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Immunogenicity testing of HIV-1 envelope glycoprotein complexes (Env-2dCD4^{S60C}) in Chinese-origin rhesus macaques

Roberto Carlos Rebelo Pereira



A thesis submitted to the Faculty of Health Sciences,

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in fulfillment of the requirements for the degree of

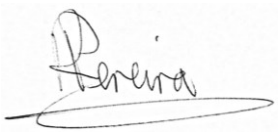
Doctor of Philosophy

Johannesburg, South Africa

2021

Declaration

I, Roberto Carlos Rebelo Pereira declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at any other university.



Roberto Carlos Rebelo Pereira

8th day of November 2021 in Johannesburg

In memory of my big brother, whom I miss dearly.

Horacio Amarildo Rebelo Pereira

1971 – 2019

Publications and presentations emanating from this research

Conference presentations:

1. **R.C.R Pereira**, M.A. Killick, M.A. Papathanasopoulos. Env-2dCD4^{S60C} complexes elicit potent, broadly neutralizing antibodies against clinically relevant HIV-1 viruses in Chinese-Origin Rhesus Macaques. HIV Research For Prevention conference. 2018. Madrid, Spain. Poster presentation.
2. **R.C.R Pereira**, M.A. Killick, M.A. Papathanasopoulos. Env-2dCD4^{S60C} complexes elicit potent, broadly neutralizing antibodies against clinically relevant HIV-1 viruses in Chinese-Origin Rhesus Macaques. Faculty of Health Sciences Research Day. 2018. University of the Witwatersrand, Johannesburg, South Africa. 2018. Poster presentation.
3. **R.C.R Pereira**, M.A. Killick, M.A. Papathanasopoulos. Env-2dCD4^{S60C} complexes elicit potent, broadly neutralizing antibodies against clinically relevant HIV-1 viruses in Chinese-Origin Rhesus Macaques. Molecular Biosciences Research Thrust Research Day. 2018. University of the Witwatersrand, Johannesburg, South Africa. Poster presentation.
4. **R.C.R Pereira**, M.A. Killick, M.A. Papathanasopoulos. Env-2dCD4^{S60C} complexes elicit potent, broadly neutralizing antibodies against clinically relevant HIV-1 viruses in Chinese-Origin Rhesus Macaques. 9th Cross-Faculty Postgraduate Symposium. 2018. University of the Witwatersrand, Johannesburg, South Africa. Oral presentation.

Abstract

A key goal in development of preventative HIV-1 vaccines is the ability to elicit broadly neutralizing antibodies (bNAbs) following vaccination. The target of these bNAbs is the only exposed protein on the surface of the HIV-1 virion, the trimeric HIV-1 envelope glycoprotein (Env). Our research group previously developed a modified two-domain CD4 molecule (2dCD4^{S60C}) that binds with greater affinity to HIV-1 Env glycoproteins through the formation of a stabilizing disulphide bridge. This allowed us to generate stable immunogen complexes suitable for immunizations in animal models. We previously showed that rabbits immunized with Env-2dCD4^{S60C} complexes elicit potent bNAbs targeting clinically relevant tier-2 HIV-1 pseudoviruses *in vitro*. These neutralizing responses were generated regardless of the form (monomeric and trimeric Env) or viral subtype (subtype B and C) of the Env glycoprotein in complex with 2dCD4^{S60C} and were noted to be anti-CD4 in nature. This study describes immunogenicity testing of the Env-2dCD4^{S60C} complex in a non-human primate model (NHP), the Chinese-origin rhesus macaque.

Trimeric HIV-1 Env glycoproteins based on a consensus sequence of subtype C founder viruses (gp140$_{FVC}$GCN4t(+)) and 2dCD4^{S60C} were expressed using mammalian and bacterial cell expression systems. The gp140$_{FVC}$GCN4t(+) and 2dCD4^{S60C} proteins were purified using lectin-affinity, nickel-affinity and size-exclusion chromatography. Immunogen complexes were then generated under reducing conditions. Immunogenicity of gp140$_{FVC}$GCN4t(+), gp140$_{FVC}$GCN4t(+)-2dCD4^{S60C} (Env-2dCD4^{S60C}) and 2dCD4^{S60C} was evaluated in genetically characterized Chinese-origin rhesus macaques (n=7) following a six-dose, four-month vaccination regimen. Breadth and potency of vaccinated macaque sera was evaluated in a TZM-bl pseudovirion neutralization antibody assay. Fourteen pseudoviruses, including the twelve tier-2 HIV-1 Env Global Panel viruses, and an infectious tier-2 SIV/HIV-1 chimeric virus (SHIV-1157ipd3N4), were tested against. Basic mapping of the target of the neutralizing responses generated in NHPs was done using various recombinant CD4 molecules to deplete sera of anti-CD4 antibody responses, and then tested in the TZM-bl pseudovirion neutralization antibody assay. Two years following the vaccination regimen, the NHPs were vaccinated with a booster immunization and subsequently challenged using a low-dose intra-rectal SHIV challenge using SHIV-1157ipd3N4.

Env-2dCD4^{S60C} complex immunized NHPs (n=4) developed bNAb responses by day 74 of the vaccination regimen following four immunizations, neutralizing 11/12 of the HIV-1 Env Global Panel of pseudoviruses with titres above 1:100, with a geometric mean titre of 1:356 for the entire group against the panel. In contrast, a single macaque that received gp140$_{FVC}$GCN4t(+)-only, developed no neutralizing titres against the HIV-1 Env Global Panel, while the 2dCD4^{S60C}-only immunized macaques (n=2) displayed similar breadth to the complex group but with lower potency (1:60). Mapping of the target of the responses of the sera using recombinant CD4 proteins to deplete sera of anti-CD4 antibodies resulted in a reduction in neutralizing potency of sera from Env-2dCD^{S60C} immunized NHPs, with a complete loss of neutralizing potency in one of the sera tested.

Two years later, neutralizing titres were no longer detectable in the NHPs, but a booster immunization returned antibody titres to neutralizing levels after two weeks, to levels previously seen. Following booster immunizations, the NHPs were administered ten low-dose intra-rectal SHIV inocula over a 10-week period. CD4+ T-cell counts were monitored using flow cytometry and SHIV viral RNA loads were monitored using RT-PCR. Infection with, or protection from the SHIV-1157ipd3N4 challenges, remained inconclusive.

The unique Env-2dCD4^{S60C} complexes elicit potent and broad neutralizing antibody responses in Chinese-origin rhesus macaques against clinically relevant tier-2 HIV-1 pseudoviruses. These neutralizing antibody responses are primarily anti-CD4 in nature, with the precise mechanism of antibody mediated neutralization requiring further delineation. Future work includes assessing whether these elicited anti-CD4 antibody responses are immunotoxic in the vaccinated subject, as well as further proof of concept SHIV-challenge studies (using vaccinated NHPs), to support the advancement of these Env-2dCD4^{S60C} immunogens into human clinical testing.

Keywords

HIV-1; SHIV; Vaccine; Envelope glycoprotein; CD4; Immunogen; Non-human primate; Rhesus macaque; Broadly neutralizing antibody; CD4i

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List of abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
ARV	Anti-retroviral
ATR	Rad3-related protein
CA	Capsid
CCNTI	Cyclin-T1
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CD4bs	CD4 binding site
CDK9	Cyclin-dependant kinase 9
CDRH3	Heavy-chain complementarity-determining region 3 loops
cGAS	Cyclic GMP-AMP synthase
CPSF6	Cleavage and polyadenylation specificity factor 6
CRF	Circulating recombinant form
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR4	C-X-C chemokine receptor type 4
DEAE	Diethylaminoethyl-dextran hydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco’s Phosphate-Buffered Saline
dUTP	Deoxyuridine triphosphate

ELISA	Enzyme-linked immunosorbent assay
Env	Envelope glycoprotein
eOD	Engineered outer domain
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
Gag	Group-specific antigen
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFI16	Gamma-interferon-inducible protein Ifi-16
IFN–γ	Interferon gamma
IL-2	Interleukin 2
LEDGF	Lens-epithelium-derived growth factor
LTR	Long-terminal repeat
MA	Matrix protein
MHC	Major histocompatibility complex
MIP–1β	Macrophage inflammatory protein 1 beta
NC	Nucleocapsid protein
Nef	Negative regulatory factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHP	Non-human primate
NK	Natural killer
NPC	Nuclear pore complex
NTB-A	NK, T- and B-cell antigen
PAMPS	Pathogen-associated molecular patterns

PBMC	Peripheral blood mononuclear cell
PIC	Pre-integration complex
PRR	Pattern recognition receptors
Rev	Regulator of virion expression
RRE	Rev-responsive element
RT	Reverse transcriptase
SCF	Skp1-Cullin1-F-Box
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SIV	Simian immunodeficiency virus
TAR	Transactivation response element
Tat	Trans-activator of transcription
Tfh-cells	T-follicular helper cells
TLR	Toll-like receptor
TNF–α	Tumour necrosis factor alpha
Ub	Ubiquitin
UNG2	Uracil DNA glycosylase
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

Chapter 1: Introduction

1.1 Disease Burden

The back and forth between human and pathogen is an age-old struggle which has shaped humanity throughout millennia. Civilizations have risen and fallen in the shadow of devastating epidemics such as the Justinian Plague of the Roman era, the Black Death of the Middle Ages, the smallpox outbreaks during colonization of the Americas, and the 1918 influenza pandemic. The latter parts of the 20th and early 21st century saw the rise of a devastatingly insidious pandemic caused by Human Immunodeficiency Virus (HIV) which leads to acquired immune deficiency syndrome (AIDS). More recently in 2020, COVID-19 plunged the world at large into chaos and uncertainty; a sobering reminder of the existential threat that infectious disease outbreaks continue to pose.

Both HIV types 1 and 2 (HIV-1, 2) lead to AIDS, with HIV-1 responsible for the current global epidemic and HIV-2 being mostly isolated to regions in western and central Africa (Popovic et al., 1984). No cure exists as of 2021 albeit for 2 patients that were confirmed cured under unique circumstances (Allers et al., 2011, Gupta et al., 2019). After several decades of research, no efficacious vaccine has yet been developed. Preventative and treatment implementations such as use of condoms, pre-exposure prophylactics and anti-retroviral (ARV) therapies have provided relief for millions while a vaccine or cure against HIV infection remains elusive.

HIV is one of the deadliest and most disruptive infectious diseases in modern history, and in 2021 is still a major public health threat despite a steady decline in infections and deaths over the last two decades (**Figure 1.1**). Currently, 37.6 million people are estimated to be infected worldwide and an estimated 34 million have died from AIDS-related illness since the start of the worldwide outbreak, with 1.5 million new infections and 690,000 deaths having occurred in 2020 alone (UNAIDS, 2021a). Southern and eastern regions of Africa currently endure the brunt of the pandemic, with an estimated 20.6 million people living with HIV-1 and death rates remaining high within these regions (**Figure 1.2**). South Africa remains the country with the highest burden with 7.5 million people currently living with the virus. Not only has HIV-1 been a detriment to the health and wellbeing of the populations of many countries, but it has also

seen a massive outlay of resources to combat it, yet these massive outlays still fall short of actual requirements for ending the pandemic. Projections earlier this past decade predicted costs for low- to middle-income regions rising to a combined US$ 30 billion per year by the start of the 2030s (Hecht et al., 2010). More recently in 2016, the United Nations committed to the "Fast-Track" strategy and "90-90-90" targets of diagnosis, treatment and prevention which would see an attempt at ending the HIV-1 pandemic by 2030 (UNAIDS, 2017). The strategy would have seen a front-loading of costs for several years from 2016 to 2020 in order to aggressively meet diagnostic and treatment targets, with an expected peak target at US$ 26 billion in 2020 (Stover et al., 2016). The costs would then decline for the next decade moving towards 2030. Since 2016, these resource targets have not been met with 2018 and 2019 seeing US$ 19 billion and US$ 18.6 billion made available to low- and middle-income countries, respectively (UNAIDS, 2021b). In 2020, the US$ 26 billion target was not met.

Despite advances in public health, pharmacological interventions and billions of US dollars spent in battling the HIV-1 pandemic, a vaccine remains a gaping hole in the fight against the virus. It is highly unlikely that the virus will ever be fully brought under control without a vaccine, particularly as nations attempt to meet "Fast-Track" targets. Modelling consistently predicts that a combination of different diagnosis and treatment scenarios coupled with a hypothetical vaccine of varying efficacy would have the most impact on the pandemic (Phillips et al., 2014, Harmon et al., 2016, Medlock et al., 2017). Moving forward, the development of a working vaccine remains one of the top priorities for infectious disease researchers and public health authorities worldwide.

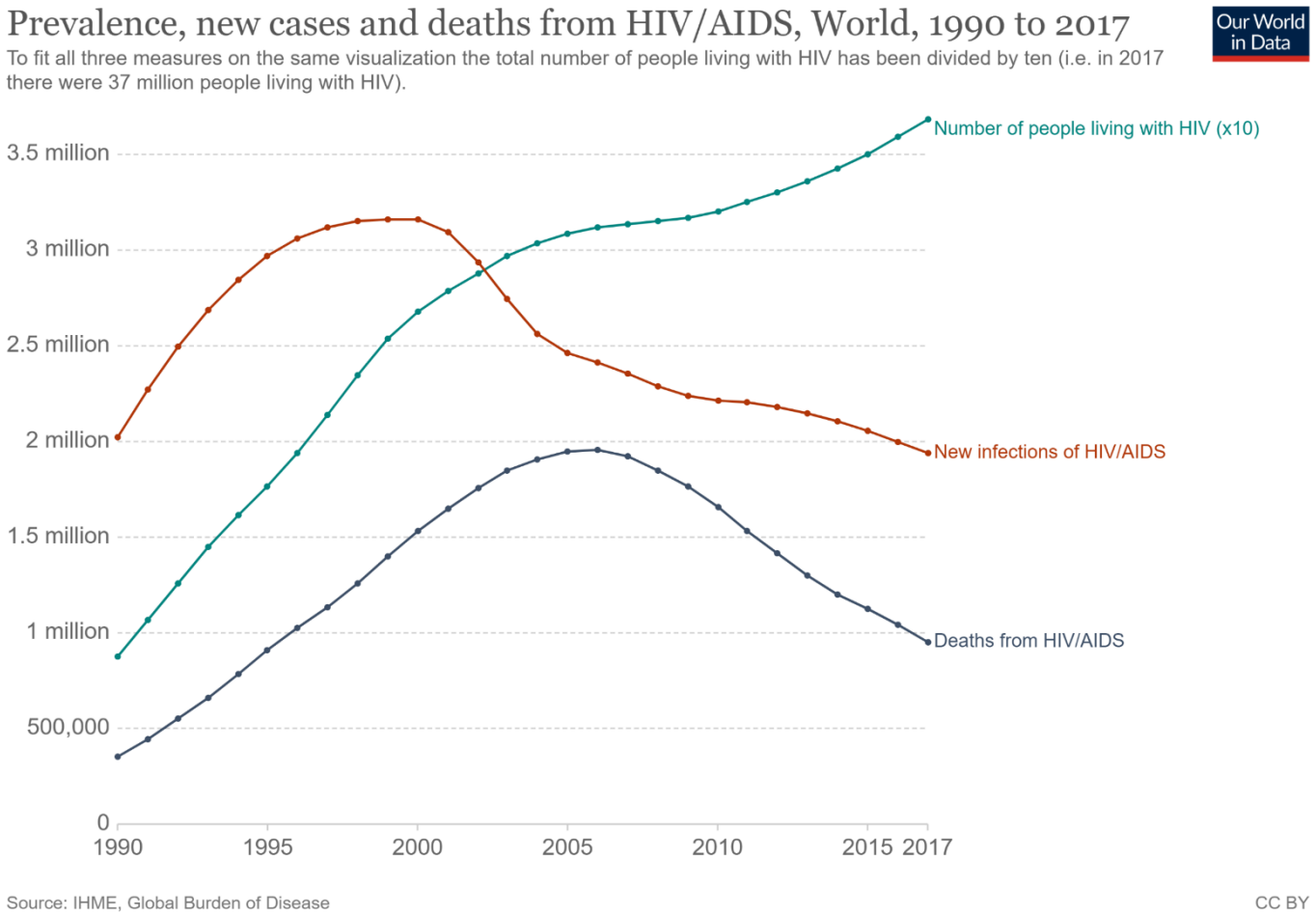


Figure 1.1: Prevalence of HIV-1 over three decades including new infections and deaths per year. Reproduced from *Our World in Data* (Roser, 2018).

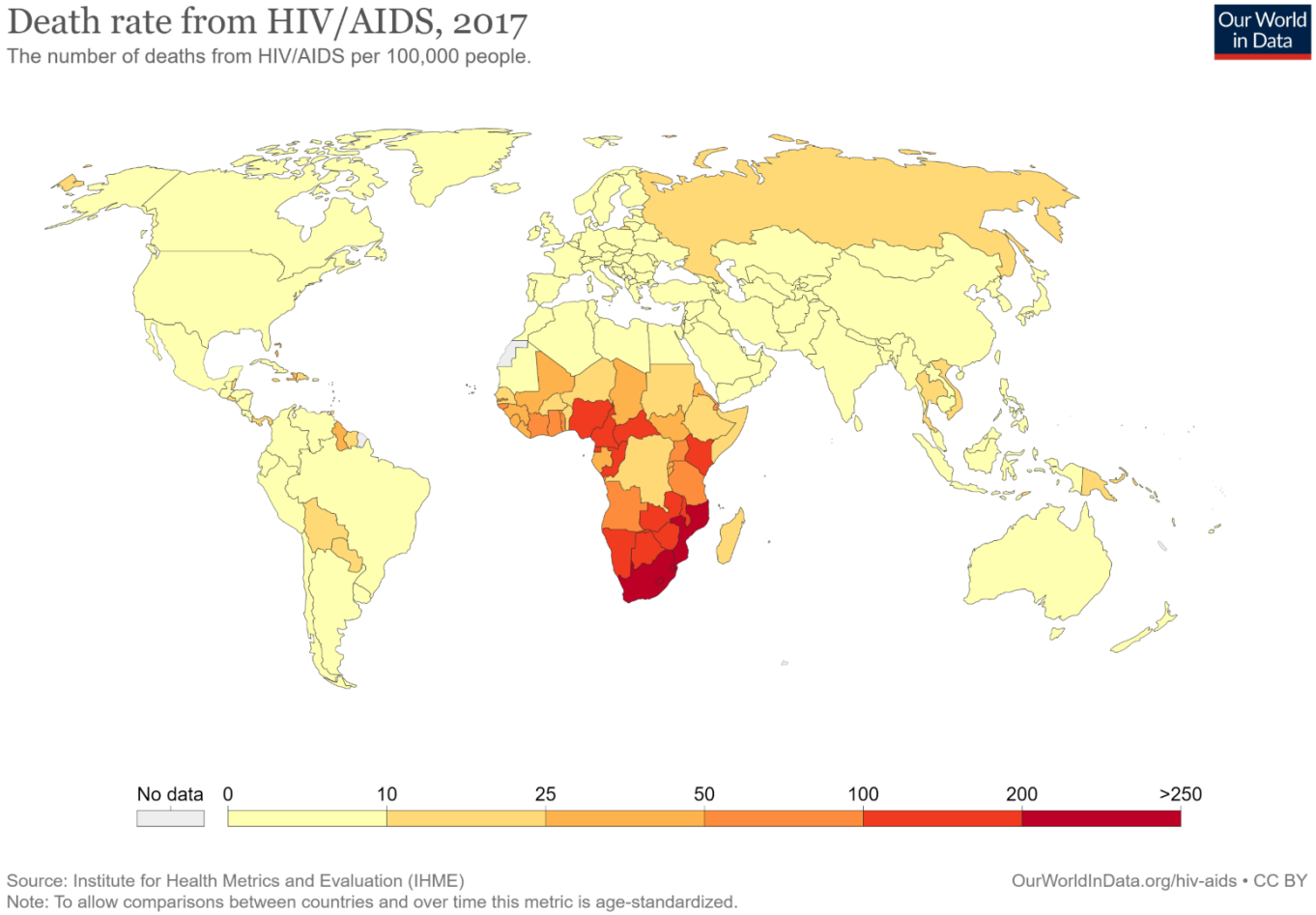


Figure 1.2: HIV-1 death rates remain high throughout Sub-Saharan Africa. Reproduced from *Our World in Data* (Roser, 2018).

1.2 Origins, classification, and diversity of HIV-1

AIDS was first described in 1981 when young, homosexual men were diagnosed in the USA with rare, opportunistic infections not commonly seen in healthy individuals (Gottlieb et al., 1981). Later, in 1984, it was found that a retrovirus (HIV-1) was responsible for the dysfunctional immune system noted in these individuals and the causative agent of AIDS (Gallo et al., 1984, Klatzmann et al., 1984). The virus was then soon found to have established itself in heterosexual populations in central Africa, with early work detecting clusters of infection in Kinshasa (Piot et al., 1984).

HIV is a lentivirus, a group of viruses which cause persistent and often debilitating infections in various mammalian species. HIV-1 may have only been described only a few decades ago, but the virus has likely persisted within the human population for at least a century (Faria et al., 2014). Phylogenetic analyses have shown that HIV-1 likely originated from cross-species transmission events of simian immunodeficiency viruses (SIV) during the late 19th to early 20th century, coinciding with the massive industrialization of central Africa. The progenitors of HIV-1 can be traced back to SIVcpz or SIVgor which infect chimpanzees (*Pan troglodytes troglodytes*) and western lowland gorillas (*Gorilla gorilla gorilla*) (Plantier et al., 2009, Gao et al., 1999). HIV-1 has been designated into four different groups (**Figure 1.3**), believed to depict four separate transmission events of SIV into the human population. These groups are M (responsible for the current pandemic), N, O and P (Sharp and Hahn, 2011). HIV-2, largely isolated to western parts of Africa, originated from the sooty mangabey (*Cercocebus atys*) strain SIVsmm, with eight different groups identified, termed groups A to H (Ayouba et al., 2013).

Various bottleneck events have occurred worldwide resulting in further complexity for the HIV-1 group M strain (**Figure 1.3**), with nine subtypes spread across different geographical regions (subtypes A to K) (Sharp and Hahn, 2011). These subtypes can also be further classified into sub-subtypes such as A1, A2, A3, A4, F1 and F2 (Vidal et al., 2000). Circulating recombinant forms (CRFs) also exist, with 102 currently known (Los Alamos National Laboratory, 2021). These CRFs occur when a patient becomes dually infected with more than one HIV-1 subtype, resulting in a recombination event and a subsequent new strain emerging (Taylor et al., 2008). If these recombinant strains infect three or more people, the strain is classified as a CRF.

1.2.1 HIV-1 error-prone replication and host factors drive diversity

The incredible diversity of the HIV-1 genome has contributed to its widespread and devastating success as an infectious disease. Like many other retroviruses, HIV-1 diversity is mainly due to the inherent error-prone nature of the virus which drives the large amounts of genomic mutations seen. Viral reverse transcriptase (RT), which is responsible for the conversion of viral RNA into cDNA which is then inserted into the host genome, does not possess exonucleolytic proofreading capabilities which can result in errors. The average number of mutations occurring for HIV-1 per base pair per replication cycle is said to be $\sim 3.6 \times 10^{-5}$ (Rawson et al., 2016). This lack of proof-reading capability is coupled with the enormous amounts of HIV-1 replication seen in an infected host, with $\sim 10.3 \times 10^{9}$ virions replicated per day (Perelson et al., 1996). RNA polymerase II is also said to have a minor contribution to the mutagenesis of the HIV-1 genome (O'Neil et al., 2002).

Intrinsic host immune factors have also played a role in the mutagenesis and diversity of the HIV-1 genome. Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) is a potent anti-viral restriction factor found in mammals and belongs to the family of APOBEC enzymes (Smith et al., 2012). APOBEC3G is a cytidine deaminase which deaminates cytosine to uracil in the minus strands of viral cDNA, which then act as a template for positive cDNA strands resulting in adenine misincorporation, and ultimately G-to-A hypermutations (Mangeat et al., 2003). Non-functionality of the provirus can occur due to shifted reading frames or the formation of termination codons.

The HIV-1 virus has evolved to combat the cytidine deaminase activity of the host, by expressing the viral infectivity factor (Vif) protein which is responsible for the ubiquitination and subsequent proteolysis of APOBEC3G (Simon et al., 2005). Mutagenesis due to APOBEC3G activity may not always result in lethal mutations though, and may in fact contribute to sublethal mutagenesis of the HIV-1 genome (Sadler et al., 2010). This is confirmed by the fact that non-functional or partially functional Vif proteins are expressed by certain viral isolates from infected individuals, which result in hypermutated virus with noticeable G-to-A substitutions, rather than destruction of those viral variants (Simon et al., 2005). Lentiviral genomes themselves are indeed adenosine rich, suggesting that APOBEC enzymes may drive diversity within lentiviral genomes (Berkhout and Hemert, 1994, Liddament et al., 2004). One study, examining frequency of premature stop codons to quantify the HIV-1 genome-wide rate of spontaneous mutation in DNA sequences in peripheral blood mononuclear cells (PBMCs), found that *in vivo* HIV-1 mutation rates may in fact be far higher

than previously shown in cell-free systems, with a majority of those mutations likely due to cytidine deaminases such as APOBEC3G, and less likely due to RT activity (Cuevas et al., 2015). It has been noted that although a large amount of the genomic HIV-1 viruses of the patient PBMCs were mutated, only a minority of these viruses are expressed and make it to the peripheral plasma, indicating that many of these mutations result in defective and non-viable virus.

1.2.2 Transmission bottlenecks and HIV-1 tropism

Transmission bottlenecks occur during HIV-1 infection from donor to new host, which would seem counterintuitive, considering the high viral loads that can occur in infectious donors and the extensive diversity seen in the HIV-1 viral population. This is also reflected by the relatively low rates of infection per sexual encounter (Patel et al., 2014). When transmission of HIV-1 occurs from donor to new host, one to several viruses go on to establish infection within the new host. In 80% of cases, a single virus is responsible for establishment of infection during heterosexual transmissions, whereas a larger percentage of transmission occurring between intravenous drug users involves more than a single virus (Salazar-Gonzalez et al., 2009, Bar et al., 2010). The phenotypic and genotypic properties of "founder" viruses have been explored previously with a few factors common between them. What has been noted consistently are founder virus' chemokine receptor 5 (CCR5) and CD4+ T-cell tropism (Keele et al., 2008, Parrish et al., 2013). Previously it has been noted that founder viruses may present a greater number of Env glycoprotein molecules on their surface overall, which also exhibit shorter variable loops, reduced glycosylation, and a propensity for neutralization by antibodies, but some of these findings have not been consistently reported within the literature (Parrish et al., 2013, Chohan et al., 2005, Curlin et al., 2010, Wilen et al., 2011).

As an infection progresses, the relatively homogenous viral population of early infection shifts towards a more varied population due to the error-prone replication of HIV-1, coupled with evolutionary pressures from the host immune system, establishing a population of quasispecies in various tissue compartments of the body (Diem et al., 2008, Boeras et al., 2011, Schnell et al., 2010). This compartmentalization already selects for certain viral species even before an infection event occurs, with further selection occurring due to; inflammatory cytokines within the vaginal tract, enhancing proteins within the male seminal fluid, or the notable increased levels of CD4+ T- cells within the penile foreskin which express CCR5 receptors, amongst other mucosal and inflammatory factors (Münch et al., 2007, Masson et al., 2015)

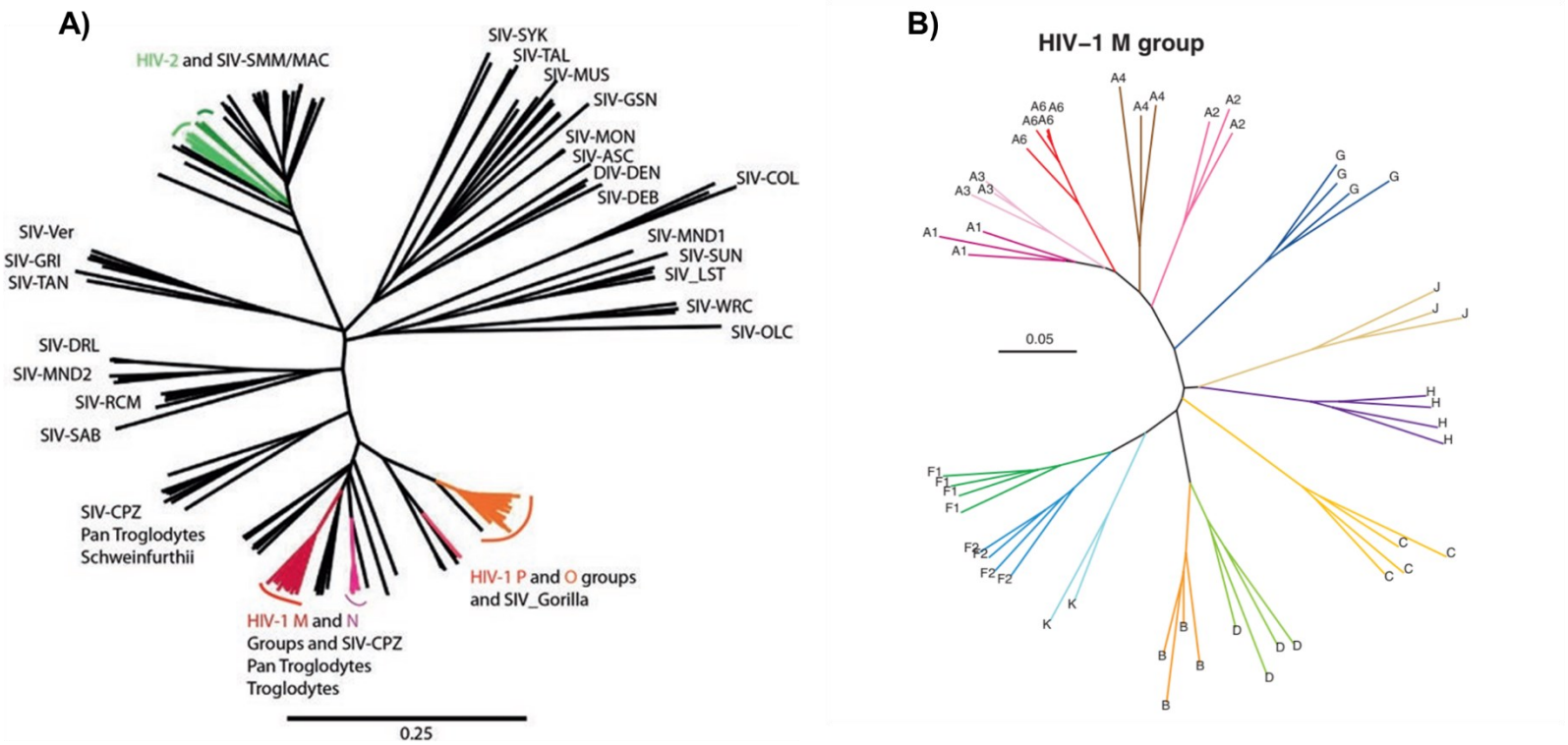


Figure 1.3: Phylogenetic trees of primate lentiviruses and HIV-1 group M. HIV-1 is closely related to lentiviruses circulating in non-human primates and likely originated from several cross-species transmission events of SIV into humans. HIV-1 group M is responsible for most infections worldwide and is classified into nine subtypes. Shaded areas on **A)** represent circulating lineages that infect humans and the primate species that they derived from (red and orange, chimpanzee and gorilla) (green, sooty mangabey). **B)** depicts all the major subtypes and sub-subtypes of HIV-1 group M. Reproduced and adapted from Foley, *Global Virology II-HIV and NeuroAIDS* (Foley, 2017).

1.3 HIV-1 genome and proteome

The HIV-1 genome consists of two, 9.8 kb RNA positive-sense strands which are capped at the 5' end and polyadenylated at the 3' end. The genome has nine open reading frames encoding for fifteen different proteins (**Figure 1.4**) (Watts et al., 2009). The structure (**Figure 1.5**) of the mature HIV-1 virion consists of the dimeric RNA genome which is encased in p24 arranged in the conical capsid (CA) (Zhao et al., 2013). Accompanying the genome in the capsid are the nucleic acid binding proteins nucleocapsid protein (NC) and important HIV-1 replicative enzymes. The inner viral core is further encased in a host-derived membrane which associates with an inner viral membrane matrix (MA) and is embedded with the Env glycoprotein spikes. Env is a trimer of heterodimers which is required for viral attachment to host-targeted cells and consists of the surface gp120 and transmembrane gp41 glycoproteins (Zhu et al., 2006). Proteins expressed by HIV-1 can be categorized into 3 broad classes: structural, regulatory, and accessory.

1.3.1 Structural proteins

The Gag (group-specific antigen) polyprotein precursor encoded by the *gag* gene accounts for a majority of the structural elements of the HIV-1 virion (Briggs et al., 2004). The 55 kilodalton (kDa) Gag polyprotein, also known as p55, associates with the host cell membrane resulting in virion assembly and is processed by viral protease into its constituent viral structural proteins MA (p17), CA (p24) and NC (p7) during virion maturation. P6 protein is also cleaved from the Gag polyprotein precursor and is important for virion budding and incorporation of accessory HIV-1 proteins (Solbak et al., 2013).

Pol encodes for the HIV-1 enzymes reverse transcriptase (transcribes the HIV-1 RNA genome into cDNA), protease (cleaves the Gag and Gag-Pol polyprotein precursors), integrase (integrates viral cDNA into the host genome) and RNase H (removes original RNA template during viral cDNA reverse transcription) (Watts et al., 2009). These enzymes are expressed from a Gag-Pol-Pro precursor which is cleaved by protease and requires ribosomal frameshifting of the overlapping *gag-pol* gene regions at the 3' end of the *gag* gene for its expression (Jacks et al., 1988).

The HIV-1 Env glycoprotein is encoded by the *env* gene and is expressed as a 160 kDa precursor which is cleaved in the endoplasmic reticulum by the host protease, furin, into the non-covalently bounded gp120 and gp41 subunits (Hallenberger et al., 1992, Kwong et al., 1998). The Env proteins are subsequently incorporated into the virion outer membrane with

gp120 on the surface and gp41 imbedded within the membrane, with the gp120-gp41 subunits forming a trimer of heterodimers (Zhu et al., 2006).

1.3.2 Regulatory proteins

The trans-activator of transcription (Tat) protein encoded by the *tat* gene is a regulator of HIV-1 gene transcription. The protein occurs in differing amino acid lengths (72-101 amino acids) dependent on the viral subtype (Roy et al., 2015). Tat ensures that full-length production of HIV-1 RNA can occur, by binding to the transactivation response element (TAR) at the 5' end of viral transcripts, along with other protein elements such as cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CCNT1) (Tahirov et al., 2010). Phosphorylation of RNA polymerase II occurs ensuring efficient and productive transcription of the entire HIV-1 genome (Edwards et al., 2005, Roy et al., 1990).

Rev encodes for the 13 kDa protein, regulator of virion expression (Rev), which binds to RNA and is vital for the transport of unspliced and partially spliced mRNA from the nucleus to cytoplasm for translation of viral proteins (Mann et al., 1994, Malim et al., 1989). Rev can enter the nuclei of the host cell due to a nuclear localization signal found on Rev, and subsequently binds to the Rev-responsive element (RRE) which is a structured sequence found within the *env* gene (DiMattia et al., 2010).

1.3.3 Accessory proteins

Four HIV-1 accessory proteins are expressed, with various functioning for each one. The accessory proteins are necessary for the persistence and virulence of the virus *in vivo*, aiding in immune evasion and infectivity.

Nef encodes for the negative factor (Nef) protein, a 27 kDa protein expressed early in viral replication and important in the pathogenicity of the virus. Interestingly, human patients and NHP test subjects that are infected with strains of the HIV-1/SIV virus that harbour dysfunctional Nef, or have it deleted outright from the genome show reduced levels of pathogenicity or even a lack thereof, highlighting the importance of Nef as an accessory protein for lentiviral infection (Daniel et al., 1992, Kirchhoff et al., 1995). Nef is known to downregulate surface proteins of infected host cells, with the main HIV-1 host cell target receptor, cluster of differentiation 4 (CD4), a principal target (Lama et al., 1999). HIV-1 co-receptors, C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4) have also been shown to be downregulated along with CD4 (Sloan et al., 2010). The downregulation of CD4 ensures that budding virions do not bind to cell-surface CD4 due to

interactions of their Env proteins with the CD4 molecules, and it may also modulate immune functioning by preventing superinfection of cells and subsequent cytotoxicity (Ross et al., 1999, Jin et al., 2005, Toyoda et al., 2015). A second major functioning of Nef is the downregulation of major histocompatibility complex class I (MHC class I) which presents internal and 'non-self' cellular proteins, including HIV-1 viral antigens, to the immune system. This downregulation results in evasion from cytotoxic T lymphocytes (CTLs) and reduced destruction of infected cells (Schwartz et al., 1996). Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a negative immune modulator, is another cell-surface receptor downregulated by Nef, allowing for continued activation of infected T-cells which is vital for HIV-1 replication (El-Far et al., 2013). Nef has also been shown to have influence on other cellular processes such as remodelling of the cellular cytoskeleton via its effects on actin, and its modulation of cellular signalling pathways to enhance HIV-1 production and increase T-cell activity (Campbell et al., 2004, Linnemann et al., 2002, Pan et al., 2012).

Vif encodes for the 23 kDa protein, Viral infectivity factor (Vif). Vif is known to restrict APOBEC3G activity by acting as an adaptor protein in the ubiquitination process of APOBEC3G (Sheehy et al., 2002). Vif interacts with cullin5, elonginB/elonginC and Rbx2 to form an E3 ubiquitin ligase complex, under the influence of the transcription factor CBF-β (Stanley et al., 2008, Jäger et al., 2012).

Vpr encodes for the 14 kDa protein, Viral protein R (Vpr), which is incorporated into virions by the interaction of Vpr with the carboxyl-terminal region of p55 Gag (Bachand et al., 1999). Vpr has been shown to have a large plethora of functions which include transport of the HIV-1 pre-integration complex (PIC) to the nucleus, cell cycle arrest and activation of the DNA damage response, amongst others. Vpr associates with the PIC, which consists of integrase, matrix and viral cDNA, and then allows for the docking of the PIC with the nuclear pore complex of the nuclear membrane (Vodicka et al., 1998, Popov et al., 1998). Vpr is able to arrest the cell cycle of infected cells in the G2 phase, allowing for sustained activity of the HIV-1 viral long terminal repeat (LTR) promoter and therefore allowing more viral replication per cell (Goh et al., 1998). The mechanisms of this G2 arrest are still being investigated, but activation of DNA repair pathways such as the Rad3-related protein (ATR) DNA repair pathway are the likely causes of this cell cycle arrest (Zimmerman et al., 2004). Vpr also has pro-apoptotic and anti-apoptotic effects on infected cells, likely dependent on the stage of HIV-1 viral infection and cell type (Zhu et al., 2003, Zimmerman et al., 2004, Murakami and Aida, 2014, Conti et al., 2000). Vpr is also said to influence HIV-1 mutation rates by its interaction

with uracil DNA glycosylase (UNG2), an enzyme that repairs nuclear DNA by removing misincorporated deoxyuridine triphosphate (dUTP), though the exact role is controversial and still being elucidated (Guenzel et al., 2012, Weil et al., 2013, Wu et al., 2016).

Vpu encodes for the 16 kDa type I integral membrane protein, Vpu, which is expressed from a bicistronic mRNA which encodes for Env as well (Schwartz et al., 1990). Vpu is capable of various functions, with the downregulation of CD4 and release of budding virions its major two functions. Vpu downregulates CD4 within the endoplasmic reticulum (ER) by high-jacking the host ubiquitin ligase machinery, via its interactions with the ubiquitin ligase βTrCP and the Skp1-Cullin1-F-Box (SCF) E3 ubiquitin (Ub) ligase complex (Binette et al., 2007). This process prevents newly formed CD4 molecules from binding the Env precursor gp160 within the ER and preventing its incorporation into newly formed virions. The other major functioning of Vpu involves the degradation of tetherin, a membrane protein which is responsible for anchoring budding HIV-1 virions to their host cell preventing viral particle release (Neil et al., 2008). Vpu is said to downregulate tetherin by mechanisms such as proteasomal degradation involving βTrCP and the SCF ubiquitin ligase complex, and blocking the recycling of tetherin within the Golgi complex (Mangeat et al., 2009). Other important functions of Vpu involve the modulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), antagonism of antibody-dependent cellular cytotoxic responses (ADCC), prevention of (NK) cell-mediated CD4+ T cell death via the downregulation of NK-T and -B cell antigen (NTB-A) (Tokarev et al., 2013, Arias et al., 2014, Shah et al., 2010). More recently it was found that Vpu can downregulate human leukocyte antigen (HLA) – C therefore reducing CTL mediated prevention of viral replication in infected CD4+ T cells (Körner et al., 2017).

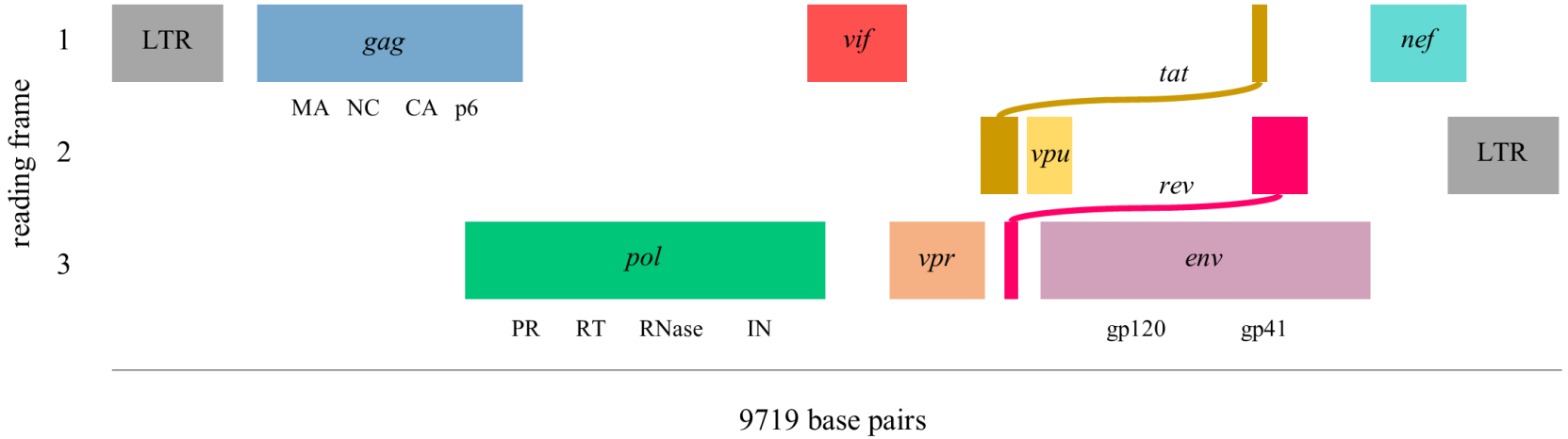


Figure 1.4: Schematic of HIV-1 genome. Organisation of the HIV-1 genome including coding regions and gene products. Adapted from Watts and Becerra et al. (Watts et al., 2009, Becerra et al., 2016).

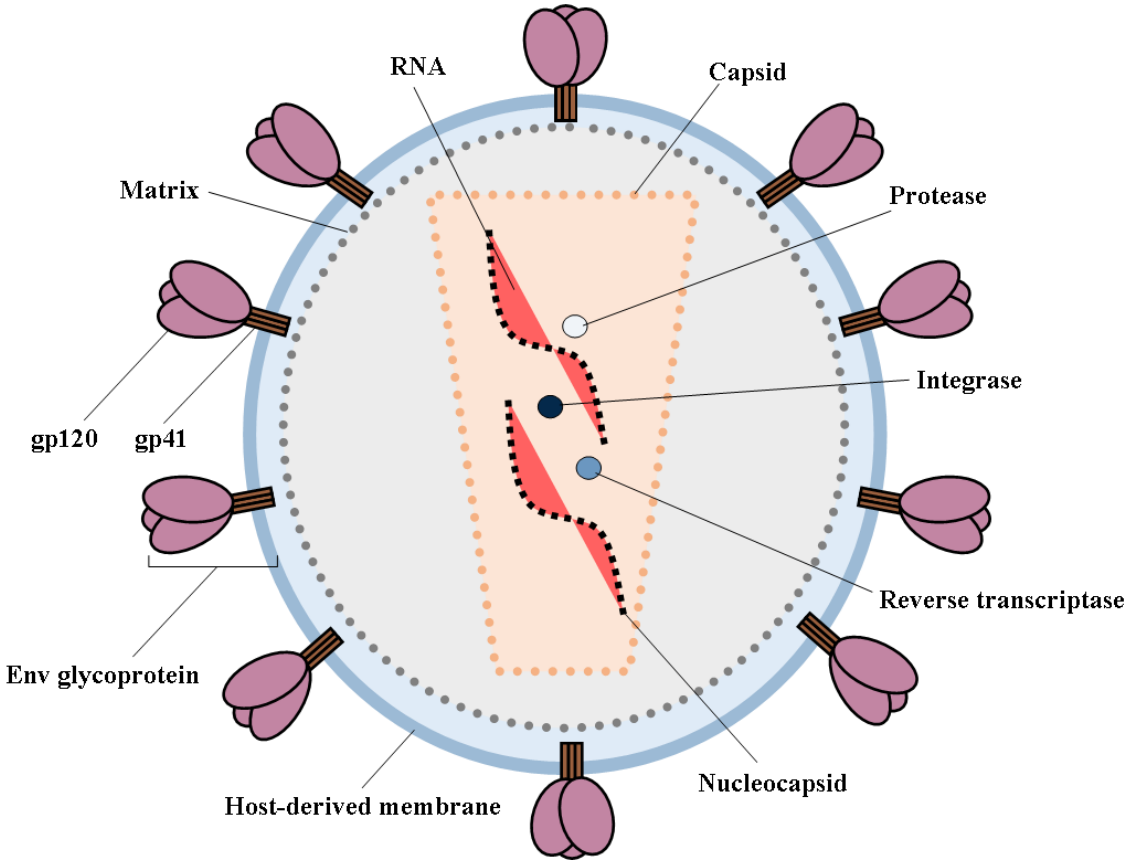


Figure 1.5: Cross-sectional model depicting the basic structure of the HIV-1 virion. The virion particle is ~120 nm in diameter. The virion is sparsely studded with the Env glycoprotein, a trimer of heterodimers, which can bind to CD4 and co-receptor CCR5 or CXCR4, allowing viral fusion with the target host cell. The capsid core contains the genome of the virus, two positive-sense strand RNA copies, and other important enzymes such as reverse transcriptase.

1.4 HIV-1 replication cycle

The HIV-1 replication cycle begins with the binding and subsequent entry of the virus into a host cell. Utilizing Env or host-cell-derived proteins that are embedded within the virion's host-derived viral membrane, the virus first attaches to the target cell through various host cell attachment factors. Receptors such as the T-cell gut mucosal homing receptor α4β7, Intercellular Adhesion Molecule (ICAM-1), dendritic cell–specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN), or even heparan sulphate proteoglycans may be involved (Guzzo et al., 2017, Bounou et al., 2002, Geijtenbeek et al., 2000, Saphire et al., 2001). These factors are non-essential but can help bring Env into proximity with CD4 and the chemokine co-receptor, enhancing infection. Once the HIV-1 virion is in proximity of the target cell, the gp120 glycoprotein subunit of the trimeric Env glycoprotein binds to the CD4 receptor by way of its CD4-binding site (**Figure 1.6**). A conformational change occurs in gp120, repositioning its 3rd variable loop (V3) and a bridging sheet for the interaction with the co-receptor (Kwong et al., 1998, Shaik et al., 2019). Co-receptor binding with gp120 then occurs, with either CCR5 or CXCR4 utilised depending on the tropism of the viral strain (Dey et al., 2009). Following co-receptor binding, viral membrane fusion then proceeds with the ectodomains of the gp41 trimeric subunits exposing their fusion peptides which then insert into the target-cell membrane (Pancera et al., 2014). Following insertion of the fusion peptide which brings the two membranes together, the interactions between the gp120 and gp41 subunits weaken resulting in the likely shedding of gp120, removing any steric hindrances for membrane fusion. The gp41 subunits in a pre-hairpin state form a six-helical bundle which drives the formation of the viral fusion pore and subsequent insertion of the HIV-1 viral contents into the target cell (Chan et al., 1997)

Once the virus enters the cytoplasm it can begin reverse transcription of its RNA genome, by the formation of the reverse transcription complex (RTC) before, during and after the uncoating of the viral capsid (Cosnefroy et al., 2016). The viral double-stranded DNA assembles into the PIC, which also consists of integrase, matrix, Vpr, reverse transcriptase and several host-derived factors (Haffar et al., 2000). The PIC travels along the microtubule network of the host cell to the nuclear pore complex (NPC) to crossover into the nucleus, with the capsid being of particular importance for the PIC entry (Yamashita and Emerman, 2004, Hulme et al., 2015, Arhel et al., 2006). Recently, it was shown that the entire capsid can enter the nucleus through the nucleopores, rupturing within the nucleus and releasing its contents (Zila et al., 2021). Other host derived proteins involved in facilitating the entry of the PIC include Cyclophilin A,

RANBP2, transportin 3, nucleoporin 153 and cleavage and polyadenylation specificity factor 6 (CPSF6) (Schaller et al., 2011, Lee et al., 2010, Rasheedi et al., 2016). Following entry of the PIC into the nucleus, integrase facilitates the docking and insertion of the viral DNA into the host genome, mediated by the high-order nucleoprotein structure formed by integrase and the viral DNA known as the intasome (Passos et al., 2017). Tethering to the nuclear chromatin occurs by interaction of integrase with lens‐epithelium‐derived growth factor (LEDGF) and is mediated by capsid's interaction with CPSF6 (Llano et al., 2006, Sowd et al., 2016). Integration does not occur randomly within the host genome as transcriptionally active regions are preferentially selected for (Schröder et al., 2002). The proviral long-terminal repeat (LTR) regions flanking either side of the provirus mediate the transcription of viral RNA destined to be assembled into viral particles as genomic RNA, or to be translated as viral proteins (Chen et al., 2009a).

The host RNA polymerase II transcribes the provirus into full-length viral RNA capable of being singly or multi-spliced, which is then exported out of the nucleus ready to be packaged as part of the viral RNA genome, or act as mRNA for translation of the various components of the HIV-1 virions (Purcell and Martin, 1993). The movement of unspliced RNA out of the nucleus is mediated by Rev and the RRE region located on the unspliced and partially spliced viral mRNA strands (DiMattia et al., 2010). Full-length unspliced HIV-1 RNA serves as a template for the Gag ($Pr55^{Gag}$) and Gag-Pro-Pol ($Pr160^{GagPol}$) precursors, or it dimerises to form the non-covalent viral genome which is then packaged in the mature virion (Chen et al., 2009a, Pettit et al., 2005). Gag is translated as a polyprotein which is later processed into matrix, capsid, SP1, SP2, nucleocapsid and p6. Interaction of Gag with full-length unspliced HIV-1 RNA results in commencement of packaging of the virion particles, with nucleocapsid playing an important role in selective packaging of the genomic HIV-1 RNA (Pettit et al., 2005, De Guzman et al., 1998). The Env precursor protein, gp160, is translated on the rough endoplasmic reticulum and is transported via the trans-Golgi network in oligomeric form, where it is glycosylated, arranged into trimers and then cleaved by furin into the gp120-gp41 subunits which are non-covalently bound (Berman et al., 1988, Schwartz et al., 1990). The virus assembles at the host cell plasma membrane and acquires its outer lipid membrane and the glycoprotein spikes as it buds from the host, managed by the endosomal sorting complexes required for transport (ESCRT) machinery which is hijacked by the virus (Morita et al., 2011).

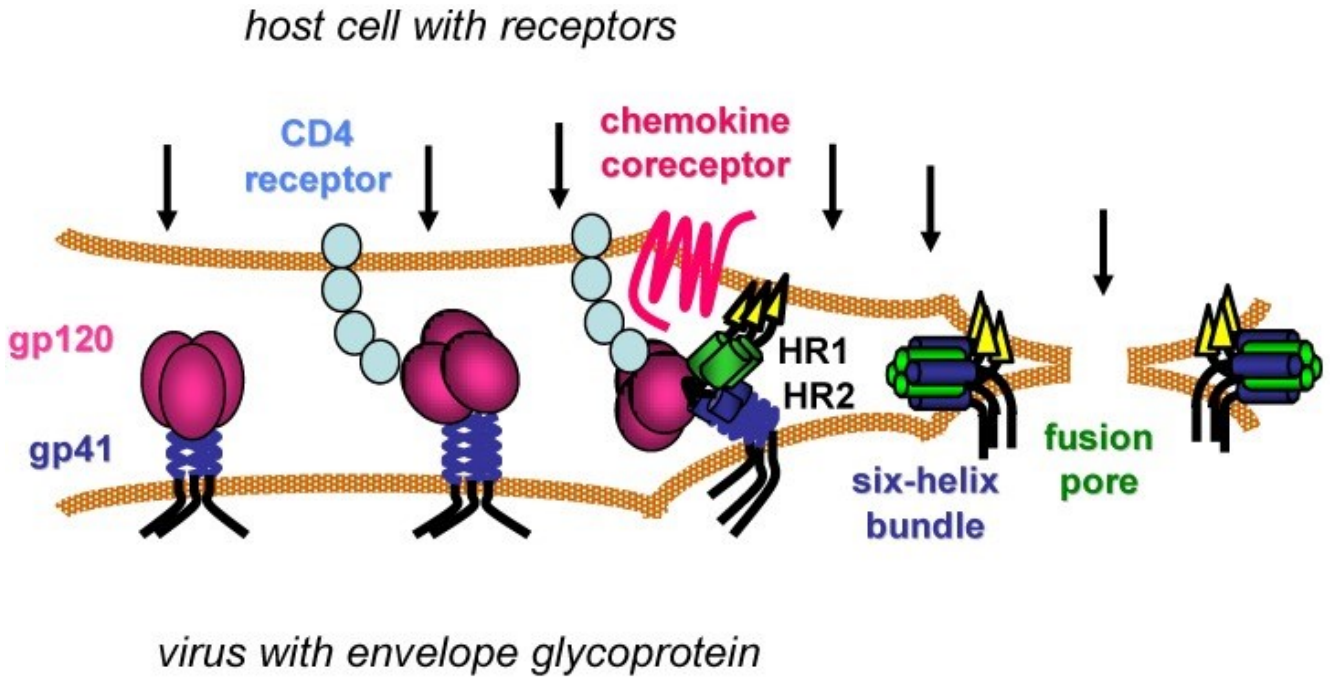


Figure 1.6: Fusion of the HIV-1 virion with target host cell. The HIV-1 Env glycoprotein binds to CD4 on the surface of a target host cell, undergoing a conformational change which exposes the co-receptor binding site. The gp41 subunit of the Env trimer forms the six-helix bundle responsible for bringing host cell and viral membranes into proximity to each other for fusion. Reproduced from De Feo and Weiss, *Viruses* (De Feo and Weiss, 2012).

1.5 HIV-1 immune response

To date, no efficacious HIV-1 vaccine has been developed, despite decades of work. The correlates of protection against HIV-1 are not yet fully understood, and the immune response against HIV-1 is complex, consisting of the entire gamut of the host innate, cellular, and humoral immune responses. HIV-1 and its interaction with the host immune system is also paradoxical, as the vast amounts of immune upregulation during infection in turn drive, rather than protect, the host from the natural disease progression of the virus. This is mirrored in early infection in NHPs where SIV transmission targets the subepithelial resting CD4+ T-cells of the genital mucosa (Zhang et al., 2004). These cells have been shown to produce fewer virions as compared to activated CD4+ T- cells, therefore implying a need for the recruitment of activated immune cells to the sites of infection to further propagate the virus. It has been shown in NHPs that following inoculation with SIV in the genital tracts of the animals, a cytokine cascade occurs, with the cervical epithelium recruiting dendritic and macrophage cells which in turn recruit activated CD4+ T-cells via a cascade of cytokine signalling (Shang et al., 2016). This is certainly noted in human patients presenting with sexually transmitted infections (STIs), which upregulates inflammation in the genital tissues and increases transmission susceptibility (Atashili et al., 2008).

1.5.1 Innate

The innate immune response to HIV-1 encompasses a large repertoire. Using experimental SIV-NHP models, some of the early mechanisms and interactions of the virus with components of the innate system have been elucidated. HIV-1 must first navigate the physical barrier of the genital epithelium, either through lesions in the epithelial lining, or inter/transcellularly (Hu et al., 2000). The virus has been shown to interact with dendritic cells of the epithelium (known as Langerhans cells), interacting with these CCR5-expressing immune cells and likely using them as a vehicle for intracellular transport to CD4+ T-cells in the sub-epithelial lymphatic tissues, or drainage into the lymph nodes, though the CD4+ T-cells are still the principal target of infection at the mucosal level (Zhou et al., 2011). The virus must overcome protective cervical or penile-urethral mucous, anti-microbial peptides which are secreted by epithelial cells and neutrophils, and interferons which are able to upregulate viral restriction factors such as APOBEC3G (Lai et al., 2009, Shapiro et al., 2001, Congote, 2007).

Vital to the innate immune system are the sensing receptors (pattern recognition receptors (PRRs)) and pathways that recognize conserved structural molecules known as pathogen-

associated molecular patterns (PAMPs) which are derived from various pathogens (Reviewed in Paludan and Bowie, 2013). Toll-like receptors (TLR) or other sensing molecules such as interferon inducible protein 16 (IFI16) and Cyclic GMP-AMP synthase (cGAS) can sense 'non-self' viral genetic material, or early viral products of reverse transcriptase within the cytosol, resulting in the release of interferons and other proinflammatory chemokines and cytokines (Jakobsen et al., 2013, Lahaye et al., 2013). Innate immune cells such as dendritic cells and macrophages are subsequently activated as well by the sensing of foreign viral material in infected cells, or apoptosis may occur in the compromised cells themselves (Doitsh et al., 2014).

1.5.2 Cellular

1.5.2.1 CD8+ T-cells

CD8+ T-cells are responsible for cytotoxic immune responses during HIV-1 infection, playing a pivotal role in the initial stages of infection and influencing the viral set-point of respective patients, with some patients such as long-term non-progressors (LTNP) and elite controllers able to control viral loads for many years under the influence of CD8+ responses (Streeck et al., 2009, Migueles et al., 2000, Ndhlovu et al., 2015, Ramsuran et al., 2018). The importance of CD8+ T-cell responses during HIV-1 infection have been illustrated in NHPs infected with SIV, with high viral loads manifesting in these animals when CD8+ T-cells begin to decline (Schmitz et al., 1999). The effectiveness with which CD8+ T-cells are able to control viral loads can be attributed to several factors including; the polyfunctionality of the CD8+ T-cells (this entails the ability of the CD8+ T-cells to release different cytokines and chemokines such as interferon (IFN–γ), tumour necrosis factor (TNF–α), interleukin-2 (IL-2), macrophage-inflammatory protein (MIP–1β)); the avidity of the CD8+ T-cells for their respective antigen; the clonality of the cells allowing differing CD8+ T-cells to recognise the same amino acid motifs; and lastly, the ability of the cells to mount an effective and protective immune response without prior exposure to HIV-1 (Almeida et al., 2007, Almeida et al., 2009).

HLA class I proteins found on the surface of all nucleated cells present small intracellular amino acid peptide sequences to CD8+ T-cells, with 'non-self' peptide sequences resulting in cytotoxic responses (Bushkin et al., 1990). Other mechanisms may also be activated such as the release of cytokines and chemokines. The earliest CD8+ responses and likely the most important, in the context of acute infection, involves the killing of HIV-1 infected cells indicated by early selective pressures on the virus selecting for escape mutants (Goonetilleke

et al., 2009). HLA-B molecules targeting epitopes in Gag seem to be the most readily associated with control of the virus, though a number of HLA alleles together may have a more additive effect than any single HLA molecule (Kiepiela et al., 2006, Leslie et al., 2010).

1.5.2.2 CD4+ T-cells

CD4+ T-cells lie at the centre of the adaptive immune response, influencing the activity of various immune cells in response to pathogens. CD4+ T-cells coordinate the functioning of B-cells and CD8+ T-cells and influence the recruitment of immune cells into various tissues. CD4+ T-cells are the principal target of HIV-1, with infection progressively destroying the cells and depleting the cell population over time resulting in a myriad of immune dysfunction (Dalgleish et al., 1984). Activated CD4+ T-cells seem to be targeted more readily by the virus rather than naïve cells, and paradoxically CD4+ T-cell depletion may in fact be occurring in the bystander population rather than the infected population with the HIV-1 Env glycoprotein one of several factors noted to have a role in apoptosis of bystander populations (Finkel et al., 1995, Muñoz-Arias et al., 2015, Cavrois et al., 2017).

Not only do CD4+ T-cells coordinate the mobilization and cytolytic responses of CD8+ T-cells by secretion of IFN-γ, and the proliferation of B-cells and secretion of their antibodies in response to HIV-1 infection, but they have also been shown to have cytolytic functioning as well. CD4+ T-cells extracted from HIV-1 infected patients have a highly cytolytic profile, with the cells expressing large quantities of granzymes (Nakanishi et al., 2009, Johnson et al., 2015). The evidence in the literature does imply that a role exists for CD4+ T-cell responses, particularly in early infection, likely aiding CD8+ T-cells in their control of early viraemia and establishment of the viral set point (Soghoian et al., 2012, Rosenberg et al., 1997). Interestingly, the profile of CD4+ T-cells collected during the only efficacious HIV-1 vaccine clinical trial to date, RV144, also displayed a cytolytic profile (de Souza et al., 2012).

An important subset of CD4+ T-cells are follicular helper T-cells (Tfh-cells). Tfh-cells are known to harbour HIV-1 virus as part of the virally persistent 'reservoir' (Perreau et al., 2013, Hufert et al., 1997). The functioning of Tfh-cells is to mediate the selection and survival of antigen-specific B-cells during affinity maturation in the germinal centres of secondary lymphoid organs such as the lymph nodes. B-cells in the germinal centres that are able to capture more antigen will receive more 'help' in the form of survival signals from Tfh-cells allowing for further mutations in order to better bind to its antigen (Reviewed in Qi, 2016). This process is thought to be critical for the generation of antibodies against HIV-1 in the

context of a potential successful HIV-1 vaccine. This is illustrated in NHP HIV-1 vaccine studies, where researchers have noted that test animals that generate more potent neutralizing antibody responses had greater ratios of Tfh-cells and B-cells within the germinal centres of their lymph nodes (Havenar-Daughton et al., 2016, Pauthner et al., 2017). Test animals with greater neutralization responses also seem to have upregulated expression of genes associated with Tfh-cells (Havenar-Daughton et al., 2016).

1.5.3 Humoral

1.5.3.1 Early antibody responses

Antibodies against HIV-1 begin to develop during the acute stages of infection, between infection and the first detectable viraemia (eclipse phase of infection). Circulating plasmablasts increase in the periphery during the early stages of infection and can account for as much as half of circulating B-cells (Buckner et al., 2013). Initial antibody responses target the gp41 subunit of the Env glycoprotein first and are highly polyreactive, likely due to the activation of memory B-cells that have previously encountered other non-HIV-1 antigens (Liao et al., 2011). Cross-reactivity has been noted in these B-cells, targeting both the gut microbiome and gp41 (Williams et al., 2015). Anti-gp120 responses follow soon after, with little or no control over escalating viraemia (Tomaras et al., 2008). Different classes of antibody are detected early at the same time including immunoglobulin M (IgM), IgA and IgG and this may be due to the initial CD4+ T-cell depletion, reducing the amount of help provided to B-cells for proliferation and appropriate class switching (Tomaras et al., 2008, Yates et al., 2013).

1.5.3.2 Autologous broadly neutralizing antibodies

The initial neutralizing antibody response against HIV-1 develops several months following infection, with antibodies developing against the autologous viral strain. A back and forth between antibody and virus ensues, resulting in numerous Env glycoprotein mutations, viral immune escape and the rise of neutralizing-resistant viral populations (Wei et al., 2003, Frost et al., 2005).Whilst antibodies develop in patients that are specific for the autologous virus, some patients develop antibodies that can neutralize a large variety of HIV-1 strains with great potency; these are known as broadly neutralizing antibodies (bNAbs) (Haynes and Mascola, 2017).

BNAbs protect by binding to the Env protein and preventing the virus from attaching to CD4 on its target cell, or prevent viral fusion with the target cell (Fouts et al., 1997, McDougal et al., 1996). Around ~20% of infected patients develop antibodies that are potent and broad, but

this is after several years of infection (Doria-Rose et al., 2009). *In vitro* viral neutralization assays have also shown that at least 50% of chronically infected HIV-1 patients develop some kind of neutralization breadth, being able to neutralize at least 50% of viruses tested against (Hraber et al., 2014). Of the ~20% that develop cross-reactive neutralizing antibodies, anywhere from 1% to 10% of these develop bNAbs that are able to neutralize hundreds of pseudoviral strains representative of circulating HIV-1 subtypes (Rusert et al., 2016, Landais et al., 2016). Interestingly, HIV-1-infected children develop bNAbs within the first 2 years of infection, with these bNAbs being more potent and broader than in most adults (Muenchhoff et al., 2016, Goo et al., 2014). It has been hypothesised that HIV-1 infected children develop favourable bNAb responses due to their unusually high viral loads and stable CD4+ T-cell numbers (McGuire et al., 2018, Muenchhoff et al., 2016, Ditse et al., 2018). It is important to note that although these antibodies can be broad and potent, they do not eradicate the virus in the hosts they originally derive from.

Recent advances in the last decade such as improved screening and *in vitro* pseudoviral assays have allowed researchers to isolate and characterise bNAbs, generating a wealth of knowledge about them. BNAbs have unusually rare, atypical traits. Somatic hypermutations are high in these immunoglobulins due to the length of time required for bNAb development and the constant viral exposure seen during chronic infection (Hwang et al., 2017). Mutations are not only noted in the variable regions of the antibodies, but also in the more conserved and stable framework regions, though this is not to say that all mutations contribute to the neutralizing potency and breadth of these immunoglobulins. Some studies have shown that insertions and deletions are required for neutralization breadth and potency, while in other cases they are not required (Liao et al., 2011, Sok et al., 2013, Liao et al., 2013b, Kepler et al., 2014). It has also been shown that very rare or 'improbable' mutations occur during somatic hypermutation, and may be required by bNAbs for the development of breadth, with even single rare amino acid mutations accounting for differences seen in neutralization breadth between antibodies of different branches in the same antibody lineage (Wiehe et al., 2018, Shen et al., 2020).

Heavy-chain complementarity-determining region 3 loops (CDRH3) of certain bNAbs such as those belonging to the CAP256-VRC26 class of antibodies are unusually long and charged, likely selected from a subset of rare naïve B-cell clones rather than developing from mutations during affinity maturation (Doria-Rose et al., 2014, Briney et al., 2012, Bhiman et al., 2015). These long CDRH3s aid some of the bNAbs in penetrating the well shielded Env glycoprotein, gaining access to the peptide sequences below (Pejchal et al., 2011).

Polyreactivity (binding of unrelated self- or foreign antigens) and autoreactivity (binding to self-antigens) have been noted in bNAbs, and this may negatively act against the development of bNAbs accounting for their rarity in HIV-1 positive patients, as autoreactivity would generally not be selected for by the immune system due to immune tolerance mechanisms. (Mouquet et al., 2010, Liao et al., 2011, Haynes et al., 2005, Liu et al., 2015). Mature B-cells that target self-antigens are fairly rare in healthy individuals, with only 20% of the mature repertoire said to be autoreactive (Wardemann et al., 2003). It has also been shown in mice that have knock-ins for various HIV-1 bNAbs that the tolerance mechanisms of these animals are highly activated, resulting in stringent control of developing B-cells such as increased deletion of clones and editing of the B-cell receptors (BCR) in the B-cell repertoire (Verkoczy et al., 2011).

1.6 The envelope glycoprotein

1.6.1 Structure

On the surface of the HIV-1 virion is the Env glycoprotein spike, a trimer of heterodimers and the sole target of bNAbs on the HIV-1 virion (**Figure 1.6 and Figure 1.7**) (Reviewed in Sanders and Moore, 2017). The gp160 precursor in the infected host cell is extensively glycosylated (with approximately 90 N-linked glycans added per trimer) before being cleaved by furin into its gp120 and gp41 subunits which remain non-covalently bound to one another (Cao et al., 2018, Hallenberger et al., 1992, Stewart-Jones et al., 2016). Three gp120-gp41 subunits then assemble with a hydrophobic tail on each gp41 unit anchoring the trimer into the host cell membrane which then becomes part of the HIV-1 virion membrane, as each viral particle buds off from its infected host cell (Zhu et al., 2006, Pancera et al., 2014, Julien et al., 2013). The gp120 subunit harbours the CD4 and co-receptor binding sites and contains five constant regions (C1-C5), and five outer variable regions or 'loops' (V1-V5), with the gp120 subunit stabilized by several disulphide bonds (Kwong et al., 1998, Pancera et al., 2014).

The gp120 subunits consist of an inner and outer domain due to their conformations and exposure in the trimeric structure, with a bridging sheet connection the two domains. The CD4 binding site (CD4bs) is a highly conserved region that has been extensively studied, and is comprised primarily of the C1, C3 and C4 regions of gp120 (Kwong et al., 1998). Recently, it was found that the quaternary structure of the Env glycoprotein is likely vital for HIV-1 infectivity, with a second CD4bs described (Liu et al., 2017). The first N-terminal domain of CD4 can bind the primary CD4bs on a single protomer of Env, with binding by a second CD4 molecule occurring on a second protomer which contains the second CD4bs which then stabilizes the interaction and allows for further coreceptor binding. The variable regions are not inherently important for CD4 binding, but the V3 loop does play an important role in membrane fusion of the viral particle and target cell by mediating binding to the co-receptor (CCR5/CXCR4) (Hwang et al., 1991, Cocchi et al., 1996, Shaik et al., 2019). The variable loops can undergo intense numbers of mutations, are heavily glycosylated and they shield the gp120 core structure underneath, contributing to immune escape of the virus (Cao et al., 2017, Tian et al., 2016, Liao et al., 2013b).

The gp41 subunits of Env are responsible for the fusing of the HIV-1 virion particle with its targeted host cell membrane. Three major domains make up gp41; the ectodomain, transmembrane domain and an intracellular cytoplasmic tail (Chan et al., 1997, Weissenhorn

et al., 1997). Gp41 wraps itself around the N and C termini of gp120 (C1 and C5) forming a four-helix collar (Pancera et al., 2014). The fusion peptide is located at the N-terminal hydrophobic region of the ectodomain and this is the region of the protein that inserts into the host cell membrane. The fusion peptide has also been shown to be conformationally flexible, and recent evidence points to the fusion peptide being exposed to solvent, rather than being buried in a hydrophobic pocket of the trimer exclusively (Kumar et al., 2019). The ectodomain is further divided into the heptad repeat regions (HR1 and HR2) which are α-helical coiled-coil structures connected by a disulphide bond (Chan et al., 1997, Weissenhorn et al., 1997). HR1 is bundled at the centre of the trimeric spike perpendicular to the viral membrane, with HR2 running diagonally to the viral membrane (Julien et al., 2013). Three HR1 and HR2 bundles within the trimer together form the six-helix bundles responsible for bringing host cell and viral membranes into proximity to each other for fusion. The final ~24 amino acids of the ectodomain of gp41 form the membrane-proximal external region (MPER) which is a highly conserved region required for viral infectivity (Carravilla et al., 2019, Fu et al., 2018).

Importantly, the Env trimeric glycoprotein is a seemingly metastable structure; occurring in closed pre-fusion states and open post-fusion states, while also occurring in various intermediate states that are in a dynamic equilibrium (de Taeye et al., 2015). The Env glycoprotein is said to 'breathe', with movement occurring within its sub-domains, particularly the apex region containing the variable loops V1-V3 (Guttman et al., 2015).

1.6.2 Targets for bNAbs

The Env glycoprotein spike bears the brunt of the humoral immune response against HIV-1. Despite regions of vulnerability existing on the Env glycoprotein, the virus is always several steps ahead of the humoral immune response, utilizing various escape and masking methods to fool the immune system. The constant evolution of the HIV-1 Env glycoprotein is driven by the error-prone RT (amongst other factors discussed above) and is in response to immune selective pressure, resulting in the accumulation of escape mutations within the sequence; the variable loop domains readily accommodate the majority of the escape mutations (O'Neil et al., 2002, Wei et al., 2003). The Env glycoprotein spike is coated with an extensive glycan shield, which makes up 50% of the mass of Env, and can conceal the protein structures underneath (Wei et al., 2003, Zhou et al., 2017). Conformational masking occurs in the pre-fusion Env glycoprotein, protecting the conserved receptor binding sites on the Env glycoprotein from antibody binding, whilst retaining the ability to bind CD4 and co-receptors;

steric hindrance is also a major concern as sites of vulnerability may be occluded from binding to neutralizing antibodies (Yang et al., 2002, Chen et al., 2009b, Pancera et al., 2014).

Although the immune system ultimately fails to deal with HIV-1 infection, advances in the last decade in B-cell culturing, sorting, and probing coupled with next-generation sequencing of evolving autologous virus and antibody in patients has yielded a trove of knowledge (Walker et al., 2009, Doria-Rose et al., 2014, Doria-Rose et al., 2009, Liao et al., 2013a). Researchers have isolated dozens of bNAbs and have mapped all the major bNAb epitopes on the pre-fusion HIV-1 Env glycoprotein spike, with antibodies assigned into six categories (**Figure 1.7**) (Chuang et al., 2019, Reviewed in McCoy and Burton, 2017, Reviewed in Kwong and Mascola, 2018).

1.6.2.1 Trimer apex – V1V2

The apex of Env is extensively covered by a glycan shield and variable loops (**Figure 1.7**). Antibodies in this category typically have long CDRH3s and can bind both the N-linked glycans of the glycan shield and the Env protein structure underneath. Antibodies most representative of this class include PG9, PGT145, CAP256-VRC26 and VRC38.01.

PG9 was described in 2009 (along with somatic variant PG19) and began the wave of discovery of new HIV-1 bNAbs. It was found to preferentially bind to conserved regions on the V1V2 loops in the trimeric conformation of Env, with a glycan at residue 160 critical for binding (Walker et al., 2009, McLellan et al., 2011). Interestingly, bNAbs in this class are sensitive even to naturally occurring heterogeneity in glycans on the Env glycoprotein (Doores and Burton, 2010). PGT145 reciprocates trimer-apex antibodies' preference for the Env trimeric structure, with the antibody shown to bind all three gp120 protomers of the native-Env complex with its long anionic CDRH3, penetrating through glycans at the so-called 'Env trimer 3-fold symmetry axis' (Lee et al., 2017). The other CDR regions of PGT145 have also been shown to stabilize the structure of the antibody while binding to Env. Both PG9 and PGT145 bind to a lysine-rich region in the V2 loop, and atypically of HIV-1 bNAbs, do not seem to display autoreactivity (Andrabi et al., 2015).

VRC38.01 is slightly different from the above two V1V2 antibodies in the way it binds the Env apex. It does not have the long and protruding CDRH3 regions of other V1V2 antibodies (such as CAP256-VRC26), exhibiting a more typical amino acid length for its CDRH3s (Cale et al., 2017, Bhiman et al., 2015). It binds via its amino acid side-chains to the side-chains in the V1V2 loops, shortening the distance that the CDRH3s need to penetrate the V1V2 glycan

shield, in contrast to PG9 and PGT145 (Cale et al., 2017). It also has a quaternary structure preference, and interestingly both its antigen-binding fragments (Fab) may bind one trimer at the same time.

1.6.2.2 High mannose patch – V3-glycan

Glycosylation of the Env protein takes place intracellularly in the infected host cell, utilising the host cell glycosylation machinery (Cao et al., 2018). This makes the glycan shield on Env human in origin and largely non-immunogenic as it is seen as 'self' by the immune system. A region of glycans near the V3 loop dubbed the 'high mannose patch' departs from this, having been noted to be a highly immunogenic site on the Env spike (**Figure 1.7**). This is likely due to the nature of the glycosylation in this region. It is thought that due to the high density of glycosylated regions on the outer portions of gp120, coupled with the way the protein folds intracellularly, the α-mannosidases in the golgi apparatus are unable to reach the mannose patch region and process these glycans efficiently (Pritchard et al., 2015). This results in a region that has a distinct glycosylation patterning from that seen on the rest of the glycan shield and is subsequently detected as less 'self' by the immune system. High mannose patch antibodies most representative of the class include 2G12, PGT121, PGT128 and PGT135.

2G12 is one of the early bNAbs that was isolated and used for many years in HIV-1 basic research before the current wave of bNAbs began in 2009 (Trkola et al., 1996). 2G12 has the unusual trait of having its heavy chain domains exchanged which enlarges its paratope and allows it to bind almost exclusively to glycans (Calarese et al., 2003). Recently, it was found that 2G12 binding to the mannose patch does indeed involve at least one amino acid, and not exclusively glycans (Chuang et al., 2019).

PGT121, PGT128 and PGT135 were all isolated from the same selection of elite neutralizers in 2011 (Walker et al., 2011). Much like 2G12 and other V3-glycan antibodies, the binding of these antibodies is largely dependent on glycans and in particular, glycan 332 (Walker et al., 2011). The motif ^{324}GDIR^{327} is also a common target in the case of PGT121 and PGT128 (Sok et al., 2016b). Each of these antibodies is differentiated by their angles of approach and individually unique sites that they bind surrounding the 332 glycan (Kong et al., 2013, Pejchal et al., 2011). These antibodies do not need to swap their heavy chain domains like 2G12 to recognise the high mannose patch. Interestingly, since the V3 loop is important for co-receptor binding, it has been speculated that this site is a particularly desirable epitope to target with

bNAbs (Shaik et al., 2019). The mannose patch itself partially shields vital regions in the V3 loop important for co-receptor binding (Sok et al., 2016b).

1.6.2.3 CD4 binding site

The CD4bs is a highly conserved region of the Env glycoprotein, integral for host cell infection (**Figure 1.7**). This would make it a sound target for bNAb generation as interfering with this region would abrogate HIV-1 binding to its target cell. The CD4bs is recessed though, making it a difficult proposition for any bNAb to bind and neutralize (Kwong et al., 1998). Nonetheless, many CD4bs bNAbs of high potency and breadth have been isolated over the years, and the first HIV-1 bNAb ever described, IgG1b12, is a CD4bs bNAb (Burton et al., 1994).

The angle of approach for these bNAbs needs to be precise to bind the CD4bs with the Env-binding angles of many CD4bs bNAbs being similar, with some slight geometrical differences between different categories (Zhou et al., 2015). CD4bs bNAbs are also some of the most somatically hypermutated of the Env bNAbs (Wu et al., 2015b). It has been proposed that CD4bs antibodies can be split into two general categories; those that bind the CD4bs with their CDRH3s or those that are genetically restricted to the V_H1-2 and V_H1-46 genes (Zhou et al., 2015, Bonsignori et al., 2016). Some CD4bs antibodies representative of the class includes CH103, VRC01, IOMA and 8ANC131.

CH103 was isolated in a landmark study in 2013 that examined the co-evolution of HIV-1 and bNAbs over time (Liao et al., 2013b). Interestingly, the founder virus in the patient that CH103 was isolated from was responsible for engaging the original naïve B-cell and unmutated BCR, which gave rise to the CH103 line as the autologous virus drove maturation in the subsequent CH103-lineage clones.

VRC01 was one of the first of the new wave of bNAbs to be described post-2009 (Zhou et al., 2010). VRC01 was shown to partially resemble CD4 in its binding of the CD4bs, yet it is distinct in its binding angle to the CD4bs (Zhou et al., 2010, Li et al., 2011). VRC01 and antibodies in its class also share common structural traits such as a five amino acid-long CDR L3 which aids in avoiding any clashes with the V5 region of Env (Wu et al., 2011, Zhou et al., 2010). The VRC01-class antibodies are also numerous and have been found in various patient donors. VRC01 and antibodies in its class have shown great potency and breadth, and VRC01 itself was chosen as the candidate antibody in the first ever large-scale passive immunization clinical trial (Gilbert Peter et al., 2017, Huang et al., 2017, Corey et al., 2021). One such antibody that was isolated and described in this class is N6, which shows extensive

neutralization breadth (98%), even against viral isolates that are resistant to other members in the class (Huang et al., 2016a). Importantly, the germline originators of the VRC01-class of antibodies show little reactivity with Env, posing many questions for vaccine researchers on how to engage these non-reactive B-cells (Wu et al., 2015b, Jardine et al., 2013).

A collection of CD4bs antibodies derived from a patient dubbed "N49", exhibited some of the highest breadth and potency recorded to date. The most potent, N49P7, was able to neutralize 100% of a 117-virus panel and 94% of a different 53-virus panel, for an overall neutralizing effectiveness of ~98% against 170 viruses tested against (Sajadi et al., 2018). These potent antibodies highlight the vulnerability and importance of the CD4bs in continued HIV-1 vaccine research.

1.6.2.4 Silent face

The 'silent face' of the Env glycoprotein is the most recent HIV-1 bNAb epitope to be described (**Figure 1.7**) (Zhou et al., 2018). This is a region that is heavily glycosylated and consists of domains V4, C4 and V5 of the gp120 monomer (Wyatt et al., 1998). This region was previously thought to be non-immunogenic and highly resistant to bNAb targeting (Wyatt et al., 1998, McCaffrey et al., 2004).

VRC-PG05 was discovered in 2018 and was the first silent face bNAb described. The antibody is not as broad as some other potent bNAbs, neutralizing 27% of HIV-1 pseudoviruses in a pseudoviral neutralization assay (Zhou et al., 2018). VRC-PG05 is a glycan-dependant bNAb with most of its targeted epitope consisting of glycans (88%), with glycan N262 of importance in addition to glycans N295 and N448. Interestingly, it is thought that VRC-PG05 interferes with CD4-induced conformational changes in Env as opposed to abrogating Env binding to CD4 (Zhou et al., 2018)

Very recently, SF12 and its related antibodies were described, adding to the number of antibodies known to target the silent face (Schoofs et al., 2019). These antibodies are far more potent and broader as compared to VRC-PG05, neutralizing 62% of HIV-1 pseudoviral strains with mean IC_{50} concentrations 4-fold more potent. SF12 contacts with more residues on Env, which accounts for its improved breadth and potency as compared to VRC-PG05.

1.6.2.5 Fusion peptide and gp120-gp41 interface

The gp120-gp41 interface region highlights the importance of studying the Env glycoprotein in its native, trimeric state as certain epitopes on Env are not evident when experimenting with

its composite gp120 and gp41 protomers in isolation (**Figure 1.7**). The interface is targeted by various antibodies that do not compete for the same binding sites and these antibodies differentially recognise Env in its pre- and post-CD4-bound states. The fusion peptide which is part of this epitope is itself a targeted epitope as well. Antibodies representative of this class include PGT151, VRC34.01, 35O22 and 8ANC195.

The first antibody described that targets the gp120-gp41 interface was PGT151 (Falkowska et al., 2014). It is an interesting bNAb as it only targets cleaved, pre-fusion, trimeric Env and is often used to stabilise the Env spike for crystallisation purposes. Typical of many bNAbs, PGT151 possesses an unusually long 28-amino-acid-length CDRH3 and glycans form part of its targeted epitope (Falkowska et al., 2014). The CDRH3 partially interacts with the fusion peptide and HR1 region locking the Env glycoprotein in its pre-fusion state, whilst also interacting with elements from the gp120 region (Lee et al., 2016). VRC34.01 also targets the fusion peptide but more directly and its discovery helped to uncover the fusion peptide as a direct target of bNAbs (Kong et al., 2016b)

35O22 and 8ANC195 represent the class the more generally recognises the gp120-gp41 interface (Huang et al., 2014, Scharf et al., 2014). Interestingly, 8ANC195 recognises the open and closed conformations of Env and can even partially reverse the CD4-induced conformational changes in Env (Scharf et al., 2015). 8ANC195 is also able to bind gp120 alone, though it was found that its epitope spans both gp120 and gp41 (Scheid et al., 2011, Scharf et al., 2014). Unlike some other bNAbs, 8ANC195 penetrates the glycan shield of Env using the entirety of its heavy chain variable region.

1.6.2.6 Membrane-proximal external region

The MPER epitope is not well recognised by antibodies on the pre-fusion trimeric Env structure and is said to be recognised in other conformations of Env, particularly the intermediate fusion conformation of gp41 (**Figure 1.7**) (Chakrabarti et al., 2011). The epitope was described well before the newer generation of bNAbs was discovered during the last decade. The MPER region is highly conserved and vital for viral fusion making it a logical target for bNAbs (Fu et al., 2018). Elicitation of antibodies against this region is complicated by the proximity of the MPER to the viral membrane. This not only reduces the accessibility of the MPER, but also results in many of the MPER bNAbs developing autoreactivity as a necessity to binding a region so close to the viral membrane (Irimia et al., 2016, Haynes et al., 2005). It is important to note that the HIV-1 lipid membrane that coats the viral particle and is embedded with the

Env spikes is derived from the human host (Welsch et al., 2007). This would make the B-cells that these bNAbs derive from rare due to immune tolerance mechanisms usually eliminating autoreactive B-cells (Verkoczy et al., 2010). Antibodies representative of this class include 2F5, 4E10 and 10E8.

2F5 along with 4E10 were some of the earliest bNAbs described (Buchacher et al., 1992, Stiegler et al., 2001). 2F5 was shown to bind to the N-terminal region of the MPER whilst 4E10 binds to a helical structure closer to the C-terminal region of the MPER (Barbato et al., 2003, Cardoso et al., 2005). These two antibodies were shown to be autoreactive, but displayed no pathogenicity when used in NHP studies (Hessell et al., 2010). Transgenic mice carrying the genes for 2F5 and 4E10 have highlighted the great difficulty in generating these bNAbs. Early immune tolerance mechanisms blocked the development of at least 95% of early 2F5/4E10 B-cell precursors, with the 5% that developed being anergic (Verkoczy et al., 2010, Verkoczy et al., 2011, Verkoczy et al., 2013). Despite the aforementioned issues with 4E10-like antibodies, there may still be cause to pursue the elicitation of related antibodies. PGZL1 was recently described, which shares similar germline genetic regions with 4E10, but has a shorter CDRH3 and exhibits less polyreactivity, likely making elicitation of this class of antibodies less difficult than initially thought (Zhang et al., 2019). More recently the highly broad and potent antibody RV217-VRC42.01, part of the same class as 4E10, was described and was shown to neutralize 96% of viruses in a 208 Env-pseudotyped virus panel (Krebs et al., 2019).

10E8 is one of the broadest known bNAbs (Huang et al., 2012). It was initially shown not to exhibit autoreactivity and binding of lipid membrane. Further testing has shown 10E8 does in fact interact with membranes albeit more weakly, and recently it was demonstrated that part of the 10E8 epitope is a portion of the gp41 transmembrane domain, positioning 10E8 parallel to the HIV-1 membrane upon binding to its epitope (Rujas et al., 2018, Rujas et al., 2016, Chen et al., 2014). More recently, LN01 was described (Pinto et al., 2019). This MPER-targeting antibody is comparable to 10E8 in its breadth and potency, and targets the same MPER epitope as 10E8, albeit with an additional target of amino acids within the transmembrane region of gp41.

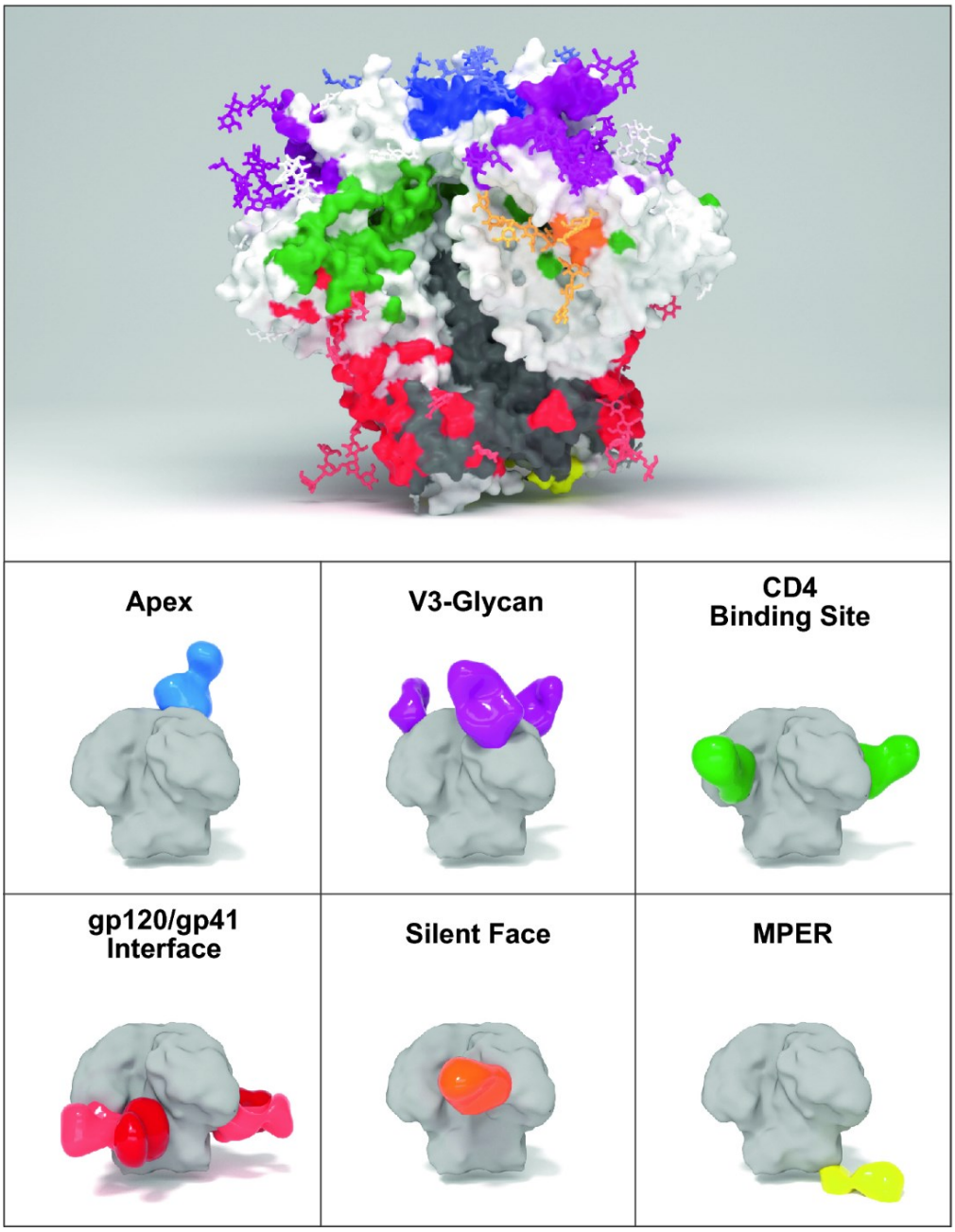


Figure 1.7: Molecular model of the HIV-1 Env glycoprotein and the principle broadly neutralizing antibody epitopes. Upper panel depicts binding locations of all the major bNAbs on the trimeric Env glycoprotein including amino acid and glycan moieties, with gp120 protomers in white and gp41 in grey. The model was built using a crystal structure of BG505 SOSIP.664 (Protein Data Bank code 4TVP). Lower panels include density maps depicting the angles of approach of the various bNAbs representative of their respective classes. Density maps depict PG9 (apex, blue), PGT121 (V3–glycan, purple), VRC01 (CD4 binding site, green), 8ANC195 (interface, pink), PGT151 (fusion peptide and gp120-gp41 interface, red), VRC-PG05 (orange, silent face), 10E8 (MPER, yellow). Reproduced from Pauthner and Hangartner, *Current Topics in Microbiology and Immunology* (Pauthner and Hangartner, 2020).

1.7 Vaccines, antibody therapies and the immune correlates of protection

To date, no efficacious HIV-1 vaccine has been developed despite decades of research and dozens of vaccine candidates tested. The immune correlates of protection for HIV-1 are still uncertain as the mere presence of anti-HIV-1 antibodies in patients following vaccination does not correspond with protection, unlike for many of the clinically approved vaccines in use today (Plotkin, 2010, Reviewed in Tomaras and Plotkin, 2017). The current body of evidence is strongly favouring bNAb responses induced by vaccination as one of the likely protective correlates, though this is inferred indirectly from passive immunization and NHP viral challenge studies, and has not been confirmed at this time in any human vaccine trials (Reviewed in Alter and Barouch, 2018, Pauthner et al., 2019). Other correlates of protection against HIV-1 acquisition such as cellular immunity or non-neutralizing antibody functionality such as Antibody-Dependent Cellular Cytotoxicity (ADCC) have been noted in clinical trials, but these are yet to be confirmed as repeatable (Rerks-Ngarm et al., 2009, de Souza et al., 2012, Haynes et al., 2012).

In vitro, standardised pseudoviral neutralization assays have helped map the potential potency and breadth of bNAbs, and assisted researchers in detecting neutralization potential in vaccinated animal and human sera (Montefiori et al., 2018). NHP studies have shown complete protection from viral challenges when bNAbs were passively immunized in these animals (Parren et al., 2001, Barouch et al., 2013b, Julg et al., 2017b, Julg et al., 2017a). In humans, the promise of bNAbs as a potential HIV-1 antiviral treatment and immunoprophylaxis has shown promise and is currently being explored in large-scale clinical trials (Caskey et al., 2015, Caskey et al., 2017, Huang et al., 2017, Mendoza et al., 2018, Crowell et al., 2019). Despite the promising evidence for the elicitation of bNAbs as an important outcome of an efficacious HIV-1 vaccine, researchers have never been able to generate broad and potent heterologous bNAb responses by immunization, either in NHPs or in humans, nor is it known if this process is even possible by traditional vaccination methodologies. It is also still unclear if results from *in vitro* pseudoviral assays and the rhesus macaque NHP model are completely valid as proxies for HIV-1 infection and vaccine efficacy in humans (Montefiori et al., 2018, Hessell et al., 2018)

1.7.1 Passive immunization and bNAb therapies

Passive immunization entails the administration of protective dosages of antibody into a subject to protect against a pathogen, circumventing the need for the subject's immune system to mount its own response. This strategy has been used for well over a century, and the use of bNAbs as monoclonal antibody therapies for HIV-1 have been explored extensively for the last two decades (The Lancet Special 1896, Reviewed in Walker and Burton, 2018). Many studies have now shown the protective properties of passive administration of bNAbs as a prophylaxis to prevent infection in animal models, and their use as a treatment therapy as well, with the bNAbs able to control viraemia for weeks at a time in Simian/Human Immunodeficiency Virus (SHIV)-infected NHPs (Mascola et al., 1999, Parren et al., 2001, Barouch et al., 2013b, Julg et al., 2017a).

A landmark study by Barouch and colleagues described the administration of a cocktail therapy consisting of PGT121, 3BNC117 and IgG1b12 bNAbs that was able to drop the viral levels of chronically infected NHPs below detectable levels within 7 days (Barouch et al., 2013b). This viral control persisted for as long as 3 months for some animals, with viral rebound corresponding with declines in serum antibody levels. Promisingly, continued virologic control was exhibited for some test animals long after serum antibody levels had declined. It has also been noted in both NHP and human trials that neutralization is not the only contributing factor of bNAbs in host viral control; Fc region functionality may also play a role in enhancing the host immune response (Barouch et al., 2013b, Schoofs et al., 2016)

In recent years, the newer generation of bNAbs have transitioned from animal to human clinical testing. Caskey and colleagues described for the first time the use of newer generation bNAbs as an HIV-1 therapeutic in humans (Caskey et al., 2015). The CD4bs bNAb, 3BNC117, was able to drop viral loads by up to 2.5 $\log_{10}$ in some test subjects, with viral loads remaining low for 28 days. In 2017, Caskey and colleagues showed that 10-1074 which is a V3 targeting bNAb could also supress viraemia in patients, though more viral escape variants were noted in patients (Caskey et al., 2017). To potentially overcome the limitations of mutating viral escape variants, Mendoza and colleagues looked at 3BNC117 and 10-1074 as a combination therapy during ARV cessation in patients (Mendoza et al., 2018). The 3BNC117 and 10-1074 cocktail showed many weeks more of viral load suppression in patients as compared to each bNAb given alone, with no viral variants noted that were resistant to both antibodies. Studies of older generation bNAb cocktail therapies in humans had shown no efficacy previously (Trkola et al., 2005).

VRC01 has also been explored as an HIV-1 treatment with promising results as well, with a single dosage of VRC01 reducing plasma viral levels in patients not on antiretroviral treatment by up to 1.8 $\log_{10}$ (Lynch et al., 2015). VRC01 has subsequently been tested in a double-blind placebo-controlled trial for its ability to sustain long-term viral control in patients who had their antiretroviral treatments interrupted. All patients bar one had to restart their antiretroviral treatment before the intended time endpoint (Crowell et al., 2019). Similar findings were also noted in an earlier open-label trial (Bar et al., 2016).

VRC01 was the candidate antibody in the important Antibody Mediated Preventation (AMP) trials which released results in early 2021 (Corey et al., 2021). The trials (HVTN 703 and 704) were a pair of randomized double-blinded placebo-controlled studies that enrolled over 4,000 individuals and looked to answer directly as to whether a bNAb can protect against HIV-1 acquisition in humans. Patients were administered low or high dosages of the antibody intravenously (10 mg/kg or 30 mg/kg) every 8 weeks for a total of 10 dosages. It was found that the antibody was able to protect against acquisition of HIV-1 strains that were already susceptible to neutralization by VRC01, with only 30% of isolates circulating in the regions of the trial-sites being sensitive to neutralization by VRC01. VRC01 was able to reduce risk of infection of HIV-1 by 75% if the infecting strain was sensitive to it but was disappointingly (but not unexpectedly) unable to significantly reduce HIV-1 acquisition overall in the trials, highlighting the limitations of a passive immunization strategy.

With mixed results emanating from the AMP trials, recent advances in antibody design will likely improve the efficacy of potential bNAb therapies and passive immunization studies in future. Far more potent bNAbs than VRC01 exist which can be used in future trials. The half-life of certain bNAbs can be extended, and researchers have managed to create bi- and tri-specific antibodies which show incredible potency and breadth as compared to their individual counterparts, while also addressing the issue of viral escape that can arise during monotherapies (Asokan et al., 2015, Xu et al., 2017, Gaudinski et al., 2018, Steinhardt et al., 2018, Moshoette et al., 2019). Although passive immunization and therapeutic strategies show promise, an efficacious immunogen-based HIV-1 vaccine is still desirable considering the high costs of manufacturing monoclonal antibodies and the logistical challenges of administrating antibodies to potentially millions of individuals.

1.7.2 Active immunization strategies to induce protective antibody responses

Current HIV-1 vaccine design aims to create immunogens that can engage with the rare B-cell precursors that eventually lead to bNAb development. The prevalent idea in the HIV-1 vaccine field is that this process will require different immunization steps and varied immunogen components that can steer these B-cells through affinity maturation and the eventual development of bNAbs.

1.7.2.1 Evolution of native-like Env trimers as vaccine immunogens

In vitro pseudoviral neutralization assays using HIV-1 pseudoviruses which display strain-specific Env glycoproteins were developed to assess the potency and breadth of sera and antibodies in HIV-1 vaccine research (Seaman et al., 2010). These assays classify different HIV-1 strains into 'tiers' based on their phenotypical susceptibility to neutralization. This is likely due to the configuration of the Env glycoprotein, with the open trimeric configuration and its intermediate state being more susceptible to neutralization (Tiers -1 and -1A), and the closed native states of the Env glycoprotein more difficult to neutralize (Tiers -2 and -3) (Reviewed in Montefiori et al., 2018). Most circulating HIV-1 strains would be classified as tier-2 strains and if the pseudoviral neutralization assay is indeed representative of what should be noted *in vivo*, a vaccine would need to elicit heterologous tier-2 antibody responses to show potential efficacy.

Vaccine strategies utilising gp120 as an immunogen have failed to elicit tier-2 antibody responses and showed no efficacy in clinical trials (Flynn et al., 2005, Pitisuttithum et al., 2006). The Env gp120 monomer presents immunodominant non-neutralizing epitopes that are inappropriate to elicit a potent and broad neutralizing antibody response against HIV-1 (Moore et al., 2006). It became apparent to researchers that development of recombinant native-like Env glycoproteins was likely a necessary step. As discussed previously (**section 1.6.2**), several of the vulnerable regions on Env targeted by bNAbs are dependent on the quaternary structure of the Env glycoprotein (Chuang et al., 2019). Different forms of the recombinant Env trimer have been developed over the years.

gp140 molecules were first developed by truncating the TM region and cytoplasmic tail of gp41 to improve solubility of the trimer (Earl et al., 1991). This had the adverse effect of destabilising the trimer. The furin-targeted cleavage site between gp120 and gp41 was modified to form uncleaved trimers which would not fall apart (Yang et al., 2000b). This had further detrimental effects of reduced expression efficiency and issues with aggregate formations

(Ringe et al., 2015). The cleavage between gp120 and gp41 is important for a fully functional Env trimer and effects the recognition of the trimer by antibodies; the gp120-gp41 interface is one of the epitopes targeted by bNAbs (Sanders et al., 2013, Ringe et al., 2013, Li et al., 2012b, Yasmeen et al., 2014). To further improve the formation of trimers, trimerization domains were added to the C-terminal ends of the truncated gp41 of gp140 recombinant proteins. The most used were the foldon domain T4 fibritin protein derived from bacteriophages, and the isoleucine zipper GCN4 derived from *Saccharomyces cerevisiae* (Yang et al., 2000a, Yang et al., 2000b, Yang et al., 2002). Some issues still existed with these early trimer constructs such as an inaccurate disulphide bond profile formation and aggregate formations (Go et al., 2016, Finzi et al., 2010). The immunogenicity was not much improved over the gp120 monomer in many animal immunization studies, with tier-1 neutralization responses generated in most cases and only a handful of NHP studies showing low-level tier-2 responses (Nkolola et al., 2010, Sanders et al., 2015, Hessell et al., 2016, Bradley et al., 2016, Saunders et al., 2017a, Saunders et al., 2017b). In humans, these trimers alone have only ever elicited tier-1 responses (Hurwitz et al., 2008, Lewis et al., 2011, Spearman et al., 2011, Cosgrove et al., 2016).

After the limitations of non-native gp140 molecules became apparent, researchers continued to develop a native-like trimer that closely resembled the Env molecule found on circulating viruses. The importance of cleaving gp120 and the truncated gp41 subunits was addressed with an optimised furin cleavage site (REKR to RRRRRR) (Binley et al., 2002). Subunit instability due to the improved cleavage was addressed with the inclusion of a disulphide bond between gp120 and the truncated gp41 subunits by mutating alanine at position 501 of gp120 and threonine at position 605 of gp41 (SOS) (Binley et al., 2000, Sanders et al., 2002). In order to stabilise the gp41 components in the trimeric pre-fusion state of the now SOS-stabilised gp120-gp41 protomers, an isoleucine to proline mutation (I559P) was introduced in the HR1 region of the truncated gp41 (Sanders et al., 2002). To further improve solubility of the trimer, part of the MPER region was removed at position 664 (Klasse et al., 2013). Finally, using all these modifications noted, the first native-like Env trimer was engineered based on an Env of a subtype A founder virus derived from a Kenyan infant known as ‘BG505’ (Wu et al., 2006, Hoffenberg et al., 2013, Sanders et al., 2013). The trimer was subsequently designated BG505 SOSIP.664.

BG505 SOSIP.664 has become the gold standard blueprint for trimer design, with much of the current Env-based vaccine research focused on it and its derivatives. The high-resolution structure of the Env glycoprotein was finally solved using BG505 SOSIP.664 as a template for

x-ray crystallography and cryogenic electron microscopy, and many of the bNAb epitopes were determined using BG505 SOSIP.664 (Lyumkis et al., 2013, Sanders et al., 2013, Julien et al., 2013). The design continues to be further improved by researchers yielding newer versions of the SOSIP trimer. Some of these newer refinements include attempts at expressing SOSIP.664 trimers from other HIV-1 subtypes, mutations in the gp41 region to further improve trimerization and improvements in stabilisation by introduction of more inter-subunit disulphide bonds (Julien et al., 2015, Kong et al., 2016a, Joyce et al., 2017, Torrents de la Peña et al., 2018). Other trimer designs have also been developed that differ from the SOSIP.664 blueprint but have been found to mimic the native trimer closely as well. These include the NFL gp140 variants that have the natural furin cleavage site replaced with a glycine-serine linker; and UFO gp140 variants that contain increased numbers of linker regions and a modified HR1 region (Kong et al., 2016a, Yang et al., 2018, He et al., 2018).

Despite the design success of modern native-like Env trimers, these Env glycoproteins have failed to yield any appreciable heterologous tier-2 antibody responses in different animal models such as rabbits, guinea pigs, mice and NHPs (Sanders et al., 2015, de Taeye et al., 2015, McCoy et al., 2016, Bradley et al., 2016, Pauthner et al., 2017, Torrents de la Peña et al., 2018). Issues have been noted such as holes in the glycan shield of the Env immunogens, and the possible influence of adjuvants or delivery methods of the immunogens, resulting in undesired immune responses. The exposed trimer base of soluble native-like Env trimeric glycoproteins (which is normally not exposed on native Env found on the virion particle) have also been noted as a problem region, with strong non-neutralizing responses elicited against that region (Nogal et al., 2020). Immunizations with BG505 SOSIP.664 in unusual animal models such as cows has shown some promise (Sok et al., 2017). Researchers were able to elicit broad and potent bNAb responses targeting the CD4bs after several immunizations in cows. Cows have a B-cell repertoire with the propensity to express bNAbs with extremely long CDRH3 loops which are a known characteristic of HIV-1-targeting bNAbs in humans. It is important to note however that this B-cell repertoire is unique to cows, and may not directly apply to humans, though it is encouraging that bNAb responses were able to be elicited in weeks rather than many months and years.

Recently, researchers were able to protect NHPs from repeated rectal challenges with a clinically relevant SHIV-BG505 following immunization with BG505 SOSIP.664 Env trimers (Pauthner et al., 2019). Although this was an autologous viral challenge, it was one of the first clear demonstrations of Env immunizations protecting NHPs from a tier-2 viral challenge. It

was found that NHPs that had higher tier-2 neutralizing antibody titres were better protected compared to animals with lower titres. Potential correlates of protection that had been noted previously in animal and human clinical trials such as ADCC and T-cell activity were not correlated with protection in this study. Interestingly and importantly, the neutralizing antibody threshold titre that correlated with protection was similar to serum antibody titres required to achieve protection in passive antibody transfer studies, and therefore sets a rough benchmark for future clinical studies. A follow up study showed that recognition of a diverse number of epitopes on Env in the high-titre NHPs may have been one of the reasons for the protection noted against SHIV challenges, with antibodies targeting the fusion peptide region thought to be responsible for the neutralizing breadth noted by the sera from some of these animals (Nogal et al., 2020).

1.7.2.2 Lineage-based designs, single-epitope immunogens, stabilised immunogen complexes and mosaics

It has become apparent from pre-clinical animal studies that immunization with the Env trimer alone may not be able to drive the development of broad and potent bNAb responses in humans. Different strategies for immunogen-based vaccines have been proposed and tested over the years with varying degrees of success. These strategies range from lineage-based immunogens to immunogens based around a single bNAb-targeting Env epitope, to stabilised immunogen complexes and multi-subtype Env cocktails.

1.7.2.2.1 Lineage / Germline vaccine designs

Lineage-based vaccine design aims to reproduce a known bNAb class (Reviewed in Kwong and Mascola, 2018, Andrabi et al., 2018). This entails engaging a naïve B-cell and its BCR known as the unmutated common ancestor (UCA), which is inferred computationally or experimentally, and then guiding the UCA towards bNAb development through a series of sequential immunizations that mimic specific intermediate Env sequences over time (Liao et al., 2013b, Doria-Rose et al., 2014). Overall, this immunization strategy aims to replicate the natural bNAb evolution process observed in a handful of human subjects where longitudinal samples were available. Interestingly, the UCAs do not usually bind most Env glycoproteins necessitating the development of unique Env-derived immunogens that can bind the UCA with sufficient affinity to activate the naïve B-cell and begin the process of affinity maturation (Xiao et al., 2009, McGuire et al., 2014, Gorman et al., 2015).

One example of lineage-based vaccine design is focused on the CD4bs bNAb lineage of CH103, which was initiated by the HIV-1 Env CH505 (Liao et al., 2013b). A series of booster gp120 immunogens were derived from various timepoints during infection in the respective patient, with the Env glycoprotein derived from the founder virus used as the priming immunogen (Bonsignori et al., 2016, Williams et al., 2017). This strategy was tested in both NHP and knock-in mice models, and it was found that the strategy initiates development of the desired bNAb lineage, but immune tolerance mechanisms restrict this development (Williams et al., 2017). This vaccine regimen is currently being tested in phase 1 human clinical trials .

It was noted that the earlier germline precursors of the VRC01-class were impeded by certain glycans from binding to Env and the CD4bs, necessitating the need to create Env immunogens with glycan and loop modifications that would allow interaction with precursor VRC01-class antibodies (Klein et al., 2013). VRC01-class alleles are commonly found in many HIV-1 infected subjects and therefore are an appealing class to target (Zhou et al., 2015). One such example of these immunogens is the development of modified gp120 monomers such as engineered outer domain (eOD) and gp120-core molecules which have parts of their glycans (e.g. N276 glycan) or variable loops (e.g. V1, V2 and V3 regions) modified or completely removed while retaining the CD4 and co-receptor binding sites, allowing for easier access and improved binding to the CD4bs (Jardine et al., 2013, Sok et al., 2016a, Havenar-Daughton et al., 2018, Duan et al., 2018).

Engineered immunogens such as eOD-GT6 and eOD-GT8 are designed to target many of the VRC01 germline antibodies and are displayed on nanoparticles to increase immunogenicity (Jardine et al., 2013). They first showed great promise in transgenic mouse models where they were able to activate VRC01-class germline precursors and drive somatic hypermutation, and they were able to bind *ex vivo* to human-derived VRC01-class B-cell precursors (Jardine et al., 2015, Briney et al., 2016, Sok et al., 2016a, Havenar-Daughton et al., 2018). There is concern that these engineered Env-based immunogens could have unspecified B-cell binding due to the presentation of non-neutralizing epitopes that are generally hidden in the trimeric native-like conformation of Env, but this unspecified binding could be prevented by adding glycans to the immunogens to mask unspecific binding and focus CD4bs binding (Jardine et al., 2015, Duan et al., 2018).

The immunogen eOD-GT8 60mer moved into phase 1 human clinical trials in 2018 to assess safety and immunogenicity in humans (ClinicalTrials.gov, 2018). Promising results of said

phase 1 trial (IAVI G001) were released in early 2021 (IAVI, 2021). The trial enrolled 48 patients who received low or high doses of eOD-GT8 60mer twice, two months apart. Almost all vaccinated participants (97%) developed detectable VRC01-class B-cells, thereby confirming germline-targeting vaccine designs as a viable and promising strategy.

1.7.2.2.2 Env epitope-based vaccine designs

Epitope-based vaccine designs look to engineer minimal immunogens that mimic the bNAb-targeting epitopes derived from full-length Env glycoprotein to focus the immune response wholly against a single epitope of Env (Reviewed in Kwong and Mascola, 2018, Andrabi et al., 2018). This form of vaccine design distinguishes itself from the lineage-based methods as elicitation of a single bNAb class is not the end-goal; the immunogens and their antigenicity are tested for binding to various classes of antibody that target the same Env region.

The most recent and successful of this design methodology was the engineering of an Env fusion peptide-based immunogen that was able to modestly induce neutralizing breadth in both mice and NHPs (Xu et al., 2018a). The immunogen was based around the target of bNAb VRC34.01, eight N-terminal amino acid residues of the Env fusion peptide (Kong et al., 2016b). The fusion peptide was chemically linked to a carrier protein, keyhole limpet hemocyanin (KLH), which improves immunogenicity. Immunization regimens consisting of the fusion peptide immunogen coupled with pre-fusion native-like Env trimer boosts, resulted in antibody induction in mice that could neutralize 31% of viruses in a 208 HIV-1 virus panel. Very modest neutralization breadth was noted in immunized NHPs, with the sera of one rhesus macaque able to neutralize 22% of viral strains tested against. In a more recent study, the same researchers were able to improve the low and inconsistent neutralization breadth noted in guinea pigs in the published 2018 study, by increasing the number of Env trimer boosts (Cheng et al., 2019). The fusion peptide has also been shown to be effective as a prime in conjunction with trimeric Env as a booster, or as a cocktail with Env in NHP immunizations (Kong et al., 2019, Cheng et al., 2020). More recently, the fusion peptide has proven useful as a vaccine prime before immunizing with whole native-like Env trimers in NHPs, reducing unwanted non-neutralizing antibody responses focused towards the base of soluble Env trimers which were shown to inversely correlate with neutralization breadth (Corrigan et al., 2021).

1.7.2.2.3 Stabilised Env-CD4 immune complexes and CD4i vaccine designs

A bulk of the current HIV-1 vaccine research looks to design immunogens based around the pre-fusion native-like Env trimer, with almost the entirety of the structure targeted in some

form by bNAbs, but it is important to note that other conformational states of the Env trimer could be vulnerable to antibody neutralization as well (Dingens et al., 2019, Chuang et al., 2019). When Env binds to CD4, a conformational change occurs. The gp120 protomers rotate and the V1V2 loops shift away from the apex of the trimer therefore opening the structure and revealing the co-receptor binding site (Wang et al., 2016, Ozorowski et al., 2017). This CD4 induced (CD4i) conformation reveals epitopes that are possible targets for antibodies and includes regions of the bridging sheet and V3 loop (Guan et al., 2013, Shaik et al., 2019). These revealed CD4i regions are relatively conserved amongst HIV-1 strains and divergent viruses, such as HIV-2 and SIV, owing to the importance of conserving functional structures such as the CD4bs and co-receptor binding sites making the CD4i regions a desirable target (Decker et al., 2005). Theoretically, the virion can still be neutralized by antibodies at this stage as the Env glycoprotein would still need to bind the co-receptor and then undergo fusion before the virion could infect its target host cell.

CD4i antibodies are often noted in many infected patients and develop early on in natural infection (Decker et al., 2005, Gray et al., 2007). Various CD4i antibodies such as 17b have been isolated over the years, but they are far less broad and potent compared to the newer generation of bNAbs (Guttman et al., 2015). There are also challenges in creating vaccine immunogens that could elicit a bNAb response against the CD4i regions as the Env glycoprotein has evolved to conceal these vulnerable regions. Steric and timing issues need to be considered for CD4i antibodies as well; cell-surface, CD4-bound Env can become inaccessible to antibodies due to the proximity of the cellular membrane, and the time frames in which an antibody would need to bind to the CD4i epitopes before the co-receptor binds and viral fusion occurs is limited (Labrijn et al., 2003, Munro et al., 2014).

Despite some shortcomings, the CD4i epitopes are still desirable and useful for HIV-1 vaccine design. CD4i antibodies could prove useful as a synergistic add-on to bNAbs such as those targeting the CD4bs, as it was shown that they could improve the binding of VRC01 to native-like Env trimers (Gardner et al., 2016). Immunizations with CD4i-presenting immunogens have shown promising results in NHPs, correlating with tissue control and clearance of viraemia following SHIV challenges (DeVico et al., 2007, Gordon et al., 2016). CD4i antibodies are often highly associated with non-neutralizing antibody effects such as ADCC in NHPs and indeed HIV-1 infected cells do regularly downregulate cell-surface CD4 via Nef and Vpu in order to prevent engagement with Env and exposure of the open Env conformation (Veillette et al., 2015, Prévost et al., 2018, Alsahafi et al., 2019). Recently, researchers noted

reduced infection risk during SHIV challenge in NHPs immunized mucosally with CD4i-presenting immunogens, with protection surprisingly correlated with microbiome modulation and accumulation of myeloid cells in the mucosal tissues (Sui et al., 2019). This may have revealed a possible novel use for these immunogens to promote mucosal innate immunity to prevent viral acquisition which is independent of anti-Env bNAb responses.

Various strategies have been developed to stabilise the CD4i-presenting gp120-CD4 complex or mimic it including: covalent cross-linking of gp120 and CD4, expressing single amino acid chains of gp120 and CD4 with flexible linker sequences, development of peptide modulators and CD4 mimetics that can induce the CD4i conformation of Env or block CD4 attachment, and stabilising of the complex using introduced disulphide bonds and remodelling of the gp120 protein structure (Vita et al., 1999, Fouts et al., 2002, Huang et al., 2005, DeVico et al., 2007, Dey et al., 2009, Cerutti et al., 2010, Roitburd-Berman et al., 2013, Ariën et al., 2016, Sui et al., 2019, Alsahafi et al., 2019).

1.7.2.2.4 Cocktails, mosaics, and consensus sequence vaccine designs

An HIV-1 vaccine will need to overcome the barrier of genetic diversity of HIV-1 if it is to be broad and efficacious. Lineage-based vaccine regimens are already looking to overcome this hurdle using sequential immunizations that mimic the natural evolution of the autologous Env glycoprotein over time in infected patients. This method seems to be superior to an Env-cocktail based approach, where several Env subtypes are administered simultaneously per immunization to mimic viral diversity encountered during natural infection (Wang et al., 2015, Williams et al., 2017, Torrents de la Peña et al., 2018). Administering too many Env glycoproteins from various viral subtypes at once seems to "de-focus" the host immune response and interfere with affinity maturation.

Mosaic vaccine strategies look to address the issues of autologous-only responses noted during HIV-1 vaccine pre-clinical and clinical trials. Mosaic vaccine design computationally eschews rare mutations or variant amino acid sequences, and looks to incorporate the most common and immunological of peptide sequences (derived from many HIV-1 subtypes and groups) at any given epitope region of an HIV-1-derived immunogen (Fischer et al., 2006). This strategy has proven useful in NHPs. Rhesus macaques immunized with Ad26 viral vectors expressing bivalent group M mosaic Env/Gag and Pol antigens were protected against SHIV-SF162$_{P3}$ challenge that was 100-fold greater in viral number than what is noted during natural infection in humans (Barouch et al., 2013a). Further testing in NHPs has noted further protection against

heterologous tier-2 SHIV challenges, and the vaccine candidate has proven safe in humans, now moving into human efficacy trials (Barouch et al., 2018, ClinicalTrials.gov, 2017b). Protection in this case is likely a combination of both humoral and cellular responses.

Another way to overcome viral diversity is the use of consensus sequence immunogens. This method is different from mosaics in that an amino acid sequence for a protein immunogen is selected by a consensus amino acid alignment, with the most common amino acid at every position included in the final sequence (Gao et al., 2005). A percentage threshold for including or excluding an amino acid is decided upon, or amino acid sequences are chosen according to phylogenetic analysis where a common ancestor sequence is chosen (or where the genetic distance between HIV-1 sequences is minimized) (Reviewed in Arenas and Posada, 2010, Korber et al., 2017). This rationale has been used to develop Env glycoproteins that have been tested in animal models, yielding tier-1 and weak heterologous tier-2 responses (Liao et al., 2013a, Hulot et al., 2015, Meyerhoff et al., 2017, Sliepen et al., 2019).

1.7.2.2.5 CD4 as an immunogen and the special case of ibalizumab

The HIV-1 Env glycoprotein is an obvious target for vaccine development, being the only exposed viral protein on the surface of the virion. Indeed, a large proportion of the vaccine research currently conducted in the field revolves around eliciting bNAb responses targeting the Env glycoprotein or developing Env-targeting bNAbs for passive infusions. Targeting the Env glycoprotein directly may not be the only strategy though in terms of development of an efficacious vaccine or prophylaxis. The host cell receptors that HIV-1 binds to, namely CD4 and co-receptors CCR5 or CXCR4, make for a logical target to prevent viral fusion with the host cells. The precedent already exists in nature, with naturally occurring mutations in the CCR5 receptor in some populations making certain individuals resistant to HIV-1 infection, dependant on the zygosity of their CCR5 allele (Novembre et al., 2005). ARV drugs such as Maraviroc target CCR5 as well (Tan et al., 2013).

CD4 is a 55 kDa immunoglobulin-like glycoprotein consisting of four extra-cellular domains and a cytoplasmic tail involved in signal-transduction. CD4 functions as a co-receptor for the T-cell receptor on T-lymphocytes during antigen presentation by major histocompatibility complex II (MHC II) on antigen-presenting cells (Wang et al., 2001, Kwong et al., 1998). Intuitively, it would seem problematic to target an important self-antigen such as CD4 to prevent HIV-1 infection, with autoimmunity and immune dysregulation a concern. During natural HIV-1 infection, a small percentage of patients do regularly develop anti-CD4

responses (Thiriart et al., 1988, Sekigawa et al., 1991, Labrijn et al., 2003). Whether these responses are associated with pathogenic or benign disease outcomes is uncertain, with the current literature still ambiguous. Immunotoxicity may depend on a number of factors such as; which epitopes of the CD4 molecule are targeted, the isotype of the generated antibodies, are these responses occurring during natural infection or due to recombinant CD4 exposure, is full length CD4 administered, and is the recipient receiving an autologous or heterologous CD4 sequence in relation to its species (Chams et al., 1988, Keiser et al., 1992, Reeves and Epstein, 1993, Müller et al., 1994, Lopalco et al., 1999, Fouts et al., 2002, Schwartz et al., 2016, Luo et al., 2017, Xu et al., 2018b). Research during the early years of the pandemic looked at soluble forms of CD4 as a possible viral inhibitor or vaccine candidate, with some promising antibody results noted in NHPs, but the particulars of such antibody responses were never explored further (Celada et al., 1990, Watanabe et al., 1991a, Watanabe et al., 1992, Schacker et al., 1994, Schacker et al., 1995). Interestingly, and perhaps in contrast to the current bulk of research in the field, the only monoclonal antibody (ibalizumab) therapy for HIV-1 that has been approved to date targets the human CD4 receptor (Emu et al., 2018).

Ibalizumab is a humanized IgG4 antibody derived from the murine antibody mu5A8 (Reimann et al., 1997, Jacobson et al., 2009). It targets the second domain of CD4 and through steric hindrance prevents viral fusion following gp120-CD4 binding. Of great importance, it does not interfere with normal immunological functioning of the CD4 receptor *in vivo* (Reimann et al., 1993). Ibalizumab has shown to be highly potent in pseudoviral assays, even more so than some Env-targeting bNAbs such as PG9 and VRC01, with IC_{50} values ten-fold less than the aforementioned bNAbs (Pace et al., 2013a). Various multi-specific bNAbs containing an ibalizumab component have shown even greater potency and breadth (Pace et al., 2013b, Huang et al., 2016b, Song et al., 2016). Ibalizumab was approved in 2018 for use as a combination therapy with ARVs in patients with multidrug resistant HIV-1 currently failing therapy (Emu et al., 2018).

1.7.3 HIV-1 vaccine clinical trials

The search for an immunogen-based HIV-1 vaccine is still ongoing as of 2020. Vaccines are one of the great advances of medicine having saved countless millions of lives during the 20th and 21st century (Orenstein and Ahmed, 2017, Wadman and You, 2017). All the infectious diseases that humans have been able to combat successfully have required an effective vaccine, and HIV-1 will likely be no different. Theoretically, if all HIV-1 positive individuals were to be on life-long antiretroviral treatment, and non-HIV-1-infected individuals at risk for infection

were to take pre-exposure prophylactics, new infections could be averted, and the course of the pandemic would be reversed (Reviewed in LeMessurier et al., 2018, Fauci, 2017). Practically, this is unlikely to happen. Human behaviour is difficult to account for when dealing with public health outcomes, coupled with the contexts that different populations reside in (ease of access to healthcare, stigma of vulnerable groups, ailing healthcare systems etc.) (Reviewed in Sengupta et al., 2011, Kranzer and Ford, 2011, Mutasa-Apollo et al., 2017). Drug regimens are a lifelong commitment, for both infected individuals and for national health services that manufacture and supply these drugs to the populace. Long-term use of pre-exposure prophylactics and ARVs are also not completely risk free with various side-effects such as renal and hepato-toxicity, and osteoporosis all a limitation; there is also the constant threat of drug resistance due to large scale usage and inadequate adherence (Reviewed in Chawla et al., 2018, Reviewed in Pennings, 2013).

An efficacious HIV-1 vaccine would be a welcome adjunct to current prevention strategies as numbers of yearly new infections remain far above 1.5 million per year, hampering attempts of ending the global outbreak by the end of this decade and controlling it in the long term. Modelling consistently predicts a positive impact for the introduction of an efficacious vaccine. Phillips and colleagues noted that even a weakly efficacious HIV-1 vaccine (~30%) introduced in Sub-Saharan Africa, which bears the brunt of the current pandemic, coupled with the continued roll-out of ARV therapy, would decrease predicted HIV-1 prevalence in 2060 by 67% (Phillips et al., 2014). Even if UNAIDS diagnosis and treatment targets for ending the HIV-1 pandemic were met in the coming years, Harmon and colleagues predict that yearly new HIV-infections would decline to 550,000 by 2070; if a 70% efficacious vaccine became available within this decade, then that number would eventually drop to 122,000 in 2070 (Harmon et al., 2016) They also predicted that if UNAIDS targets were not met and prevailing ARV trends noted in the early 2010s continued forward, then the introduction of said hypothetical vaccine could prevent a massive 42 million total new infections between 2027 and 2070. Other modelling by Medlock and colleagues using a hypothetical 50% efficacious vaccine introduced in 2020 and eventually scaled up to 70% coverage (and assuming treatment and diagnosis trends noted in the 2010s), could potentially result in 17 million averted infections by 2035 (Medlock et al., 2017). Several million of these infections averted would be in southern and eastern parts of Africa. Meeting UNAIDS diagnosis and treatment targets or even stricter targets coupled with a vaccine could see even larger reductions.

As of the second quarter of 2021, six HIV-1 vaccine efficacy trials have been fully completed. These clinical trials explored different combinations of viral vectors and recombinant HIV-1 proteins. Three vaccine trials are currently in progress as of the second quarter of 2021; HVTN 702 testing a canarypox vector-based vaccine was halted in 2020, whilst HVTN 705 and 706 are testing adenoviral vector/mosaic-based vaccine strategies.

1.7.3.1 AIDSVAX B/E (VAX003) and AIDSVAX B/B (VAX004)

VAX003 was a double-blinded, randomized, phase 3 trial which enrolled 2,546 injection drug users from Thailand and ran between 1999 and 2003 (Pitisuttithum et al., 2006). The vaccine consisted of two recombinant gp120 molecules derived from a subtype B and subtype CRF01_AE virus, administered in formulation with the adjuvant alum. Anti-gp120 responses did develop in the vaccinated subjects, but no protection was found in placebo and vaccine arms of the clinical trial. VAX004 was a double-blinded, randomized, phase 3 trial which enrolled 5,403 subjects and ran simultaneously with VAX003 (Flynn et al., 2005). The test groups consisted of men who have sex with men and high-risk female groups. The vaccine consisted of recombinant subtype B gp120 molecules formulated in alum. No protection was found much like VAX003, with only an estimated 6% efficacy noted.

1.7.3.2 HVTN 502 (STEP) and HVTN 503 (Phambili)

The failures of previous Env-based vaccines, coupled with compelling research based on HIV-1 cellular immune responses, encouraged researchers to design trials around eliciting said responses. LTNPs and cellular immunity are associated with one another, and similar findings had been noted in NHP models. The STEP and Phambili trials were both double-blinded, randomized phase 2b trials that took place in the mid-2000s (Buchbinder et al., 2008, Gray et al., 2011). The vaccines contained no Env glycoproteins and instead aimed to induce protective T-cell responses that could control viral load during infection. The vaccine regimen for both trials consisted of three separate adenoviral vectors (Ad5) expressing the *gag, nef,* and *pol* genes administered as a single mixture per immunization (MRKAd5). Interim analysis during the STEP trial yielded worrying results; the vaccine arm had an increase incidence of HIV-1 acquisition vs the placebo arm and both STEP and Phambili studies were subsequently stopped. The increase in HIV-1 infections in the vaccine arm was associated with Ad5 antibody seropositivity and the circumcision status of males. Interestingly, follow up testing of the subjects involved in the study noted that the increased HIV-1 risk that was noted in uncircumcised and Ad5 seropositive vaccinated men had waned over time (Duerr et al., 2012).

1.7.3.3 RV144

The RV144 trial or 'Thai Trial' ran from 2003 to 2006 with results published in 2009 (Rerks-Ngarm et al., 2009). This was the first and only HIV-1 vaccine clinical trial completed that showed any sort of significant efficacy. The trial was a randomized, multi-centre, phase 3 trial that enrolled 16,402 heterosexual participants at risk of HIV-1 infection. The vaccine administered in the test arm consisted of a recombinant canarypox vector (ALVAC-HIV) expressing HIV-1 Gag, Pro and gp120 linked to the TM region of gp41; this was followed by two recombinant gp120 protein boosts (AIDSVAX B/E). The vaccine efficacy at 42 months in the trial using modified intent-to-treat analysis was 31.2%. Interestingly, vaccine efficacy at 12 months was estimated as high as 60% using a post-hoc analysis and then subsequently began to decrease over time. Protection was associated with non-neutralizing antibody responses against the V1/V2 region of Env and ADCC functionality, with a surprisingly low titre of neutralizing antibodies noted. Other potential correlates such as CD4+ T-cell responses have been noted since then (Haynes et al., 2012, Corey et al., 2015).

1.7.3.4 HVTN 505

HVTN 505 was stopped after an interim analysis found lack of efficacy (Hammer et al., 2013). This was a randomized, double-blinded phase 2b trial that enrolled 2,504 men and transgender women who have sex with men. At baseline participants had to be below a threshold of anti-Ad5 antibody titres and men had to be circumcised, addressing the issues noted in the Phambili and Step trials. The DNA prime vaccine consisted of subtype B *gag / pol / nef* and subtype A, B and C *env*. The boost consisted of Ad5 vectors expressing subtype B Gag-Pol and Env from subtypes A, B and C.

1.7.3.5 HVTN 702

HVTN 702, a phase 2b/3 trial, was the most recent and far along HIV-1 vaccine clinical trial being conducted, but was stopped in early 2020 after an interim analysis found a lack of efficacy (NIAID, 2020, Gray et al., 2021). It was analogous to RV144 and looked to confirm the immunological correlates noted in RV144 (Bekker et al., 2018). Env immunogens were adapted from RV144 for the predominant HIV-1 subtype in South Africa and the adjuvant was changed to the more potent MF59. Initial phase 1/2 safety trials showed the vaccine was safe and elicited IgG antibody responses in all vaccinated individuals though V1V2 responses were lower than what was recorded in RV144 (Bekker et al., 2018) A total of 5,407 individuals were enrolled in the phase 2b/3 trial, with 2,704 receiving the vaccine and 2,700 receiving the

placebo. During the first 2 years of monitoring, 138 HIV-1 infections occurred in the vaccinated group as compared to the placebo group's 133 HIV infections over the same trial period. As data continues to be released and analysed from the trial, reasons put forward for the vaccine's lack of efficacy include differences in HIV-1 incidence between the RV144 and HVTN 702 study populations, correlates of protection being specific only to RV144 due to Thailand's circulating HIV subtypes, or differences in host genetic factors between study populations (Dieffenbach and Fauci, 2020, Gray et al., 2021).

1.8 Non-human primate models for HIV-1 vaccine research

An animal model which recreates all the important features of HIV-1 infection does not exist. NHP models have been in use for decades now in HIV-1 vaccine research and have become the standard animal model for HIV-1 vaccine testing. Chimpanzees were used in the earlier days of the pandemic, with HIV-1 shown to replicate in these animals (Fultz et al., 1986, Hu et al., 1987, Fultz et al., 1992, Gao et al., 1999). The virus was poorly pathogenic though, and with chimpanzees becoming unavailable for medical research due to their conservation status, the model was abandoned (Koopman et al., 1999, O'Neil et al., 2000, Knight, 2008). Researchers looked to the SIV-NHP model as a replacement, particularly the use of different Old-World macaque species. SIV is closely related to HIV-1, and when infected in non-natural NHP hosts of the virus (such as macaques), an AIDS-like pathology develops which closely mimics that seen in humans (Letvin et al., 1983, Letvin et al., 1985, Reimann et al., 1996). Macaques are also physiologically and immunologically similar to humans, albeit for some important differences genetically in terms of MHC-types and other immunological restriction factors (Blokhuis et al., 2010, Doxiadis et al., 2011, Reviewed in Estes et al., 2018). To test antibody responses to the HIV-1 Env glycoprotein as SIV and HIV differ in this respect, chimaeras of SIV/HIV (SHIV) were developed (Shibata et al., 1991, Reimann et al., 1996).

Rhesus macaque (*Macaca mulatta*) is the NHP species most often used for HIV-1 vaccine research. Indian-origin rhesus macaques have been used extensively for this type of research, but in recent decades have been more difficult to import due to restrictions imposed in India, with researchers using Indian-origin rhesus macaques from already established colonies outside of India (Cohen, 2000). Chinese-origin rhesus macaques are being utilised more often in recent years due to easier import (Reviewed in Zhou et al., 2013). There are some differences with regards to disease progression between these two types; SIV viral set-points for Chinese-origin rhesus macaques are lower, CD4+ T-cell counts remain higher during chronic infection, antibody responses appear to be stronger and the animals survive longer as compared to their Indian-origin counterparts (Marthas et al., 2001, Ling et al., 2002, Trichel et al., 2002, Zhou et al., 2013). Chinese-origin rhesus macaques appear to mimic the disease progression seen in humans more closely in terms of CD4+ T-cell decline and overall lower viral loads, as compared to Indian-origin rhesus macaques.

The immunogenetic profiles of rhesus macaques are important when challenging rhesus macaques with SIV or SHIV, as various restriction factors could bias viral challenge studies with some test animals being more, or less susceptible to viral challenge (Chaichompoo et al.,

2010, Albrecht et al., 2014). For many years, the immunogenetic profiles of Chinese-origin rhesus macaques was less known compared to their Indian-origin counterparts, but in recent years more data has been generated on this subject (Hernandez et al., 2007, Chen et al., 2011a, Li et al., 2012a, Liu et al., 2013, Karl et al., 2013, Jiang et al., 2019).

1.8.1 MHC class I

MHC class I molecules on the surface of nucleated cells present intracellular-derived peptide chains to the immune system (Doherty and Zinkernagel, 1975). The immune system recognises these peptide chains as 'self' or 'non-self' and deploy CTL and NK cell responses when foreign, and likely pathogenic material, is recognised. In humans, three primary MHC class I genes exist namely *HLA-A, -B* and *-C* with hundreds of polymorphisms occurring throughout different human populations (Buhler and Sanchez-Mazas, 2011). Rhesus macaque MHC class I genes referred to as *Mamu-A* and *-B* are highly polymorphic and are the *HLA-A* and *-B* equivalents. The macaque MHC class I regions differ from humans as far more combinations and copy numbers can be expressed at one time, particularly for the *Mamu-B* genes (Otting et al., 2005, Doxiadis et al., 2011). Some SIV epitopes recognised by these MHC class I molecules have been identified, and certain *Mamu* alleles such as *Mamu-A*01, Mamu-B*08* and *Mamu-B*17* have been noted to influence outcomes of viral challenge studies in rhesus macaques (Allen et al., 1998, Mothé et al., 2003, Yant et al., 2006, Loffredo et al., 2007b)

1.8.2 KIR

Killer-cell immunoglobulin-like receptors (KIR) are found on NK cells of the innate immune system and regulate NK cell-mediated destruction of pathogenic cells, either through inhibitory or activating signalling (Moretta and Moretta, 2004). These KIRs interact with major histocompatibility complex (MHC) class I found on nucleated cells and can determine the presence or absence of viral infection of those cells that express lowered levels of MHC class I. In humans, the polymorphic complexity of KIR alleles reflects that of the MHC class I regions, and this complexity exists in rhesus macaques as well albeit in the form of extensive copy number expression of *KIR* genes. Hundreds of genotype and haplotype combinations have also been noted in rhesus macaques, in contrast to humans where *KIR* genes have been designated into two haplotypes (Sambrook et al., 2005, Blokhuis et al., 2010, Blokhuis et al., 2011). Rhesus macaques from different geographical regions such as China and India also differ in their presence or absence of certain *KIR* genes (Blokhuis et al., 2011). Viral challenge studies have noted associations between certain *KIR* haplotypes and viral control. The presence

of *KIR3DL05–KIR3DS05–KIR3DL10* was associated with quicker progression to AIDS in test animals, where as in other studies *KIR3DL02* and *KIR3DSW08* was associated with improved viral control and disease progression (Bostik et al., 2009, Chaichompoo et al., 2010, Albrecht et al., 2014).

1.8.3 TRIM5α

Tripartite motif protein 5α (TRIM5α) is a cytoplasmic restriction factor that can make NHP cells resistant to lentiviral infection (Stremlau et al., 2004). Human cells express TRIM proteins as well, but they are ineffective at preventing HIV-1 infection. TRIM proteins consists of a RING, B-box2 and coiled-coil domains, as well as a C-terminal B30.2 (SPRY) domain for theTRIM5 α-isoform that is important for antiviral functioning (Lim et al., 2010b). The SPRY domain can recognise the capsid core of certain infecting lentiviruses, accelerating uncoating and destabilising the viral core (Takeda et al., 2015). TRIM5α has been shown to influence SIV infectivity in rhesus macaques dependant on the infecting SIV strain, *TRIM5* genotype (homozygous restrictive, heterozygous permissive, and homozygous permissive) and occurring polymorphisms in the SPRY domain of the macaques (Lim et al., 2010b) (Yeh et al., 2011, Wu et al., 2015a, Kasturi et al., 2017). Interestingly, a variant of the TRIM5α protein can occur when the *TRIM5* gene harbours a retro transposed *cypA* pseudogene, resulting in expression of a TRIM5α-cyclophilin A fusion protein (TRIMCypA) (Yu et al., 2013). This variant does not protect Old World monkeys from HIV-1 infection in contrast to TRIM5α. TRIMCypA occurs in pig-tailed macaques and to lesser extents in other macaque species, and is extremely rare in Chinese-origin rhesus macaques (Yu et al., 2013)

1.8.4 CCL3

Chemokine (C-C motif) ligand 3 (CCL3), or formally known as macrophage inflammatory factor-1α (MIP-1α), is a chemokine that can interact with CCR5 (Cocchi et al., 1995, Menten et al., 1999). Two genes encode for CCL3, *CCL3* and *CCL3-like 1* (*CCL3L1*), with copy number variations occurring for *CCL3L1* likely due to segmental duplication events (Townson et al., 2002). Copy number variation in *CCL3L1* genes was shown to be associated with decreased infection rates and HIV-1 viral control in humans (Gonzalez et al., 2005, Dolan et al., 2007). An increased copy number of the *CCL3L1* is said to result in increased expression of the CCL3 chemokine and protect subjects from HIV-1 infection, by either competitively binding CCR5 or through other means such as modulating cell-mediated immune responses (Moore and Klasse, 2007, Dolan et al., 2007). Lowered copy numbers associated with

increased risks of infection, high viral loads and quicker progression to AIDS (Townson et al., 2002, Gonzalez et al., 2005, Dolan et al., 2007). Controversy exists for the exact role that CCL3 plays in HIV-1 susceptibility and viral control (Shrestha et al., 2009). Some researchers have found no correlations between the chemokine and HIV-1 control, and questions have been raised as to the methodology used previously to detect associations between CCL3 and HIV-1 (Urban et al., 2009, Field et al., 2009, Bhattacharya et al., 2009). A 2010 meta-analysis looking at 9 studies and incorporating 6000 subjects did indeed find an association between *CCL3L1* copy number variation and HIV-1 susceptibility (Liu et al., 2011).

Rhesus macaques carry an ortholog of *CCL3L1* on their chromosome 16. CCL3 is expected to act similarly to what is noted in humans, with copy number variation resulting in variable levels of CCL3 expression, and subsequent control of SIV infection (Taormina et al., 2012). Lower copy numbers have been associated with more rapid progression to simian AIDS in rhesus macaques (Degenhardt et al., 2009). Chinese-origin rhesus macaques also seem to carry more copy numbers than their Indian-origin counterparts, which could potentially contribute to their improved SIV disease control. *CCL3L1* copy number has also been found not to associate with SIV control in rhesus macaques (Lim et al., 2010a). The research for CCL3 effects on HIV-1/SIV control therefore remains uncertain, with conflicting data existing.

1.9 Justification of current study

HIV-1 vaccine research has made significant strides in the past decade; from the moderately positive results of the RV144 trial and wealth of immunological correlates garnered from it; to the isolation of hundreds of potent monoclonal antibodies and the elucidation of their development during natural infection; to the solving of the native-like Env structure and the subsequent Env-based immunogens that have been developed (Rerks-Ngarm et al., 2009, Haynes et al., 2012, Julien et al., 2013, Chuang et al., 2019). Yet, in 2021, researchers are still without a vaccine, with immunogen-based vaccine strategies to elicit bNAbs still making their way through early pre-clinical trials. Env-based immunogens have yet to induce consistent, potent and broad tier-2 neutralizing responses in animal models (Sanders et al., 2015, Pauthner et al., 2017, Zhou et al., 2017, Saunders et al., 2017b, Pauthner et al., 2019). The fusion peptide has recently emerged as a possible target for a vaccine, but despite the moderately positive results obtained in NHPs, consistency of breadth and potency of the neutralizing responses will need to be improved (Xu et al., 2018a).

A typical immune strategy will likely not suffice for HIV-1. It will need to be elucidated how a naïve B-cell and its UCA can be activated by an immunogen, and then subsequently be affinity maturated into a broad and potent antibody. This would need to occur within a reasonable time frame and not the several years that it takes during natural infection for a bNAb to arise (Landais et al., 2016). Sequential immunization schemes to steer the affinity maturation of potential bNAbs towards the high levels of somatic hypermutation required will likely need to be incorporated, as well as accounting for the lack of focused immunogenicity that has been noted with typical Env immunogens (Klasse et al., 2016, Escolano et al., 2016, McCoy et al., 2016, Williams et al., 2017, Mohan et al., 2018). Immune tolerance mechanisms will also need to be overcome to ensure bNAb development in a large proportion of those vaccinated (Verkoczy et al., 2010, Verkoczy et al., 2011, Kelsoe and Haynes, 2017).

Alternative Env-based immunogens may prove to be useful in creating new vaccine targets and overcoming the noted barriers to bNAb development. Immunogen complexes which expose CD4i epitopes are one such alternative. As described previously (**section 1.7.2.2.3**) stabilised immunogen complexes of Env and CD4 mimic a key step in the HIV-1 entry process and expose conserved epitopes that could potentially be exploited as a vaccine target. Env-CD4-based immunogens such as full-length single chains (FLSC) or cross-linked Env to CD4-mimetics have proven to be promising immunogen candidates (DeVico et al., 2007, Thomas et al., 2014, Shen et al., 2017).

Our research group has previously tested Env-CD4 subunit immunogens in New Zealand white rabbits and we were able to elicit tier-2 neutralizing responses against a panel of clinically relevant HIV-1 subtype B and C pseudoviruses (Killick et al., 2015). gp140 sequences based on a consensus of subtype C founder viruses with a trimeric GCN4 leucine zipper, and two-domain CD4 with a serine to cysteine mutation at position 60 were complexed (Env–2dCD4^{S60C}) and administered to New Zealand white rabbits. The gp140 sequences were developed with the rationale that an Env-based HIV-1 vaccine would need to be representative of the viral diversity of the specific region that the vaccine would be introduced; in this case South Africa, which is predominately subtype C (Killick et al., 2014).

Sera from complex-immunized rabbits showed greater neutralization activity as compared to those rabbits that were administered monomeric Env, trimeric Env or 2dCD4^{S60C} alone. 2dCD4^{S60C} (**Figure 1.8**) consists of the outer two domains of CD4, with the S60C mutation positioning a thiol in proximity of the Env V1/V2 loop disulphide (Cys126–Cys196) allowing for a formation of an interchain disulphide bond and therefore a highly stable Env-CD4 immunogen complex (Cerutti et al., 2010). This highly stable complex could allow for the exposure of unique neutralizing-antibody-targeted epitopes not present on unliganded Env proteins. Mapping of the immunized rabbit sera revealed the protective immune responses of Env–2dCD4^{S60C}-vaccinated rabbits to be targeted towards the CD4 component of the immunogen; a surprising result not previously seen by other research groups working on similar immunogen concepts (Killick et al., 2015). This unexpected result is potentially promising despite autoimmunity concerns, considering the recent approval of the anti-CD4 antibody ibalizumab for HIV-1 combination therapy (Emu et al., 2018).

The next step for Env–2dCD4^{S60C} following the promising results in our rabbit immunization studies was to evaluate the complex in NHPs, specifically in Chinese-origin rhesus macaques. Thus, the goal of the current project was to initiate a pilot study for the preclinical development of Env-2dCD4^{S60C} in Chinese-origin rhesus macaques to establish safety, immunogenicity and ultimately the efficacy of these immunogens as a potential HIV-1 protective vaccine.

The overall aim of the study was therefore to test the immunogenicity and protective efficacy of the Env-2dCD4^{S60C} complex in NHPs. This was achieved by:

1. Expression and purification of trimeric gp140 envelope glycoproteins and 2dCD4^{S60C}
2. Generation and biochemical characterization of Env-2dCD4^{S60C} complexes
3. Immunization of Chinese-origin rhesus macaques

4. Evaluation of total Env and/or 2dCD4^{S60C} binding antibody titres, HIV-1 pseudoviral neutralization potency and breadth of vaccinated macaque sera
5. Evaluation of the target of the neutralizing antibody titres using antibody depletion assays
6. Mucosal viral challenges of immunized Chinese-origin rhesus macaques with a subtype C virus (SHIV-1157ipd3N4)
7. Monitoring of systemic SHIV infection using viral load assays
8. Monitoring of CD4+ T-cell numbers using flow cytometry

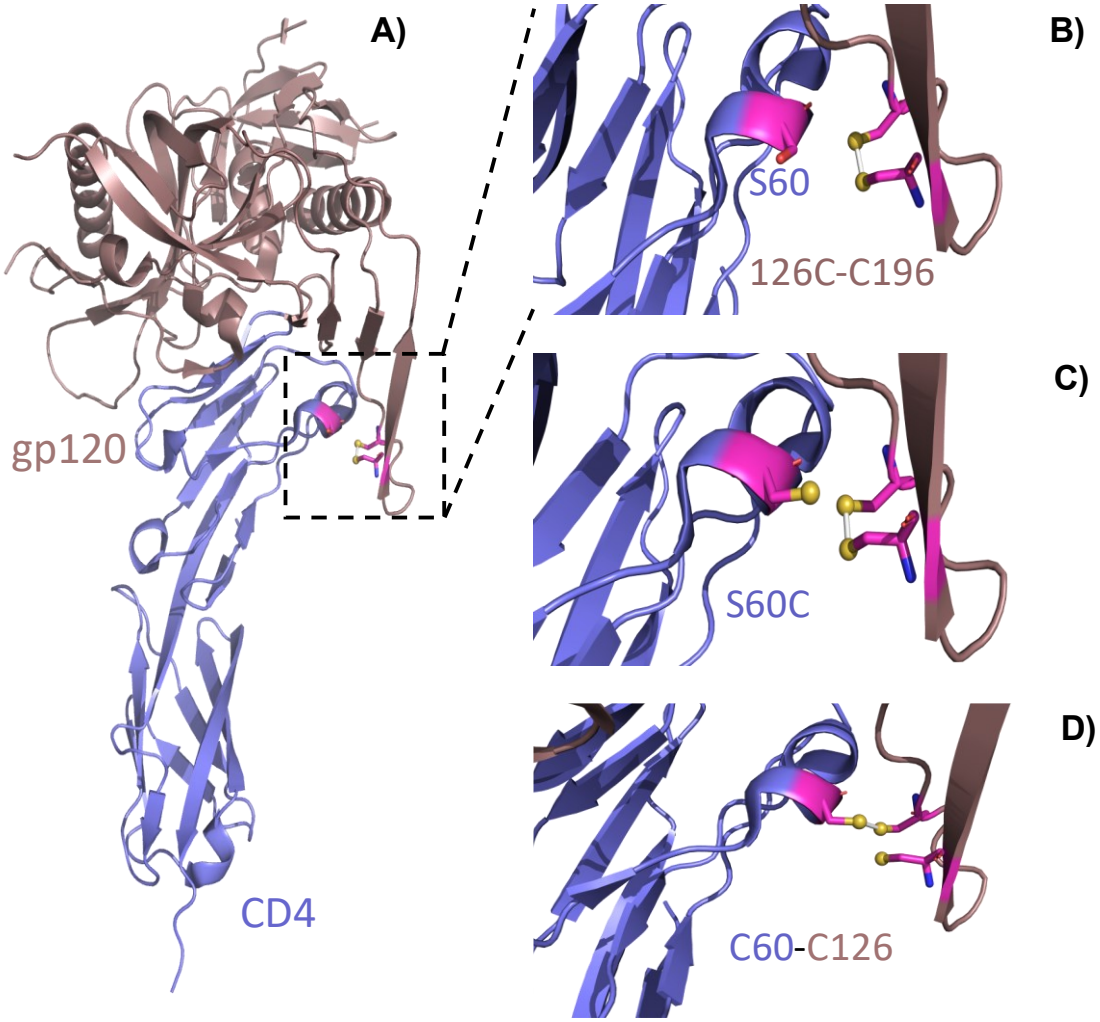


Figure 1.8: Molecular model depicting interaction of gp120-CD4 receptor complex and S60C mutation (Protein Data Bank code 1G9M). A) gp120 (copper) and CD4 (purple) are shown in ribbon representation, with Ser60 on the CD4 D1 α-helix shown in ball-and-stick representation. **B)** Enlarged image depicting the proximal positioning of Ser60 on CD4 to the Cys126–Cys196 disulphide at the base of the gp120 V1/V2 loop. **C)** Introduction of a Cys residue at position 60 of CD4, replacing Ser. **D)** Modelling predicts the formation of an intermolecular CD4 Cys60–Cys126 gp120 disulphide bond, due to its Cys60 side-chain thiol positioning within 2 Å of gp120. Adapted from Cerutti et al., *The Journal of Biological Chemistry* (Cerutti et al., 2010).

Chapter 2: Methods

2.1 Experimental model and subject details

Chinese-origin rhesus macaques (*Macaca mulatta*; #57, #58, #65, #66, #67, #68, #69; n=7) were bred and sourced from the Primate Unit and Delft Animal Centre (PUDAC) of the South African Medical Research Council (SAMRC) (Cape Town, South Africa). Animals were housed at the Central Animal Service (CAS) of the University of the Witwatersrand, Faculty of Health Sciences (Johannesburg, South Africa). This animal study was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand with animal ethics approval codes 2015/11/25/C and 2019/11/66/C (**Appendix F: Clearance certificates**). SHIV challenge studies were approved by the Department of Agriculture, Forestry and Fisheries (Pretoria, South Africa) in accordance with Section 20 of the Animal Diseases Act of South Africa (**Appendix F: Clearance certificates**). None of the animals were previously enrolled in other studies. Animals were monitored daily by competent CAS staff who were sensitized to the needs of NHPs. Housing, husbandry, and environmental enrichment of the animals was according to internationally accepted standards.

The Chinese-origin rhesus macaques were genotyped for restrictive *Mamu-A* and *Mamu-B* alleles such as *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17* which can influence SIV/SHIV challenge study outcomes in NHPs (Mothé et al., 2002, Mothé et al., 2003, Yant et al., 2006, Loffredo et al., 2007b, Loffredo et al., 2007a). No restrictive *Mamu* alleles were detected in most of the test animals, with only one NHP having a single restrictive *Mamu-A* allele. All animals were genotyped for restrictive TRIM5α polymorphisms as well with all test animals presenting with restrictive *TRIM5α* genotypes, balancing the restrictive polymorphisms amongst all test groups (Lim et al., 2010a, Lim et al., 2010b, Letvin et al., 2011). A summary table of the genotyping data is available in the appendices (**Appendix B: Supplementary figures, Supplementary Figure 6**).

2.2 Constructs, antibodies, and reagents

HIV-1 subtype C Env sequences were inferred from an Env founder virus sequence (Env_{FVC}) and used for the design of codon-optimised (human) Env expression cassettes (GeneArt) cloned in pcDNA3.1(-) expression vectors (Invitrogen) (Killick et al., 2014). Constructs were

already available in our laboratory before commencement of the current study. *Env* constructs expressed trimeric gp140 glycoproteins with a trimerizing GCN4 leucine zipper at the C-terminus of gp41 and an intact cleavage site ($gp140_{FVC}$GCN4t(+)) (Yang et al., 2000b). Stable cell lines expressing $gp140_{FVC}$GCN4t(+) were already available in our laboratory before commencement of the current study.

Additionally, constructs expressing codon-optimised (*Escherichia coli*) two-domain CD4 ($2dCD4^{S60C}$) were already available in our laboratory before commencement of the current study. $2dCD4^{S60C}$ has a serine to cysteine mutation at position 60 allowing for a stabilising disulphide bridge formation with HIV-1 Env glycoprotein (Cerutti et al., 2010).

Constructs expressing 4dCD4-WT and 4dCD4-Mut, and the purified recombinant proteins were already designed, expressed and available in our laboratory before commencement of the current study (work not published). The 4dCD4-Mut protein contains the F43A and R59A mutations which reduces the protein's affinity for Env binding (Moebius et al., 1992, Fontenot et al., 2007). A summary of the design, expression and purification of these proteins can be found in the appendices (**Appendix A: Expression and purification of recombinant CD4 proteins for anti-CD4 antibody depletion work**).

1dCD4 was used as a control for protein expression and characterisation work, and has previously been described elsewhere (Chen et al., 2011b). The protein was already available and expressed in our laboratory before commencement of the current study.

The challenge SHIV used in this study was SHIV-1157ipd3N4 and has been described previously (Song et al., 2006). The virus was provided by our collaborator Dr. Gerald Chege at PUDAC and the SAMRC. The viral stock was expanded using methods as described by Chege and colleagues (Chege et al., 2021).

VSV-G was used as a specificity control in pseudoviral neutralization assays and was provided by Prof. Penny Moore at the National Institutes for Communicable Diseases (NICD) (Johannesburg, South Africa).

Ibalizumab was used during antibody depletion work as a control, and was already available and expressed in our laboratory before commencement of current study (Moshoette et al., 2019).

Lyophilized lectin from *G.Nivalis* was used for enzyme-linked immunosorbent assays (ELISA) (#L8275) (Sigma-Aldrich).

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (catalogue number and contributor in parenthesis); TZM-bl reporter cell line (JC53-bl, Cat#8129) (Drs. J.C. Kappes, X. Wu, and Transzyme Inc.), gp120BaL (Cat#4961) (Dr. M. Reitz), IgG1b12 (Cat#2640) (Drs. D. Burton and C. Barbas), 17b (Cat#4091) (Dr J.E. Robinson), pSG3Δ*env* molecular clone (Cat#11051) (Drs. J.C. Kappes and X. Wu), ZM53M.PB12 molecular clone (Cat#11313) (Drs E. Hunter and C. Derdeyn), HIV-1 12-virus Env Global Panel (Cat#12670) (Dr. D. Montefiori), SF162.LS molecular clone (Dr. D. Montefiori), VRC01 (#ARP-12033) (Dr. X. Wu).

2.3 Transformation of recombinant plasmids into *Escherichia (E.) coli* DH5α and plasmid preparations

All recombinant constructs were previously available in our laboratory and were stored at 0.1 µg/µl in sterile water (pcDNA3.1(-)-gp140$_{FVC}$GCN4t(+) and pET15b-2dCD4^{S60C}). Chemically competent DH5α *E.coli* cells were transformed using 100-200 nanograms of DNA and 30 µl of competent cells. Samples were stored on ice for 20 minutes, heat shocked for 2 minutes at 42°C, and then incubated on ice for a further 2-5 minutes. Transformed cells were plated on lysogeny broth (LB) ampicillin-positive (100 µg/ml) agar plates and incubated overnight at 37°C.

Recombinant plasmid was extracted using a Plasmid Midi Preparation Kit (Qiagen) according to manufacturer's instructions (see **Appendix C: Bacterial and DNA work** for protocol). Spectrophotometry and agarose gel electrophoresis were used to assess concentration and purity of recombinant plasmids (see **Appendix C: Bacterial and DNA work** for reagents). Plasmids were stored at -20°C.

2.4 Expression, purification, and characterization of gp140$_{FVC}$GCN4t(+), 2dCD4^{S60C} and Env-2dCD4^{S60C} complexes

2.4.1 General mammalian cell culture

All mammalian tissue culture experiments and maintenance of cultures, unless otherwise stated, were carried out at 37°C using a humidified, water-jacketed incubator (Thermo Fisher Scientific) at 5% CO_2.

HEK 293T and TZM-bl adherent cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with; 10% fetal bovine serum (FBS) (Gibco, Life Technologies), 2 mM GlutaMax (Gibco, Thermo Fisher Scientific), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Sigma-Aldrich). Cell passaging was done twice a week using TrypLE Express (Gibco, Thermo Fisher Scientific) to dissociate adherent cells from tissue culture flask surface and re-seeded between 1/5 and 1/10.

The 293-F suspension cell line (FreeStyle 293-F) (Invitrogen, Life Technologies) was maintained in FreeStyle 293 Expression Medium (Gibco, Thermo Fisher Scientific). Adherent 293-F cells were mechanically dislodged from the tissue culture plate surface by pipetting action during sub-culturing procedures and seeded at a ratio of 1/10 in an appropriate volume of FreeStyle 293 Expression Medium. Viability and cell concentration were determined by adding equal parts of cells and 0.4% trypan blue solution (Sigma-Aldrich), and then analyzed using the Countess II Automated Cell Counter (Thermo Fisher Scientific).

Long-term freezer stocks were prepared at 1×10^6 cells/ml in freezing mix (HEK 293T and TZM-bl cell lines – 90% [v/v] FBS and 10% [v/v] dimethyl sulphoxide (DMSO) (Sigma-Aldrich); 293-F cell line – 90% [v/v] Freestyle 293 expression medium and 10% [v/v] DMSO), aliquots were placed in cryovials (Nunc) and cooled at a rate of -1°C/minute using a CoolCell LX freezing container (Corning) according to the manufacturer's instructions. The working cell-line was generally discarded at passage 30.

2.4.2 Env expression

$gp140_{FVC}$GCN4t(+) was expressed in stably transfected 293-F cells under selection by 0.25-0.5 mg/ml of G418 sulphate (Gibco, Thermo Fisher Scientific). Stably transfected cell stocks expressing $gp140_{FVC}$GCN4t(+) were already available in our laboratory and expression was previously described (Killick et al., 2015). Briefly, to transfect 293-F cells, T25 flasks were seeded 24 hours before transfection with 1×10^6 cells and 5 ml of FreeStyle 293 Expression Medium (Gibco, Thermo Fisher Scientific). On day of transfection, overnight T25 tissue culture flasks were assessed for 60%-80% cell confluency before transfection. Transfections were set-up as follows; 4 µg of pcDNA3.1(-)-$gp140_{FVC}$GCN4t(+) plasmid construct was combined with 40 µl of Polyfect transfection reagent (Qiagen) up to a final volume of 500 µl using a serum-free and antibiotic-free tissue culture media. Transfection complex was thoroughly mixed and incubated for 5-10 minutes before being added to tissue culture flask and incubated at 37°C for 8 hours. Following incubation, media containing transfection mix

was aspirated off and 10 ml of fresh FreeStyle 293 Expression Medium (Gibco, Thermo Fisher Scientific) was added and cells were incubated for 48 hours. Media was harvested, centrifuged at 1,000×g for 10 minutes, and stored at -20°C. Expression was assessed through standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (see **Appendix D: Recombinant protein work** for reagents).

To upscale production of Env protein, stable cell lines expressing gp140$_{FVC}$GCN4t(+) were generated. Following transfections as described previously, cells were expanded into larger T150 tissue culture flasks and 0.25-0.5 mg/ml of G418 sulphate (Gibco, Thermo Fisher Scientific) was added to select for stably transfected cells. Stably expressing 293-F cells were then further upscaled into 100 ml of FreeStyle 293 Expression Medium, supplemented with 0.25-0.5 mg/ml of G418 sulphate, in 500 ml disposable Erlenmeyer flasks (Corning). Cells were maintained on shaking platforms at 130 rpm positioned within standard mammalian cell culture incubators. Cells were passaged as required and maintained below 3×10^6 cells/ml, with the viability monitored using the Countess II Automated Cell Counter. Harvested culture supernatant containing expressed Env glycoprotein was centrifuged at 1,000×g for 10 min, filtered through PALL Acrodisc filters (0.45 µm) and stored at -20°C until purification. Protein expression was continually assessed by SDS-PAGE and Western blot analysis.

2.4.3 Confirmation of HIV-1 Env glycoprotein expression

Expression of gp140$_{FVC}$GCN4t(+) in cell culture medium was confirmed by 10% acrylamide SDS-PAGE followed by Coomasie blue staining, or by Western blot analysis (see **Appendix D: Recombinant protein work** for reagents). For Western blot analysis, gels were equilibrated in transfer buffer for 10 minutes. Protein bands were transferred onto poly-C nitrocellulose membrane (Amersham Bio Sciences) using the Trans Blot SD Semi Dry transfer cell (30 minutes, 15 V) (BioRad). The nitrocellulose membrane was washed 2× for 5 minutes in 1×Tween-Tris Buffered Saline (T-TBS) and then blocked for 1 hour (or overnight at 4°C) in T-TBS containing 1% of lyophilized bovine serum albumin (BSA). The membrane was washed 2× for 5 minutes in T-TBS and probed for 45 min with sera from rabbits previously immunized with gp140$_{FVC}$GCN4t(+) (1:2,000) (Killick et al., 2015). Unbound primary antibody was removed by washing the membrane 3× in T-TBS for 5 minutes and then probed for 45 min with a donkey anti-rabbit-HRP-linked whole antibody (1:2,000; GE Healthcare). The membrane was then washed 3× in T-TBS for 10 minutes. SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) reagents in a 1:1 ratio was added to the

membrane. Luminescence was visualized using the ChemiDoc XRS+ Gel Imaging System on ChemiHi Sensitivity mode (BioRad).

2.4.4 HIV-1 Env glycoprotein purification

$gp140_{FVC}$GCN4t(+) was purified as previously described (Killick et al., 2015). Briefly, approximately 500 ml of culture supernatant and 4 ml of lectin cross-linked to 4% beaded agarose (Sigma-Aldrich) were added together and incubated overnight at 4°C while constantly stirring. The following day supernatant was added to a liquid chromatography column (2.5×10 cm) (Sigma-Aldrich) and resin bound to $gp140_{FVC}$GCN4t(+) was packed. Resin was then washed with 200 ml (50×column bed volume) respectively of 0.5 M and 1 M of NaCl made up in 1×Dulbecco's Phosphate-Buffered Saline (DPBS) (Thermo Fischer Scientific), followed with a final 200 ml wash with only 1×DPBS. The Env protein was then eluted using 7-10×column bed volumes of 1×DPBS containing 1 M methyl α-D-mannopyranoside (MMP) (Sigma-Aldrich). The eluted protein was sterile filtered using PALL Acrodisc filters (0.45 µM), and then buffer exchanged and concentrated in 1×DPBS down to 1 ml using Amicon Ultra Centrifugation Filters with a 50 kDa cut-off (Merck Millipore, now "Merck Sigma"). On average, 2-4 mg of total protein was obtained from 1 L of cell-culture supernatant. Env glycoprotein concentration and purity was determined by Pierce BCA Protein Assay (Thermo Fisher Scientific) (see **Appendix D: Recombinant protein work** for protocol) and SDS-PAGE, respectively. Protein was snap-frozen in liquid nitrogen and stored at -80ºC.

Env protein was further purified using size-exclusion chromatography (SEC, HiPrep 16/60 Sephacryl S-200 HR, GE Life Sciences) according to in-house protocols using the ÄKTA FPLC platform (GE Healthcare) to isolate the trimeric form and remove aggregates/monomeric forms. Lectin-purified samples were loaded on to a pre-equilibrated column in volumes of 500 µl and resolved at a flow rate of 1 ml/min in sterile 1×DPBS. Fractions containing the trimeric forms of Env were collected, pooled, and concentrated using Amicon Ultra Centrifugation Filters with a 50 kDa cut-off. The column was cleaned as recommended by the manufacturer (GE Life Sciences) using 0.5 M NaOH and then equilibrated using 1×DPBS. Concentration and purity were assessed using Pierce BCA Protein Assay and SDS-PAGE, respectively, and concentration of $gp140_{FVC}$GCN4t(+) was adjusted accordingly to produce stocks of 1 mg/ml. Protein was snap-frozen in liquid nitrogen and stored at -80°C.

2.4.5 2dCD4^{S60C} expression

2dCD4^{S60C} was expressed and purified as previously described (Cerutti et al., 2010). Briefly, pET15b constructs coding for 2dCD4^{S60C} were transformed in chemically competent BL21* *E. coli* cells and expressed in standard LB medium using an isopropyl 1-thio-β-galactopyranoside-free induction protocol. Cultures were grown in shaking-incubators at 37°C until mid-log phase (OD_{600} of 0.7) and then transferred to shaking-incubators at 20°C for 20 hours. Bacterial cells were pelleted, re-suspended in 1/10th the original culture volume in 1×DPBS containing chicken-egg lysozyme (0.5 mg/ml, Sigma-Aldrich) and incubated on a shaking-incubator for 1 hour. Cells were then lysed using 3× freeze-thaw cycles in liquid nitrogen and then sonicated 3× (80% amplitude, 1 second on/0.5 second off, 1 minute each round). Cell lysate was centrifuged at 20,000×g at 4°C for 45 minutes.

2.4.6 2dCD4^{S60C} purification

2dCD4^{S60C} was purified from inclusion bodies using the incorporated C-terminal polyhistidine-tag and was purified using a denaturing (urea) protocol and established Ni^{2+}-affinity chromatography protocol (see **Appendix D: Recombinant protein work** for reagents). Following clarification of the cell lysate by centrifugation, pellets were re-suspended in solubilisation buffer and mechanically homogenized using a tissue homogenizer. Homogenate was incubated for 30 minutes on a shaker and then centrifuged at 20,000×g for 45 minutes at 4°C. Supernatant was carefully collected and added together with 2-3 ml of resin charged using $NiSO_4$ and allowed to stir overnight at 4°C. Resin was harvested the next day by centrifugation and purified by Ni^{2+} affinity-chromatography under denaturing conditions. Resin was washed with increasing concentrations of imidazole-containing wash buffers (10 mM or 20 mM imidazole) and then protein was eluted using a high imidazole concentration elution buffer (500 mM imidazole).

2.4.7 2dCD4^{S60C} refolding

Eluted 2dCD4^{S60C} was refolded using a dialysis protocol. Eluted 2dCD4^{S60C} samples were transferred to SnakeSkin Dialysis Tubing (MWCO 10,000, Thermo Fisher Scientific) overnight at 4°C in folding buffer A, followed by overnight dialysis at 4°C in folding buffer B (see **Appendix D: Recombinant protein work** for reagents). The purified 2dCD4^{S60C} was then dialyzed for 2 rounds in 20×volumes of 1×DPBS at 4°C for at least 2 hours each round and then overnight for a final round. Precipitate was removed by centrifugation at 3,200×g for 10 min at 4°C followed by filtration through PALL Acrodisc filters (0.45 µm). Protein samples

were concentrated using an Amicon Ultra Centrifugation Filter with a 10 kDa cut-off. Concentration and purity were assessed using Pierce BCA Protein Assay and SDS-PAGE, respectively. Protein was snap-frozen in liquid nitrogen and stored at -80ºC.

2.4.8 Characterization of gp140$_{FVC}$GCN4t(+) and 2dCD4^{S60C}

ELISA was used to confirm the conformational and functional integrity of the purified gp140$_{FVC}$GCN4t(+) and 2dCD4^{S60C} proteins (see **Appendix E: Serology work** for reagents). Briefly, Nunc MaxiSorp ELISA plates (Thermo Fisher Scientific) were coated overnight at 4°C with 100 µl of 2 µg/ml of lyophilized lectin dissolved in 1×DPBS. Following coating overnight, the plates were aspirated and blocked for 1 hour at room temperature with 250 µl of assay buffer/well, and then washed 3×300 µl/well with PBS-T. A five-fold dilution series of gp140$_{FVC}$GCN4t(+) or control protein gp120BaL was prepared in assay buffer at concentrations of 1,000-8 ng/ml. Each Env dilution was incubated with an equal volume of 2 µg/ml 2dCD4^{S60C} made up in assay buffer. This would yield a final assay concentration range of 500-4 ng/ml of Env and a final concentration of 1 µg/ml of 2dCD4^{S60C}. Any controls were adjusted accordingly to match the dilution range. Following dilution of Env/CD4 proteins, 100 µl of each dilution was transferred in duplicate to respective wells and incubated for 1 hour at room temperature. Following incubation, plates were washed 5×300 µl/well with PBS-T and plates were probed with 100 µl of IgG1b12 or 17b diluted in assay buffer (1:2,000), respectively, and incubated for 1 hour at room temperature. Following incubation, plates were washed 5×300 µl/well with PBS-T and plates were probed with 100 µl of a sheep anti-human-HRP-linked whole antibody diluted in assay buffer (1:2,000; GE Healthcare) and incubated for 1 hour. Plates were washed 5×300 µl/well with PBS-T and then developed using the Sure Blue TMB peroxidase substrate (KPL) and stopped with 0.2 M sulphuric acid, per manufacturer's instructions. Plate signal was measured using an absorbance value of 450 nm. Results were analysed using GraphPad Prism 8 (www.graphpad.com).

2.4.9 Generating Env-2dCD4^{S60C} complexes

Env-2dCD4^{S60C} complexes were generated by incubating 2 mg of gp140$_{FVC}$GCN4t(+) with 1 mg of 2dCD4^{S60C} (approximately 3-fold molar excess of 2dCD4^{S60C} to Env) under reducing conditions (1 mM β-mercaptoethanol final concentration) for 1 hour in order to facilitate disulphide exchange between C60 of 2dCD4^{S60C} and C126 of gp140$_{FVC}$GCN4t(+). Following complexing, samples were centrifuged at 16,000×g for 10 minutes and protein complexes were purified using SEC (HiPrep 16/60 Sephacryl S-200 HR) using the ÄKTA FPLC platform.

Elution peaks corresponding to the trimeric Env conformation were collected and concentrated using Amicon Ultra Centrifugation Filter with a 50 kDa cut-off. Concentration and purity were assessed using Pierce BCA Protein Assay and SDS-PAGE, respectively.

To confirm that complexing and disulphide stabilization of $gp140_{FVC}$GCN4t(+) and $2dCD4^{S60C}$, standard SDS-PAGE and Western blot analysis were performed under reducing/non-reducing conditions with known concentrations of each sample. Standard SDS-PAGE (12.5%) was done under reducing conditions with approximately 5× the amount of the protein complex and Env-alone (5 µg total), compared to $2dCD4^{S60C}$-alone (1 µg total), to better compare the molar quantities that were used in the complexing reaction.

Western blot analysis was conducted in similar fashion using 5× the amount of protein complex and Env-alone (1 µg total), compared to $2dCD4^{S60C}$-alone (200 ng total). Western blots were done under both reducing and non-reducing conditions. Western blot procedure was used as described above (**section 2.4.3**), with the protein detected using a primary Penta·His Antibody (Qiagen) specific only for his-tagged $2dCD4^{S60C}$. This would allow detection of the $2dCD4^{S60C}$ protein bound or unbound to Env under reducing or non-reducing conditions.

2.5 Rhesus macaque immunizations

Chinese-origin rhesus macaques were immunized six times over a 4-month period; day 0, day 14, day 30, day 60, day 90 and day 120. The animals were administered a 7^{th} booster immunization two weeks before SHIV viral challenges commenced, consisting of the same formulations previously administered. Recombinant proteins including $2dCD4^{S60C}$, $gp140_{FVC}$GCN4t(+) and Env-$2dCD4^{S60C}$ complexes were formulated in Adjuplex adjuvant (Sigma-Aldrich) which consists of a nanoliposomal mixture of carbomer homopolymer and highly purified natural phospholipids (Chakrabarti et al., 2013). Four animals received Env-$2dCD4^{S60C}$, two received $2dCD4^{S60C}$ and one received $gp140_{FVC}$GCN4t(+). NHPs were sedated with 0.25 mg/kg of midazolam and 10 mg/kg of ketamine before each immunization and blood draw. Each rhesus macaque received a total of 100 µg of respective recombinant protein (Env or $2dCD4^{S60C}$ only) or recombinant protein complex (Env-$2dCD4^{S60C}$), made up to 500 µl with 5×Adjuplex and 1×DPBS. Immunizations were administered as two subcutaneous injections (250 µl, 50 µg) in both inner medial-thigh areas of each rhesus macaque using a 1 inch 25-gauge needle. Skin was pulled up to make a tent-like shape and injections administered. Blood samples of 10 ml were collected at; day 0, day 14, day 30, day 44, day 74, day 104 and day 134. Blood was also collected pre, and two weeks post the 7^{th} immunization booster shot.

Whole blood was collected in red-topped Vacutainer tubes (BD Biosciences) for serum isolation, then frozen and stored at -80°C.

2.6 HIV-1 Env and CD4 end-point titres

Total Env and 2dCD4^{S60C} end-point binding titres were determined by ELISA, in duplicate (See **Appendix E: Serology work** for reagents), for all the immunized rhesus macaques using Nunc MaxiSorp ELISA plates (Thermo Fisher Scientific) coated overnight at 4°C with 50 µl of 1 µg/ml of matched recombinant protein (gp140$_{FVC}$GCN4t(+) or 2dCD4^{S60C}) in 1×DPBS. Following coating overnight, the plates were aspirated and blocked for 1 hour at room temperature with 250 µl of assay buffer/well, and then washed 3×300 µl/well with PBS-T. Heat-inactivated sera (see **Appendix E: Serology work** for protocol) from immunized rhesus macaques were prepared in a two-fold dilution series starting from 1:500 to 1:2,048,000 made up in assay buffer, and then 50 µl of 1:16,000 to 1:2,048,000 dilutions were transferred to the ELISA plates and incubated for 1 hour at room temperature. End-point titres were determined as the reciprocal of the dilution required to obtain an absorbance value of 1 unit at 450 nm. This value represents a minimum point in the linear range of the dilution curve, allowing comparison across all samples. Following incubation, plates were washed 5×300 µl/well with PBS-T and bound antibodies were detected using 50 µl/well of a sheep anti-human-HRP-linked whole antibody diluted in assay buffer (1:2,000; GE Healthcare) and incubated for 1 hour at room temperature. Plates were washed 5×300 µl/well with PBS-T and then developed using the Sure Blue TMB peroxidase substrate (KPL) and stopped with 0.2 M sulphuric acid, per manufacturer's instructions. Plate signal was measured using an absorbance value of 450nm. Results were analysed using GraphPad Prism 8.

2.7 HIV-1 pseudoviral neutralization assays

2.7.1 Pseudovirus production

Replication-incompetent HIV-1 pseudoviruses were generated as previously described (Montefiori, 2020b). Briefly, 1×10^6 HEK 293T cells were seeded 24 hours before transfection so that cell confluency on day was 50-80%. Cells were co-transfected with *env*-deficient backbone expression plasmid (pSG3Δ*env*) and respective *env* plasmid in a 1:2 ratio, for a total of 10 µg of DNA. For every 1 µg of DNA, 3 µl of Fugene HD transfection reagent (Promega) was added. Transfection reactions were made up to 500 µl using Opti-MEM (Gibco, Thermo Fisher Scientific) and allowed to incubate for up to 15 minutes at room temperature. Transfection mix was then added to HEK 293T cells in T25 culture flasks and incubated at

37ºC, 5% CO_2. After eight hours supernatant was removed and 5 ml of fresh DMEM was added. Virus-containing media was harvested 48 hours post-transfection, centrifuged, adjusted to 20% FBS and sterile filtered (0.22 µm) before being stored at -80ºC.

2.7.2 Titrating pseudoviruses

Median Tissue Culture Infectious Dosage ($TCID_{50}$) was determined as previously described (Montefiori, 2020b). Briefly, viral stocks were prepared in a five-fold dilution series in a final volume of 100 µl of DMEM/well in Nunc 96-well tissue culture plates (Thermo Fisher Scientific). Following viral dilution, 100 µl of freshly trypsinized TZM-bl cells were added to each well at a concentration of 0.1×10^6 cells/ml for a final assay volume of 200 µl/well. Additional diethylaminoethyl-dextran hydrochloride (DEAE Dextran, Sigma-Aldrich) was supplemented for a final concentration in each well of 20 µg/ml. A single column was assigned as a cell-only control and titrations were done in duplicate. Plates were incubated for 48 hours at 37ºC, 5% CO_2.

Following incubation, media was aspirated from the tissue culture plates and cells were lysed with 80 µl/well of Glo-Lysis buffer (Promega) for 5 minutes. Following lysis, 50 µl of lysate was transferred to white 96-well luminometer plates, and 50 µl of Bright-Glo Luciferase reagent (Promega) was added to each well. Plates were read within 15 minutes on the GloMax Discover Microplate Reader and Luminometer (Promega). $TCID_{50}$ was determined using the assay Excel macro that can be obtained on the Los Alamos National Laboratory HIV Immunology database. (www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm)

2.7.3 Viral neutralization assays

Heat-inactivated sera from immunized rhesus macaques from day 0, day 74, day 104 and day 134 of the immunization regimen, and sera from pre- and post-7th immunizations (pre-SHIV challenge) were analysed for neutralization breadth and potency using a standardised neutralization assay, as described previously (Montefiori, 2020a). The panel of pseudoviruses tested against were the HIV-1 pseudoviruses ZM53M.PB12 (tier-2, subtype C), SF162.LS (tier-1, subtype B), the 12-virus Env Global Panel consisting of 12 heterologous tier-2 Env clones representative of global HIV-1 subtype diversity (deCamp et al., 2014, Seaman et al., 2010). The challenge virus, SHIV-1157ipd3N4, was also tested against.

Briefly, heat-inactivated rhesus macaque sera were prepared in a three-fold dilution series in a final volume of 100 µl of DMEM/well in Nunc 96-well tissue culture plates (Thermo Fisher Scientific). Following sera dilution, 50 µl of respective virus diluted to 4000 $TCID_{50}$ was added

per well and the 150 µl of sera/virus per well was incubated for 1 hour at 37°C. Following incubation, 100 µl of freshly trypsinized TZM-bl cells were added to each well at a concentration of 0.1×10^6 cells/ml for a final assay volume of 250 µl/well. Additional DEAE Dextran was supplemented for a final concentration in each well of 20 µg/ml. Two single rows were assigned as cell-only and cell/virus-only controls, and all dilutions were done in duplicate. Plates were incubated for 48 hours at 37ºC, 5% CO_2.

Luminescence was quantified using the Glo-Lysis buffer / Bright-Glo Luciferase reagent system (Promega) and GloMax Discover Microplate Reader and Luminometer (Promega) as described in section above (**section 2.7.2**). Luciferase reporter gene expression is induced by the viral Tat protein and is directly proportional to the number of infectious pseudoviral particles added at the beginning of the assay. The reciprocal of serum dilution required to achieve 50% inhibition (ID_{50}) relative to the virus control wells, after the control background has been accounted for, was calculated by fitting a variable-slope, non-linear regression curve using GraphPad Prism 8. ID_{50} values were recorded, and colour coded in Excel using "colour scales".

2.8 Anti-CD4 antibody depletion of immunized rhesus macaque sera

Sera from immunized rhesus macaques was depleted of anti-CD4 antibodies using $2dCD4^{S60C}$ or mutated 4dCD4 (4dCD4-Mut) and the impact on neutralizing potency of sera was measured in a viral neutralization assay as described above (**section 2.7.3**). Recombinant wild-type 4dCD4 (4dCD4-WT), and antibodies ibalizumab and VRC01 were all used as controls in the antibody depletion assay described below.

Briefly, heat-inactivated rhesus macaque sera were prepared in a three-fold dilution series in a final volume of 100 µl of DMEM/well in Nunc 96-well tissue culture plates (Thermo Fisher Scientific). $2dCD4^{S60C}$ or 4dCD4-Mut were prepared in DMEM at a concentration of 2.5 µM and 50 µl was added per well for a final assay concentration in each well of 0.5 µM. Plates were incubated for 1 hour at 37°C to allow for depletion of anti-CD4 responses within the rhesus macaque sera. Following incubation, 50 µl of respective virus diluted to 4,000 $TCID_{50}$ was added per well and the plates were incubated for a further 1 hour at 37°C. Following incubation, 50 µl of freshly trypsinized TZM-bl cells were added to each well at a concentration of 0.2×10^6 cells/ml for a final assay volume of 250 µl/well. Additional DEAE Dextran was supplemented for a final assay concentration in each well of 20 µg/ml. Two single rows were assigned for the cell-only and cell/virus-only controls, and all dilutions were done in duplicate. Plates were incubated for 48 hours at 37ºC, 5% CO_2.

Luminescence was quantified using the Glo-Lysis buffer / Bright-Glo Luciferase reagent system (Promega) and GloMax Discover Microplate Reader and Luminometer (Promega) as described in section above (**section 2.7.2**).

2.9 SHIV challenges

All 7 rhesus macaques were inoculated intrarectally over a 10-week period with the subtype C-derived virus, SHIV-1157ipd3N4 (Song et al., 2006). NHPs were sedated with 0.25 mg/kg of midazolam and 10 mg/kg of ketamine. A 2 ml dilution of 5,000 $TCID_{50}$ in RPMI-1640 (Gibco, Thermo Fisher Scientific) was administered per viral challenge per animal, diluted from a 34,939 $TCID_{50}$ viral stock titrated in TZM-bl cells as described above (**section 2.7.2**). Following intra-rectal inoculation, the animals were rested in sternal recumbency position with their pelvis slightly elevated for 10 min. Blood samples were collected at baseline and at weekly intervals following each challenge in red-topped and purple-topped Vacutainer tubes (BD Biosciences) for serum, plasma and PBMC isolation.

2.10 Viral Load Assay

Viral loads were monitored using RT-PCR and were conducted by our collaborators at PUDAC in the Western Cape, South Africa. Briefly, RNA was extracted from plasma samples using QIAamp viral RNA mini kit (Qiagen). The SIV *gag* gene was used as a standard with a 10^{10} gag copies/ml stock (Resource of Nonhuman Primate Immune Reagents, Emory University) serially diluted in nuclease-free water down to 1 copy/ml to prepare standards for the RT-PCR assay. The TaqMan RNA-to-Ct 1-Step Kit (Life technologies) was used to amplify RNA standards and viral samples. RT-PCR reactions were run on the QuantStudio 7 Real-Time PCR machine (Applied Biosystems).

Primers used:

PDGAGF (forward primer): (5'd GTCTGCGTCATCTGGTGCATTC 3')

PDGAGR (reverse primer): (5'd CACTAGGTGTCTCTGCACTATCTGTTTTG 3')

PDGAGP (TaqMan probe): (5'd FAM-CTTCCTCAGTGTGTTTCACTTTCTCTTCTGCG-BHQ-1 3')

Cycling conditions:

Stage	Step	Temperature	Time
Holding	Reverse transcription	48°C	15 minutes
Holding	Activation of DNA polymerase	95°C	10 minutes
Cycling (40 cycles)	Denature	95°C	15 seconds
	Anneal/Extend	60°C	1 minute

2.11 CD4+ T-cell counts

2.11.1 Fluorescence compensation

CD4+ T-cell absolute counts were determined using the FACS Aria (BD Biosciences) flow cytometer. Instrument quality control is performed weekly on the instrument using BD CS&T beads (BD Biosciences) to monitor the fluidics, optics, and electronics according to manufacturer's recommendations.

Before sorting of cells from whole blood of NHPs, a compensatory step was performed to adjust for spectral overlap between fluorescent dyes. A 3-colour kit of BD Calibrite beads (BD Biosciences) containing unstained beads, PerCP beads (red), FITC beads (green-yellow) and PE beads (red-orange) were used for compensation. Beads were prepared as single stains in separate tubes (1 drop in 350 µl of BD FACS sheath fluid) and used to adjust voltages accordingly using the compensation protocol in the FACSDiva software (V6.1.3).

2.11.2 Preparing whole blood from NHPs for CD4+ T-cell counts

CD4+ T-cell absolute counts were determined using whole uncoagulated blood from NHPs. Samples were stained using three different antibodies: BD PerCP Mouse Anti-NHP CD45 Clone D058-1283 (Cat# 558411), BD PE Mouse Anti-Human CD4 Clone L200 (Cat# 550630) and BD FITC Mouse Anti-Human CD3ε Clone SP34 (Cat# 556611). Briefly, 5 µl of each antibody and 5 µl of 1×DPBS (total volume of 20 µl) were added to BD Trucount tubes (BD Biosciences) and 50 µl of whole rhesus macaque blood was then added. The samples were vortexed and incubated for 15 min at room temperature in the dark. Following incubation, 450 µL 1×BD FACS lysing solution (BD Biosciences) was added to the sample, vortexed and incubated a further 15 min in the dark at room temperature.

Samples were then analysed on the FACS Aria using FACSDiva software (V6.1.3). Separate gates were drawn around Trucount beads (PE and FITC positive) and the CD45+ lymphocyte population (PerCP positive). The CD45+ lymphocyte population was divided into sub-populations using a quadrant using PE and FITC parameters, with positive CD4+ T-cell events in the CD3+ CD4+ quadrant (PE and FITC positive). A total of 15,000 CD45+ lymphocyte events were then recorded per sample tube. An example of the gating strategy used can be found in the appendices (**Appendix B: Supplementary figures, Supplementary Figure 7**).

CD4+ T-cell counts were determined using the following equation and shown as number of CD4+ T-cells/µl blood: $A = X/Y \times N/V$

Absolute count of the cell population (A), number of positive cell events (X), number of bead events (Y), BD Trucount bead concentration (N/V, where N = number of beads per test and V = test volume).

Chapter 3: Results

3.1 Expression, purification, and characterization of gp140$_{FVC}$GCN4t(+), 2dCD4^{S60C} and Env-2dCD4^{S60C} complexes

3.1.1 Expression and purification of gp140$_{FVC}$GCN4t(+)

Recombinant gp140$_{FVC}$GCN4t(+) was expressed and purified in sufficient quantities and purity for NHP immunizations. Env protein expression was continually assessed using Western blot analysis and samples were probed with sera from rabbits previously immunized by our laboratory with Env (**Figure 3.1, B**) (Killick et al., 2015). Stably transfected 293-F cells expressing gp140$_{FVC}$GCN4t(+) were grown in batches and media harvested every several days. Following lectin-affinity chromatography and concentrating, the relative molecular weight, purity and concentration of gp140$_{FVC}$GCN4t(+) was assessed by SDS-PAGE. A single distinct band of approximately 140 kDa corresponding to the protein under reducing conditions was noted on Coomassie stained gels, with several unknown lower molecular weight contaminants detected and is consistent with results of previous purifications of gp140$_{FVC}$GCN4t(+) in our laboratory (**Figure 3.1, A**). The gp140$_{FVC}$GCN4t(+) protein has an intact proteolytic cleavage site, therefore a second band of furin-cleaved gp120 was observed and corresponds to gp120BaL, used as a sizing control. On average, 1 L of harvested 293-F gp140$_{FVC}$GCN4t(+) culture supernatant would yield 2-4 mg of protein following lectin-affinity chromatography.

Trimeric conformations of gp140$_{FVC}$GCN4t(+) Env glycoproteins were isolated using SEC, with a distinct peak noted for the trimeric conformation between 42.5 ml and 55 ml with the peak retention volume at 46 ml (**Figure 3.3, A**). On average, 60% of total protein loaded for SEC was recovered, corresponding to the fractions containing trimeric Env conformations. Peaks likely corresponding to oligomeric and monomeric conformations were noted but were subsequently excluded by fractionation of the sample following SEC.

3.1.2 Expression and purification of 2dCD4^{S60C}

Recombinant 2dCD4^{S60C} was expressed and purified in sufficient quantities for complexing to gp140$_{FVC}$GCN4t(+), and subsequent immunizations, using Ni^{2+}-affinity chromatography. Following Ni^{2+}-affinity chromatography, refolding under oxidative conditions and concentrating, the protein was assessed by SDS-PAGE (**Figure 3.2**). A single distinct band of

approximately 22 kDa corresponding to 2dCD4^{S60C} protein under reducing conditions was noted on Coomassie stained gels, with several unknown contaminants detected. On average, 1 L of bacterial cell culture expressing 2dCD4^{S60C} would yield 0.5-1 mg of protein following Ni^{2+}-affinity chromatography and oxidative refolding procedures.

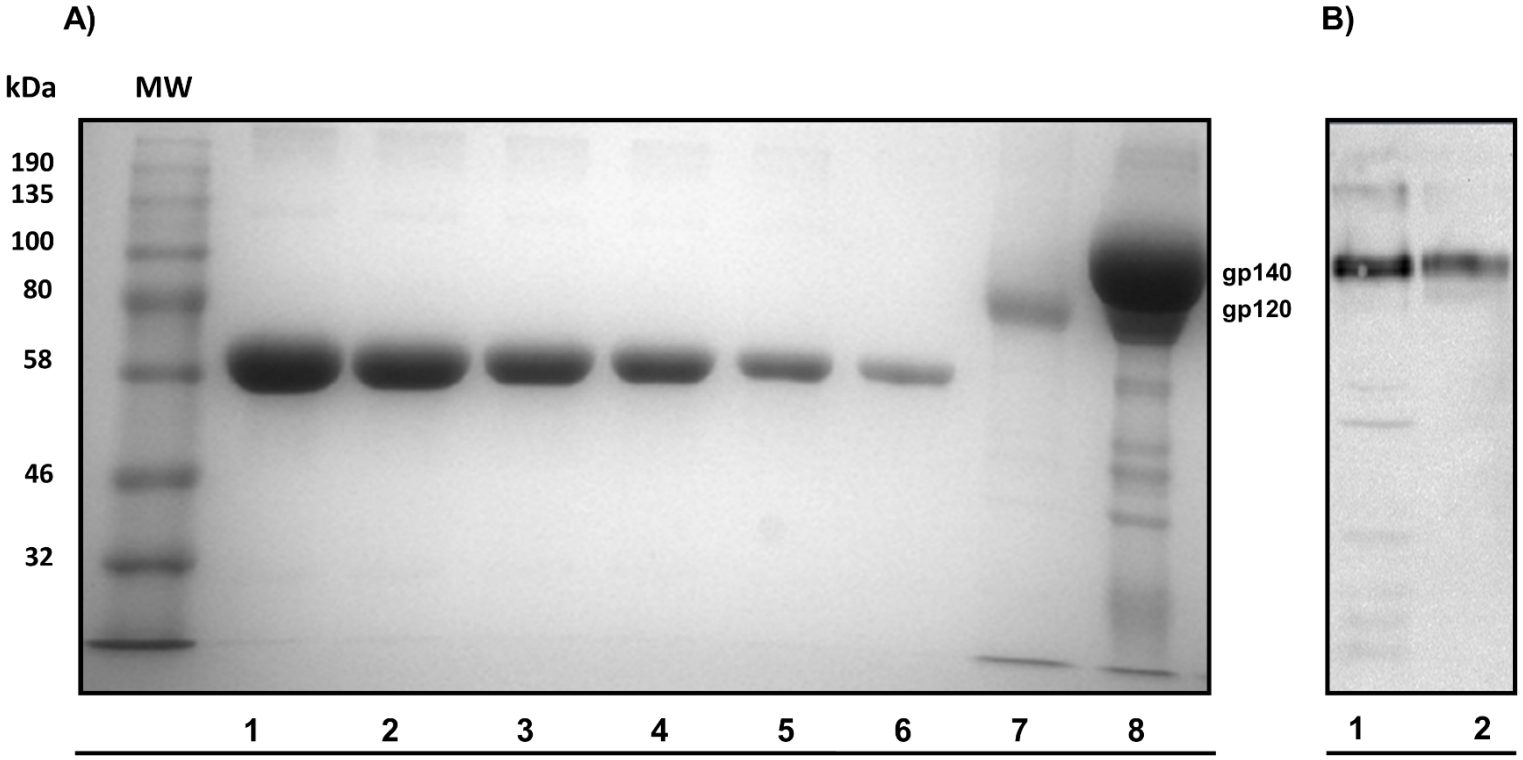


Figure 3.1: SDS-PAGE and Western blot analysis of purified and unpurified gp140$_{FVC}$GCN4t(+). The Env protein gp140$_{FVC}$GCN4t(+) was expressed in stably transfected 293-F cells and was purified using *G. nivalis* lectin-affinity chromatography. **A)** Purified gp140$_{FVC}$GCN4t(+) was analysed using SDS-PAGE (10%), alongside a gp120 sizing control and BSA protein standard series; **lanes 1-6**, BSA standards (1.5, 1, 0.75, 0.5, 0.25, 0.125 mg/ml); **lane 7**, gp120BaL; **lane 8**, purified gp140$_{FVC}$GCN4t(+). **B)** Culture media containing gp140$_{FVC}$GCN4t(+) expressed by stably transfected cells (in suspension or adhered) was probed on a Western blot using rabbit sera previously immunized with Env and detected using a secondary donkey anti-rabbit-HRP-linked whole antibody; **lane 1**, stably transfected cell culture media from Erlenmeyer flask with cells expressing in suspension; **lane 2**, cell culture media from T75 tissue culture flask with adherent cells expressing. **MW**=molecular weight marker (#P7712, 11-245 kDa, New England BioLabs).

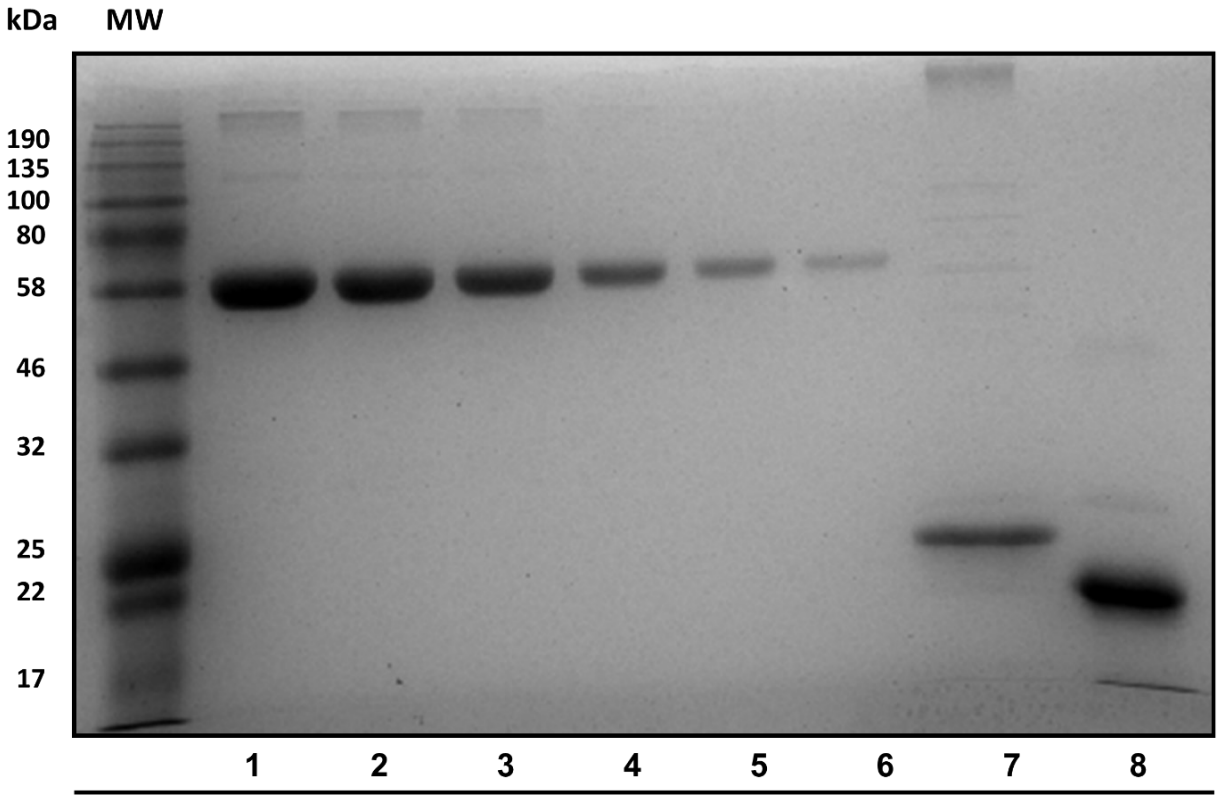


Figure 3.2: SDS-PAGE of purified 2dCD4^{S60C}. The CD4 protein 2dCD4^{S60C} was expressed in transformed BL21* *E.coli* and was purified using Ni^{2+}-affinity chromatography. Purified 2dCD4^{S60C} was analysed using SDS-PAGE (12.5%), alongside a 1dCD4 sizing control and BSA protein standard series; **lanes 1-6**, BSA standards (1.5, 1, 0.75, 0.5, 0.25, 0.125 mg/ml); **lane 7**, 2dCD4^{S60C}; **lane 8**, 1dCD4. **MW**=molecular weight marker (#P7712, 11-245 kDa, New England BioLabs).

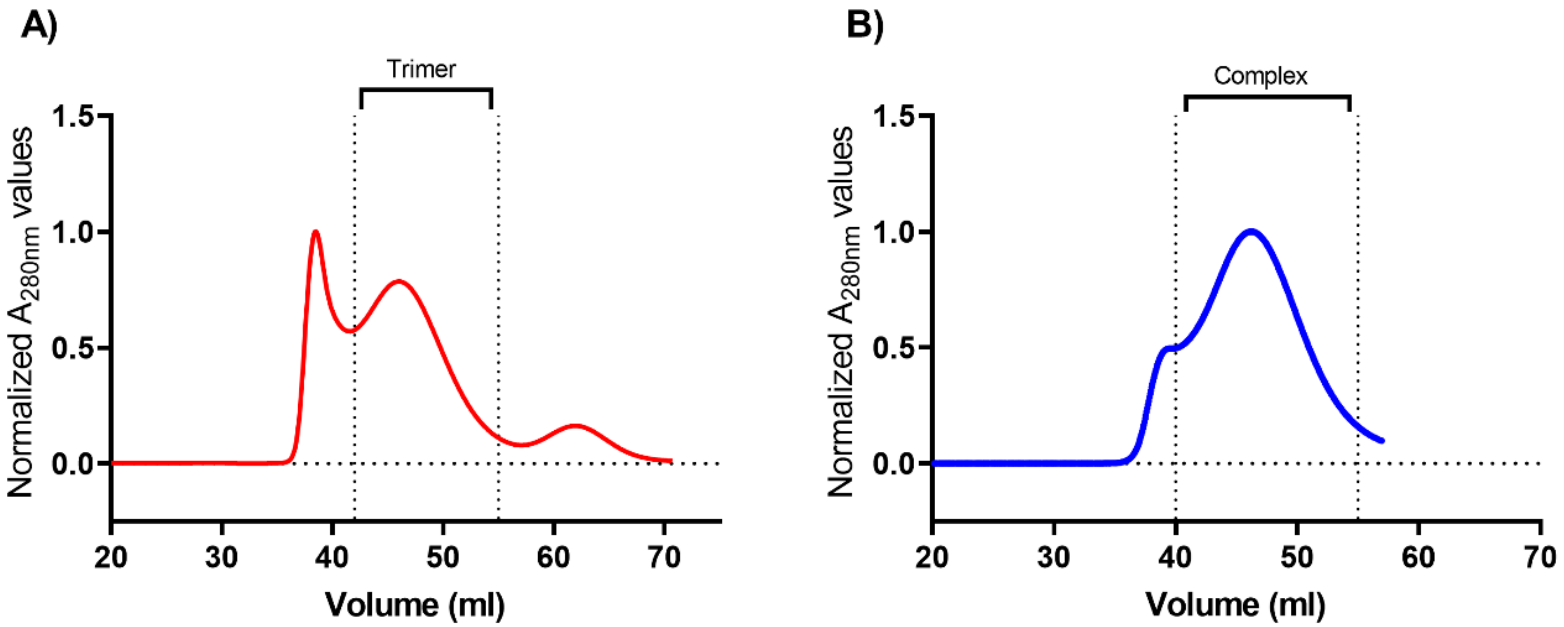


Figure 3.3: Size exclusion chromatography elution profiles of $gp140_{FVC}$GCN4t(+) Env glycoproteins and Env-2dCD4^{S60C} complexes. Normalized elution chromatograms of purified trimeric $gp140_{FVC}$GCN4t(+) **A)** and Env-2dCD4^{S60C} complexes **B)** showing retention volumes. Vertical dotted lines indicate the collected fractions.

3.1.3 Recombinant gp140$_{FVC}$GCN4t(+) and 2dCD4^{S60C} are conformationally and functionally intact

Conformational and functional integrity of the gp140$_{FVC}$GCN4t(+) and 2dCD4^{S60C} recombinant proteins were analysed using ELISA (**Figure 3.4**). The antibodies IgG1b12 and 17b recognise the CD4bs and CD4i epitopes on Env, respectively. The Env protein gp120BaL was used as a control for comparison. gp120BaL alone and gp140$_{FVC}$GCN4t(+) alone (dark blue) showed no signal as expected. 2dCD4^{S60C} alone showed no signal as expected (data not shown). Increased IgG1b12 binding was observed with an increase in Env concentration (gp120BaL/gp140$_{FVC}$GCN4t(+) + IgG1b12 (purple)). Confirmation of binding of 2dCD4^{S60C} to Env proteins, in particular gp140$_{FVC}$GCN4t(+), was implied by the increased signal of 17b binding to the Env proteins in the presence of 2dCD4^{S60C} (gp120BaL/gp140$_{FVC}$GCN4t(+) + 2dCD4^{S60C} + 17b (magenta)), indicative of 2dCD4^{S60C} inducing the CD4i epitope recognised by 17b.

3.1.4 gp140$_{FVC}$GCN4t(+) and 2dCD4^{S60C} form stable complexes

Env-2dCD4^{S60C} complexes were successfully generated, with unbound 2dCD4^{S60C} removed by a further round of SEC. SEC elution curves depicted a more homogeneous profile as compared to the first round of SEC of Env alone, with greatly reduced oligomeric or monomeric conformations of Env, with Env bound to 2dCD4^{S60C} in this case (**Figure 3.3, B**). The peak retention volume was noted at 46 ml. On average, 40% of total protein loaded for SEC was purified as the Env-2dCD4^{S60C} complex (corresponding to the trimeric Env peak on the elution curve). SDS-PAGE analysis under reducing conditions confirmed the presence of a band at the ~22 kDa mark, corresponding to 2dCD4^{S60C} in the trimeric Env fractions following SEC, and in the expected concentrations following complexing and loading of samples accounting for molar quantities of the proteins (**Figure 3.5, A**). Western blot analysis was performed using a Penta·His Antibody (Qiagen) to detect his-tagged 2dCD4^{S60C} (**Figure 3.5, B**). The 2dCD4^{S60C} protein was shown to be bound to Env under non-reducing conditions following electrophoresis and this binding interaction was disrupted upon use of the reducing agent DTT. Under non-reducing conditions, 3 bands are noted in lane 6 (2dCD4^{S60C} only); these may correspond with different redox states of CD4, or dimeric conformations (Cerutti et al., 2010, Cerutti et al., 2014). Taken together these results confirm Env-2dCD4^{S60C} complex formation, likely through the engineered inter-disulphide exchange between Env and 2dCD4^{S60C} described previously by our research group.

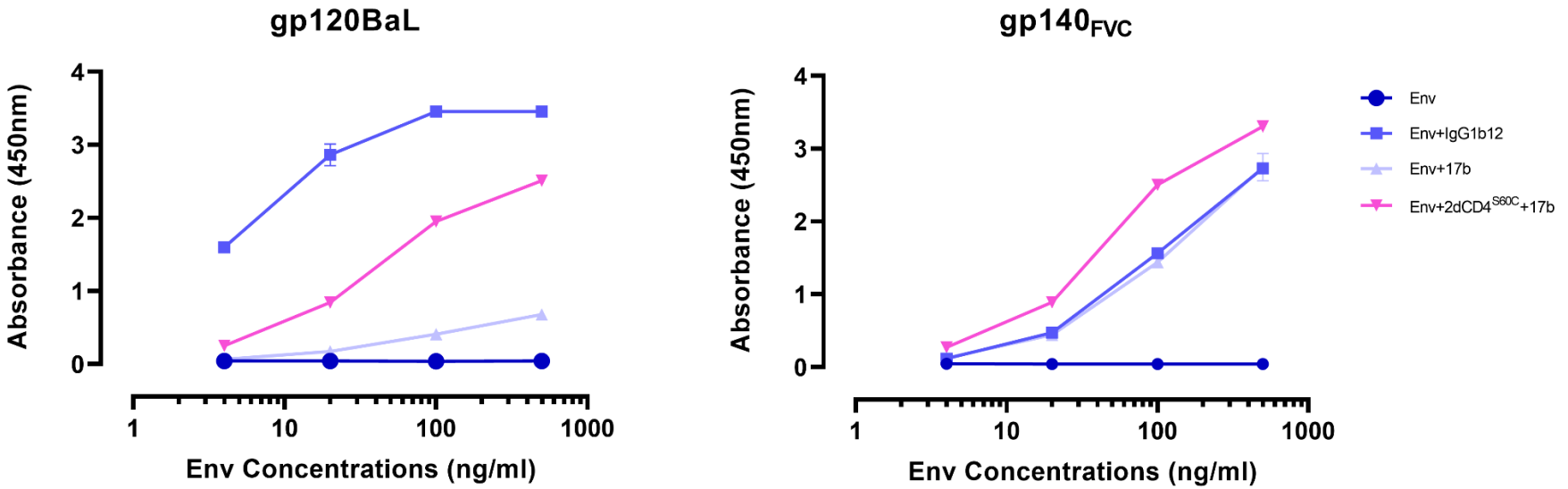


Figure 3.4: ELISA testing functionality of recombinant Env and CD4 proteins. Binding of $gp140_{FVC}$GCN4t(+) to $2dCD4^{S60C}$ was tested and compared to gp120BaL. Env proteins were prepared in a 5-fold dilution series and captured on plates by lectin. A constant amount of $2dCD4^{S60C}$ was incubated with the Env proteins and probed with CD4i antibody 17b. Env proteins alone were probed with CD4bs antibody IgG1b12. Samples were probed with a secondary sheep anti-human-HRP-linked whole antibody. All antibodies were added at a 1:2,000 dilution.

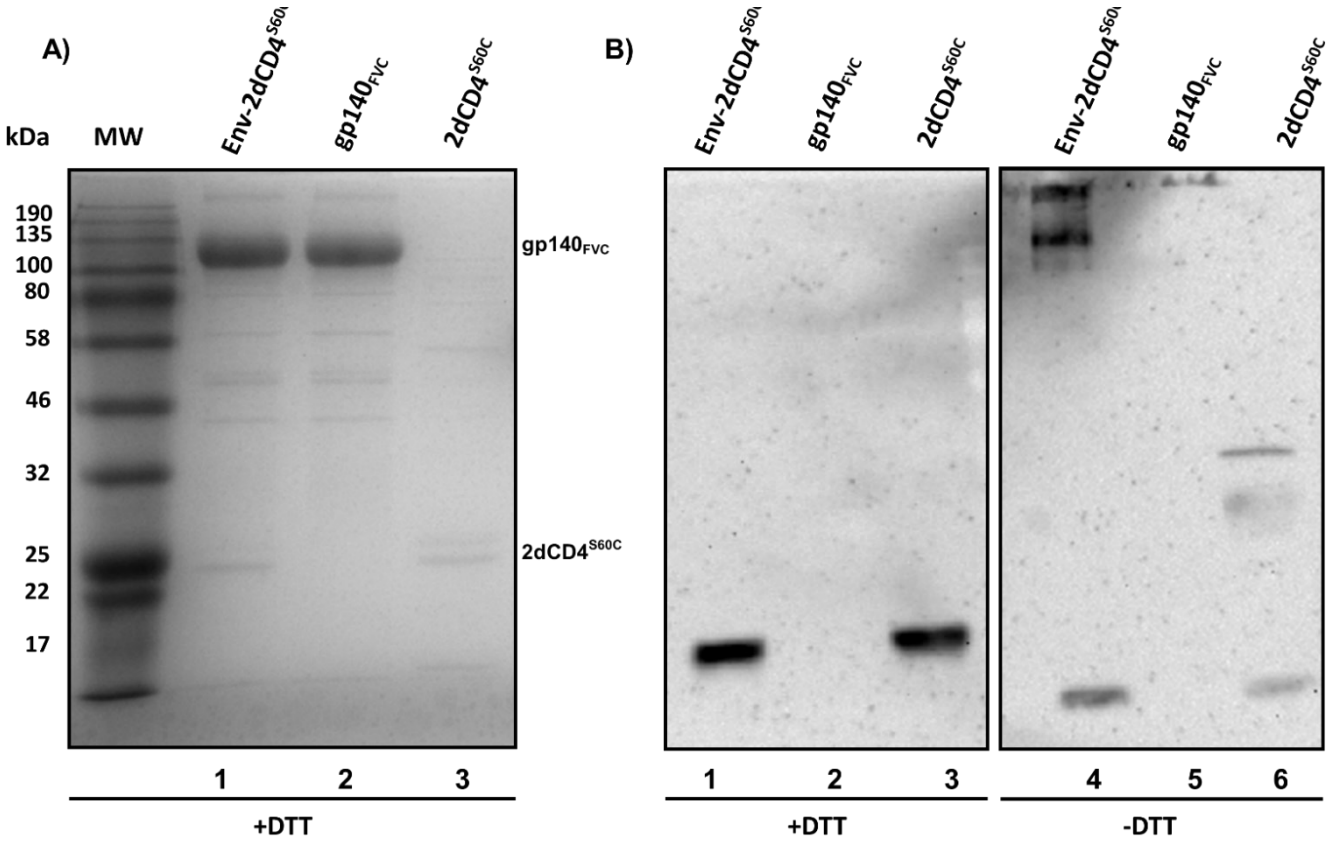


Figure 3.5: SDS-PAGE and Western blot analyses of purified trimeric gp140$_{FVC}$GCN4t(+), 2dCD4^{S60C} and Env-2dCD4^{S60C} complexes. The gp140$_{FVC}$GCN4t(+) and 2dCD4^{S60C} proteins were complexed under reducing conditions and purified using SEC. **A)** Reducing SDS-PAGE (12.5%) indicates Env-2dCD4^{S60C} complex formation. **Lane 1** depicts 5 µg of Env-2dCD4^{S60C} collected from SEC, following complexing, and separated into its constituent protein components due to reducing conditions. **Lane 2** and **3** depict 5 µg and 1 µg of purified gp140$_{FVC}$GCN4t(+) and 2dCD4^{S60C}, respectively. **B)** Western blot analysis using a Penta-His antibody (1:2,000) to detect his-tagged 2dCD4^{S60C} liganded to Env and formation of inter-disulphide linkage (**Lane 1, 4**). Individual Env and 2dCD4^{S60C} (**Lane 2, 3, 5, 6**) were used as sizing and negative controls. 2dCD4^{S60C} bound to Env was detected exclusively under non-reducing conditions (**Lane 4**). **MW**=molecular weight marker (#P7712, 11-245 kDa, New England BioLabs).

3.2 NHP immunizations with Env, 2dCD4^{S60C} and Env-2dCD4^{S60C}

3.2.1 Immunizations induce robust anti-CD4 and anti-Env antibody binding titres

Seven Chinese-origin rhesus macaques were immunized six times over the course of 120 days with gp140$_{FVC}$GCN4t(+) (n=1), 2dCD4^{S60C} (n=2), or Env-2dCD4^{S60C} (n=4) formulated with Adjuplex adjuvant (**Figure 3.6**). A 7th booster immunization consisting of the same immunogens was administered to all animals two years after the initial immunization regimen had ended (day 1000), and two weeks before commencement of SHIV mucosal challenges (**Figure 3.13, A**). The NHPs were immunized subcutaneously and bilaterally in both legs to potentially improve immune responses, as noted previously in other studies (Pauthner et al., 2019, Pauthner et al., 2017).

All Env-based immunogens (gp140$_{FVC}$GCN4t(+) and Env-2dCD4^{S60C}) induced anti-Env antibody titres as early as 14 days after the first immunization with anti-Env antibody titres similar for all animals vaccinated with Env-based immunogens over the course of the regimen (**Figure 3.7, A**). As Expected, CD4-only vaccinated animals generated no anti-Env antibody responses. All CD4-based immunogens (2dCD4^{S60C} and Env-2dCD4^{S60C}) eventually induced anti-CD4 antibody responses (**Figure 3.7, B**). Anti-CD4 antibody titres developed at different time points between different animals and immunization groups. In the CD4-only immunized group, animal #65 developed anti-CD4 antibody titres earlier than animal #67, with titres reaching similar levels at day 30 and maintaining similar rises and falls in levels between both animals throughout the initial immunization regimen.

In the Env-2dCD4^{S60C} group, anti-CD4 responses were detected only from day 44 onwards, two weeks after the 3rd immunization. Interestingly, anti-CD4 antibody titres of the Env-2dCD4^{S60C} group were on average 10-fold lower as compared to the CD4-only animals over the course of the vaccine regimen. One potential reason for differences in titres between groups could be due to the CD4-only animals receiving more overall 2dCD4^{S60C} per dose of immunization as compared to the Env-2dCD4^{S60C} group. All antibody titres for all test animals followed the same pattern of peaking at day 74, two weeks after the 4th immunization.

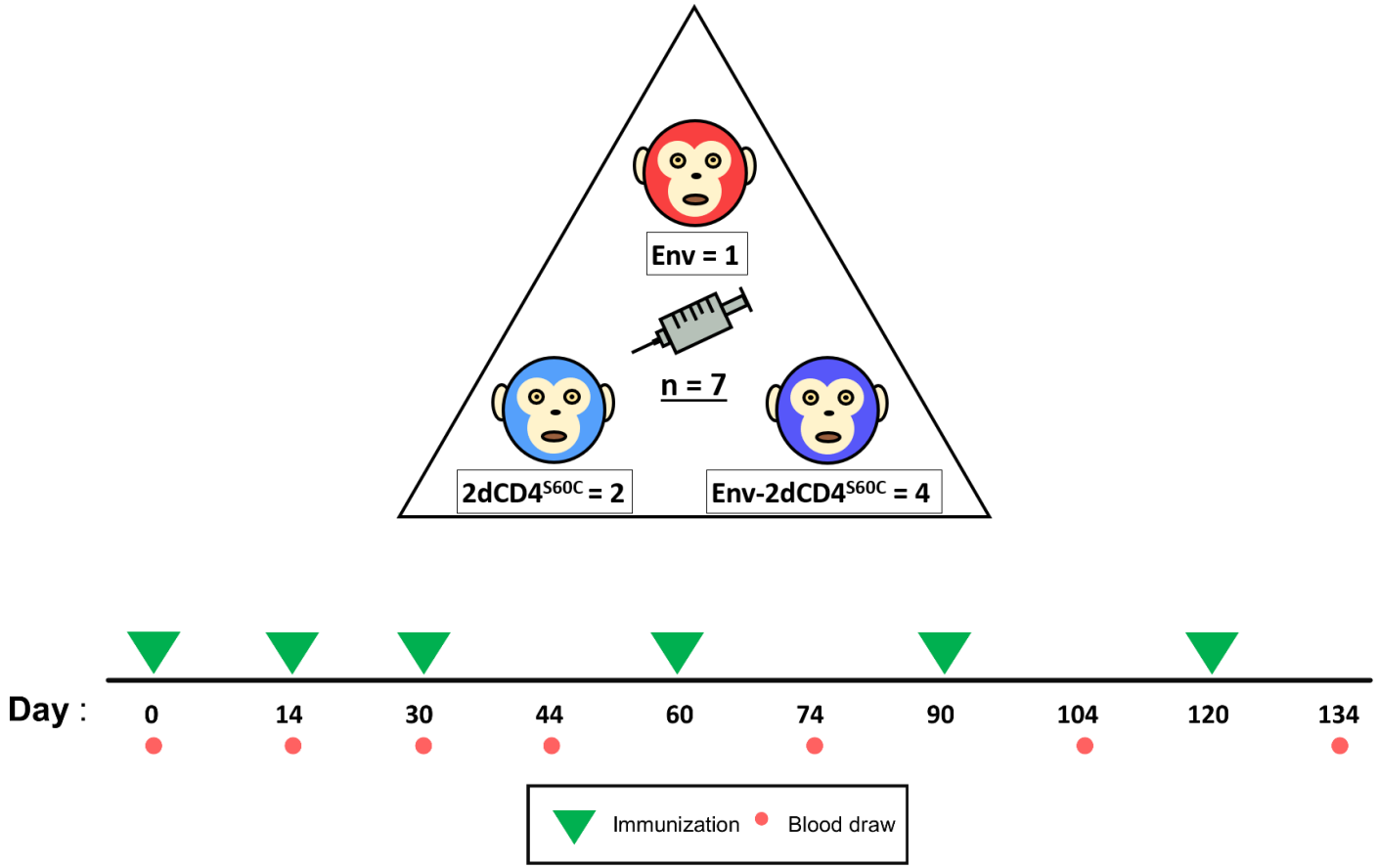


Figure 3.6: Immunization schedule for NHPs. Timeline depicting immunization and blood sampling schedule for Chinese-origin rhesus macaques (n=7). NHPs each received 100 µg total of protein immunogen per immunization, split and administered bi-laterally in each inner thigh (250 µl, 50 µg). gp140$_{FVC}$GCN4t(+) (red; #69; n=1), 2dCD4^{S60C} (blue; #65, #67; n=2), Env-2dCD4^{S60C} (purple; #57, #58, #66, #68; n=4).

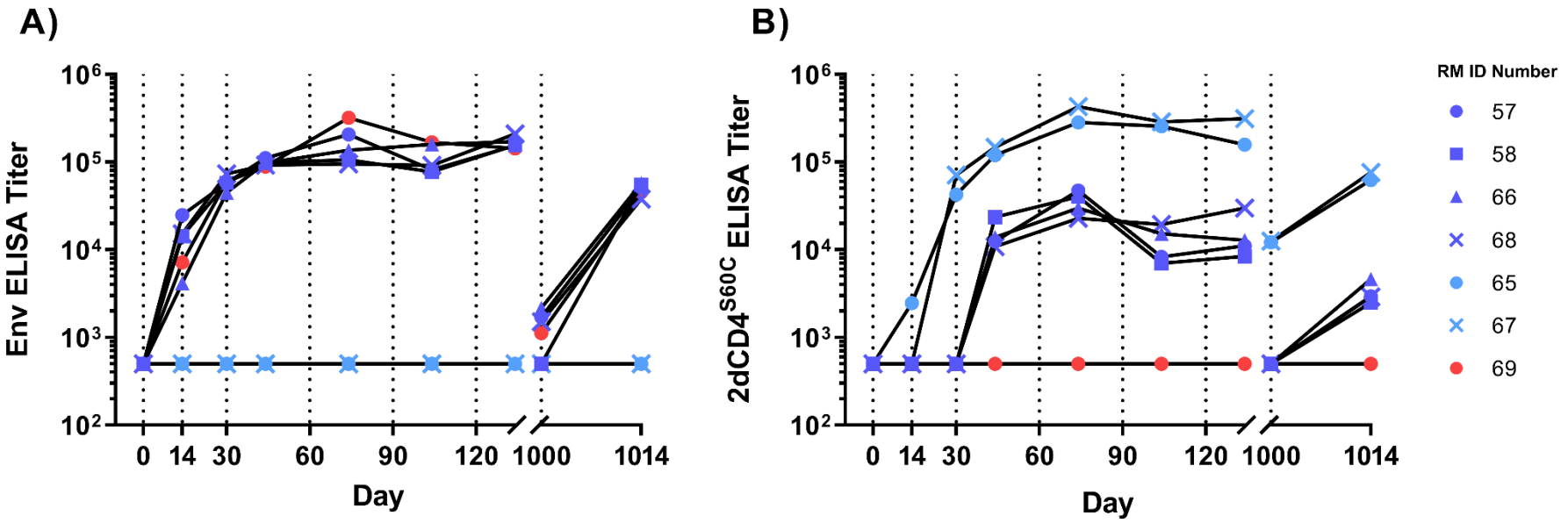


Figure 3.7: End-point ELISA antibody titres. A) Anti-Env antibody titres were determined by titrating all 7 sera samples extracted over the course of the immunization schedule against gp140$_{FVC}$GCN4t(+) immunogen. **B)** Anti-CD4 antibody titres were determined by titrating all 7 sera samples extracted over the course of the immunization schedule against 2dCD4^{S60C} immunogen. Dotted lines indicate day of vaccination. Titres are depicted on the y-axis (logged). ELISA titres are represented as the dilution factor required to obtain an absorbance value of 1 at 450 nm. Env-2dCD4^{S60C} group (purple, n=4); 2dCD4^{S60C} group (blue, n=2); Env rhesus macaque (red, n=1).

3.2.2 Env-2dCD4^{S60C} immunizations in NHPs induces broad and potent neutralizing responses against clinically relevant HIV-1 pseudoviruses

Using the HIV-1 Env Global Panel, which is representative of global HIV-1 viral subtype diversity (tier-2), and the tier-1 and tier-2 viruses SF162 and ZM53, respectively, we tested the neutralization breadth and potency of the sera from our immunized NHPs (**Table 3.1**). Pre-immunization neutralizing titres were determined for one animal from each test group and used as the baselines for all the animals (ID_{50} under 1:20 was considered non-neutralizing**)**. As expected, no neutralizing activity was detected at baseline (**Appendix B: Supplementary figures**, **Supplementary Table 1**). Furthermore, no neutralizing activity against specificity control virus VSV-G was detected at baseline (**Appendix B: Supplementary figures, Supplementary Table 1**), nor at day 74 which were the peak of neutralizing titres (**Table 3.1**). One of the NHPs had low level cross-reactivity against VSV-G at day 74 but the titre was near the cut-off for non-neutralization of 1:20. The animal that received Env-only (#69) developed no neutralizing responses against any of the tier-2 viruses at any of the time points tested, but developed high neutralizing titres against the tier-1 virus SF162 (**Table 3.1**). Animals vaccinated with 2dCD4^{S60C}-only (#65, #67) developed neutralizing titres with breadth but lower potency (**Table 3.1**), with the geometric mean titre for the entire group against the Env Global Panel remaining below 1:100 for day 74, 104 and 134 (**Figure 3.8**).

The Env-2dCD4^{S60C} immunized NHPs (#57, #58, #66, #68) developed potent bNAb responses at day 74, neutralizing at least 11/12 of the tier-2 HIV-1 pseudoviruses of the Env Global Panel with titres above 1:100, with serum ID_{50} values peaking over 1:1,000 for some viruses tested against (**Table 3.1**). The geometric mean titre for the Env-2dCD4^{S60C} immunized group at day 74 was 6-fold greater as compared to the 2dCD4^{S60C} immunized group (1:356 and 1:60, $p < 0.001$) (**Figure 3.8**). Following day 74, the potency of the serum ID_{50} values for Env-2dCD4^{S60C} immunized animals began to wane with a significant decrease in the geometric mean titres noted between day 74 and day 134 (1:356 and 1:149, $p<0.001$), despite two additional immunizations. Importantly, the geometric mean titre for the Env-2dCD4^{S60C} immunized group at day 104 was still 2.5-fold greater as compared to the 2dCD4^{S60C} immunized group (1:184 and 1:73, $p<0.001$) and 2-fold greater as compared to the 2dCD4^{S60C} immunized group at day 134 (1:149 and 1:77, $p<0.002$) (**Figure 3.8**).

Animal #68 still showed excellent breadth and potency by day 134 and was able to neutralize 11/12 of the Env Global Panel and tier-2 virus ZM53 with a serum ID_{50} greater than 1:100, with several still above 1:500 (**Table 3.1**). Animals #58 and #66 also retained some potency and

breadth, neutralizing 9/12 viruses with titres above 1:100, with animal #57 losing much of its breadth and potency by day 134, neutralizing 2/12 viruses with high potency. All Env-2dCD4^{S60C} immunized animals continued to neutralize tier-1 virus SF162 with high potency throughout until day 134, while two of the animals (#57, #58) lost neutralizing potency against tier-2 virus ZM53 with titres comparable to the animals immunized with 2dCD4^{S60C} only.

Interestingly, geometric mean titres against the Env Global Panel for the 2dCD4^{S60C} immunized animals showed a modest but significant increase between day 74 and day 134 (1:60 and 1:77, $p<0.02$) (**Figure 3.8**).

Table 3.1: Pseudoviral neutralization assay ID_{50} profile of immunized NHPs. Potency and breadth of NHP sera, tested against the 12-virus HIV-1 Env Global Panel. Each row represents one virus and each column a single rhesus macaque, the immunogen it was vaccinated with, and the time point the sera was collected from. Neutralization data is indicated as the reciprocal dilution of rhesus macaque sera required to achieve 50% inhibition of respective HIV-1 pseudoviruses. VSV-G was used as a specificity control and infects cells independently of CD4, CCR5 or CXCR4. ***Appendix A** for baseline bleed ID_{50}.

			Day 74						
			Env	2dCD4^{S60C}		Env-2dCD4^{S60C}			
		Clade	#69	#65	#67	#57	#58	#66	#68
HIV-1 Global Reference Strains	398F1	A	<20	105	129	685	1053	1256	2346
	246F3	AC	<20	62	73	168	207	237	473
	CNE55	AE	<20	84	93	178	216	328	450
	CNE8	AE	<20	65	71	295	485	872	656
	X2278	B	<20	68	64	278	476	622	489
	TRO11	B	<20	123	85	518	677	1032	1034
	BJOX2000	BC	<20	47	25	128	218	277	273
	CH119	BC	<20	62	70	565	1229	1720	685
	CE0217	C	<20	62	<20	154	168	287	383
	CE1176	C	<20	63	65	153	746	217	369
	25710	C	<20	54	73	211	304	366	589
	X1632	G	<20	<20	<20	31	40	101	65
Tier-2	ZM53	C	<20	49	137	97	151	117	176
Tier-1	SF162	B	1154	<20	<20	590	411	1512	660
Control	VSV-G		<20	<20	<20	<20	<20	28	<20

			Day 104						
			Env	2dCD4^{S60C}		Env-2dCD4^{S60C}			
		Clade	#69	#65	#67	#57	#58	#66	#68
HIV-1 Global Reference Strains	398F1	A	<20	190	214	346	692	883	791
	246F3	AC	<20	63	66	60	119	109	220
	CNE55	AE	<20	97	110	186	269	476	691
	CNE8	AE	<20	181	175	169	319	293	520
	X2278	B	<20	88	74	128	338	314	441
	TRO11	B	<20	101	116	207	338	509	743
	BJOX2000	BC	<20	75	<20	37	77	138	156
	CH119	BC	<20	56	47	49	88	102	178
	CE0217	C	<20	65	55	74	264	324	393
	CE1176	C	<20	60	62	59	62	66	161
	25710	C	<20	68	81	154	266	273	577
	X1632	G	<20	<20	23	50	43	48	30
Tier-2	ZM53	C	<20	28	43	61	132	160	282
Tier-1	SF162	B	962	<20	<20	551	756	1210	647

			Day 134						
			Env	2dCD4^{S60C}		Env-2dCD4^{S60C}			
		Clade	#69	#65	#67	#57	#58	#66	#68
HIV-1 Global Reference Strains	398F1	A	<20	187	212	228	717	363	1166
	246F3	AC	<20	66	57	68	199	208	473
	CNE55	AE	<20	102	136	77	140	140	530
	CNE8	AE	<20	162	215	79	108	203	382
	X2278	B	<20	63	131	94	290	248	579
	TRO11	B	<20	96	129	185	420	468	669
	BJOX2000	BC	<20	48	<20	55	100	141	232
	CH119	BC	<20	56	37	47	130	119	282
	CE0217	C	<20	52	66	53	116	115	240
	CE1176	C	<20	33	49	53	51	<20	188
	25710	C	<20	71	106	58	74	92	239
	X1632	G	<20	49	55	51	49	57	56
Tier-2	ZM53	C	<20	61	86	44	46	116	214
Tier-1	SF162	B	846	<20	<20	1372	1377	1614	966

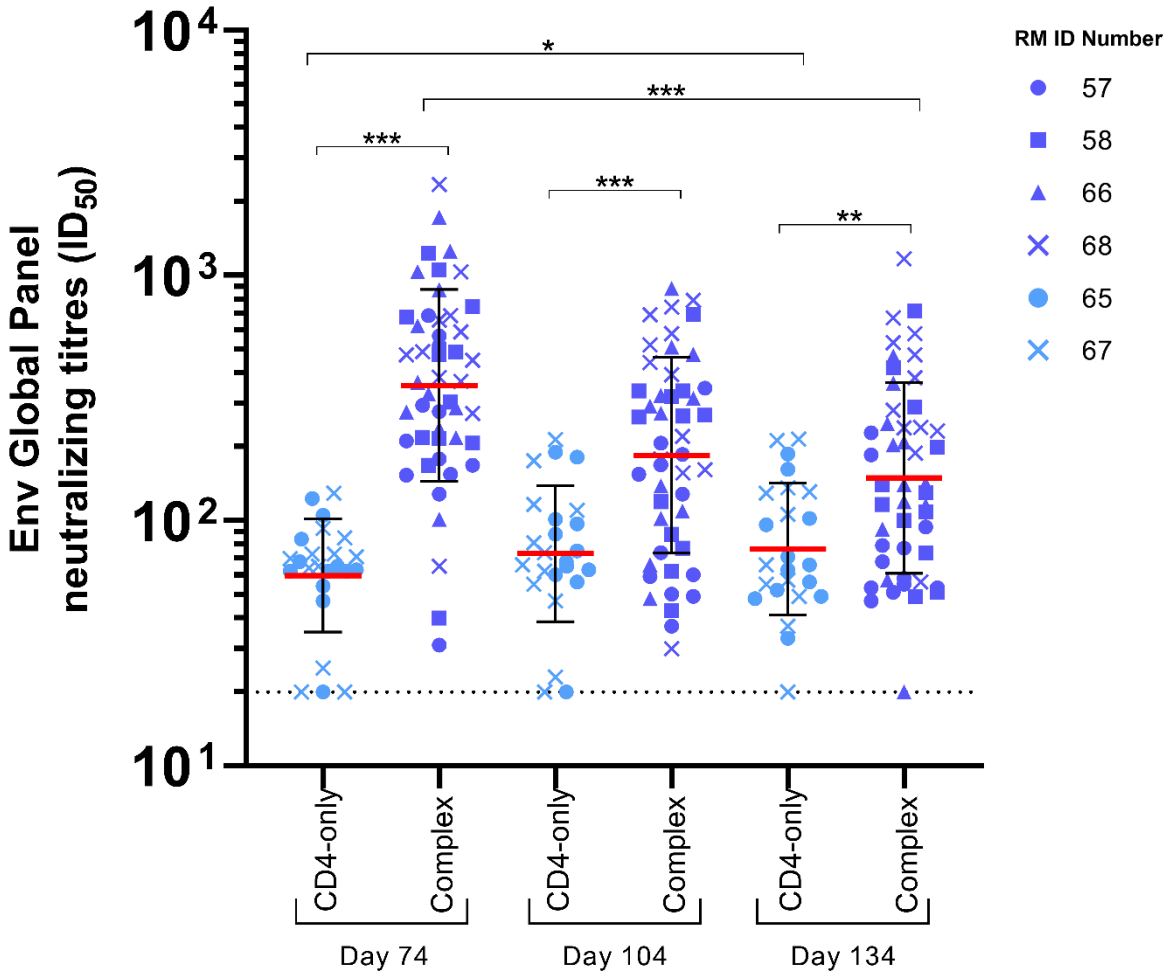


Figure 3.8: Average neutralizing titres of Env-2dCD4^{S60C} and 2dCD4^{S60C} immunized NHP groups. All serum ID_{50} titres of 2dCD4^{S60C} group (blue, n=2), or Env-2dCD4^{S60C} group (purple, n=4) animals against the entire 12-HIV-1 Env Global Panel for day 74 (post-4th immunization), day 104 (post-5th immunization) and day 134 (post-6th immunization). Statistical differences in neutralization between CD4-only and complex groups was calculated using an unpaired, two-tailed Mann-Whitney test; $^{**}p < 0.002$ and $^{***}p < 0.001$. Statistical differences in neutralization within same groups between day 74 and day 134 in the complex and CD4-only immunized groups was calculated using a Wilcoxon matched-pairs signed rank test; $^{*}p < 0.02$ and $^{***}p < 0.001$. Geometric mean titres are depicted in red and black bars indicate geometric SD. Dotted line indicates cut-off for neutralizing titres (<1:20).

3.2.3 Neutralizing antibody titres in NHPs immunized with Env-2dCD4^{S60C} wane completely after 2 years, but can be restored to detectable and neutralizing levels by a single boosting immunization

Following a two-year gap, all test animals received a 7th booster immunization (day 1000, -2 weeks before SHIV mucosal challenges). Antibody binding titres dropped considerably between day 134 and day 1000 as expected (**Figure 3.7**). Anti-Env titres were near undetectable levels at day 1000, with animal #58 having no detectable levels according to our own cut-offs for detection (dilution factor required to obtain an absorbance value of 1 at 450 nm) (**Figure 3.7, A**). Anti-Env titres saw a decrease from the last recorded titres at day 134 to day 1000 of 90-fold (1:154,372 to 1:1,708), 79-fold (1:170,551 to 1:2,162), and 139-fold (1:209,842 to 1:1,512) for complex immunized animals #57, #66 and #68, respectively. The Env-only immunized animal #69 saw a similar drop of 128-fold (1:143,118 to 1:1,121). Two weeks following booster immunizations (day 1014), anti-Env titres increased, but were on average 3-fold lower than the last recorded titres (day 134) of the initial immunization regimen for all animals (**Figure 3.7, A)**. As expected, CD4-only immunized animals continued to show no anti-Env titres after the 7th booster immunization.

Anti-CD4 titres showed a similar decrease two years post-final vaccination (**Figure 3.7, B**). All complex immunized animals had no detectable anti-CD4 titres at day 1000 according to our own cut-offs for detection. Interestingly, CD4-only immunized animals still had detectable anti-CD4 titres at day 1000 as compared to day 134, with a 13-fold (1:157,993 to 1:12,150) and 25-fold (1:313,597 to 1:12,523) reduction for #65 and #67, respectively. Two weeks following booster immunizations (day 1014), anti-CD4 titres were detectable again for the complex immunized group (**Figure 3.7, B**). Already detectable anti-CD4 titres for the CD4-only immunized group increased further following the booster immunization. The anti-CD4 titres were on average 3.5-fold lower following booster immunizations, as compared to the last recorded titres (day 134) of the initial immunization regimen for all animals. The Env-only immunized animal continued to show no anti-CD4 titres, even after 7th immunization booster shot.

Neutralization assays were conducted to evaluate neutralizing titres two years following vaccinations, and in preparation for SHIV challenges (**Table 3.2**) (**Figure 3.9**). Two viruses previously tested against were selected for follow up, tier-1 and tier-2 viruses SF162 and ZM53, respectively. Using both viruses would give an indication as to the robustness of potency and breadth of tier-1 and tier-2 neutralizing antibody responses over time. Pre-booster

sera (day 1000) no longer showed any neutralizing activity, in line with data showing that end-point antibody titres had decreased substantially as well (**Figure 3.7**).

Two weeks following booster immunizations (day 1014), neutralizing activity against tier-2 virus ZM53 was restored for 3/4 of the complex immunized animals, comparable to titres noted at day 134, and in line with rises in end-point antibody titres (**Table 3.2**) (**Figure 3.9**) (**Figure 3.7**). Animal #57 was unable to neutralize tier-2 virus ZM53 following the booster immunization. CD4-only immunized animals had initially showed low-level neutralizing activity against ZM53 until day 134, but it was not restored following the booster immunization at day 1000. Interestingly, the Env-only immunized animal had initially shown high neutralizing activity against tier-1 virus SF162, but two years on and following a booster immunization, that potent neutralizing activity was not restored.

3.2.4 Env-2dCD4^{S60C} immunizations in NHPs induce neutralizing titres against a tier-2 heterologous subtype C SHIV

Before commencement of SHIV challenges in NHPs, neutralizing sensitivity of SHIV-1157ipd3N4 was assessed. Surprisingly, sera from day 0 baseline bleeds for animals #58, #67 and 69# showed low-level neutralizing potency against SHIV-1157ipd3N4 (**Table 3.2**). Day 74 sera from all animals showed neutralizing activity against SHIV-1157ipd3N4, but most notably 3/4 complex immunized animals showed a clear increase in neutralizing activity, with the rest having titres comparable to the day 0 baseline sera (**Table 3.2**) (**Figure 3.9**). By day 1000 sera neutralizing titres for these three rhesus macaques (#58, #66 and #68) had dropped to comparable day 0 levels, consistent with the general waning in anti-Env and anti-CD4 antibody end-point titres and neutralizing activity observed against SF162 and ZM53. Animals #57, #65, #67 and #69 retained their low-level activity, displaying neutralization titres comparable to day 0 baseline titres. Following booster immunizations, day 1014 sera for 3/4 complex immunized animals that had previously demonstrated neutralizing titres (#58, #66 and #68) showed a clear increase in neutralizing activity comparable to day 74 sera, with the rest of animals showing no further increases from their baseline levels of activity.

Table 3.2: Pseudoviral neutralization assay ID_{50} profile of immunized NHPs pre- and post-booster immunizations. Potency and breadth of NHP sera, tested against ZM53, SF162 and SHIV-1157. Each row represents one virus and each column a single rhesus macaque, the immunogen it was vaccinated with, and the time point the sera was collected from. Neutralization data is indicated as the reciprocal dilution of rhesus macaque sera required to achieve 50% inhibition of respective HIV-1 pseudoviruses.

			Day 0 Pre-Bleed		
			Env	2dCD4^{S60C}	Env-2dCD4^{S60C}
		Clade	#69	#67	#58
Tier-2	ZM53	C	<20	<20	<20
Tier-1	SF162	B	<20	<20	<20
	SHIV 1157	C	40	63	51

			Day 74						
			Env	2dCD4^{S60C}		Env-2dCD4^{S60C}			
		Clade	#69	#65	#67	#57	#58	#66	#68
Tier-2	ZM53	C	<20	49	137	97	151	117	176
Tier-1	SF162	B	1154	<20	<20	590	411	1512	660
	SHIV 1157	C	49	63	61	81	111	202	174

			Pre-Boost (Day 1000)						
			Env	2dCD4^{S60C}		Env-2dCD4^{S60C}			
		Clade	#69	#65	#67	#57	#58	#66	#68
Tier-2	ZM53	C	<20	<20	<20	<20	<20	<20	<20
Tier-1	SF162	B	<20	<20	<20	<20	<20	<20	<20
	SHIV 1157	C	40	24	27	66	78	54	85

			Post-Boost (Day 1014)						
			Env	2dCD4^{S60C}		Env-2dCD4^{S60C}			
		Clade	#69	#65	#67	#57	#58	#66	#68
Tier-2	ZM53	C	<20	<20	<20	<20	52	99	153
Tier-1	SF162	B	80	<20	<20	240	680	1311	450
	SHIV 1157	C	77	63	32	79	197	152	149

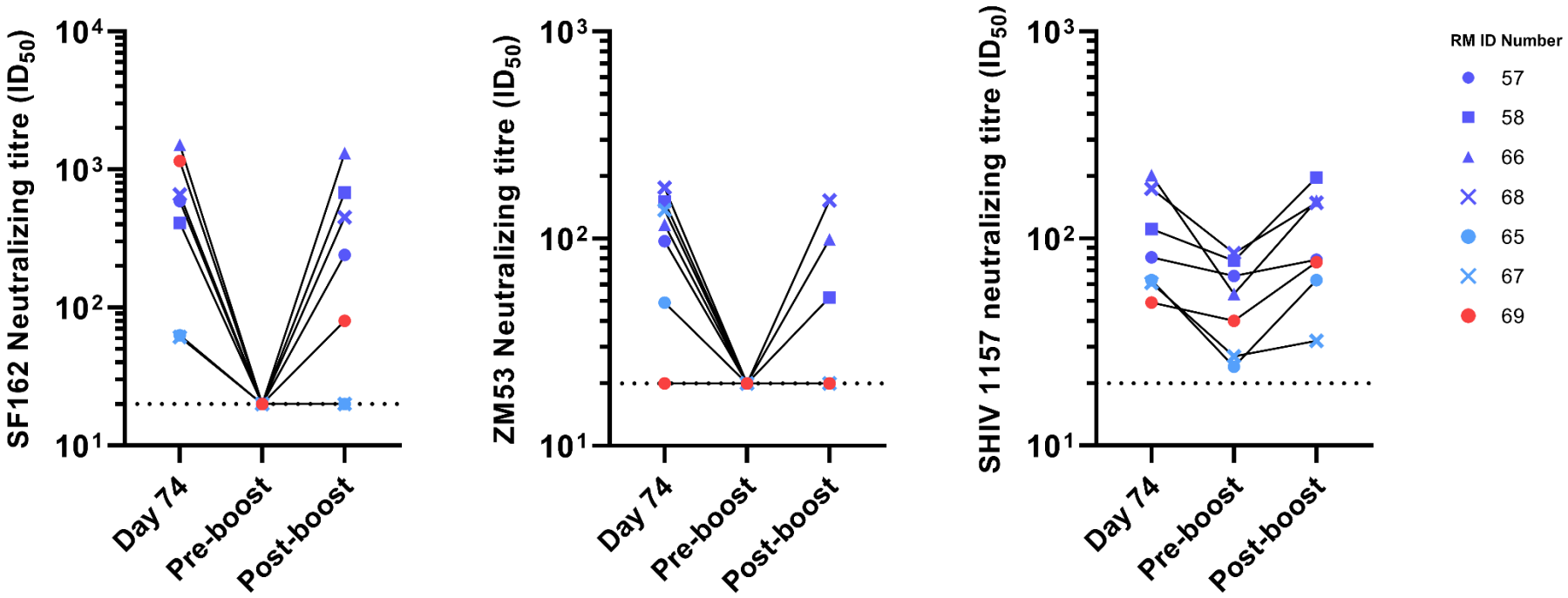


Figure 3.9: Serum neutralizing titres of NHPs pre- and post-booster immunizations. Rhesus macaques were immunized two years after a six dose immunization regimen, and two weeks prior to commencement of SHIV mucosal challenges. HIV-1 pseudoviruses tested against included ZM53, SF162 and the challenge virus SHIV-1157. Neutralizing titres were compared between peak of previous six dose vaccine regimen (day 74), two years after end of sixth vaccine dose (pre-boost, day 1000) and two weeks following administration of booster immunizations (post-boost, day 1014). Env-2dCD4^{S60C} group (purple, n=4), 2dCD4^{S60C} group (blue, n=2), Env rhesus macaque (red, n=1). Dotted line indicates cut-off for neutralizing titres (<1:20).

3.3 Anti-CD4 antibody depletion assays

3.3.1 CD4-directed antibodies are responsible for the bulk of neutralizing activity noted in sera from Env-2dCD4^{S60C} vaccinated NHPs

Previous work by our research group indicated that neutralizing antibody responses generated in rabbits after immunization with the Env-2dCD4^{S60C} complex were driven by anti-CD4 antibody responses, according to various antibody depletion work conducted (Killick et al., 2015). Basic mapping of the antibody responses generated in the current study in immunized rhesus macaques aimed to identify if similar anti-CD4 directed antibody responses were responsible for the broad neutralizing activity noted in this study. Whereas previous depletion work done on rabbit sera used bead-based absorption methods for depletions, the current study employed a competition-based neutralization assay that utilized different concentrations of various recombinant CD4 proteins to deplete sera responses *in vitro*.

A set of control assays were first conducted to establish that the CD4 proteins to be used for depletion of the immunized rhesus macaque sera were specific only for antibodies in the sera and were not inhibiting the viruses used in the various assays (**Figure 3.10**). Two versions of soluble 4dCD4, wild-type (4dCD4-WT) and mutant (4dCD4-Mut), were first tested for neutralizing activity against two viruses that would be used for all the antibody depletion work. The CD4 protein 4dCD4-Mut contains the F43A and R59A mutations which reduces the protein's affinity for Env binding (Moebius et al., 1992, Fontenot et al., 2007), therefore it can be incorporated directly into the pseudovirus inhibition assay to deplete anti-CD4 responses in immunized NHP sera while having minimal binding/inhibitory effects on the pseudoviruses. As expected, 4dCD4-Mut had no neutralizing effect against tier-1 virus SF162 and tier-2 virus ZM53, whereas 4dCD4-WT was able to neutralize SF162 potently while showing some neutralizing activity against ZM53 at its highest concentration (100 µg/ml) (**Figure 3.10**, **A and B**). Data for a third recombinant CD4, 2dCD4-WT, was available in our laboratory (work unpublished), and was used to predict the activity of the 2dCD4^{S60C} protein against SF162 and ZM53. 2dCD4-WT shows inhibitory activity against tier-1 virus SF162 when used at concentrations greater than 1 µg/ml, whereas it shows minimal inhibitory activity with concentrations up to 100 µg/ml against tier-2 virus ZM53. The activity of 2dCD4-WT against SF162 and ZM53 predicted accurately the inhibitory effects of 2dCD4^{S60C} in the subsequent assays detailed in the rest of this section, with 2dCD4^{S60C} suitable only for depletion work against tier-2 virus ZM53 (**Figure 3.11**).

The specificity of the depletion of anti-CD4 responses by incorporation of 4dCD4-Mut into the pseudovirus assay was evaluated against purified HIV-1 bNAbs, VRC01 and ibalizumab (**Figure 3.10, C**). When 4dCD4-Mut was added in combination with the HIV-1 CD4bs directed bNAb, VRC01, no interference was noted on the inhibition curve of VRC01 as compared to the antibody alone against ZM53. However, when 4dCD4-Mut is added in combination with the monoclonal antibody ibalizumab which is a CD4-targeting HIV post-attachment inhibitor, a reduction in inhibitory potency of approximately 11-fold was noted when compared to ibalizumab alone (a shift in IC_{50} of 0.19 µg/ml to 2.2 µg/ml). This reduction in potency indicates that 4dCD4-Mut is sequestering ibalizumab and reducing the overall inhibitory effect of the antibody in the assay.

Rhesus macaque sera #66 and #68 were selected from the Env-2dCD4^{S60C} complex immunized group for the depletion experiments as they consistently demonstrated the highest neutralizing titres over the duration of vaccine regime. More specifically, day 74 sera corresponding to maximum neutralising titres were used in the subsequent depletion experiments. Initial depletion of the NHP sera was done with a standard concentration of 4dCD4-Mut per well (10 µg/ml final concentration in assay). Only a modest reduction in neutralization activity was noted (**Appendix B: Supplementary figures, Supplementary Figure 4**). The effect of increasing concentrations of 4dCD4-Mut on the depletion of sera was then tested to find an optimal concentration of CD4 to be used that could fully deplete the neutralizing ability of the NHP sera (**Figure 3.10, D**). Three concentrations of 4dCD4-Mut (10, 20, 30 µg/ml) were used to deplete sera from animal #66. Increasing amounts of 4dCD4-Mut resulted in marginal increases in reduction of neutralizing activity of the sera against ZM53, with the ID_{50} of sera alone being 1:195 and dropping to 1:55 when using 30 µg/ml 4dCD4-Mut to deplete sera. The other two concentrations of 10 and 20 µg/ml resulted in reduced titres of 1:124 and 1:82, respectively.

A final set of controls compared the depletion abilities of 4dCD4-Mut and 2dCD4^{S60C} (the CD4 immunogen that was used in the NHP vaccinations) (**Figure 3.10, E and F**). Conservative equimolar amounts (0.1 µM) of 4dCD4-Mut and 2dCD4^{S60C} were used to deplete sera from animal #66. For 2dCD4^{S60C}, a final 0.1 µM concentration as used in assay equates to approximately 2 µg/ml, whereas a 0.1 µM concentration of 4dCD4-Mut equates to approximately 4 µg/ml. The 2dCD4^{S60C} was superior in its ability to deplete animal #66 sera as compared to 4dCD4-Mut, at the same molar concentration (**Figure 3.10, E**). Sera alone neutralized ZM53 in this assay at an ID_{50} of 1:592, with 4dCD4-Mut reducing it to 1:168, and 2dCD4^{S60C} resulting in more than a 10-fold drop to 1:56. When added alone against virus

(**Figure 3.10, F**), 2dCD4^{S60C} had an elevated low-level inhibitory effect against ZM53 (~30%) when compared to 4dCD4-Mut (~10%), but this low-level inhibition was likely not high enough to have any appreciable effect on any of the depletion assays as a decrease in the neutralization titres was observed rather than enhancement in neutralizing activity. Subsequent assays confirmed inhibitory effects were lower than described above and in line with the 2dCD-WT assay on which the concentrations were initially based (**Figure 3.10, B)**.

Having performed preliminary optimization of the anti-CD4 antibody depletion assays, day 74 sera (peak of neutralizing titres) of animals #66, #68, #65, #67 and #69 were chosen for antibody depletion and then tested against ZM53 (**Figure 3.11**). As mentioned, sera from the two NHPs that showed the greatest potency and breadth at day 74 from the Env-2dCD4^{S60C} immunized group (#66 and #68) were chosen and compared against sera from the 2dCD4^{S60C} (#65 and #67) and Env (#69) immunized animals. Sera was depleted with 4dCD4-Mut, 2dCD4^{S60C} or both in combination, using equimolar concentrations. For 2dCD4^{S60C}, a final 0.5 µM concentration as used in assay equates to approximately 10 µg/ml, whereas a 0.5 µM concentration of 4dCD4-Mut equates to approximately 20 µg/ml. Complex-immunized animal #66 sera showed a near 3-fold decrease in neutralizing activity when depleted with 4dCD4-Mut with a drop in ID_{50} of 1:221 to 1:77, but had a near complete reduction in neutralizing activity ($ID_{50} < 20$), with an approximately 8-fold reduction to an ID_{50} of 1:28 when depleted with 2dCD4^{S60C} alone, with no further benefit noted in depletion when using both CD4 proteins in combination. Complex-immunized animal #68 sera had a complete reduction in neutralizing activity of 1:187 when depleted with 4dCD4-Mut, 2dCD4^{S60C} or both in combination. CD4-only immunized animals #65 and 67# showed low-level neutralizing activity against ZM53 with ID_{50} titres of 1:35 and 1:24, respectively. The activity was reduced following depletion with 4dCD4-Mut, 2dCD4^{S60C} and both in combination. Interestingly, an enhancement in viral infectivity in the assay was noted for animal #67, particularly in the presence of 4dCD4-Mut. It is unclear if this apparent enhancement in infectivity is non-specific, as day 0 sera in combination with 4dCD4-Mut was not evaluated. The Env immunized NHP, #69, showed no neutralizing activity against tier-2 virus ZM53, with no further effect noted with the addition of 4dCD4-Mut, 2dCD4^{S60C} or both in combination.

To confirm that the anti-Env responses did not contribute significantly to the neutralisation breadth, depletions were conducted and sera tested against tier-1 virus SF162 as described above, but only using 4dCD4-Mut (**Figure 3.12**). The Env-2dCD4^{S60C} immunized sera of animals #66 and #68 showed no appreciable reduction in neutralization potency following

depletions, with approximately a 1.5-fold reduction noted in ID_{50} titres (1:1,567 to 1:953, and 1:706 to 1:452, respectively). The sera of 2dCD4^{S60C} immunized animals #65 and #67 showed no activity against tier-1 virus SF162, as expected, with no further effect noted with the addition of 4dCD4-Mut. For Env immunized animal #69, no appreciable reduction in neutralization potency of sera was noted following depletion with 4dCD4-Mut, with the same 1.5-fold reduction in ID_{50} titres (1:764 to 1:507) noted as compared to the complex immunized sera. Separate assays were conducted only using 2dCD4^{S60C} to deplete sera and tested against tier-1 virus SF162, but inhibition in the assay was noted due to the presence of 2dCD4^{S60C}. Near 100% neutralization of SF162 was detected at all depleted serum dilutions (**Appendix B: Supplementary figures, Supplementary Figure 5**).

In summary, depletion of the anti-CD4 responses in the complex immunized animal sera resulted in a loss of activity against the heterologous, tier-2 viral isolate ZM53, whilst maintaining neutralizing activity against the tier-1 isolate SF162 through the retained anti-Env specificity following anti-CD4 specific depletion. Combined, these results reaffirm the contribution of the anti-CD4 responses to the breadth and potency of the observed anti-HIV responses following immunization of animals with Env-2dCD4^{S60C} complex and suggest that the elicited anti-Env responses do not predominate the neutralizing activity.

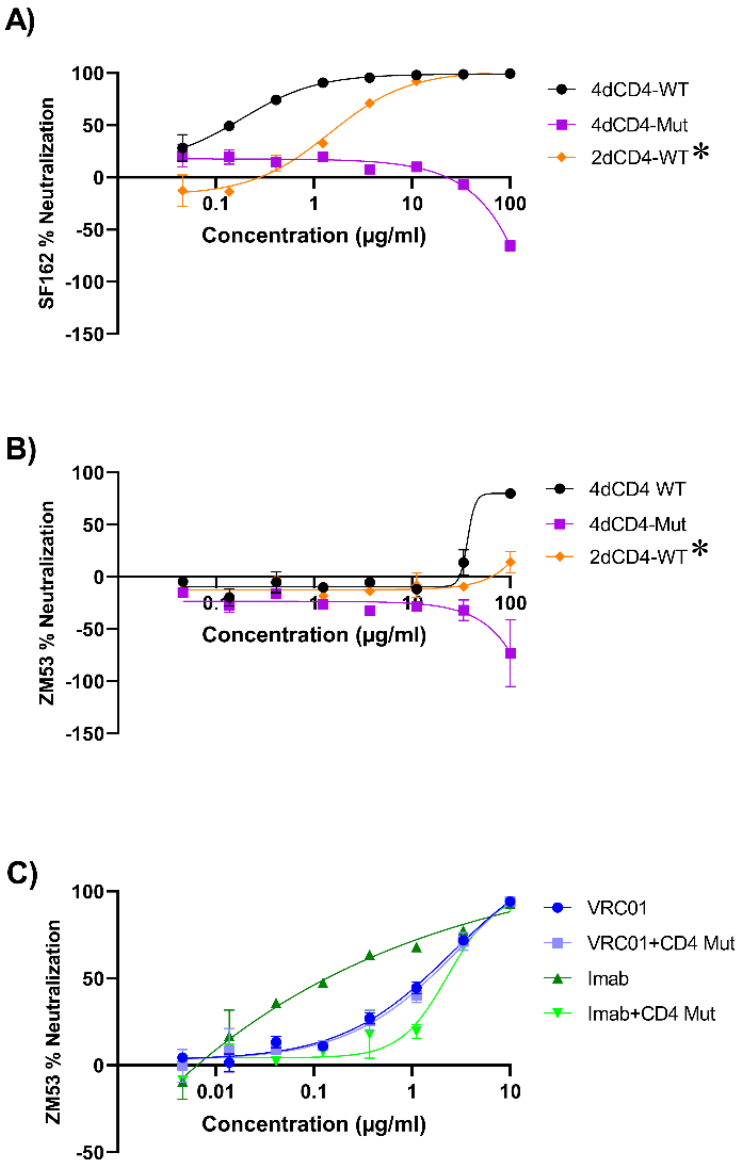


Figure 3.10: Control assays for anti-CD4 antibody depletion work of immunized NHP sera. Control assays were conducted to establish that recombinant CD4 proteins can deplete NHP sera of anti-CD4 antibodies successfully. **A)** and **B)** depict the inhibition activity of recombinant CD4 proteins alone against SF162 and ZM53. Starting concentration of 100 µg/ml and diluted in a 3-fold dilution series (black, 4dCD4-WT), (pink, 4dCD4-Mut), (orange, 2dCD4-WT*). **C)** depicts the interaction of 4dCD4-Mut with the Env CD4bs-targeting antibody VRC01, and CD4-targeting antibody ibalizumab. Antibodies were diluted in a 3-fold dilution series starting at 10 µg/ml, 4dCD4-Mut was added at a constant final concentration of 10 µg/ml within the assay (blue, VRC01), (light blue, VRC01+4dCD4-Mut), (green, ibalizumab), (light green, ibalizumab+4dCD4-Mut).*=data was obtained from previous experiments (unpublished) and used as a proxy for 2dCD4^{S60C} activity against SF162 and ZM53. Figures **D)-F)** on next page.

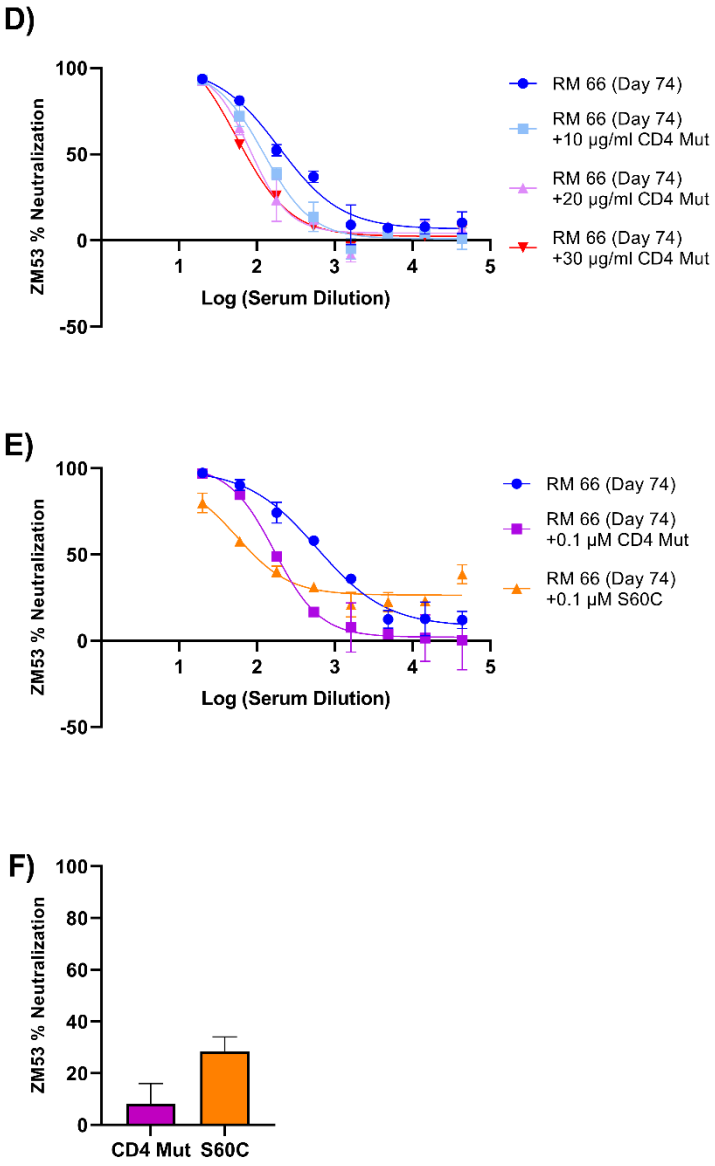


Figure 3.10: Continued. D) compares the anti-CD4 depleting effect on day 74 sera of rhesus macaque #66 immunized with Env-2dCD4^{S60C} at three different concentrations of 4dCD4-Mut. (blue, sera only), (light blue, sera+10 µg/ml 4dCD4-Mut), (lilac, sera+20 µg/ml 4dCD4-Mut), (red, sera+30 µg/ml 4dCD4-Mut). **E)** depicts the anti-CD4 antibody depleting effect of 4dCD4-Mut vs 2dCD4^{S60C} on day 74 sera of rhesus macaque #66 immunized with Env-2dCD4^{S60C} (blue, sera only), (purple, sera+0.1 µM 4dCD4-Mut), (orange, sera+0.1 µM 2dCD4^{S60C}). **F)** is a control for **E)** and depicts the inhibition activity of 0.1 µM of 4dCD4-Mut alone or 2dCD4^{S60C} alone against ZM53 (purple, 4dCD4-Mut), (orange, 2dCD4^{S60C}).

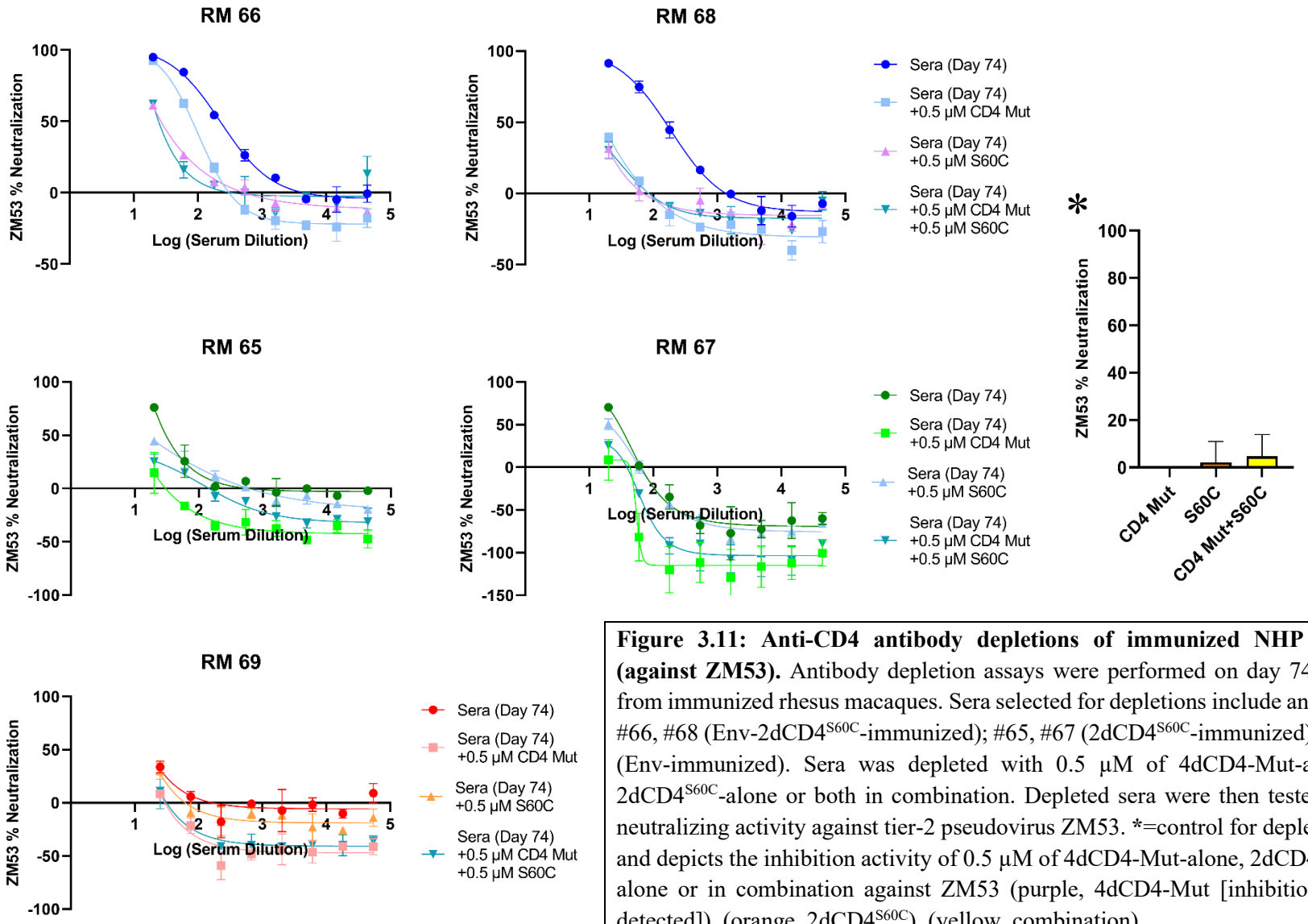


Figure 3.11: Anti-CD4 antibody depletions of immunized NHP sera (against ZM53). Antibody depletion assays were performed on day 74 sera from immunized rhesus macaques. Sera selected for depletions include animals #66, #68 (Env-2dCD4^{S60C}-immunized); #65, #67 (2dCD4^{S60C}-immunized); #69 (Env-immunized). Sera was depleted with 0.5 µM of 4dCD4-Mut-alone, 2dCD4^{S60C}-alone or both in combination. Depleted sera were then tested for neutralizing activity against tier-2 pseudovirus ZM53. *=control for depletions and depicts the inhibition activity of 0.5 µM of 4dCD4-Mut-alone, 2dCD4^{S60C}-alone or in combination against ZM53 (purple, 4dCD4-Mut [inhibition not detected]), (orange, 2dCD4^{S60C}), (yellow, combination).

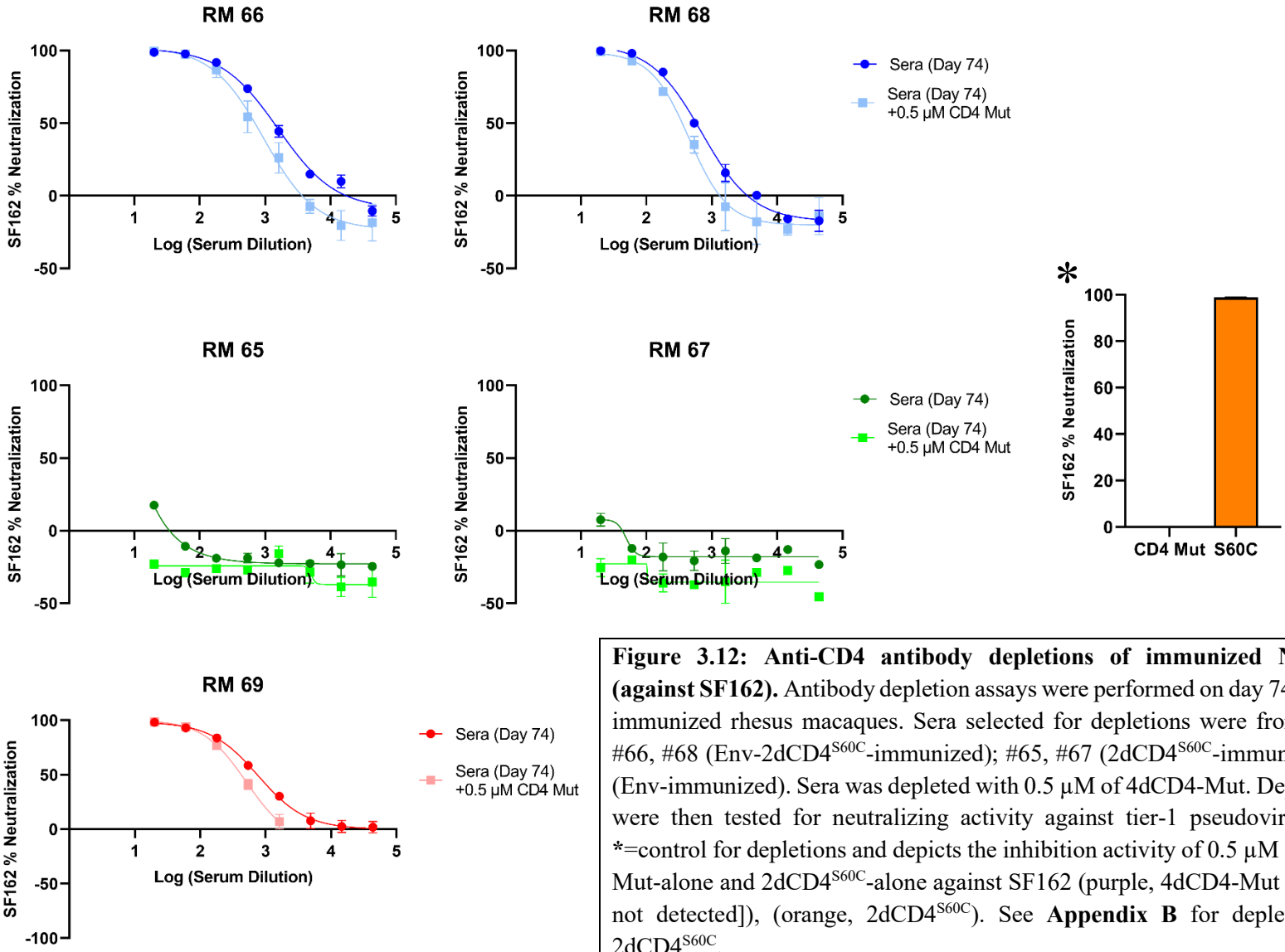


Figure 3.12: Anti-CD4 antibody depletions of immunized NHP sera (against SF162). Antibody depletion assays were performed on day 74 sera from immunized rhesus macaques. Sera selected for depletions were from animals #66, #68 (Env-2dCD4^{S60C}-immunized); #65, #67 (2dCD4^{S60C}-immunized); #69 (Env-immunized). Sera was depleted with 0.5 µM of 4dCD4-Mut. Depleted sera were then tested for neutralizing activity against tier-1 pseudovirus SF162. *=control for depletions and depicts the inhibition activity of 0.5 µM of 4dCD4-Mut-alone and 2dCD4^{S60C}-alone against SF162 (purple, 4dCD4-Mut [inhibition not detected]), (orange, 2dCD4^{S60C}). See **Appendix B** for depletions with 2dCD4^{S60C}.

3.4 NHP intra-rectal SHIV challenges

3.4.1 Data from SHIV challenge in NHPs remains inconclusive

Seven Chinese-origin rhesus macaques were challenged intra-rectally with 5,000 $TCID_{50}$ of SHIV-1157ipd3N4 once weekly over a 10-week period (**Figure 3.13**). Two weeks before commencement of viral challenges the animals received booster immunizations as mentioned previously. Viral loads and CD4+ T-cell counts were monitored weekly (**Figure 3.13**).

Viraemia above the threshold of detection was noted at week 2 in 6/7 of the animals (#66, #68, #65, #67, #69), seven days after the 2nd intra-rectal challenge had been administered (**Figure 3.13**). Viral loads were atypical as compared to previously published work regarding SHIV-1157ipd3N4 challenges in both Indian- and Chinese-origin rhesus macaques with researchers noting mean peak viral loads of 4.05×10^7 and 5.69×10^5 viral RNA copies/ml, respectively. Animal #67 (immunized previously with 2dCD4^{S60C} alone) showed the highest viral load peak at only 648 viral RNA copies/ml (Song et al., 2006, Wang et al., 2014). Following the week 2 peak, viral loads dropped to undetectable levels by week 5 for all the animals that had detectable viraemia previously. A second viral load peak was noted in NHP #67 at week 6 (7 days after 6th intra-rectal challenge), with the gradual increase peaking during week 8 at 58 viral RNA copies/ml and viraemia remaining detectable until end of the study. A second rise was also noted in NHP #69 with detectable viral loads from the 7th week onward, peaking at 85 viral RNA copies/ml by the end of the study. Interestingly, median peak viral loads for the Env-2dCD4^{S60C} immunized group were far lower as compared to the 2dCD4^{S60C}-only immunized group (31 viral RNA copies/ml vs 459 viral RNA copies/ml (**Appendix B: Supplementary figures, Supplementary Figure 8**), with 1/4 of the Env-2dCD4^{S60C} immunized group never developing detectable viraemia above threshold of detection.

Median CD4+ T-cell counts remained in the expected range for a rhesus macaque of between 500-2,500 cells/µl for 6/7 of the animals over the 10-week challenge period, with animal #65 having an atypical median count of 3,860 cells/µl over the challenge period (**Figure 3.13**) (Chege et al., 2021). All animals had CD4+ T-cell counts notably peak at week 3, which was 7 days following the week 2 peak in viraemia detected in 6/7 of the animals. Animals #57, #66, #68, #65 and #69 had peaks at week 3 that exceeded CD4+ T-cell counts expected for the species, with levels returning to more typical ranges the following week. A second peak in CD4+ T-cell counts was observed at week 7, which coincided with a second spike in viraemia detectable in 2/7 animals, but on average was not as high as the peak noted in week 3. The

highest increase at week 7 was observed in animal #66, with CD4+ T-cell counts decreasing back into typical ranges the following weeks.

It is difficult to make any firm conclusions regarding the viral challenge data as viral loads were atypical and far below what has been reported in the literature for a challenge study using this SHIV in Chinese-origin rhesus macaques. While there is evidence of systemic infection due to detection of plasma viral load, this was transient in nature and failed to achieve exponential viral expansion *in vivo* across all seven immunized animals. Although viral loads were lower on average in the Env-2dCD4^{S60C} immunized macaques compared to the 2dCD4^{S60C}- and Env-only groups, it is not definitively clear as to whether the complex immunized rhesus macaques were afforded superior protection from SHIV challenge. The limited numbers of animals, particularly in the control groups (2dCD4^{S60C}- and Env-only groups), may have masked any low-level protective signal. Furthermore, confirmation of systemic infection could not be made as sampling for proviral integration in tissues was not performed.

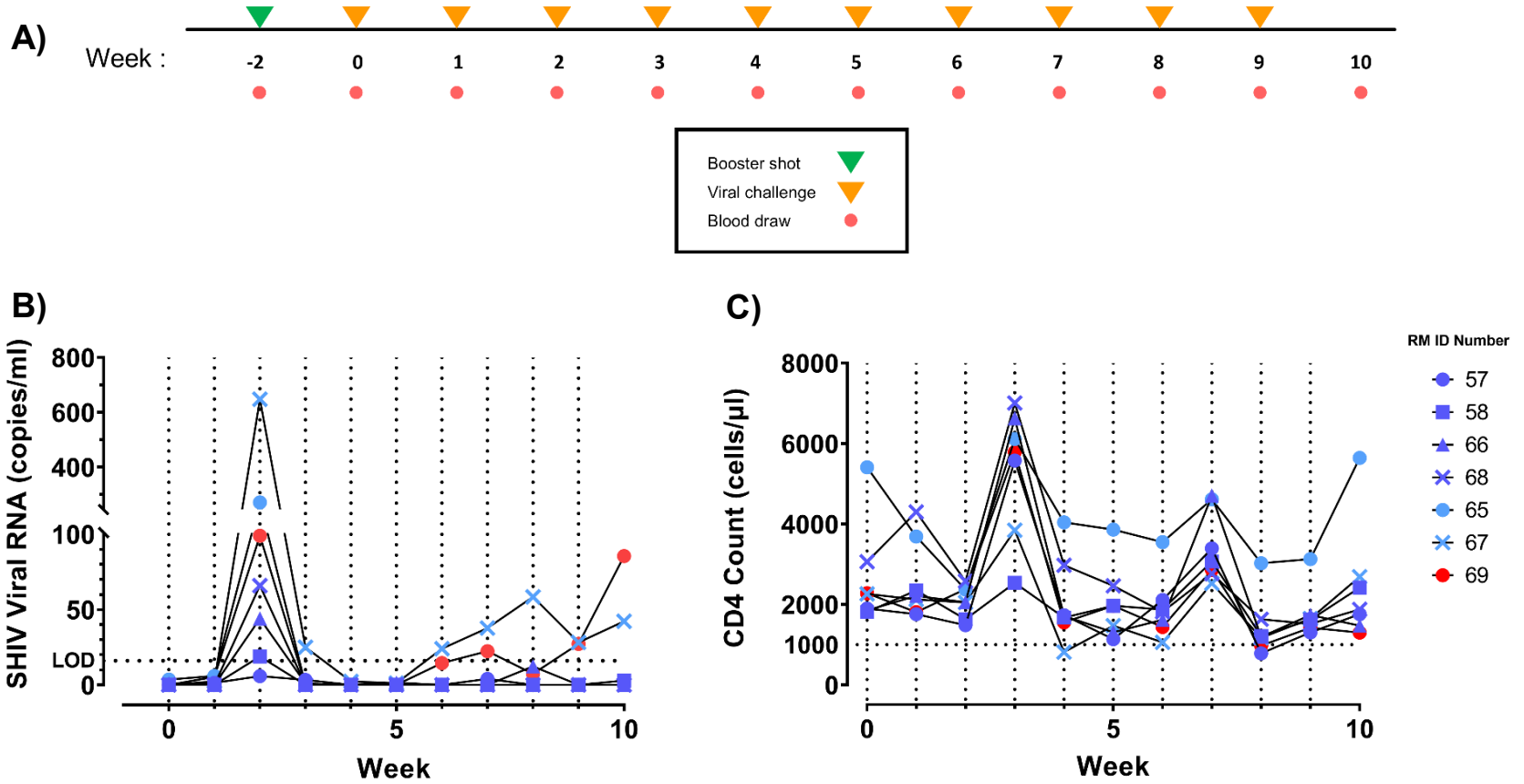
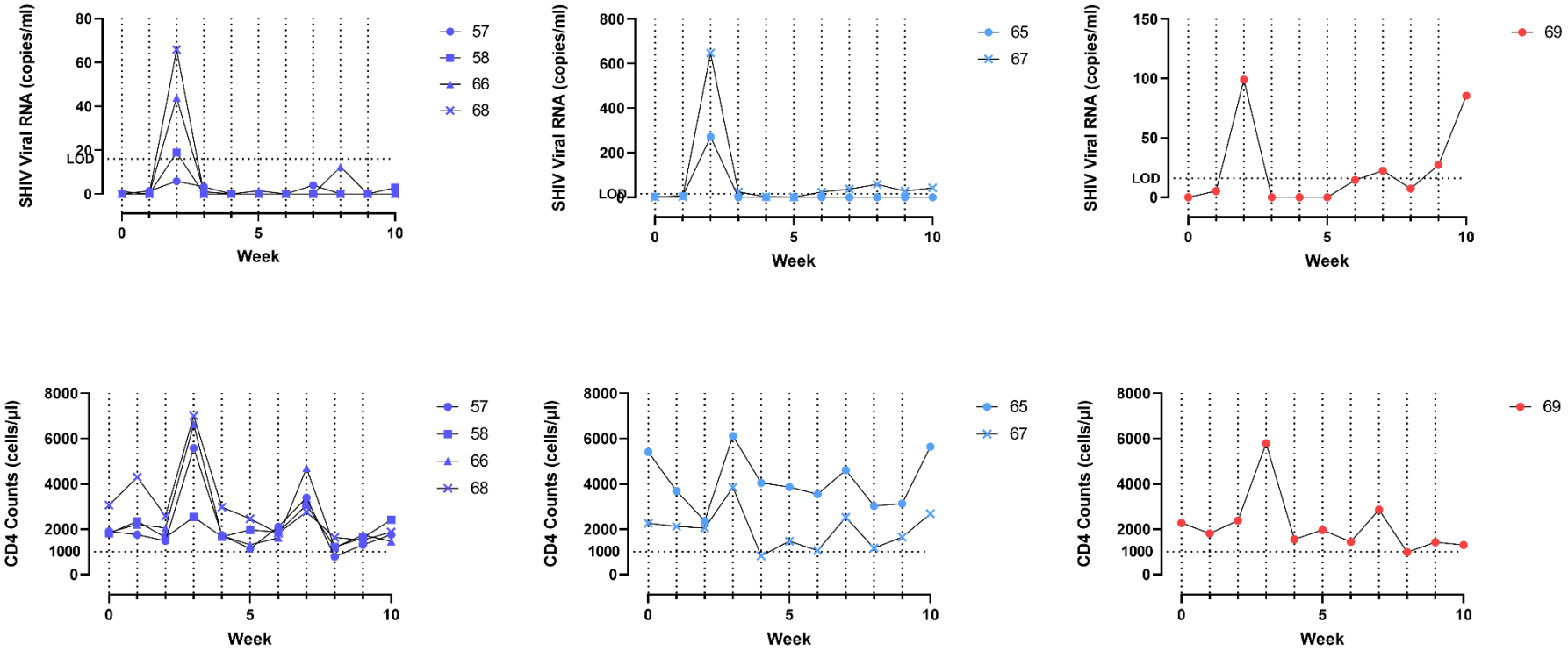


Figure 3.13: SHIV-1157 challenge in NHPs. All previously immunized animals were challenged with the subtype C derived SHIV-1157ipd3N4 with CD4+ T-cell counts and viral loads monitored. **A)** Pre-challenge immunization boosters were administered 2-weeks before commencement of challenge. Weekly SHIV inoculum (5,000 $TCID_{50}$) was administered intra-rectally for a total of 10 weeks. **B)** Peripheral blood viral loads were monitored weekly throughout the duration of the challenge study. **C)** Absolute CD4+ T-cell counts were monitored weekly throughout the duration of the study. Env-2dCD4^{S60C} group (purple, n=4), 2dCD4^{S60C} group (blue, n=2), Env rhesus macaque (red, n=1). Vertical dots indicate administration of viral inoculum. **LOD**=limit of detection (~16 SHIV viral RNA copies/ml). **D)** viral loads and CD4 counts depicted graphically according to immunization groups, on next page.

D)



*** Figure 3.13: continued.**

Chapter 4: Discussion

In 2021, an efficacious HIV-1 vaccine remains elusive. Six HIV-1 vaccine efficacy clinical trials have been fully completed to date with five showing no efficacy (Pitisuttithum et al., 2006, Gray et al., 2011, Buchbinder et al., 2008, Rerks-Ngarm et al., 2009). The RV144 trial showed a modest signal of efficacy indicative that the development of a vaccine to end the global outbreak was not entirely out of reach. Disappointingly, HVTN 702, which looked to reproduce the RV144 trial, albeit with some slight modifications to the Env immunogen subtype and adjuvant, was stopped prematurely due to a lack of efficacy (Gray et al., 2021). The recent failure of HVTN 702 is once again a sobering reminder of the monumental task at hand of developing an HIV-1 vaccine. It is still unknown as to whether vaccination against HIV-1 infection will be possible.

A neutralizing antibody response will likely be required for sterilizing protection like most other commercially approved vaccines for other viruses (Reviewed in Tomaras and Plotkin, 2017, Plotkin, 2010, Alter and Barouch, 2018). Passive immunization studies including the AMP trial, recent SHIV challenge studies in NHPs and *in vitro* pseudotyped viral assays all point towards neutralizing antibody responses being vital for an HIV-1 vaccine to be efficacious in humans (Schoofs et al., 2016, Caskey et al., 2017, Mendoza et al., 2018, deCamp et al., 2014, Pauthner et al., 2019, Corey et al., 2021). In the last decade, much work has been done to elucidate the processes involved in the evolution of bNAb responses against HIV-1 by the human immune system. From the isolation of rare bNAbs generated naturally in a small percentage of HIV-1 infected patients, to the development of native-like Env glycoproteins, and the unpacking of the immunological responses that take place within germinal centres, a clearer picture is beginning to form as to how protective antibody responses against HIV-1 infection could be generated, though much remains unclear.

In this study, we examined the potential of an Env-CD4-based immunogen to elicit neutralizing antibody responses in Chinese-origin rhesus macaques against clinically relevant HIV-1 viruses *in vitro*. This built on earlier work by our research group, which showed that the Env-2dCD4^{S60C} immunogen complex is unique in its ability to generate potent and broad neutralizing antibody responses in a smaller animal model (Killick et al., 2015, Grant, 2017).

This earlier work did some initial mapping of the target of these neutralizing antibody responses and surprisingly the data was indicative of the responses being anti-CD4 in nature. In the current study, we conducted preliminary mapping of the target of these antibody responses following successful induction of neutralizing antibody responses in the rhesus macaque model using Env-2dCD4^{S60C}. We looked to answer a basic question; are these neutralizing antibody responses once again CD4-directed, or are they targeting regions exposed on the transitionary state of Env following complexing to CD4, in the rhesus macaque animal model? Finally, in the current study, we sought to evaluate whether these *in vitro* neutralizing responses could in fact translate to a viral challenge model and protect our Env-2dCD4^{S60C} vaccinated NHPs against mucosal viral challenges using an infectious tier-2 SHIV.

4.1 gp140$_{FVC}$GCN4t(+) does not elicit tier-2 neutralizing antibody responses on its own

The Env immunogen used in our study was the trimeric gp140 glycoprotein, gp140$_{FVC}$GCN4t(+), based on a consensus sequence of subtype C founder viruses (Killick et al., 2014). The concept of consensus sequence use in HIV-1 vaccine design is certainly not a novel idea, having been investigated for many years now (Gaschen et al., 2002). HIV-1 infection is established with one to several "founder" virus/viruses infecting the susceptible host, and rational vaccine design would entail targeting conserved Env epitopes that are shared between these viruses (Keele et al., 2008). Group M viruses constitutes the bulk of infections worldwide, with subtype C strains dominating in Sub-Saharan Africa and India, therefore the rationale exists to develop immunogens specifically targeting the most widely circulating strains.

The diversity between subtypes is immense and remains a massive hurdle for HIV-1 vaccine development, with up to a third of amino acid sequences differing between subtypes (Gaschen et al., 2002). The current known bNAbs all target the Env glycoprotein spike of HIV-1, but even a small number of mutations in the spike can confer immunological escape (Reviewed in Bonsignori et al., 2017, Dingens et al., 2019). This is seen in the spike proteins of other viruses as well, but with far less diversity occurring between strains than what is seen with HIV-1. A small number of mutations in the influenza spike protein can confer resistance to antibodies and reduce efficacy of yearly vaccines, and most recently mutations in the SARS-CoV-2 spike protein conferring resistance to neutralization by convalescent sera and sera from vaccinees

has been widely publicised during the COVID-19 pandemic (Doud et al., 2018, Wibmer et al., 2021, Wu et al., 2021).

HIV-1 Env immunogens based around consensus sequences indeed show an increased propensity to induce tier-2 neutralizing titres (albeit sporadically), along with improvements seen in cellular immunity, which may be critical for an effective vaccine should the antibody response fail to prevent infection (Liao et al., 2013a, Hulot et al., 2015, Sliepen et al., 2019). Consensus sequence-derived Env proteins also eliminate strain-specific immunological responses that occur due to unique immunodominant epitopes found within specific subtypes, which may be useful in targeting of autologous virus but may hinder the development of broad cross-reactive antibody responses against many viral strains (Sliepen et al., 2019, McCoy et al., 2016).

Despite being based on a consensus sequence, $gp140_{FVC}$GCN4t(+) alone was unable to elicit clinically relevant tier-2 neutralizing titres in a NHP (animal #69). This was expected, as previous work by our research group noted it was a poor immunogen in eliciting tier-2 neutralizing responses when used alone as a vaccine immunogen in rabbits (Killick et al., 2015, Grant, 2017). It is unlikely that the lack of ability of $gp140_{FVC}$GCN4t(+) protein to induce neutralizing antibody responses is due to its *in silico* design, resulting in an Env molecule that is not conformationally intact and is antigenically inert. Previous work by our research group showed that the genetic sequence of Env_{FVC} contained important amino acid residues required for CD4 binding and binding of the CD4bs-targeting bNAbs (Killick et al., 2014). Also, previous and current study work using ELISA showed that $gp140_{FVC}$GCN4t(+) can bind CD4, inducing the CD4i epitope which 17b can bind. $gp140_{FVC}$GCN4t(+) also binds to IgG1b12 in both older work and in the current study (**section 3.1.3**) indicative of a conformationally intact CD4bs (Killick et al., 2015). It is important to note though that in designing of the Env_{FVC}, the V1/V2, V4 and V5 loops were significantly shortened as compared to subtype C founder virus Env sequences that were used to design the Env_{FVC}.

Potent neutralizing responses against tier-1 virus SF162 were generated in NHP #69, as seen previously in rabbits. Immunizations in both animal models and humans with gp120 monomers or non-native gp140 trimers have failed to induce clinically relevant tier-2 neutralizing antibody responses over the years due to exposure of immunodominant, non-neutralizing epitopes and instability of the structures themselves (Flynn et al., 2005, Moore et al., 2006, Li et al., 2006, Kovacs et al., 2012, Hessell et al., 2016, Cosgrove et al., 2016). Indeed it is then

not unexpected that the gp140$_{FVC}$GCN4t(+) trimer fails to elicit any relevant tier-2 neutralizing responses in NHPs, as the immunogen is based on the non-native GCN4 trimer design. We continued to use this Env-immunogen design in the current study though, as previous work by our group showed that the Env-2dCD4^{S60C} complex is effective at eliciting broadly neutralizing antibodies regardless of the type of Env-immunogen that is bound to 2dCD4^{S60C}, whether it is monomeric or trimeric forms of Env used. There may be a slight boost in neutralizing titres though when using a trimeric Env in the immunogen complex, as will be explained later in this discussion (Killick et al., 2015, Grant, 2017).

Since the elucidation of the structure of native-like Env glycoproteins in 2013, the HIV-1 vaccine field has gravitated towards the use of native-like Env trimers due to their structural stability and solubility, and their accurate quaternary structures that engage the major bNAbs (Julien et al., 2013). Despite the structural improvements of Env-based immunogens, typical immunization protocols using whole native-like trimers still routinely fail to elicit tier-2 heterologous neutralizing responses in animal models, with sporadic elicitation of heterologous tier-2 responses occurring in only a handful of animals, or only autologous tier-2 responses occurring in a larger swath of test animals more consistently (Sanders et al., 2015, Pauthner et al., 2017, Torrents de la Peña et al., 2018, Pauthner et al., 2019). Our research group is currently exploring the immunogenicity of Env-2dCD4^{S60C} complexes that use native-like Env trimers.

4.2 Env-2dCD4^{S60C} is superior in its ability to induce a broad and potent neutralizing antibody response

Using the Env glycoprotein alone as an HIV-1 vaccine immunogen has posed a difficulty for researchers. The conserved and vulnerable regions on the Env glycoprotein such as the CD4bs or co-receptor binding site are shielded from the immune system. BNAbs towards such regions develop in a minority of patients after some time has passed during natural infection, with those naturally occurring bNAbs unable to neutralize the autologous viral strains found within the host (Doria-Rose et al., 2009). The strategy employed by our laboratory to overcome such limitations was to stabilize the Env glycoprotein (in this case, gp140$_{FVC}$GCN4t(+)) with the recombinant CD4 protein 2dCD4^{S60C} developed by our laboratory, with the goal of generating neutralizing antibody responses against regions exposed in the transitionary state of the Env glycoprotein. As described earlier (**Figure 1.8**) mutating the serine residue at position 60 of domain 1 of CD4 enhances the overall affinity of the Env-CD4 interaction due to the formation

of a intramolecular disulphide bridge between the cysteine at position 126 of the V1/V2 loop of Env and the introduced cysteine at position 60 of CD4 (Cerutti et al., 2010).

The CD4i conformation of Env is induced when CD4 binds to the CD4bs on gp120. A host of conformational changes occur in the core domains and bridging sheet of the gp120 protomers including the rotation of the gp120 protomers outwards from the trimeric structure, a shifting of the V1V2 loops away from the trimer apex and formation of the four-stranded gp120 bridging sheet (Ozorowski et al., 2017, Wang et al., 2016, Wang et al., 2018). The resultant epitopes exposed have been characterized according to different antibodies that bind each of them with three "clusters" of binding noted (A, B and C) which includes the gp120 face occluded by gp41 in trimeric conformation of Env, a region proximal to the co-receptor binding site with V1/V2 involvement, and the co-receptor binding site itself (Guan et al., 2013). These epitopes are conserved across many HIV-1 subtypes therefore making for a promising vaccine target, though some challenges remain such as access to sterically restricted regions and the transitory exposure of the targeted epitope (Labrijn et al., 2003, Rizzuto et al., 1998, Decker et al., 2005, Mengistu et al., 2015).

Our Env-2dCD4^{S60C} immunogen has consistently generated broad and potent neutralizing antibody responses in rabbits, and now in a NHP animal model in the current study. Though researchers such as Montefiori, Mascola and colleagues have attempted to standardize the HIV-1 pseudoviral neutralization assay, it remains important to be cautious in comparing neutralizing titre data between different research groups (Mascola et al., 2005). With this caveat in mind, the neutralizing responses generated by Env-2dCD4^{S60C} are unlike anything seen in the HIV-1 vaccine literature to date, particularly now that neutralizing responses have been induced in the rhesus macaque model.

Responses induced by our immunogen complex in four of the NHPs (#57, #58, #66 and #68) were potent and broad enough to neutralize the entirety of the tier-2 HIV-1 Env Global Panel during the peak of neutralizing titres, with neutralizing potency remaining until end of the vaccination regimen, albeit diminished (**Table 3.1**) (**Figure 3.8**). Previous work in rabbits by our research group showed potent neutralizing activity against other tier-2 viruses, and rabbit sera independently assessed by Monogram Biosciences Inc. using their in-house neutralization assay showed similar potent neutralizing activity (Killick et al., 2015, Grant, 2017). Furthermore, the NHP immunization data presented here is consistent with our group's previously published findings that a four-dose regimen administered biweekly over a 12-week

period is suitable in eliciting broadly neutralizing responses within different animal models. While the waning in the neutralization responses in the NHPs following the 5th and 6th immunizations is discussed in more detail later, four doses of the Env-2dCD4^{S60C} complex are sufficient and further boosting immunizations provide little benefit in broadening the response.

Stabilizing the Env glycoprotein in a transition state for use as a vaccine immunogen has been employed by various research groups, showing promising data from immunogenicity testing and NHP viral challenge work. Examples include the use of various CD4 mimetics such as the well-described M64U1 (Shen et al., 2017, Dey et al., 2012, Jette et al., 2021) or chemical crosslinking of Env to CD4 (Fouts et al., 2002, DeVico et al., 2007). One such strategy that has been under development by Fouts, DeVico and colleagues for many years is the full-length single chain (FLSC) (Fouts et al., 2000, DeVico et al., 2007, Thomas et al., 2014, Fouts et al., 2015, Schwartz et al., 2016, Sui et al., 2019). Much like the Env-2dCD4^{S60C} complex, the FLSC stabilizes gp120 in a transition state using the first two domains of CD4. In the case of FLSC, CD4 and gp120 are expressed in a single polypeptide chain with a linker sequence between the Env (gp120BaL) and CD4 (domain 1 and 2) components. Immunizations in NHPs with these FLSC proteins have resulted in broad neutralizing responses against pseudotyped viruses *in vitro*, an accelerated decline and subsequent clearance of viraemia in the tissues of animals infected with a tier-2 SHIV, and it has been shown to protect against infection during intra-rectal viral challenges with a SHIV or SIV (Fouts et al., 2015, DeVico et al., 2007). The immunogen has moved into clinical trials and was shown to be immunogenic in humans in a phase 1a trial (Chua et al., 2021). As expected, CD4i antibody responses have consistently been shown to be responsible for the neutralizing and protective effects noted in these studies.

Despite the similarities between FLSC and our own Env-2dCD4^{S60C}, there is a divergence in the target of the antibody responses generated by either immunogen. Interestingly, and surprisingly to our research group, it has become clear that the CD4 component of Env-2dCD4^{S60C} is likely responsible for the bulk of the neutralizing potency and breadth induced in both rabbits and in the current study using NHPs. In particular, the use of 2dCD4^{S60C} in complex with gp120 or trimeric Env seems to be a key requirement for consistently inducing these unique responses. Regardless of the form of Env glycoprotein used (monomeric or trimeric), or the subtype of the Env molecule (subtype B or C), neutralizing antibody responses have been consistently induced in rabbits, and now in NHPs (Killick et al., 2015, Grant, 2017).

4.3 Preliminary antibody mapping experiments reaffirm the contribution of anti-CD4 in the observed neutralizing responses

We attempted to map the target of the neutralizing antibody responses in the Env-2dCD4^{S60C} immunized NHP sera by depleting the sera of CD4-directed antibody responses using various forms of recombinant CD4 proteins (**Figure 3.11**) (**Figure 3.12**). A clear drop in neutralizing potency against the tier-2 virus ZM53 was noted in these assays after depleting with CD4 proteins, particularly when using 2dCD4^{S60C}. These results are comparable to previous work done by our laboratory using CD4- or Env-coated magnetic beads to deplete rabbit sera. In the current study, some neutralizing activity remained against ZM53 following anti-CD4 antibody depletion using 2dCD4^{S60C}, but only at the lowest dilutions of sera. When sera depleted with 4dCD4-Mut were tested against tier-1 virus SF162 (both Env immunized and complex immunized sera), no large reduction in neutralization titres was noted. We were able to show in this study indirectly that the tier-2 neutralizing responses induced by the complex immunogen are likely CD4-directed, whereas the remaining anti-Env responses following CD4 depletion are only capable of neutralizing tier-1 pseudoviruses. This is further implied by the neutralizing titres generated by gp140$_{FVC}$GCN4t(+) alone, 2dCD4^{S60C} alone or the Env-2dCD4^{S60C} complex. The Env-only immunized NHP (#69) generated neutralizing titres against tier-1 virus SF162 throughout the vaccine regimen and after a booster immunization (though titres never recovered to previous levels) before commencement of SHIV challenges. The CD4-only immunized NHPs (#65 and #69) generated low-level neutralizing titres against tier-2 viruses but generated no neutralizing titres against the tier-1 virus SF162. The complex immunized animals (#57, #58, #66 and #68) generated neutralizing titres against all the viruses tested against, regardless of tier-level. Though not completely conclusive, there is a strong implication that anti-CD4 antibody responses are responsible for most of the tier-2 neutralization potency of the sera from Env-2dCD4^{S60C} immunized NHPs.

We did not deplete the NHP sera of Env-directed responses, so it is difficult to make a firm conclusion on the role that Env-directed antibodies have in the complexed immunized NHP sera, but some indirect evidence already mentioned, and previous depletion work done by our research group on vaccinated rabbit sera implies a far smaller effect for Env-directed antibody responses (Killick et al., 2015) (unpublished work from our laboratory). Though not directly measured in this study, there is the implication that CD4i responses are not responsible for the neutralizing potency noted against the tier-2 viruses, at least not at the level that would account for the bulk of the neutralizing effect. Depleting the sera of NHPs vaccinated with Env-

2dCD4^{S60C} of anti-CD4 antibodies almost fully abrogated the neutralizing potency of the sera against the tier-2 virus ZM53, with neutralizing potency maintained against tier-1 virus SF162, implying that if CD4i antibodies are induced they are likely only effective against more easily neutralized tier-1 viruses, and/or not potent or present in sufficient concentrations to neutralize tier-2 viruses. A further point is that if CD4i antibodies were indeed responsible for the bulk of the tier-2 neutralizing activity noted, the addition of 2dCD4^{S60C} to the competition neutralization assays used in this study would likely have enhanced the neutralizing activity against the tier-2 virus ZM53, and not decreased it as noted. 2dCD4^{S60C} binding to ZM53 in the neutralization assay would have exposed the CD4i moieties of the Env spikes of the ZM53 virions, potentially enhancing the neutralizing effect of the sera in the assay further.

If anti-CD4 responses are indeed responsible for the broadly neutralizing activity observed in the Env-2dCD4^{S60C} complex immunized animals, then this raises the critical question as to why similar neutralizing activity was not observed in the 2dCD4^{S60C}-only immunized group animals? Immunizing NHPs (#65 and #67) with 2dCD4^{S60C} alone generated low-level neutralizing responses against the HIV-1 Env Global Panel despite high anti-CD4 antibody binding titres (**Figure 3.7**) (**Table 3.1**). This contrasts with the observed broadly neutralizing antibody responses elicited by the Env-2dCD4^{S60C} immunogen even though anti-CD4 antibody binding titres are 10-fold less than the 2dCD4^{S60C}-only immunized group. Though not tested in the current study, previous work established that any of monomeric gp120 or trimeric forms of the Env glycoprotein spike bound to 2dCD4^{S60C} can induce broad and potent neutralizing responses, though the Env trimer bound to 2dCD4^{S60C} seems to generate slightly higher neutralizing titres, at least in rabbits. A few hypotheses may explain such discrepancies.

One possible reason for the increase in total anti-CD4 antibody titres noted in the 2dCD4^{S60C} immunized NHPs versus the ones that received Env-2dCD4^{S60C} is the amount of total CD4 protein administered per immunization. Each animal received 100 µg total of Env, 2dCD4^{S60C} or Env-2dCD4^{S60C} complex per immunization. Although total concentrations of protein administered are the same it does not account for the molar quantities of each protein that would be received by each vaccination group, with the 2dCD4^{S60C} animals receiving far more CD4 protein molecules per vaccine dose versus the complex group.

As for why 2dCD4^{S60C} administered alone generates low-level neutralizing titres, whilst the Env-2dCD4^{S60C} complex generates highly potent neutralizing responses, it can perhaps be explained by a stabilizing effect of Env on CD4, or a selection bias of certain redox states of

the 2dCD4^{S60C} protein. Our laboratory has done extensive research on the redox states of CD4 and its implications on binding to gp120. We previously showed that gp120 binding is influenced by the oxidative state of monomeric 2dCD4, and the disulphide bonds in domains 1 and 2 can result in metastability of the surrounding regions resulting in overall structural changes (Cerutti et al., 2014, Owen et al., 2016, Owen et al., 2018). The 2dCD4^{S60C} protein (and 2dCD4-WT) has been shown to occur in several isoforms due to different oxidative states, depending on which cysteine pairs in domains 1 and 2 form disulphide bonds, which is proposed to affect the folding of the protein (Cerutti et al., 2010). During complex formation, Env "selects" for the relevant 2dCD4^{S60C} isoforms with the correct disulphide pairing in domains 1 and 2 required for gp120 binding/engagement. Selection and stabilization of this "relevant" 2dCD4^{S60C} isoform within the immunogen complex allows for the exposure of crucial epitopes and the development of neutralizing antibody responses in rabbits, and now in NHPs.

As part of domain 1 of CD4 remains buried in the CD4bs when bound to Env, regions exposed on the rest of domain 1 and the entirety of domain 2 are more likely to be accessible (Kwong et al., 1998, Wang et al., 2016). There may be an "immuno-focusing" effect at play which further aids the increase of neutralizing antibody responses directed towards 2dCD4^{S60C} when complexed to gp120 and may be further enhanced when using trimeric forms of Env. Domain 1 of 2dCD4^{S60C} binds to the CD4bs which can occlude part of domain 1, resulting primarily in domain 2 being exposed and theoretically "focusing" the immune responses towards this region. This may be enhanced further with a single Env glycoprotein trimer being able to bind and present three 2dCD4^{S60C} molecules at once. When the complex immunogen is generated under reducing conditions, excess 2dCD4^{S60C} is added to the reaction in an attempt to bind every free CD4bs on the gp140$_{FVC}$GCN4t(+) trimer. Non-neutralizing epitopes that may be immunodominant on gp120 alone, may be hidden in the trimeric conformation as well, further focusing an immune response towards the attached CD4 molecules (Liu et al., 2008, Wang et al., 2016, Wang et al., 2018, Yang et al., 2019). The role of redox states of CD4 and targeting of certain regions of bound versus unbound CD4 are merely speculative at this stage and will require further work to elucidate the role played by any of the above, if any.

In contrast to our work, it has been consistently shown that the protective immune responses elicited by FLSC are CD4i directed. Recent work found that the FLSC does not elicit anti-CD4 antibody responses when using a CD4 sequence that is autologous to the vaccine recipient (Schwartz et al., 2016). In the mentioned study, cynomolgus macaques were immunised with

FLSC that used the rhesus macaque 2dCD4 sequence which is identical to that of cynomolgus macaques. When vaccinated with FLSC carrying the human 2dCD4 sequence (88% identical) an elevated anti-CD4 immune response was noted by ELISA as compared to animals that received the autologous sequence. When human or rhesus FLSC vaccinated sera was analysed for binding to primary CD3+ T-cells by flow cytometry, none was detected. This perhaps partially explains the immune responses generated by the NHPs in our study when vaccinated only with 2dCD4^{S60C} or Env-2dCD4^{S60C}, which saw high anti-CD4 antibody binding titres generated, and low- to high-levels of neutralizing titres induced. We did not however check for binding of our vaccinated sera to primary rhesus macaque T-cells. Interestingly and in contrast to the above, older studies using whole soluble human CD4 as an immunogen in rhesus macaques and chimpanzees generated antibodies that were cross reactive with macaque cells, or digitonin-treated chimpanzee lymphocytes (Watanabe et al., 1991a, Watanabe et al., 1992). Further, immunizing rhesus macaques with soluble rhesus macaque CD4 developed autologous anti-soluble rhesus macaque CD4 antibody responses in these animals (Watanabe et al., 1992). It would have been illuminating had we incorporated a rhesus macaque CD4 sequence into the current study and it remains important to explore in future work.

Although the Env-2dCD4^{S60C} immunogen induces potent and broad antibody responses not seen in the literature to date, it is not necessarily the first of an Env-CD4 immunogen strategy to induce anti-CD4 antibody responses that may be neutralizing against HIV-1. Two studies by DeVico et al. and Varadarajan et al. are such examples (Devico et al., 1996, Varadarajan et al., 2005). Both showed evidence of anti-CD4 antibody responses being partially responsible for antiviral activity detected in either study, respectively. DeVico and colleagues immunized a goat with a covalently crosslinked gp120-CD4 immunogen which induced antibody responses capable of neutralizing primary HIV-1 isolates *in vitro.* They found that the antibody responses elicited were polyclonal in nature, targeting gp120, CD4 and complex-specific moieties. Using CD4+ SupT1 cells to deplete vaccinated goat serum of cell-surface CD4-directed responses, they noted a decrease in cell surface reactivity with SupT1 cells, with a sera dilution of 1:20 resulting in a detection signal that was 16% of sera that was not depleted. A drop in neutralizing titres against primary HIV-1 was noted as well. It was speculated that the goat sera contained two populations of neutralizing antibodies, with the CD4-directed responses likely responsible for preventing viral syncytia formation. Varadarajan and colleagues used DNA or DNA-protein boost vaccine regimens to vaccinate guinea pigs with a two-domain CD4 based immunogen

gp120-CD4$_{D1D2}$ single chain. The gp120-CD4$_{D1D2}$ single chain elicited neutralizing antibody responses that could be depleted from sera using two-domain CD4 immobilized on a column.

4.4 Env-2dCD4^{S60C}, autoimmunity and CD4-targeting as an HIV-1 vaccine strategy

If the neutralizing antibody responses induced in our NHPs are indeed anti-CD4 in nature, it raises an important concern regarding deleterious autoimmunity that could occur in the host. The current study was not designed to detect immunotoxic effects of the Env-2dCD4^{S60C} immunogen. There were no aberrations detected in CD4+ T-cell data collected before (2 years post-vaccine regimen, 2 weeks post-booster vaccinations) commencement of SHIV intra-rectal challenges in the current study, with CD4+ T-cell counts within normal ranges for the species (Chege et al., 2021). We would be remiss though in making any firm conclusions from such data on any deleterious autoimmune effects of the CD4-based immunogens administered to the NHPs. There is a plethora of related research though that can inform on the possible safety and rationale of using such vaccine strategies.

The use of CD4 as both an HIV-1 therapeutic and vaccine immunogen has been studied in some detail. Studies by Watanabe and colleagues explored the use of soluble CD4 as an immunogen and found immunizations in rhesus macaques with whole soluble human CD4 generated anti-CD4 antibody responses that prevented infection of macaque lymphocytes and macrophages with SIV *in vitro*, with no immune dysregulation detected (Watanabe et al., 1991a, Watanabe et al., 1991b). Similar effects were noted when chimpanzees were immunized, and when autologous soluble CD4 was administered to rhesus macaques (Watanabe et al., 1992). Much of this research seemingly ends in the 1990s and the particulars of such responses were never explored further. Two phase I clinical trials from the same period found that low and high dose infusions of soluble CD4 over 10 weeks in HIV-1 positive individuals had a favourable safety profile with no notable immunological aberrations detected, such as drops in CD4+ T-cell counts (Schacker et al., 1995).

During natural HIV-1 infection, a small percentage of patients develop detectable anti-CD4 antibody responses (~10%), with some data suggesting that such antibody responses are detectable in HIV-1 infected patients even before HIV-1 seroconversion occurs (Kowalski et al., 1989, Celada et al., 1990, Keiser et al., 1992). The data also suggests that these responses are likely directed to domains 3 and 4 which are further away from the gp120-binding site located in domain 1 of CD4, with anti-CD4 antibodies generated during natural infection able

to bind recombinant CD4 proteins, but unable to bind cellular-bound CD4 (Kowalski et al., 1989, Moore et al., 1990, Denisova et al., 2003). More recent studies have found a deleterious association between naturally occurring anti-CD4 antibodies during HIV-1 infection in patients who are virally supressed but fail to recover their CD4+ T-cell counts (Luo et al., 2017, Jiang et al., 2018). Currently, it remains inconclusive as to the effect that anti-CD4 antibodies play in both natural infection and in vaccine-induced settings.

It is perhaps serendipitous that the Env-2dCD4^{S60C} immunogen induces anti-CD4 antibodies with broad and potent neutralizing ability. Targeting the CD4 protein to treat or prevent HIV-1 infection has gained traction in recent years with the approval of ibalizumab, the first and only monoclonal antibody approved to date for the treatment of HIV-1, currently used in patients failing ARV therapy and suffering with multidrug resistant HIV-1 (Jacobson et al., 2009, Emu et al., 2018, Markham, 2018, Gathe et al., 2021). A highly potent entry-inhibitor, ibalizumab exhibits an excellent safety profile with no immunosuppressive activity detected in humans. It is important to point out that ibalizumab was developed by humanizing a murine monoclonal antibody and switching the subclass to that of IgG4 (which has a lower likelihood of inducing an ADCC response when bound to CD4+ T-cells); these factors improve the safety profile of the antibody and any anti-CD4 antibody induced *in vivo* by immunization would be different in this regard (Reimann et al., 1997). A different anti-CD4 antibody, UB-421, was recently evaluated in a small phase 2 clinical trial and showed some promising results, maintaining suppressed HIV-1 viral loads in patients in the absence of ARV therapy for up to 16 weeks (Wang et al., 2019). The researchers did detect a concerning decrease in regulatory CD4+ T-cells during the study period, though the clinical significance was unclear.

The Env-2dCD4^{S60C} immunogen may very well induce an "ibalizumab-like" response in both rabbits and NHPs, with antibodies targeting CD4 receptors on the TZM-bl cell line that our laboratory uses for pseudoviral neutralization assays, preventing infection of the reporter cell line. Depletion work in the current study was unable to fully deplete the neutralizing effect of the NHP sera at its highest concentrations. This may imply a small role for anti-Env antibody responses, or we were unable to deplete antibodies that target epitopes that overlap both Env and CD4 components of Env-2dCD4^{S60C}. A crystal structure of a subtype C gp120 bound to soluble CD4 and the Fab region of CD4i antibody 21c revealed the antibody to be bimolecular, binding regions on both gp120 and CD4 (Diskin et al., 2010). This is not surprising as various HIV-1 bNAbs exhibit autoreactivity (Mouquet et al., 2010, Verkoczy et al., 2013, Liu et al., 2015, Finney and Kelsoe, 2018). Interestingly, sera from all our NHPs immunized with the

Env-2dCD4^{S60C} immunogen were unable to neutralize subtype G pseudovirus X1632 of the Env Global Panel with high potency. This virus was noted to be highly resistant to neutralization by ibalizumab in previous work done by our laboratory, perhaps further pointing towards a link between anti-CD4 responses induced by the immunogen complex and its relation to an "ibalizumab-like" response (Moshoette et al., 2019).

It remains unclear in the literature as to whether a vaccine immunogen could consistently and safely induce an anti-CD4 response that is "ibalizumab-like" and which could remain robust and protective over a clinically relevant time frame. Celada and colleagues were able to induce anti-CD4 antibody responses in mice using a rgp120-CD4 immunogen, with the sera able to prevent syncytia formation, with a particular monoclonal antibody isolated from sera able to prevent syncytia formation but not prevent gp120 binding to CD4 (Celada et al., 1990). Although ibalizumab was originally derived from mouse monoclonal antibody 5A8 after induction by vaccination in BALB/c mice, the vaccination protocol used was vastly different as compared to immunizing with an Env-CD4-based immunogen (Burkly et al., 1992).

It is important to note that neutralizing responses for the NHPs in the current study peaked at day 74, following four immunizations (**Table 3.1**) (**Figure 3.8**). An additional two immunizations did not increase the potency of the elicited responses further nor was an improvement in breadth against resistant strain X1632 of the Env Global Panel noted, with neutralizing titres waning following these final two immunizations. Whether these responses would have declined regardless of the final two immunizations is unknown, but it does raise the concern that if these neutralizing antibody responses are anti-CD4 in nature, there may be immune tolerance mechanisms at work which would select out B-cells over time which have a propensity for targeting self-antigens. Though in the context of anti-Env directed responses, issues with autoreactivity have been one of the noted roadblocks for developing bNAbs against the HIV-1 Env spike (particularly MPER-targeting bNAbs), with B-cells that are autoreactive occurring rarely in healthy humans (Mouquet et al., 2010, Liu et al., 2015, Verkoczy et al., 2013).

4.5 Results of Intra-rectal viral challenges with SHIV-1157ipd3N4 remain inconclusive

Rhesus macaques remain the standard animal model for HIV-1 vaccine research. Though it is unknown as to whether the model is an accurate surrogate for testing potential HIV-1 vaccines

as no successful vaccine has been designed to date, the model remains an important tool for immunogenicity and efficacy testing of potential HIV-1 vaccine candidates.

Following promising immunization results in our NHPs, the animals were tested for protection against infection from a SHIV primary isolate challenge. There was a two-year gap between end of the initial vaccine regimen and commencement of the viral challenge. We assessed the longevity of the neutralizing antibody responses generated during the initial vaccine regimen. As expected, neutralizing responses fell in tandem with total antibody binding titres for Env and CD4 (**Figure 3.7** and **Figure 3.9**). It was encouraging that a single booster immunization was able to return neutralizing titres to similar levels noted previously within two weeks before commencement of challenge.

The virus used to challenge the NHPs was SHIV-1157ipd3N4, a R5-tropic virus that encodes an HIV-1 subtype C Env glycoprotein spike derived from a founder virus isolated from a Zambian child (Song et al., 2006, Wang et al., 2014). Importantly, this virus has been shown to replicate vigorously in both Indian and Chinese-origin rhesus macaques, hence one of the reasons this strain was chosen for viral inoculations. The virus is also categorized as tier-2, therefore it would prove to be a clinically relevant virus in the current study (Siddappa et al., 2010). The NHPs used in this study were Chinese-origin rhesus macaques which have been shown to have different SIV/SHIV disease progression vs their Indian-origin counterparts (Reviewed in Zhou et al., 2013). SIV/SHIV viral loads in Chinese-origin rhesus macaques are typically lower compared to Indian-origin rhesus macaques. In addition, slower and reduced rates of lymphocyte depletion are noted and are more comparable to that seen in HIV-1 infected humans who eventually develop AIDS. Despite the bulk of HIV-1 vaccine work using Indian-origin rhesus macaques, the Chinese-origin counterparts may in fact more accurately resemble the viral kinetics of HIV-1 in humans (Veazey and Ling, 2017, Ling et al., 2002, Trichel et al., 2002, Reviewed in Zhou et al., 2013).

Before commencement of challenges, the neutralizing potency of the immunized NHP sera was tested against the challenge SHIV stock. A favourable neutralizing profile was detected against SHIV-1157ipd3N4 with 3/4 of the Env-2dCD4^{S60C} animals able to neutralize the SHIV with titres above 1:100 following booster immunizations. The correlates of protection for a protective HIV-1 vaccine are currently unknown, therefore, it is difficult to gauge as to whether a certain neutralizing ID_{50} titre would be protective in a viral challenge study. A balance needs to be struck between providing an adequate and clinically translatable challenge inoculum

within the NHP model whilst not unrealistically overwhelming the immune system and masking any beneficial protection that a potential working vaccine may have induced. Pauthner and colleagues recently found that an *in vitro* neutralizing ID_{50} titre of ~1:500 against the autologous pseudovirus (~1:30 against the Env-matched challenge virus), following immunizations with native-like Env trimers based on the SOSIP design, corresponded with 90% protection against a medium-dosage intra-rectal autologous SHIV viral challenge in NHPs (Pauthner et al., 2019). In this study a challenge dosage consisted of 1.4×10^7 SHIV-BG505 virions which approximated to an animal infectious dose of 75% i.e. 75% of animals become infected after a single challenge. They were able to establish a rough benchmark for a protective ID_{50} titre for NHP SHIV challenges within their study and provide a benchmark for similar studies in the HIV-1 vaccine field. It is difficult to compare the titres from the above study with the titres obtained in our own study though; our NHPs were not immunized with the same immunogens, we suspect differences in the targeted neutralizing responses (CD4-directed versus Env-directed), the SHIV strain is different and our NHPs were not challenged with a virus matched for the same Env trimer that they were immunized with (autologous versus heterologous strains).

Disappointingly, the 10-week viral challenge conducted by our laboratory yielded atypical results with which we could not make any further determinants regarding any potential protective effect of the Env-2dCD4^{S60C} immunogen in NHPs (**Figure 3.13**). When comparing the weekly viral titres and peak viral loads of the NHPs, with titres obtained by other research groups testing SHIV-1157ipd3N4 in Chinese-origin rhesus macaques, our peak viral loads were far lower, by at least ~1,000 to ~10,000-fold (Song et al., 2006, Wang et al., 2014). We did not sample tissues for proviral DNA and subsequent detection of a latent viral reservoir; therefore, it was difficult to assess which of the animals became systemically infected. However, the detection of low-level viral loads within plasma samples of all the NHPs suggest some of the animals may have been infected.

NHPs in the current study were genotyped for restrictive *Mamu* and *TRIM5α* genotypes that can influence SIV/SHIV challenge outcomes (Letvin et al., 2011, Lim et al., 2010b, Lim et al., 2010a). Only animal #69 presented with a restrictive *Mamu-A* allele (**Appendix B: Supplementary figures, Supplementary Figure 6**). All animals had restrictive *TRIM5α* genotypes (heterozygous or homozygous restrictive) that had the potential to contribute to the observed atypical viral loads noted in this study. It is unlikely though that these genetic factors played a part in the low-level viraemia noted in the current study. Restriction of viral replication

has been studied extensively in the Indian-origin rhesus macaque model with less genotype data being available for Chinese-origin rhesus macaques. Chinese-origin rhesus macaques presenting with similar restrictive *Mamu* haplotypes as seen in Indian-origin rhesus macaques may restrict virus replication if infected, though previous data from other groups remains inconclusive (Wambua et al., 2011). Importantly, the SHIV-1157ipd3N4 virus used in this study was originally generated using a SIVmac239 backbone, with this macaque-adapted virus shown to be resistant to the restrictive effects of TRIM5α, therefore it is unlikely that restrictive *TRIM5α* genotypes contributed to the atypical viral loads noted in this study (Kirmaier et al., 2010, Fenizia et al., 2011, Eudailey et al., 2018).

A further limitation was the number of animals we had access to in this study. We were only able to source seven animals for use in the current study. Though useful for immunogenicity testing, it becomes difficult to assess vaccine efficacy with an underpowered group. Repeated low-dosage challenges such as we attempted in this study can increase the statistical power required to detect a true vaccine effect with less animals; had we access to ten animals, two test groups of five animals each would have achieved statistical power of 95% to detect a vaccine effectiveness of 90% (Regoes et al., 2005). Testing of the Env-2dCD4^{S60C} immunogen using a larger number of NHPs to allow for adequate statistical power remains a priority moving forward. Additionally, a change in viral stock or use of a different viral strain will likely yield improved results in future challenge studies.

Chapter 5: Conclusion

The world continues to grapple with the devastating HIV-1 outbreak that began its relentless worldwide spread four decades ago. The progress in development of ARV therapies and pre-exposure prophylactics have been a boon in the fight against HIV-1. ARV programs around the world, particularly in South Africa, have had significant impacts on the spread of the virus whilst improving the quality of life of those living with the disease. Worryingly though, this will not be enough to end the global pandemic, with over 1.5 million infections still taking place per year. The development of an efficacious HIV-1 vaccine is needed now more than ever.

In the current study, the groundwork was laid for the continued study and development of the Env-2dCD4^{S60C} immunogen complex. Once again, the immunogen was able to elicit broad and potent neutralizing antibody responses, this time in the NHP model, and is unlike anything noted in the HIV-1 vaccine literature to date. Although an imperfect model, rhesus macaques remain vital for the study and development of new HIV-1 therapeutics and potential vaccine candidates.

Immunizations with native-like Env glycoprotein trimers in animal models have yielded lackluster and sporadic clinically relevant neutralizing antibody responses to date, with researchers having to devise novel ways to engage the B-cell lineages and UCAs likely required to develop bNAbs that can effectively protect individuals from infection. The development of a working HIV-1 vaccine will require an approach not seen before for other infectious diseases.

Our Env-2dCD4^{S60C} immunogen complex was initially developed with the goal of eliciting neutralizing CD4i antibody responses. Data generated in the current study in NHPs and in previous work in rabbits has continually pointed towards anti-CD4 antibody responses being responsible for the bulk of neutralization noted in sera from these immunized animals. This will require continued study as to the feasibility of such a strategy for an HIV-1 vaccine design, with the potential for deleterious autoimmunity a theoretical possibility. The approval of the safe and highly potent anti-CD4 antibody ibalizumab in recent years has added credence to the idea of targeting the host receptor to prevent HIV-1 infection.

Future research will need to interrogate the nature of the antibody responses that are occurring in these vaccinated animals with work already underway to isolate and characterize the neutralizing antibodies generated by the Env-2dCD4^{S60C} complex (unpublished work). The type of Env glycoprotein complexed with 2dCD4^{S60C} has been shown not to impact the neutralizing responses generated, though trimeric forms seem to slightly improve titres; our research team is already in the process of elucidating if use of native-like Env trimers in complex with 2dCD4^{S60C} provide any further improvements in neutralizing titres (unpublished work). Further testing of the potential efficacy of the Env-2dCD4^{S60C} complex in NHPs will be required, particularly with a modified SHIV challenge model using a larger and more statistically powered group of animals; this can then decisively inform as to the potential of the immunogen to eventually move into early-stage human trials.

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Appendix A: Expression and purification of recombinant CD4 proteins for anti-CD4 antibody depletion work

Subcloning of 4dCD4-WT and 4dCD4-Mut constructs

Plasmid constructs containing mammalian, codon-optimized 4dCD4-WT and 4dCD4-Mut sequences were obtained from GeneArt (**Supplementary Figure 1**). The F43A/R59A mutant in 4dCD4-Mut replaces the phenylalanine (F) and arginine (R) residues at their respective positions, eliminating binding to HIV-1 gp120. This eliminates the inhibitory effects observed in HIV-1 pseudoviral assays when using 2dCD4-WT, 2dCD4^{S60C}, and 4dCD4-WT. The 4dCD4-WT and 4dCD4-Mut constructs contained an N-terminal, membrane-localization signal that in the absence of the CD4 transmembrane and cytoplasmic region, allows for efficient secretion of the expressed proteins. Using the 4dCD4-WT and 4dCD4-Mut constructs available, primers were designed to amplify the corresponding 4dCD4 (~1200bp) amplicons. The forward primer incorporated an XbaI restriction site, while the reverse primer included 6×histidine residues that would be incorporated in frame on the C-terminus of 4dCD4 proteins, followed by a stop codon and NotI restriction site. Amplicons were amplified by standard PCR using Q5 High-Fidelity 2×Master Mix (New England BioLabs) using the manufacturer's recommended 50 µl reaction volumes. Amplicons were resolved on a 0.8% agarose gel and visualized using Ethidium Bromide/UV light staining (data not shown).

Amplicons corresponding to the correct size of ~1200 bp were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Subcloning of the amplicons into the mammalian protein expression vector pCI-neo (Promega), were carried out by directional restriction site cloning using the XbaI and NotI sites, respectively. The pCI-neo backbone and 4dCD4 amplicons (both WT and Mut) were digested using the XbaI and NotI FastDigest enzymes (Thermo Scientific) according to the manufacturer's protocol. Digested products were resolved on 0.8% agarose gel, corresponding bands were excised and purified as previously described above. Ligation reactions were performed using a 3:1 ratio of amplicon (insert) to backbone (combined final DNA 100 ng) and ligated using the T4 DNA ligase (New England BioLabs). Ligation reactions were transformed into DH5α *E. coli* and cultured on ampicillin-positive LB agar plates. Positive colonies were screened using XbaI and NotI double restriction digests (**Supplementary Figure 2**). Successful cloning was confirmed by restriction digest using XbaI and NotI. Positive clones contained the appropriately sized insert, ~1200 bp

for 4dCD4-WT (data not shown) and 4dCD4-Mut (a total of three clones were screened per construct). A single clone for each construct was selected and large-scale DNA plasmid isolations were performed to obtain enough quantities for mammalian transfections.

Expression and purification of 4dCD4-WT and 4dCD4-Mut

Small-scale mammalian transfections were carried out in the 293-F cell line using a standardized 3:1 ratio of polyethylenimine (PEI) to DNA. Culture supernatants containing the expressed protein were evaluated by Western blotting using Penta·His Antibody (Qiagen) to detect his-tagged 4dCD4-WT and 4dCD4-Mut (data not shown). To facilitate large-scale expression of the constructs, 293-F stable cell lines were generated by transient transfection protocols, followed by selection of stable transformants with an optimized G418. After approximately 2 weeks of selection pressure, G418 resistant-mammalian colonies appeared and were cultured until confluent. Media samples were collected and expression of the 4dCD4 proteins were assessed by Western blotting (**Supplementary Figure 3**, **B**).

Large-scale production of the 4dCD4 (WT and Mut) proteins were carried out with a combination of transient transfection and collection of supernatants from stable expressing cell lines. Stable cell lines were sub-cultured to a concentration of 0.5×10^6 cells/ml and cultured in appropriate disposable Erlenmeyer flasks at 130 rpm until the culture reached 4×10^6 cells/ml in the presence of G418, before harvesting the culture media. Cells were removed by centrifugation at 350×g for 10 minutes, and culture supernatant containing the expressed protein was stored at 4ºC until purification. Transient transfections were carried out as previously described and scaled-up accordingly. Culture supernatant was collected and replaced every several days, for a maximum of 14 days.

Combined culture supernatants were purified using Ni^{2+}-affinity chromatography, using methods as described for $2dCD4^{S60C}$ in sections (**2.4.6**) and (**2.4.7**). Purified 4dCD4-WT and 4dCD4-Mut were analyzed by SDS-PAGE (**Supplementary Figure 3, A**).

Appendix B: Supplementary figures

>2dCD4_WT

MKKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILGNQGSFLTKGPSKLNDRADSRRSLWDQGNF
PLIIKNLKIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQSLTLTLESPPGSSPSVQCRSPRG
KNIQGGKTLSVSQLELQDSGTWTCTVLQNQKKVEFKIDIVVLAFQKLEHHHHHH

>2dCD4_S60C

MKKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILGNQGSFLTKGPSKLNDRADSRRCLWDQGNF
PLIIKNLKIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQSLTLTLESPPGSSPSVQCRSPRG
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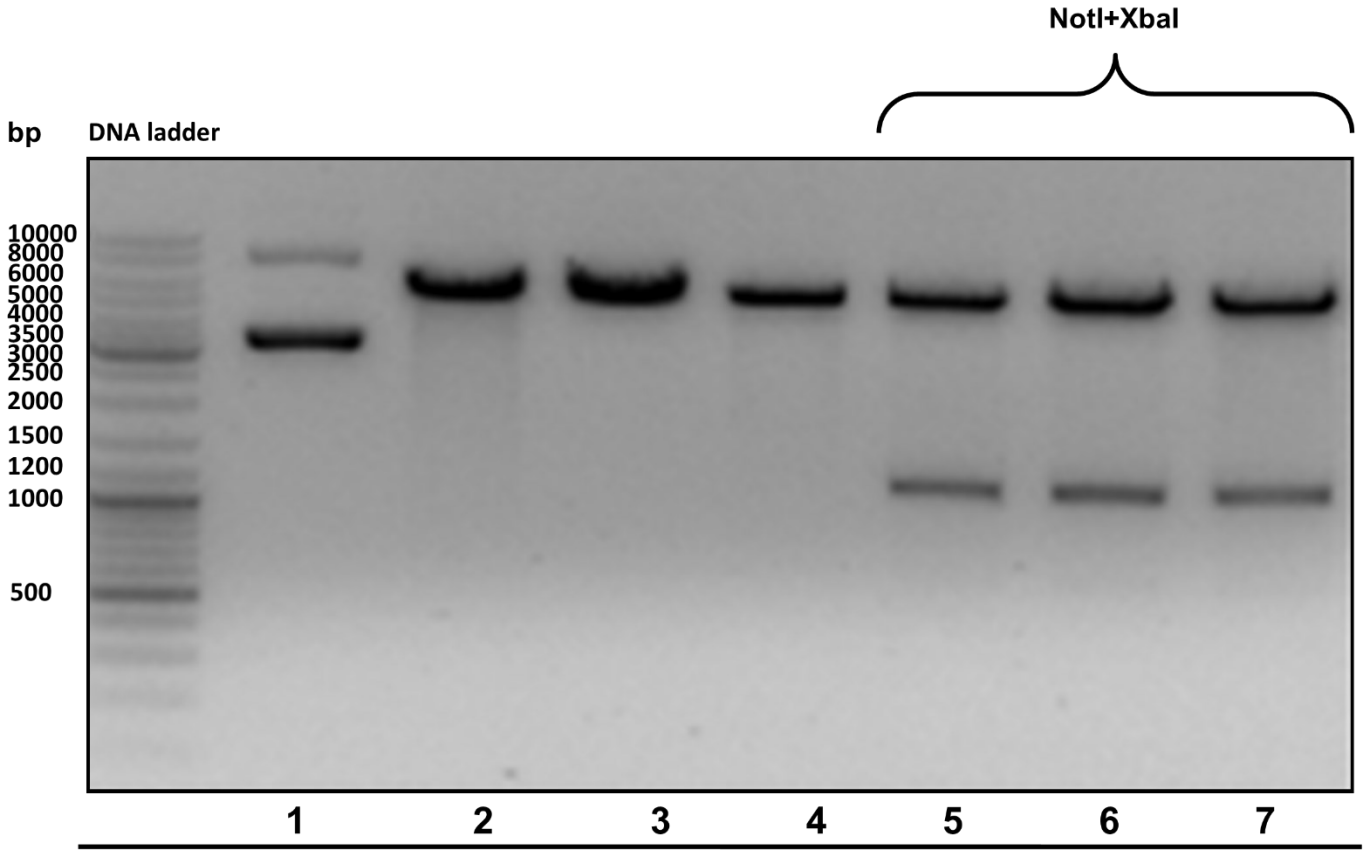
>4dCD4_WT

MDRGVPFRHLLLVLQLALLPAATQGKKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILGNQGSF
LTKGPSKLNDRADSRRSLWDQGNFPLIIKNLKIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQG
QSLTLTLESPPGSSPSVQCRSPRGKNIQGGKTLSVSQLELQDSGTWTCTVLQNQKKVEFKIDIVVLAF
QKASSIVYKKEGEQVEFSFPLAFTVEKLTGSGELWWQAERASSSKSWITFDLKNKEVSVKWVTQDPKL
QMGKKLPLHLTLPQALPQYAGSGNLTLALEAKTGKLHQEVNLVVMRATQLQKNLTCEVWGPTSPKLML
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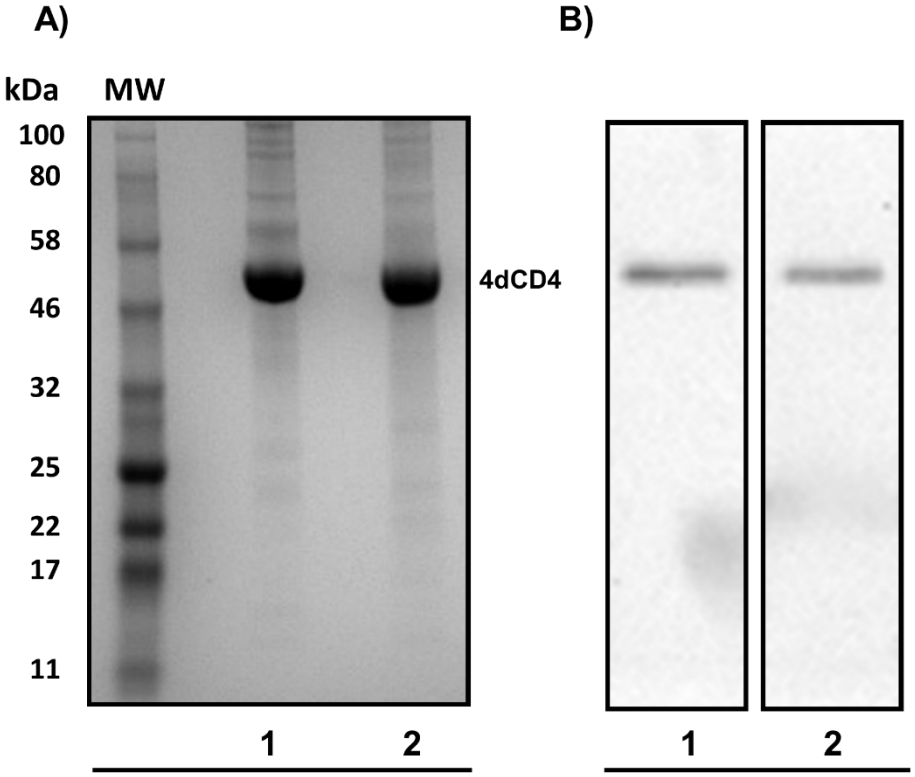
>4dCD4_MUT

MDRGVPFRHLLLVLQLALLPAATQGKKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILGNQGSA
LTKGPSKLNDRADSRASLWDQGNFPLIIKNLKIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQG
QSLTLTLESPPGSSPSVQCRSPRGKNIQGGKTLSVSQLELQDSGTWTCTVLQNQKKVEFKIDIVVLAF
QKASSIVYKKEGEQVEFSFPLAFTVEKLTGSGELWWQAERASSSKSWITFDLKNKEVSVKWVTQDPKL
QMGKKLPLHLTLPQALPQYAGSGNLTLALEAKTGKLHQEVNLVVMRATQLQKNLTCEVWGPTSPKLML
SLKLENKEAKVSKREKAVWVLNPEAGMWQCLLSDSGQVLLESNIKVLPTWSTPVHHHHHH

Supplementary Figure 1. Amino acid sequences of recombinant CD4 proteins depicted in FASTA format. Recombinant CD4 proteins used in this current study for depletion of anti-CD4 antibodies in vaccinated NHP sera. Above sequences are for **2dCD4-WT**, **2dCD4^{S60C}**, **4dCD4-WT** and **4dCD4-Mut**.



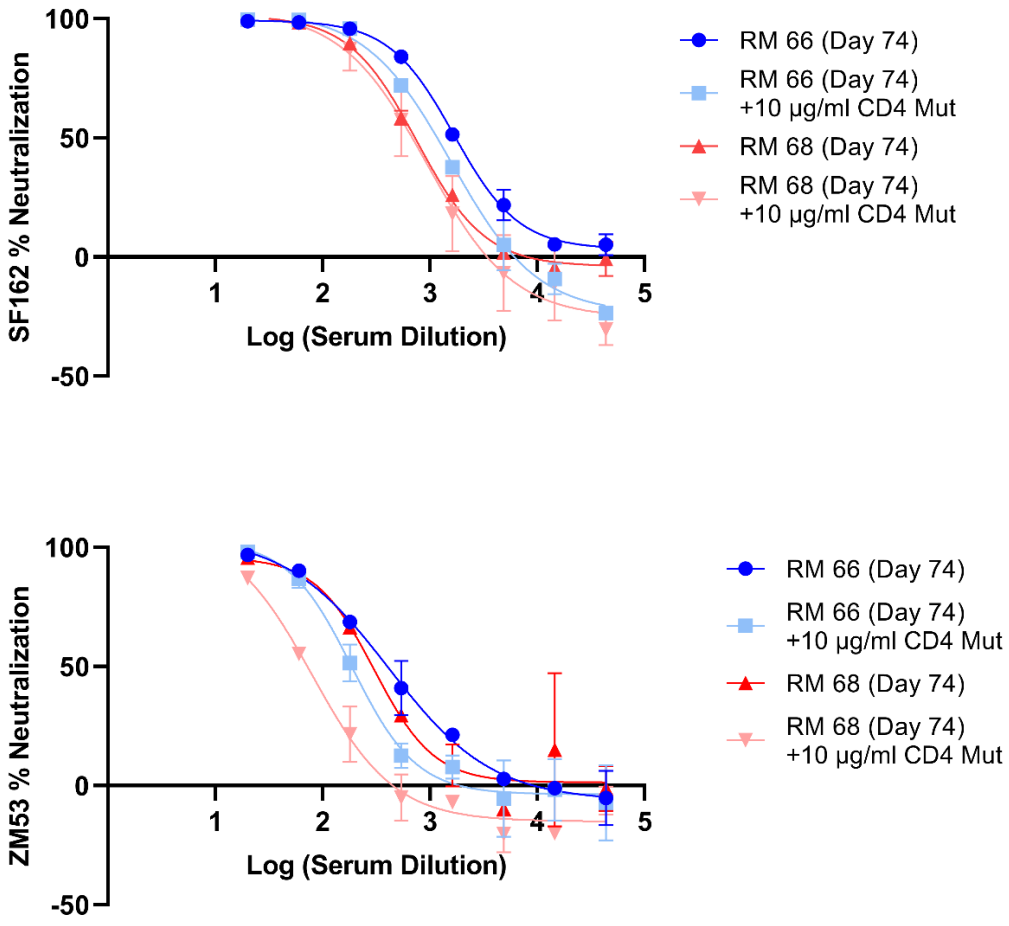
Supplementary Figure 2. Restriction enzyme digest screening of selected 4dCD4-Mut clones. Ampicillin-resistant colonies were cultured in minimal volumes followed by mini-DNA plasmid preparations of each clone. Isolated plasmid DNA was digested with NotI and XbaI to excise the 4dCD4-Mut (~1200 bp) inserts; **lanes 5-7**, pCI-neo 4dCD4-Mut clones 1-3. The pCI-neo plasmid back bone was included as an undigested control or digested individually with NotI or XbaI; **lane 1**, pCI-neo undigested; **lane 2**, pCI-neo (NotI); **lane 3**, pCI-neo (XbaI); **lane 4**, pCI-neo). Digested samples were resolved on 0.8% agarose gel. **DNA ladder** (#SM1173, O'GeneRuler DNA Ladder, Thermo Fischer Scientific).



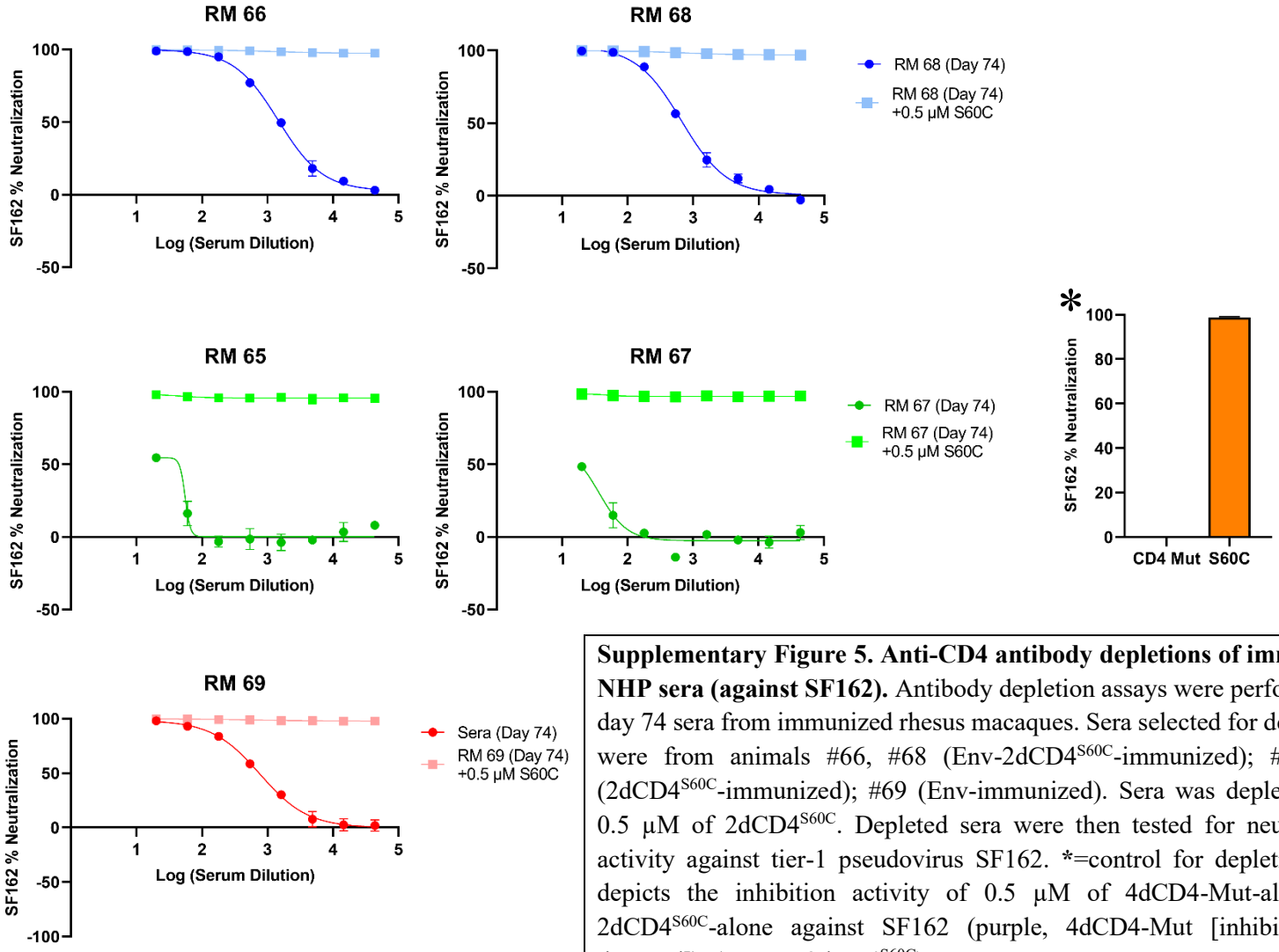
Supplementary Figure 3. SDS-PAGE and Western blot analysis of purified and unpurified 4dCD4-WT and 4dCD4-Mut. The 4dCD4-WT and 4dCD4-Mut proteins were expressed in transient and stable 293-F cell lines and were purified using Ni^{2+}-affinity chromatography. **A)** Purified 4dCD4-WT and 4dCD4-Mut were analysed using SDS-PAGE (12.5%); **lane 1**, 4dCD4-WT; **lane 2**, 4dCD4-Mut. **B)** Western blotting confirming stable cell lines expressing 4dCD4-WT and 4dCD4-Mut, probed with a Penta·His Antibody (1:2,000, Qiagen); **lane 1,** 4dCD4-WT; **lane 2,** 4dCD4-Mut. **MW**=molecular weight marker (#P7712, 11-245 kDa, New England BioLabs).

Supplementary Table 1. Pseudoviral neutralization assay ID_{50} profile of immunized NHPs (baselines). Potency and breadth of NHP sera, tested against the 12-virus HIV-1 Env Global Panel. Each row represents one virus and each column a single rhesus macaque, the immunogen it was vaccinated with, and the time point the sera was collected from. Neutralization data is indicated as the reciprocal dilution of rhesus macaque sera required to achieve 50% inhibition of respective HIV-1 pseudoviruses. VSV-G was used as a specificity control and infects cells independently of CD4, CCR5 or CXCR4.

			Day 0 Pre-Bleed		
			Env	2dCD4^{S60C}	Env-2dCD4^{S60C}
		Clade	#69	#67	#58
HIV-1 Global Reference Strains	398F1	A	<20	<20	<20
	246F3	AC	<20	<20	<20
	CNE55	AE	<20	<20	<20
	CNE8	AE	<20	<20	<20
	X2278	B	<20	<20	<20
	TRO11	B	<20	<20	<20
	BJOX2000	BC	<20	<20	<20
	CH119	BC	<20	<20	<20
	CE0217	C	<20	<20	<20
	CE1176	C	<20	<20	<20
	25710	C	<20	<20	<20
	X1632	G	<20	<20	<20
Tier-2	ZM53	C	<20	<20	<20
Tier-1	SF162	B	<20	<20	<20
Control	VSV-G		<20	<20	



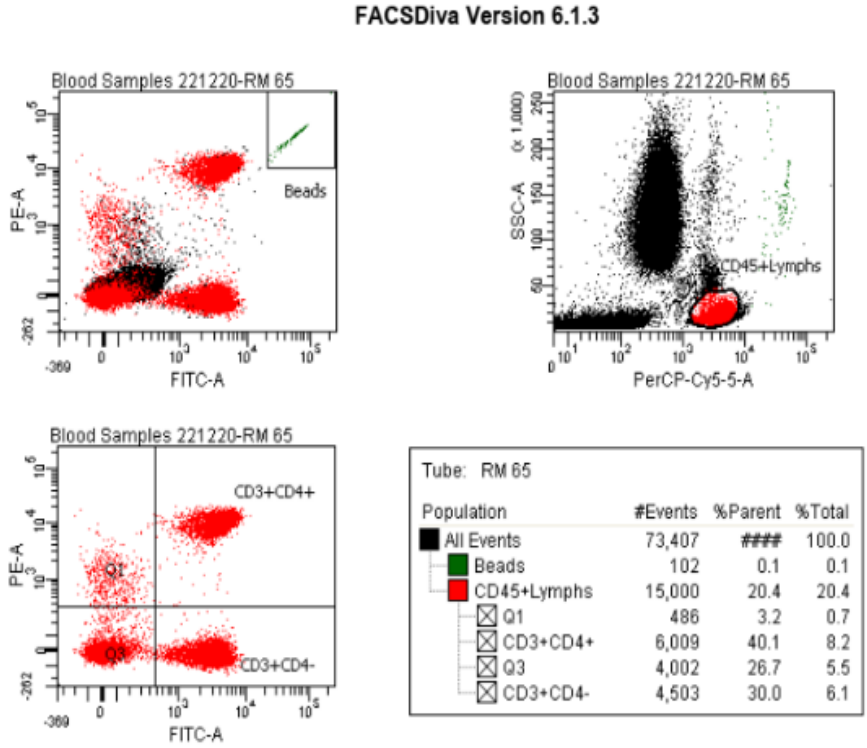
Supplementary Figure 4. Preliminary depletions of NHP sera. Antibody depletion assays were performed on day 74 sera from immunized rhesus macaques. Sera selected for depletions were from animals #66, #68 (Env-2dCD4^{S60C}-immunized). Sera was depleted with 10 µg/ml of 4dCD4-Mut. Depleted sera were then tested for neutralizing activity against tier-1 pseudovirus SF162 or tier-2 pseudovirus ZM53.



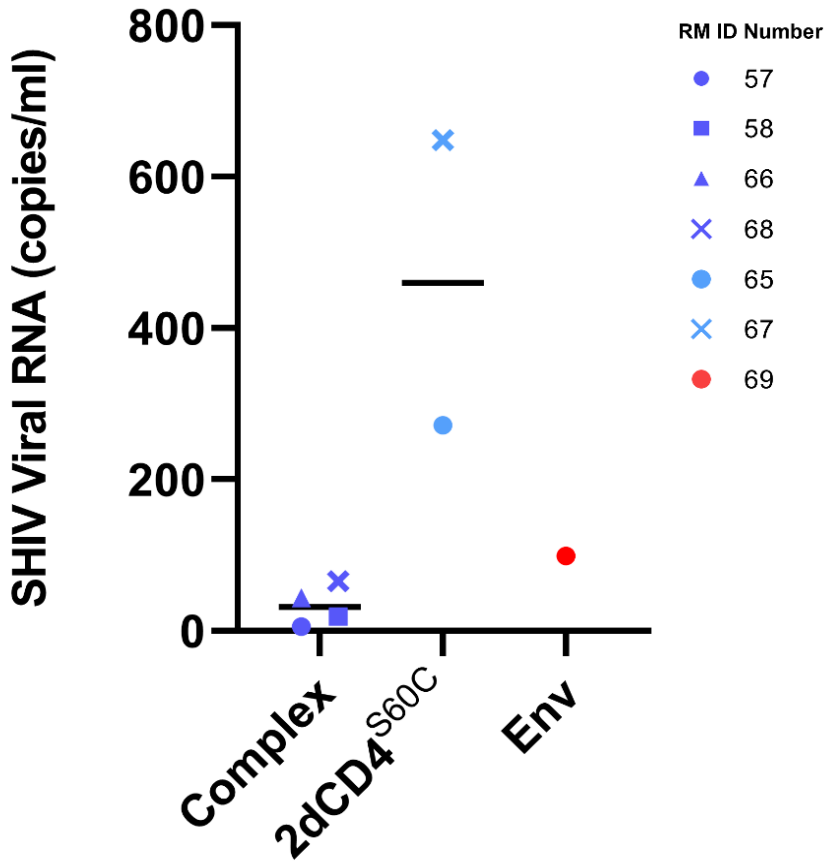
Supplementary Figure 5. Anti-CD4 antibody depletions of immunized NHP sera (against SF162). Antibody depletion assays were performed on day 74 sera from immunized rhesus macaques. Sera selected for depletions were from animals #66, #68 (Env-2dCD4^{S60C}-immunized); #65, #67 (2dCD4^{S60C}-immunized); #69 (Env-immunized). Sera was depleted with 0.5 µM of 2dCD4^{S60C}. Depleted sera were then tested for neutralizing activity against tier-1 pseudovirus SF162. *=control for depletions and depicts the inhibition activity of 0.5 µM of 4dCD4-Mut-alone and 2dCD4^{S60C}-alone against SF162 (purple, 4dCD4-Mut [inhibition not detected]), (orange, 2dCD4^{S60C}).

Rhesus macaque number	*Mamu-A*		*Mamu-B*		*Trim5α*	
#57	A701b	A023	B003a	B003a	TFP/Q	Heterozygous restrictive
#58	A007	A007	B039a	B013a	TFP/Q	Heterozygous restrictive
#65	A007	A007	B001a	B043b	TFP/TFP	Homozygous restrictive
#66	A224	A043	B056a	B056a	TFP/Q	Heterozygous restrictive
#67	A224a	A224a	B001a	B001a	TFP/Q	Heterozygous restrictive
#68	A019	A043	B003a	B003b	TFP/Q	Heterozygous restrictive
#69	A007	A010	B001a	B001d	TFP/TFP	Homozygous restrictive

Supplementary Figure 6. Summary of genotyping results of Chinese-origin rhesus macaque *MHC class I* and *TRIM5* genes. All 7 NHPs used in the current study were genotyped for restrictive *Mamu* and *TRIM5α* alleles known to be associated with spontaneous virological control during SIV/SHIV challenge studies in NHPs. Most of the animals did not have restrictive *Mamu* alleles such as *Mamu-A*01*, *Mamu-B*08* and *Mamu-B*17* except for #69 which harboured a single allele copy of *Mamu-A*01*. All animals had restrictive *TRIM5α* genotypes, with animals #65 and #69 presenting with a highly restrictive homozygous genotype. Colours indicate immunization groupings; Env-2dCD4^{S60C} group (purple, n=4), 2dCD4^{S60C} group (blue, n=2), Env rhesus macaque (red, n=1).



Supplementary Figure 7. Gating strategy for absolute CD4+ T-cell counts of whole blood from NHPs. Whole blood from rhesus macaques was stained using three different antibodies: BD PerCP Mouse Anti-NHP CD45 Clone D058-1283 (Cat# 558411), BD PE Mouse Anti-Human CD4 Clone L200 (Cat# 550630) and BD FITC Mouse Anti-Human CD3ε Clone SP34 (Cat# 556611). The BD Trucount beads and CD45+ lymphocyte populations were gated, and 15,000 CD45+ lymphocyte cell events recorded. The absolute CD4+ T-cell count was determined by dividing the number of positive CD4+ T-cell events (CD3+ CD4+ sub-population) by the number of bead events and then multiplying by the BD Trucount bead concentration.



Supplementary Figure 8. Peak viral loads recorded during 10-week intra-rectal SHIV challenge in NHPs. Peak viral load for each NHP was recorded and ordered by immunization group. Median viral load for each group is depicted by a black line.

Appendix C: Bacterial and DNA work

LB medium (1 L)

- Measure out 10 g tryptone, 10 g yeast extract and 5 g NaCl and add all ingredients into a beaker, add 1 L of dH_2O, autoclave and store at room temperature.

Ampicillin 1000× stock (10 ml)

- Dissolve 1 g of ampicillin in 3 ml of ddH_2O by vortexing. Top up with 100% ethanol. Store at -20°C.

Transformation buffer (100 ml)

- Measure out 1.47 g $CaCl_2.H_2O$ and 0.3024 g PIPES. Top up with ddH_2O to 80 ml.
- Add 15 ml Glycerol, pH to 7.0 with NaOH and top up to 100 ml with ddH_2O. Autoclave and store at 4°C.

LB Agar (200 ml)

- Measure out 7 g of nutrient agar and top up with 200 ml of ddH_2O. Autoclave and store at room temperature.

Competent bacterial cells

- Prepare 10 ml LB medium cultures with bacterial cells diluted 1/10.
- Culture cells overnight at 37°C in shaking incubator. Do not add antibiotic.
- Dilute 1 ml of overnight culture in 40 ml of sterile LB medium.
- Grow culture in shake incubator at 37°C until an A_{600} of 0,4 is reached.
- Pellet cells by centrifugation at 1,500 RPM for 10 min.
- Add 10 ml of transformation buffer to pelleted cells.
- Incubate on ice for 20 min.
- Centrifuge again at 1,500 RPM for 10 min.
- Resuspend in 1 ml of transformation buffer.
- Make 100 ul aliquots and store in freezer at -80°C.

50×TAE buffer (1 L)

- Weigh out 242 g tris base. Add 57.1 ml glacial acetic acid and 100 mL 0.5 M EDTA. Add 800 ml of distilled water. Adjust pH to 8.0 with HCl. Top up to 1 L with dH_2O. Store at room temperature.

EDTA 0.5 M (500 ml)

- Measure out 93.05 g of $Na_2EDTA.H_2O$ and dissolve in 350 ml of dH_2O. Bring to pH 8.0 with 10 M NaOH. Top up to 500 ml with dH_2O and autoclave. Store at toom temperature.

1% Agarose gel (200 ml)

- Add 2 g of agarose to 200 ml of 1×TAE buffer.

Plasmid Extraction/Midi Prep (Qiagen, USA)

- Pick a bacterial colony from a freshly grown bacterial plate and inoculate a starter culture of 5 ml LB medium containing selective antibiotic. Incubate overnight at 37°C on shaking platform.
- Dilute the starter culture 1 / 500 in 25 ml selective LB medium and incubate overnight at 37°C on shaking platform.
- Harvest bacterial cells by centrifugation at 6,000×g for 15 min at 4°C.
- Resuspend pellet in 4 ml of Buffer P1 thoroughly until no clumps remain.
- Add 4 ml of Buffer P2 and mix thoroughly to remove any clumping. Incubate at room temperature for 5 min.
- Add 4 ml of pre-chilled Buffer P3 and mix by inverting vessel 4-6 times. Incubate on ice for 15 min.
- Centrifuge at 20,000 x g for 30 min at 4°C. Supernatant containing plasmid DNA should then be removed swiftly.
- Re-centrifuge the supernatant at 20,000×g for 15 min at 4°C. Supernatant containing plasmid DNA should then be removed swiftly.

- Equilibrate a QIAGEN-tip 100 by adding 4 ml of Buffer QBT and allow the column to empty.
- Add plasmid-containing supernatant to column and allow to enter resin by gravity.
- Wash the QIAGEN-tip with two 10 ml washes with Buffer QC.
- Elute DNA with 5 ml of Buffer QF.
- Precipitate the DNA by adding 3.5 ml of room-temperature isopropanol and centrifuge immediately at ≥15,000×g for 30 min at 4°C and carefully remove the supernatant.
- Wash the DNA pellet with 2 ml of room-temperature 70% ethanol and centrifuge at 15,000 g for 10 min. Remove supernatant without disturbing the DNA pellet.
- Air-dry the pellet for 10 min and then redissolve the DNA in high quality sterile water or any other suitable buffer.
- Quantify by spectrophotometry and store at -20°C until required.

Appendix D: Recombinant protein work

SDS-PAGE

Monomer solution (30% Acrylamide/ 0.8% Bisacrylamide, 100 ml)

- Measure and add together 30 g of acrylamide and 0.8 g of Bis-acrylamide. Top up to 100 ml with dH_2O.

4×Tris-Cl, pH 6.8 (Stacking buffer, 100 ml)

- Measure out 6.05 g of Tris and add to 40 ml of dH_2O. Bring pH to 6.8 with HCl. Top up to 100 ml with dH_2O and store at 4°C.

4×Tris-Cl, pH 8.8 (Running buffer, 500 ml)

- Measure out 91 g of Tris and add to 300 ml of dH_2O. Bring pH to 8.8 with HCl. Top up to 100 ml with dH_2O and store at 4°C.

10% SDS (100 ml)

- Measure out 10 g of SDS and top up to 100 ml with dH_2O. Store at room temperature.

10% Ammonium Persulphate (1 ml)

- Measure out 0.1 g of ammonium persulphate and top up to 1 ml with dH_2O.

2×Sample Buffer (10 ml)

- Measure out and add together 2.5 ml of 4×stacking buffer, 4 ml of 10% SDS, 2 ml glycerol, 0.2 ml of β-Mercaptoethanol, and 2 mg of bromophenol blue. Top up to 10 ml with dH_2O. Make 1 ml aliquots and store at – 20°C.

10×Electrophoresis buffer (1 L)

- Measure out and add together 30.28 g Tris, 144.13 g glycine and 10 g of SDS. Top up to 1 L with dH_2O. Store at room temperature.

Gel staining and Western blotting

Transfer buffer (1 L)

- Add 200 ml of methanol in 800 ml of 1×Electrophoresis buffer. Store at room temperature.

Coomassie Blue (1 L)

- Add 500 ml methanol and 100 ml acetic acid into 400 ml of dH_2O. Dissolve in 0.25 g Coomassie R250. Store at room temperature.

Destain (1 L)

- Add 50 ml ethanol and 70 ml acetic acid into 850 ml of dH_2O. Store at room temperature.

10×TBS (1 L)

- Measure out and add together 10 g NaCl, 30 g Tris and 2.0 g of KCl. Dissolve into 950 mL of dH_2O. Adjust pH to 7.4 with HCl. Make up to 1 L with dH_2O. Store at 4ºC.

T-TBS (1 L)

- Add 500 µl of Tween20 into 1L of 1×TBS. Dissolve. Store at 4ºC.

1% BSA blocking solution

- Mix 1 g of lyophilized BSA in 100 ml 1×T-TBS.

Protein purification

Solubilisation buffer

- Add together 1×DPBS, pH 7.4, 8 M urea, 50 mM glycine, 2 mM- β-Mercaptoethanol, 20 mM imidazole. Store at room temperature.

Wash buffer (0 mM imidazole)

- Add together 1×DPBS, pH 7.4, 8 M urea, 50 mM glycine, 2 mM- β-Mercaptoethanol. Store at room temperature.

Elution buffer (500 mM imidazole)

- Add together 1×DPBS, pH 7.4, 8 M urea, 50 mM glycine, 2 mM- β-Mercaptoethanol, 500 mM imidazole. Store at room temperature.

Wash buffers (10 mM, 20 mM, 50 mM imidazole)

- Utilize 0 mM and 500 mM imidazole buffers to make desired imidazole concentrations of wash buffers. Store at room temperature.

Folding Buffer A

- Add together 50 mM glycine, 10% sucrose, 1 mM EDTA, 1 mM reduced glutathione (GSH), 0.1 mM oxidized glutathione (GSSG), 4 M urea (pH 9.6).

Folding Buffer B

- Add together 50 mM sodium carbonate, 10% sucrose, 1 mM EDTA, 0.1 mM GSH and 0.01 mM GSSG (pH 9.6).

Pierce BCA Protein Assay

- Make up a protein standard range of lyophilized BSA diluted in 1×PBS (2000 µg/ml-25 µg/ml) with the addition of a blank as well.
- Pipette 25 µl of each standard in duplicate onto a 96-well Nunc PolySorp microwell plate (Sigma-Aldrich).
- Add 25 µl of your protein of interest in duplicate to plate.
- Add 200 µl of working reagent to each well containing standard or sample (50:1 mix of BCA reagent A and BCA reagent B).
- Incubate plate at 37ºC on a microplate shake incubator for 20-30 minutes.
- Remove and let cool to room temperature.
- Measure absorbance on a microplate reader at 562nm.
- Prepare a standard curve and determine protein concertation of samples of interest.

Appendix E: Serology work

ELISA Assay Buffer

- 1×DPBS containing 0.05% Tween 20 and 1% lyophilized BSA.

PBS-T

- 1×DPBS containing 0.05% Tween 20.

Diethylaminoethyl-dextran hydrochloride (DEAE Dextran)

- Make up a 10 mg/ml stock of DEAE Dextran in 1×DPBS, mix well until dissolved.
- Sterile filter (0.22 μm) the mix and store at 4°C.

Heat inactivation of rhesus macaque sera

- Thaw samples at room temperature and mix gently.
- Heat inactivate for 30 minutes at 56°C in water bath or in heat block.
- Mix gently and spin down condensation.
- Store at 4°C for up to 2 weeks, or at -20°C for longer periods.

Appendix F: Clearance certificates

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/11/55/C

APPLICANT: Professor M Papathanasopoulos

SCHOOL: Molecular Medicine and Haematology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Immunogenicity testing of HIV-1 envelope glycoprotein vaccine complexes (Env-2dCD4^{S60C}) in Chinese origin rhesus macaques

Number and Species

7 Young Chinese Origin Rhesus macaque

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/11/24. This approval remains valid until 2017/12/01.

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

None

Signed: _____ (Chairperson, AESC) Date: 1st Dec 2015

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: 1 December 2015

cc: Supervisor: N/A
Director: CAS

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Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Maria Papathanasopoulos

b. Department: Molecular Medicine and Haematology

c. Experiment to be modified / extended

				AESC NO
Original AESC number	**2015**	**11/25**	**C**	
Other M&Es : ~~Not Applicable~~				1

d. Project Title: Immunogenicity testing of HIV-1 envelope glycoprotein vaccine complexes (Env-2dCD4^{S60C}) in Chinese Origin rhesus macaques

		No.	Species
e.	Number and species of animals originally approved:	7	*Macaca mulatta (Rhesus Macaque)*
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	7	Rhesus Macaque
h.	Number of animals used to date:	7	Rhesus Macaque

i. Specific modification / extension requested:

We are requesting an additional bleed at day 30 for the immunization protocol submitted for the rhesus macaques.

j. Motivation for modification / extension:

Leaving out a bleed at day 30 for the proposed immunization protocol was an oversight from our research group. Early titer amounts, particularly in the first month of immunizations are important for tracking antibody responses to our immunogens and was always required in our protocol.

The new proposed immunization schedule (extra bleed added) will now be as follows:

Day 0: Pre-immune bleed -10ml of blood will be collected
Immunizations: Each macaque will be immunized with 100 µg relevant immunogen and adjuvant, intramuscularly/subcutaneously (1 or 2 sites).
Day 14: Macaques will be bled - 10ml of blood will be collected.
Immunizations: Each macaque will be immunized with 100µg relevant immunogen and adjuvant, intramuscularly/subcutaneously (1 or 2 sites).

AESC 2017 M&E

Day 30: Macaques will be bled - 10ml of blood will be collected

Immunizations: Each macaque will be immunized with 100µg relevant immunogen and adjuvant, intramuscularly/subcutaneously (1 or 2 sites).

Day 44: Macaques will be bled - 10ml of blood will be collected.

Day 60: Immunizations: Each macaque will be immunized with 100µg relevant immunogen and adjuvant, intramuscularly (1 or 2 sites).

Day 74: Macaques will be bled - 10ml of blood will be collected. Rectal and vaginal lavage samples will be collected.

Day 90: Immunizations: Each macaque will be immunized with 100µg relevant immunogen and adjuvant, intramuscularly/subcutaneously (1 or 2 sites).

Day 104: Macaques will be bled - 10ml of blood will be collected. Rectal and vaginal lavage samples will be collected.

Day 120: Immunizations: Each macaque will be immunized with 100µg relevant immunogen and adjuvant, intramuscularly/subcutaneously (1 or 2 sites).

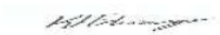
Day 134: Macaques will be bled - 10ml of blood will be collected. Rectal and vaginal lavage samples may be collected

Date: 18 December 2017

Signature: 

RECOMMENDATIONS: Approved. Collection of blood on day 30.

Date: 20 December 2017

Signature: 

Chairman, AESC

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2019/11/66/C

APPLICANT: Dr M Killick
School: School of Pathology; Department: Molecular Medicine and Haematology; Location: CAS

PROJECT TITLE: Protective efficacy studies with HIV-1 Env/2dCD4S60C vaccine immunogen in genetically characterised Chinese origin macaques

Category: C; Species and Numbers involved: *7X male/female, Chinese origin Rhesus Macaques*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 26 Nov 2019. This approval remains valid until 11 Mar 2022.

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ Date: 12/03/2020
(Chairperson of the AREC)

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

Signed: _____ Date: 12/03/2020
(Registered Veterinarian)

CC: Student supervisor: Prof M Papathanasopoulos
Director Central Animals Service: Dr Kim Jardine

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference:

Prof Maria Papathanasopoulos
HIV Pathogenesis Research Unit (HPRU)
Department of Molecular Medicine and Haematology
Faculty of Health Sciences
University of Witwatersrand

CC: Dr Mark Killick
Email: Mark.Killick@wits.ac.za

Dear Prof Papathanasopoulos,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your application, submitted on 02 September 2019, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, "*Protective efficacy studies with the HIV-1 Env/2dCD4S60C vaccine immunogen in genetically characterized Chinese origin rhesus macaques*" with the following conditions:

Conditions:

1. This study is approved as per the application form dated 02 September 2019 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za
2. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

3. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
4. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
5. Samples to be transported must be packaged in compliance with the Regulations of the National Road Traffic Act, 1996 (Act No 93 of 1996) or IATA requirements;
6. The study may only start after Ethics approval was obtained;
7. The study must be conducted in full compliance with *Veterinary and Para-Veterinary Professions Act 1982 (Act no 19 of 82)* at all times;
8. Only rhesus macaques housed within the Central Animal Service (CAS), University of Witwatersrand, may be used in this study;
9. Viral manipulation and processing of potentially SHIV-infect rhesus macaque blood samples must to be completed in the access-controlled HPRU, Faculty of Health Sciences, University of Witwatersrand;
10. Rhesus macaque plasma samples may be transported to the Delft Centre and Primate Unit, Western Cape, for viral load analysis;
11. Rhesus macaque serum samples may be stored at at – 80C at the HPRU, Faculty of Health Sciences, University of Witwatersrand;
12. The stored serum samples may only be used for further research after having obtained new Section 20 approval and may not be outsourced without prior written approval from DAFF;
13. This section 20 expires on 30 October 2020.

Title of research/study: "Protective efficacy studies with the HIV-1 Env/2dCD4S60C vaccine immunogen in genetically characterized Chinese origin rhesus macaques."
Researcher (s): Prof Maria Papathanasopoulos

Institution: HIV Pathogenesis Research Unit, Department of Molecular Medicine and Haematology, Faculty of Health Sciences, University of Witwatersrand

Your Ref./ Project Number:
Our ref Number:

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -09- 05

CLASSIFICATION: CONFIDENTIAL

SUBJECT: Protective efficacy studies with the HIV-1 Env/2dCD4S60C vaccine immunogen in genetically characterized Chinese origin rhesus macaques

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@dalrrd.gov.za
Reference: 12/11/1/1/20A (JD)

Responsible person(s): Prof Maria Papathanasopoulos
Institution: HIV Pathogenesis Research Unit, Department of Molecular Medicine and Haematology, Faculty of Health Sciences, University of Witwatersrand
Email: Mark.Killick@wits.ac.za

Dear Prof Papathanasopoulos and Dr Killick

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) – EXTENSION OF THE EXPIRY DATE

Title of research project / study: "Protective efficacy studies with the HIV-1 Env/2dCD4S60C vaccine immunogen in genetically characterized Chinese origin rhesus macaques"

An amendment is hereby granted on the attached Section 20 approval that was issued for the above mentioned study on 2019-09-05 and with expiry date of 30 October 2020.

i) As requested, the validity of the section 20 approval is extended to 31 March 2021;

ii) All other conditions as specified in the Section 20 approval of 2019-09-05 remain in full effect. This includes the validity of laboratory approvals in terms of SANAS and DALRRD.

Kind regards,

Mdluli pp

DIRECTOR: ANIMAL HEALTH
Date: 2020 -08- 04