Molecular Characterization and Immunogenicity of an HIV-1 subtype C founder Consensus viral envelope SOSIP.664 Trimer covalently bound to 2dCD4^{S60C}

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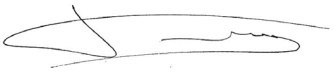
A thesis submitted to the School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, October 2022

Declaration

I, **Nancy Lola Tumba** (student number: 1594387), hereby declare that the work encompassed in this Ph.D. thesis was undertaken by myself. Where others' work, thoughts and ideas have been mentioned, I have used conventional referencing to acknowledge their sources. A Turnitin plagiarism report indicating the calculated level of plagiarism according to an internet-based software has been included in the appendix section.

The work is being submitted for the degree of Doctor of Philosophy to the University of the Witwatersrand, in Johannesburg, South Africa. The present thesis, in part or as a whole, has not been previously submitted for examination or for a degree at this or any other institution.

Signature: ___  _ Date: 19 October 2022

Dedication

This work is dedicated to:

My marvelous parents, *Bob Tumba* and *Lola Mitheo*, who have always encouraged, supported, and loved me unconditionally.

My wonderful husband *Alef Michel Meulenberg*, who believes in the best version of myself.

My precious daughter *Imani Alkje Meulenberg*, who can always bring a smile to my face.

"Each day we live at the mercy of organisms
one-trillionth our size."

Dr. Paul Brand / Philip Yancey

Publications arising from this thesis

1. **Nancy L. Tumba**, Previn Naicker, Stoyan Stoychev, Mark A. Killick, Gavin R. Owen, Maria A. Papathanasopoulos. Covalent binding of human two-domain CD4 to an HIV-1 subtype C SOSIP.664 trimer modulates its structural dynamics. *Biochemical and Biophysical Research Communications*, Volume 612, 2022, Pages 181-187, ISSN 0006-291X,

   https://doi.org/10.1016/j.bbrc.2022.04.101.

   (https://www.sciencedirect.com/science/article/pii/S0006291X2200643X)

2. **Nancy L. Tumba**, Gavin R. Owen, Mark A. Killick, Maria A. Papathanasopoulos. Immunization with HIV-1 trimeric SOSIP.664 BG505 or Founder Virus C (FVC_{Env}) covalently complexed to two-domain $CD4^{S60C}$ elicits cross-clade neutralizing antibodies in New Zealand white rabbits. *Vaccine: X*, Volume 12, 2022, 100222, ISSN 2590-1362,
   https://doi.org/10.1016/j.jvacx.2022.100222.
   (https://www.sciencedirect.com/science/article/pii/S2590136222000821)

Acknowledgments

I am indebted to my supervisors for the crucial role they collectively played in the supervision of this doctoral work.

◊ Prof. Maria Papathanasopoulos, thank you for the guidance, expertise and assistance offered from before I enrolled as a Ph.D. student and throughout the years of my studies and career. Thank you for providing the platform and opportunities that made this work possible as well as for organizing the training fellowship at Grenoble. Your feedback and encouragement have been invaluable and your advice and remarkable ability to find solutions to problems has been instrumental in navigating the ups and downs of Ph.D. work.

◊ Dr Gavin Owen, words cannot express the gratitude I have for your input towards this work. You have gone beyond your supervisory duties in ensuring my work was substantial in terms of scope and collaborative opportunities while bringing me back to earth whenever I went beyond striving for 'perfection' into the realm of the 'impossible.' I thank you for your patience with me when I did not see eye-to-eye regarding science that is not overtly translational. Thank you for your open door policy and how you availed yourself to answer my questions and offer support. And of course, thank you for helping me to master the Oxford comma.

◊ Dr Mark Killick, thank you for being so level-headed and occasionally pointing out potential pitfalls. Your expertise and wealth of knowledge have been tremendously resourceful. As the more established post doc in the lab, I am glad I could rely on your experience both administratively and scientifically. You have played a significant role in guiding me on how to juggle the pressures of Ph.D. work and the requirements of a new academic, particularly with regards to supervision of students. Thank you.

My deepest appreciation go to my family:

My husband Alef, thank you for the love, support and sacrifice that did not go unnoticed. If I made it this far, it was because I knew you were by my side every step of the way. Thank you for partnering with me in life and for accepting me in all my quirkiness through the good and

bad times. I truly appreciate your non-judgmental attitude and by your own admission- how you “believe more for me than I sometimes do.”

My parents who always encouraged and provided the means for me to pursue the best education possible, and for demonstrating how to work hard and smart. *A ma chère maman Lola, je te remercie de tout cœur pour ta camaraderie, ton aide avec Imani, tes conseils, ton encouragement, ton amour, tes prières, et nos fous rires. Je suis très reconnaissante pour ton soutien et encore merci pour tout ce que tu as fait pour moi.* To my brother Mike, thank you for embodying the belief that it’s never too late to achieve anything and that the spotlight should not be on those who shout the loudest but those who have endured the most and survived.

I am grateful for the financial support received through the Wits University Staff-linked bursary and the National Research Foundation (NRF) Thuthuka grant.

To our CSIR collaborators Dr Previn Naicker and Dr Stoyan Stoychev, thank you for the use of your facilities and equipment and for training me on all things HDX-MS.

To all my friends and spiritual family (too many to list), thank you for the support, prayers, encouragement, and the occasional “escape.” A special mention goes to Kim Wong, for the Asia-Africa soul friendship which started about twenty years ago and will continue for eternity. Apart from my husband and my mother, you know me best. I am so grateful for your friendship, support and love.

Special thanks go to the HPRU family (present and past members): As colleagues and students, you have helped more than you know in creating the kind of atmosphere where we find joy in each other’s successes and commiserate over failed experiments. Thuli, thank you for all the “chats” and for a being a like-minded friend on this transformation journey. Adri- our paths keep crossing and I like it- may there be many more “good mornings” and impromptu chats.

Last yet most importantly, to GOD who has given me purpose, meaning, and life “twice.” Thank You for not shying away from the humility of the cross so we could profess your Righteousness as our own.

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List of Abbreviations

2dCD4	two–domain CD4
Ad5	adenovirus serotype 5
ADCC	antibody–dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ARV	antiretroviral
BCR	B-cell receptor
bNAb	broadly neutralizing antibody
bp	base pairs
CCR5	chemokine (C–C motif) receptor type 5
CD4	cluster of differentiation 4
CD4bs	CD4–binding site
CD4i	CD4–induced
CDRH3	complementarity determining region 3 of the heavy chain
CXCR4	chemokine (C–X–C motif) receptor 4
DEAE Dextran	diethylaminoethyl dextran
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein–Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FVC_{ENV}	Envelope glycoprotein of the founder virus consensus C
Fab	antigen binding fragment region
Fc	fragment crystallizable region
FCS	fetal calf serum
Gag	group associated antigen
GALT	gut-associated lymphoid tissue

gp120	envelope glycoprotein 120
gp41	envelope glycoprotein 41
HAART	highly active antiretroviral therapy
HEK	human embryonic kidney
HIV-1	human immunodeficiency virus type–1
HLA	human leukocyte antigen
HR1	heptad repeat 1
HRP	horseradish peroxidase
IC_{50}	50% inhibitory concentration
ID_{50}	50% inhibitory dilution
IFN	interferon
Ig	immunoglobulin
IgM, G, and A	immunoglobulin M, G, and A
IL-1, 2, 12, 15, 17, and 22	Interleukin–1, 2, 12, 15, 17, and 22
kDa	kilodalton
LTR	long terminal repeat
MA	matrix
mAb	monoclonal antibody
MMP	methyl α–D–mannopyranoside
MPER	membrane proximal external region
Nab	neutralizing antibody
NHP	non–human primate
NZW	New–Zealand white
PBMCs	peripheral blood mononuclear cells
PEI	polyethylenimine
Pol	polymerase
PR	protease
PrEP	pre-exposure prophylaxis
PSV	pseudovirus

Rev	regulator of expression of viral proteins
RT	reverse transcriptase
sCD4	soluble CD4
SEC	size exclusion chromatography
SHIV	simian–human immunodeficiency chimeric virus
SHM	somatic hypermutation
SIV	simian immunodeficiency virus
Tat	trans-activator of transcription
$TCID_{50}$	50% tissue culture infectious dose
T/F	transmitted/founder
V1V2	variable regions 1 and 2
V3	variable region 3
VH	variable heavy chain gene
VL	variable light chain gene
VLP	virus–like particle
Vif	viral infectivity factor
Vpr	viral protein R
Vpu	viral protein U

Abstract

Research towards an effective HIV-1 vaccine has been ongoing for almost four decades with no vaccine candidate showing a clear ability to induce a protective immune response. The main challenge has been the inability of currently tested immunogens to elicit potent, broadly neutralizing antibodies (bNAbs). Conventional immunogen approaches are based on HIV-1 envelope glycoproteins (Env) that are structurally stable and as close to the native HIV-1 trimer conformation as possible, for example SOSIP trimers. In addition, such immunogens should have antigenic capabilities of displaying the correct neutralizing antibody (nAb)-inducing epitopes common among the extremely high genetically diverse HIV-1 isolates, while at the same time, masking immunodominant but irrelevant sites. As such, novel insights into HIV-1 immunogen designs, in particular the differential display of conserved epitopes and the resulting effect on the immune system is beneficial to vaccine development.

Work done previously has shown that covalently complexing various HIV-1 Env to two-domain $CD4^{S60C}$ ($2dCD4^{S60C}$) has resulted in immunogens capable of eliciting a consistent antibody-mediated immune response in small animals, that is broad and potent. The aim of this work was to develop a next generation immunogen composed of a SOSIP trimer covalently complexed to $2dCD4^{S60C}$. An HIV-1 founder virus consensus C Env (FVC_{ENV}) which epitomizes all the collective properties of subtype C transmitted/founder (T/F) viruses) and the well characterized subtype A BG505 Env (control) were used in this study and each were covalently coupled to a 2dCD4 protein to form Env:2dCD4 complexes. The structural and conformational stability of the complexes were tested by evaluating the ability of broadly neutralizing antibodies to effectively recognize the immunogens in ELISA. Conformational dynamics studies - used to probe some of the attributes of the covalently complexed immunogen – were done on the FVC_{ENV} covalent immunogen complex (FVC_{Env}:$2dCD4^{S60C}$)

in comparison to its non-covalently bound equivalent complex (FVC_{Env}:2dCD4^{WT}). Lastly, the immunogenicity of the covalent complexes (FVC_{Env}:2dCD4^{S60C} and $BG505_{Env}$:2dCD4^{S60C}) was established in rabbits by testing the presence and scope of antigen recognizing antibodies in the rabbit sera following immunization and using the Env-pseudotyped neutralization assay against a panel of heterologous Tier 2 viruses.

The newly created covalent bond effectively locks the FVC_{Env}:2dCD4^{S60C} immunogen in the open, CD4-bound conformation thereby exposing the highly conserved CD4 induced epitopes. Hydrogen-deuterium exchange experiments revealed that covalent linkage between Env and 2dCD4 result in a drastic change in the dynamics and solvent exposure of domain 2 of CD4, which advances our knowledge of the improved immunogenicity of the complex. The founder virus consensus C envelope (FVC_{ENV}) trimer covalently complexed to 2dCD4 remarkably displayed optimal immunogenicity, with all the rabbits who received the complex showing the ability to elicit antibodies with up to 76,5% breadth (13 out of 17 heterologous viruses neutralized). Such consistently elicited immune responses from a singular adjuvanted recombinant protein immunogen administered in a simple regimen are encouraging and underscore the potential of this SOSIP immunogen as a prime candidate for further developments of vaccine regimens.

The findings highlighted in this study provide evidence that an efficient bNAb response can be successfully elicited in a pre-clinical setting using SOSIP.664 Env-based immunogens designed to expose conserved epitopes. The high success rate (5/5 animals) in inducing a strong immune response and the magnitude of the response (high titers) against some of the viral isolates show the potential sustainability of the antibody response and the feasibility that small improvements to the immunogen complex and/or the immunization strategy could yield a ‘panacea’ immunogen.

Chapter 1 Introduction and integrative narrative

1.1 The Human Immunodeficiency Virus

1.1.1 Human Immunodeficiency Virus pandemic

Human Immunodeficiency Virus types 1 and 2 (HIV-1, HIV-2) are the causative agent for the acquired immunodeficiency syndrome (AIDS), leading to the global pandemic which has claimed 36.3 million lives to date (World Health Organization, 2021). There were 37.7 million people living with the virus at the end of 2020 (UNAIDS, 2021), the overwhelming majority infected with HIV-1. The uptake of antiretroviral drugs has tremendously decreased AIDS-related morbidity and mortality and global HIV infection, however, the rate of new infections remains extremely high at 1.5 million in the year 2020 alone (UNAIDS, 2021). Sub-Saharan Africa carries the heaviest HIV disease burden, with two-thirds (25.4 million) of the global cases (World Health Organization, 2021).

1.1.2 Genomic Organization and Structure of HIV-1

HIV-1 is a retrovirus of the lentivirus genus. Its genome is composed of two identical positive single-stranded RNA molecules encapsulated in the core of the virus. When reverse-transcribed and integrated into the host genome, the proviral DNA has a length of 9.8 kb and consists of the common retrovirus genomic structure, made up of *gag-pol-env* genes with six additional genes, flanked at both ends by long terminal repeat (LTR) sequences [1] (Fig. 1.1). In the 5' to 3' direction, the genes encoded are *gag* (codes for matrix, capsid, nucleocapsid, and p6 proteins); *pol* (codes for the protease, reverse transcriptase, Rnase H and integrase enzymes); *Vif* (viral infectivity factor); *Vpr* (virus protein r); *Tat* (transactivator protein); *Rev* (RNA splicing regulator); *Vpu* (virus protein unique); *Env* (which codes for the envelope glycoproteins) and *nef* (negative regulating factor) (Fig. 1.1). While Gag, Pol, and Env-common to all retroviruses- are essential structural proteins, Tat and Rev are regulatory

proteins and Vpu, Vpr, Vif and Nef are referred to as accessory proteins. Two of these accessory proteins are encoded in a succession of two open reading frames with the first exons of *tat* and *rev* located between *vpr* and *vpu* while their second coding exons are found near the end of the *env* gene (Fig. 1.1).

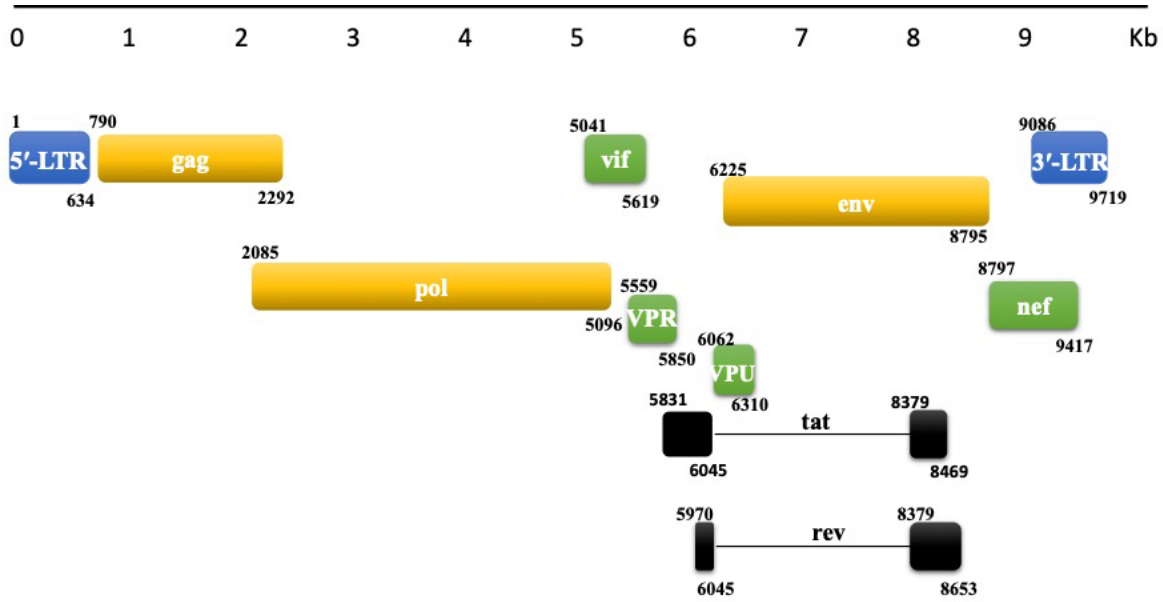

Figure 1.1. Organizational map of the HIV-1 genome. Reading frames of the genes coding for structurally essential (orange), regulatory (black) and accessory (green) proteins are shown as rectangular blocks, with the start and stop codons indicated at the upper left and bottom right of each rectangle. 5' and 3' long terminal repeats (LTR) regions are also boxed though these are not translated into proteins. The spliced exons of *tat* and *rev* are shown linked by a black line. The genome consists of just over 9,700 nucleotides based on the HXB2 sequence (K03455). Figure modified from Lutz Gürtler [2].

Each virion measures approximately 145 nm in diameter [3] and is composed, at its outer surface, of spikes made up of three surface envelope glycoproteins (gp120) non-covalently linked to three transmembrane envelope glycoproteins (gp41) (Fig. 1.2). These proteins which are encoded by the *env* gene play a role in facilitating attachment of the virus to the host cell [4,5]. The core proteins are made by translation of the *gag* gene and include the matrix protein p17 which forms a layer underneath the envelope with the viral conical core or capsid (p24) embedded within it (Fig. 1.2) [4,5]. The nucleocapsid protein p7 which binds to the RNA

strands is located inside the capsid core, along with reverse transcriptase and integrase enzymes. Reverse transcriptase, integrase and protease are enzymes necessary for replication [4,5].

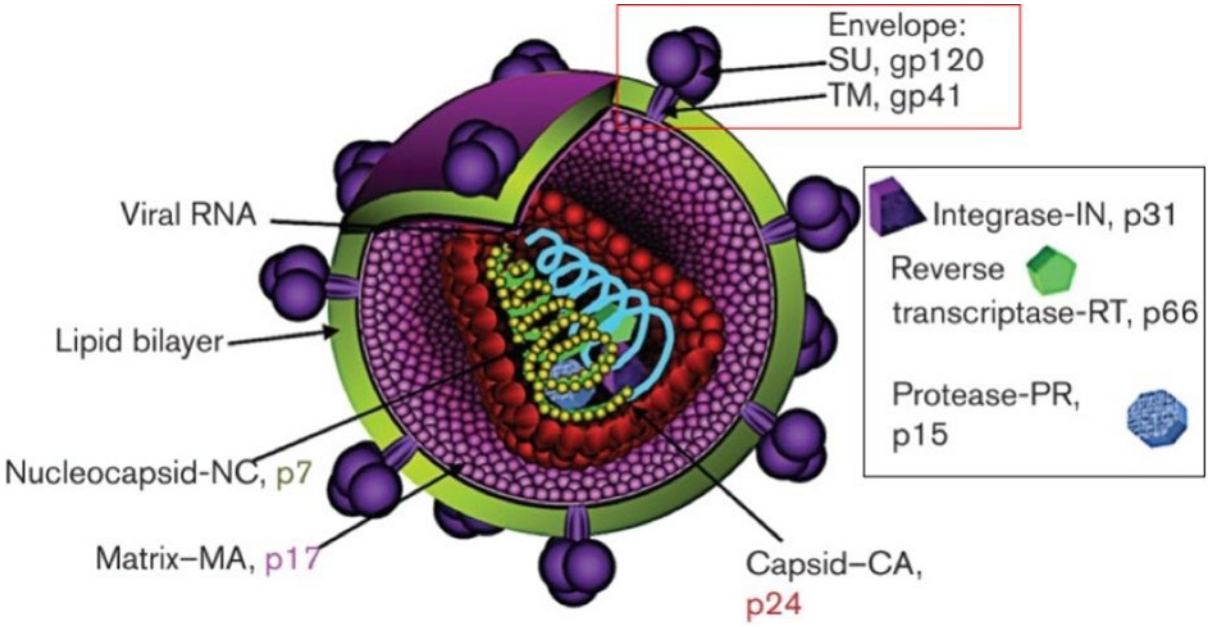

Figure 1.2. Structure of the HIV-1 virion. Schematic of HIV-1 showing location of viral proteins. One of the envelope glycoprotein trimeric spikes composed of gp120 and gp41 subunits is boxed in red. These form the viral outer membrane proteins. The Gag polyprotein processed into the matrix, capsid, and nucleocapsid elements form the viral inner core. The protease, reverse transcriptase and integrase enzymes, products of the Pol polyprotein are shown encapsulated in the core of the virus with their visual representation and names in a black box. This depiction is not to scale. Figure reproduced from Steckbeck *et al.*, 2013 [6] with slight modifications.

1.1.3 HIV Replication Cycle

The HIV replication cycle begins when the virus binds to and fuses with the surface of the host cell. The outer surface gp120 component of the viral Env recognizes CD4 receptors found on activated $CD4^+$ T lymphocytes and monocytes/ macrophages [7,8]. Binding of gp120 to CD4 induces Env trimer conformational changes that enable gp120 to interact with the co-receptors CCR5 (R5) and/or CXCR4 (X4). Engagement of the co-receptor leads to further modifications ultimately exposing the gp41-contained fusion peptide that drives fusion of the viral with the

host membranes [7]. This in turn leads to the release of the viral core (genetic material and viral proteins) into the host cell cytoplasm, a process referred to as entry (Fig. 1.3). Following the uncoating of the capsid, the HIV reverse transcriptase transcribes the single-stranded viral RNAs to double-stranded cDNA (Fig. 1.3) (reviewed in [9]). Subsequently, translocation of the viral cDNA into the nucleus occurs, a process facilitated by the Vpr accessory protein which mediates the assimilation of a preintegration complex, which includes the viral integrase, through its nuclear localization signal [10].

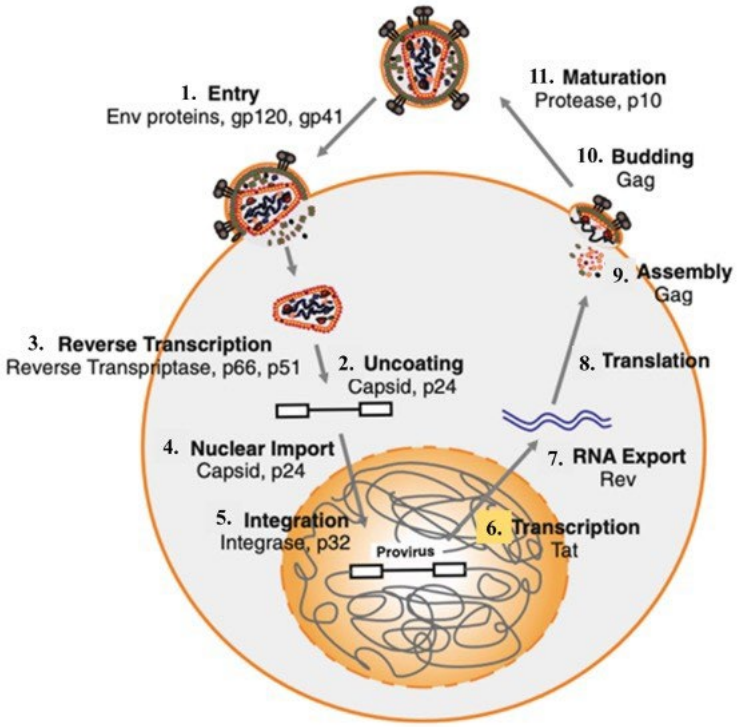

Figure 1.3. The typical HIV-1 replication cycle. Illustration of the main viral replication stages: 1 – HIV-1 entry into the host cell; 2 – disintegration and uncoating of the capsid; 3 – reverse transcription of viral RNA into cDNA; 4 – passage of proviral genome into the nucleus; 5 – integration of proviral DNA into the host DNA; 6 – transcription of HIV DNA into multiple RNA transcripts; 7 – Export of singly-spliced and unspliced viral messenger RNA (mRNA); 8 – mRNA translation into viral proteins; 9 – assembly of viral genomic RNA and proteins at the plasma membrane; 10 – release of new immature viral particle by budding; 11 – catalysis and cleavage events leading to maturation into an infectious virus. Figure reprinted from Kirchhoff *et al.*, 2013 [11].

Productive infection and expression of the genetic material require integration of the proviral DNA, a process driven by the integrase enzyme which involves cleavage of the DNA and

insertion into the host genome (Fig. 1.3) [12,13]. Replication of the proviral DNA occurs alongside the host DNA replication and involves co-operative regulation by host transcription factors as well as the viral Tat protein (Fig. 1.3) [14]. The HIV provirus is transcribed into multiple RNA species which are transported (partially spliced or unspliced) out of the nucleus and into the cytoplasm, a process known as nuclear export (Fig. 1.3) [15]. The Rev protein acts as the vehicle which transports intron-containing mRNAs from the nucleus to the cytoplasm [16-20] (Fig. 1.3). Once translation of the RNA species has resulted in all the necessary viral proteins, a new virus particle is ready to be assembled at the plasma membrane (Fig. 1.3). Virus assembly is a highly ordered yet complex process in which Gag binds to cell membranes [21,22]. Viral structural proteins, including Env, proteins required for capsid formation, and viral genetic material assemble at the surface of the host cell. As viral proteins accumulate at the plasma membrane, they induce the curvature of the membrane which progresses into a spherical membrane-coated shape, a process known as budding (Fig. 1.3) [23-25]. At this point, the immature virion begins the process of maturation in which the protease enzyme completes cleavage of the Gag and Gag-Pol polyproteins to produce the viral proteins required to form the matrix and the core of the virus [3,26]. The fully cleaved, mature virion is now capable of subsequent rounds of infection.

1.1.4 HIV Pathogenesis and Infection

HIV infection most commonly occurs at mucosal surfaces, following sexual transmission, and involves cell-free and cell-associated virus in body fluids [27]. Despite the multitude of viral quasispecies (mix of different strains) transferred to the mucosal area, research has shown that in most transmission cases, a single virus quasispecies is responsible for establishing productive infection across the mucosal surface [28-30]. This infecting virus is known as the founder virus [31]. Founder viruses display specific phenotypic qualities such as preferential usage of the CCR5 coreceptor [31], resistance to interferon-γ and improved interaction with

dendritic cells [32] (Founder viruses are discussed in additional details in chapter 1.3.4). Not surprisingly, the risk of HIV-1 transmission is directly correlated to the amount of plasma HIV-1 RNA (the viral load) in the HIV-infected individual, with a 2-4 fold increase of transmission for every 1 $\log_{10}$ rise in viral load [33]. Among the factors that increase the risk of HIV-1 sexual transmission and/or the infectiousness of the virus are the presence of other sexually transmitted diseases in either sexual partner [34]. These include genital ulcers [35], bacterial vaginosis [36] and herpes simplex type-2 infections [37], to name a few [38]. This increased infectiousness is not simply a result of an impaired mucosal barrier but also of immune cellular activation which presents a permissive environment for HIV to replicate [38].

Primary or acute HIV-1 infection is often associated with a high level of virus replication evidenced by a rapid peak in plasma viral load (Fig. 1.4), followed by a decline concurrent with the appearance of HIV-specific $CD8^+$ cytotoxic T lymphocytes (CTL) [39,40]. This early phase of infection is also characterized by virus dissemination to regional lymphoid tissues as shown by the lymphadenopathy [41,42] evidenced by lymph nodes inflammation and increased expression of cytokines (IFN-γ, IL-1, IL-2, and IL-12) [43-46]. The local expansion of the virus spans 1 to 2 weeks post-infection [47,48]. Once local virion production has reached high levels, the virus disseminates to the peripheral draining lymph nodes. This stage marks the official acute phase of infection, it is characterized by the appearance of the detectable virus in the plasma and lasts 2-4 weeks [47]. The continuous virus replication counteracts antiviral immunity by the high frequency of mutations it incurs combined with a low expression of viral antigens and the persistence of virus in lymphoid compartments, thus establishing a systemic infection. Eventually, circulating lymphocytes become sequestered in these sites, with the high-level of viral replication ultimately resulting in the destruction of lymphoid tissue architecture and a depletion of lymphocytes both at the lymphoid tissues as well as in circulation [49]. Circulating virus disseminate to the whole lymphoid environment with extensive replication

seen in the gut-associated lymphoid tissues (GALT), spleen and brain, where persistent viral reservoirs are established [50-52], thus preventing HIV cure.

This allows progression to the chronic phase of infection (Fig. 1.4), marked by sustained viral replication in all secondary lymphoid tissues and general immune activation as well as cellular exhaustion (senescence) (reviewed in [53,54]). The immune dysfunction associated with this phase of infection affects all aspects of the immune system: a progressive and gradual depletion in circulating $CD4^+$ T lymphocytes (Fig. 1.4) is seen alongside the increase in viral load [47,55]. This is partially accounted for by the virus directly infecting $CD4^+$ T-cells but also by the infection causing uninfected bystander T-cells to be activated and eventually eradicated through apoptosis [56-58]. In addition, the pool of resting memory and naïve $CD4^+$ T-cells are sapped and this leads to T-cell homeostasis failure [59]. Virus specific $CD8^+$ T-cells develop in acute infection and persist throughout the advanced stages of HIV disease, even when overall T cell numbers wane [60]. The initially homogeneous virus starts evolving rapidly at the peak of the early HIV-specific $CD8^+$ T cell response as evidenced by the presence of mutations in $CD8^+$ T cell epitopes [30]. This causes a selection of viral variants that contribute to viral persistence [61].

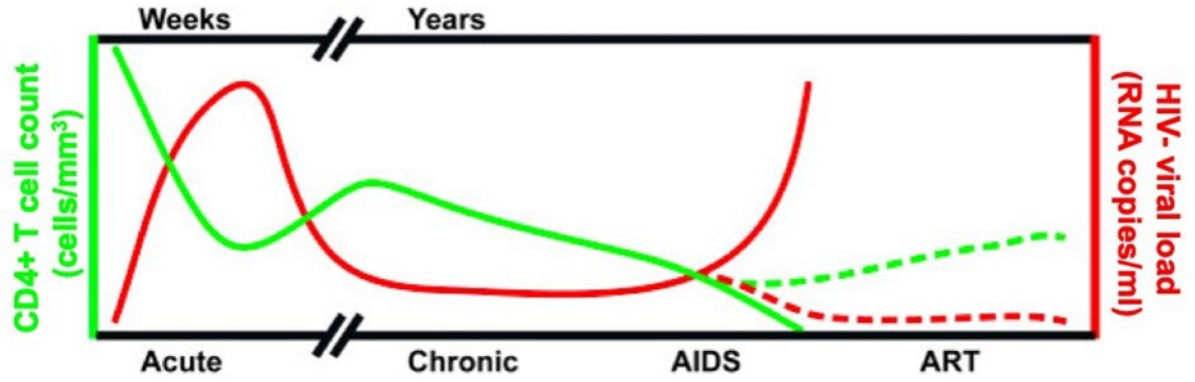

Figure 1.4. Stages of HIV-1 disease progression. Schematic representation of the amount of $CD4^+$ T-cells and HIV viral load during the three main stages of HIV-1 infection. Solid lines represent overall changes of $CD4^+$ T-cells (green) and HIV-1 viral load (red) over time. Dotted lines show the early effects of introducing highly active antiretroviral therapy (HAART). Figure modified from [62].

In addition to the immunological dysfunction of T lymphocytes, HIV also affects B cells and natural killer (NK) cells through a paradoxical immune activation, homeostatic responses and hyporesponsiveness [63-65]. HIV disease progression towards the chronic phase with ongoing viral replication and high levels of inflammatory cytokines provide a context in which NK cells develop impaired antiviral effector functions and dysfunctional engagement of antibody-dependent cell-mediated cytotoxicity (ADCC) [66]. In this context, the immune system shows an expansion of $CD56^{neg}$ NK cells with functional anergy, displaying an aberrant collection of activating and inhibitory receptors [67]. Certain KIR genes expressed on NK cells have a role in elite control of HIV [68,69] as well as lowering viral set-point thereby slowing HIV disease progression [70,71]. Furthermore, NK cells have the ability to modulate T and B cell responses by hampering the generation of long-lived memory B cells and eliminating T follicular helper cells (Tfh) which alter germinal centres formation and shape the induction of antibodies (via B cells cross-talk) [72]. Neutralizing antibodies generally arise approximately 12 weeks post-transmission but this immune response is rapidly overcome by the evolution of neutralization-sensitive viruses into neutralization-resistant variants [73,74].

Monocytes and macrophages also express CD4 receptors and are, therefore, susceptible to HIV infection [75,76]. Monocyte-derived dendritic cells (mDC) express a restriction factor called SAMHD1 which impairs HIV-1 reverse transcription by keeping intracellular dNTPs at low levels and through its ribonuclease activity [77,78]. In addition, mDCs express lectins such as dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and the sialic acid-binding immunoglobulin-type lectin 1 (SIGLEC-1) which facilitate capture of virions and their transmission to bystander $CD4^+$ T-cells [79,80]. As such, monocytes have opposing functions during HIV-1 disease pathogenesis, fighting the virus yet contributing to its dissemination. Latency, defined as irreversible viral DNA integration in the host genome in the absence of virion production, has been shown for monocytes and macrophages. The

infected monocytes/macrophages are believed to be the vehicles through which HIV enters the central nervous system (CNS) [81,82], as these activated leukocytes move from peripheral blood to become brain-resident or perivascular macrophages [83,84]. The non-infected monocytes/macrophages engulf HIV-1 virions, process these into peptides and present them on the monocytes/macrophages cell surfaces in association with major histocompatibility complex II (MHC-II) molecules. Infection of these cells would dramatically impair their ability to ingest foreign microbes [81,85] and present the antigens to T-cells, thereby contributing to the immune dysfunction seen in HIV infection and disease progression [86,87].

The final and most advanced stage of HIV-1 infection is the onset of Acquired Immunodeficiency Syndrome (AIDS). It is characterized by great impairment to the immune system evidenced by a marked reduction of naïve as well as memory $CD4^+$ T-cells and a corresponding increase in HIV viral load (Fig. 1.4). An AIDS diagnosis includes a CD4 T cell count decline below 200 cells/mm^3 and/or the presence of opportunistic infections (Table 1.1). Opportunistic infections arise at this stage due to the immunosuppression and range from bacterial, viral, fungal, and protozoan origins (Table 1.1). Some malignancies such as invasive cervical cancer (from HPV co-infection), lymphomas, and cancers associated with oncogenic herpesviruses are also common at this stage (Table 1.1). In the absence of treatment, patients die within two years of an AIDS diagnosis.

Table 1.1: Opportunistic infections associated with HIV-1 disease progression to AIDS

PATHOGEN	ASSOCIATED INFECTIONS	CLINICAL MANIFESTATION	REFS.
BACTERIAL			
Mycobacteria	*Mycobacterium avium* complex (MAC) *Mycobacterium tuberculosis* (TB)	Pulmonary disease; osteomyelitis, synovitis, and disseminated disease in the lymph nodes, and bone marrow, cough	[88,89] [90-95]
Salmonella	Salmonellosis	Intestinal tract infection; diarrhea, abdominal cramps, fever, thyroid abscess	[96,97]

Treponema pallidum	Syphilis	Infected sores, ulcers and blisters in the genital, mouth or rectum areas	[98,99]
Bartonella henselae	Bartonellosis	Macule/papule, lymphadenopathy, aches, malaise, fatigue, headache, fever	[100-102]
VIRAL			
Hepatitis	Hepatitis B virus (HBV)	Liver inflammation and cirrhosis, extreme fatigue, nausea, jaundice, lethargy, malaise	[103-105]
	Hepatitis C virus (HCV)	Liver inflammation and failure (dark urine, fever, fatigue, jaundice), hepatomegaly	[106-108]
Herpesvirus	Cytomegalovirus (CMV)/ human herpes virus 5 (HHV-5)	Mononucleosis, retinitis, blindness, diarrhea, hepatitis, petechiae, jaundice	[109-118]
	Epstein-Barr virus (EBV)/ human herpes virus 4 (HHV-4)	Mononucleosis, fatigue, fever, throat inflammation, Burkitt lymphoma	[119-121]
	Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2)	Oral herpes; cold sores, fever blisters on skin, lips, mouth, eyes. Genital herpes	[122-125]
	Varicella zoster virus (VZV)/ human herpes virus 3 (HHV-3)	Varicella (chickenpox), herpes zoster (shingles), fever, vesicular rash, malaise	[126-136]
Human papilloma virus (HPV)	HPV infection	Genital warts, cervical cancer, oral and upper respiratory lesions	[137-140]
FUNGAL			
Pneumocystis jiroveci	*Pneumocystis jiroveci* Pneumonia (PCP)	Fever, cough, difficulty breathing	[141,142]
Aspergillus spp.	Aspergillosis	Allergic sinusitis, invasive aspergillosis	[143-145]
Candida spp.	Mucocutaneous candidiasis Oropharyngeal candidiasis	Pseudomembranous (thrush), erythematous (atrophic), lesions, fatigue	[115,116,146-150]
Cryptococcus neoformans	Cryptococcal meningitis	Pneumonia-like symptoms, skin lesions, meningoencephalitis	[115,116,151-157]
Coccidioides spp.	Coccidioidomycosis (valley fever)	Respiratory infection, bone and joint infection, cough, meningitis	[117,158-164]
Histoplasma capsulatum	Histoplasmosis	PDH, prolonged fever, splenomegaly, headache, skin lesion, cough, chest pain	[115,116,165-171]
PROTOZOAL			
Cryptosporidium spp.	Cryptosporidiosis	Diarrhea, abdominal cramps, weight loss	[172-174]
Toxoplasma gondii	Toxoplasmosis	Swollen lymph nodes, CNS infection	[175-178]
Isospora belli	Isosporiasis	Chronic diarrhea, eosinophilia, flatulence	[179-187]
Microsporidia	Microsporidiosis	Diarrhea, abdominal pain, dehydration	[181,188-195]
Plasmodium	Malaria	Fever, headache, weakness, insomnia	[196,197]
Leishmania infantum	Leishmaniasis	Fever, hepatosplenomegaly, pancytopenia	[198-202]
Pneumocystis spp.	Pneumocystis pneumonia	Fever, dyspnea, tachypnea, cough, diarrhea	[117,203-211]
Trypanosoma cruzi	Chagas disease	Fever, myocarditis, meningoencephalitis	[212-214]

The introduction of Highly Active Antiretroviral Therapy (HAART; also known as combination antiretroviral therapy) has significantly reduced the mortality and morbidity

associated with HIV-1 infection [215,216]. HAART has been successful in reducing HIV-1 transmission and delaying onset of AIDS by decreasing the viral load and allowing reconstitution of $CD4^+$ T-cells [217,218]. Yet, even with plasma viremia successfully suppressed for years, the viral reservoirs in the latently infected, resting $CD4^+$ T-cells persist [219,220]. This implies that upon cessation of HAART, viral rebound is an inevitable eventuality [219,221]. As a drive towards eradication, novel therapeutic approaches aimed at targeting the viral reservoirs are under study (reviewed in [222,223]). The combination of immune-based therapies with HAART could provide promising and viable avenues to the eradication of HIV. However, these strategies would not be complete without investigating novel vaccine candidates as a vaccine offers the best solution to effectively curb the rate of new infections.

1.1.5 Host Immune Responses to HIV Infection

The host immune system is capable of mounting a potent response to HIV infection. Even in the absence of HAART, a very small percentage (less than 0.2%) of infected individuals have the ability to spontaneously control viral replication [224-226]. These individuals, called elite controllers or long-term non progressors, are the archetypal demonstration of how host genetics, host restriction factors, and other as yet unidentified factors, control HIV-1 replication and ultimately, disease progression [227,228]. As a first line of defence against invading pathogens, the innate immune system constitutes an early and rapid response during the acute phase of HIV-1 infection. The cells of the innate immune system recognize pathogens by pattern recognition receptors such as the Toll-like receptors (TLRs) that detect viral nucleic acids and proteins [229,230]. HIV-1 ssRNA encodes some TLR7 and TLR8 ligands [227,231] which, when stimulated, lead to the activation of dendritic cells (DCs) and the production of pro-inflammatory cytokines like interferons (IFN) and tumor necrosis factor α (TNF-α) [232-

235]. Innate cells such as dendritic cells (DCs) and monocytes produce these cytokines and shape the early immune response to acute HIV-1 infection.

Among the cells of the innate immune system, NK cells play a key role in antiviral control. It is understood that NK cell activation is triggered by the cytokine storm [233] (high amounts of pro-inflammatory cytokines, including IL-15 and IFNα) [236] that follows acute HIV-1 infection. The heterogeneity of NK cells arises from the differential receptors they express, characterized by the network of activating and inhibitory killer cell immunoglobulin-like receptors (KIRs) on their surface which associate with MHC class I (MHC-1 or HLA) ligands. Genome-wide association studies as well as epidemiological data have shown that specific MHC-I/HLA alleles, in combination with KIR genotypes, play a role in modulating HIV-1 disease progression [237,238]. Particularly, expression of the KIR3DS1 activating receptor, in combination with HLA class I of the Bw4 serological group, proved to be protective against HIV disease progression compared to having the HLA class I molecule alone [239]. As such, the HIV elite controllers mentioned above have an overrepresentation of HLA-Bw4, specifically of the B*57 alleles [70].

In addition to the effect of the innate immune cells, there are germline encoded host restriction factors that have demonstrated restriction of HIV-1 infection. These include the constitutively expressed apolipoprotein B mRNA editing calatytic polypeptide-like 3G (APOBEC3G), the tripartite motif-containing 5 (TRIM5) and tetherin proteins (Fig. 1.5). APOBEC3G specifically acts against HIV-1 by incorporating into the virus through its interaction with Gag, deaminating the viral cDNA and making it vulnerable to nuclease degradation [240-243]. However, the virus overcomes this host defense mechanism by causing the viral infectivity factor (Vif) protein to mediate proteosomal degradation of APOBEC3G [244,245] (Fig. 1.5). TRIM5, specifically the TRIM5α variant, acts in the early stage of the viral replication life cycle by recognizing the motifs in the viral capsid lattice, interfering with the normal uncoating of the

capsid (Fig. 1.5), preventing reverse transcription and, therefore, protecting the cell from productive infection [246]. HIV-1 counterstrategy in overcoming this host defence mechanism is the introduction of variants that have evolved Gag mutations (such as the H87Q mutant) thus containing capsid proteins that are resistant to the TRIM5α protein inhibition [247]. Finally, tetherin (also known as bone marrow stromal antigen 2; BST2) tethers fully formed enveloped HIV-1 particles on the plasma membrane of the infected cells crippling the release of the virus and inhibiting their spread [248,249] (Fig. 1.5). The virus counteracts the action of tetherin through its viral protein U (Vpu) by acting as a BST-2 antagonist [250] (Fig. 1.5).

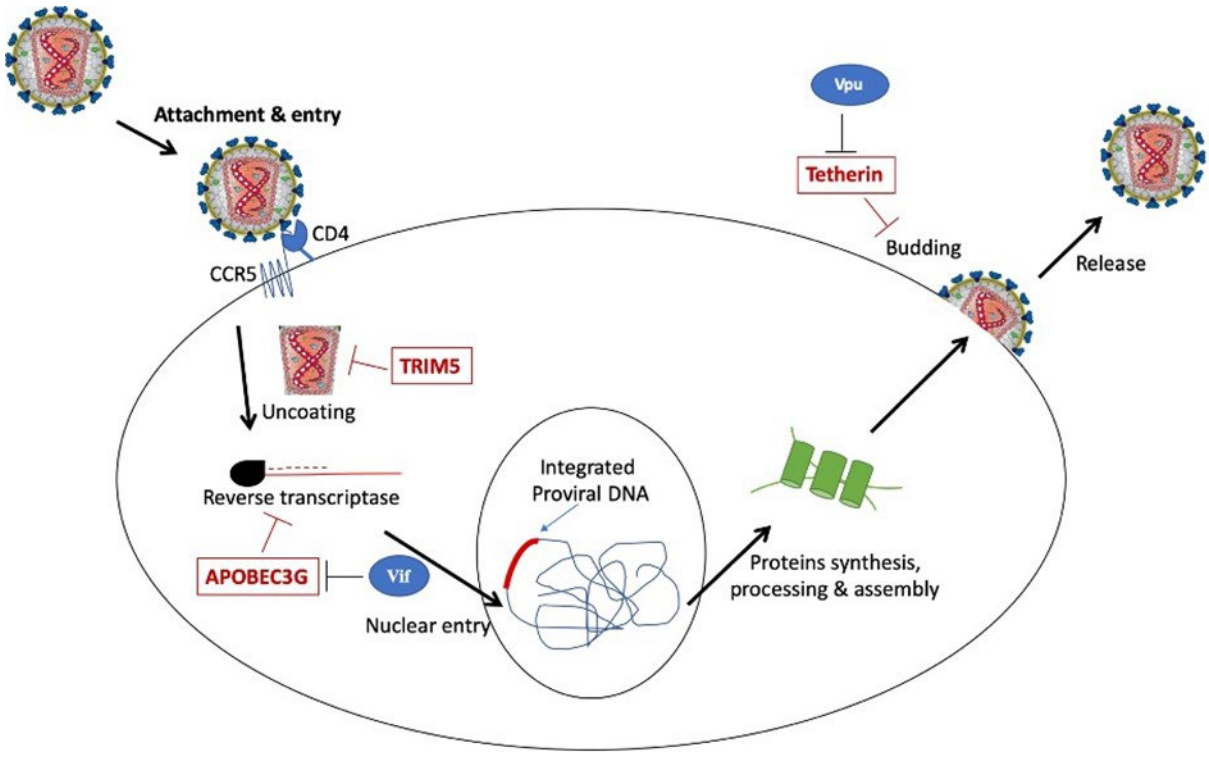

Figure 1.5. Schematic representation of restriction factors in HIV-1 replication cycle. Constitutively expressed antiviral factors APOBEC3G, TRIM5, and Tetherin (all boxed in red) and their link to restriction of HIV-1 infection. The packaged APOBEC3G causes hypermutations in reverse transcription; TRIM5 causes uncoating of the capsid in a manner not conducive to reverse transcription; and Tetherin prevents successful viral budding. Viral agonists are depicted as blue circles with the name of the protein (Vif or Vpu). Drawing of virion from Fenstermacher, Katherine (2013) https://doi.org/10.6084/m9.figshare.862069.v2

Evidently, the innate immune response alone is insufficient to inhibit HIV pathogenesis. Therefore, priming of the adaptive immune response follows to supplement the response with antiviral T-cells and the development of HIV-specific antibodies. The humoral response to HIV is complex, partly because of the constantly changing nature of B cell epitopes due to the virus mutating, and partly as a reflection of the waning numbers of $CD4^+$ T-cells and how their depletion indirectly affects B cell maturation. The HIV-1 envelope glycoprotein (Env) is the only target for neutralizing antibodies (Abs). Functional Ab responses to Env can be organized into three categories: Non-neutralizing Ab responses; neutralizing Ab responses; and broadly-neutralizing Ab responses. Non-neutralizing Abs form the initial antibody response and come from short-lived plasmablasts arising prior to GC formation [251,252]. HIV-specific, virion-binding IgM Abs are detected within the first few weeks of infection, followed by the enrichment of IgG3 antibodies that have undergone GC-independent class switching and no affinity maturation [251], and finally anti-gp41 antibodies. These early antibodies bind to the Env even though they do not neutralize the virus, but have antiviral activity such as phagocytic activity mediated through the Ab Fc fragment. The majority of these antibodies have their epitopes on the gp41 portion of Env [253].

In the chronic phase of HIV-1 infection the Ab isotype is predominantly IgG, in contrast to the IgA Abs that predominate in early infection despite having a short half-life [254,255]. These IgG antibodies are neutralizing Abs that are specific to the infecting strain or 'autologous' virus [73,255-260]. These Abs generally have their epitopes on gp120 and they persist throughout the course of infection [261,262]. It is against this strain-specific response that the virus gains Env mutations in order to escape the host antibody recognition [73]. A fraction of HIV-infected individuals (10-30%) develop broadly neutralizing Abs that are able to neutralize a broad spectrum of diverse HIV-1 viral strains or 'heterologous' isolates [263,264]. These Abs generally take a few years to evolve the breadth and potency of their response [265-267], they

have epitopes on conserved regions of the Env trimer [268], and they have distinct structural features such as some degree of somatic hypermutations, polyreactivity, and an unusually long heavy-chain complementarity-determining region 3 (HCDR3) [269]. The unusual features of these Abs account for some of the challenges faced in trying to elicit these type of Abs in the context of vaccination [270-272]. In addition, the immune pressure exerted on the virus by these antibodies leads to the accumulation of escape mutants which become neutralization-resistant viruses.

Cellular adaptive immunity has a crucial role in HIV-1 disease progression. T follicular helper (T_{FH}) cells assist antigen-exposed follicular B cells to stimulate memory B cells, B cell affinity maturation and the generation of virus-specific antibodies. T-cells also contain T-cell receptors (TCR) on their surface which recognize antigens displayed on antigen presenting cells (APC) [273]. Major histocompatibility complex class I (MHC I) and II (MHC II) molecules present T-cell epitopes which are recognized by $CD8^+$ and $CD4^+$ T-cells, respectively. Naïve $CD8^+$ T-cells activation requires the recognition of an 8-10 amino acid antigen by the TCR, a costimulatory signal expression in the form of CD80/CD86 binding of a CD28 molecule (on the $CD8^+$ T-cell) to the APC, and a pathogen induced signalling molecule such as IL-12 or type I IFN [274,275]. Following epitope recognition, $CD8^+$ T-cells become cytotoxic T lymphocytes. HIV has evolved numerous strategies to escape $CD8^+$ T-cell responses by introducing mutations in regions around CTL epitopes which impact recognition of these epitopes [276-278]. In addition, HIV also disrupts proper $CD8^+$ T-cell signalling by down-regulating MHC I expression via its *nef, tat, and vpu* gene products [279-281], by modifying cytokine production [282-284] and by altering engagement of cellular receptors in infected cells [285-287]. Improper TCR stimulation results in abnormal functioning of the $CD8^+$ T-cells. In addition to their role in initiating $CD8^+$ T-cell responses, $CD4^+$ T-cell subsets release IL-17 (Th17 $CD4^+$ T-cells) and IL-22 (Th22 $CD4^+$ T-cells) which play a role in the mucosal host

defense against bacteria by reducing microbial translocation and resulting in mucosal damage [288-290].

1.2 HIV-1 Vaccines

1.2.1 Rationale for Preventative Vaccines

As mentioned above, HAART has been effective in controlling viremia in HIV-1 infected individuals [55,291-293]. These drugs successfully target various stages of the viral replication cycle. While very effective against circulating virus, HAART cannot eliminate latent viral reservoirs which results in viral rebound upon discontinuation of the drugs [294]. Nonetheless, there has been large improvements to HAART such as cabotegravir, a long-acting injectable antiretroviral drug (integrase inhibitor) used to prevent HIV-1 infection, where an injection every two months of cabotegravir is 69% more effective than the current pre-exposure prophylaxis drug (Truvada) which is orally administered daily [295]. In December 2021, Cabotegravir was FDA approved for use in HIV-1 pre-exposure prophylaxis (PrEP) (UNAIDS 2021).

Various preventative approaches, other than HAART, are being explored to treat HIV-1 infection, such as the use of antibodies to complement small-molecule based therapies. Antibodies are appealing as therapeutic agents because they can directly neutralize the virus through their antigen binding fragment (Fab) but they can also act in conjunction with diverse effector cells to clear the virus through their Fc domain [296,297]. One such effector function is the antibody-dependent cellular cytotoxicity (ADCC) through which NK cells, activated macrophages, and monocytes can be engaged to kill infected target cells [298-300]. However, antibodies have their drawbacks; their short half-life in humans (12-23 days) would require continuous administration (every 2-4 months) in order to be effective [301]. Antibody therapy

is continuously being improved by using more potent antibodies, bi- and tri-specific antibodies as well as trying different delivery routes such as infusion or gene therapy with adeno-associated virus [302-308]. While antibody therapy has shown sustained viremic suppression with no viral escape (no emergence of resistant viral variants) in non-human primate studies [309,310], clinical trial results have yet to prove similar effects in HIV-infected individuals. In addition, further research is needed to overcome the high cost of antibody therapy and the challenge of having to continuously administer the therapy. As such, an efficacious preventative HIV-1 vaccine is the most promising strategy in controlling the HIV pandemic since an effective vaccine should provide sterilizing immunity (complete prevention of HIV infection).

1.2.2 Challenges to Vaccine Design

Vaccines work by priming the immune system to generate a rapid recall response with lymphocytes attacking and eliminating the pathogen upon exposure, thus protecting the individual from becoming infected. Due to the challenges in developing an HIV-1 vaccine, it has been suggested that first generation HIV-1 vaccines may provide only partial protection. While not ideal, such a vaccine would assist in curbing viral replication thereby delaying the time between infection and progression to AIDS [311]. Based on modelling studies, an HIV vaccine that offers partial protection would still be beneficial to public health as a lower viral load results in a lower risk of transmission, and prolonged survival. The caveat with a partially efficacious vaccine is the possibility of viral rebound through escape mutations as has been seen in pre-clinical trials with non-human primates [312,313]. Therefore, the development of a prophylactic vaccine which prevents infection remains the penultimate strategy towards restricting the pandemic.

An effective vaccine works by stimulating protective immunity in a similar manner to naturally acquired immunity. However, the same challenges that cause the immune response to fail in

clearing an HIV infection have also caused unprecedented obstacles in the development of a safe and effective vaccine against HIV. Despite the lack of clear immune correlates of protection and the ineffectiveness of classical vaccine approaches against HIV-1, extensive research has gone into various types of candidate vaccines, discussed in this chapter. HIV-1 vaccine development has been difficult for multiple reasons, some of the major ones are highlighted in Table 1.2. These include the fact that HIV-1 infection of $CD4^+$ T-cells, memory T-cells and antigen presenting cells seriously cripples the immune system by depleting these immune cells, altering their antigen presentation, and by allowing viral persistence through latently infected cells [314,315]. In addition, the HIV-1 virion has extensive genetic hypervariability within the host, between hosts, and at a global level, which is a result of rapid replication (10^{10} new virions a day) [316], mutations, and recombination brought about by its error-prone reverse transcriptase enzyme which has no proof-reading ability. It is estimated that the viral diversity in a person infected with HIV-1 for only 6 months equates to the annual global diversity of circulating strains of influenza A [317,318]. This genetic heterogeneity and high antigenic diversity allows the virus to evade CD8+ cytotoxic T cell (CTL) immune responses [319]. Due to the subtype specificity of CTL and some HIV neutralizing antibodies, the sequence variation of HIV-1 also poses a challenge in terms of vaccine development. In addition, the non-covalently bound trimeric structure of the Env masks key epitopes and the substantial glycosylation covering the surface of the virus forms a glycan shield making access by neutralizing antibodies challenging. These are some of the obvious obstacles to the development of an HIV-1 vaccine that targets the Env.

Table 1.2: Challenges to the development of an HIV vaccine

Properties of the virus	Significance
Infects and destroys $CD4^+$ T-cells	Dysfunction of $CD8^+$- and B-cells immune response
Infects antigen presenting cells	Alters the presentation of HIV antigens
Infects resting memory T-cells	Sequesters the virus in latent reservoirs

Has extensive subtype and sequence hypervariability	Escapes from cellular and humoral immunity
Has complex evolving quasispecies within the host	Introduces variants that escape T cell recognition
Has high rate of replication combined with genetic mutations	Rapidly introduces variants with selective advantage
Has a highly glycosylated envelope (glycan shield)	Shields protein epitopes from antibody recognition
Shows conformational masking of conserved sites	Exposes extraneous immunodominant epitopes
Integrates into the host genome	Establishes permanent infection

The current consensus is that engagement of all arms of the immune system will be required to achieve HIV-1 sterilizing protection. Humoral immune responses have proven critical in restricting viral entry into target cells, in controlling some of the viral replication, and in providing selection pressure through neutralizing antibodies [73]. Cellular immune responses have shown evidence of protection in early vaccine studies in non-human primates [319,320], using replication competent as well as CMV-based vectors [321].

Another obstacle to HIV vaccine development is the lack of an adequate animal model. While non-human primates have been used in HIV vaccine development and have been beneficial as predictors of the Merck Ad5 vaccine failure [322], they have shortcomings when it comes to addressing the variability of the HIV-1 Env since chimeric S/HIVs (Simian/HIV Env) are limited. As a result, the efficacy of an HIV vaccine can only be assessed in human clinical trials. The afore-mentioned hindrances to vaccine development notwithstanding, research towards an effective HIV vaccine continues as a preventative vaccine would provide one of the most feasible means of halting the spread of HIV.

1.2.3 Vaccine Design Strategies

Since the protective mechanism of an HIV-1 vaccine are unknown, researchers have used both classical and innovative methods to try and develop comprehensive vaccines which engage various aspects of the immune response (Fig. 1.6). Some conventional and commercially available vaccines such as those for rabies [323], hepatitis A [324], yellow fever [325], Salk polio [326], Varicella Zoster Virus (VZV) [327], and influenza [328], as well as the trivalent

Mumps/Measles/Rubella (MMR) vaccine [329] use whole inactivated or live-attenuated viruses. Inactivated SIV has been tested in non-human primates (NHPs) and was shown to effectively protect monkeys challenged with the same SIV included in the vaccine, albeit the response was not broad nor potent and was combined with disappointing CTL responses (i.e., no protein synthesis in cells) [330]. However, an HIV-1 live-attenuated vaccine would pose safety concerns so it has not been evaluated in humans, despite the SIV equivalent having been shown to induce cellular and humoral immunity in NHPs [331]. The main concern with whole inactivated and live-attenuated viruses is safety- the potential risk that the virus would revert to a non-attenuated, pathogenic strain due to the high mutation rate of the viral reverse transcriptase [332]. While these vaccine modalities are not viable in humans, their use in NHPs have informed on the mechanisms underlying their effectiveness which is beneficial to the HIV vaccine design field.

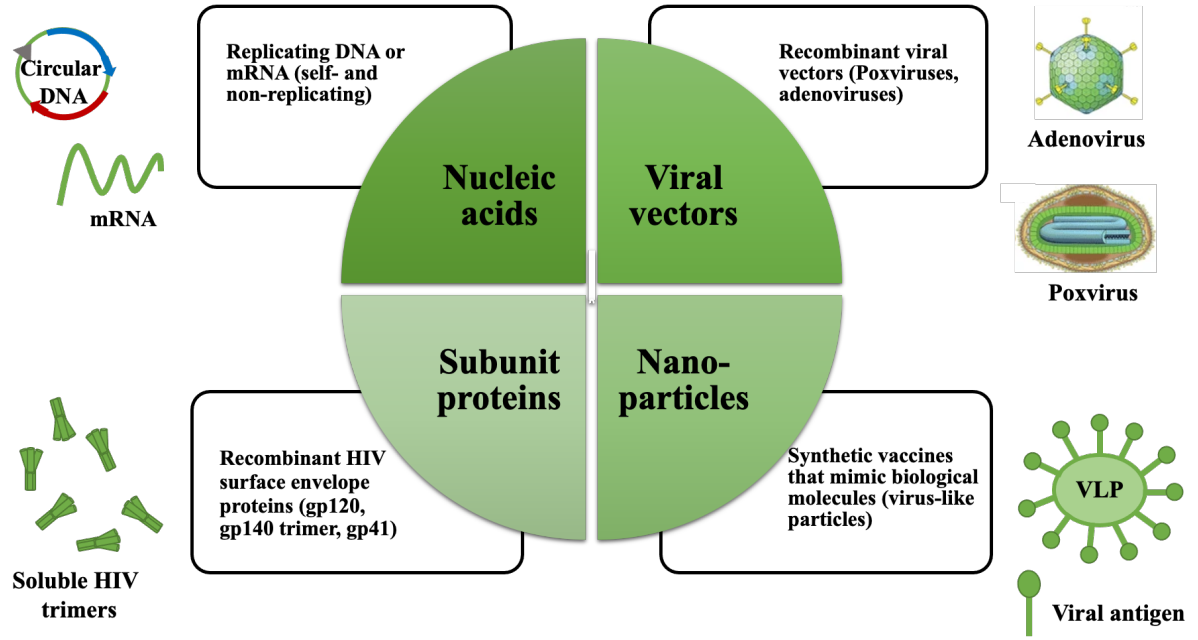

Figure 1.6. HIV-1 vaccine and immunogen design strategies. The various strategies employed in designing HIV vaccines are indicated, grouped based on main immunogen components, and include: Replicating DNA or self- and non-replicating mRNA vaccines; viral vectors used alone or as boosters to DNA plasmids; HIV-1 subunit proteins or peptides; and synthetic vaccines that include viral like particles, viral capsids, cargo proteins, and other self-assembling proteins. Adenovirus and poxvirus drawings reprinted from [333].

DNA vaccines (Fig. 1.6) also have the ability to induce both cellular and humoral immune responses but they carry additional advantages, such as favourable thermostability, affordable cost and ease of manufacture, and no anti-vector response which permits repeated administration. One drawback to DNA vaccines is their poor immunogenicity when used alone [334]. As such, they have been tested in prime-boost vaccination regimens. One such strategy involved the HIV-DNA prime and HIV-Modified Vaccinia Ankara (MVA) boost which elicited strong cellular and antibody-mediated responses in a phase I randomized trial [335]. In addition to potent heterologous prime-boost strategies, various approaches aimed at improving the immune responses from DNA vaccines have included better DNA delivery systems and the co-administration of molecular adjuvants [336-338].

Viral vectors have also been traditionally used as a preventative T cell-based vaccine strategy to deliver HIV vaccine antigens (Fig. 1.6). Their appeal is in their ability to induce cell-mediated responses as well as significant antibody responses. As mentioned above, viral vectors have been used in combination with DNA vaccines because T-cells can specifically target the vector. Therefore, when the viral vector is used as a heterologous boost, the immune response to the viral vector is not boosted but the antigen-specific T-cell population is [339-341]. Viral vectors can be replication-competent or non-replicating. Of the non-replicating kind are adenoviruses, adeno-associated viruses, recombinant MVA vectors, alphaviruses, as well as canarypox (ALVAC) vectors. Adenovirus vectors are five- to ten-fold stronger in inducing T cell responses and are the most promising vaccine vectors for HIV [342-344]. The most popular of the adenovirus vectors is the human Adenovirus serotype 5 (Ad5) which was used, after DNA priming, as an immunization boost in the STEP HIV vaccine study [345,346]. This regimen elicited strong $CD8^+$ T cell responses in the study vaccinees but pre-existing cellular immunity and the presence of neutralizing antibodies to the vector considerably compromised clinical efficacy [347-350]. The pre-existing immunity associated with Ad5 vectors limits

transgene expression and potentially exacerbates HIV acquisition, and as such, newer adenovirus vectors such as Ad26 and Ad35 that have lower pre-existing immunity are under study [347,351].

The interest in recombinant, replication-competent or live delivery vectors for HIV vaccine work came about with the unprecedented success of the SIVmac239 Δnef vaccine used in rhesus macaques, which was shown to elicit broadly effective and durable immunity [352,353]. Replication-competent vectors also include the Adenovirus family, particularly Ad4, Ad5, and Ad6 viral vectors [354-356], the recombinant human CMV (rhCMV) [357], the NYVAC vector [358], the recombinant VSV [359,360], the Sendai virus [361] and the herpes simplex virus [358]. Another advantage to the use of replication-competent viruses, particularly those that naturally infect cells at the mucosal sites, is the targeted mucosal immunity and long-term persistence of vaccine-induced responsive cells at the transmission 'hot-spots' areas such as the genito-rectal mucosae [362].

Nanoparticle-based vaccines (Fig. 1.6) offer a flexible approach to vaccine design with many benefits: they are engineered to protect the antigen from degrading prematurely, they induce sustained immunity against infections, and they facilitate intracellular uptakes. Various nanoparticle platforms, such as virus-like particles (VLPs), liposomes, and self-assembling polypeptide nanoparticles, have been recently used to display HIV-1 antigens [363-370]. VLPs are carriers that display the natural structure and intrinsic immunogenic properties of viruses. These spherical nanoparticles range between 20-200 nm in diameter [371] and are generally composed of purified viral protein devoid of the virus genetic material [372]. The native HIV-1 Env is the only target for neutralizing antibodies [373,374] and is therefore the main antigen for HIV-1 VLPs. HIV-1 Gag polyproteins as well as combinations of Gag-Pol and Env have also been used as components of HIV-1 VLPs where the Gag-VLP elicited the activation and maturation of dendritic cells as well as the activation of pro-inflammatory cytokines leading to

an NK immune response [375]. A Gag-Pol-Nef VLP was tested in conjunction with an Env trimer-expressing VLP in mice and the vaccine candidate was shown to generate high levels of Gag-specific $CD8^+$ T-cell responses in addition to polyfunctional Env-specific CD4 responses. This showed that combinations of VLPs expressing different antigens provide a balanced $CD4^+$ and $CD8^+$ T cell responses and an effective induction of humoral and cellular immune responses [376-378].

Liposomes are spherical lipid-based vesicles with an aqueous cavity. They have the advantage of antigen presentation on the surface or encapsulated within the cavity and thus offer an appealing and versatile platform for the presentation of different HIV-1 immunogens. Liposomes were first used in HIV-1 vaccine delivery 26 years ago [379] and they have since been used to present a high density of Env trimers [380] as well as short epitope peptides [367,381]. HIV-1 Env vaccines in liposomal formulation have been tested in mice and have been shown to induce better B cell activation [380,382]. Naturally occurring, non-viral, self-assembling protein scaffolds have also been used to deliver vaccine antigens. These nano-scale protein scaffolds (nanocages) include encapsuling and chaperones, among others [383-385].

The naturally occurring protein assemblies have inspired synthetic, *de novo* designed nanocages that also depend on protein oligomerization motifs with highly ordered architectures and improved functional versatility. Man-made mimics of natural microbe architecture, such as the self-assembling 60-subunit and 120-subunit nanostructures [386-389] and the self-assembling 24-subunit ferritin nanocage have been used to present HIV-1 trimers in mice and rabbit immunization regimens [366,369,390]. While the use of ferritin nanoparticles generally yielded higher HIV-1 trimer-binding antibodies titers, there was no improvement in rabbit sera neutralization titers against the Env used in the scaffold in one study [390,391] and significant improvement in another study with a different Env [369]. The contradicting results could be purely Env dependent or could be due to the combined restrictions of the number of antigens

(eight Env trimers) that can be displayed on the ferritin octahedrons which might limit their immunogenicity [392]. Unlike liposomes, a shortcoming of self-assembling protein immunogens is they can induce antibody responses against the scaffold [393,394].

Subunit HIV-1 vaccine candidates were the first to be used in human clinical trials. They were composed of monomeric gp120 and gp160 surface Env glycoproteins and provided proof-of-principle for protection from HIV-1 infection [395,396]. The Env protein is expressed as gp160 and gets proteolytically cleaved into the gp120 and gp41 subunits by the cellular furin protease [397]. One trial which included HIV-1 subunit proteins was VAX004 where a recombinant clade B antigen was used in a cohort of men who have sex with men as well as women at high risk of HIV-1 transmission [398]. The results displayed neutralizing antibody responses against the homologous strain used in the vaccine, showing immunogenicity but not the type of protection required against genetically diverse HIV-1 strains [399]. The results from such studies led to research into improved Env structures that are better representations of the HIV-1 functional spike. A number of antigenic structures resulted from these efforts and included structurally polymorphic gp120, Env-based epitope scaffolds, and near native trimers. Of note, the SOSIP.664 trimer design was iteratively constructed over the years and, to date, is considered the best soluble, recombinant representation of the HIV-1 trimeric spike [400] (Fig. 1.7). A SOSIP.664 trimer consists of a disulphide bond (SOS) which covalently-links gp120 and gp41 subunits [401], an I559P (IP) mutation designed to stabilize the prefusion conformation of Env [402], an improved cleavage site [403], and is truncated at position 664 which prevents aggregation [404] (Fig. 1.7).

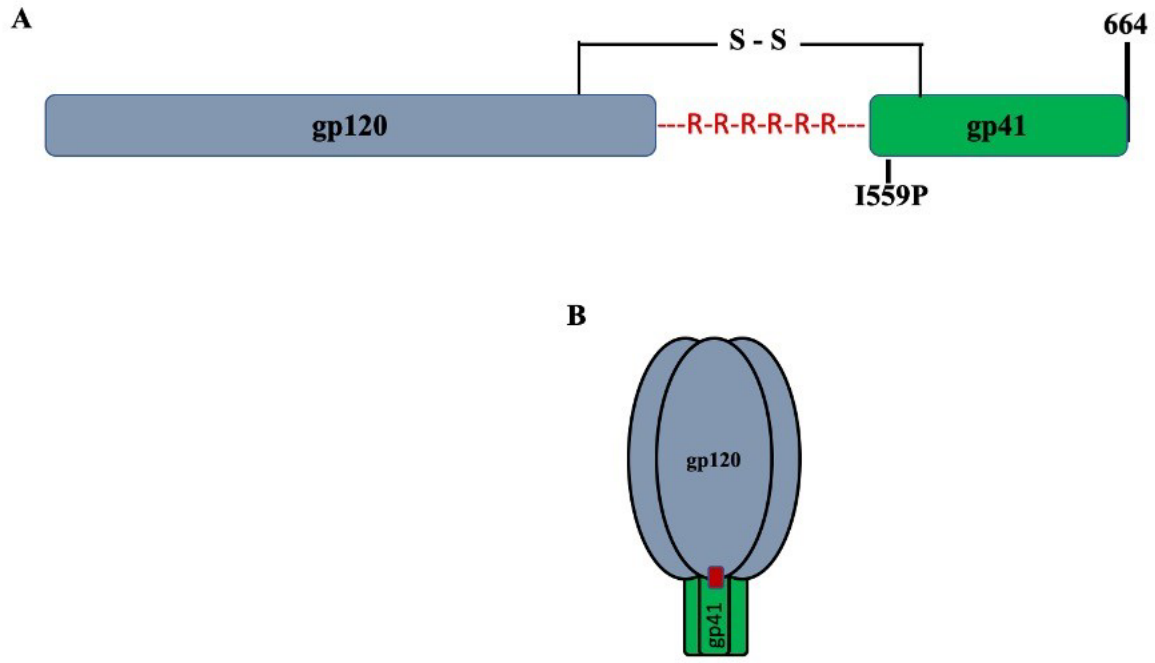

Figure 1.7. Structure of the SOSIP.664 gp140 trimer. (A) Linear representation of gp120 and gp41 with disulphide link (S-S), the six arginine furin cleavage site, the isoleucine to proline substitution at position 559, and the truncation at position 664 are shown. (B) The assembled Env trimeric spike with one gp120 and one gp41 heterodimers labelled. Furin cleavage site is shown in red.

These SOSIP.664 trimers were the first immunogens capable of inducing moderate to strong neutralizing antibody responses in preclinical trials against a tier-2 pseudovirus [405], a virus with a neutralization phenotype similar to clinical isolates. However, as was the case for all previously-mentioned vaccine candidates, the responses failed in terms of coverage against heterologous Tier-2 pseudoviruses. As will be discussed in chapter 1.3.1, initial modifications to SOSIP trimers came in the form of stabilizing mutations aimed at improving their thermostability and antigenic profile with some improvement to the immunogenicity of the trimers. Additional SOSIP trimers variants were introduced with germline targeting mutations and modified N-linked glycosylation sites resulting in moderate improvements to the immunogenicity.

A significant breakthrough in Env immunogenicity was, however, seen when various Env conformations were complexed to two-domain CD4 [406] but this modification has not been attempted with SOSIP trimers prior to the work described herein.

1.2.4 HIV-1 Vaccine Clinical Trials

There has been a number of HIV-1 vaccine trials conducted to date in humans with results ranging from ineffective to increased risk of acquisition to a moderate but promising protection. The most relevant clinical trials will be reviewed here. Two HIV-1 vaccine clinical trials (VaxGen) were the first Phase III HIV-1 trials initiated in a cohort composed of men who have sex with men, intravenous drug users, and women at high risk of contracting HIV-1 [398,407]. Vaxgen efficacy trials tested the hypothesis that neutralizing antibodies against the gp120 Env could be induced and would protect against HIV-1 infection. The rationale for these trials came from preclinical, non-human primate studies that showed efficient prevention of infection in the primates using a recombinant HIV-1 gp120 protein [408-410]. However, the challenges in terms of immunogen design combined with the failure of these studies and others like them to confer protection [407,411] redirected the vaccine trial efforts towards those that would potentially induce T-cell immunity.

The STEP and Phambili companion studies were Phase IIb trials involving high risk individuals who received an adenovirus type 5 (Ad5) vector encoding HIV-1 proteins (Gag, Pol and Nef) with no Env component. These efficacy trials were designed for induction of a $CD8^+$ T-cell immune response. The studies were terminated before the anticipated end when preliminary results revealed an increased rate of HIV-1 infection among the vaccine recipients for those that were Ad5 seropositive. *Post hoc* analysis revealed that anti-adenovirus pre-existing immunity was the cause of the increased HIV-1 infection [345,412]. In these recipients, vaccination promoted Ad5-specific memory T-cells activation and their trafficking to mucosal sites resulting in a high accessibility of $CD4^+$ T-cells for HIV-1 to infect [413]. As such, live

vectors have come under scrutiny to ensure their suitability for the general population and to limit their use to boost immunizations, thereby reducing the risks of antivector immunity [364].

RV144 was a Phase III clinical trial, the first to demonstrate 31.2% efficacy in vaccine mediated immune protection [414]. It consisted of a recombinant canarypox vector prime expressing HIV-1 Gag, Pol, and Env proteins followed by a recombinant gp120 bivalent Env subunit boost based on clades B and E [414]. This vaccine was intended to induce both cellular and humoral immune responses. The vaccine showed efficacy in its ability to reduce HIV infection rates but exhibited no impact on HIV-1 disease progression in those vaccinees who became infected (no significant difference in $CD4^+$ T-cell count), except for a perceived reduction in viral load in the genital mucosa of the vaccinees, which may have lowered their ability to transmit HIV-1 [415]. *Post hoc* analyses revealed that binding, but non-neutralizing plasma IgG antibodies with epitopes in the variable 1 and 2 (V1V2) loop of the HIV-1 Env was a positive correlate of protection [254,416,417]. At the same time, plasma IgA antibodies correlated with increased infection (i.e., lowered vaccine efficacy) [416]. Of interest, the *post hoc* analysis also showed that after the first year, vaccine efficacy was higher (60%) which translates into protection that was not sustained during the three-year duration of the study [414,416,418].

In an effort to replicate and possibly improve on the RV144 results, a phase II/III clinical trial, named HVTN702, was started in South Africa with a vaccine based on the RV144 regimen but tailored to clade C – the most prevalent in the sub-Saharan African population [419]. It also consisted of a canarypox vector and a bivalent gp120 protein subunit, both modified and combined with the MF59 adjuvant used to enhance the immune response [419]. Additionally, this vaccine regimen had two extra booster shots at 12-months and 18-months, included in an effort to extend the early protective effects seen in RV144. At the scheduled interim review, it

was found that this vaccine regimen did not prevent HIV-1 acquisition, and as such, the trial was discontinued due to lack of efficacy [419].

While the HVTN702 results were disappointing, they highlighted the need for an effective HIV-1 vaccine and for research efforts to continue in order to find innovative ways to curb HIV-1 transmission. Of the remaining HIV-1 vaccine trials, two large-scale studies are worth mentioning: the Mosaico trial and the Imbokodo trial [420]. Both trials are exploring novel mosaic vaccines. The Imbokodo trial was terminated in August 2021 due to lack of efficacy (no protection conferred), however, the vaccine was found to be safe and did not incur any serious adverse side effects (NIH.gov). A new wave of antibody-based therapies are also being explored: the Antibody Mediated Protection (AMP) programme has two ongoing safety and efficacy trials, namely HVTN 703 and HVTN 704, where passively administered broadly neutralizing antibodies (bNAb) are being tested as a preventative method [420]. The results of the AMP trials reinforce the "proof-of-concept" that antibodies can prevent infection, however, the single antibody (VRCO1) used could not offer the desired protection [295].

1.3 HIV-1 Envelope Glycoprotein Trimer-based Immunogens

1.3.1 Stabilized recombinant HIV-1 envelope glycoprotein trimers

The understanding that the functional Env trimer is the only target for neutralizing antibodies spurred the production of recombinant Env in soluble form. In a manner typical of type I fusion machinery, the functional mature form of the HIV-1 Env requires proteolytic cleavage and folding. Env arises from an immature, uncleaved gp160 precursor which undergoes oligomerization, glycosylation and cleavage into mature and non-covalently associated gp120 and gp41 subunits [421-423]. An additional, truncated Env subunit is found in natural infection and can be expressed from mammalian cells – the gp140 Env trimer refers to a gp160

ectodomain with a deletion of the cytoplasmic and transmembrane gp41 domains [424,425]. Expressing the three noncovalently linked gp120-gp41 heterodimers outside of the context of a membrane proved challenging as the resultant trimers are structurally unstable in solution. Prior to the development of SOSIP.664 trimers, elimination of the gp120-gp41 cleavage site improved the stability of the first generation Env trimers (gp140 constructs), some of which contained additional trimerization domains [426,427]. The immunogenicity of these trimers showed some improvement over the use of soluble gp120 [428]; however, the trimers consistently failed to induce Tier-2 neutralizing antibodies or bNAbs [405,429,430]. We now understand through electron microscopic data and biochemical analyses that those first generation trimers were not the best antigenic mimics of the functional HIV-1 trimer [429,431,432].

The rise in isolation and characterization of bNAbs from HIV-infected individuals has also informed our understanding of their interaction with Env trimers (Fig. 1.8). Of note, many bNAb epitopes rely on the correct folding of the trimer, from the trimer apex to the gp120-gp41 fusion peptide epitopes which are only properly presented in the quaternary structure of the trimer, with the different protomers in close proximity (Fig. 1.8) [433].

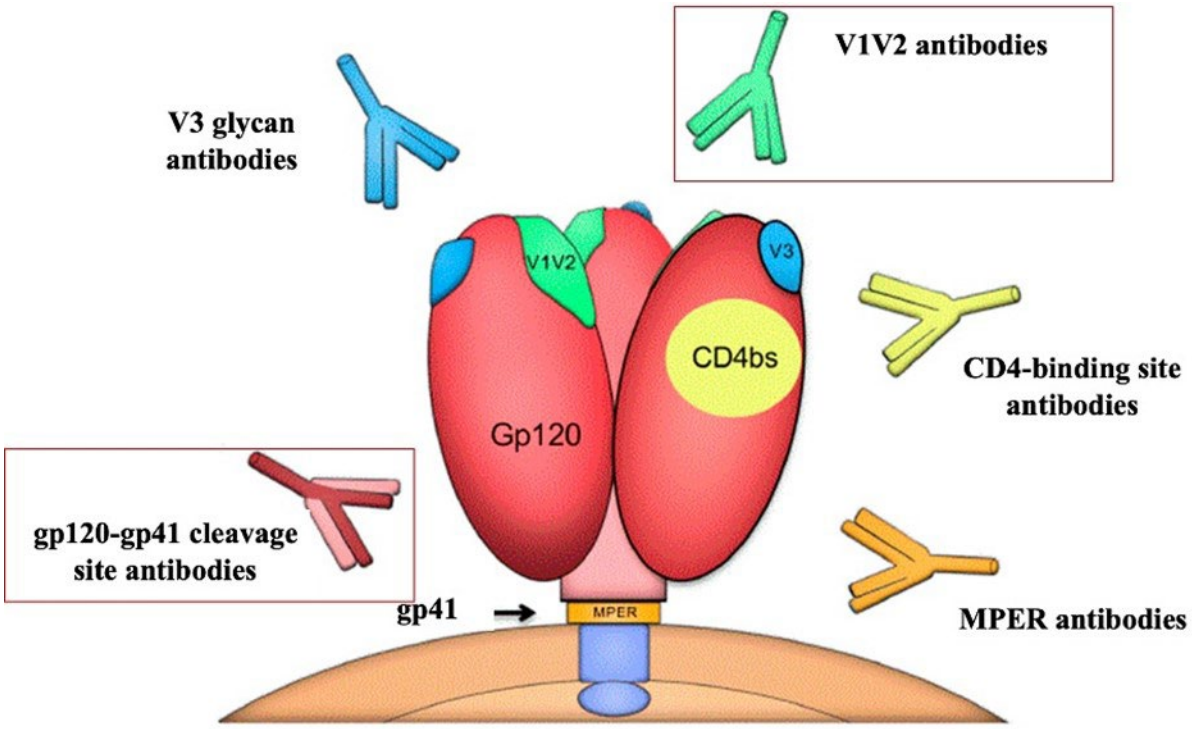

Figure 1.8. Major targets of bNAbs on the HIV-1 trimeric spike. HIV-1 Env trimer with the three gp120 shown in red and gp41 embedded in the membrane. Main antibody targets are also shown; namely, the CD4-binding site (CD4bs) in yellow; the V1V2 trimer apex site in green; the V3 glycan in blue; the membrane proximal external region (MPER) in orange; and the gp120-gp41 epitope which is made up of residues in gp120 and gp41 shown in red and pink, respectively. Antibodies targeting the various epitopes are shown in the same colour as their respective targets. Antibodies dependent on the quaternary conformation of the trimer are boxed in red. For simplicity, the extensive glycosylation on the trimer spike is not shown. Figure modified from Stefic *et al.* [434]

Following the failure of cleavage-site removal to provide trimer immunogens with the desirable antigenicity, different stabilization methods were attempted which included the introduction of an intermolecular disulphide bond (SOS gp140), the substitution of an isoleucine to a proline (I559P or IP) to improve trimerization and the truncation at residue 664 (Fig. 1.7) [435-437]. This gave rise to the aforementioned SOSIP.664 gp140 trimer, which was assessed with the BG505 sequence and confirmed by electron microscopy to be "native-like" and to present the correct bNAb epitopes [404,438,439]. The SOSIP.664 trimers were constructed from multiple isolates and have been used in crystallization studies to elucidate the Env structure in complex, unliganded, and with various ligands [440-448]. The atomic-level information of the liganded SOSIP trimers helped in identifying and characterizing bNAb epitopes and the role that glycans

play towards immune evasion and antibody recognition [440,442-444,449-454]. Construction of SOSIP.664 trimers from different isolates involved the introduction of stabilizing mutations, which are discussed below. When the immunogenicity of the SOSIP trimers was assessed in preclinical studies involving rabbits and non-human primates, they were able to induce neutralizing antibodies against autologous Tier-2 viruses, a first for Env-based immunogens, and a step in the right direction for immunogen design [405].

Another generation of cleavage-independent native trimers, where a flexible linker replaced the furin cleavage site, was produced to circumvent the proteolytic processing and to improve the stability of the trimers by inhibiting the conformational changes induced by cleavage. These include Native Flexible Linker (NFL) trimers [455]; Single Chain gp140 (SC-gp140) trimers [456]; and Uncleaved pre-Fusion Optimized (UFO) trimers [457], among others. While antigenically relevant, these trimers have also yielded mainly autologous, neutralizing antibody responses when used in animal immunizations [458-460].

Despite this progress, all Env trimers display some conformational metastability evidenced by the conformational change that occurs when the virus engages with the CD4 receptor as the virus and cell membrane fuse [461]. Five different conformational states have been described for HIV-1 Env [462]. The presence of “open” or CD4-bound and “closed” or unbound states and intermediary conformations were demonstrated with hydrogen-deuterium exchange, structural analyses, and smFRET experiments for both membrane-bound and soluble Env [462-464]. This conformational heterogeneity was bound to affect the neutralization potential of the Env since different epitopes are exposed during the various states. In an effort to reduce the exposure of immunodominant epitopes, such as the V3 loop, which leads to non-neutralizing antibodies or those that neutralize only Tier-1 viruses, additional SOSIP.664 trimer stabilization modifications that reduce their exposure have been assessed [433]. While the BG505 SOSIP.664 was relatively easy to design and formed native-like trimers, other isolates

initially failed to form stable trimers. Whether the modifications were premeditated or not, Torrents de la Pena *et al.* have shown a link between the changes made to stabilize soluble HIV-1 Env trimers and the naturally occurring traits of stable proteins found in extremophile bacteria and archea [465].

Thermophilic proteins contain more hydrophobic residues compared to their mesophilic homologues which results in more condensed packing of the protein domains [466,467]. This strategy was employed with HIV-1 Env trimers by introducing leucine residues in the V3 loop thereby increasing the hydrophobicity of this immunodominant domain [468]. Similarly, the addition of hydrophobic mutations in the rest of the trimer (core or stem) also increase the amount of Env protein packing [469,470]. This resulted in a reduction in flexibility (increased stabilization) of the trimer resulting in lower exposure of non-neutralizing epitopes [468,471]. In addition to the hydrophobic residues, charged amino acids are instrumental to proteins found in thermophiles as they provide stability by ion pairing internally and at the surface exposed to solvents [472]. Similarly, charged residues introduced in the cleavage site (the interface between gp120 and gp41) increased the thermostability of the trimers and produced well-ordered trimers [473,474]. It has also been shown that aromatic amino acids are abundant in extremophile proteins as their ring stacking ability and hydrophobic packing increase thermostability [472,475]. In the same manner, the introduction of aromatic residues in the HIV-1 Env trimer has resulted in higher stability of the trimer apex, the trimer base, and the trimer interface [469-471,476]. The aromatic and hydrophobic residues account for approximately 45% of the mutations that have contributed to the Env trimer stability [465].

Generally, a higher number of proline and glycine residues are identified in loops of proteins found in thermophilic organisms, which contribute to their conformational stability [477]. The SOSIP ‘I559P’ mutation acts in a similar manner for the loop of heptad repeat 1 (HR1) in the HIV-1 trimer. The HR1 is partially disordered in the pre-fusion conformation but forms a

helical structure in the post-fusion state [461]. Introducing the I559P substitution causes destabilization of the post-fusion state while stabilizing the pre-fusion conformation [402]. Finally, the abundance of predicted disulphide bonds in proteins from thermophiles have been shown to increase the rigidity of those proteins [478-480]. Certain viruses (vaccinia and influenza) naturally have disulphide bonds between their Env subunits but the lack of natural disulphide bonds between the HIV-1 Env subunits results in gp120 shedding [402]. As such, one of the first modifications towards the development of a stable Env trimer was the SOSIP disulphide bond (A501C-T605C) linking the gp120 and gp41 subunits [401]. Additional disulphide bonds have been added to further stabilize the trimer, including an interprotomer disulphide bond (E49C-L555C) [481], a different intersubunit disulphide (A73C-A561C) [452], and an intrasubunit disulphide bond (I201C-A433C) used to lock the trimer in the pre-fusion conformation [441,482]. These new generation trimers with the A501C-T605C bond, the intersubunit disulphide bond, and either the interprotomer or the intrasubunit disulphide have shown hyperstability at temperatures reaching 92 °C and 81 °C, respectively [481].

The ability to develop stable, soluble native-mimics of HIV-1 Env trimers (Fig. 1.7) and use them as immunogens has dramatically advanced the field of antibody-based vaccine design. While this progress has been significant, additional strategies to elicit bNAbs such as the selection of immunogens that target naïve B cell receptors and directing B cell lineages to recognize conserved Env epitopes have also been explored and are discussed in the next section. Overall, Env trimer developments represent one of the biggest scientific advances in the progress towards the development of a promising HIV-1 vaccine.

1.3.2 Rational Env vaccine design to broaden neutralizing antibody responses

The 10-30% of HIV-1 infected individuals who develop bNAbs do so after 2-3 years of infection, when virus and B cells have had time to co-evolve and drive antibody maturation and the acquisition of somatic hypermutations (SHM) [262]. A few strategies have been tested

in order to attempt replicating this natural infection effect in the context of vaccination. The strategies include using empirical (i.e., inductive) vaccine regimens; rationally designing immunogens that target desired germlines; using regimens based on patient lineages; and the targeted iterative design of vaccines whereby structural improvements are based on the lessons learnt, including failures of previous vaccine regimens. Empirical approaches to vaccine design maximize on the genetic diversity of HIV-1 by administering native-like trimer of different clades. One such study tested a clade A, a clade B, and two clade C trimers either combined or sequentially in rabbit immunizations [483]. They observed that the antibodies produced in the rabbits could neutralize the Tier-2 pseudoviruses from which the immunogen was composed (autologous) but other Tier-2 pseudoviruses were either weakly or not neutralized, and as such, the breadth was limited [483]. While these empirical approaches inform us on the B cell responses shaped by related but different antigens, these experiments lack the bNAbs development process that lends to epitope variation which enables antibodies to distinguish multiple viruses in natural infection [484]. The germline-targeting immunogen design came about following the understanding that previous immunogens such as BG505 SOSIP.664 failed to activate bNAbs germline precursors [485-487]. Since a limited set of naïve B-cell receptors give rise to bNAbs, rationally designing immunogens to activate these receptors seemed to be a logical next step. This process involves engineering immunogens that can trigger an immune response from knock-in mice which express identified germline precursors of HIV-1 antibodies, in particular VRC01-class of antibodies [383,384,469,488-491]. Two such immunogens, namely eOD-GT6/8 and 426c.TM4ΔV1-V3, as well as a version of BG505 SOSIP engineered to target germlines (BG505 SOSIP.v4.1-GT1) have been shown to successfully engage the correct germline precursors [384,484,488,490]. However, once germline precursors have been engaged, a step-wise immunization schedule is required to steer the antibody response towards affinity maturation. For this, researchers have studied the

phylogenetic lineage of HIV-1 Env sequences found in infected individuals and traced the co-evolutionary pathways of virus and Env over time [492-495].

In-depth studies looking at virus and antibodies co-evolution have been done with HIV-1 infected individuals having dominant CD4-binding site, V1V2, and V3 glycan bNAbs, as well as membrane-proximal external region (MPER)-directed responses [263,496-500]. The CD4-binding site antibody CH103 was isolated at 136 weeks post-infection with 55% breadth [499]. Analysis of the CH103 lineage revealed ancestral transcripts as early as 14 weeks post-infection which had no neutralization potential [499]. In this instance it took 100 weeks to reach the breadth of CH103 and they found evidence of co-evolution with another antibody lineage (CH235) [499]. The study which looked at the V1V2 targeting antibody (CAP256-VRC26) traced its lineage through twelve antibodies from 30 weeks to 206 weeks post-infection [498]. These antibodies had limited SHM but boasted of unusually long CDRH3 (35 residues), thus showing the possibility of relative rapid development of bNAbs through correct selection of B cell with fewer SHM [498]. The study looking at MPER-directed antibodies involved an HIV-1 infected individual who developed breadth 81 weeks after infection, with the virus species in this individual showing an accumulation of mutations in the gp41 MPER region [263]. Finally, longitudinal analysis of serum and B cells of an HIV-1 infected individual from 5 to 38 months post-infection revealed an antibody lineage that targets a V3 region glycan site [496]. This lineage surfaced shortly after 10 months post-infection and developed breadth of neutralization around month 38 post-infection [496]. The level of SHM in this antibody reached approximately 15% and the lineage was shown to involve a complex pathway of sub-lineages with early diversification and diverse antibodies [496]. While maturation enables expansion of lineages to the point of dominating the neutralizing response, the examples above show that each site of vulnerability dictates the degree of SHM required and the time to the development of breadth, which could range from 6 months to multiple years [501]. The aim of vaccine

development here is to identify the viral sequences which determined the maturation pathways in those individuals. Once sequences have been identified, longitudinally sampled variants are selected and used in sequential immunizations with individual (in sequence) or mixed (swarm) immunogens representing evolving viral sequences. The proof-of-concept of this strategy has been shown using germline knock-in mice where sequential immunizations with gp120 proteins that covered a specific B cell ontogeny yielded weak Tier 2 neutralization against the autologous virus [502]. While these results in animal studies are promising, these lineage-based immunogens need to be tested in human clinical trials to determine their efficacy. Many of these immunization strategies used to drive the B cell response towards the development of CD4-binding site, MPER-directed, V1V2, and V3 glycan bNAbs have reached phase 1 clinical trials or are set to enter these trials in the next two years [503].

Finally, vaccine designs based on specific epitopes have been attempted for CD4-induced and MPER antibodies. The rationale behind these approaches is based on the understanding that different Env conformations (closed/prefusion; pre-hairpin intermediates; open/CD4-bound; postfusion) expose various sites of vulnerability on the virus (Fig. 1.9) [504]. bNAbs that target epitopes optimally exposed at each of these conformations have been isolated from HIV-1 infected individuals (Fig. 1.9). Structural information based on bNAbs complexed to HIV-1 have guided this vaccine design strategy. Researchers have made use of stabilized trimers, as well as domain and epitope scaffolds in a targeted approach to elicit these types of antibodies in a vaccine context. However, the strategy requires a two-pronged approach of masking non-neutralizing epitopes while selectively exposing the required sites. This includes the stabilization of trimers described above [405,470] as well as the manipulation of glycosylation sites to mask or expose various underlying protein sites [505-507]. The role of glycans in vaccine design is discussed in further details in section 1.3.3. The minimalistic, targeted approach to vaccine design has been used to induce MPER-directed immunity in rabbits by the

simple introduction of two mutations which expose an epitope in the fusion machinery within the MPER [508]. In addition, stable scaffolds used to display MPER epitopes on a framework [509,510] have also been used to elicit MPER-directed antibodies with limited success [511-513]: while these antibodies had similar epitopes as naturally occurring MPER bNAbs, they lacked broadly neutralizing capabilities [509,514,515]. One of the drawbacks to vaccine design based on epitopes is that such immunogens should ideally induce a polyclonal response, with the additional disadvantage that many of the broadly neutralizing epitopes have targets encompassing protein and glycan components [400,443,444].

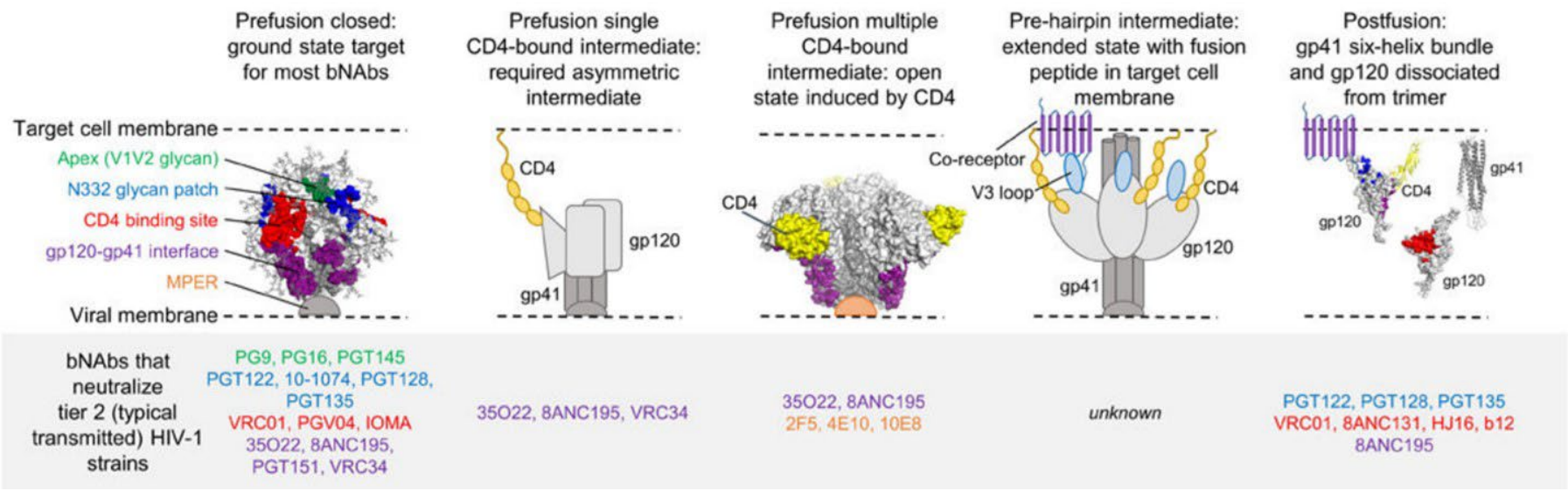

Figure 1.9. Conformational changes to HIV-1 Env binding and entry. The various states of conformational rearrangement to the HIV-1 Env during binding and entry are depicted with the known broadly neutralizing antibodies that target each state listed under and colour coded based on the epitope targeted. gp120 is in light grey, gp41 is shown in dark grey, and the glycans as grey sticks. Image modified from Pancera *et al.* [504].

1.3.3 The function of Env glycans

Another hindrance to the development of bNAbs in a vaccine context is the extensive glycosylation found on native HIV-1 virions, with glycans accounting for approximately 50% of the Env mass. The glycans on the surface of HIV-1 form a shield hindering naïve B cells from recognizing the underlying protein targets. In addition, these glycans are host-derived [516] and so they are used by the virus to evade the immune system. However, as demonstrated for bNAbs in natural infection, vaccine induced bNAbs should recognize glycans as well as protein components of the virus [503]. Due to the abundance of glycans on the prefusion trimeric conformation, it is not surprising to see antibodies developing strategies to facilitate penetration of the glycan shield: antibodies which target the V1V2 apex such as PGT145 and PG9 have developed anionic loops that pierce the glycan shield to connect with the underlying protein component of Env (Fig. 1.9) [448,517-519]. Similarly, antibodies that recognize V3 glycans such as PGT121 and PGT128 also have protruding loops (long CDRH3) that allow access to their epitopes (Fig. 1.9) [520-522]. The V3 glycan epitope is composed of a high mannose patch with the N332 glycan as a key residue. An antibody like 2G12 recognizes the glycan cluster with no protein component [523,524]. The antibody VRC-PG05 was described to use a double-protruding loop to target the N262 glycan as well as the N295 and N448 glycans on Env [525]. This was the first and currently only antibody described to recognize an epitope on the trimer 'silent face'. The VRC-PG05 epitope is composed of 88% glycans; however, the antibody has limited breadth (neutralizes 27% of HIV-1 virions) [525].

Antibodies to the gp120-gp41 interface have also developed recognition of protein and glycans. The well-characterized antibodies from this group are 35O22 and 8ANC195 which recognize

various conformations of the Env trimer (Fig. 1.9) [526,527] as well as Env glycans N88, N234, N276, and N637 [442,528,529]. CD4-binding site (CD4bs) bNAbs are not exempt from glycan recognition. The conserved CD4bs is particularly recessed and densely shielded by glycans, and, therefore antibodies have developed approach angles and glycan recognition strategies for potent and broad neutralization. There are two classes of CD4bs bNAbs; antibodies that use the same heavy chain germline gene lineage, also known as the VH1-2*02-gene usage restricted antibodies [444,530,531] and antibodies that have a dominant 3rd heavy chain complementarity determining region (CDR H3) loop [532,533]. The VRC01 family of bNAbs fall under the VH1-2*02-gene restricted category; their germline encoded CDR-H2 R71 allows them to mimic the interaction of CD4 R59 with the D368 residue on the virus and as a result these antibodies display astonishing breath (98% for the N6 antibody) [487,534]. Among the CDR H3 dominant group, some antibodies (HJ16 and 179NC75) interact differently with the virus, whereby the D368 residue is irrelevant but the N276 glycan is crucial to the antibody epitope [535,536]. Because of the partially buried nature of MPER which is embedded in the membrane, MPER-directed antibodies can only access their epitope when the virus has undergone some of the conformational changes required for fusion [400,537]. In particular, these antibodies recognize the transiently exposed prefusion intermediate following the binding of several CD4 molecules (Fig. 1.9) [537,538]. Unlike the other regions of Env, the MPER is relatively glycan-free. Therefore some but not all of the antibodies targeting this region have developed recognition of the lipid membrane in addition to a linear sequence in the MPER [539,540].

The importance of glycans is seen in the number of bNAbs that target these carbohydrates. As a result, there is a vaccine strategy that includes glycan hole engineering whereby HIV-1 Env

glycosylation is deliberately removed and reintroduced in sequential immunizations in a manner that allow antibodies to recognize the proteome and subsequently acquire recognition of the glycan for improved neutralization [491,505,506,516]. Similar to the immune escape mechanism HIV-1 employs in natural infection, glycans have also been used to conceal sites that would otherwise be immunodominant but non-neutralizing in a vaccine context [505,506]. Unfortunately, due to their conformational heterogeneity and complexity (variance in sugars, branching patterns, and linkage) glycoproteins are known to be averse to crystallization and, therefore, structural studies [541-543]. Stewart-Jones *et al*. (2016) managed to resolve the crystal structure of fully glycosylated trimers [443] and this, combined with the characterization of bNAbs which recognize glycans, has informed the design of Env-based immunogens with glycan patterns similar to circulating viruses. The glycopeptide antigen and glycan restoration approaches were tested in rabbit immunizations and found to elicit non-neutralizing, glycan-dependent antibodies [544,545], as well as neutralizing antibodies that have strong similarities in terms of angle of approach and epitope recognition with antibodies from natural infection [516]. Similarly, an immunogen (RC1) that uses glycans to conceal immunodominant epitopes was able to induce glycan-targeting antibodies in mice, rabbits, and macaques, albeit non-neutralizing ones .

1.3.4 Transmitted founder viruses signatures

There is a large population of diverse quasispecies (related but non-identical viral sequences) at mucosal surfaces at the time of HIV-1 transmission. However, 60-80% of new infections occur from a single transmitted/founder (T/F) virus as evidenced by the homogeneous population of virus in the newly infected individual soon after transmission [28,31,546-548]. T/F viruses have specific phenotypic and genetic determinants that give them the advantage

needed to successfully establish infection and to disseminate in the new host [31,549,550]. Phenotypically, the traits that differentiate T/F Envs from chronic Envs are the almost exclusive use of the CCR5 co-receptor by T/F Envs [31,551-553] and their infection of T-cells and not macrophages [30,31,549,554] due to the high requirement for CD4-mediated virus entry [555-557]. Genetically, T/F Envs bear shorter variable loops and less N-linked glycans than chronic viruses, but the fewer glycans have only been evidenced for T/F viruses of clades A and C [29,74,547,558], not clade B [29,549,559]. The narrow window in genetic diversity at transmission is known as the 'transmission bottleneck' [560,561]. An additional determinant to the transmission bottleneck is the route of transmission. Heterosexual transmission generally shows the most stringency in terms of selective pressure exerted on transmitted viruses, followed by transmission cases involving men who have sex with men, and finally injection drug users whereby only half the new infections are established by a single variant [562,563]. Vertical transmission between mother to child also displays a strong genetic bottleneck with a single virus transmitted in most cases [564].

Understanding T/F virus signatures and developing immunogens that elicit antibodies which target these types of viruses (those that establish infection) is an important step to vaccine design work. The characterization of T/F viruses has allowed tracing of virus evolution and, indirectly, antibody ontogeny in studies that looked at virus-antibody interactions [28,30,61]. In addition, transmitted/founder virus sequences have been used to prime the immune system in prime/boost vaccination strategies [565]. In a similar approach, a clade C based consensus sequence generated from 1,894 transmitted/founder sequences (FVC_{ENV}) was used to maximize the qualities of T/F viruses in a clade- and region-specific manner [566] which would

prove beneficial in regions such as sub-Saharan Africa and India where clade C is the predominant circulating strain.

1.4 Complexed Env immunogens: a model for the exposure of conserved epitopes

1.4.1 Stabilization of trimer-based protein complexes

HIV-1 Env is required to have some metastability in order to accommodate the functional folding and structural reassembly needed for fusion. However, this metastability also assists with immune evasion by allowing shedding of gp120 [567] and generating various Env conformations during the course of infection that act as immune decoys [568]. The creation of recombinant, stable, soluble homogeneous Env trimers as an attempt to overcome the inherent metastability of Env was described above. Additional attempts have been made in further reducing the trimeric Env metastability by replacing the gp41 ectodomain with a version that is "uncleaved prefusion-optimised" (UFO), which when tested in rabbits, was better able to induce a Tier-2 neutralizing response than other soluble trimers tested [390]. "Native flexibly linked" (NFL) trimers were also designed to minimize metastability by maintaining the I559P mutation found in SOSIP trimers, but replacing the furin cleavage site with a flexible linker which covalently holds the Env subunits [455]. NFL trimers are antigenically and biochemically similar to SOSIP trimers although they do not necessitate cleavage of the Env [455,482].

In the closed, prefusion conformation of Env, interdomain interactions bring the first two variable loops (i.e., V1V2) of each protomer in close proximity at the apex of the trimer [442,449,451], with the V3 loop sheltered underneath (Fig. 1.10). The closed form also shields

the bridging sheet which forms quaternary contacts underneath the V1V2 loop and which contains components of the co-receptor binding site [471-473]. Upon binding to CD4, the allosteric changes push the gp120 lobes away from each other, resulting in an opening of the trimer, disruption of V1V2 contacts with V3 (Fig. 1.10), and the convergence of constituents that make up the coreceptor binding site [463,469]. The closed, prefusion state of the Env trimer has been preferred for vaccine immunogen studies [569] due to the evidence that many bNAbs target the V1V2 and other quaternary epitopes.

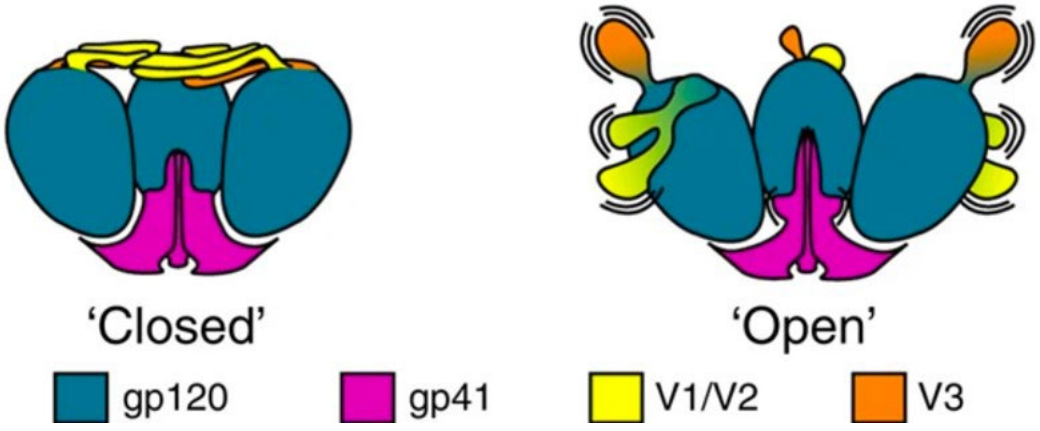

Figure 1.10. Differences between closed and open conformations of the Env trimer. Closed Env trimer conformation showing inter-protomeric interactions of V1V2 loops at the trimer apex and shielding of the V3 loop. The open, CD4-bound conformation is shown with the outward displacement of the protomers and disruption of the variable loop interactions. Figure modified from Guttman *et al.* [570]

Upon binding of HIV-1 Env to CD4, the coreceptor binding site (CoRBS) becomes exposed with epitopes in and near it providing templates for the elicitation of bNAbs. Over 40 of the known HIV-1 specific antibodies have their epitopes exposed following CD4 binding to the Env trimer [571]. These can be grouped as (1) recognizing the gp120 face normally shielded by gp41; (2) recognizing V1V2 including regions close to the CoRBS; and (3) recognizing the CoRBS [571], therefore displaying a range of specificities. While some of these antibodies

display neutralization breadth [572,573], the majority have limited neutralization. Nonetheless, almost all show evidence of Fc-mediated humoral effector functions [571] against infected cells [574,575] and cell-bound viruses [576-578].

CD4i antibodies have the majority of their epitopes in the V1V2 stem β-strands and gp120 C4 domain, regions necessary for Env binding to chemokine co-receptors once bound to CD4 [579-582]. These epitopes are therefore highly conserved, and associated with T/F signatures [583]. It was initially believed that these CD4i antibodies were non-neutralizing or weakly neutralizing [579], however, the finding that single-chain and Fab fragments of the same antibodies neutralized primary isolates [584,585] revealed that the full IgG antibodies may experience steric occlusions in reaching their epitopes, in natural infection. This is due to the majority of CD4i epitopes being near the bridging sheet and in close proximity to the viral Env and the cell membrane, following attachment of HIV-1 Env to cellular CD4 [586,587]. The ability to generate CD4i antibodies in infection has been clearly demonstrated [579,584,588-590]. CD4i antibodies appear early in infection and unlike other bNAbs, do not require extended B cell maturation [517,591,592]. A study designed to elicit CD4i antibodies in macaques made use of a vaccine composed of Bal gp120 and domains 1 and 2 of macaque CD4 fused as a single-chain complex [593]. The study showed the presence of CD4i antibodies post-vaccination and in the macaques who were vaccine-naïve but challenged with a simian/human immunodeficiency virus (SHIV) [593].

CD4i is also a main target of antibodies with ADCC effector function as was shown in a study that revealed over 90% of the HIV-1 infected participants had plasma containing ADCC activity in CD4i antibodies [575,594,595]. CD4i antibodies have also been isolated from individuals who naturally suppress viremia, and control HIV infection [593] and from the

RV144 trial participants [416,596]. As such, stabilization of Env-CD4 complexes that expose previously shielded epitopes have been evaluated as potential immunogens with encouraging neutralizing responses [590,597], however, stabilization of the complex by cross-linking has become a necessity [597] which is discussed below.

1.4.2 Replicating the HIV-1 Env-CD4 receptor complex model

The HIV-1 Env bound to CD4 provides a stable complex which boasts of conserved epitopes essential for replicative functions such as engagement of the coreceptor and viral entry [593]. These functions being a necessity for the majority of HIV-1 strains, the use of CD4-bound conformation immunogens in a vaccine context should theoretically provide broad humoral immunity against a very diverse pool of HIV-1 strains. One of the studies showing the ability of Env-CD4 complexes to generate a strong yet diverse immune response directed to epitopes other than Env alone involved testing the immunogenicity of the complexes in rabbits [598]. This study showed that the antibody response targeted both Env and CD4 [599] but the Env-specific antibodies were able to neutralize some primary isolates from clades B and C [598]. What this study and others like it demonstrated was that inclusion of the full-length (4 domain) CD4 increases the potential for an autoimmune response [598]. As a result, efforts were directed towards defining the minimal required CD4 domains [598], which was aided by the solved crystal structure of gp120-CD4 (with the 17b Fab) [600].

It was determined that the 22 residues in domain 1 of CD4 which form the binding interface with gp120 is composed of a mixture of H-bonds, electrostatic, and hydrophobic interactions. This prompted further efforts towards finding CD4 mimetics of only the functional CD4 domain 1 which are able to induce Env transition to an open/CD4-bound conformation. The first CD4 mimic was adapted from the Scyllatoxin (scorpion toxin) backbone fragment which

showed structural similarities to CD4. It was used to engineer an effective CD4 structural mimic, named CD4M3, albeit with two to four orders lower affinity for Env than soluble CD4 [601]. An optimized version of CD4M3 called CD4M9 was developed, which bound even better to Env compared to native CD4, and was able to inhibit viral infection of cells [602]. In the same vein, other CD4 mimetics called miniCD4s were engineered, coupled to Env and tested as immunogens in rabbits [603]. These miniCD4s complexed to HIV-1 Env could induce CD4i antibodies with neutralization potential against some HIV-1 strains [604]. In another approach, Cerutti *et al.* covalently complexed monomeric gp120 to the first two N-terminal domains of CD4 creating a gp120-CD4 intermolecular disulphide bond which could potentially enhance the strength of the complex [605]. The complex contains a recombinant form of two-domain CD4 with an S60C mutation which facilitates covalent linkage of the V1V2 loop of gp120 to CD4 via formation of a disulphide bridge from the Cys^{126} on Env to the Cys^{60} on CD4 [605]. This method of covalently linking Env to two domain CD4 proved to elicit superior neutralizing responses in rabbit immunizations when compared to Env only or Env differentially complexed to CD4 [406]. The CD4 complexing was also done with GCN4 trimeric Envs with similar remarkable results [406].

1.4.3 Potential limitations to including the HIV-1 primary receptor CD4 in vaccine immunogens

The advantages to using Env-CD4 complexes have been highlighted above; however, a potential caveat rests in the belief that vaccine formulations which include CD4 pose safety concerns as they have the potential to elicit autoimmunity by breaking tolerance (i.e., the failure of the mechanisms that maintain immune tolerance to self-antigens). Approximately 10% of HIV-1 infected individuals generate autoantibodies against CD4 presumably due to the natural

formation of gp120-CD4 complexes [606-614]. However, these antibodies do not react with CD4 expressed on cell surfaces [608,609,611,615]. While some anti-CD4 antibodies mediate a decline in CD4 expressing T-cells in various clinical settings [616-618], no causal link has been established between this autoreactivity and AIDS [619-623]. Of interest, CD4 specific autoantibodies have also been found in HIV-1 exposed yet uninfected individuals [614,619,621-623]. In these individuals who are naturally-resistant to HIV-1 infection, the anti-CD4 antibodies do not discriminate between cell-bound and soluble CD4 [614,620]. In pre-clinical studies, Env-CD4 complexes do induce antibodies against CD4 and, therefore, these antigens could result in autologous immunogenicity if they recognize the host $CD4^+$ lymphocytes, but this has not yet been demonstrated. On the contrary, immunotoxicologic work done on cynomolgus macaques have demonstrated that anti-CD4 autoantibodies are not as readily elicited by vaccination [624].

In addition, the successful use of therapeutic anti-CD4 monoclonal antibodies in clinical trials has shown that anti-CD4 antibodies are well tolerated [625-627]. Anti-CD4 antibodies have been previously used as therapeutics to treat inflammatory diseases, malignancies, and autoimmune diseases [628,629]. In the context of HIV-1, Ibalizumab – an anti-CD4 humanized murine antibody – was shown to be well tolerated and was able to block infection with no autoimmunological reactions [630,631]. Ibalizumab has been approved by the FDA for use in combination with HAART against multidrug resistant HIV-1 [632,633]. Collectively, the above studies demonstrate that CD4-containing Env complex antigens remain valuable as potential HIV-1 vaccine immunogens which stabilize the Env trimer transition state and expose conserved CD4i and coreceptor epitopes, without the fear of inducing immunotoxic responses.

1.5 Study rationale and outcome

Since the search towards an effective HIV-1 vaccine began, and as it became evident that conventional vaccination approaches would not work, multiple efforts have been directed towards developing potential vaccine candidates that target conformation-specific epitopes. We have learnt of the protective effects of anti-Env responses in blocking HIV-1 acquisition and of the ability of cellular immunity to regulate infection and viral loads. We have seen a myriad of Env structures designed in various combinations of subunit vaccines, trimeric forms, nanoparticulate diplays, included in vectors, engineered to over-emphasize certain epitopes, deglycosylated, glycan-controlled, and cross-linked to CD4 and CD4 mimetics. We have seen these Env structures paired with advances in immunogen design and tested in a plethora of immunization regimens in small animals, NHPs and humans. While all of these efforts have resulted in some of the best, structurally stable recombinant HIV-1 Env vaccine candidates, improvements to the immunogenicity of these vaccine candidates has been met with varying levels of success in human clinical trials.

The work outlined in this thesis presents the characterization and immunogenicity of a novel covalently linked "native-like" Env trimer-CD4 complex. This immunogen complex was constructed using the SOSIP.664 trimer version of a founder virus consensus C Env (FVC_{ENV}) covalently linked to two-domain CD4 (FVC_{ENV}:$2dCD4^{S60C}$).

Published article 1 (Chapter 2) delineates the conformational dynamics of the covalent immunogen complex as compared to the non-covalently bound counterpart, with particular emphasis on the changes observed in the CD4 portion of the complex. HDX-MS is a beneficial technique in determining differences in protein dynamics and their ensuing conformational stability. Since previous work had shown the necessity of covalently complexing the Env to

2dCD4^{S60C} in order to consistently obtain the cross-neutralizing response [406]; we exploited the HDX-MS technique to delineate the conformational differences in the receptor portion between SOSIP FVC$_{ENV}$ covalently and non-covalently complexed to 2dCD4^{S60C} .

Very few differences were observed in the first domain of CD4 between the covalently bound SOSIP FVC$_{ENV}$:2dCD4^{S60C} and the non-covalently bound SOSIP FVC$_{ENV}$:2dCD4^{WT}. The first domain of CD4 is involved in interaction between the receptor and binding partners (HIV-1 Env as well as host MHCII). Residues shown to be crucial for these interactions remained stabilized as we observed little to no differences between the covalent and non-covalent complexes. The rest of CD4 domain 1 was relatively unchanged aside from a couple of allosteric effects seen in some residues. Markedly, the interconnecting strand between the two domains of 2dCD4 displayed slight differences between the complexes with greater exchange seen in the covalent complex for some of the residues. The increased solvent accessibility in this interface region reveals the manner in which changes may be propagated from the mutated S60C residue in the first domain along the strands to the second domain.

We showed that the second domain of 2dCD4 exhibited a large-scale destabilization in the disulphide-stabilized SOSIP FVC$_{ENV}$:2dCD4^{S60C} complex as many of the residues became more solvent exposed. The lack of apparent intramolecular interactions such as hydrogen bonds between these residues and the evident stability maintained in the non-covalent SOSIP FVC$_{ENV}$:2dCD4^{WT} complex led to the conclusion that the observable disordering of D2 residues in FVC$_{ENV}$:2dCD4^{S60C} resulted from the covalent coupling. After analysing all the differences seen in CD4 between the two complexes, we developed a model describing the propagation of changes from the disulphide link at position 60 of domain 1 in FVC$_{ENV}$:2dCD4^{S60C} through the interface via the interconnecting residues in a somewhat

transient or undetectable manner which result in the dramatic disordering observed in domain 2 (published article 1). We hypothesize that the tremendous difference in dynamics of 2dCD4 in SOSIP FVC_{ENV}:$2dCD4^{S60C}$ contributes to the improved immunogenicity of this covalent complex (shown in submitted article 2, Chapter 3) by allowing the presentation of antigenic residues which are novel or differentially exposed in SOSIP FVC_{ENV}:$2dCD4^{S60C}$. Our experimental setup led to constraints which impaired our ability to analyse the changes in the FVC_{ENV} portion of the complexes. However, previous mapping of the bNAb responses showed they are largely directed to CD4 and/or novel epitopes spanning both Env and CD4, not Env alone. As such, future work will require a broader examination of the conformational dynamics in both Env and CD4, in order to address possible differences in the SOSIP FVC_{ENV} portion of the complexes.

In Chapter 3, submitted article 2 describes the antigenicity of the covalently bound SOSIP FVC_{ENV}:$2dCD4^{S60C}$ (termed FVC_{ENV} SOSIP.664-$2dCD4^{S60C}$ in the publication) complex, as compared to the unliganded Env trimer. Furthermore, the immunogenicity of FVC_{ENV}SOSIP.664-$2dCD4^{S60C}$ and the well characterized BG505 SOSIP.664-$2dCD4^{S60C}$ was evaluated and compared in a preclinical context in New Zealand white rabbits. In this work, we used an Env based on sub-Saharan African T/F sequences to which we included the SOSIP.664 trimerization mutations. The FVC_{ENV} SOSIP.664 trimer was recognized by a variety of anti HIV-1 bNAbs indicating its structural integrity. For the trimer complex, we observed that binding of some antibodies remained unchanged while two antibodies (one that targets the CD4-binding site and one which targets the V3 loop) lost their binding potential. This loss of some epitopes and the markedly improved binding of a CD4-induced antibody

confirmed that the purified immunogen complexes were in the CD4-bound, open trimer conformation.

The rabbit immunizations show that in accord with other works using individual protein immunogens, even those in the SOSIP.664 format, our FVC_{ENV} SOSIP.664 immunogen alone failed to produce the much-coveted broad and potent bNAb response until it was covalently complexed to $2dCD4^{S60C}$. Modification of the FVC_{ENV} SOSIP.664 immunogen by covalent complexing with a two-domain CD4 receptor with an S60C modification allows for high affinity binding using an intermolecular disulphide bridge between the gp120 component on Env and $2dCD4^{S60C}$ [605]. Of note, the BG505 SOSIP Env used in other studies was also tested here, with our keynote modification and the remarkably different outcome of this complex showing breadth and potency of neutralization for the first time. To our knowledge, consistent elicitation of such a potent and broad neutralizing antibody response using first-generation, homogeneous SOSIP Env trimers in a simple immunization protocol is unprecedented in the field.

The two studies presented in Articles 1 and 2 complement each other and advance the HIV-1 vaccine field by displaying the substantial difference a subtle modification in immunogen design could have in providing a paradigm shift towards soluble SOSIP Env immunogens, and more importantly, in describing SOSIP complex immunogens capable of eliciting tier-2 neutralizing antibodies with high potency. The type of characterization of these immunogens described in this study opens potential avenues in designing even more advanced Env immunogens and demarcating the type of antibodies prompted by these immunogens. Specifically, the observation that covalently-linking SOSIP Env trimers to $2dCD4^{S60C}$ provides novel evidence that the covalent-linkage causes changes in D2 of CD4 which likely contributes

to the variances in antigenicity seen. The work done here demonstrates the value of complementary studies looking at protein dynamics combined with preclinical work that provides information on the immunogenicity of the complexes and their ability to generate bNAbs. Essentially, it suggests a novel manner of displaying SOSIP trimer immunogens for improved antigenicity and immunogenicity using a targeted disulphide resulting in a CD4-bound, open Env conformation with a destabilized D2 in the CD4 component. Designing an effective HIV-1 vaccine would require the proverbial "village" - concerted efforts from various groups and scientific disciplines which allow cumulative knowledge and understanding in order to curb the HIV-1 pandemic. Some study limitations include the failure to test the immunogencity of unliganded BG505 SOSIP.664 in the rabbit immunizations. We justified that unliganded BG505 SOSIP.664 has been tested multiple times in rabbit immunizations [459,483,505,634] with similar results. As such, the use of additional rabbits for testing unliganded BG505 SOSIP.664, although a beneficial control, was considered contrary to the Replacement, Reduction and Refinement principles in animal experimentation.

Identifying the antibodies targets on the SOSIP Env:2dCD4 complex is an integral part of future work as it would inform whether the antibodies responsible for the breadth and potency seen are solely CD4-directed or bind both Env and CD4 exposed epitopes in the covalenly bound complex, and whether the epitopes are novel or differentially exposed in the covalent complex. For this, antibody depletion experiments would allow for broad mapping in assessing whether the antibodies target the Env trimer, CD4 or the complex with the possibility of determining the contribution of conformation-dependent antibodies to the breadth of neutralization from the rabbits antisera. Finer mapping could be achieved with depletion experiments using the same immunogen envelopes (SOSIP FVC_{ENV} and/or SOSIP BG505)

with specific mutations such as D368R which abrogates the majority of CD4-binding site antibodies, or mutations in the Phe43 pocket in loop D, or the removal of glycans which some antibodies depend on such as N160 in V2 and N301/N332 in V3. Ultimately, the isolation and characterization of antibodies from immunized rabbits PBMCs would ideally complete the picture in determining the exact epitopes of these antibodies, their sequences, and the differences in their maturation pathway. Insight into the above will allow comparison of these rabbit-derived antibodies to bnAbs from HIV-1 infected individuals, knowing that substantial differences exist in heavy and light chain gene usage between the two species [635]. In addition, somatic hypermutations (SHMs) and gene conversion form a major part of rabbits Ab diversity and many bnAbs from natural infection have shown high levels of SHMs [636]. As such, the differences and similarities in these HIV-1 targeting antibodies from rabbits and humans need to be explored.

Chapter 2

Covalent binding of human two-domain CD4 to an HIV-1 subtype C SOSIP.664 trimer modulates its structural dynamics.

Published in *Biochemical and Biophysical Research Communications*, Volume 612, 2022, Pages 181-187, ISSN 0006-291X.

Biochemical and Biophysical Research Communications 612 (2022) 181-187

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

Covalent binding of human two-domain CD4 to an HIV-1 subtype C SOSIP.664 trimer modulates its structural dynamics

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a r t i c l e i n f o

Article history:

Received 1 April 2022

Accepted 21 April 2022

Keywords:

Hydrogen-deuterium exchange

HIV-1 subtype C SOSIP.664 trimer

Cluster of differentiation 4

HIV-1 vaccine immunogen

Protein dynamics

a b s t r a c t

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) mediates host cell infection by binding to the cellular receptor CD4. Recombinant Env bound to CD4 has been explored for its potential as an HIV vaccine immunogen as receptor binding exposes otherwise shielded, conserved functional sites. Previous preclinical studies showed an interchain disulphide linkage facilitated between Env and 2dCD4^{S60C} generates an immunogenic complex that elicits potent, broadly neutralizing antibodies (bNAbs) against clinically relevant HIV-1. This study investigated conformational dynamics of 2dCD4^{WT} and 2dCD4^{S60C} bound to an HIVϵ1C SOSIP.664 Env trimer using hydrogen-deuterium exchange mass spectrometry. The Env:2dCD4^{S60C} complex maintains key contact residues required for MHCII and Env/gp120 binding and the residues encompassing Ibalizumab's epitope. Important residues remaining anchored, with an increased flexibility in surrounding regions, evidenced by the higher exchange seen in flanking residues compared to Env:2dCD4^{WT}. While changes in Env:2dCD4^{S60C} dynamics in domain 1 were moderate, domain 2 exhibited greater variation. Lack of stability-inducing H-bonds in these allosteric sites suggest the improved immunogenicity of Env:2dCD4^{S60C} result from exposed CD4 residues providing diverse/novel antigenic targets for the development of potent, broadly neutralizing Ibalizumab-like antibodies.

1. Introduction

Human Immunodeficiency Virus type 1 (HIV-1) is composed *inter alia* of the envelope glycoproteins (Env) extracellular gp120 and transmembrane gp41 components non-covalently associated as heterodimeric trimers into the viral lipid bilayer [1]. HIV-1 infects host cells by initially binding of gp120 to the CD4 receptor on the surface of CD4^{+} T-cells. CD4 binding induces considerable conformational changes in gp120, enabling binding to CCR5 or CXCR4 [2]. From a therapeutic and vaccine development perspective, these co-receptor binding site epitopes as well as other CD4-induced (CD4i) sites are favourably exposed in the CD4-bound state.

Vaccine constructs based solely on Env monomers, trimers or SOSIP.664 trimers have poor immunogenicity in preclinical and human trials [3-9]. This is largely attributed to the extent of antibody somatic hypermutation required to generate potency and breadth over time, an invariably strong feature of HIV-1 bNAbs not yet replicable in a vaccine context. Thus, novel immunogen strategies and immunization protocols are being interrogated. While soluble CD4 (sCD4) in various forms has been investigated as candidate vaccines and therapeutics [10,11], a noteworthy vaccine strategy involves the use of Env:CD4 complexes. Covalently cross-linked complexes of Env to sCD4 did not generate autoimmunity but generated neutralizing antibodies against primary HIV-1 isolates following immunization of NHPs [12]. Similarly, we reported vaccine immunogens based on gp120 or gp140 conformations covalently complexed to a S60C mutant (2dCD4^{S60C}) eliciting bNAbs targeting the more neutralization-resistant (Tier-2 and -3) viruses

Abbreviations: 2dCD4, two domain CD4; CD4, cluster of differentiation 4; CD4bs, CD4 binding site; HDX-MS, hydrogen-deuterium exchange mass spectrometry; HIV-1, human immunodeficiency virus type 1; HPLC, High performance liquid chromatography; gp120, envelope glycoprotein 120 kDa; MHCII, major histocompatibility complex class II.

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https://doi.org/10.1016/j.bbrc.2022.04.101

in New Zealand white rabbits [13]. Interestingly, the bNAb activity was largely directed towards Env:2dCD4^{S60C}, whereas the matched Env:2dCD4^{WT} showed a less efficient antibody response [13]. As determined by computational modelling, the substituted cysteine in 2dCD4^{S60C} enables a targeted disulphide exchange with the Cys126-Cys196 disulphide bridge on gp120, forming a Cys60- Cys126^{gp120} bond [14], which likely locks Env:2dCD4^{S60C} complexes in a conformation displaying conserved or novel epitopes. To understand the mechanisms underlying the improved antigenicity of the Env:2dCD4^{S60C}, we need to interrogate its conformational dynamics.

Thus, this study elucidated differences in conformational dynamics of SOSIP.664 trimers non-covalently bound to 2dCD4^{WT} or covalently bound to 2dCD4^{S60C} to provide conformational insights contributing to improved immunogenicity of Env:2dCD4^{S60C} in the context of 2dCD4, as determined by amide hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS).

2. Materials and methods

2.1. Hydrogen-deuterium exchange mass spectrometry (HDX-MS)

Differences between the non-covalently bound HIV-1C SOSIP.664gp140:2dCD4^{WT} complex (hereafter referred to as Env:2dCD4^{WT}) and covalently bound Env:2dCD4^{S60C} were compared by HDX-MS, using the automated LEAP PAL HDX system (Leap Technologies) coupled to an Agilent 1100 HPLC system and Sciex 6600 TripleTOF mass spectrometer. The amide hydrogen-deuterium exchange reaction was initiated by mixing 4 μl of Env:2dCD4^{WT} or Env:2dCD4^{S60C} with 16 μl 100% deuterated water at 4 °C. Three labelling time-points were used between 15 and 3600 s of exchange, after which 20 μl of the reaction mix was added to 30 μl of 1.7 M guanidium hydrochloride with 20 mM TCEP and 0.1% (v/v) formic acid to quench the reaction. Non-deuterated controls included substituting deuterated water with MilliQ water, and fully deuterated controls included proceeding with the exchange reaction overnight. Quenched samples were passed through a Poroszyme pepsin column (Life Technologies) (2.1 x 5 mm; flow rate, 100 ml/min) for proteolytic digestion, and the peptic fragments were desalted and separated on a Aeris peptide XBC18 column (2.1 x 5 mm) (linear gradient of 10e40% acetonitrile at a flow rate of 200 ml/min for 10 min). Eluted peptides were analysed on the AB Sciex 6600 TripleTOF mass spectrometer. For peptide identification the 6600 TripleTOF was operated in Data Dependant Acquisition (DDA) mode while for deuterium labelling only a precursor scan was collected. In DDA mode precursor scans were acquired from *m/z* 360e1500 using an accumulation time of 250 ms followed by 30 product scans, acquired from *m/z* 100-1800 at 100 ms each, for a total scan time of 3.3 s. Charge ions (1+ - 5+, that fall in the mass range 360-1500 *m/z*) were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Collision energies were chosen automatically as function of *m/z* and charge. Dynamic exclusion was set to 15 s. HDX-MS experiments were done in duplicate.

Data analysis and other methods can be found in supplementary methods.

3. Results

Partially deglycosylated HIV-1C SOSIP.664 gp140 was successfully expressed, purified and complexed to 2dCD4^{WT} or 2dCD4^{S60C} (Supplementary Fig. S1), and the conformational dynamics of the purified Env:2dCD4^{WT} or Env:2dCD4^{S60C} complexes were analysed by HDX-MS. Sequence coverage of 87% from 55 unique peptides were obtained for the 2dCD4 portion of the complexes (Supplementary Figs. S2 and S6). Conclusive analysis was not achieved for the Env portions of the complexes as the peptic fragments obtained for Env were insufficient for detailed interrogation.

3.1. Minimal changes in D1 of Env:2dCD4^{S60C} do not map to functionally important residues

The 2dCD4 portion of the complexes displayed differences in the rate of exchange between Env:2dCD4^{WT} and Env:2dCD4^{S60C} complexes in D1, particularly over one peptide (47-56) (Fig. 1). In this peptide, there was a 28% difference in deuterium incorporation along residues Gly41 to Leu44 and a 58% difference in Gly47 with the Env:2dCD4^{S60C} displaying rapid exchange from the first (15s) time-point compared to a more gradual deuteration in Env:2dCD4^{WT} (Supplementary Figs. S3 and S4). This implies that these residues are relatively buried in the non-covalent complex but patently solvent exposed in the covalent complex.

Some residues showed little to no difference between covalently and non-covalently bound complexes. This was the case for the mutated S60C residue having no deuteration for any of the time-points (Supplementary Fig. S3), while Lys35- a residue essential for MHCII binding- showed marginal deuterium uptake in both 2dCD4^{S60C} and 2dCD4^{WT} (0-16% and 11-29%, respectively) (Supplementary Figs. S3 and S4). Overall, changes in D1 indicate conformational differences resulting from a subtle loss of structural integrity in a few residues.

Full deuteration was observed in Env:2dCD4^{S60C} for the aromatic Phe43 residue (critical for Env binding) for all time-points assayed, while this residue displayed high (but not full) deuteration in Env:2dCD4^{WT} (68% - 15s, 71% - 5 min, and 72% - 60 min) (Supplementary Figs. S3 and S4). This lack of extensive differences in deuterium uptake between Env:2dCD4^{S60C} and Env:2dCD4^{WT} in D1 signals that the changes in dynamics are localised. Thus, the shift from protection to exposure in and around β-strand C″, triggered by the covalent bond formation between 2dCD4^{S60C} and Env is confined to the single peptide (47-56) notwithstanding a few allosteric effects.

3.2. Changes in conformational dynamics of residues at the 2dCD4 D1 and D2 interface induced by the Cys60-Cys126^{gp120} bond reveal how changes could be propagated across the domains

Within the interconnecting CD4 strand, where the G strand of D1 leads into the A strand of D2, three peptides (96-106; 103-111; and 105-113) show significant differences in deuteration (Fig. 1). These changes were mapped to Phe98, Thr101, Ser104, Asp105 and Gly111 (Fig. 2 and Supplementary Figs. S3 and S4). All these residues were fully deuterated for Env:2dCD4^{S60C} from the earliest timepoint and although all show 100% deuteration at the highest timepoint (60 min) for Env:2dCD4^{WT}, levels of deuteration varied widely at 15s mark (0% for Phe98 and Gly111; 61% for Thr101) (Supplementary Figs. S3 and S4). Other differences included Ser104 and Asp105, where there was no exchange for Ser104 except at 60 min (only 55%) in Env:2dCD4^{WT}, in contrast to full deuteration for all time-points in Env:2dCD4^{S60C} and comparatively less exchange for Asp105 (41-61%) in Env:2dCD4^{WT} (Supplementary Figs. S3 and S4). Differences in deuterium uptake on these three residues indicate this local environment experienced changes allowing for greater solvent accessibility in Env:2dCD4^{S60C}.

3.3. Covalent binding of Env:2dCD4^{S60C} increases the flexibility of CD4 D2

Several changes in deuteration levels was seen in D2, particularly in and between strands A and B, strand E, and in the G strand

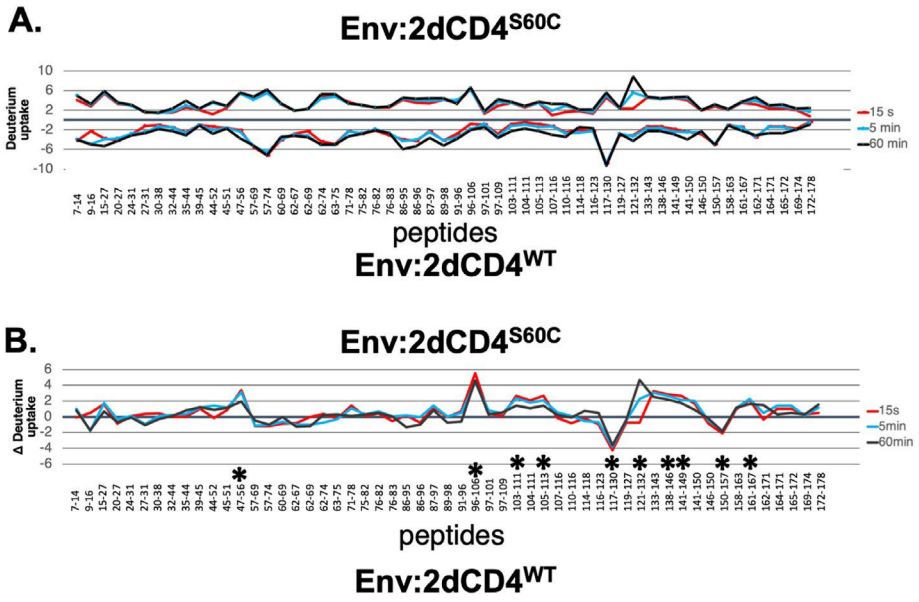

Fig. 1. Deuterium exchange profile of Env:2dCD4^{S60C} and Env:2dCD4^{WT} across all peptides. (A) Butterfly mirror and (B) difference plots comparing average deuterium incorporation for each peptide after 15s (red), 5 min (blue) and 60 min (black) deuteration. Lines above zero show uptake in Env:2dCD4^{S60C} while lines below zero show uptake for Env:2dCD4^{WT}. * denote peptides showing differences >2 Da. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Supplementary Figs. S3 and S4). Differences in the exchange rate of the D2 residues was extensive but concentrated in three regions: strands A and B with the loop between (region 1); strands C and E and a few adjoining residues (region 2); and strand G (region 3) (Figs. 1 and 3). Structurally, the amides in these residues do not appear to be involved in hydrogen bonding outside of the secondary structures where they reside, as such, electrostatic effects and solvent accessibility may be responsible for the protection seen in Env:2dCD4^{WT}. Noticeably, six peptides (117-130; 121-132; 138-146; 141-149; 150-157; and 161-167) displayed significant heterogeneity between Env:2dCD4^{S60C} and Env:2dCD4^{WT} (Fig. 1). Of these, 117-130 and 150-157 showed greater solvent exposure in Env:2dCD4^{WT} compared to Env:2dCD4^{S60C}.

The first region encompasses some of the residues discussed in the interdomain section above along with Leu116 whose solvent exposure occurred more rapidly in Env:2dCD4^{WT} than in Env:2dCD4^{S60C} (only at 15s), in contrast to the other changes seen in D2 (Supplementary Figs. S3 and S4). In addition, Thr111 was fully exposed (100% exchange) for the duration of the experiment in Env:2dCD4^{S60C} with full protection for this residue in the first two time-points for Env:2dCD4^{WT} (100% deuteration at 60 min). Similarly, Leu114 also supported full protection in Env:2dCD4^{WT} but was solvent exposed at 60 min for Env:2dCD4^{S60C} (Supplementary Figs. S3 and S4). This indicates that Env:2dCD4^{WT} sustained some level of protection in this region whereas Env:2dCD4^{S60C} exposed these residues except for Leu116 which remained anchored.

The second region includes residues surrounding Cys130 (a residue involved in a -RHstaple disulphide bond with Cys159 (Fig. 4) whose redox state dictates the functionality of CD4 [14,15,16]). No differences were seen for Cys130. Levels of deuteration in Arg131 and Ser132 was higher for the first two time-points in Env:2dCD4^{WT} compared to Env:2dCD4^{S60C} (Fig. 4), indicating structural protection in the covalent complex. Peptic fragments covering Cys159 were insufficient to conclusively determine its level of deuteration. Ser145, Ser147, and Glu150 were fully deuterated from 15s in Env:2dCD4^{S60C} while all three residues gained deuterons relatively slowly over time in Env:2dCD4^{WT} reaching 78% (Ser145), 67% (Ser147), and 100% (Glu150) deuterium uptake (Supplementary Figs. S3 and S4). This indicates Env:2dCD4^{WT} is protected in this D2 region whereas Env:2dCD4^{S60C} exposed these residues while maintaining the adjoining F strand static/shielded (Fig. 4).

The third region contains D2 β-strand G where several peptides show increased deuterium uptake in Env:2dCD4^{S60C} relative to Env:2dCD4^{WT} (Figs. 1, 3 and 4). Predominantly, Lys167 and Ile172 were fully deuterated in Env:2dCD4^{S60C} with gradual increases in deuteration in Env:2dCD4^{WT}. Leu177 displayed gradual deuteration for both Env:2dCD4^{S60C} and Env:2dCD4^{WT} with the covalent complex reaching full deuteration compared to the non-covalent complex only reaching 14% deuteration at the highest timepoint. Overall, the higher deuterium incorporation in the G strand on Env:2dCD4^{S60C} indicates higher solvent accessibility or enhanced flexibility compared to Env:2dCD4^{WT}. The lack of potential H- bonding interactions in this region indicates a general trend towards the G strand being less rigid or having a diminished burial depth in the Env:2dCD4^{S60C}.

From an immunological standpoint, Ibalizumab binds to CD4 D2 and of the four amino acids comprising the epitope (Glu77, Pro121, Pro122, Gln163), only Gln163 showed differential exchange- gradual deuteration in Env:2dCD4^{WT} (39%, 42%, 75%) compared to rapid exchange in Env:2dCD4^{S60C} (Figs. 1 and 2). This indicates that Gln163 is slightly more exposed in Env:2dCD4^{S60C}. The lack of difference in deuteration for the Ibalizumab epitope (aside from the Gln163 residue) between the two 2dCD4 variants indicates that the introduction of the covalent bond with 2dCD4^{S60C} did not structurally impede the presentation of the antigenic residues comprising the Ibalizumab epitope.

4. Discussion

HIV-1 vaccine design studies encourage the presentation of immunodominant epitopes that are conserved amongst clinically relevant circulating HIV-1 subtypes worldwide. Our previous studies revealed that covalently complexed Env:2dCD4^{S60C} resulted

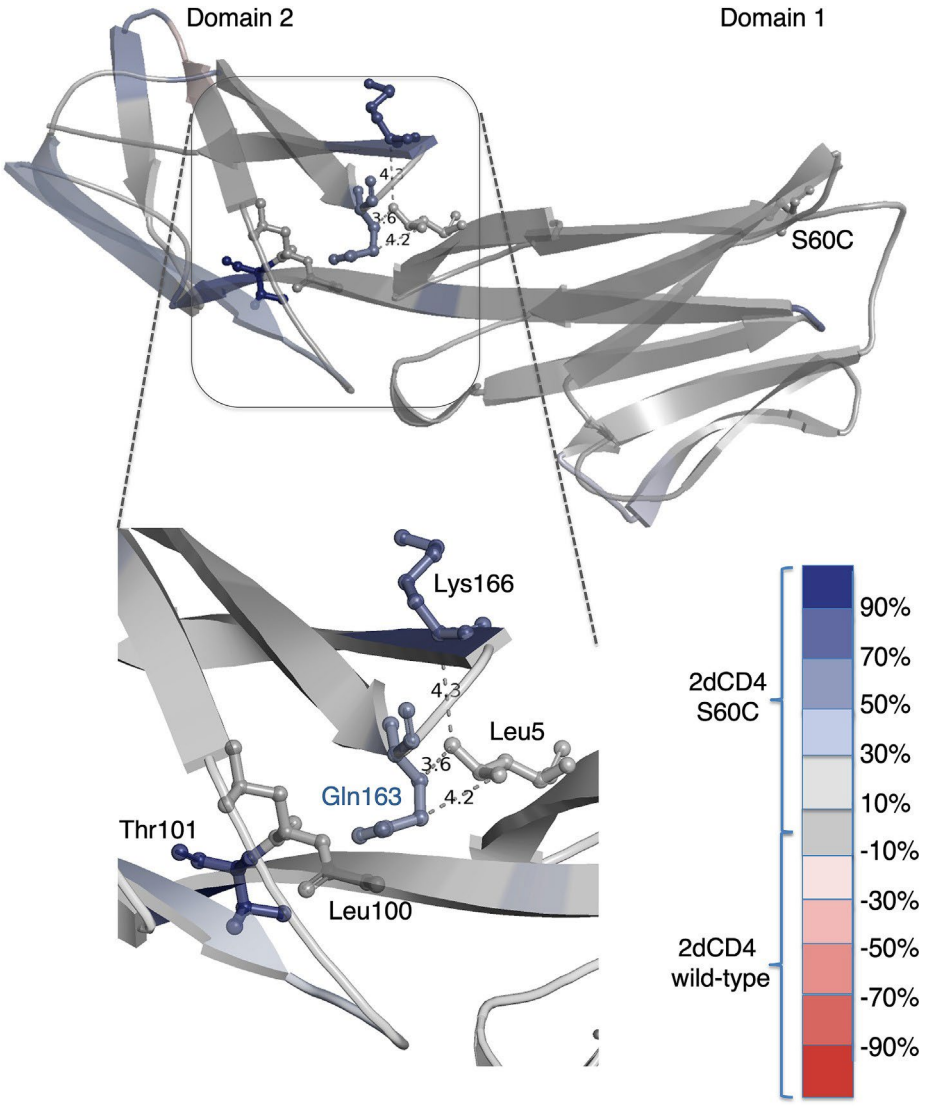

Fig. 2. Deuterium uptake differences between Env:2dCD4^{WT} and Env:2dCD4^{S60C} at the CD4 D1 and D2 interface. Relative deuteration (at 5 min) mapped onto the 2dCD4 crystal structure (PDB: 1CDJ**). Some residues are shown as ball-and-stick and labelled with amino acid and residue number. Distances between Leu5 in D1 and the closest D2 residues are shown. A colour gradient was used where red and blue represent higher relative deuteration in Env:2dCD4^{WT} and Env:2dCD4^{S60C}, respectively.** (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in highly desirable bNAb activity [13]. Whether the targeted epitopes are novel or presented differently in Env:2dCD4^{S60C} versus Env:2dCD4^{WT} remains to be determined and may involve an increase in the stability of the complex, a change in conformational landscape, or epitope enhancement/diversification through an undetermined mechanism.

This study compared the conformational dynamics of Env:2dCD4^{S60C} and Env:2dCD4^{WT}. HIV-1C gp140 Env complexed to 2dCD4^{S60C} shows improved immunogenicity over the 2dCD4^{WT} complex in rabbits [13]. We hypothesised that the disulphide linkage in Env:2dCD4^{S60C} impacted structure-function by changing specific states of the Env:2dCD4^{S60C} complex thereby exposing unique epitopes by inducing different conformational dynamics to the Env:2dCD4^{WT}.

2dCD4 has disulphide bonds in both D1 and D2. Reducing the metastable, allosteric D2 disulphide is necessary for Env binding, evidenced by the fact that it is impossible for Env to bind a CD4 molecule with an oxidized D2 disulphide [14,16]. Also, the D1 disulphide is a structural bond whose reduction has deleterious effects on CD4 structure [17]. The low concentration (1 mM) of β-mercaptoethanol used to facilitate exchange and formation of the Cys60-Cys126^{gp120} bond in Env:2dCD4^{S60C} was unlikely to alter the gp120 bound redox state of either D1 or D2 disulphides. If it had, a reduced D1 disulphide would result in a conformationally disrupted protein unable to interact with Env, while a reduced D2 disulphide was congruent with the binding of CD4 to gp120. Thus, addition of 1 mM β-mercaptoethanol to Env:2dCD4^{S60C} has not incurred changes to the complex outside of facilitating the Cys60-Cys126^{gp120} disulphide link.

Published studies examining dynamics and immunogenicity of gp120:CD4 complexes have focused on gp120 [18-20]. By contrast, this study evaluated the structural dynamics of 2dCD4 in the context of covalent and non-covalent binding to an HIV-1C SOSIP.664 gp140. Analysis did not extend to the Env portion due to low peptide coverage for those regions. Another acknowledged limitation in the experimental setup was the failure to include unliganded 2dCD4$^{WT/S60C}$ as controls. Substantial information is available on the conformational dynamics of unliganded 2dCD4,

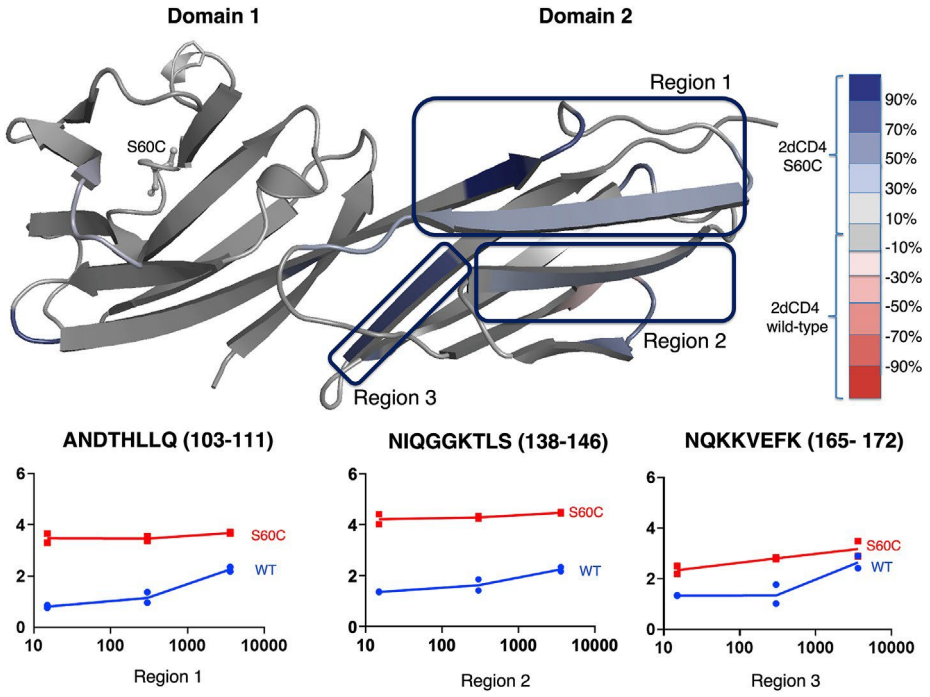

Fig. 3. Deuterium uptake differences between Env:2dCD4^{S60C} and Env:2dCD4^{WT} after 5 min labelling mapped onto the 3D structure of 2dCD4. Regions with the highest amount of variations in deuterium incorporation are boxed and labelled. Selected plots showing relative deuterium content for representative peptic fragments are shown. The 2dCD4^{WT} curves are shown in blue and 2dCD4^{S60C} curves in red. Each replicate bullet point is represented. Position of S60C is shown in ball and stick. For the ribbon structure, the colour scale represents change in percentage deuteration from Env:2dCD4^{S60C} to Env:2dCD4^{WT} with higher relative deuteration indicated in red in Env:2dCD4^{S60C} and blue in Env:2dCD4^{WT}. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

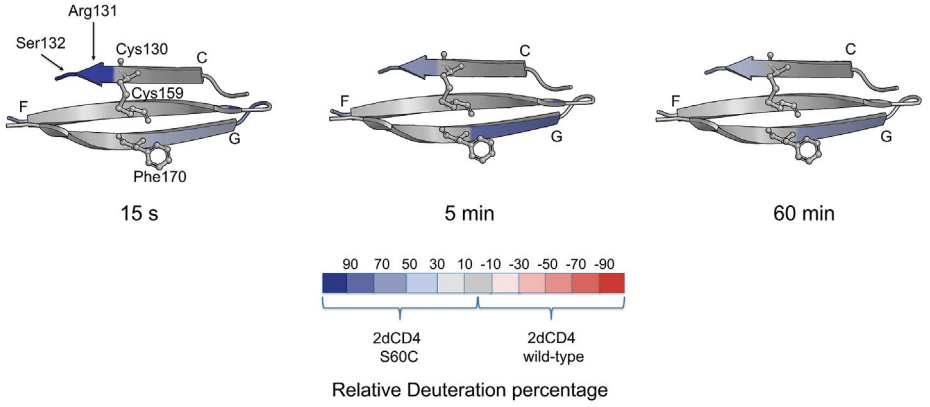

Fig. 4. Deuterium incorporation differences in the C, F, and G strands of D2 for Env:2dCD4^{S60C} and Env:2dCD4^{WT}. β-strands (labelled C, F and G) are depicted with cysteine residues making up the allosteric bond and the G-strand phenylalanine represented in ball-and-stick format. Also labelled are C-strand residues Arg131 and Ser132. Colours show difference in deuteration according to the scale, with higher relative deuteration in red in Env:2dCD4^{WT} and blue in Env:2dCD4^{S60C}. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

including the effects of D1 and D2 disulphide ablation on the structural conformation of 2dCD4 [14,17,21]. As such, this study focused on the differences observed between Env:2dCD4^{WT} and Env:2dCD4^{S60C} for the CD4 component, in the context of an intact D1 disulphide and a reduced D2 disulphide.

Most functionally relevant residues displayed little to no change between Env:2dCD4^{S60C} and Env:2dCD4^{WT}. This was the case for Lys35 and Phe43 (a residue involved in binding gp120) had slight differences in deuteration, although deuteration was high in this residue for both Env:2dCD4^{S60C} or Env:2dCD4^{WT}. Thus, the formation of the directed disulphide bond, likely does not affect the integrity of interaction between gp120 and 2dCD4.

Most differences in dynamics between Env:2dCD4^{S60C} and Env:2dCD4^{WT} were seen in D2. D1 had minor differences observed in a single peptide whose comparatively small fluctuations may be indicative of changes prompted by the introduction of the cysteine at position 60 and the resultant covalent linkage. These oscillations were propagated through the network of interactions across the length of the protein with changes in interdomain, bridging residues (Fig. 2). This assumedly resulted in the evident alterations seen in D2. Changes in D2 were evidenced by its acquired solvent exposure and deduced enhanced flexibility (Supplementary Fig. S5).

The redox biology of CD4 in the context of Env binding is well characterised [14,15,16]. D2 of CD4 has an unusual Cys130-Cys159 allosteric disulphide bond that connects adjacent strands [16,22],

and is reduced natively by thioredoxin secreted on $CD4^+$ T-cells [15]. Reduction of the Cys130-Cys159 disulphide causes the β-strands surrounding it to slightly collapse inwardly leading to a spatially diminished, stable molecule with decreased dynamics [21]. Both protein complexes were size-exclusion purified as 2dCD4-bound SOSIP.664 trimer complexes and as such, the D2 Cys130-Cys159 disulphide would have been reduced in both as required for gp120 binding. The lack of dynamic changes for Cys130 in Env:2dCD4^{S60C}, combined with the changes for the two residues directly following, is interesting as it indicates that disruption by the Cys60-Cys126^{gp120} covalent bond had no detectable effect on Cys130 yet caused a shift in the network of residues directly following Cys130. Thus the S60C-induced covalent bond may act as a stabilizing disulphide, allowing the shielding of some residues while exposing surrounding ones for a more dynamic presentation of additional conformations. Thus Env:2dCD4^{S60C} presents the 2dCD4 component in its reduced isoform (similar to Env:2dCD4^{WT}) but the covalent complex displays the increased dynamics of an oxidized D2 disulphide, generating an attractive vaccine immunogen target. Flexibility of D2 is well described in the context of gp120 binding and the high dynamics of D2 observed in Env:2dCD4^{S60C} was fitting for this flexible domain. These changes may be directly causative or may contribute to the difference in immunogenicity of Env:2dCD4^{S60C} compared to Env:2dCD4^{WT}.

Results imply that covalently linking 2dCD4^{S60C} with Env resulted in effective stabilization of conformation(s) that, albeit more transiently present in Env:2dCD4^{WT} may cause prolonged exposure of epitopes in Env:2dCD4^{S60C}. The potent bNAb responses from rabbits immunized with Env:2dCD4^{S60C} were largely attributed to the 2dCD4-directed component [13], in agreement with the abovementioned hypothesis. Thus, covalently linking the two molecules further increases the dynamics and conformational flexibility of D2 in 2dCD4, conducive with the generation of bNAbs that are potentially directed against novel 2dCD4^{S60C} epitopes.

Ibalizumab is a humanized Mab, a CD4-directed post-attachment HIV-1 inhibitor that is FDA approved for use in treatment-experienced adults with multidrug resistant HIV-1 infection. The Ibalizumab epitope is formed by amino acid residues in D2 of CD4: Leu96, Pro121, Pro122 and Gln163 as well as Glu77 and Ser79 in D1, opposite the site where gp120 and the MHC class II molecule bind in D1. We hypothesize the potent, bNAb activity elicited by our Env:2dCD4^{S60C} immunogen is Ibalizumab-like. The HDX-MS data supports this hypothesis by confirming the stable exposure of relevant Ibalizumab epitopes yet a disruption of networks surrounding this epitope in Env:2dCD4^{S60C}. This result was slightly unexpected as residues involved in intermolecular interactions with binding partners tend to be solvent exposed, enabling hydrogen binding and proton transfer. However, maintaining the integrity of the Ibalizumab epitope and the interface for binding partners such as HIV-1 gp120 and MHCII suggests the immunogen would not likely cause immunosuppression.

We set out to determine differences in conformational dynamics of Env:2dCD4^{S60C} and Env:2dCD4^{WT}. In this scenario, the S60C-assisted covalent bond triggers a network of changes that slightly disrupts D1 stability but increases the dynamics of D2 of 2dCD4. These results highlight the conformational dynamics differences between Env:2dCD4^{S60C} and Env:2dCD4^{WT} and reveal the structural mechanisms behind these novel CD4-bound immunogens which consistently induced potent, HIV-specific bNAbs in preclinical testing. Our findings provide important insights into the highly

Funding

Research was supported by grants from the Department of Molecular Medicine and Haematology, the Poliomyelitis Research Foundation, and the South African National Research Foundation through a Thuthuka grant.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.04.101.

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Author Contributions. Experimental setup and analysis, N.T. and P.N.; Conceptualization, G.O., N.T., P.N., and S.S. Funding acquisition, G.O., M.P., and N.T.; Supervision, G.O., M.P., and M.K.; Validation, P.N., G.O., M.K., M.P. and N.T.; Writing (original draft), NT; writing (review and edits), G.O., P.N., M.P., S.S., M.K. and N.T. All authors have read and agreed to the final version of the manuscript.

Addendum:

The low peptide coverage obtained for the Env portion of the complexes could be improved by optimizing the quenching conditions which greatly impact digest results and thus has a direct effect on the sequence coverage. Optimization would involve buffer composition and pH adjustments.

Article 1 supplementary:

Supplementary Methods

Reagents and Recombinant plasmids used in this study

The 2dCD4^{WT} and 2dCD4^{S60C} recombinant plasmids were used for protein expression in *Escherichia* (*E.)* coli BL21 (DE3) (Novagen), as described previously (Cerruti *et al.,* 2010). Two domain CD4 represents a truncated version of UniProtKB entry P01730 (four domain CD4). HEK-293S Gnt I$^{-/-}$ mammalian cell lines (ATCC^{Ò} CRL-3022) which are deficient in the glycosyl transferase I enzyme, were used for the expression of partially deglycosylated recombinant HIV-1 Env. A codon optimized HIV-1 subtype C SOSIP.664 gp140 construct based on the previously described consensus Founder Virus subtype C (FVC) sequence (Killick *et al.,* 2014) was used to design an HIV-1C SOSIP.664 gp140 including the trimer stabilizing I559P and disulphide bridge forming A501C/T605C mutations, and the gp120 C-terminus hexa-arginine motif (Binley *et al.,* 2000; Sanders *et al.,* 2002; Binley *et al.,* 2002; Klasse *et al.,* 2013). HIV-1C SOSIP.664 gp140 was synthesized by GeneArt (Regensburg, Germany) and cloned into the pCDNA3.3 expression vector (Invitrogen, CA, USA) to create pNTFV$_{SOSIP.664}$. The human furin sequence (NCBI accession number (X17094.1) was codon optimized, synthesized by GeneArt (Regensburg, Germany) and cloned into the pCDNA3.3 expression vector (Invitrogen, CA, USA) to create pNT$_{furin}$. Co-transfection of the Furin-encoding plasmid optimizes cleavage of the HIV-1C SOSIP.664 gp140 into the gp120 and gp41 components, ensuring optimal protein folding (Binley *et al.,* 2000; Binley *et al.,* 2002).

Recombinant protein expression and purification

Recombinant his-tagged 2dCD4^{WT} and 2dCD4^{S60C} proteins were expressed and purified using nickel affinity chromatography followed by oxidative refolding as described previously

(Cerruti *et al.,* 2014). The $pNTFV_{SOSIP.664}$ and and pNT_{furin} were used to transiently co-transfect HEK-293 Gnt $I^{-/-}$ mammalian cells (Kwon *et al.,* 2015). Supernatant containing soluble, cleaved HIV-1C SOSIP.664 gp140 Env was collected every 48 hours for a total of six days. Supernatant containing soluble proteins were centrifuged at 3220g for 10 min at 4°C to remove cell debris, filtered through a 0.22 mm polyethersulfone membrane (Merck Millipore, Darmstadt, Germany), and stored at -20 ºC until use.

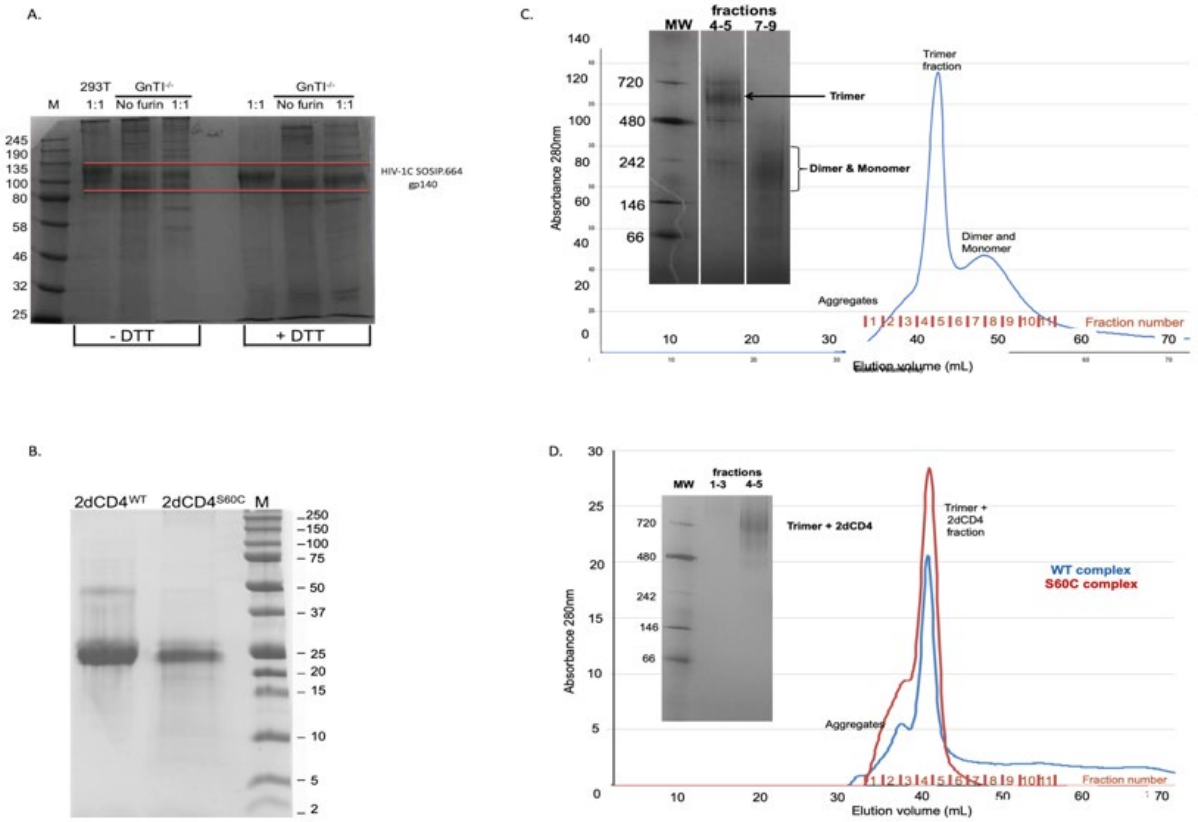
The pooled, clarified supernatant was incubated overnight with *Galanthus nivalis* lectin agarose (Sigma-Aldrich, Sweden) at 4 °C, while stirring, then passed through an empty luer-lock, non-jacketed liquid chromatography column (dimensions: 2.5 x 10 cm; Sigma-Aldrich, St. Louis, MO, USA). The recombinant Env bound resin was then washed with 20 column bed volumes (±80 ml) of 1 x PBS containing 1 M NaCl, followed by two additional washes of the same volume with 0.5 M NaCl in PBS, then PBS only. Recombinant Env was subsequently eluted by incubating for 30 min with 50 ml 1 M methyl-a-D-mannopyranoside (MMP, Sigma-Aldrich, Steinheim, Germany). Eluted SOSIP.664 gp140 were concentrated with a 50 kDa molecular weight cut-off Amicon Ultra-50 (Sigma-Aldrich, Steinheim, Germany) concentrator and washed three times (50 ml/wash) with 1 x Dulbecco PBS. Concentrated SOSIP.664 gp140 was further purified by gel filtration using a Hiload Superdex (26/60) S-200 size-exclusion chromatography (SEC) column (Amersham) with an AKTA FPLC (GE Healthcare Life Sciences, New York, USA). The SEC column was equilibrated in Dulbecco 1 x PBS buffer at a flow-rate of 1 mL/min, trimer fractions were collected and concentrated using a 50 kDa molecular weight cut-off Amicon Ultra-50 (Sigma-Aldrich, Steinheim, Germany) concentrator, washed three times (50 ml/wash) with 1 x PBS and quantified with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford IL, USA), according to manufacturer's instructions. The SOSIP.664 gp140 expression, size and identity were confirmed by SDS-PAGE, Western blot and blue-native (BN) PAGE analyses.

Preparation of Env:2dCD4 complexes was conducted as follows: 4-fold molar excess of purified $2dCD4^{WT}$ or $2dCD4^{S60C}$ was added to the partially deglycosylated HIV-1C SOSIP.664 gp140 for 60 minutes at room temperature. For the covalently-linked complex, a 4-fold molar excess of $2dCD4^{S60C}$ in the presence of a reducing agent 1 mM b-Mercaptoethanol (β-Me) was added to the partially deglycosylated HIV-1C SOSIP.664 gp140 (hereafter referred to as Env:$2dCD4^{S60C}$). The β-ME concentration was previously determined to allow an equilibrium of complex formation without the deleterious breakage of the newly-formed disulphide bond (Cerruti *et al.,* 2010). Partially-deglycosylated Env:$2dCD4^{S60C}$ complexes were purified by gel filtration chromatography (Hiload 26/60 Superdex S-200; Amersham), as described above, to remove excess CD4.

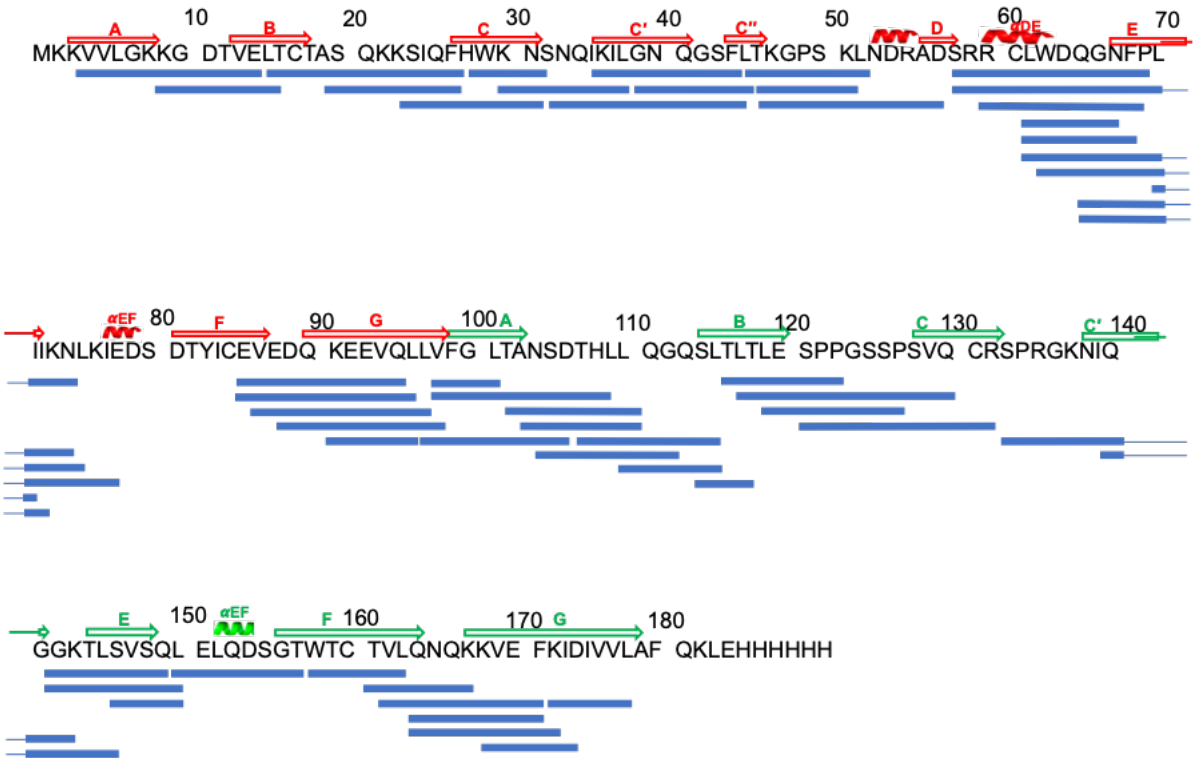
Hydrogen-deuterium exchange mass spectrometry (HDX-MS) method can be found in main text

Data analysis

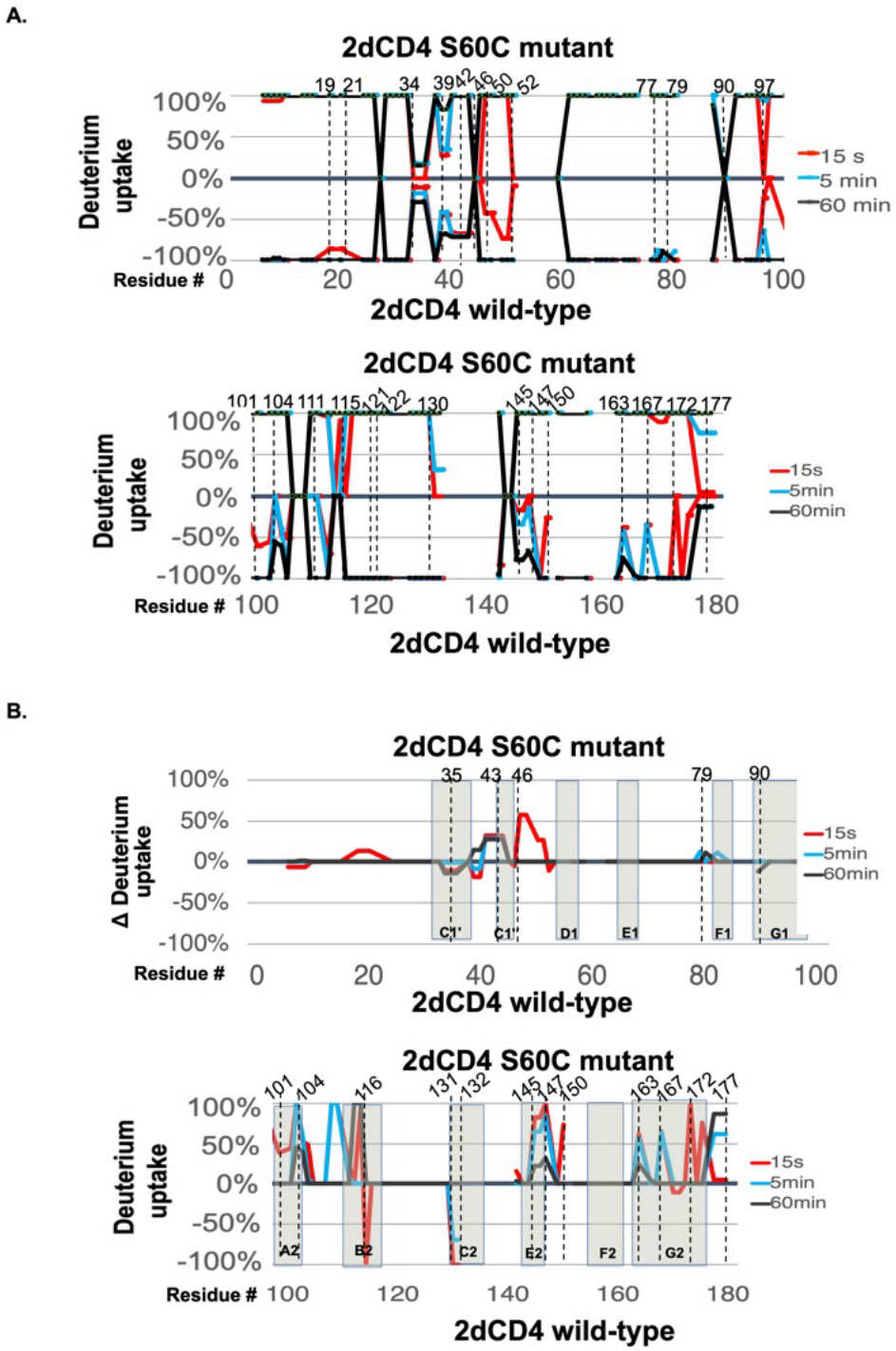
The quality of the peaks and the frequency of deuterium incorporation was measured in duplicate for each time-point and for each peptide of the Env:$2dCD4^{WT}$ or Env:$2dCD4^{S60C}$ complexes using the HDExaminer 1.3 software (Sierra Analytics, Modesto, CA). The average frequency of peptides was determined, mapped onto the protein sequence and used to obtain the distributions of exchange using a maximum entropy method. The theoretical percentage exchange of each peptic fragments in light of their primary sequence, also known as the intrinsic exchange, was automatically calculated, whereas the weighted average exchange was manually determined over the exchange rates distribution for each peptide. Percentage exchange were mapped onto the crystal structure of 2dCD4 (PDB ID: 1CDJ) using PyMOL (Molecular Graphics System, DeLano Scientific system, LLC, Palo Alto, CA).



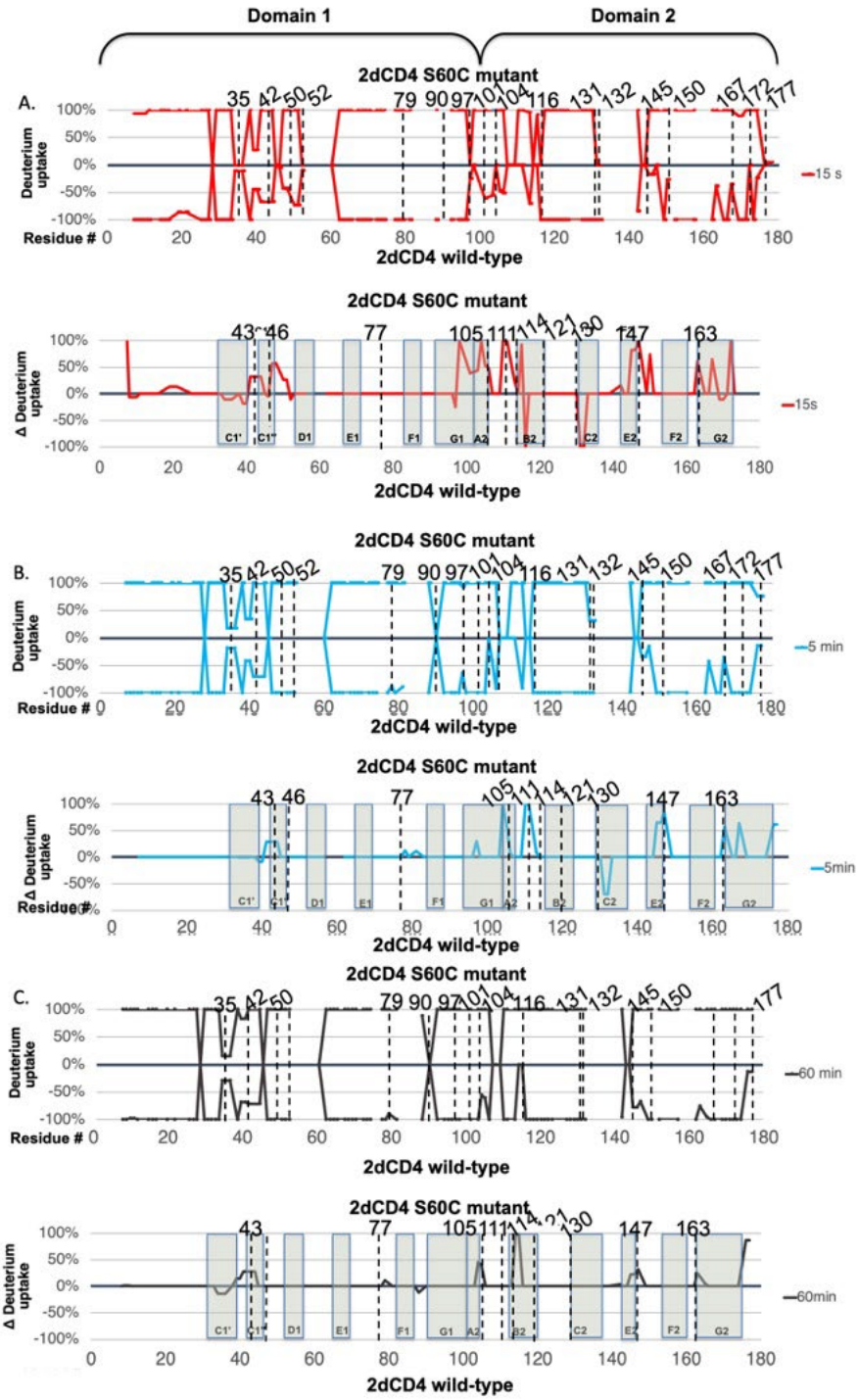
Supplementary Figure S1. Purification and characterization of HIV-1C SOSIP.664 trimer. (A) SDS-PAGE analysis of lectin-purified HIV-1C SOSIP.664 trimer under non-reducing (- DTT) and reducing (+ DTT) conditions, followed by Coomassie blue R250 staining. Ratio of Furin to Env plasmid used for the transfection is indicated. M- Prestained protein standards, broad range (Thermo Fisher Scientific, MA, USA). (B) SDS-PAGE analysis of 2dCD4 proteins post-purification and refolding. Representative size exclusion profiles and blue native (BN)-PAGE gels of homogeneously glycosylated HIV-1C SOSIP.664 trimer expressed in HEK-293S Gnt $I^{-/-}$ cells, before (C) and after (D) complexing with $2dCD4^{WT}$ and $2dCD4^{S60C}$. MW- Nativemark™ Unstained Protein Standard (Thermo Fisher Scientific, MA, USA).



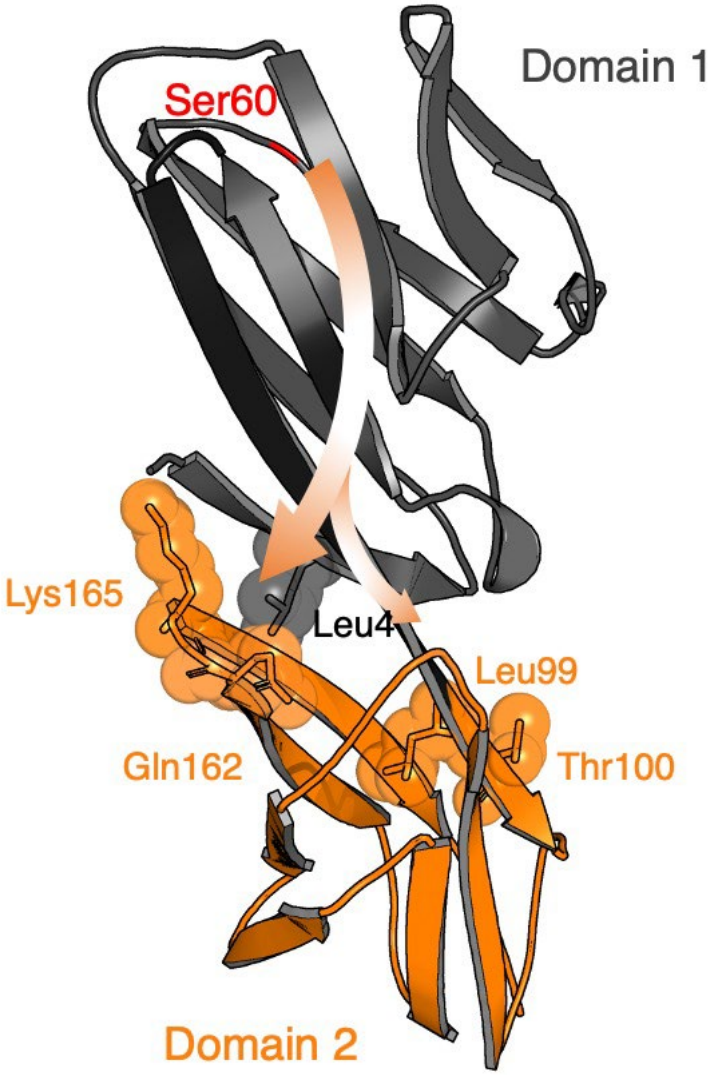
Supplementary Figure S2. HDX peptide coverage map of 2dCD4. Sequence coverage showing identified peptic fragments in blue with secondary elements identified above the sequence in red for domain 1 and in green for domain 2.



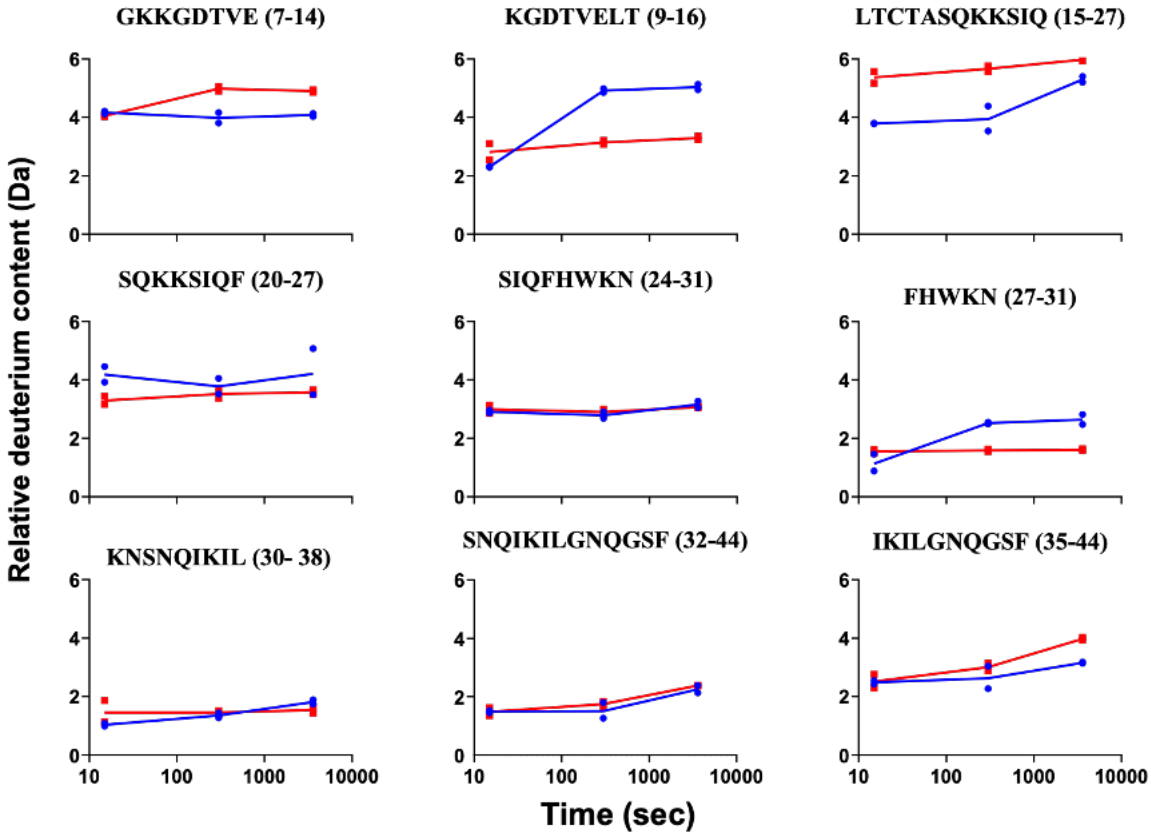
Supplementary Figure S3. Deuterium exchange profile and important structural elements of 2dCD4^{S60C} and 2dCD4^{WT} complexed to HIV-1C SOSIP.664 gp140 (Env). Butterfly mirror plots comparing relative deuterium incorporation as percentage deuteration for each peptide along the 2dCD4 protein primary sequence (top) and difference plot in deuterium incorporation between 2dCD4^{S60C} and 2dCD4^{WT} (bottom) at 15 s (A), 5 min (B) and 60 min (C). For all, the lines above zero show uptake in the Env:2dCD4^{S60C} while the lines below zero show uptake for the corresponding Env:2dCD4^{WT}. Residues specifically mentioned in the text are indicated above the plot with vertical dotted lines to demarcate their position. Selected b-strands are shown on the deuterium difference plot as grey boxes with the strand notation and domain indicated (for example, C1β refers to b strand Cβ of domain 1). Individual curves of this figure represented in supplementary figure S3.

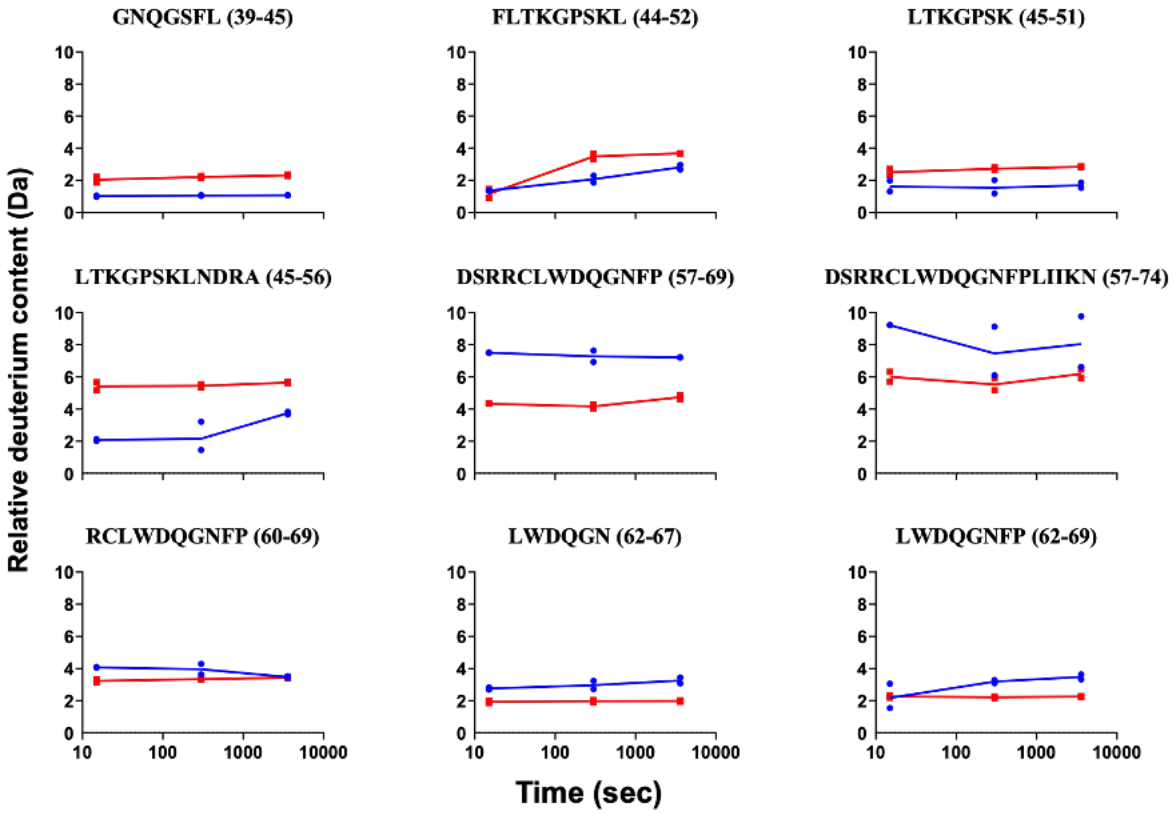


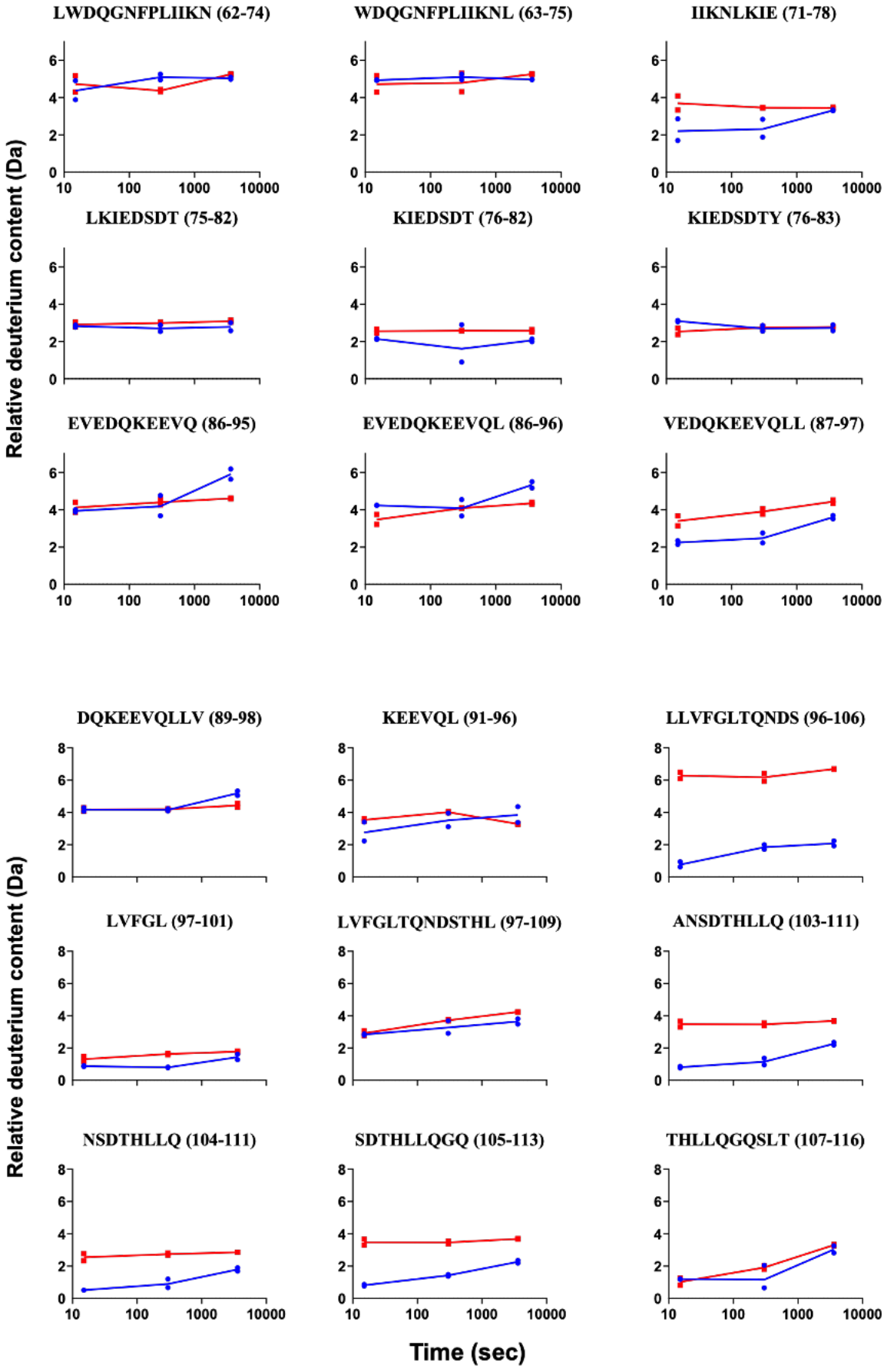
Supplementary Figure S4. Deuterium exchange profile and important structural elements of 2dCD4^{S60C} and 2dCD4^{WT} complexed to HIV-1C SOSIP.664 gp140 (Env). Butterfly mirror plots comparing relative deuterium incorporation as percentage deuteration for each peptide along the 2dCD4 protein primary sequence (top) and difference plot in deuterium incorporation between 2dCD4^{S60C} and 2dCD4^{WT} (bottom) after 15 s (A), 5 min (B) and 60 min (C) deuteration. For all, the lines above zero show uptake in the Env:2dCD4^{S60C} while the lines below zero show uptake for the corresponding Env:2dCD4^{WT}. Residues specifically mentioned in the text are indicated above the plots with vertical dotted lines to demarcate their position. β-strands are shown on the deuterium difference plots as grey boxes with the strand notation and domain indicated (for example, C1′ refers to β-strand C′ of domain 1).

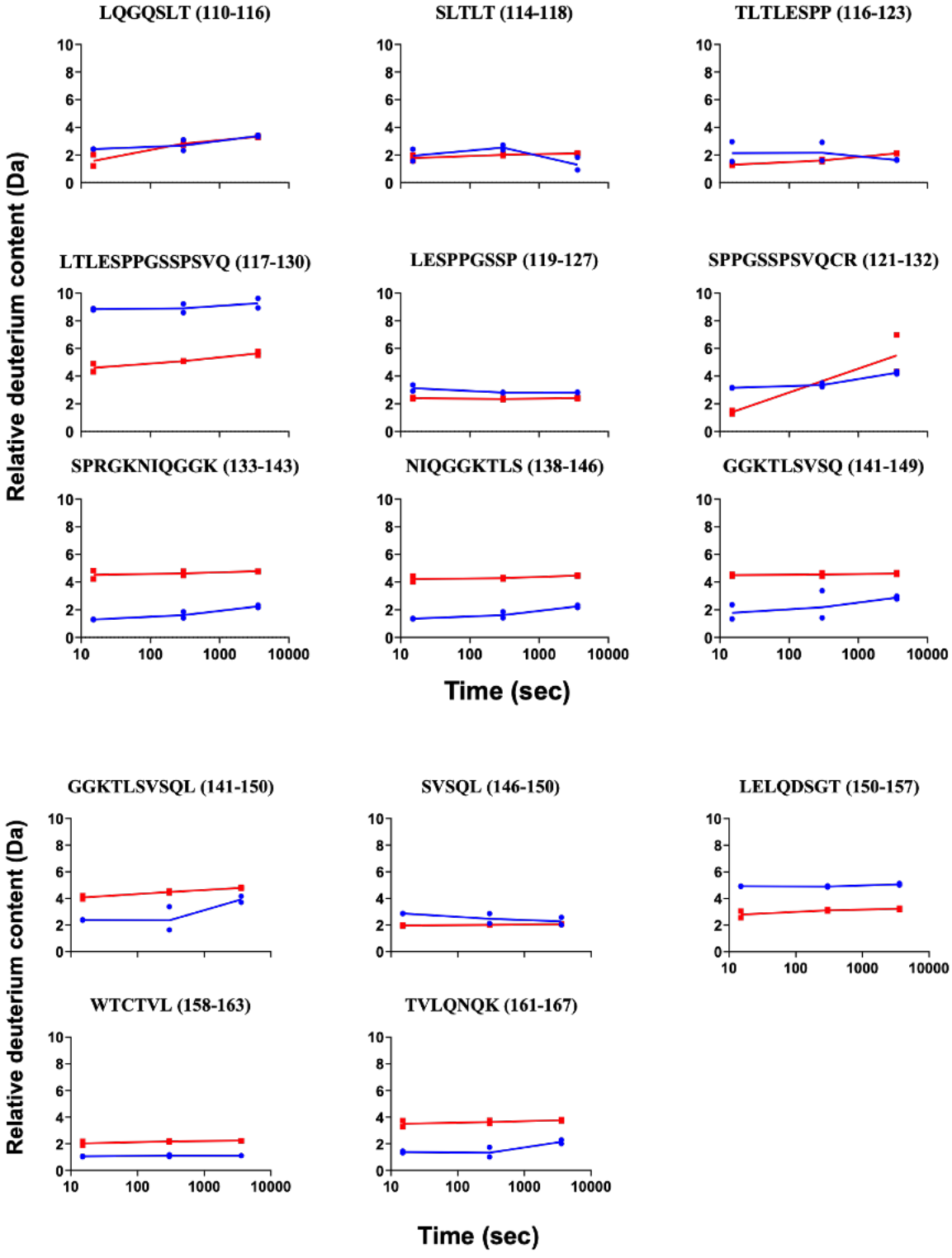


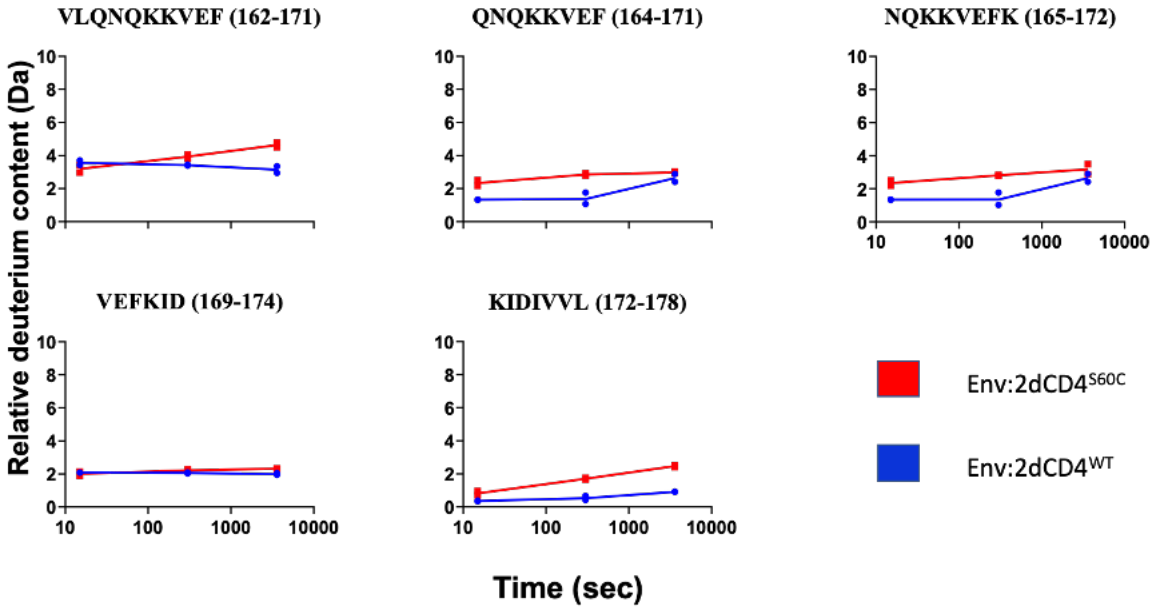
Supplementary Figure S5. Proposed model of the dissemination of changes from the Cys60-Cys126^{gp120} covalent bond through the network of interacting residues to domain 2. The premise of this model resides in the propulsion of changes created by covalently linking 2dCD4^{S60C} to HIV-1 Env. The increased dynamics seen in domain 2 could be explained by a cascade of changes from Cys60 which likely propagates through domain 1 and is transmitted across the domains via the interdomain and bridging residues (orange transparent spheres; arrows). This model assumes the cascade travels through a network of interacting residues that include subtle changes to residues internal to the protein that either could not be readily assessed and detected by HDX-MS or do not involve changes to their dynamics.


GKKGDTVE (7-14)
KGDTVELT (9-16)
LTCTASQKKSIQ (15-27)
SQKKSIQF (20-27)
SIQFHWKN (24-31)
FHWKN (27-31)
KNSNQIKIL (30- 38)
SNQIKILGNQGSF (32-44)
IKILGNQGSF (35-44)
Relative deuterium content (Da)
Time (sec)


GNQGSFL (39-45)
FLTKGPSKL (44-52)
LTKGPSK (45-51)
LTKGPSKLNDRA (45-56)
DSRRCLWDQGNFP (57-69)
DSRRCLWDQGNFPLIIKN (57-74)
RCLWDQGNFP (60-69)
LWDQGN (62-67)
LWDQGNFP (62-69)
Relative deuterium content (Da)
Time (sec)


LWDQGNFPLIIKN (62-74)
WDQGNFPLIIKNL (63-75)
IIKNLKIE (71-78)
LKIEDSDT (75-82)
KIEDSDT (76-82)
KIEDSDTY (76-83)
EVEDQKEEVQ (86-95)
EVEDQKEEVQL (86-96)
VEDQKEEVQLL (87-97)
DQKEEVQLLV (89-98)
KEEVQL (91-96)
LLVFGLTQNDS (96-106)
LVFGL (97-101)
LVFGLTQNDSTHL (97-109)
ANSDTHLLQ (103-111)
NSDTHLLQ (104-111)
SDTHLLQGQ (105-113)
THLLQGQSLT (107-116)
Relative deuterium content (Da)
Relative deuterium content (Da)
Time (sec)


LQGQSLT (110-116)
SLTLT (114-118)
TLTLESPP (116-123)
LTLESPPGSSPSVQ (117-130)
LESPPGSSP (119-127)
SPPGSSPSVQCR (121-132)
SPRGKNIQGGK (133-143)
NIQGGKTLS (138-146)
GGKTLSVSQ (141-149)
GGKTLSVSQL (141-150)
SVSQL (146-150)
LELQDSGT (150-157)
WTCTVL (158-163)
TVLQNQK (161-167)
Relative deuterium content (Da)
Time (sec)



Supplementary Figure 6. Deuterium uptake curves for all peptides. HDX-MS analysis of 2dCD4 in complex with HIV-1C SOSIP.664 gp140, comparing the covalently bound 2dCD4^{S60C} to the non-covalently bound 2dCD4^{WT}. Deuterium uptake curves for all peptides are shown in red for Env:2dCD4^{S60C} and blue for Env:2dCD4^{WT}. The change in centroid mass of each deuterated peptide, compared to its undeuterated equivalent, was used to plot the relative deuterium content incorporated using the HDExaminer software v1.3. Residue numbers are indicated at the top of each plot. Replicates from two independent experiments are plotted for each peptide.

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Chapter 3

Immunization with HIV-1 trimeric SOSIP.664 BG505 or FVCEnv (Founder Virus C) covalently complexed to two-domain $CD4^{S60C}$ elicits cross-clade neutralizing antibodies in New Zealand white rabbits.

Published in *Vaccine: X*, Volume 12, 2022, ISSN 2590-1362.

Immunization with HIV-1 trimeric SOSIP.664 BG505 or Founder Virus C (FVC_{Env}) covalently complexed to two-domain $CD4^{S60C}$ elicits cross-clade neutralizing antibodies in New Zealand white rabbits

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ABSTRACT

Background: An ongoing challenge in HIV-1 vaccine research is finding a novel HIV-1 envelope glycoprotein (Env)-based immunogen that elicits broadly cross-neutralizing antibodies (bnAbs) without requiring complex sequential immunization regimens to drive the required antibody affinity maturation. Previous vaccination studies have shown monomeric Env and Env-GCN4 trimers which contain the GCN4 leucine zipper trimerization domain and are covalently bound to the first two domains of CD4 ($2dCD4^{S60C}$) generate potent bnAbs in small animals. Since SOSIP.664 trimers are considered the most accurate, conformationally intact representation of HIV-1 Env generated to date, this study further evaluated the immunogenicity of SOSIP.664 HIV Env trimers (the well characterized BG505 and FVC_{Env}) covalently complexed to $2dCD4^{S60C}$. *Methods:* Recombinant BG505 SOSIP.664 and FVC_{Env} SOSIP.664 were expressed in mammalian cells, purified, covalently coupled to $2dCD4^{S60C}$ and

antigenically characterized for their interaction with HIV- bnAbs. The immunogenicity of BG505 SOSIP.664-2dCD4^{S60C} and FVC$_{Env}$ SOSIP.664-2dCD4^{S60C} was investigated in New Zealand white rabbits and compared to unliganded FVC$_{Env}$ and 2dCD4^{S60C}. Rabbit sera were tested for the presence of neutralizing antibodies against a panel of 17 pseudoviruses. *Results:* Both BG505 SOSIP.664-2dCD4^{S60C} and FVC$_{Env}$ SOSIP.664-2dCD4^{S60C} elicited a potent, HIV-specific response in rabbits with antibodies having considerable potency and breadth (70.5% and 76%, respectively) when tested against a global panel of 17 pseudoviruses mainly composed of harder-to-neutralize multiple clade tier-2 pseudoviruses. *Conclusion:* BG505 SOSIP.664-2dCD4^{S60C} and FVC$_{Env}$SOSIP.664-2dCD4^{S60C} are highly immunogenic and elicit potent, broadly neutralizing antibodies, the extent of which has never been reported previously for SOSIP.664 trimers. Adding to our previous results, the ability to consistently elicit these types of potent, cross-neutralizing antibody responses is dependent on novel epitopes exposed following the covalent binding of Env (independent of sequence and conformation) to 2dCD4^{S60C}. These findings justify further investment into research exploring modified open, CD4-bound Env conformations as novel vaccine immunogens.

Keywords: HIV-1 vaccine immunogens; envelope glycoprotein trimers; covalent complexes; broadly neutralizing antibodies; New Zealand White rabbits; immunogen design

INTRODUCTION

The HIV/AIDS pandemic has now continued for four decades, with approximately 37.7 million individuals living with HIV globally, and 1.5 million new infections reported in 2020 [1]. While these numbers have vastly decreased from 1997 when the new infections peaked at 2.9 million [1], a decisive halt to this spread can only be achieved with an effective prophylactic vaccine. The broad diversity of HIV-1 has contributed to the difficulty in identifying an Env-

based vaccine immunogen capable of inducing immunity against all circulating HIV-1 groups and clades. To address viral diversity, researchers have tried to design immunogens that direct the immune responses towards regions of the viral envelope glycoprotein (Env) that are highly conserved, such as the CD4 receptor binding site (CD4bs), the gp120-gp41 interface, the V1V2 trimer apex, and the membrane proximal external region (MPER) [2]. A number of broadly neutralizing antibodies (bnAbs) that target these conserved sites have been isolated from HIV-infected individuals thereby showing the possibility of their induction in humans. However, if HIV-infected individuals develop bnAbs, it is too late to impact on disease progression. By contrast, their neutralizing potential, and their ability to protect against infection in animal challenge models has also been studied [3-5], providing evidence of the importance of preexisting humoral immune responses in preventing transmission.

In an effort to improve immunogens, the HIV-1 vaccine research field has made use of multiple techniques such as mutations, cleavage site disruption and the introduction of trimerization domains to create stabilized, recombinant HIV-1 Env trimers [6, 7]. This gave rise to SOSIP trimers, the first Env-based immunogens with antigenic and topological structures that best represent the native HIV-1 virion trimeric spike [7-9]. When assessed in rabbits, the stabilized SOSIP trimers induce autologous neutralizing antibodies as well as neutralization of the more sensitive Tier-1 viruses [10]. Env trimers can be stabilized in the closed, prefusion conformation or the open, post-CD4 engaged state. These different Env conformations expose diverse epitopes and ultimately impact the elicitation of neutralizing antibodies, relevant for HIV-1 vaccine design [10, 11]. Some of the broadest and most potent antibodies characterized from HIV-infected individuals target epitopes presented in the closed, prefusion conformation, when the trimer is in its quaternary fold. As a result, the majority of trimer and immunogen design efforts have been directed towards fastening the Env in the closed conformation, with

studies on SOSIP trimers being the most advanced. However, not much research has been directed towards the use of SOSIP trimers in the CD4-bound, open conformation.

It is believed that sites of vulnerability of HIV-1 may be contextual and that the different Env conformations (i.e., prefusion, CD4-bound intermediates, pre-hairpin intermediate, and postfusion) structurally expose different epitopes for antibodies to target [2]. The CD4-bound conformation seems to contain less neutralization epitopes than the closed, prefusion conformation of Env, evidenced by the fewer number of antibodies identified from natural infection that specifically target this state [2]. The CD4-bound Env conformation exists in two distinct states- when a single gp120 monomer is engaged by CD4 (asymmetric trimer) or when all three gp120 monomers are bound to CD4 (open trimer) [12]. The transient nature of these CD4-bound conformations may contribute to the lack of antibodies to these epitopes in natural infection [13].

Transmitted/founder (TF) HIV-1 virions are defined as the viruses that establish productive infection following mucosal transmission [14, 15]. They exhibit certain characteristics such as shorter variable loops, less putative N-linked glycans, enhanced interactions with dendritic cells, better infectivity as cell-free virions, and a preference for the chemokine receptor 5 (CCR5) [14]. Of interest, the shorter variable loops and reduced number of glycans have been linked to a modest increase in susceptibility to neutralizing antibodies [14, 16]. Furthermore, TF viruses have displayed better interaction with the integrin pair $\alpha4\beta7$ compared to chronic viruses [17]. $CD4^+$ T-cells that express $\alpha4\beta7$ are preferentially targeted in acute infection [18] and the binding of TF viruses to them could result in more efficient virus entry as well as cell-to-cell spread. The differences inherent in TF viruses compared to chronic ones highlight the significant role of TF viruses in HIV-1 infection and the need to develop immunogens that elicit antibodies able to recognize these ‘fitter’ viruses.

Previous work using an Env immunogen composed of the consensus sequence obtained from over 1,800 subtype C TF envelopes [19]; namely, the Founder Virus C Env (FVC_{ENV}), covalently complexed to the first two domains of CD4 (2dCD4) via cysteine linkage through targeted intermolecular binding of gp120 to CD4 by introduction of a serine to cysteine substitution in CD4 [20] ($2dCD4^{S60C}$) gave rise to exceptional immunogens [21]. The former study made use of various Env conformations, from monomeric to leucine-zipper linked gp140 trimers, with the same outcome: all consistently elicited potent, cross-neutralizing antibody activity against clinically-relevant HIV-1 isolates in New Zealand white rabbits [21] and rhesus macaques (Pereira *et. al.* unpublished data).

Here we describe the SOSIP trimerization of FVC_{ENV} as compared to the well characterized BG505. SOSIP trimers constitute the best antigenic mimics of HIV-1 in its native state and, therefore, provide an excellent immunogen model. Moreover, by locking the BG505 and FVC_{ENV} SOSIP.664 trimer in the CD4-bound, open state by covalently complexing our SOSIP Env trimer to $2dCD4^{S60C}$, we interrogated whether the CD4-bound conformation of SOSIP trimers is antigenically compromised or would replicate and possibly improve on results seen with other SOSIP.664 and Env-$2dCD4^{S60C}$ conformations. Consequently, the immunogenicity of BG505 SOSIP.664-$2dCD4^{S60C}$ and FVC_{ENV} SOSIP.664-$2dCD4^{S60C}$ was evaluated in New Zealand white rabbits and the antisera response characterized and compared for neutralization potential.

MATERIALS AND METHODS

Ethics statement. Twenty female New Zealand white rabbits were maintained at the Wits Research Animal Facility (WRAF) in a pathogen-free environment, where all animal work was conducted in accordance with the Animal Research Ethics Committee (AREC) directives and National/International recommendations. Animal housing, care and the immunization and bleed protocols were all conducted in accordance with the institutional requirements set by

AREC and WRAF. All AREC comply with the South African National Standard on the care and use of animals for scientific research. Clearance was obtained from the Animal Ethics Screening Committee, University of the Witwatersrand (certification #: 2018/02/10B).

Recombinant plasmids description. The BG505 SOSIP.664 clone in the VRC3831 plasmid was kindly provided by Dr. Peter Kwong (VRC, NIH). The subtype C derived FVC *env* gene constructed from the consensus sequence of TF viruses [19] was used to incorporate the SOSIP.664 mutations: I559P, A501C, T605C, ^{508}REKR^{511} to R6, and MPER truncation at residue 664 [7]. The codon-optimized FVC_{ENV} SOSIP.664 clone was synthesized by GeneArt (Life Technologies, Regensburg, Germany) and cloned into the pCDNA3.3 expression vector (Invitrogen, Grand Island, NY). The plasmid containing the coding-sequence of the furin protease, i.e., pcDNA3.1-Furin, was obtained from Dr. J.P. Moore [8]. Two-domain CD4 (2dCD4) plasmids- pET15b-2dCD4$^{(WT/S60C)}$ were used for the expression of wild-type (WT) and S60C mutant versions of 2dCD4. The pET15b (Novagen, Germany) plasmids contain a 6x polyhistidine (His) tag at the 3′ terminus in frame with the 2dCD4 sequence to allow for Nickel affinity purification.

Mammalian cell lines and antibodies. The CD4- and CCR5- positive TZM-bl cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (ARRRP), (Contribution of Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc.). The HEK293T cells were obtained from the American Type Culture Collection, also through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. TZM-bl and HEK293T cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM Glutamax (Gibco/Invitrogen, Waltham, MA, USA), and 100 U/mL penicillin-streptomycin (Gibco/Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to prepare the complete

growth medium. Cells were incubated at 37 °C in 5% CO_2 and 80% relative humidity. Purified mAbs PG9 (Dr. D. Burton), VRC01 & VRC03 (Xueling Wu, Zhi-Yong Yang, Yuxing Li, Gary Nabel, John Mascola) [22], HJ16 (Dr. Antonio Lanzavecchia), and 10e8 (Dr. Mark Connors) were obtained from ARRRP. Expression plasmids encoding heavy- and light-chain sequences of the monoclonal antibodies 17b and PGT145 were a generous gift from Dr. Pascal Poignard (IAVI, Scripps Research Institute).

Subcloning of FVC_{Env} into the VRC3831 backbone. To improve the expression of FVC_{ENV} recombinant protein, the FVC_{ENV} coding region from the pCDNA3.3 plasmid was subcloned into the VRC3831 plasmid backbone using the Gibson Assembly cloning method (New England Biolabs, USA), according to manufacturer's instructions. The integrity of the resultant clones was verified by sequencing along the 5′- and 3′-end portions of the inserted gene using vector-specific primers (SeqinF: 5′TGTGATCAGATATCGCGG´3 and SeqinR: 5′AAGGCACTGGGGAGGGGC´3) with the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) using an automated ABI 3100 genetic analyzer. Chemically competent *E. coli* DH5α bacterial cells were transformed with the final DNA constructs as previously described [23]. Transformants were selected on Luria Bertani agar plates supplemented with 50 μg/mL kanamycin. Plasmid DNA was isolated and purified using QIAGEN™ plasmid purification kits (Qiagen, Maryland, USA) according to the manufacturer's instructions.

Expression and purification of Env SOSIP.664 trimers. HIV-1 Env were expressed in HEK293T cells following transient co-transfection with the Env plasmids and pcDNA3.1-Furin as described previously [22]. Supernatants containing the secreted Env recombinant proteins were clarified by centrifugation at low speed and filtration through a 0.22 μm filter. Purification of the SOSIP.664 trimers from the supernatant was performed using agarose beads-conjugated *Galanthus nivalis* lectin (Sigma-Aldrich) column chromatography with

affinity for mannose glycans, as previously described [24]. A second purification by gel filtration chromatography (HiPrep Sephacryl S-300; GE Healthcare, Piscataway, NJ) with an AKTA FPLC (GE Healthcare) was performed to isolate the trimers from aggregates, dimers, and monomers, followed by concentration using centrifugal filtration with a 50 kDa MWCO Amicon Ultra filter (Millipore, Billerica, MA, USA). The final protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL). The purity of expressed proteins was evaluated by reducing and non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blotting to confirm size and identity, as well as by blue native (BN)-PAGE to confirm the trimeric state.

Expression and purification of 2dCD4. Competent BL21* *E. coli* (DE3; Invitrogen) cells (New England Biolabs, Ipswitch, MA, USA) were transformed with pET15b-2dCD4$^{WT/S60C}$ plasmids using a standard protocol (Novagen, USA). Bacterial cells were propagated at 37 °C under ampicillin selection pressure in LB medium (1% w/v tryptone, 0.5% w/v yeast, 1% w/v NaCl, pH 7.3). Recombinant 2dCD4$^{WT/S60C}$ proteins were expressed and purified as described previously [20]. Briefly, the 2dCD4 insoluble fraction was purified by denaturing the proteins using chaotropic agents, capturing the his-tagged 2dCD4 using Nickel affinity, and refolding by a gradual removal of chaotropes in buffer-exchange dialysis. Following refolding of the expressed 2dCD4 proteins using a series of dialysis steps, the proteins were concentrated with an Amicon Ultra filter 10 kDa MWCO (Millipore, Billerica, MA, USA) and stored at -80 °C.

Env:CD4 immunogen complex formation and purification. The purified, recombinant Env proteins (BG505 SOSIP.664 or FVC$_{ENV}$ SOSIP.664) were complexed to the purified 2dCD4 proteins (2dCD4^{WT} or 2dCD4^{S60C}) by overnight incubation at 4 °C using a 3-fold molar excess of the soluble 2dCD4 proteins. Formation of the intermolecular disulphide bridge in the complexes with the mutant 2dCD4^{S60C} was facilitated by addition of 1 mM β-mercaptoethanol as a reducing agent. The complexes were centrifuged at 3,500 x g for 5 min to remove

precipitates. The complexes were purified from unbound 2dCD4 by size exclusion chromatography using a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare), followed by concentration with a 50 kDa MWCO Amicon centrifugal filter.

Enzyme-Linked Immunosorbent Assay (ELISA). The monoclonal antibodies 17b and PGT145 were expressed and purified as described previously [25, 26]. To determine the structural integrity and conformational antigenicity of the recombinant BFG505 SOSIP.664 and FVC_{ENV} SOSIP.664 trimers and the complexes, the reactivity of the Env to human-derived HIV-1 specific mAbs was evaluated in ELISAs as previously described [9]. Immuno MaxiSorp 96-well plates (Nunc) were coated with Env or Env-2dCD4^{S60C} complexes used in the vaccinations at 2 μg/ml. The following HIV-1 specific mAbs were added in a 3-fold serial dilution starting at 10 μg/ml: PG9, 10-1074, IgG1b12, VRC01, A32, 35O22, PGT145, 10e8, and 17b. The presence of bound antibodies was detected with a horseradish peroxidase-conjugated anti-human IgG antibody (GE Healthcare).

Immunizations and serum collection. New Zealand white rabbits were divided into four groups with five animals in each group. All experimental animals weighed between 3 to 5 kg at the commencement of the study. The rabbits were given intramuscular injections of 50 μg total protein/animal of either FVC_{ENV} SOSIP.664 (group A), 2dCD4^{S60C} (group B), BG505 SOSIP.664-2dCD4^{S60C} complex (group C), or FVC_{ENV}-2dCD4^{S60C} complex (group D). Each injection was adjuvanted with Adjuplex Adjuvant (Advanced BioAdjuvants, Omaha, Nebraska) comprising of detergent-free lecithin and the carbomer homopolymer. The immunization schedule consisted of multiple doses with injections on days 0, 28, 56, and 84 with bleeds on days 0 (prior to immunization), 14, 42, 70, and 98.

Pseudovirion production. The plasmid encoding the HIV-1 *env* gene of strains QH0692.42, 398_F1, 246F3, TRO.11, X2278, CAP210.2.00.E8, Du422.12, 246F, 25710, CE0217, ZM53.12, CNE8, CNE55, CH119.10, BJOX2000, CE1176, X1632, and the *env* gene of the

vesicular stomatitis virus glycoprotein (VSV-G; control) were obtained from the ARRRP and used in the production of pseudovirions. Recombinant pseudoviruses were produced in HEK293T cells as previously described [27]. The $TCID_{50}$ of each pseudovirus batch was determined, as described previously [28].

TZM-bl neutralization assay. Neutralization of pseudovirions was measured with the TZM-bl cells-based luciferase assay as previously described [29]. Neutralization was determined as the difference in RLU between the test wells and the cells-only control wells divided by the difference in RLU values between the virus-only control and cells-only control wells [30]. Each serum was tested against the VSV-G pseudoviruses to confirm the HIV-1 Env specificity of neutralization. Positive controls included human monoclonal antibodies (mAbs) PG9 and VRC03 at concentrations ranging between 0.005 and 10 μg/mL. Neutralization was reported as the 50% inhibitory dilution (ID_{50}) for sera, or 50% inhibitory concentration (IC_{50}) for mAbs which represent the dilution of serum (or mAbs) resulting in a 50% reduction in RLU.

RESULTS

FVC_{ENV} SOSIP.664 amino acid sequence comparison to BG505 and other Env sequences

The BG505 SOSIP.664 modifications [9] were introduced in the original FVC_{ENV} sequence [19] to produce the FVC_{ENV} SOSIP.664 gp140 protein. The changes included the A501C and T605C mutations to create the interprotomer SOS disulphide link, the I559P mutation to stabilize the trimer, the replacement of the R517, E518, K519, R520 to to 6xArg (R517-R522) for the furin cleavage site, and finally the sequence truncation after the Asp at position 664 to delete the MPER region of gp41 and to enhance the solubility of the protein (Supplementary Fig. S1). An Asn at position 332 and a Ser at position 334, the potential N-linked glycosylation site on which certain V3-directed antibodies are highly dependent, was already present in the FVC_{ENV} sequence. Comparatively, the FVC_{ENV} sequence, which is a consensus envelope of subtype C founder viruses [19], contained shorter V1V2 and V4 loops (Supplementary Fig.

S1). The FVC_{ENV} SOSIP.664 sequence contains 17 *N*-linked glycosylation sequons (Supplementary Fig. S1).

FVC_{ENV} SOSIP.664 and BG505 SOSIP.664 express native-like trimers and form protein complexes with 2dCD4^{S60C}

The FVC_{ENV} SOSIP.664 and BG505 SOSIP.664 trimers were successfully expressed in HEK293T cells and purified via lectin-affinity chromatography followed by size-exclusion chromatography (SEC) (Fig. 1). While the trimer fraction constituted the largest peak, there was a proportion of dimers and monomers formed for both SOSIP.664 Envs (Fig. 1A). Reducing and non-reducing SDS-PAGE confirmed successful cleavage of the FVC_{ENV} and BG505 trimers as observed by the transition of gp140 to gp120 in the presence of DTT (Fig. 1B). This indicates that the optimal 6-arginine cleavage site and co-transfection with furin provided efficient cleavage. Purified FVC_{ENV} and BG505 SOSIP.664 trimers were complexed to 2dCD4^{S60C} in the presence of a low concentration of reducing agent, to facilitate reduction of the native disulphide bond and subsequent formation of the intermolecular covalent bond between Env and 2dCD4 locking the FVC_{ENV} and BG505 SOSIP.664 Envs in the open, CD4-bound conformation. SEC purification of the FVC_{ENV}-2dCD4^{S60C} and BG505-2dCD4^{S60C} complexes showed elution of the higher molecular weight complexes (Fig. 1A) whose increased molecular mass was confirmed by BN-PAGE (Fig. 1C).

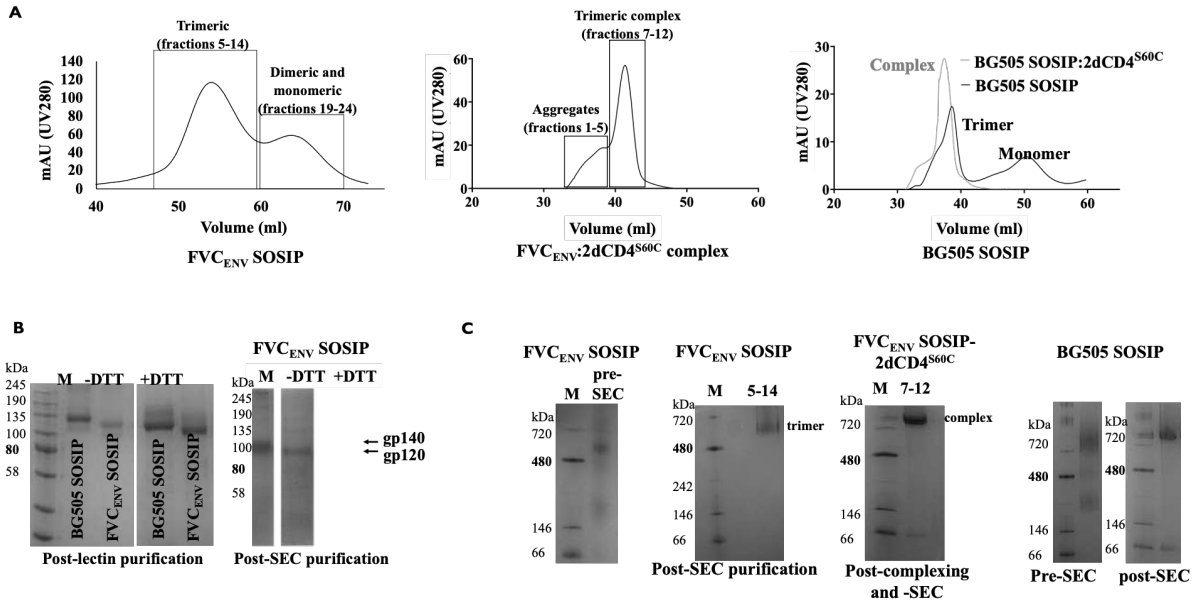


Figure 1. Biochemical characterization of FVC_{ENV} and BG505 SOSIP.664 trimers covalently complexed to $2dCD4^{S60C}$. FVC_{ENV} and BG505 SOSIP.664 trimers complexed to $2dCD4^{S60C}$ were purified using size-exclusion chromatography and analysed by SDS-PAGE and BN-PAGE. (A) HiPrep Sephacryl S-300 HR SEC profile of lectin-purified FVC_{ENV} SOSIP.664 and BG505 SOSIP.664 gp140 expressed in HEK293T cells before and after complexing to $2dCD4^{S60C}$ with peaks correlating to aggregates, trimers and monomers annotated. (B) Reducing (+DTT) and non-reducing (-DTT) SDS-PAGE analysis with 10% Tris-Glycine gels of lectin-purified FVC_{ENV} SOSIP.664 gp140 and BG505 SOSIP.664, following Coomassie blue R250 staining. Subsequent SEC-purified FVC_{ENV} SOSIP.664 gp140 also shown. The shift of the gp140 band to gp120 under reducing conditions indicates furin-mediated cleavage to allow gp120-gp41 dissociation. The molecular weight marker (M), indicated on the left, is the broad-range, colour prestained protein standard (NEB). (C) Coomassie-stained BN-PAGE analysis of FVC_{ENV} and BG505 pre- and post-SEC purification as well as the FVC_{ENV}:$2dCD4^{S60C}$ complex post- SEC pooled fractions. The molecular weight marker (M), indicated on the left, is the NativeMark™ protein standard (Thermo Fisher Scientific).

FVC_{ENV} SOSIP.664 produces native-like trimers with preserved antigenic qualities, similar to BG505 SOSIP.664

We used ELISAs to quantify the binding of various HIV-1 neutralizing antibodies to the SOSIP.664 gp140 trimers in the unliganded and $2dCD4^{S60C}$ complexed forms. The SEC-purified SOSIP.664 trimers (FVC_{ENV} and BG505) were immobilized onto ELISA plates and we monitored the binding of serially-diluted mAbs that recognize various regions of the Env, namely, the quaternary epitope V1V2 apex (PG9 & PGT145), a V3 glycan epitope (10-1074),

the CD4 binding site (VRC01 & IgG1b12), the first and second constant region C1-C2 (A32), the gp120/gp41 interface (35O22), and the MPER (10e8) (Fig. 2A). Antibodies whose epitopes are only formed when the trimer is in the quaternary native fold- such as PG9, PG16 and PGT145- are used to confirm trimers have adopted the correctly folded, quaternary conformation. All three of these antibodies recognize the V1V2 crown of the trimer when the protomers are in close proximity to each other in the closed, prefusion trimeric conformation (Fig. 2B). This epitope is compromised in the open, CD4-bound conformation although the PG9 and PG16 somatically-related antibodies indiscriminately bind to the monomeric versions of Env albeit with lower affinity [31]. These antibodies are also dependent on N160 or N156 glycans which are present in the FVC_{ENV} and BG505 trimers based on the amino acid sequence analysis (Supplementary Fig. S1). We observed strong binding of the VRC01, IgG1b12, 10-1074 and PG9 antibodies and weaker binding of the A32, 35O22 and PGT45 antibodies for the FVC_{ENV} SOSIP.664 trimer (Fig. 2A). Similarly, VRC01, 10-1074 and PG9 bound well to the BG505 SOSIP.664 trimer, however, the binding of IgG1b12 - while detectable - was significantly lower than its recognition of FVC_{ENV} (Fig. 2A). In contrast, antibodies A32 and 35O22 displayed more pronounced binding to the BG505 SOSIP.664 Env compared to the FVC_{ENV} SOSIP.664 trimer (Fig. 2A). 10e8 was included as a negative control as its epitope is absent in the SOSIP.664 trimers and it showed no binding to the FVC_{ENV} SOSIP.664 trimer (Fig. 2A).

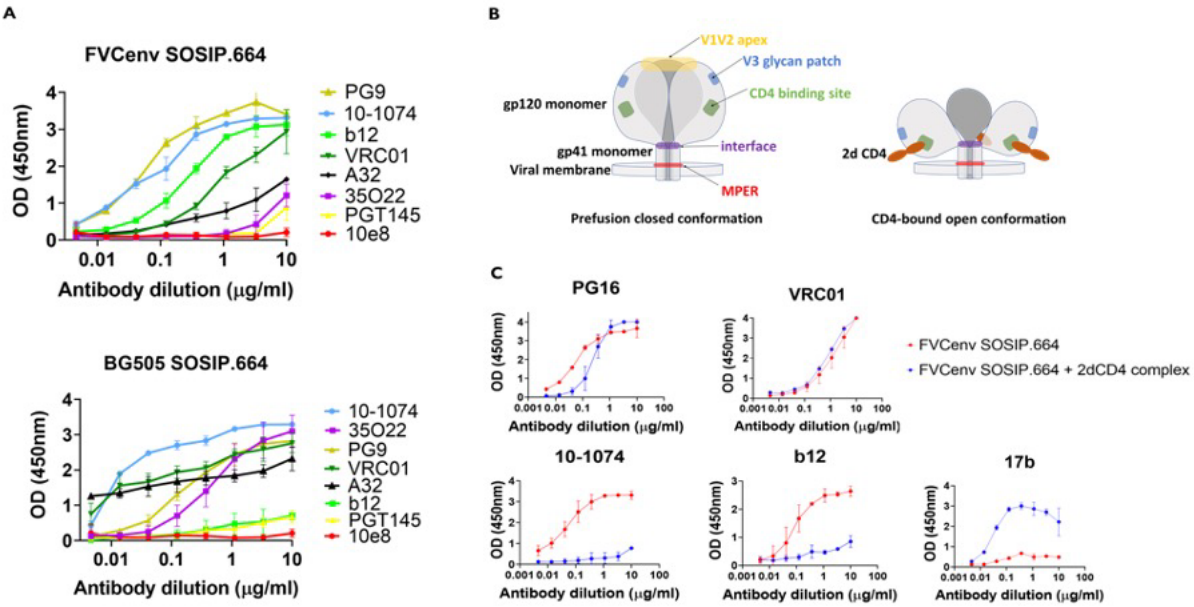


Figure 2. Antigenicity of SOSIP.664 trimers (FVC_{ENV} and BG505) and complexes. (A) Recognition of FVC_{ENV} and BG505 trimers by a selection of antibodies targeting the V1V2 apex (yellow curves- PG9 & PGT145), the CD4 binding site (green curves- IgG1b12 & VRC01), the V3 region (blue curve- 10-1074), the gp120/gp41 interface (purple curve- 35O22), the C1-C2 region (black curve- A32), and an MPER epitope (red curve- 10e8) used as a negative control. (B) Model of unliganded SOSIP.664 trimer in the closed, prefusion conformation (left) and the open, CD4-bound conformation (right). The trimer spike is represented with two of the three protomers shown in light grey and the third protomer shown in dark grey with broadly neutralizing antibodies epitopes indicated with arrows and coloured in orange for the V1V2 apex, blue for the V3 glycan patch, green for the CD4 binding site, purple for the gp120/gp41 interface, and red for the MPER region. (C) Effect of complexing FVC_{ENV} to $2dCD4^{S60C}$ on the high-binding antibodies PG16, VRC01, IgG1b12, and 10-1074 as well as the CD4-induced mAb 17b. Red curves represent FVC_{ENV} SOSIP.664 trimers and blue curves the FVC_{ENV} SOSIP.664 trimers complexed to $2dCD4^{S60C}$.

We then tested the effect of complexing FVC_{ENV} SOSIP.664 to $2dCD4^{S60C}$ on the antibodies which bound well to the trimer. We observed that the binding of the CD4bs antibody IgG1b12 was substantial for the FVC_{ENV} SOSIP.664 trimer but was significantly reduced in FVC_{ENV} SOSIP.664-$2dCD4^{S60C}$ (Fig. 2C). The binding of the other CD4bs mAb VRC01 remained unchanged between FVC_{ENV} SOSIP.664 and FVC_{ENV} SOSIP.664-$2dCD4^{S60C}$ (Fig. 2C). Recognition of the V1V2 apex mAb PG16 was also similar for the unliganded FVC_{ENV} SOSIP.664 and the $2dCD4^{S60C}$-complexed trimer (Fig. 2C). Another monoclonal antibody that lost most of its binding capacity in FVC_{ENV} SOSIP.664-$2dCD4^{S60C}$ was the V3 glycan binding mAb 10-1074 (Fig. 2C). In addition, we were able to confirm CD4 binding through preferential

recognition of the CD4-induced epitope by the 17b mAb in the FVC_{ENV} SOSIP.664-2dCD4^{S60C} complex (Fig. 2C).

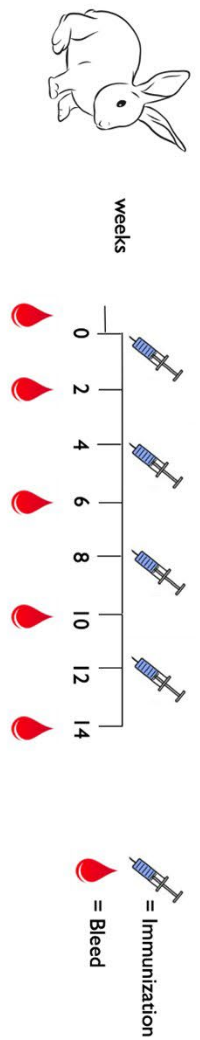
Immunization with BG505 SOSIP.664 and FVC_{ENV} SOSIP.664-2dCD4^{S60C} elicits potent, cross-neutralizing antibodies in rabbits.

To evaluate the ability of the covalently complexed BG505 SOSIP.664-2dCD4^{S60C} and FVC_{ENV} SOSIP.664-2dCD4^{S60C} to induce humoral immune responses, we conducted immunogenicity studies with the purified recombinant proteins (expressed in mammalian cells with natural glycosylation preserved) using rabbits as the pre-clinical hosts. Each group of immunized animals was given either the 2dCD4^{S60C} protein, unliganded FVC_{ENV} SOSIP.664, BG505 SOSIP.664-2dCD4^{S60C} or FVC_{ENV} SOSIP.664-2dCD4^{S60C}, as outlined in the immunization schedule in Figure 3A.

The presence of neutralizing antibodies in the immunized rabbit sera was determined using the standard TZM-bl neutralization assay against 17 Tier 2 pseudoviruses comprising clades A, AC, B, C, AE, BC, E, and G (Fig. 3B). These Tier 2 pseudoviruses are considered as excellent determinants of antibodies neutralizing potential as they provide an exceptional representation of biologically relevant strains that have Env conformations of most circulating viruses [32]. We used sera collected two weeks after the last immunization as the antibody responses are shown to be at the highest titres following the fourth longitudinal immunization and neutralizing antibodies PG9 and VRC03 were used as controls (Fig. 3B). Results demonstrate that the covalently complexed Env SOSIP trimers (both FVC_{ENV} and BG505) were able to elicit antibodies that display robust neutralization of 12 (BG505 complex) and 13 (FVC_{ENV} complex) of the seventeen pseudoviruses tested (Fig. 3B). Comparatively, sera from the rabbits immunized with the unliganded FVC_{ENV} (Env control) were only able to weakly neutralize two of the pseudoviruses (398_F1 and CH119.10) and the sera from animals who received the

2dCD4^{S60C} show negligible neutralization with only two rabbits able to neutralize one pseudovirus (QH0692.42) at titers below ID_{50} values of 240 (Fig. 3B). Of interest, all five animals who received FVC_{ENV} SOSIP.664-2dCD4^{S60C} generated a cross-neutralizing antibody response against twelve of the thirteen pseudoviruses neutralized with two antisera - from rabbits 0289 and 0330 - failing to neutralize the Du422.12 pseudovirus (Fig. 3B). Similarly, all the animals who received BG505 SOSIP.664-2dCD4^{S60C} elicited cross-neutralizing antibodies against ten of the twelve neutralized pseudoviruses, with two rabbits (0300 and 0329) whose sera could not neutralize two of the clade C pseudoviruses (CAP210.2.00.E8 and Du422.12) (Fig. 3B). Overall, the cross-neutralizing response was consistently elicited in all the rabbits who received the covalent complexes with a few exceptions for one or two pseudoviruses. As expected, PG9 and VRC03 displayed different neutralization profiles against the panel of 17 pseudoviruses. The neutralization (IC_{50}) values of PG9 and VRC03 against the panel of viruses was compared to the antibodies' respective neutralization profile data on the bNAber repository (bNAber.org).

A



B

	Rabbit Serum	Clade A: QH0692.42	Clade A: 398_F1	Clade AC: 246F3	Clade B: TRO.11	Clade B: X2278	Clade C: CAP210.2.00.E8	Clade C: Du422.12	Clade C: 246F	Clade C: 25710	Clade C: CE0217	Clade C: CE1176	Clade C: ZM53.12	Clade AE: CNE8	Clade AE: CNE55	Clade BC: CH119.10	Clade E: BJOX2000	Clade G: X1632	(non-HIV) VSV-G
		Tier-2 Pseudoviruses																	
		Sera ID$_{50}$																	
FVCenv SOSIP.664	0333	<40	61	<40	<40	<40	<40	<40	<40	<40	161	<40	<40	<40	<40	61	<40	<40	<40
	0291	<40	124	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	284	<40	<40	<40
	0286	<40	148	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	73	<40	44	<40
	0270	<40	49	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40
	0401	<40	49	74	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	65	<40	<40	<40
2dCD4_S60C	0321	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40
	0305	237	<40	<40	<40	<40	<40	<40	<40	57	86	<40	<40	49	<40	<40	<40	<40	<40
	0283	167	<40	<40	45	<40	<40	<40	93	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40
	0372	<40	<40	<40	62	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40
	0431	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40
BG505 SOSIP.664-2dC4_S60C complex	0284	<40	5970	954	2037	<40	197	281	4830	1335	641	<40	<40	3708	2760	1418	<40	868	<40
	0300	<40	834	299	649	<40	<40	<40	1312	315	153	<40	<40	571	1522	293	<40	282	<40
	0328	<40	2238	768	1425	<40	83	78	3485	744	537	<40	<40	1544	12689	1059	<40	1575	<40
	0329	<40	3960	1348	1443	<40	540	235	2838	514	424	<40	<40	1675	747	1397	<40	403	<40
	0268	<40	464	60	204	<40	<40	<40	393	108	68	<40	<40	230	106	145	<40	174	<40
FVCenv SOSIP.664-2dC4_S60C complex	0289	769	1150	1206	3102	<40	724	<40	3586	887	506	<40	<40	2400	889	1058	<40	1500	<40
	0306	209	707	346	1687	<40	296	540	1756	341	215	<40	<40	994	1572	415	<40	164	<40
	0342	298	740	487	1968	<40	395	175	2155	603	278	<40	<40	1257	4500	601	<40	1321	<40
	0275	52	547	288	858	<40	145	118	1037	239	164	<40	<40	816	4207	314	<40	394	<40
	0330	130	831	611	1454	<40	568	<40	1259	490	330	<40	<40	989	2189	671	<40	957	<40
	pre-bleed Serum*	<40	<40	<40	<40	50	<40	<40	<40	62	<40	<40	<40	<40	<40	<40	<40	<40	<40
		MAbs IC$_{50}$ (µg/mL)																	
	PG9	>50	>50	9,64	>50	0,02	0,17	>50	0,04	0,05	0,01	<0,005	0,04	0,19	0,26	0,79	0,88	0,03	N/A
	VRC03	0,95	0,16	>50	0,09	0,02	>50	>50	>10	0,17	>10	0,42	10,3	>10	0,42	>50	6,45	0,01	N/A

Sera

> 500
250 to 499
100 to 249
41 to 99
< 40

mAbs

<1
1 to 9
10 to 24
25 to 50
> 50

Figure 3. Rabbit sera neutralization of a Tier 2 pseudovirus panel. (A) Schematic of rabbit immunization schedule with FVC_{ENV} SOSIP.664, $2dCD4^{S60C}$, BG505 SOSIP.664-$2dCD4^{S60C}$ and FVC_{ENV} SOSIP.664-$2dCD4^{S60C}$. The rabbits were immunized four times, monthly, with 50 μg of the soluble proteins adjuvanted with Adjuplex. Two weeks prior to the first immunization and two weeks following each immunization, blood samples were collected. Immunizations and bleeds time-points are indicated in weeks. (B) Neutralization titers of the rabbit sera in each of the four immunization groups. Sera obtained prior to immunizations (pre-bleed) and at week 14, following the final immunization, were tested in a TZM-bl reporter cell assay against the VSV-G specificity control pseudovirus and HIV-1 pseudoviruses QH0692.42, 398_F1, 246F3, TRO.11, X2278, CAP210.2.00.E8, Du422.12, 246F, 25710, CE0217, ZM53.12, CNE8, CNE55, CH119.10, BJOX2000, CE1176, and X1632. Monoclonal antibodies (mAbs) PG9 and VRC03 were included as neutralization controls for each pseudovirus except the non-HIV VSV-G pseudovirus. Numerical values represent ID_{50} (for sera) and IC_{50} (for mAbs) neutralization titers. $ID_{50}<40$ and $IC_{50}>10$ indicate no detectable neutralization for the rabbit sera or mAbs, respectively.

DISCUSSION

An important milestone in HIV- vaccine development is the ability to elicit potent, bNAbs following vaccination. Our results demonstrate the ability to induce antibodies that neutralize heterologous HIV-1 tier 2 pseudoviruses in rabbits, with a single immunogen complex composed of trimeric SOSIP.664 envelopes covalently coupled to $2dCD4^{S60C}$. Sera from rabbits immunized with the FVC_{ENV} SOSIP.664-$2dCD4^{S60C}$ displayed neutralization against 13/17 (76,5%) of tier 2 HIV-1 isolates from clades A, AC, B, C, AE, BC, and E, at notably high titers. Similarly, sera from rabbits immunized with the BG505 SOSIP.664-$2dCD4^{S60C}$ complex were also able to neutralize the same pseudoviruses, with the exception of the clade A QHO692.42 pseudovirus, with comparatively high titers. The foundation of this work was accomplished in a previous study where various HIV-1 Env conformations (monomeric gp120 and GCN4 trimeric Env) covalently complexed to $2dCD4^{S60C}$ elicited potent, broadly neutralizing antibodies against 100% of twelve subtype B and C isolates made up of tier-1, tier-2 and a single tier-3 pseudoviruses [21]. This study further investigated the impact of SOSIP.664 (the archetype HIV-1 trimer mimic) on the immunogenicity of the covalently bound complexes locked in an open, 2dCD4-bound conformation in small animal models, comparing the well characterized BG505 sequence to FVC_{Env}.

Overall, sera from rabbits immunized with BG505 SOSIP.664-2dCD4^{S60C} and FVC$_{ENV}$ SOSIP.664-2dCD4^{S60C} neutralized over 70% of the Tier-2 pseudoviruses tested, at exceptionally high potencies. While these results are significant, the spectrum of neutralization cross-reactivity of the SOSIP.664-2dCD4S60C complexes is narrower compared to the Envs in the previously reported study. This could be attributed to the difference in Env conformation or to the inclusion of pseudoviruses from clades A, AC, AE, BC, E and G which were not tested in the previous study [21]. Of note, one clade B (X2272) and two clade C (CE1176 and ZM53.12) pseudoviruses were resistant to neutralization in this study while all clade B and C pseudoviruses tested were neutralized in the Killick *et al.* study [21]. However, no direct comparison can be made as the pseudovirus panels used in the antibody neutralization assay varied between the two studies.

As expected, the rabbit sera from the groups immunized with either FVC$_{ENV}$ SOSIP.664 or 2dCD4^{S60C} exhibited low potency, antibody neutralization potential, with narrow breadth. A limitation of this study is that we did not include immunization with unliganded BG505 SOSIP.664 as a control. However, multiple groups have previously tested unliganded BG505 SOSIP.664 in rabbit immunizations, and shown its immunogenicity is limited to eliciting antibodies capable of neutralizing the autologous, immunogen-matched pseudovirus with the occasional cross-neutralization but of very limited breadth and potency [11, 33]. Thus, in an effort to reduce the number of animals used in experimentation, the ethical decision was taken not to include an unliganded BG505 SOSIP.664 group, particularly since an unliganded FVC$_{Env}$SOSIP.664 group was included. In summary, this is the first report of covalently bound SOSIP.664-2dCD4^{S60C} complexes capable of eliciting potent, broadly neutralizing antibodies in small animals, and further expands our knowledge on SOSIP.664 immunogenicity.

The observation that neither the Env sequence (BG505 or FVC_{ENV}), nor the Env conformation (SOSIP trimer in this study versus monomeric or GCN4-linked Env trimers described in Killick *et al.*, 2015) used influences the ability to elicit the potent, heterologous neutralizing response is suggestive that covalently complexing the Env with $2dCD4^{S60C}$ creates the auspicious conformational change responsible for the elicitation of the potent bnAbs displayed here.

The CD4-bound conformation has not been widely explored, particularly in terms of immunogen development, as prefusion (ligand-free) states are known to favour neutralizing antibodies epitopes [34, 35], including conformational ones [9, 36] while non-neutralizing antibodies sometimes target epitopes exposed upon CD4 binding such as the CD4-induced (CD4i) and the crown of the third hypervariable (V3) loop [37, 38] epitopes. As such, immunogen development strategies have sought to maximize prevention of immunodominant (neutralization-hypersensitive) regions, targets of non-neutralizing antibodies, by engineering Envs with increasing mutations for structural stability of the trimer in its native state with much reduced CD4 affinity [39, 40]. While it cannot be argued that the preferential antigenic properties of these improved trimers have been finely mapped and their improved immunogenicity in preclinical studies well demonstrated [11, 41], the limitations of focusing on the closed, ligand-free trimer as an immunogen have been highlighted here and in other studies [21].

The CD4-bound conformation of Env has been shown to exhibit some conformational plasticity [42] as intermediate transitional conformations occur when the various loops engage with CD4 [43]. We have shown that the CD4-bound FVC_{ENV} displays the open trimer conformation as evidenced by the improved binding of CD4-induced mAb 17b. We have also observed the loss of some bnAb epitopes in the CD4-bound conformation, most of which could be explained by either steric hindrance or epitope loss due to the conformational changes involved in binding to CD4 (as may have been the case for PGT145; however, the ELISA

binding was not substantial enough to perform a comparative assessment between CD4-liganded and -free conformations). In the first instance, we observed the contrast between two CD4 binding site antibodies whereby IgG1b12 seemed to compete with the bound CD4 molecule evidenced by the reduced binding of the mAb in the CD4-bound conformation and VRC01 whose binding remained unaffected in the CD4-liganded and -unliganded conformations. This is in accord with other studies that have shown VRC01 to have less steric hindrance than IgG1b12 in the way they approach their cognate epitopes, with IgG1b12 having a loop-proximal angle (similar to soluble CD4) and being more sensitive to loop packing, whereas VRC01 has a different angle of approach and its interaction with the functional virus spike is amenable to some alteration of the spike configuration [44]. The loss of recognition of the V3-targetting antibody 10-1074 was unexpected as the V3 loop exposure should be heightened post-CD4 attachment with the removal of the V2 glycan (N197) which normally occludes V3 antibodies from accessing their epitopes [9]; however, a study looking at cell surface expressed Env conformational states with increasing CD4 engagement has shown that antibodies targeting the V3 high mannose patch can drastically differ in their reactivity to unbound- and CD4 induced triggered conformations [45]. Specifically, of the four V3-directed bNAbs they tested, PGT128 and PGT135 bound better to the CD4 engaged, open trimer compared to the closed trimer, the binding of 2G12 was largely unchanged for all Env conformations, while PGT121 preferentially bound to the ligand-free, closed trimer with a noticeable decrease in binding potential for the CD4-bound Env [45]- similar to our result for 10-1074. Future studies to validate the different structural conformations between our FVC_{ENV} SOSIP.664-2dCD4$^{WT/S60C}$ complexes can be conducted using cryoelectron microscopy.

Notably, the conformational difference between closed and open states could expose additional epitopes and may explain the potent cross-neutralizing response seen here and similar studies [21]. Depletion of the antisera response in the Killick *et al.* study showed that the neutralizing

response could be broadly mapped to the CD4 portion of the immunogen [21]. Exposure of previously shielded residues could explain the improved immunogenicity seen with Env covalently complexed to 2dCD4^{S60C} as the epitopes may be presented in the Env and CD4 components of the complex. The potential concern in the vaccine field with using CD4-bound HIV-1 Env immunogens is that following vaccination, they could induce "self" antibodies that target the host CD4+ T-lymphocytes. This concern was fueled by research conducted in the 1990's showing the presence of CD4-targeting autoantibodies in HIV-1 infected individuals which presumably arise from the presence of naturally occurring gp120-CD4 complexes during disease progression [46-55]. In a variety of clinical settings, anti-CD4 antibodies result in a decrease in CD4 expressing T cells [56-58]. However, in HIV-1 preclinical studies, anti-CD4 autoantibodies are rarely elicited by vaccination as evidenced by immunotoxicologic studies conducted in immunized cynomolgus macaques [59].

Of interest, antibodies which target host receptors have been discovered and engineered- Ibalizumab is an example of such a monoclonal antibody, which targets host CD4, and has shown efficiency in reducing viral load when administered as an antiviral therapeutic [60, 61]. Furthermore, Ibalizumab has been FDA approved for use in combination antiretroviral treatment against multi-drug resistant HIV-1 in treatment experienced patients [62, 63]. While Ibalizumab is a humanized monoclonal antibody, its approved use in a clinical setting sets a precedent for looking at modifying the current Env-2dCD4^{S60C} immunogen using rational design to ensure we only elicit Ibalizumab-like antibodies. Such an immunogen would be capable of consistently eliciting potent, broadly neutralizing antibodies against a range of clinically relevant HIV-1 strains, without the requirement of complex, sequential Env immunization regimens currently being tested for driving appropriate neutralizing antibody affinity maturation.

FUNDING

This study was supported in part by grants from the department of Molecular Medicine and Haematology, HIV Pathogenesis Research Unit (HPRU) and the South African National Research Foundation (Thuthuka 113998 & 129859).

Conflict of Interest statement: The authors declare no competing interests, financial or otherwise.

Author Contributions: Experimental setup and analysis, N.T.; Conceptualization, M.P., N.T., G.O. and M.K.; Funding acquisition, M.P. and N.T.; Supervision, M.P., M.K. and G.O.; Validation, M.P., G.O., M.K. and N.T.; Writing (original draft), NT; writing (review and edits), G.O., M.P., M.K. and N.T. All authors read and agreed to the final version of the manuscript.

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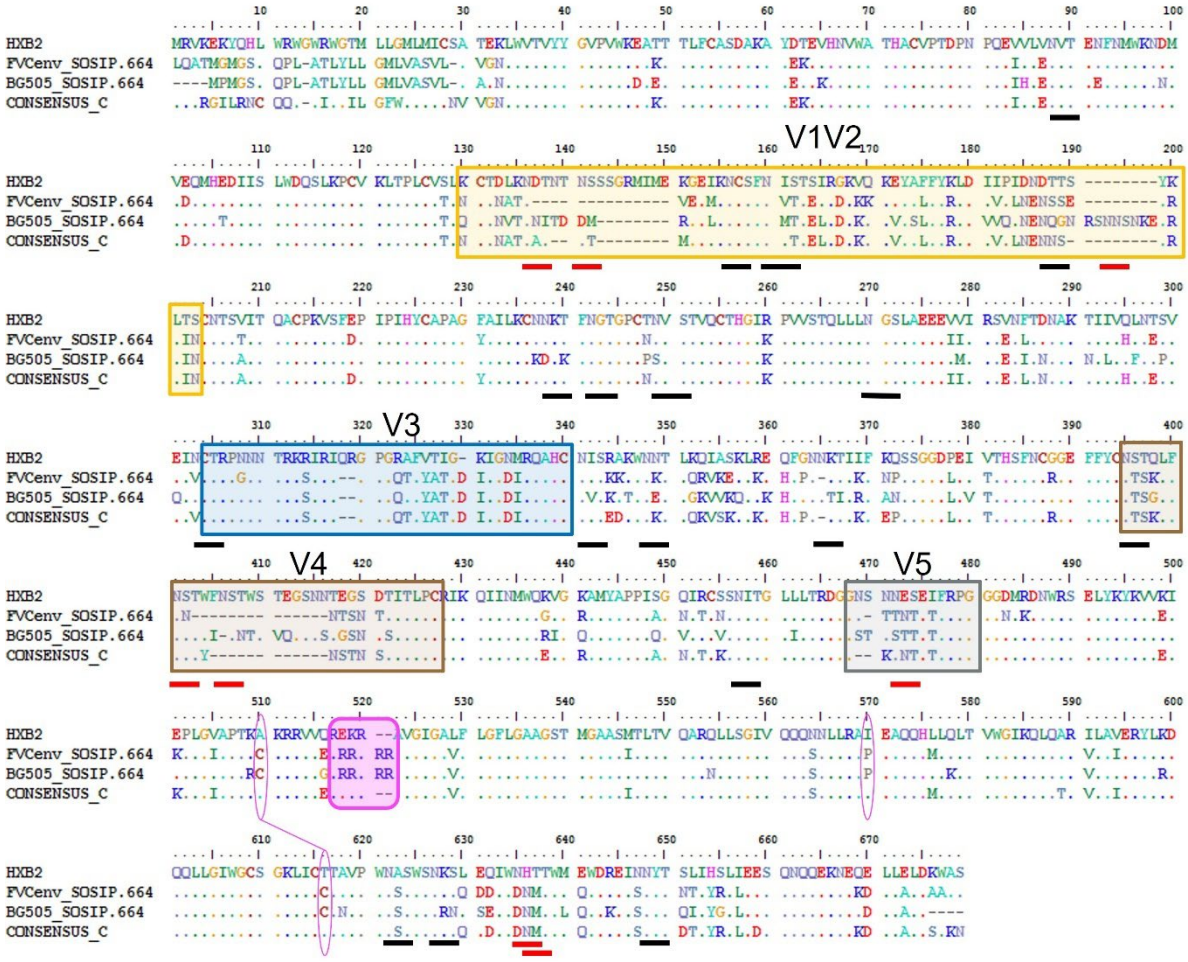
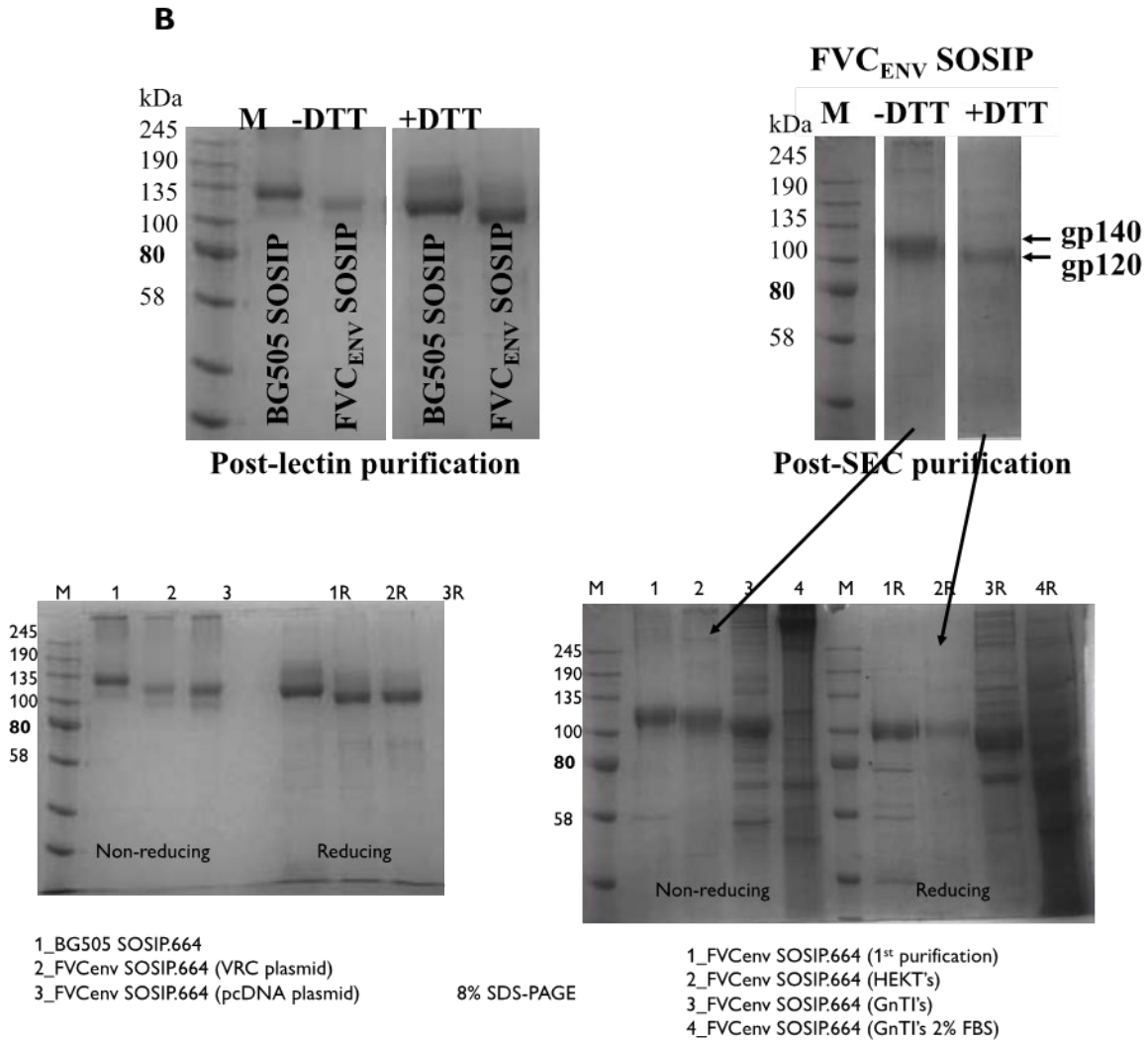
Article 2 supplementary:



Supplementary Figure S1. Amino acid sequence and antigenic characteristics of the FVC_{ENV} SOSIP.664 immunogen. Amino acid sequence alignment and comparison of FVC_{ENV} SOSIP (GenBank accession no. MW269981) with HXB2 (GenBank accession no. K03455, BG505 SOSIP (GenBank accession no. ABA61515), and the Consensus C SOSIP (GenBank accession no. DQ401076) sequences. The variable regions are boxed: V1V2 in orange, V3 in blue, V4 in brown, and V5 in grey and labelled above each box. SOSIP stabilization mutations are circled pink with the two residues comprising the disulphide bond (A501C-T605C) linked with a line. The modified furin cleavage site is boxed in pink. The three amino acids (NXT/S) making up all potential N-linked glycosylation sites are underlined in black if present in the FVC_{ENV} sequence and at least one other sequence, and in red if absent from FVC_{ENV} but present in at least one of the other sequences. Putative glycans were assigned based on Hamby & Hirst, 2008 [637]. Alignment was performed with DNAMAN 6.0 software.



Supplementary Figure S2. Uncropped gel pictures from Figure 1B

Chapter 4_ References

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Appendix

Appendix A: Human Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG | HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Ref: W-CP-180322-2 22 March 2018

TO WHOM IT MAY CONCERN:

Waiver:	This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).
Investigator:	**Ms Nancy Tumba (student no.1594387)**
Supervisor:	**Dr Gavin Owen**
Project title:	**Molecular Characterisation and immunogenicity of an HIV-1 subtype C founder virus consensus viral envelope SOSIP.664 Trimer**
Reason:	This is an invitro cell culture based study to express proteins in bacteria and HEK293T and HEK2935 GnTI- cells

Professor Clement Penny

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Rhulani Mkansi, Iain Burns

Research Office Secretariat: Faculty of Health Sciences, Phillip Tobias Building, 3rd Floor, Office 301, 29 Princess of Wales Terrace, Parktown, 2193 Tel +27 (0)11-717-1252 /1234/2656/2700 Private Bag 3, Wits 2050, email: zanele.ndlovu@wits.ac.za Office email: hrec-medical.researchoffice@wits.ac.zaWebsite:

Appendix B: Animal Ethics Clearance Certificates

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2018/02/10/B

APPLICANT: Ms N Thumba

SCHOOL: Molecular Medicine and Haematology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Immunization of New Zealand white rabbits with HIV-1 SOSIP.664 HIV-1 trimeric envelope complex for the analysis of the serum antibody response

Number and Species

20X 3-6 months female and male New Zealand White rabbits

Approval was given for the use of animals for the project described above at an AESC meeting held on 2018/02/27. This approval remains valid until 2020/04/05.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

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:

Signed: _____ (Chairperson, AESC) Date: 6/04/2018

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

Signed: _____ (Registered Veterinarian) Date: 9/4/18

cc: Supervisor: Professor M Papathanasopoulos
Director: CAS

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AREC 2017 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL RESEARCH ETHICS COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Nancy Tumba

b. School and email address: School of Pathology. Nancy.tumba@wits.ac.za

c. Experiment to be modified / extended **AREC/AESC NO**

Original AESC number	**2018**	**02**	**10B**
Other M&Es :			

d. Project Title: Immunization of New Zealand white rabbits with HIV-1 SOSIP.664 HIV-1 trimeric envelope complex for the analysis of the serum atibody response

		No.	**Species**
e.	Number and species of animals originally approved:	20	*Oryctolagus cuniculus*
f.	Number of additional animals previously allocated on M&Es:	-	
g.	Total number of animals allocated to the experiment to date:	20	*Oryctolagus cuniculus*
h.	Number of animals used to date:	-	

i. Specific modification / extension requested:

The addition of 3 personnel to assist with the animals daily monitoring and weighing as required by CAS regulations. The individuals are Dr. Gavin Owen (co-supervisor on this project), Ms Simone Smith (MSc student affiliated with the project and already trained in animal handling by the CAS) and Ms Ashlyn Davis (MSc student in the department).

j. Motivation for modification / extension:

The rabbits to be used in this study will only come of age (3months) and be acquired at the end of July 2018. Although these experiments will commence shortly following this, it will result in the experiments proceeding while I- the main investigator (Nancy Tumba)- will be on maternity leave (mid-September to mid-January). While this will not affect the smooth progression of the fortnightly blood draws and immunizations, I will need help with the daily monitoring and weighing of the animals. I have approached the above-mentioned individuals and they are more than willing to get trained and help during my maternity leave. In addition, the reason I am requesting the addition of 3 people is partly because a shared load would be easier with everyone's current work load and partly due to the assistance being required during the December holidays when many will be on leave and some out of the province.

Date: 2018-07-22 Signature:

RECOMMENDATIONS

Date: 6/08/2018 Signature: Chairman, AREC PP

Appendix C: Turnitin report

Turnitin Originality Report

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