<20
Group 2	prime - 20ug ODECCOBPICS-C protein (2) boost - 20ug hCMP V1cyc 144-142 protein (2)	393	36	<20	<20
		<20	<20	<20	<20
		74	<20	<20	<20
		320	<20	<20	<20
Group 3	prime - 20ug 1WR2 peptide (3) boost - 20ug $gp140^{FV}$ protein (2)	1221	74	<20	<20
		906	<20	<20	<20
		511	<20	<20	<20
Group 4	prime - 20ug 10RC peptide (3) boost - 20ug $gp140^{FV}$ protein (2)	4572	22	<20	<20
		2060	<20	<20	<20
		348	51	<20	<20
		42	<20	<20	<20
Group 5	prime - 2mg Wt-JRFL Env DNA (3) boost - 45 ug hCMP-JRFL-V1 cyc protein (2)	350	<20	<20	<20
		<20	<20	<20	<20
		52	<20	<20	<20
		198	23	<20	<20
Group 6	prime - 2mg JRFL Env-V570D DNA (3) boost - 45 ug hCMP-JRFL-V1 cyc protein (2)	113	<20	<20	<20
		101	<20	<20	<20
		1157	32	<20	<20
		986	33	<20	<20
Group 7	prime - 2mg JRFL Env-SEKS DNA (3) boost - 45 ug hCMP-JRFL-V1 cyc protein (2)	280	28	<20	<20
		1839	24	<20	<20
		814	<20	<20	<20
Group 8	prime - 2mg JRFL Env-V570D DNA (3) boost - 45ug $gp140^{FV}$ protein (2)	435	<20	<20	<20
		689	<20	<20	<20
		490	<20	<20	<20
		606	<20	<20	<20
Prebleed controls		<20	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20
		<20	<20	<20	<20

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

Chapter 4 : Discussion

Challenges facing the development of an effective HIV-1 prophylactic vaccine include extensive global-viral diversity, early establishment of latent viral reservoirs, limited small-animal models and immune evasion strategies of the virus including: low density expression of Env spikes; antigenic variability; glycan shielding; transient exposure of epitopes and conformational masking, and the correlates of immune protection are not well defined (Barouch, 2008; Chun et al., 1997a). Recent characterization of bNAbs isolated from HIV-1 infected patients, has facilitated the identification of vulnerable epitopes on Env. Their neutralization mechanisms can be exploited by novel HIV-1 vaccine strategies to elicit effective B-cell immunity using reverse vaccinology (Corti et al., 2010; de Taeye et al., 2016; Lanzavecchia et al., 2016; Van Regenmortel, 2016).

In an attempt to overcome these challenges and elicit bNAbs, the HIV-1 vaccine field has developed several stabilized Env immunogens that aim to expose neutralization-sensitive epitopes, while at the same time minimizing the exposure of epitopes that elicit non-neutralizing Abs (de Taeye et al., 2015; Sanders et al., 2015). However, in recent years it has become unclear as to the precise Env immunogen required to engage the naïve B population and the subsequent immunization protocol that will drive the extensive somatic hypermutation necessary for antibody affinity maturation in humans (Doria-Rose et al., 2014; Klein et al., 2013; Liao et al., 2013b; Mascola and Haynes, 2013).

In this thesis, we aimed to evaluate subtype B and C Env-based immunogens stabilized to expose a CD4bs or CD4i epitope using various immunization regimens in small animals. In addition, we compared the antigenicity and immunogenicity of these Env-based immunogens between those of South African and Indian origin in order to elucidate any differences between HIV-1 subtype C Envs from these geographically distinct regions.

4.1 Analysis of selected HIV-1 Env derived sequences from Indian and South African origin.

Designing an immunogen to represent the vast HIV-1 diversity is a challenging process, and several approaches have been proposed. One approach is to use the sequence of a transmitted founder virus, the strain of the single viral variant from which the quasispecies in an infected individual evolved (Abrahams et al., 2009). However, using a variant from a newly infected individual excludes variants that have evolved in the presence of adaptive immune responses over time (Haynes et al., 2016). It could be argued that using a sequence of Env from late-stage infection could better represent the evolution of the virus over time however would not include the specific intermediaries. Another approach is to derive an amino acid consensus sequence that is representative of a vast number of circulating isolates within a certain population or a particular geographical region (Gaschen et al., 2002).

HIV-1 subtype C is the most dominant subtype globally, with almost half of people infected with HIV-1 worldwide, being infected with this subtype (Hemelaar, 2012). South Africa and India are estimated to have a combined burden of approximately 9 million people living with HIV-1, predominantly infected with subtype C (UNAIDS, 2015). As such, a subtype C based HIV-1 Env immunogen would likely be key to vaccine strategies and approaches carried out in these regions. HIV-1 subtype B has a wider geographic distribution than subtype C, but accounts for approximately 11 % of the global HIV-1 burden (Hemelaar, 2012). We included a comparison of the immunogenicity of subtype B stabilized Env derivatives from our collaborators in this study. Although these were based on a different subtype, the aim was to provide proof-of-concept that presenting conserved regions of Env to the immune system would elicit bNAbs independent of subtype, and allowed us to compare different immunogen designs in this regard.

Here, we selected six Env sequences based on their geographical origin, three variants derived from South African HIV-infected patients and three variants from Indian HIV-1 infected patients. These variants were selected as they were isolated fairly early following infection (Fiebig stages I-V (up to 129 days post infection) – Table 2.1) and thus are representative of the transmitted founder virus population. In particular, the South African Env's (ZACAP45, ZACAP210 and ZA706010164) were selected because they had been reported by Abrahams et al. (2009) to be derived from patients with low diversity *env* genes, suggesting that they followed a neutral evolution model from a single founder virus and were under early selection pressure, therefore representing a founder-type virus. In addition the selected variants were derived from individuals infected via heterosexual transmission, and thus represent the viral strain that was able to overcome the mucosal bottleneck and lead to the establishment of productive infection (Haynes et al., 2016; Joseph et al., 2015; Keele et al., 2008; Shaw and Hunter, 2012).

For the Indian variants, we included two Env sequences (IN25925 and IN25710) that have previously shown an inherent resistance to neutralization by bNAb IgG1b12, one of the first bNAbs characterized (Kulkarni et al., 2009; Patil et al., 2014; Ringe et al., 2010) and one reported to be sensitive to IgG1b12 neutralization (IN26191) (Kulkarni et al., 2009). The precise mutations responsible for IgGb12 resistance are discussed in greater detail in the SPR analysis section (section 4.4).

The CD4bs epitope on HIV-1 Env is an attractive target vaccine design due to its high degree of conservation and it's critical role in viral entry (Lynch et al., 2012; Mascola and Haynes, 2013)Li et al 2007). BNAbs targeting the CD4bs epitope display potent neutralization and extensive breadth (Diskin et al., 2011; Scheid et al., 2016; Wu et al., 2010), however the CD4bs may not be readily accessible to naïve B cell surface-receptors, as it is recessed

in a pocket on Env and further masked by an evolving shield of N-linked glycans and variable sequence loops of Env.

The CD4bs epitope is discontinuous and distributed over six segments in all five of the conserved regions of gp120 (Kwong et al., 1998). Critical contact residues on gp120 include Asp 368, Glu 370 and Trp 427 and these amino acids are conserved amongst all the Env's in this study (Figure 3.2), which was expected since they are conserved among all primate immunodeficiency viruses (Foley et al., 2015; Kwong et al., 1998). CD4 also binds to regions on gp120 that are variable, particularly the large interfacial cavity. Variable amino acid residues in this region (Ala 281, Ser 364, Ser 365, Thr 455 and Arg 469) are surrounded by the three conserved residues mentioned above (Kwong et al., 1998).

Analysis of the V1 and V2 loops of the six Env sequences used in this study found that the V1 loop ranged between 16 – 20 amino acids, consistent with a study by Yao et al. (2015) (15 - 40 amino acids in length) for subtype C Env's. The V2 loop length was relatively conserved between the Env variants of 45 amino acids in length for all the Env's except Env variant IN26191, which has a shorter V2 loop containing 40 amino acids (Figure 3.2). Again, this was within the expected range as reported by Yao et al. (2015) of 33 to 57 amino acids for this variable loop. The length of these variable loops has been shown to affect bNAbs targeting the CD4bs (VRC01 and IgGb12) and the quaternary V1/V2 loop epitope (e.g. PG9/PG16) by masking crucial amino acid residues (Patil et al., 2014).

The V3 loop length is highly conserved in HIV-1 Env's in general and has been attributed as an important region for HIV-1 infection and a critical determinant of co-receptor usage (Regoes and Bonhoeffer, 2005). In addition, PGT monoclonal antibodies 121–123, 125–128, 130, 131 are directed against the V3 loop region (Walker et al., 2011). With the exception of Env ZA706010164, which contained 34 amino acids in the V3 loop, the

Env variants in this study had a V3 loop length of 35 amino acids, consistent with the report for HIV-1 subtype C gp120's by Yao et al. (2015).

The V4 loop lengths varied between 22 – 39 amino acids (within the reported range of 18 – 44 amino acids) by Yao et al. (2015). Env ZACAP210 has the longest V4 loop and ZACAP45 the shortest V4 loop. The number of amino acids in the V5 loop ranged between 12 – 15, again within the expected range of 10 – 23 amino acids as reported by Yao et al. (2015). Env ZACAP210 has the longest V5 loop, whereas Env's ZA706010164 and IN26191 contained the least number of amino acids in this region.

Although the six Env sequences selected each contain the critical amino acids for CD4 binding and their variable loop lengths were found to be within the expected ranges ultimately, antigenic and functionality assessment of the HIV-1 Env requires empirical testing of the expressed recombinant HIV-1 Env glycoproteins. Thus, the selected Env sequences were designed and codon optimized for mammalian expression, for downstream biochemical characterization and small animal immunization studies.

4.2 Design and expression of matched monomeric and trimeric HIV-1 Envs from South African and Indian origin

Using monomeric gp120 as a subunit vaccine has had limited success since it resulted in Abs directed against non-neutralizing epitopes of the hidden face of gp120 (Sanders et al., 2013; Schultz and Bradac, 2001; VanCott et al., 1995a; Yang et al., 2000). This failure, coupled to the discovery of quaternary specific antibodies has driven the field to consider alternative stabilization methods that better mimic the native trimeric structure. However, soluble gp140 trimers are labile in nature and disintegrate into their respective gp120 and gp41 subunits (Earl et al., 1994; Sanders et al., 2013). Therefore, stabilization methods of the trimer were designed and included elimination of the cleavage site, cross-linking gp120 and gp41 and introducing trimerization motifs (Chen et al., 2004; Harbury et al., 1993;

Saha et al., 2012; Sanders et al., 2013; Selvarajah et al., 2008; Yang et al., 2000). Further studies have shown that proteolytic cleavage is essential for the native conformation of gp140, which led to the development of the SOSIP trimer. More recently, and after inception of this study, the SOSIP stabilizing mutations were shown to be the most promising method of stabilizing the trimer in a native-like conformation (Beddows et al., 2007; Sanders et al., 2013; Sanders et al., 2015; Sanders et al., 2002).

In this study, we employed the use of the GCN4 motif to stabilize the trimeric gp140 constructs and compare the antigenicity and functionality to their matched gp120 monomeric conformations.

All six Env constructs were used to successfully express recombinant Env proteins following transfection of HEK293T as confirmed by reducing-SDS-PAGE and Western blot analyses. The trimeric conformation in this study was stabilized using the GCN4(+) trimerization motif, since it was a well-established protocol in our laboratory and shown to generate stable trimers in mammalian cell culture (Killick et al., 2015). Western blot analysis using HIV-1 infected patient sera as a source of primary antibody, confirmed the presence of expressed HIV-1 Env glycoproteins that resolved to within the expected molecular weight range of approximately 120 kDa and 140 kDa for the gp120 and gp140GCN4(+) Env constructs, respectively (Figure 3.3). The slight variations in the migration profile between the different Env's could be attributed to slight differences in glycosylation patterns and variation in the amino acid sequences (Figure 3.2) which have been reported to affect the molecular weight of Env (Benureau et al., 2016). Inefficiency of gp140 cleavage by host-furin protease was also noted, as bands corresponding to gp140 and gp120 were present (Figure 3.3, 3.4). This inefficiency has been reported previously by Binley et al. (2000) who suggested that in natural infection *in vivo*, unprocessed gp160 is sorted into the lysosomal pathway meaning that no uncleaved gp160 is expressed on the virion surface. In mammalian cell expression however, furin proteases

may become saturated resulting in uncleaved gp160/gp140 expression (Binley et al., 2000).

The presence of uncleaved trimers in our study was of concern since Ringe et al. (2013) cautioned using uncleaved trimers in antigenicity and immunogenicity studies because they have been found to expose non-neutralizing gp120 and gp41 epitopes. Since the SOSIP trimer has proven to be an improved method for stabilizing Env in a native-like conformation (Julien et al., 2015; Klasse et al., 2013; Klasse et al., 2016; Sanders et al., 2013), future work could look at incorporating the SOSIP mutations in the immunogen construct design. The SOSIP trimer contains an intermolecular disulphide bond between gp120 and the gp41 ectodomain and an isoleucine to proline substitution at residue 559 of gp41, which stabilizes the interaction between the gp41 subunits. Mutation of the native cleavage site to 6 consecutive arginine residues results in efficient cleavage following co-transfection with a furin-encoding plasmid. These SOSIP-stabilized Envs show improved antigenicity profiles particularly through the stabilization of quaternary structure-dependent epitopes (Sanders et al., 2013).

Large-scale recombinant Env expression and purification was carried out according to protocols previously established in our laboratory (Killick et al., 2014). Following preliminary purification using *G. nivalis* Lectin affinity chromatography, SEC was used as a “polishing step” to improve the purity and isolate specific monomeric and trimeric conformations of the various Env’s expressed (Figure 3.5 and Figure 3.6). The South African and Indian gp120 Env constructs produced a larger proportion of dimers than monomers compared to our previous findings using the founder virus gp120 construct (Killick et al., 2015). Dimer formation has been reported to occur when over-expressing soluble gp120 in the absence of gp41 (Finzi et al., 2010). A mutation of leucine to alanine at residue 111 results in a significant decrease of gp120 dimer formation. Leucine was conserved across all six sequences at this residue (Figure 3.2) including the founder virus sequence

(Killick et al., 2015). The formation of dimers has also been associated with the V1V2 region, and perhaps a reduction in the size of the V1V2 region may result in less propensity of the gp120 to form dimers (Finzi et al., 2010; Guo et al., 2013). Therefore the dimerization may be as a result of differences in the length of the V1V2 loop regions, which were shorter in the founder virus construct. Future work could look at the impact of the length of the V1V2 loops on dimerization.

The trimeric gp140GCN4(+) constructs produced similar elution profiles to our previous findings, with the presence of oligomeric, trimeric and monomeric conformations. The gp140GCN4(+)$_{IN25710}$ Env construct that produced a higher molecular weight band at approximately 260 kDa (Figure 3.8) showed a distorted SEC profile (Figure 3.6F and Figure 3.7B) with the majority of the conformation appearing to be oligomeric in nature This could be attributed to the formation of dimers of trimers, as reported by Feng et al. (2012).

Interestingly, the isolation of fractions corresponding to the trimeric conformation for Env variants IN26191, ZACAP45, ZACAP210, ZA706010164 and to a lesser extent, IN25710, show the presence of low molecular weight bands when resolved under reducing SDS-PAGE at approximately 30, 50 and 60 kDa (Figure 3.8). As these bands are visibly present under reducing SDS-PAGE it suggests that they are associated with the trimeric conformation and could possibly be cleaved gp41-GCN4(+) products. Further studies could use a gp41 specific Ab to confirm this using Western blot analysis. Encouragingly our SPR analysis using PGT145 BNAb, which recognizes a quaternary, trimeric specific epitope on Env, bound favourably to the gp140GNC4(+)$_{IN25925/IN25710}$ trimeric conformations compared to the matched gp120 monomers (Figure 3.20). This suggests that the combination of the gp41 ectodomain and GCN4 motif assembles these Env's into a native-like trimer (Yasmeen et al., 2014). Unfortunately our analysis in this regard was limited due to insufficient protein quantity

during the SPR analysis and provides an opportunity for future characterization of the remaining protein conformations.

Variable amounts of the gp120/gp140GCN4(+) Env's were purified to homogeneity for antigenicity and immunogenicity studies. Generally, higher yields were obtained for the monomers compared to the trimers. Very low yields were obtained for Env's gp140GCN4(+)$_{ZACAP210/IN25710/IN26191}$ and gp120$_{IN25710/IN26191}$ (Table 3.1) and could be attributed to differences in amino acid sequences (Gustafsson et al., 2012). Large scale protein expression resulted in sufficient quantities of these Env variants for limited biochemical characterization studies.

4.3 Expression and purification of recombinant 2dCD4$^{Wt/FD/S60C}$

In addition to Env purification, three variants of 2dCD4$^{Wt/FD/S60C}$ were expressed and purified to homogeneity and confirmed to be of correct molecular weight (Figure 3.9). The 2dCD4^{S60C} variant contains a serine-cysteine mutation at position 60 which, when liganded to Env, forms an improved stabilized Env/2dCD4 complex compared to the wild-type 2dCD4 as a result of the formation of a intramolecular disulphide bridge presumably between cysteine residues 126 on gp120 and cysteine 60 in CD4 (Cerutti et al., 2010). The 2dCD4$^{Wt/S60C}$ variants were used for SPR experiments and for generation of the relevant Env/2dCD4^{S60C} complexes for immunogenicity testing. The 2dCD4-folding defective (FD) was used as a control in the immunization studies and will be discussed in more detail in the relevant section below.

4.4 Antigenicity analysis of the gp120/gp140GCN4(+) Env variants

4.4.1 Surface plasmon resonance as an aid in vaccine design

We next sought to characterize the gp120/gp140GCN4(+) Env variants by comparing their binding kinetics to 2dCD4$^{Wt/S60C}$ and a panel of six CD4bs-directed bNAbs. This experiment was necessary to confirm the effect of any amino acid differences between the sequences empirically, and the kinetic

information obtained from SPR would enable the quantification of the binding interaction between macromolecules.

In SPR, a ligand is immobilised onto a sensor surface chip containing a thin metal film (usually gold) coated with a suitable surface chemistry that allows for direct coupling of the ligand (e.g. carboxyl groups). A solution containing analyte is passed over this surface and binding of the ligand and analyte results in a change in mass concentration on the chip. This change is measured as a change in refractive index on the sensor surface with light that is reflected off the metal film using a glass prism (Nguyen et al., 2015). Therefore a change in the mass concentration results in a shift in the angle of incidence required for resonance, and therefore a change in the reflected light intensity. A sensorgram is generated from a plot of the changes in reflected light (designated an arbitrary unit termed response units (RU)) over time, and this enables the real-time monitoring of biomolecular interactions occurring on the surface of the chip (Lang et al., 2005).

The advantage of using SPR in Env-Ab interaction analyses, as opposed to Enzyme-linked immunosorbent assays (ELISA's), is that it can be monitored in real-time, using label-free technology (Tudos and Schasfoort, 2008). Labels have the disadvantage of potentially causing steric hindrance or changing the structural configurations of the proteins being studied. Therefore label-free strategies, including SPR, enable better simulation of native biomolecule interactions (Nguyen et al., 2015).

A further advantage of SPR is that all bound analyte can be removed and the chip surface regenerated, while leaving the active ligand intact. This regeneration step allows repeated binding experiments using different analytes or different conditions/concentrations of the analyte (Lang et al., 2005). Specific to the ProteON XPR36 system platform is interspot referencing (Bio-Rad Laboratories Inc., 2009). Interspots are positioned adjacent to the interaction surfaces. These interspots do not have ligand

immobililized onto their surface and are used as a reference to control for non-specific binding.

SPR data obtained for biochemical interactions are routinely fitted using the Langmuir 1:1 model, which assumes that one molecule of ligand binds to one molecule of analyte. However, interactions cannot always be described using this model and a multitude of factors and the complexity of interactions may result in reduced fitting confidence to this model. Experimental considerations include the following; protein samples may not be homogenous; proteins may aggregate; multivalent interactions (e.g. bivalent antibodies); ligand may be immobilised in a sub-optimal orientation on the chip; mass transport effects during the experiment; steric hindrance; bulk refractive index changes; nonspecific binding of the analyte to the chip surface; experimental conditions including pH and temperature; complex binding mechanism and drift, which can occur if the immobilised ligand escapes from the capture surface, causing a drift in the baseline RU prior to injection of the analyte (Bio-Rad Laboratories Inc., 2013; Lang et al., 2005).

Experimental procedures can be implemented to correct for some of these problems, e.g. baseline drift can be corrected for by referencing with a blank buffer to improve accuracy (Bio-Rad Laboratories Inc., 2013; Lang et al., 2005); the effect of mass transport, which occurs when the analyte diffuses onto the chip surface from the solution at a slower rate than the rate of binding between the analyte and ligand creating a shortage of analyte at the ligand surface, can be reduced through immobilization of less ligand, or through an increase in the analyte flow rate. Mass transfer can be corrected for post-experimentation, by analysing the kinetic data, using the 1:1 Langmuir mass transport model (Bio-Rad Laboratories Inc., 2013; Lang et al., 2005). Although it is possible to correct for many of the experimental problems using reference blanks, in some cases the data simply does not fit a one-on-one model and further experiments to elucidate the cause of the lack of fit need to be investigated.

To date, the majority of published SPR data of HIV-1 Env-Ab interactions has been performed using GE Healthcare's Biacore platforms. We were concerned that results obtained from different SPR platforms may not be comparable. However a recent comparison of the performance of four biosensor instruments: GE Healthcare's Biacore T100, Bio-Rad's ProteOn XPR36, ForteBio's Octet RED384, and Wasatch Microfluidics's IBIS MX96, in which ten mAbs were evaluated against the same antigen under the same experimental conditions, has been published (Yang et al., 2016). When comparing the Biacore and ProteON, they found the data comparable within the working limits of the instruments, however it was noted that the Biacore instrument generated more consistent data. The authors of this comparative study attributed deviations in published binding kinetics to differences in methodologies, rather than differences in the platforms used. These differences include inconsistencies in ligand capture formats and ligand capture levels. The ProteON XPR36 is to be discontinued in the near future (Yang et al., 2016), for this reason, as well as the improved consistency of the Biacore instruments one could expect publications to continue to rely on the Biacore platforms for kinetics analysis.

Overall, SPR provides a reliable, high-throughput method for characterizing and quantifying biomolecule interactions and was applied to Ab-Antigen binding in this instance. However, the data needs to be supported with additional biochemical characterization including sample purity analysis, the stoichiometry of the paratope-epitope interaction, and the ligand capture formats and levels which may reduce inter-laboratory variation.

4.4.2 Surface plasmon resonance interactions of Env gp120/gp140GCN4(+) variants with 2dCD4$^{Wt/S60C}$

To confirm the integrity of the CD4bs on the Env variants, we performed SPR with two variants of the first two domains of CD4 (2dCD4). The 2dCD4^{S60C} variant was previously designed in our laboratory and shown to have an increased affinity (K_D) for gp120 (6 nM) compared to 2dCD4^{Wt} (53

nM) and was attributed exclusively to the significantly slower off-rate of 2dCD4^{S60C}, as a result of an intermolecular disulphide exchange between gp120 and the mutant 2dCD4 (Cerutti et al., 2010).

Pertinent to this study, the incorporation of HIV-1 Envs of Indian origin (IN25925 and IN25710) that have reported resistance to IgG1b12 neutralization, may also show decreased affinity for binding to CD4, due to the shared, partial epitope overlap. This could impact down-stream Env/2dCD4 liganded complex formation. Although the study by Kulkarni et al. (2009) showed no correlation between IgG1b12 resistance and resistance to sCD4, we sought to confirm the binding of CD4 to our Env variants.

While our SPR experiments showed that all the Env variants were able to bind both variants of 2dCD4, disappointingly non-specific binding was too high (exceeding 10% R_{MAX}) to accurately calculate binding affinities (Appendix - Figures B1 and B2) (personal communication, Dalia Shezifi, PhD, Applied Biology Group Leader, Bio-Rad Laboratories, Haifa). Non-specific binding can be corrected by capturing a reference protein, such as BSA, to equivalent levels of the ligand on the chip surface and subtracting this interaction as a reference surface. Alternatively non-specific binding interactions can be limited by adjusting the buffer conditions including salt concentration, pH or the addition of a surfactant (Bio-Rad Laboratories Inc., 2012). The non-specific binding of soluble CD4 has been reported by Yasmeen et al. (2014), who aimed to compare binding of a monomeric and trimeric HIV-1 Env to sCD4. The high background binding in their experiments, despite using different SPR sensor chips, resulted in them drawing the same conclusion as we have done here, that their HIV-1 Envs were able to bind to soluble CD4, however they were unable to assess the binding kinetics. Unfortunately in our experiments, limited amounts of Env glycoproteins prohibited further optimization in an attempt to obtain accurate binding affinities. Despite this, we were able to ascertain that all the Env

variants had an accessible and functional CD4bs as shown by the SPR sensorgrams (Figures 3.10 and 3.11).

4.4.3 Surface plasmon resonance binding kinetics of the CD4bs directed bNAbs

In an effort to further characterize the CD4bs of the Indian and South African purified Envs (gp120/gp140GCN4(+)) we performed additional SPR experiments against a panel of CD4bs directed bNAbs (IgG1b12, VRC01, HJ16, VRC-CH31, NIH45-46^{G54W}, VRC03). The partial overlap of epitopes shared between CD4 and CD4bs-directed bNAbs may reveal slight differences that could contribute to future vaccine immunogen design.

Sequence analysis of Env variants does not necessarily predict neutralization sensitivity of HIV-1 Envs. Published findings by Wu et al. (2009) and Li et al. (2011), have shown how non-contact residues outside the bNAb binding site epitope can affect tertiary folding of Env and therefore binding and neutralization CD4bs-directed bNAbs. Therefore it is critical the antigenicity of the expressed Envs be empirically determined and contextualized in relation to the amino acid sequences.

A summary of the SPR experiments conducted in this thesis is provided for initial screening experiments using single analyte concentrations in Table 3.2 and binding kinetic experiments in Table 3.3. The initial single analyte screening experiments (Figure 3.12 and 3.13) showed binding of the Env variants to all six immobilized bNAbs. This confirmed that the paratopes of the bNAbs immobilized were accessible to the Env variants, although ligand accessibility could not be quantified. However, a comparison of the binding affinities between the Env's could be made since the same chip, with the immobilized ligand, was used for all the Env variants tested and was regenerated between Env analyses. In addition, the single analyte experiments provided a basis for subsequent Env concentration ranges that would yield accurate kinetic data. In general, a serial dilution series

consisting of four to five concentrations of analyte, with the highest concentration returning no more than 200 RU, is required for accurate kinetic calculations (Bio-Rad Laboratories Inc., 2009).

BNAb IgG1b12 is directed against a discontinuous epitope overlapping the CD4 binding region of HIV-1 Env and is reported to neutralize 30 – 40 % of primary isolates of subtype B and C viruses (Bures et al., 2002; Georgiev et al., 2013; McInerney et al., 1997; Zwick et al., 2003). Envs of Indian origin have shown some resistance to neutralization by IgG1b12 (Kulkarni et al., 2009). Binding kinetics were only obtained for the gp140GCN4(+)$_{ZACAP45}$ Env interaction with IgG1b12. Data was fitted to a 1:1 Langmuir model with an on-rate (Ka: 8.12 x 10^5 $M^{-1}s^{-1}$) and off rate (Kd: 3.41 x 10^{-4} s^{-1}) forming a very stable complex with a binding affinity of 0.419 nM (Table 3.4). This Env showed a slightly more stable interaction compared to that reported by Hoorelbeke et al. (2013) for a subtype B Env $gp140^{JRFL}$ (Ka: 1.17 x 10^6; Kd: 1.89 x 10^{-3}; K_D: 1.6) as well as that of monomeric $gp120^{YU-2}$ (K_D: 46.6) (Gift et al., 2011).

We were unable to calculate accurate binding kinetics for the other gp120/gp140GCN4(+) variants tested with IgG1b12 since they did not fit the 1:1 Langmuir model (Figure 3.14). We expected IgG1b12 to bind poorly to Indian Env variants IN25925 and IN25710 due to their reported resistance to neutralization by IgG1b12 (Kulkarni et al., 2009). Consistent with the reported neutralization resistances we found that single analyte analysis using 1000 nM concentrations of each Env, showed that the monomeric gp120 Envs IN25925 and IN25710 showed the lowest binding levels (Figure 3.12). However improved binding for IN25710 to IgG1b12 was noted for the trimeric conformation compared to IN25925 and ZA706010164 (Figure 3.13). In addition SPR sensorgrams depicted an exponential decay in the dissociation phase suggesting an overall lower binding affinity of the other Env variants tested (gp120$_{ZACAP210/ZACAP45/IN25710/IN25925}$) and (gp140GCN4(+)$_{IN25710/IN25925}$). The exponential decay resembles an

apparent biphasic response and has been reported previously for a IgG1b12 Fab fragment binding to gp140 Env (Feng et al. (2012)).

It may be tempting to try and explain lack of fits to the 1:1 Langmuir model as a result of bivalent binding in which a biphasic dissociation curve is observed with a rapid first dissociation phase, followed by a second slower dissociation phase, ending in a curve almost parallel to the baseline (Homola et al., 1999; Zeng et al., 2014). However, one cannot simply apply the bivalent model to an interaction without confirming the stoichiometry of the paratope-epitope interaction first, by using a technique such as Isothermal titration calorimetry (ITC) (Yasmeen et al., 2014). Whilst determining the stoichiometry fell outside the scope of this thesis, future work could address this and also compare whole IgG binding versus using the Fab fragment of the IgG molecule in SPR experiments (Yasmeen et al., 2014).

We then evaluated whether each Env amino acid sequence could predict binding to specific bNAbs, and compared that to the actual SPR data obtained. IgG1b12 resistance is regulated by multiple regions of gp120 (Utachee et al., 2010) and several factors contribute to this resistance including differences in amino acid residues, glycosylation and conformation. The mutations Y173H/S and D/E/N185G/S/T in the V2 loop, E/S268K/R (HXBc2 numbering) in the outer domain of the CD4bs have been reported to confer resistance to IgG1b12 neutralization (Gnanakaran et al., 2010). Evaluation of the amino acid residues confirmed that the three Env variants of Indian origin evaluated in this study do indeed contain a histidine (H) or serine (S) at position 173, and the 273K (HXBc2 268) mutation is present in the Indian Env variant IN25925. Interestingly only the susceptible Env variant IN26191 was found to contain the resistance conferring amino acid glycine (G) at position 185. None of the above signature amino acids were found to be present in the South African Env variants. While these amino acids have been shown to impact on IgG1b12

resistance, any single mutation alone or in combination with other documented resistance mutations may not necessarily confer a resistance phenotype (Li et al., 2011; Wu et al., 2009).

The glycosylation of Env also plays an important role in the correct folding of gp120 as well as the structural rearrangements upon CD4 binding, directly affecting Ab recognition of gp120, viral entry and infectivity of the host cell (Huang et al., 2012; Kumar et al., 2011). Glycosylation sites (N197 located at the V1/V2 stem and N186 located near the V2 tip overlapping the CD4bs) have been implicated in the resistance of HIV-1 (CRF01_AE) to IgG1b12 neutralization (Utachee et al., 2010; Wang et al., 2013). Together, these two N-linked glycans are thought to play a role in stabilizing the structure of the V1/V2 regions of Env and interfere with IgG1b12 binding by masking the epitope on gp120 (Utachee et al., 2010). Bearing in mind that the glycan composition of the Env variants in this study was predicted by the N-Glycosylate tool and that differential glycosylation patterns occur depending on the cell type used to express the Env (Raska et al., 2014), the glycosylation of our Env's cannot be confirmed. In our analysis both IgG1b12 sensitive and resistant Envs were found to have PNLG's present within the 185-190 region of the V2 loop, thus reinforcing the inconsistency of using amino acid/glycosylation profiles as a predictor of neutralization phenotype. However, the absence of PNLG at position 186 in the V2 loop for Env variants gp120/gp140GCN4(+)$_{ZACAP45}$ and gp120/gp140GCN4(+)$_{IN26191}$ may indeed increase their susceptibility to IgG1b12 binding, by reducing the steric hindrance inferred by this glycan (Figure 3.2) (Utachee 2010).

Conformational changes in the V2C2 region of gp120 have been suggested as another possible factor for IgG1b12 resistance (Patil et al., 2014). The V1V2 domain is important for recognition by CD4bs-directed bNAbs, including IgG1b12. Variation in the V2 loop length may change the conformation of this region, resulting in the inaccessibility of the epitope

(Patil et al., 2014). Analysis of the V2 loop of the Env variants in this study reveals that Env variant IN26191 has a V2 loop containing 40 amino acids, whereas the V2 loop of the other Env variants comprise 45 amino acids (Figure 3.9). The shorter V2 loop length for Env IN26191 may have a conformational effect on the CD4bs epitope accessibility compared to the other Indian Env variants.

VRC01 shares overlapping binding sites with CD4 and IgG1b12, however has been found to be far more potent, neutralizing over 90% of circulating HIV-1 variants compared to IgG1b12 and CD4-Ig. An important contact residue for VRC01 to gp120 is an N-linked glycan at position 276 on gp120 (Wu et al., 2010; Zhou et al., 2010). Sequence analysis of the Env variants did not show the presence of any documented VRC01 resistance mutations, including PNLG at position 460 and 475, and the crucial PNLG for VRC01 binding to gp120 at position 281 (HXBc2 276) was found to be conserved in all the Env variants (Figure 3.2) (Guo et al., 2012; Zhou et al., 2010).

Single analyte screening experiments revealed that the Env's of Indian origin showed favourable binding to VRC01 compared to the South African Env's (Figures 3.12-3.13). Sequence analysis of Env IN26191 revealed the absence of the PNLG at position 294 (HXBc2 289), which has previously been reported to increase susceptibility to VRC01 (Wang et al., 2013). Our single analyte binding experiments confirmed preferential binding of this Env to VRC01 compared to the other monomeric gp120 tested (Figure 3.12). Binding kinetics obtained using VRC01 yielded the most consistent and reliable binding kinetic results against the South African and Indian Env variants tested. All binding kinetics experiments fitted the 1:1 Langmuir binding model and achieved a Chi^2 value of less than 10% of R_{max}. However, Env's of Indian origin showed very little dissociation when binding to VRC01 (Figure 3.15) compared to the South African Env's. Dissociation rates could only be accurately measured for the South African Env's ($gp120_{ZACAP210/ZACAP45}$ and $gp140GCN4(+)_{ZACAP45}$) tested and one Indian

Env ($gp120_{IN25925}$) (Figure 3.15, Table 3.4 and 3.5). The off-rates (Kd) for monomeric $gp120_{IN25710}$, and trimeric $gp140GCN4(+)_{IN25710/IN25925}$, could not be determined as these did not obtain the minimal 5% dissociation decay required for accurate measurement (Bio-Rad Laboratories Inc., 2011; Marquart, 2006-2016). The recommended dissociation time for Kd values lower than 1×10^{-5} s^{-1} and 1×10^{-6} s^{-1} should be extended to 85 and 850 minutes, respectively to ensure adequate decay (>5%) and accurate Kd determination. The 10 minute dissociation times employed in our SPR study are thus too brief to accurately determine the Kd values, however the following conclusions can still be applied. Overall the Indian Env showed higher binding affinities for VRC01 compared to South African Envs, as a consequence of their lower dissociation rates (Kd $<1.0 \times 10^{-5}$). This markedly slow dissociation has been reported in other studies for VRC01 Fab regions, with dissociation rates of $<1 \times 10^{-5}$ s^{-1} (Kovacs et al., 2012; Yasmeen et al., 2014).

Comparison of the binding kinetics of VRC01 with the gp120/gp140GCN4(+) Env variants showed an overall higher binding affinity for the trimers compared to the monomers (Figure 3.15 and Tables 3.4-3.5). Interestingly this was a result of both a favourable on-rate and off-rate, which is in contrast to the results reported by Yasmeen et al. (2014), who showed a ten-fold difference in overall binding affinity of the BG505 $gp120\text{-}gp41_{ECTO}$ monomer compared to the BG505 SOSIP 664 trimer. Our data is similar to those reported previously for subtype C monomers and trimers with an overall binding affinity ranging between 20 – 139 nM, consistent with the findings of (Kovacs *et al.*, 2012 and Scheid *et al.*, 2011 who reported a 0.4 – 96 nM binding affinity range when using VRC01 Fab fragment.

VRC-CH31 is a VRC01-like antibody that recognizes the CD4bs epitope. Critical contact residues on gp120 for VRC-CH31 binding include 279N/D, 280N, 456R, 458G and 459G and were found to be conserved in all Env

variants (West et al., 2012). The on-rates (Ka) were similar for all the VRC-CH31 gp120 and gp140GCN4(+) Env's tested and were comparable to those obtained for VRC01 (less than a 3-fold difference). Only $gp120_{ZACAP210}$ showed negligible binding to VRC-CH31. However the overall binding affinities (KD) were at least five-fold greater than those observed for the South African Env variants. Similar to VRC01, Indian Env IN25710 (monomeric and trimeric) showed no detectable dissociation and thus the dissociation rate (Kd) could not be accurately determined. However, this highly stable interaction suggests an overall binding affinity in the sub-nano molar range for Env IN25710 to VRC-CH31. The South Africa $gp140GCN4(+)_{ZACAP45}$ showed a four-fold increase in binding affinity over the matched monomer, however the same pattern was not observed for the Indian IN25925 monomeric and trimeric Envs which showed equivalent binding affinities. Our binding affinities of the Env variants to VRC-CH31 ranged between 9.8 – 200 nM. The published findings by Kovacs et al. (2012) showed different binding affinities of VRC-CH31 to an HIV-1 subtype C monomer (C977ZA012), with a K_D of 4.6×10^{-6} M, whereas the matched trimer had an overall binding affinity of 13 nM, consistent with our study.

VRC03 is somatically related to VRC01 and also binds to the CD4bs of gp120, but its neutralization is less broad than that of VRC01 (Wu et al., 2010). The gp120 amino acid contact residues for VRC03 binding are strikingly similar to VRC01 (Figure 3.2) and are largely conserved among the Env variants in this study. However VRC03 is able to neutralize 50 % of HIV-1 isolates, compared to 90 % by VRC01. The amino acid residue alteration at position 441 (HXBc2 position 429) in Env variant $gp120/gp140GCN4(+)_{ZACAP45}$ has a glycine (G) whereas the other Env's have a negatively charged glutamic acid (E). This affects the charge of the protein in a region important for VRC03 binding (Wu et al., 2011) (Figure 3.2). The SPR experiments showed negligible binding to both the monomeric and trimeric conformations of Env ZACAP45, and is most likely due to this mutation.

Binding of VRC03 with Env IN25925 was also poor, however we were able to calculate the binding affinity for the monomeric conformation ($gp120_{IN25925}$). The binding affinity of $gp120_{IN25925}$ was determined to be K_D: 37.6 nM (Figure 3.19 and Tables 3.4-3.5), approximately two-fold higher than that reported with Env $gp120^{YU-2}$ (K_D: 16.1 nM) (Wu et al., 2010). The difference could be attributed to the different gp120 isolate (YU-2) used in the report by Wu et al. (2010). Overall VRC03 binding was poor for the Env variants compared to the binding observed with VRC01, which is consistent with the lower neutralization profile of VRC03 than VRC01.

HJ16 is a glycan dependent CD4bs antibody that recognizes an epitope on the CD4bs that is related, but non-overlapping, to IgG1b12 and is also dependent on the N276 glycan located on the outer domain of gp120 (Balla-Jhagjhoorsingh et al., 2013; Corti et al., 2010). In addition, amino acid residues important for HJ16 binding include D489, R490 (HXBc2 positions 474 and 476) and a combination of M490 and R491 (HXBc2 position 475 and 476) (Pietzsch et al., 2010). The amino acid residue M490 and N276 glycan are conserved in all the Env variants. However, Env ZACAP45 contains both D489N and R491K mutations, and Env ZACAP210 contains the R491K mutation (Figure 3.2).

The kinetic data obtained for the Env's tested (ZACAP45 and IN25925) supported these amino acid mutations and revealed insufficient binding of HJ16 with $gp120_{ZACAP45}$ (RU binding signal < 10, (Yang et al., 2016)), and minimal binding to the trimeric conformation $gp140GCN4(+)_{ZACAP45}$. Env $gp140GCN4(+)_{IN25925}$ formed the most stable complex with a 4.3-fold greater affinity compared to its matched monomers.

$NIH45\text{-}46^{G54W}$ is a more potent clonal variant of the VRC01 CD4bs-directed antibody, in which a single substitution (Glycine to Tryptophan at position 54) gave rise to increased contact with the gp120 bridging sheet and thus improved breadth and potency of the Ab (Diskin et al., 2011). Kinetic data

was obtained for NIH45-46^{G54W} binding to Envs gp120$_{ZACAP210/ZACAP45/IN25710/IN25925}$ and gp140GCN4(+)$_{ZACAP45/IN25925,}$ which all had similar association and dissociation rates (Figure 3.18 and Tables 3.4 – 3.5). The dissociation rate for gp140GCN4(+)$_{IN25710}$ was extremely slow, but could not be validated due to the limited dissociation time of the experiment. Binding affinities ranged between 6.74 – 24.1 nM, consistent with a published findings by Diskin et al. (2011), with the exception of gp120$_{ZACAP210}$ which had an approximately ten-fold lower binding affinity of 273 nM. Comparison of the amino acid sequences in which the substitutions D/N279K, Q456S, Q458Y and Q459E (Diskin et al., 2011) (Figure 3.2) have been suggested to confer resistance to NIH45-46^{G54W} revealed that none of these resistance mutations occurred in any of the Env variants in this study.

Poor binding was observed for the monomeric Env variants to bNAb PGT145 compared to the trimeric Env variants tested (Figure 3.20). This was expected since this bNAb binds to an epitope that is presented in the context of the trimeric spike (Yasmeen et al., 2014). This confirmed that the gp140 Env variants tested (gp140GCN4(+)$_{IN25925/IN25710}$) were successfully stabilized by the GCN4 trimerization motif and were in a native-like conformation. Unfortunately insufficient quantities of the other Env variants prohibited binding analysis with bNAb PGT145, limiting the evaluation of the native-like conformation to the two Indian Env's IN25925 and IN25710.

Overall, the trimeric gp140GCN4(+) Env variants showed higher binding affinities to the bNAbs evaluated compared to the matched gp120 monomers (Table 3.4 and 3.5). This observation has been reported for several bNAbs in the literature including IgG1b12, 2G12, VRC01, VRC-CH31 and 2F5, which have a stronger affinity for trimeric Env's presumably due to their resemblance to the native trimer, than for the gp120 monomer (Fouts et al., 1997; Kovacs et al., 2012; Sanders et al., 2015; Zolla-Pazner and Cardozo, 2010). Whether this is due to increased binding stoichiometry or the overall quaternary conformation of the trimeric Env is unclear. BNAbs

recognizing quaternary epitopes formed on a mature HIV-1 virion include 2909, PG9 and PG16. These Abs generally do not bind to gp120 monomers, and if they do, it is a weak interaction because these Abs only recognize epitopes on a quaternary structure on Env, which develop as a result of multimerization of the protein-protein Env subunits (Fouts et al., 1997; Kovacs et al., 2012; Sanders et al., 2015; Zolla-Pazner and Cardozo, 2010).

The trimeric Env spike consists of three gp120 monomers, therefore it has three equivalent CD4 binding sites. In a study of a subtype C trimer, it was found that all three binding sites were accessible to CD4, but that each binding site had a different affinity for CD4 compared to a subtype B trimer in which the binding affinities were the same for each CD4bs (Saphire et al., 2001). Another study using a subtype C trimer determined that only two of the three CD4 binding sites were accessible for CD4 binding (Pancera et al., 2005). It has been suggested that the orientation of the gp120 monomer determines the CD4bs accessibility, which in turn is dependent on the trimerization motif used to stabilize the three gp120 monomers into a trimeric conformation (Saphire et al., 2001). In addition, the Fab region of VRC01 has been shown to bind to gp140 in a 3:1 stoichiometry (Kovacs et al., 2012) but a single antibody has been found to inactivate an Env trimeric spike suggesting that the efficiency of antibody binding is the important determinant of neutralization potency (Yang et al., 2005). Based on this it is likely that the improved binding of bNAbs to the trimers compared to the monomers is a result of improved presentation of the discontinuous CD4bs epitope in the context of a trimeric Env spike which presents the epitope in a native-like conformation.

The comparison of the CD4bs-directed bNAbs with our Env's confirm that resistance to an Ab is influenced by a combination of factors including amino acid mutations within the targeted epitope or of non-contact residues, variations in glycosylation and size of the variable loops. Together, these

factors contribute to the overall conformation of Env, ultimately determining the accessibility of epitopes targeted by bNAbs (Wu et al., 2009), whereas a single difference may be tolerated. Moreover, although they appeared functional and conformationally correct, the impact of imperceptible differences during the expression and purification of these recombinant proteins on appropriate epitope exposure cannot be excluded.

With the exception of VRC03 and IgG1b12, the Indian Env variants displayed higher affinities for the bNAbs compared to the South African Env variants. Generally, this is a consequence of the slower dissociation rates observed for binding of the bNAbs with the Indian Env variants. An Env with a higher avidity could stabilize the Env-naïve B cell interaction and could trigger the appropriate maturation pathway, driving the evolution of bNAbs over time, potentially making it a better Env for vaccine immunogen design (Liao et al., 2013b). Having said this, the SPR binding studies were performed with Abs that have already undergone high affinity maturation, and Env's with a high avidity for these Abs may not be sufficient to engage the naïve B cell and drive the appropriate response (Bhiman et al., 2015; Liao et al., 2013b).

Altogether, this suggests that the Env variants are antigenically correct and suitable for complex formation and immunogenicity testing in small animals. The immunogenicity study was carried out with one representative Env from each region namely IN25925 for India and ZACAP45 for South Africa. The 2dCD4-liganded gp120/gp140$_{ZACAP45/IN25925}$ Env variants were successfully generated and purified for the immunogenicity assays. SDS-PAGE analysis (Figure 3.23) confirmed the successful formation of the complexes.

4.5 Comparative Immunogenicity of recombinant gp120/gp140GCN4(+) Env variants, peptides, DNA vaccines and cyclic permutants

As mentioned earlier, HIV-1 subtype C has developed into the most significant subtype worldwide and is associated with almost half of the HIV-

1 infections worldwide. It accounts for the majority of infections in South Africa and India and is mainly acquired via heterosexual transmission, with the infections in India having originally been introduced by a single South African lineage (Hemelaar, 2012; Kulkarni et al., 2009; Shen et al., 2011; UNAIDS, 2015; van Harmelen et al., 1997; van Harmelen et al., 1999). Early research using T-cell line adapted strains of HIV-1 Env in vaccine design failed to elicit bNAbs following vaccination. Given the appreciation in the difference of neutralizing sensitivity between laboratory-adapted and primary viral strains, the majority of HIV-1 Env subunit vaccines are based on sequences derived from primary HIV-1 infected patients and thus better represent viral strains circulating within the general population (Mascola et al., 1996; Sawyer et al., 1994; Wrin et al., 1995).

Numerous strategies aimed at better mimicking the native-like trimeric conformation of Env, or improving the exposure of bNAb epitopes using cyclic permutants, crosslinking Env with CD4, or using epitope scaffolds are gaining significance (Correia et al., 2014; Devico et al., 1996; Ofek et al., 2010; Saha et al., 2012; Sanders et al., 2015). To this end, we compared a number of different vaccine strategies, including monomeric Envs ($gp120_{ZACAP45/IN25925}$), trimeric stabilized Envs ($gp140GNC4(+)_{ZACAP45/IN25925}$), gp120 outer domain fragments (ODECCOPBPICS gp120), monomeric and trimeric cyclic permutants (Cyc4OD $gp120^{JRFL}$, and $gp120^{JRFL}$ h-CMP V1cyc 144-142), VRC01 engrafted epitope scaffolds (1ORC and 1WR2), heterologous DNA prime (Wt-JRFL-Env-DNA, JRFL Env-570D DNA, JRFL Env-SEKS DNA), protein boost ($gp140^{FV}$, $gp120^{JRFL}$ h-CMP V1cyc 144-142) immunization protocols and CD4-liganded gp120/gp140GCN4(+) complexes (using Env's ZACAP45 and IN25925) to elicit neutralizing antibody's following vaccination of rabbits. The focus of the first arm of the immunization study was on HIV-1 subtype C sequences, the second arm of immunogens were designed using an HIV-1 subtype B sequence.

Env/2dCD4^{S60C} liganded complexes were successfully generated as shown by SDS-PAGE analysis (Figure 3.22). The presence of contaminating bands was noted as discussed in section 4.2, and could possibly be cleaved gp41-GCN4(+) products. The 2dCD4^{S60C} variant, used to stabilize the various Env's to expose a CD4i epitope, was used to control for neutralization breadth obtained by NAbs directed towards 2dCD4^{S60C} rather than the desired CD4i epitope of the Env/2dCD4^{S60C} liganded immunogens. The 2dCD4^{Wt} variant provided a comparison to the 2dCD4^{S60C} variant. The 2dCD4^{FD} variant control does not fold correctly and has previously been shown to be unable to bind to gp120 (Cerutti et al., 2010), therefore no neutralizing response was expected from this group. These 2dCD4 variant controls would also demonstrate the importance of using a correctly folded 2dCD4 in these immunogen complexes.

Midpoint antibody titre's were determined for the second arm of immunogens (stabilized gp120/gp140 derivative immunogens) in the heterologous prime, protein boost immunized rabbits at week 10 (two weeks after the last prime immunogen and two weeks prior to the first boost immunogen) by midpoint titre ELISA's against their cognate antigens (Figure 3.25). In addition, terminal antibody titres elicited in the rabbits were determined by endpoint titre ELISA's for all the immunogens (first and second arm) against their cognate antigens (Figures 3.24 and 3.25).

Due to differences in immunogen types, doses administered to rabbits, immunization protocols and titre proteins used in ELISA assays, direct comparison of midpoint and endpoint titres should be considered with caution. However, several observations were made. Midpoint anti-gp140^{FV} mean antibody serum titres were higher for groups immunized using proteins primes (ranging between 10^3 – 10^4) compared to the rabbits that were primed using DNA (10^2 for all DNA immunogens). Anti-matched peptide titres (1ORC and 1WR2) were found to be the higher for 1ORC (10^6) compared to 1WR2 (10^4) (Figure 3.25).

Compared to the anti-gp140^{FV} titres, midpoint anti-hCMP-JRFL-V1 cyc titres were approximately ten-fold higher for Cyc4OD (10^5), 10-fold lower for ODECCOBPICS-C (10^2), but similar for the DNA prime immunogens (all ranging between 10^2 and 10^3). The low Ab titres detected using DNA immunogens compared to protein immunogens was consistent with a study in Indian rhesus macaques, which compared immunization regimens using trimeric JRFL DNA and protein immunogens in various prime-boost combinations (Chakrabarti et al., 2013). They reported detecting much lower Ab titres after DNA only immunizations compared to immunizing with the corresponding protein only. However, the Ab titres of the macaques immunized with DNA prime vaccines increased 20-fold after a single protein boost (Chakrabarti et al., 2013). In contrast, Vaine et al. (2010) compared different vaccination regimens in rabbits using JRFL gp120 DNA and protein immunogens alone or in combination and found no differences in the terminal bleed Ab titres. However, their titres were detected after five immunizations, compared to only three DNA immunizations in our study, which may account for the difference. Despite the low levels, the Ab titres observed suggest that JRFL Env expression *in vivo* was induced by the DNA prime immunogens, and the responses observed were detectable by the HIV-1 subtype B Env JRFL as well as the subtype C Env gp140^{FV}.

In order to compare the endpoint titres of the terminal bleed sera obtained from rabbits immunized with the stabilized gp120/gp140 Env derivative immunogens, they were titred against hCMP-JRFL-V1 cyc (Figure 3.25). The animals primed with protein and DNA immunogens showed a marked increase in the Ab titres after the protein boosts compared to the week 10 sera as follows: approximately five-fold increase for Cyc4OD (1.00×10^6), approximately 1×10^4 fold increase for ODECCOBPICS-C (5.76×10^6), approximately 4.0×10^3 times increase for WT-JRFL Env DNA (3.2×10^6); 1.2×10^3 times increase for JRFL-Env-V570D (1.28×10^6) and 3.84×10^3 times increase for JRFL Env-SEKS DNA (1.28×10^6). A smaller increase of approximately 400 times was observed for group 8 (JRFL-Env-V570D

prime, gp140^{FV} boost). This lower increase compared to the other groups can be attributed to the use of the gp140^{FV} protein used as a boost immunogen whereas the other groups were boosted with the hCMP-JRFL-V1 cyc protein. This was expected since one would expect to obtain higher titres when using a matched immunogen compared to that obtained using a different Env isolate. The Ab titres of the terminal bleeds for the animals immunized with the VRC01 epitope engrafted scaffold peptides 1WR2 and 1ORC, followed by gp140^{FV} protein boosts had similar anti-hCMP-JRFL-V1 cyc titres (10^5). Our terminal bleed end-point titres were comparable to the findings of a study by Kesavardhana et al. (2016) in which a DNA prime-protein boost guinea pig immunization study using hCMP-V1-cyc-144-142 reported end-point titres ranging between 10^6 and 10^7.

The marked increase in endpoint titres between those detected after boost immunizations compared to those detected after prime immunizations is similar to the NHP study comparing immunization with JRFL gp140 DNA-prime, protein-boost regimens by Chakrabarti et al. (2013) mentioned above. Although exact values cannot be compared between the studies due to the use of different immunogens and a different animal model, the same trend was observed of a marked increase in Ab titres (approximately 100-fold in their case) post boosting with three JRFL gp140 protein inoculations.

The matched terminal anti-Env responses that were elicited in the rabbits for the South African Env ZACAP45 (6.4×10^5 for the monomer and 8.5×10^5 for the trimer) were approximately 10-fold lower than those detected in the Indian Env IN25925 (4.3×10^6 for the monomer and 3.4×10^6 for the trimer). Interestingly there was little difference in the endpoint titres for the monomeric compared to the matched trimeric Env conformations (Figure 3.24). This is in contrast to published studies which have found that trimers generally induce higher titres of binding antibody responses than their corresponding monomers (Kim et al., 2005; Kovacs et al., 2012). In addition, these anti-Env endpoint titres determined for the South African and Indian

Envs are approximately 10-fold to 100-fold, higher than those obtained in a previous study in our laboratory (Killick et al., 2015) and can be attributed to the different cut-off value determination used in the ELISA.

For the purposes of comparison, anti-2dCD4^{S60C} responses were determined by ELISA for rabbits immunized with all the 2dCD4$^{S60C/Wt/FD}$ control groups. All the 2dCD4 variants elicited fairly high titres against 2dCD4^{S60C} ranging between 10^6 and 10^7, with the 2dCD4^{FD} variant eliciting the highest titres (Figure 3.24 A).

When comparing the endpoint titres of the Env/2dCD4 liganded groups, the sera obtained from the rabbits of groups immunized with these complexes elicited similar matched anti-Env titres, with the exception of gp140GCN4(+)$_{IN25925}$/2dCD4 which elicited remarkably lower matched anti-Env titres (Figure 3.24 B). However, the anti-2dCD4^{S60C} responses in rabbit sera immunized with the Env/2dCD4 liganded groups were ten-fold higher for the gp120/2dCD4 liganded groups compared to the gp140GCN4(+)/2dCD4 liganded groups (Figure 3.24 A). In contrast to our previous findings (Killick et al., 2015), the gp120/2dCD4 liganded groups elicited similar anti-2dCD4^{S60C} titres to the 2dCD4^{S60C} control group, however the lower anti-2dCD4^{S60C} titres elicited for the gp140/2dCD4 liganded groups is consistent with this study.

We compared the ability of the Ab's elicited with the different immunogens to neutralize a diverse panel of HIV-1 strains encompassing different HIV-1 subtypes with different neutralization sensitivities. As mentioned in section 3.5.2, terminal bleed sera from rabbits immunized with Env gp120$_{IN25925,}$ liganded and unliganded with 2dCD4^{S60C}, the three 2dCD4 variant control groups, as well as the control in which the reducing agent was omitted during complex formation of Env/2dCD4^{S60C}, were sent to Monogram BioSciences Inc. who performed the PhenoSense Neutralizing antibody assay against a panel of 15 HIV-1 pseudotyped viruses from different tiers

(Tier 1 – 3, with Tier 1 ranked as being the most sensitive and Tier 3 being the least sensitive to neutralization by NAbs (Seaman et al., 2010)), as well as HIV-2 and SIV (Table 3.6) (Killick et al., 2015). The sera of rabbits immunized with the remaining immunogens of the first arm of immunizations and all the sera from rabbits immunized with the second arm of immunogens were assayed in-house against a smaller panel of four HIV-1 pseudoviruses representing Tier 1 and 2 (Table 3.7 and 3.8). Prebleed sera were also assayed and found to have no neutralization activity (Tables 3.6 – 3.8).

Despite having high endpoint titres, overall the Env variants (gp120 and gp140GCN4(+)) and the stabilized gp120/gp140 Env derivatives elicited Abs with very low neutralization responses, limited to Tier 1 neutralization of isolate SF162 and virtually no neutralization of isolate BaL (Tables 3.6 – 3.8). Although our comparison of these immunogens is limited to SF162, a subtype B isolate, varying potencies were observed between these groups.

The Abs raised against the monomeric and trimeric Env immunogens of South African and Indian origin were similar in their neutralization responses, both when tested at Monogram Biosciences Inc. or in-house (Tables 3.6 and 3.7). This is in contrast to published studies which have found that trimers generally induce higher titres of neutralizing antibody responses than their corresponding monomers (Kim et al., 2005; Kovacs et al., 2012; Li et al., 2006; Zhang et al., 2007), however is consistent with our previous findings (Killick et al., 2015).

The rabbits primed with protein immunogens (Groups 1 and 2, Table 3.8) elicited a very low neutralization response with an average ID_{50} neutralization titre against SF162 of 89 and 196, respectively. Cyclic permutants provide a method of stabilizing gp120 while maintaining its native fold by effectively moving the N- and C-termini of gp120 into one of the variable loops by complete or partial deletion of that loop. Cyclic permutants further stabilized with the addition of a trimerization domain,

have been shown to bind to sCD4 as well as bNAbs IgG1b12, PG9 and PG16 (Saha et al., 2012). Unfortunately, this alternative method of Env stabilization did not elicit the desired NAbs following immunization of rabbits.

This poor neutralization response was also observed with the gp120 outer domain fragment (OD_{EC}COBPICS) immunogen. The OD_{EC}COBPICS fragment protein aimed to present non-glycosylated conserved epitopes of gp120, found on the outer domain, to the immune system. Since this fragment-based immunogen excludes the inner domain of gp120, it would limit the non-neutralizing Abs that are normally directed towards this region of gp120 (Bhattacharyya et al., 2010). A gp120 outer domain fragment based on the HXBc2 isolate was shown to elicit Abs with moderate neutralization against one subtype C (Tier 1) and three subtype B (Tier 2) pseudoviruses (Bhattacharyya et al., 2010). Similarly, the neutralization response in our immunization experiments using this fragment immunogen (based on the JRFL sequence) was directed towards Tier 1 pseudoviruses, despite several boost immunizations with the gp140 cyclic permutant protein.

A small increase in neutralization of SF162 was observed in sera of rabbits primed with a DNA immunogen. Compared to protein-based immunogens, DNA-based immunogens are simple to produce, stable and easily characterized (Kalams et al., 2013). They are also regarded as a safer option compared to attenuated viral vectors (Kalams et al., 2013). DNA vaccines have been reported to generally be unable to elicit bNAbs when used in isolation, however, when immunized in combination with an Env protein (boost) a more robust response is observed (Kalams et al., 2013; Leung et al., 2004; Munseri et al., 2015; Richmond et al., 1998). We evaluated three DNA-based immunogens in a heterologous prime-boost regimen, each one expressing Env based on the JRFL isolate. These included an Env in which a V570D mutation was introduced to stabilize the trimer, an uncleaved Env (SEKS) as well as the wild-type Env. These were

followed by protein boosts using the subtype B stabilized gp140 cyclic permutant or the subtype C $gp140^{FV}$. The use of DNA-immunogens has been shown to reduce viremia in NHP models (Patel et al., 2013; Rosati et al., 2009) and over 70 human phase I and II trials have been completed in which a DNA-based immunogen either in isolation or in combination with a protein or viral vector have been tested (IAVI, 2017). However, a phase III human efficacy trial (HVTN 505) testing a heterologous prime-boost regimen in which the DNA immunogen expressed *gag*, *pol, nef and env* genes as well as two Env proteins from subtype A and C, was prematurely withdrawn due to futility and safety concerns (Excler and Michael, 2016; Sheets et al., 2016; Shin, 2016). A comparison between our DNA immunogen groups showed a four-fold greater neutralization of SF162 when immunized with the JRFL-Env-570D DNA prime (average ID_{50} of 588) compared to the wild type (average ID_{50} of 149). The uncleaved DNA prime JRFL-Env-SEKS DNA elicited an even higher neutralization response to SF162 (average ID_{50} of 977). This is probably due to an improved native-like structure of the JRFL-Env-570D and the uncleaved JRFL-Env-SEKS trimers compared to the WT-JRFL Env. Boosting with $gp140^{FV}$ compared to h-CMP-JRFL-V1 cyc 144-142 after priming with JRFL-Env-V570D DNA resulted in a similar neutralization response to SF162 (average ID_{50} of 544) (Table 3.5). The increased neutralization response seen in our DNA prime, protein boost regimens compared to protein prime, protein boost regimens has been reported previously (Vaine et al., 2008; Vaine et al., 2010; Wang et al., 2005). It has been suggested that immunizing with a DNA vaccine induces a higher quality initial Ab response compared to immunizing with a protein vaccine. A possible explanation for this has been suggested by Vaine et al. (2010) to be due either to the induction of Abs with a superior biophysical quality or to the DNA immunogen presenting an improved antigen conformation to the immune system compared to a protein immunogen.

The VRC01 scaffold peptides, in particular 1ORC, designed to focus the Ab response to the VRC01 epitope showed improved neutralization of SF162 compared to the other immunogens. The neutralization response of rabbits immunized with 1WR2 was comparable to those immunized with the uncleaved DNA prime JRFL-Env-SEKS DNA. However rabbits immunized with 1ORC elicited an average two to four-fold increase in ID_{50} neutralization titre against SF162 compared to the gp120/gp140GCN4(+) Env variants as well as the stabilized gp120/gp140 Env derivative immunogens. This shows anti-HIV Ab responses can be induced by presenting only a specific epitope to the immune response. This is consistent with published findings which report that although a low neutralization response is seen with scaffold peptides, they are able to direct the immune response to various epitopes on Env (Jiang et al., 2016; Morris et al., 2017; Ofek et al., 2010).

Altogether, these results are consistent with the ongoing frustration in the HIV-1 vaccine field in which immunogens are typically unable to induce NAbs that display the neutralization breadth across heterologous primary isolates of HIV-1 required for an effective vaccine (Beddows et al., 2007; Cho et al., 2001; Sanders et al., 2015). The findings of a study in which guinea pigs were immunized with the h-CMP v1cyc 144-142 immunogen, were shown to generate a gp120 directed neutralizing response able to neutralize Tier 1 isolates but displayed weak neutralizing activity against Tier 2 isolates (Kesavardhana et al., 2016). This response was also reported by Vaine et al. (2010) who compared JRFL gp120 DNA and protein prime-boost vaccination regimens in rabbits.

Using gp120 in immunizations typically elicits Abs targeted to linear epitopes in the V3 loop, and SF162 is extremely sensitive to neutralization by V3 loop directed antibodies (Pinter et al., 2004; Sanders et al., 2015; Vaine et al., 2010; Vancott et al., 1995b). It is therefore possible that most of the Abs elicited in our study using the (gp120 and gp140GCN4(+)) Env

variants and stabilized gp120/gp140 Env derivatives are directed to the V3 loop, however epitope mapping experiments would have to confirm this.

More promising neutralization responses were obtained from the gp120/gp140GCN4(+)/2dCD4^{S60C} liganded immunogens aimed to expose cryptic epitopes on Env that are only exposed post binding to CD4. A direct comparison between the neutralizing responses induced by the 2dCD4-liganded immunogens is limited since the first immunization experiment's rabbit sera, which was tested by Monogram Biosciences Inc., was assayed against as panel of 12 HIV-1 pseudovirus isolates (Table 3.6) whereas the subsequent terminal bleed rabbit serum samples, were tested in-house and only assayed against a panel of four HIV-1 pseudovirus isolates (Table 3.7).

However, the same trend was observed and in addition, the trends were consistent with the previous findings in our laboratory (Killick et al., 2015), in which the 2dCD4-liganded Envs complexes elicited a bNAb response capable of cross-subtype neutralizing response of subtype B and C isolates from Tiers 1 and 2. The neutralizing response was improved for the trimeric-liganded complexes (gp140GCN4(+)/2dCD4^{S60C}) compared to the monomeric liganded complexes (gp120/2dCD4^{S60C}) regardless of the Env sequence used.

We were concerned that the use of the reducing agent, β-mercaptoethanol (to facilitate the formation of inter-molecular disulphide stabilization of the complex), during complex formation may have had an aberrant effect on intra-molecular disulphide bond formation of Env, resulting in a conformational change in the Env variants. We therefore included a control using the gp120$_{IN25925}$/2dCD4^{S60C} complex in which no β-mercaptoethanol was used during complex formation. No difference was observed on SDS-PAGE analysis (Figure 3.21) and the difference in immunogenicity as tested in neutralizing antibody assays was negligible (Table 3.3).

We included a set of 2dCD4$^{Wt/FD/S60C}$ variant controls to control for 2dCD4 only NAbs in downstream pseudovirion assays. Three of the five rabbits immunized with 2dCD4^{Wt} as well as three of the five rabbits immunized with 2dCD4^{S60C} showed activity in the pseudovirion assays (Table 3.6). Of these, three showed ID_{50} neutralization values greater than 100 of Tier 2 pseudoviruses, whereas none of the rabbits immunized with the 2dCD4^{FD} control elicited neutralizing Abs. This was expected since 2dCD4^{FD} does not fold correctly and has been shown to be unable to bind to gp120 (Cerutti et al., 2010). This shows that the 2dCD4$^{Wt/S60C}$ variants were correctly folded and in the correct conformation and displayed the correct epitopes. We therefore hypothesised that the Abs elicited with the 2dCD4$^{Wt/S60C}$ variants target the CD4 receptors on TZM-bl cells in the pseudovirion assays and block infection, although this was not tested. Despite this, not every rabbit in these groups responded, even though they received 2dCD4 immunogens. On average, a more consistent neutralization response was achieved from groups immunized with the Env/2dCD4 liganded complexes.

One would expect to see Ab responses against the human 2dCD4 in rabbit sera as it differs significantly from rabbit 2dCD4 and only shares 55 % similarity (Liu et al., 1999) and is thus recognized as foreign. This was noted in a similar study by Schwartz et al. (2016) who assessed a gp120-CD4 immunogen in cynomolgus macaques and compared the use of different CD4 molecules complexed to gp120 in order to assess whether a response is induced when heterologous CD4 molecules are used. They compared three gp120-CD4 complexes, one using a human CD4, one using a rhesus macaque CD4 and one using a cynomolgus macaque CD4. They detected anti-CD4 Abs against the human CD4, but not the rhesus or cynomolgus macaque CD4. The latter two CD4 sequences are 100 % homologous, therefore confirming that the elicitation of heterologous anti-CD4 titres occurs in immunization studies when using interspecies immunogens. This raises the question of whether the same neutralizing response would be induced if humans were vaccinated with our Env/2dCD4 liganded complex.

An anti-2dCD4 response raises the concern of autoreactivity. Anti-CD4 Abs have been detected early in HIV-1 infected individuals (Keiser et al., 1992) and have been implicated in neutralizing HIV-1 infection via post-attachment activity (Hou et al., 2015). Approximately 10-15 % of HIV-1 infected individuals contain Abs against CD4 which target domains three and four of CD4 (Chams et al., 1988; Kowalski et al., 1989). In addition, Abs directed against the extracellular portion of CD4 have been detected in approximately 10 % of HIV-1 infected patients and are thought to be directed to a transiently exposed epitope on CD4 on binding to gp120 (Kowalski et al., 1989).

Human phase 1 clinical trials in which HIV-1 infected patients were administered CD4 were found to be safe and well tolerated (Hodges et al., 1991; Kahn et al., 1990; Schacker et al., 1995). By the same token, HIV-1 bNAbs are often autoreactive (Mascola and Haynes, 2013). As an example, the bNAb VRC01 has been found to bind human ubiquitin ligase E3A, an enzyme involved in normal cell functioning (Liu et al., 2015b). Therefore, preclinical studies and early clinical trials will have to assess the safety and tolerance of any Abs regardless of their target epitope.

Interestingly, the end-point titre data does not correlate with the pseudovirus neutralization data, and there appears to be an inverse relationship between the anti-Env and anti-2dCD4^{S60C} endpoint titre and the neutralization data. For example, the gp140$_{IN25925}$/2dCD4^{S60C} complex generated very low anti-2dCD4 and anti-Env titres (Figure 3.24), yet showed very high neutralization of pseudoviruses Sf162, BaL, ZM53 and CAP210 (Table 3.7). Whereas the 2dCD4^{S60C} immunogen resulted in approximately ten-fold higher end-point titres compared to gp140$_{IN25925}$/2dCD4^{S60C} but only two of the five rabbits immunized with this immunogen showed high neutralization of the pseudoviruses (Table 3.6). This is possibly due to the NAb response being directed to one or more novel epitopes exposed either on gp120 or 2dCD4 or both, post binding of these two molecules. This indicates that the use of

the matched Env or 2dCD4 molecules to determine the antibody titres in end-point ELISA's would not quantify the response appropriately. Rather, the neutralization-directed epitope, which has not yet been ascertained, would give a more accurate indication of the neutralizing response titres. Efforts to explore this are ongoing.

In summary, all the immunogens assessed in this study induced an Ab response capable of neutralizing Tier 1 pseudovirus SF162. The only immunogens that stood out in terms of neutralization breadth and potency were the 2dCD4^{S60C}-Env liganded immunogens, which was improved for the trimeric Env conformations. This response was consistent with both Env isolates (ZACAP45 and IN25925) tested as well as our previous findings (Killick et al., 2015). This data suggests that the Ab responses induced by the different gp120/gp140GCN4(+) Env variant immunogens and stabilized gp120/gp140 derivatives with limited neutralization are possibly directed towards one or more regions on Env, whereas those induced by the Env/2dCD4^{S60C} liganded immunogens could be directed towards one or more transiently exposed epitopes either on Env post binding to 2dCD4, or as suggested by Kowalski et al. (1989), transiently exposed epitopes on 2dCD4 post binding to gp120. However, our previous findings suggest that the NAb response is directed against 2dCD4, since neutralization activity was diminished post depletion of anti-CD4 antibodies (Killick et al., 2015).

Despite this, it is possible that the Env/2dCD4-liganded immune response may be directed to a region on CD4, distinct from the region to which gp120 binds, and may be "Ibalizumab" in nature. The mAb Ibalizumab, a humanized mAb engineered from its mouse progenitor, blocks HIV-1 entry by binding to domain 2 of human CD4 (Song et al., 2010). Ibalizumab has been reported to reduce viral loads in HIV-1 infected patients approximately ten-fold with no serious side effects, as determined in a phase 1b safety, pharmacokinetics and antiviral activity trial (Jacobson et al., 2009). Ibalizumab-treated patients showed no indication of CD4+ T-cell depletion

and Ibalizumab was not immunogenic. Since Ibalizumab binds to an epitope on the opposite side of the CD4 molecule to which gp120 and the MHC class II molecule bind, it does not interfere with the “normal” immune response (Song et al., 2010). The response could also be similar to that of 21c, a bimolecular Ab isolated from an HIV-1 infected individual which targets domain one of the CD4 molecule and binds to a subtype C gp120 (CAP210) in the presence of sCD4 (Diskin et al., 2010).

Ongoing studies in our laboratory are attempting to map the epitopes responsible for the neutralization breadth observed when immunizing rabbits with Env/2dCD4^{S60C} liganded immunogens to elucidate the neutralization mechanism observed.

Overall, the immunogens tested in this study all elicited anti-Env Abs in rabbits, however the majority of the activity was non-neutralizing. This was not unexpected since during the course of the study emerging evidence on the extent of affinity maturation required for the evolution of an anti-HIV bNAb response in an infected patient emerged (Bhiman et al., 2015; Liao et al., 2013b; Moore et al., 2012). These studies propose an interplay between constant antigenic challenge and resulting immune escape by Env concurrently with an increased percentage of affinity maturation of the B cells over time. This implies that multiple sequential immunizations with Env immunogens would be required to stimulate the appropriate B cell precursor and to drive the affinity maturation process. These findings could explain why numerous immunization experiments with Env, in this study and internationally (Bhattacharyya et al., 2010; Bowles et al., 2014; Burke et al., 2009; Celada et al., 1990; Cho et al., 2001; Derby et al., 2006; DeVico et al., 2007; Earl et al., 1994; Hammonds et al., 2005; Heyndrickx et al., 2013; Kim et al., 2005; Klasse et al., 2016; Kulkarni et al., 2013; Leung et al., 2004; Liao et al., 2013a; Narayan et al., 2013; Rosati et al., 2009; Sanders et al., 2013; Schwartz et al., 2016; Sneha et al., 2015; VanCott et al., 1995a; Wang et al., 2005), have never elicited bNAbs targeting clinically relevant HIV-1.

Thus future work could build on using the vaccine immunogens described in this study and design sequential immunization regimens using an evolving Env sequence over time to try and elicit bNAbs. Moreover, the "Ibalizumab-like" bNAbs described here warrant further investigation.

4.6 Concluding remarks

It is likely that an effective vaccine would require the elicitation of bNAbs in order to be protective against HIV-1. An Env based-immunogen has been the focus of intensive vaccine efforts since it is the primary viral component exposed on the virus. A region-specific Env immunogen could address the large diversity challenge of HIV-1 vaccine design. Here we compared the antigenicity of HIV-1 subtype C Env's from South Africa and India using isolates representative of these geographically distinct regions. The ability of these Envs, liganded and unliganded to 2dCD4^{S60C}, as well as Env-based immunogens designed as stabilized Env derivatives (based on HIV-1 subtype B isolates) to elicit bNAbs were evaluated and compared in rabbits.

While differences in amino acid sequences, PNLG's and variable loop lengths were noted between the Env's of South African and Indian origin, the SPR experiments showed that a combination of these factors influence the overall exposure of relevant epitopes, and therefore binding of bNAbs. SPR was found to be a useful tool for quantifying and comparing the Ab-Env interactions. It would be interesting to determine the stoichiometry of the Ab-Env interaction to understand the Ab-Env interaction in more detail. Future work could look into this as well as compare Env binding with the Fab region of the Ab to the whole immunoglobulin.

The Env's of Indian origin had higher affinities for the CD4bs bNAbs VRC01, VRC-CH31, HJ16 and NIH45-46^{G54W} and elicited approximately ten-fold higher anti-Env titres when immunized in rabbits compared to the South African Env's. However, immunization of rabbits with the South African and Indian Env's were equivalent in their neutralization responses. In addition,

the Env only neutralization responses were comparable to those obtained with the JRFL stabilized Env derivatives. However, of these immunogens the VRC01 engrafted scaffold peptide 1ORC induced an improved neutralization response against Tier 1 pseudoviruses, presumably indicating a focusing of the immune system to a predetermined epitope on the scaffold peptide. Future directions using this approach could possibly present an epitope based on an intermediary epitope, (rather than one based on an affinity matured bNAb) that better engages the naïve B cell and could be used to drive the evolution of bNAbs.

In comparison to the Env based immunogens, the Env/2dCD4^{S60C} liganded complexes elicited a potent and broadly neutralizing response and reinforced our earlier findings (Killick et al., 2015). Interestingly, this response was observed regardless of the Env isolate used. Although this neutralization response may be directed against 2dCD4 (Killick et al., 2015), it may target a region on 2dCD4 distinct to that which binds gp120 and MHC class II molecules. If the response is directed to a transiently exposed epitope on 2dCD4 post binding to gp120, it warrants further investigation as to the feasibility of such a strategy in HIV-1 vaccine design. Ongoing studies in our laboratory are attempting to elucidate the neutralization mechanism induced by our Env/2dCD4 liganded complexes. In addition, studies striving to replicate this response in a NHP model using a homologous NHP 2dCD4 to cancel out the effects of using a ‘foreign” heterologous 2dCD4 from a different species, are ongoing.

Overall, the findings in this study lay the groundwork for further testing of these HIV-1 Env based immunogens in various combinations using sequential prime-boost strategies to optimally drive affinity maturation of the appropriate B cell lineages.

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Appendices

Appendix A

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND
ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Maria A. Papathanasopoulos

b. Department: Molecular Medicine and Haematology

c. Experiment to be modified / extended

				AESC NO
Original AESC number	**2010**	**12**	**03**	
Other M&Es :				A

d. Project Title: **Immunization of New Zealand white rabbits with Novel HIV-1 subtype C founder virus envelope glycoprotein complexes and evaluation of anti HIV-1 immune responses**

		No.	**Species**
e.	Number and species of animals originally approved:	60	*New Zealand White Rabbits*
f.	Number of additional animals previously allocated on M&Es:		N/A
g.	Total number of animals allocated to the experiment to date:	60	*New Zealand White Rabbits*
h.	Number of animals used to date:	60	*New Zealand White Rabbits*

i. Specific modification / extension requested:
Additional 40 Rabbits required/ extension of 1 year

j. Motivation for modification / extension:

The results obtained from our original immunization experiments showed a marked difference between rabbits immunized with Env immunogens alone, vs Env-CD4 complexed immunogens. We obtained broadly neutralizing antibody responses that are desirable for an HIV vaccine candidate. We have established that a proportion of the broadly neutralizing antibody response seen was directed against the CD4, and the remainder against the Env-CD4 complex. We would like to repeat one of the experiment sets, and include additional controls (see groups 6 and 7 below) to confirm this. If the data are verified, we would require further testing in non human primate models.
The proteins to be immunized (5 rabbits per group) with the same adjuvant as used previously (Adjuplex) include:
1. HIV-1 Envelope glycoprotein (Env) alone
2. Env complexed to mutated 2dCD4(S60C)
3. 2dCD4(S60C) alone
4. Env complexed to HelC peptide
5. Hel C alone
6. Env complexed with wildtype 2dCD4 (new control)
7. wildtype 2dCD4 alone (new control)
8. control (no protein)

AESC 2012 M&E

Date: 25 June, 2012

Signature: 

RECOMMENDATIONS
Approved as requested.

Date: 29 June 2012

Signature: 

Chairman, AESC

AESC 2013 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Maria A. Papathanasopoulos

b. Department: Molecular Medicine and Haematology

c. Experiment to be modified / extended

	AESC NO		
Original AESC number	**2010**	**12**	**03**
Other M&Es :			20101203A

d. Project Title: **Immunization of New Zealand white rabbits with Novel HIV-1 subtype C founder virus envelope glycoprotein complexes and evaluation of anti HIV-1 immune responses**

		No.	Species
e.	Number and species of animals originally approved:	60	*New Zealand White Rabbits*
f.	Number of additional animals previously allocated on M&Es:	40	*New Zealand White Rabbits*
g.	Total number of animals allocated to the experiment to date:	100	*New Zealand White Rabbits*
h.	Number of animals used to date:	100	*New Zealand White Rabbits*

i. Specific modification / extension requested:
Additional 20 Rabbits required. Extension of 4 months. Repeat one experimental set to include erythropoietin.

j. Motivation for modification / extension:
Overall, the results obtained from our immunization experiments show a marked difference between rabbits immunized with Env immunogens alone, vs Env-CD4 complexed immunogens. We obtained broadly neutralizing antibody responses that are desirable for an HIV vaccine candidate. We would like to repeat one of the experiment sets, and include an Envelope glycoprotein based on an HIV-1 subtype C isolate from India to determine whether there are differences in the immunogenicity between South African and Indian Envs (we have shown there are differences in antigenicity). The proteins to be used (5 rabbits/group) with the same adjuvant as previously (Adjuplex) include:
1. HIV-1 Envelope glycoprotein alone (Env, based on IN26191 isolate)
2. Env complexed to mutated 2dCD4(S60C)
3. 2dCD4(S60C) alone
4. uncomplexed Env and 2dCD4(S60C)

Date: 30 April, 2013

Signature:

RECOMMENDATIONS
Approved: additional 20 rabbits, extension of time by 6 months; modification of protocol to include Indian glycoprotein.

AESC 2013 M&E

Date: 3 May 2013

Signature: 

Chairman, AESC

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/51/B

APPLICANT: Prof M Papathanasopoulos

SCHOOL: Molecular Medicine and Haematology

LOCATION: Faculty of Health Sciences

PROJECT TITLE: *Comparitive immunogenicity of novel Indian and South African HIV-1 vaccine immunogens in New Zealand white rabbits and evaluation of anti HIV-1 immune responses*

Number and Species

48 New Zealand white rabbits

Approval was given for to the use of animals for the project described above at an AESC meeting held on 26 August 2014. This approval remains valid until 25 August 2016.

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 is subject to any additional conditions listed below:

None.

Signed: _____ Date: 12th September 2014
(Chairperson, AESC)

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: _____ Date: 12th September 2014
(Registered Veterinarian)

cc: Supervisor: N/A
Director: CAS

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Appendix B

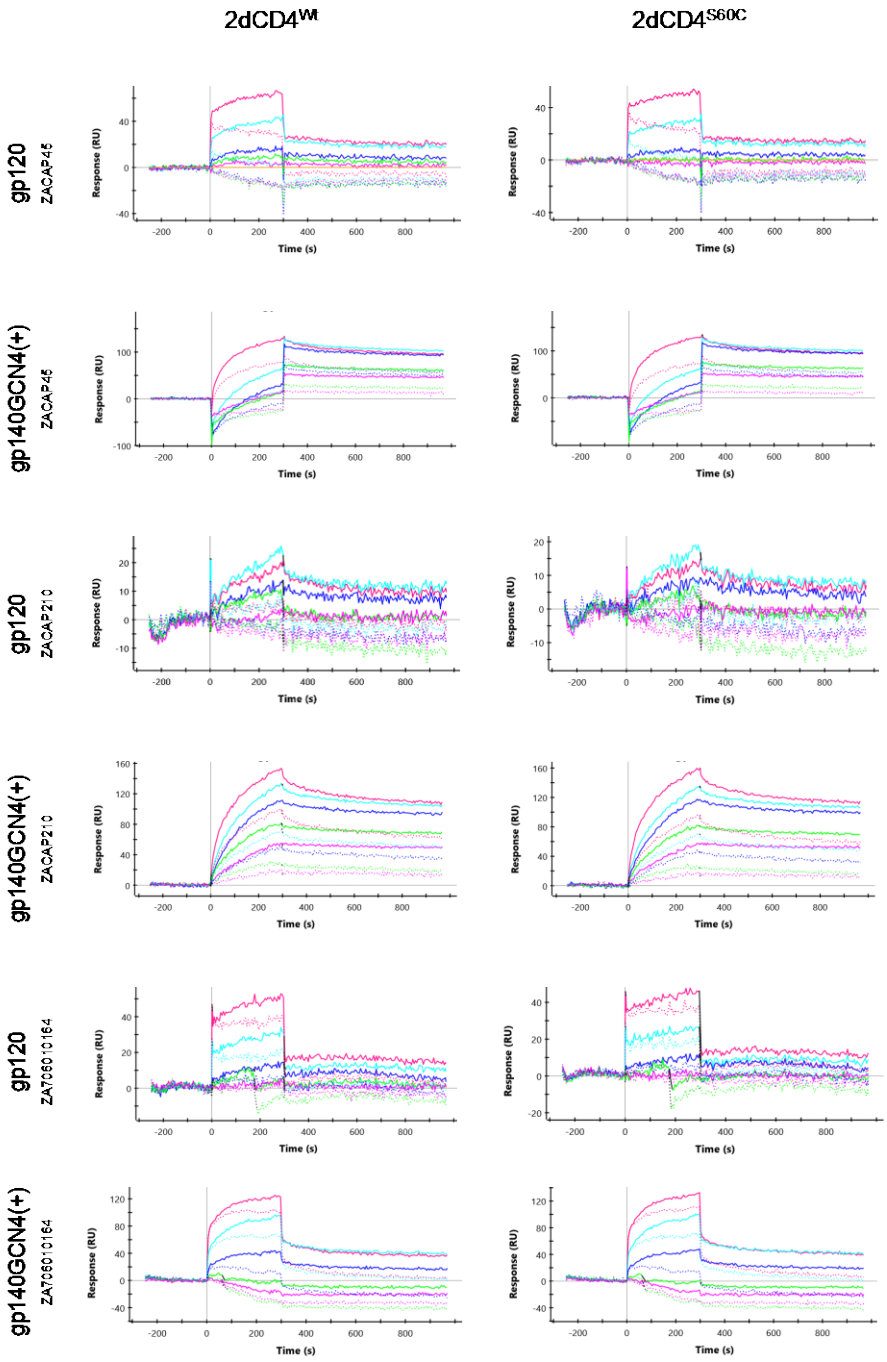

Figure B1: SPR sensorgrams showing binding and interspot data of increasing concentrations of gp120 and gp140GCN4(+) conformations of the HIV-1 envelope glycoproteins of South African origin to 2dCD4^{Wt} and 2dCD4^{S60C}. 2dCD4^{Wt} and 2dCD4^{S60C} were covalently captured onto separate flow cells on the chip at a concentration of 0.02 mg/ml at a flow rate of 30 µl/min for 300 s to a final RU of 2000. The gp120 and gp140GCN4(+) Env's were passed over the ligands at a flow rate of 30 µl/min for 300 s with a dissociation time of 600 s. Binding kinetics could not be accurately determined due to non-specific binding (dotted curves) exceeding10% R_{MAX} as per manufacturer instructions (Bio-Rad Laboratories Inc., 2009).

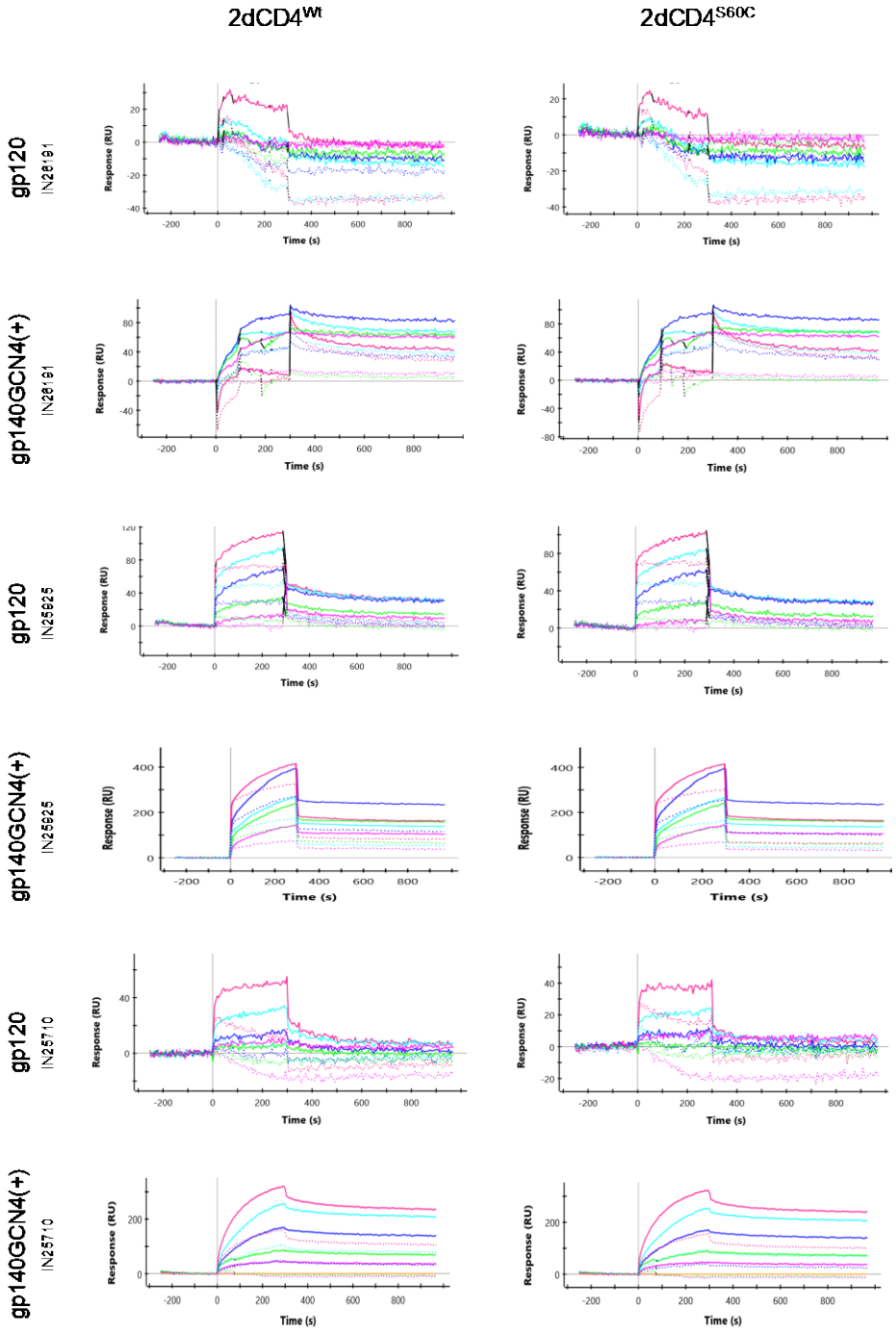

Figure B2: SPR sensorgrams showing binding and interspot data of increasing concentrations of gp120 and gp140GCN4(+) conformations of the HIV-1 envelope glycoproteins of Indian origin to 2dCD4^{Wt} and 2dCD4^{S60C}. 2dCD4^{Wt} and 2dCD4^{S60C} were covalently captured onto separate flow cells on the chip at a concentration of 0.02 mg/ml at a flow rate of 30 µl/min for 300 s to a final RU of 2000. The gp120 and gp140GCN4(+) Env's were passed over the ligands at a flow rate of 30 µl/min for 300 s with a dissociation time of 600 s. Binding kinetics could not be accurately determined due to non-specific binding (dotted curves) exceeding 10% R_{MAX} as per manufacturer instructions (Bio-Rad Laboratories Inc., 2009).