

Identification of novel viral mutations in HIV-1 subtype C conferring resistance to the broadly neutralizing antibody, VRC01

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DECLARATION

I, Asanda Zintle Mbadu, declare that this dissertation entitled: Identification of novel viral mutations conferring resistance to the broadly neutralizing antibody, VRC01, submitted for the degree of Master of Science in Biology (Microbiology) at the North-West University, has not previously been submitted by me to this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

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DEDICATION

For my parents, Nomsa and Wiseman Mbadu and Akhanyise.

PRESENTATIONS

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) subtype C remains a global concern with HIV-1 subtype C being the most prevalent form of HIV-1, accounting for approximately 50% of infections worldwide. HIV-1 bNAbs are the most promising preventive HIV vaccine, however, there are challenges in the elicitation of bNAbs by vaccination, and thus the field has moved towards assessing passive immunization using isolated bNAbs. South Africa was one of the participating countries in the recent vaccine trial HVTN 703/HPTN 081 of the Antibody Mediated Prevention (AMP) studies, which showed that the CD4bs antibody VRC01 effectively protected against the acquisition of HIV-1 in only 30% of virus isolates that were highly sensitive to VRC01, with no protection against resistant isolates. One of the challenges in using VRC01 for passive immunization is resistance to neutralisation due to naturally occurring mutations in the HIV envelope (Env) protein. The aim of this study was to identify mutations in HIV-1 subtype C viruses that contribute to VRC01 resistance. Seven Env sequences of VRC01-resistant HIV subtype C viruses were analysed to identify putative neutralisation escape mutations. HIV LANL database and Aliview software were used to align the resistant HIV Env sequences to HXB2. Putative escape mutations were identified in four regions of the Env sequences: the C1 region, loop D, and the β 23 and β 24 regions. Nine Env-pseudotyped viruses were generated from mutated envelopes and were analysed for their neutralisation sensitivity to VRC01 using a pseudovirus-based neutralisation assay. The mutant pseudoviruses were also assessed for sensitivity to four other bNAbs, that is, VRC07-523LS and 3BNC117 which bind on the same epitope as VRC01 and, 10E8 and PG9 that bind to different epitopes on the Env gene. Neutralisation data was compared with *in silico* data obtained from machine learning analyses performed at the Fred Hutchinson Cancer Research Center (FHCRC) (Seattle, Washington, USA) which predicted the probability of sensitivity and IC_{80} values for VRC01. Four Envs bearing single mutations (H0902_K279D, V0217_E279D, V1298_E455T, and V1255_D99N) became sensitive to VRC01 and 3BNC117 compared to their respective wildtype strains. Both VRC01-resistant clones and reversion mutations remained sensitive to VRC07-523LS except for H0902_K279D, highlighting possible cross-resistance among antibodies binding to the same epitope. When machine learning was used to predict the impact of each of the identified mutations on VRC01 sensitivity, the predictions matched the experimental findings in 3/5 cases. Generally, 3 mutations that conferred VRC01 sensitivity in the neutralisation assay, were predicted to be sensitive by machine learning as well, excluding V703_1255_D99N which was found slightly sensitive to VRC01 by neutralisation assay, and resistant by machine learning prediction. Interestingly the single mutation, E455T in the V703_1298 mutant conferred complete sensitivity to VRC01. However, in the H703_1798 Env, the same mutation (E455T) did not change the sensitivity to VRC01. Similarly, a single mutation, D99N in V703_1255 caused partial sensitivity to VRC01, while V703_1298_D99N was resistant. Thus, our neutralisation assay showed partial concordance with the *in-silico* predictions. In conclusion, more neutralisation data is needed to prove that machine learning may be a useful tool to

identify and screen potential mutations that contribute to VRC01 resistance. Our data identified some specific residues important for VRC01 resistance in HIV-1 subtype C. Some of these features may result in cross-resistance to other antibodies that bind to CD4bs which is clinically significant for the testing of other CD4bs antibodies. Moreover, the sensitive mutant V703_1298_E455T warrants further investigation as this is the first report on mutation E455T to show VRC01 sensitivity in HIV-1 subtype C isolates.

Keywords: HIV-1, Broadly neutralizing antibody, VRC01, Viral mutations, Resistance

ABBREVIATIONS

6HB:	Six-helix bundle
ADCC:	Antibody dependent cellular cytotoxicity
ART:	Antiretroviral therapy
ARV:	Antiretroviral
BME:	β -mercaptoethanol
bNAb:	Broadly neutralising antibody
bp:	Base pairs
C1-C5:	Constant regions 1 – 5
CCR5:	C-C chemokine receptor type 5
CD4:	Cluster of differentiation 4
cDNA:	Complementary DNA
CDR:	Complementary-determining region
CDRH3:	Complementarity-determining region-3 of the heavy chain
CHR:	C-terminal heptad repeat
CXCR4: C-X-C	chemokine receptor type 4
DC:	Dendritic cells
DEAE-dextran:	Diethylaminoethyl dextran
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxynucleoside triphosphates
ddNTPs:	Dideoxynucleoside triphosphates
DMEM:	Dulbecco's Modified Eagle Medium

dsDNA:	Double-stranded deoxyribonucleic acid
<i>E. coli</i> :	<i>Escherichia coli</i>
EDTA:	Ethylenediaminetetraacetic acid
Env:	Envelope
Fab:	Fragment antigen binding region
Fc:	Crystallisable fragment
FBS:	Foetal bovine serum
Fp:	Fusion peptide
Gag:	Group-associated antigen protein
GlcNAc:	N-acetyl glucosamine
GnTI:	N-acetylglucosamine transferase I
GnTII:	N-acetylglucosamine transferase II
Gp120:	Glycoprotein 120 kDa
Gp41:	Glycoprotein 41 kDa
HEK:	Human epithelial kidney
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1:	Human Immunodeficiency Virus Type 1
HIV-2:	Human Immunodeficiency Virus Type 2
HPTN:	HIV Prevention Trials Network
HR1:	Heptad repeat 1
HR2:	Heptad repeat 2
HVTN:	HIV Vaccine Trials Network

ID50:	50% inhibitory dilution
LB:	Luria Bertani
LC:	Langerhans cells
LTR:	Long terminal repeat
mAb:	Monoclonal antibody
Man:	Mannose
MPER:	Membrane proximal external region
nAb:	Neutralising antibody
NK:	Natural killer
NHP:	Nonhuman primate
NHR:	N-terminal heptad repeat
PCR:	Polymerase chain reaction
PEP:	Post-exposure prophylaxis
PNG:	Potential N-linked glycan
Pol:	Polymerase
PrEP:	Pre-exposure prophylaxis
PSV:	Pseudovirus
RLU:	Relative light units
RNA:	Ribonucleic acid
RT:	Reverse transcriptase
sCD4:	Soluble cluster of differentiation 4
SHIV:	Simian-human immunodeficiency chimeric virus

SIV:	Simian Immunodeficiency Virus
SOC:	Super optimal broth with catabolite repression
STI:	Sexually transmitted infections
SU:	Surface
TAE:	Tris-acetate acid EDT
Tat:	transcriptional transactivator protein
TCID:	tissue culture infectious dose
TM:	Transmembrane
UNAIDS:	United Nations programme on HIV/AIDS
V1-V5:	Variable regions 1 -5
WHO:	World Health Organisation

AMINO ACID ABBREVIATIONS

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

SYMBOLS AND UNITS

β :	Beta
bp, kb:	Base pairs and kilobases
$^{\circ}\text{C}$:	Degrees Celsius
Mg:	Magnesium
mM, M:	Millimolar and molar
ng, μg , mg:	Nano-, micro- and milligram
nm, μm , cm:	Nano-, micro and centimetre
rpm:	Revolutions per minute
μl , ml:	Micro- and millilitre

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Background

Human Immunodeficiency Virus (HIV) was identified approximately 40 years ago as the causative agent of AIDS, and yet there is no licensed vaccine (Burton *et al.*, 2012). Two types of HIV were discovered, HIV-1 which contributes to the worldwide AIDS epidemic and HIV-2 which is less pathogenic than HIV-1 (Ricard *et al.*, 1994; Gilbert *et al.*, 2003). While after the mid-1990s new infections declined considerably, there were still 1.5 million people who became newly infected with HIV in 2021 with about 54% of the new infections being detected in women and girls (UNAIDS, 2021). HIV continues to be a global health concern with about 40.1 million people who have died from AIDS-related illnesses since the beginning of the epidemic (UNAIDS, 2022b) .

HIV-1 has a high mutation rate due to the lack of a proof-reading mechanism in RNA reverse transcriptase during replication, and a high replication rate with rapid viral turn-over (Drake, 1993; Abram *et al.*, 2010). As a result, there is high diversity in HIV which resulted from different transfers of HIV to humans. The high rate of diversity in HIV led to several HIV-1 groups (M, N, O, P) that have been identified. Within these groups exist diverse subtypes which differ in their sequence, spread across the globe and in their antibody reactivity (Moore *et al.*, 1990; Choisy *et al.*, 2004; Gartner *et al.*, 2020). Group M contributes predominantly to the epidemic, with its high diversity leading to multiple subtypes (A, B, C, D, F, G, H, J and K) (Lynch *et al.*, 2009), including subtype C which accounts for roughly half of the current HIV-1 infections globally making it a focus for vaccine development and testing (Pegu *et al.*, 2014; Hraber *et al.*, 2017). It is abundant in sub-Saharan Africa, especially in South Africa (Kharsany *et al.*, 2019), followed by countries such as the Philippines, India, Indonesia in Asia, and New Zealand and Papua New Guinea in the Pacific (UNAIDS, 2022a). Although the highest priority of public health is to develop a vaccine for HIV, identifying a preventive vaccine has proven to be a challenge to the biomedical research community mainly because of significant scientific obstacles presented by the virus (Ng'uni *et al.*, 2020). The major obstacle in vaccine development is the high level of mutation and recombination of the virus which results in novel variants that escape the immune response (van Heuvel *et al.*, 2022).

Immunization efficacy trials are of great importance in regions that are highly affected by HIV-1 such as South Africa (Rademeyer *et al.*, 2016). Although some of the challenges that affect vaccine design exist, such as low potency and breadth demonstrated by vaccine candidates, and safety and affordability (Kennedy *et al.*, 2020; Ng'uni *et al.*, 2020), the exceptional diversity of HIV-1 presents the greatest challenge in accomplishing protection in both active and passive immunization studies (Rademeyer *et al.*, 2016). The HIV-1 Env gene, which encodes the Env glycoproteins gp120 and gp41, is the most genetically variable gene that contributes to the diversity of the virus (Coffin *et al.*, 1997; Burton *et al.*, 2012).

Over the years researchers have identified various prevention methods that reduced HIV-1 acquisition. These include the use of condoms, male circumcision, and pre/post-exposure prophylaxis in healthy individuals (Peterson *et al.*, 2007; Cork *et al.*, 2020). These methods managed to reduce HIV infections however, they require adherence and are most likely to be discontinued by the user (Abdool Karim *et al.*, 2010) and that might lead to a loss of control of the infections hence the need for an HIV vaccine. However, the development of a vaccine is a possible solution as vaccines require less adherence since they are not administered daily. A major research goal is the development of immunogens that can induce broadly neutralising antibodies (bNAbs) (Burton *et al.*, 2012) that can neutralise a wide range of different virus isolates (Walker & Burton, 2008). There is abundant research that is focused on broadly neutralising antibodies as potential prevention therapy and several bNAbs have progressed to human clinical trials (Mahomed *et al.*, 2021). However, vaccine strategies to elicit bNAbs have failed (Andrabi *et al.*, 2018; del Moral-Sánchez & Sliepen, 2019; Williams *et al.*, 2021) and thus the field has moved towards testing isolated bNAbs. One of the bNAbs that has been tested for efficacy in HIV prevention is VRC01 antibody (Corey *et al.*, 2021).

The VRC01 monoclonal antibody was isolated by Wu *et al.* (2010) at the Vaccine Research Center (VRC), National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH). VRC01 is directed against the CD4 binding site (CD4bs) of the HIV-1 Env glycoprotein (Wu *et al.*, 2010) and is the furthest experimental candidate for the evaluation of HIV prevention in clinical trials (Snow, 2018). Antibodies like VRC01 are of interest because they target the highly conserved CD4 receptor binding site on gp120, which attaches the virus to the target cells during viral entry (Lynch *et al.*, 2015b). VRC01 neutralised about 90% of all the major circulating viral strains and was observed, in a non-human model, to confer protection against Simian/HIV (SHIV) targeting the CD4bs (Pegu *et al.*, 2014). VRC01 prevention was further tested in the Antibody Mediated Prevention (AMP) study which was undertaken in two parallel studies: (1) HVTN 703/HPTN 081 which was based in sub-Saharan Africa (HIV Prevention Trials Network) and (2) HVTN 704/HPTN 085 based in United States, Peru, Brazil, and Switzerland (HIV Prevention Trials Network), the largest phase 2b trial to test antibodies for prevention of HIV infection (clinicalinfo.hiv.gov, 2021). The two studies evaluated the safety, tolerability and efficacy of VRC01 for the prevention of HIV-1 infection (Snow, 2018), and viral features that contribute to natural resistance to the antibody. In the AMP trial, VRC01 demonstrated safety and tolerability but was not highly effective in preventing HIV-1 acquisition, however, the data provided proof-of-concept that bnAb prophylaxis can be effective (Corey *et al.*, 2021). However, protection by VRC01 is challenged by viral isolates that escape neutralisation by this antibody (Wu *et al.*, 2010; Lynch *et al.*, 2015b; Bar *et al.*, 2016; Rademeyer *et al.*, 2016; Otsuka *et al.*, 2018).

HIV-1 Env has multiple mechanisms of immune evasion – including conformational masking, glycosylation and sequence variation by substitution of amino acids and adding and shifting glycans mostly

in its regions of interactions (Cuevas *et al.*, 2015; Zhou *et al.*, 2017; Otsuka *et al.*, 2018). Although there are several ways that HIV-1 can escape VRC01, mutations in contact regions such as loop D, CD4 binding site, β 23- loop V5 of the gp120 have been described to contribute highly to VRC01 escape (Li *et al.*, 2011; Lynch *et al.*, 2015b; Otsuka *et al.*, 2018). VRC01 does not protect individuals from infection with VRC01-resistant strains (Wu *et al.*, 2010). This was confirmed by the results of the AMP Phase 2b prevention study mentioned above where only 30% of the virus isolates were sensitive to VRC01, and the remaining 70% were resistant (Corey *et al.*, 2021; Williamson *et al.*, 2021). Moreover, subtype C viruses are naturally more resistant to VRC01 compared to other subtypes (Rademeyer *et al.*, 2016; Bricault *et al.*, 2019). A previous study observed the development of resistance of HIV-1 isolates towards VRC01 and other bNAbs (Rademeyer *et al.*, 2016). However, the observed reduction of viral plasma levels in isolates that are sensitive to VRC01 (Lynch *et al.*, 2015a; Lynch *et al.*, 2015b) indicates that individuals exposed to VRC01-sensitive viruses are likely to be safe from HIV infection when administered with VRC01 antibodies. Thus, in order to use bNAbs in humans or to design bNAb-based vaccines, it is crucial to understand how resistant viruses escape antibody neutralisation (Otsuka *et al.*, 2018). In addition, data that describes the contribution of viral features to resistance and how this changes over time is important in vaccine design (Rademeyer *et al.*, 2016). Method such as machine learning have been useful in identifying sites that are more associated with neutralisation resistance. This method is a computer program used to analyse the virus sequence of a participant and predict the extent of resistance to a drug (Blassel *et al.*, 2021). The software measures the predicted probability that a virus will be sensitive to a specific drug. Some of the sites identified by this method have not been categorized before, thus there is no experimental data to confirm their contribution towards VRC01 potency (Magaret *et al.*, 2019).

Given the resistance that a proportion of HIV-1 subtype C viruses demonstrate against the VRC01 bNAb, this study sought to validate known mutations that confer resistance towards VRC01 and identify novel mutations that contribute to VRC01 resistance in HIV-1 subtype C isolates.

1.2 Problem statement

The rate of evolution of HIV-1 poses a considerable challenge in the development of a vaccine, as it results in high levels of viral variability (Wu *et al.*, 2010; Hemelaar *et al.*, 2019). Therefore, a vaccine that will protect against the various HIV-1 strains is vital. Passive immunization of several broadly neutralising antibodies has demonstrated the safety and tolerability of these antibodies with stable pharmacokinetics and efficiency in protecting against HIV-1 infection (Mayer *et al.*, 2017; Pegu *et al.*, 2017; McCoy, 2018). As observed in the AMP trial, VRC01 did not prevent individuals from being infected by VRC01-resistant HIV-1 viruses. It has also been noted that the rate of natural resistance towards VRC01 is higher in HIV-1 subtype C which is the dominant strain in South Africa than in subtype B (Rademeyer *et al.*, 2016; Bricault

et al., 2019). It is therefore important to update all the emerging mutations resulting in viral resistance. This will consequently update the methods of treatment and prevention.

1.3 Study aims and objectives

1.3.1 Aims

The aim of the study is to validate known VRC01 resistance mutations in HIV-1 clade C Env pseudoviruses and identify novel mutations that may contribute to resistance.

1.3.2 Objectives:

The objectives of the study were to:

- Analyse resistant HIV-1 subtype C virus Env sequences and compare them to known VRC01 resistance mutations to find previously identified and putative novel resistance mutations.
- Revert the VRC01 resistant HIV-1 subtype C virus Env sequences into sensitive sequences by substituting the identified and putative novel VRC01 resistance residues with VRC01 sensitive residues using site-directed mutagenesis.
- Conduct neutralisation assays to assess the sensitivity of mutated viruses to determine whether the mutations alter/abrogate resistance to VRC01 and other related CD4bs directed bNAbs.

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CHAPTER 2 LITERATURE REVIEW

2.1 The Origin, Classification and Global Distribution of Human Immunodeficiency Virus type 1 (HIV-1)

HIV-1 evolved from the Simian Immunodeficiency Virus (SIV), a virus infecting chimpanzees (SIVcpz) (Levy, 1993). According to the phylogenetic analysis, SIVcpz genome sequences revealed that all HIV-1 strains were closely related to the SIVcpz lineage, which indicates that they are the reservoirs of HIV-1 (Gao *et al.*, 1999). Simian retroviruses are reported to have been transmitted to humans through contact with infected blood or other fluids through bites or scratching during the hunting and butchering of infected African nonhuman primates (Peeters *et al.*, 2014). Thus, after its discovery, HIV-1 was classified as a primate *Lentivirus* belonging to the family *Retroviridae* and found to be biologically related to HIV-2 that belongs to the same genus and family but evolved from the sooty mangabeys (Levy, 1993; Keele *et al.*, 2006; Sharp & Hahn, 2011). HIV-1 is widely spread amongst different regions of the North America, the Caribbean, South America, Western Europe, Central Asia, East Asia, Southeast Asia, North Africa and the Middle East, sub-Saharan Africa, and Australia, while HIV-2 is confined in West Africa (McCutchan, 2006). HIV-1 has increased levels of viral replication and disease progression compared to HIV-2 (Marlink *et al.*, 1994; Gao *et al.*, 1999; Popper *et al.*, 1999). It is further divided into phylogenetic groups M (main), N (outlier) originating from a chimpanzee species (*Pan troglodytes troglodytes* (Ptt)), O (non-M) and P showing a close relation with gorillas than chimpanzees (D'arc *et al.*, 2015). These groups arose as a result of independent cross-species transmission events (Thomson *et al.*, 2002; Plantier *et al.*, 2009; Sharp & Hahn, 2010). Although these groups belong to HIV-1, they have been shown to have occurred through different evolution routes. Phylogeographic analysis revealed that Group M and N occurred due to transmission of SIVcpz to humans (Keele *et al.*, 2006; Sharp & Hahn, 2011). Phylogenetic studies suggest that HIV-1 group O viruses were transmitted from chimpanzees to gorillas before they were then transmitted to humans (D'arc *et al.*, 2015). The discovery of group P, a non-pandemic group that was discovered in Cameroon (Plantier *et al.*, 2009; Vallari *et al.*, 2011), showed a close relation to the gorilla virus (SIVgor) and possibly resulted from transmission between gorillas and humans (Takehisa *et al.*, 2009). It is described to be less adapted to humans similar to groups O and N, and biologically distinct from the other three HIV-1 groups since it is more related to SIVgor (Plantier *et al.*, 2009). It is believed that the evolution of the four groups resulted in differences in their genetic makeup and subsequently contributed to the AIDS pandemic (Lythgoe & Fraser, 2012).

Group M is largely responsible for the global pandemic and while groups N and O are confined to West Africa, group M has spread throughout Africa (Figure 2-1) and globally with nine subtypes (A, B, C, D, F, G, H, J, K) and sub-subtypes belonging to subtypes A (A1-A3) and F (F1-F2) (Tebit *et al.*, 2007; Sharp &

Hahn, 2011). The great diversity in the genomes of HIV-1 Group M viruses led to more diverse HIV-1 subtypes (Choisy *et al.*, 2004; Travers *et al.*, 2005; Lynch *et al.*, 2009). HIV-1 diversity largely occurs within the envelope protein (Starcich *et al.*, 1986; Burton *et al.*, 2012). Subtype A is most common in East Africa, Eastern Europe, and Central Asia as well as parts of Central Africa although other subtypes D and C viruses circulate in these regions as well (Billings *et al.*, 2017). Subtype B is the dominant subtype in regions such as Central and Southern America, Western and Central Europe (Gartner *et al.*, 2020). However, there have been reports of an increase in subtype C compared to subtype B among HIV/viral hepatitis coinfecting patients in Southern Brazil (Avanzi *et al.*, 2017). Subtype C is present worldwide however, it is more prevalent in Africa and India, and accounts for about half of global infections (Hemelaar *et al.*, 2006; Gartner *et al.*, 2020). It dominates in South Africa where it reaches more than 98% of overall infections (Wilkinson *et al.*, 2015; Wilkinson *et al.*, 2016; Hemelaar *et al.*, 2019). Moreover, South Africa has a high level of subtype C diversity due to subtype C viruses being introduced from surrounding countries (Wilkinson *et al.*, 2015; Wilkinson *et al.*, 2016). Although an effective global vaccine candidate should ideally protect against all circulating variants (Burton *et al.*, 2012), the global abundance of subtype C highlights the need to focus on this subtype for vaccine development and testing (Pegu *et al.*, 2014; Hraber *et al.*, 2017) along with studies on broadly neutralising antibodies (bNAbs) that show broader and more potent neutralisation of subtype C viruses (Taylor *et al.*, 2008; Shaw & Hunter, 2012).

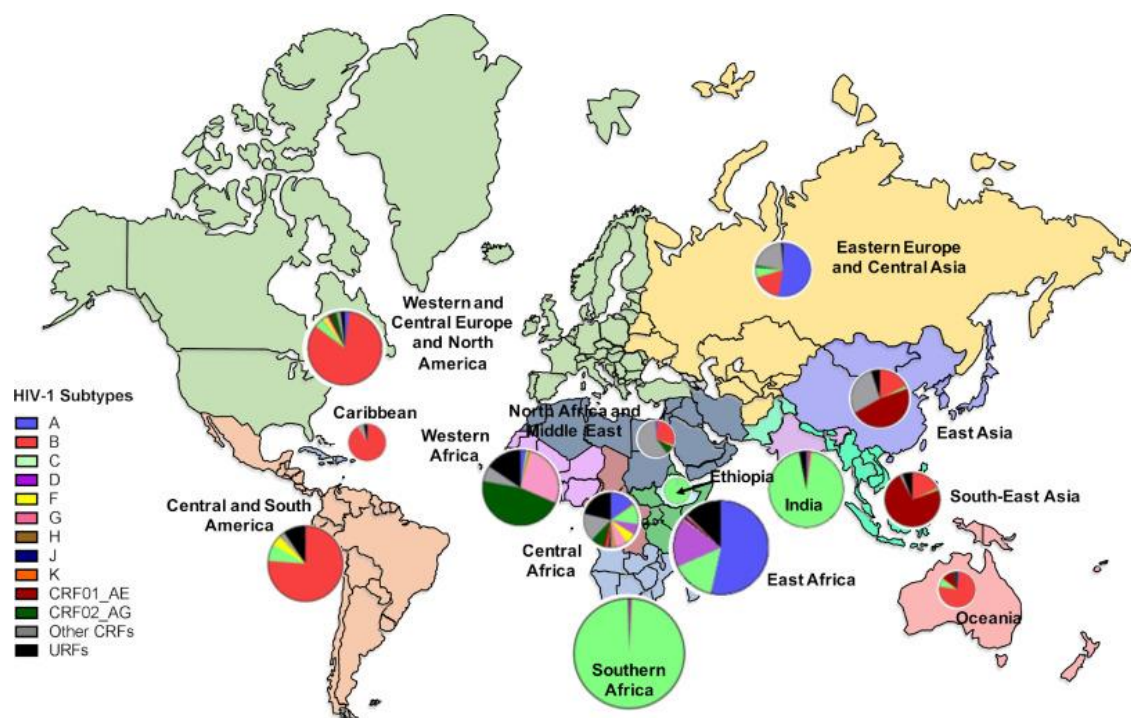


Figure 2-1: Map showing the prevalence of HIV-1 group M subtypes globally. The pie graphs show the percentage of each subtype, circulating recombinant forms (CRFs) and unique recombinant forms (URFs) that circulate within a region and the size of each pie is the representation of the total infections in that region. Adapted from Gartner *et al.* (2020).

Currently, there are over 100 CRFs (Los Alamos National Laboratory, 2021). CRFs emerged as a result of the recombination and ongoing spread of viruses belonging to different subtypes (Umviligihozo *et al.*, 2021) of global importance (Korber *et al.*, 2001).

2.1.1 HIV structure

A mature HIV-1 virus is round and measures approximately 100 nm in diameter. It has two copies of a single-stranded RNA genome incorporated within a conical capsid surrounded by a plasma membrane containing outer viral envelope proteins. The RNA genome consists of about 9,750 nucleotides (Wain-Hobson, 1989; Blood, 2016). HIV-1 genome encodes nine genes which are translated into proteins (Levy, 1993). Six of these genes (*vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*) are smaller accessory genes and three are structural genes (*Gag*, *Pol*, and *Env*, Figure 2-2) that encode for proteins necessary for the replication of HIV-1. Amongst the major genes, *Gag* encodes a polyprotein precursor Pr55^{Gag} which when cleaved by viral protease generates mature gag proteins of the outer core membrane (MA, p17), the capsid protein (CA, p24), the nucleocapsid (NC, p7) and a smaller, nucleic acid-stabilising protein p6 (Figure 2-2). Then follows the *Pol* gene that codes for the enzyme, protease, (PR), reverse transcriptase (RT) and integrase (IN), important for viral replication in the host cell, which results from the cleavage of the precursor Pr160^{GagPol} by viral protease (Freed, 2001). The *Env* gene encodes for the viral envelope glycoproteins, which bind to the receptors on the surface of the host cell (Gelderblom *et al.*, 1989; Fanales-Belasio *et al.*, 2010). *Env* gene is also cleaved from the precursor polyprotein gp160, however, unlike *Gag* and *Pol*, *Env* is cleaved by a cellular protease, resulting in two glycoproteins surface (SU) *Env* glycoprotein gp120 and the transmembrane (TM) glycoprotein gp41 found on the viral envelope (Figure 2-2) (Kirchhoff, 2013; Sanders & Moore, 2017). Inside the virus envelope is the capsid, which encloses two copies of the viral RNA as well as proteins that are crucial for the replication and survival of the virus in the host (Turner & Summers, 1999).

HIV-1 genome



HIV-1 mature virion

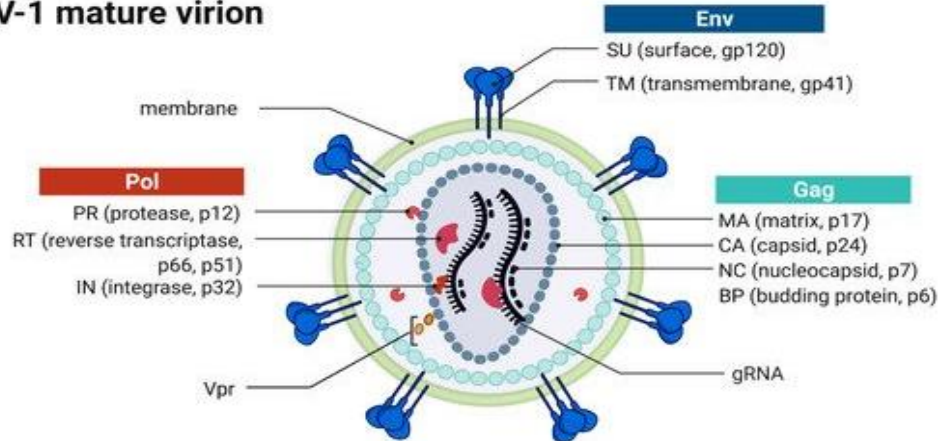


Figure 2-2: HIV-1 genome and a structure of an HIV-1 virion. Depiction of the HIV-1 genome and a structure of an HIV-1 virion which contains two identical copies of single-stranded RNA molecules which are responsible for encoding all the genetic information needed during the viral life cycle. The diagram shows the three large genes of the HIV-1 genome (Gag, Pol, and Env) and their respective proteins. Adapted from van Heuvel *et al.* (2022).

2.1.2 HIV replication

Human Immunodeficiency Virus infects CD4⁺ T lymphocytes, dendritic cells and macrophage-lineage cells of the immune system (Levy, 1993). For HIV to replicate, the gp120 component of the envelope must bind to the primary viral receptor, CD4 (Figure 2-3). As revealed by single-molecule fluorescence resonance energy transfer (smFRET), the engagement of the CD4 leads to different states of conformation of the Env (Ma *et al.*, 2018). When Env is CD4-bound, gp120 assumes a conformation suitable for CCR5/CXCR4 to interact with the V3 variable region exposed and the bridging sheet formed (Klimas *et al.*, 2008; Sanders & Moore, 2017; Wang *et al.*, 2020). Once the gp120-coreceptor bond is established, additional Env conformational transitions are formed which result in the fusion of the host membrane and the viral membrane (Briggs *et al.*, 2009; Mailler *et al.*, 2016). During the process of receptor binding, the hydrophobic fusion peptide at the N terminus of gp41 inserts into the target cell membrane. Thereafter, the heptad repeat 2 (HR2) region of gp41, located near the viral membrane, binds to the hydrophobic groove on the HR1 trimeric coiled coil in an anti-parallel manner. This results in the formation of an energetically stable six-helix bundle (6HB), driving the viral and target cell membranes into proximity (Wang *et al.*, 2020).

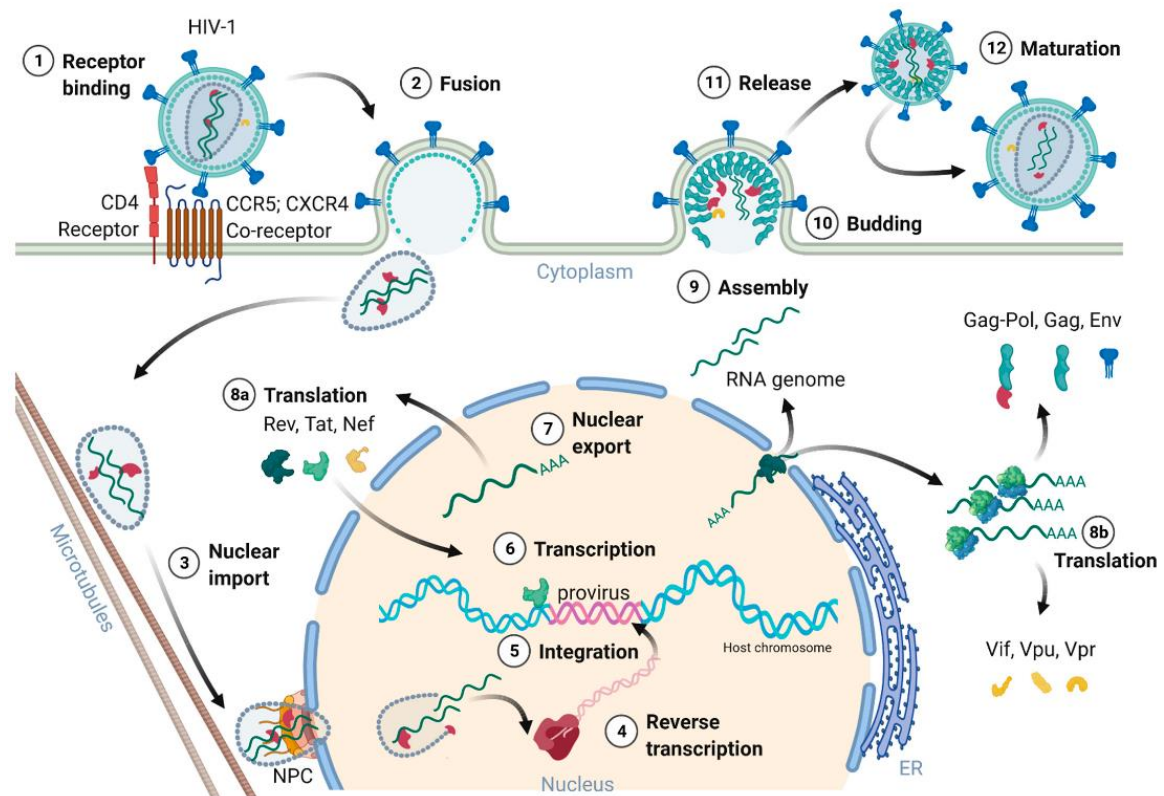


Figure 2-3: Replication cycle of HIV-1 in the host cell. The numbers indicate each stage of the life cycle as follows: 1) Binding of gp120 to the receptor and co-receptor, 2) Fusion and entry of viral contents into the host cell, 3) Import of viral RNA into host nucleus, (4) Reverse transcription of viral RNA into dsDNA, followed by 5) Integration of viral dsDNA into the host genome, 6) Transcription of proviral DNA into RNA, 7) Nuclear export, 8a & 8b) Translation of viral RNA into viral proteins, 9) Assembly of new, immature virion, 10 & 11) Budding and release of the immature virion, 12) Maturation into an infectious virion. Adapted from van Heuvel et al. (2022).

Upon entry to the cell, the viral RNA is converted to double-stranded DNA (dsDNA) by the action of the enzyme reverse transcriptase (RT) (Hu & Hughes, 2012). This is the enzyme that contributes to the high mutation rate in HIV-1 because of its lack of a proof-reading mechanism (Drake, 1993; Abram *et al.*, 2010), consequently enabling HIV-1 to escape the host immune response and develop resistance against antiviral drugs (Freed, 2001). The viral DNA ultimately gets transported into the nucleus and integrase (IN) merges the viral genome with the host DNA (Smith & Daniel, 2006; Klimas *et al.*, 2008). Studies have reported three proteins (matrix (MA), vpr and IN) that play a significant role in viral DNA transport (Freed, 2001). Then the proviral DNA (viral DNA that has been integrated into the host genome) is transcribed into the HIV RNA by the host DNA polymerase II and subsequently translated into viral proteins (Klimas *et al.*, 2008). Transcription is controlled by both the host cell and the viral genes. The RNA transcribed from DNA, called messenger RNA (mRNA), is then transported from the nucleus of the cell to the cytoplasm.

Once in the cytoplasm, proteins of the HIV virus are made using the HIV mRNA as the template. The mRNAs are categorized into three classes: (I) full-length, unspliced ~9 kb mRNA, (II) intron-containing, partially spliced ~4 kb mRNAs, and (III) intronless, fully spliced ~2 kb mRNAs (Sertznig *et al.*, 2018; van Heuvel *et al.*, 2022). The *Gag* and *Pol* gene products are translated from the unspliced full-length mRNA. *tat*, *rev*, and *nef* are translated from fully spliced mRNAs, whereas *vif*, *vpr*, *vpu*, and the Env precursor protein gp160 are translated from partially spliced transcripts (van Heuvel *et al.*, 2022). Further posttranscriptional modifications take place in proteins containing signal peptide such as *vpu* and Env upon entering the endoplasmic reticulum (ER). Glycosylated Env passes through the Golgi apparatus and is cleaved by the cellular furin-like proteases into gp120 and gp41 (van Heuvel *et al.*, 2022). These proteins, together with the viral RNA are assembled at the cell membrane and bud off as non-infectious, immature forms of new virions (Klimas *et al.*, 2008; Kirchhoff, 2013). Immediately after the budding, the protease is activated to cleave *Gag* (group specific antigen) and *Gag* polyprotein precursor Pr55^{Gag} into mature components (Klimas *et al.*, 2008; Bhatti *et al.*, 2016). Pr55^{Gag} forms one of the most important viral structural proteins since its expression is necessary for the assembly, budding, and release of immature particles (Checkley *et al.*, 2011). Protein configuration is reorganized and a mature, infectious virion results (Klimas *et al.*, 2008; Bhatti *et al.*, 2016). The assembly of viral genomic RNA, Env glycoprotein complex, and *GagPol* precursor protein (Pr160^{GagPol}) into virus particles takes place at the plasma membrane (Checkley *et al.*, 2011). The incorporation of the Env glycoprotein complex into virus particles is a major step in the production of infectious HIV-1 virions (Checkley *et al.*, 2011).

2.2 HIV-1 envelope (Env) structure and glycoproteins processing

2.2.1 HIV-1 envelope glycoprotein

The HIV envelope glycoprotein is generated when the nascent Env glycoprotein160 (gp160) is modified within the Endoplasmic Reticulum (ER) simultaneously with the process of translation during replication. The unprocessed gp160 is glycosylated with N-linked and some O-linked oligosaccharide side chains in the ER, following cleavage of its leader peptide sequence at its N-terminus region (Kornfeld & Kornfeld, 1985; Leonard *et al.*, 1990; Coffin *et al.*, 1997). Thereafter, gp160 monomers oligomerize mainly into trimers, with possible dimers and tetramers that end up in the Golgi complex and the trans-Golgi network (TGN) for further modifications of oligosaccharide side chains (Earl *et al.*, 1990; Lu *et al.*, 1995; Checkley *et al.*, 2011). At this stage, the premature interactions of the envelope protein with CD4 receptors are prevented by the *vpu* protein which suppresses its expression via ubiquitin-mediated proteasomal degradation (Schubert *et al.*, 1998). The maturation of gp160 completes when the enzyme furin mediates cleavage of gp160 at the Arg-X-Lys/Arg-Arg site into two glycoproteins, gp120 and gp41 (Figure 2-4) (Veronese *et al.*, 1985; McCune *et al.*, 1988; Hallenberger *et al.*, 1992).

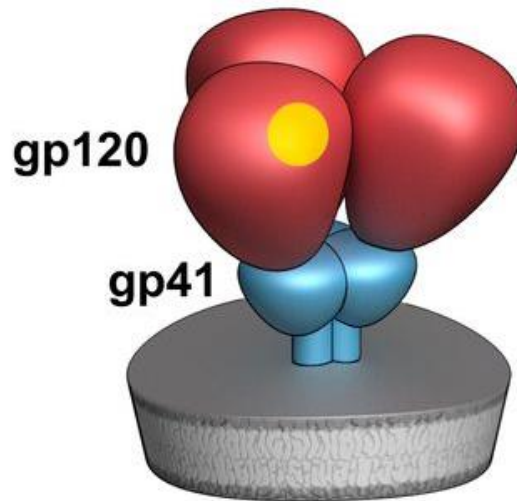


Figure 2-4: Quaternary structure of trimeric gp120 and gp41 in the unliganded form on the surface of the virion. The gp120 is in red, gp41 in blue and the CD4 binding site (yellow). Adapted from Harris *et al.* (2013).

The gp160 cleavage results in three non-covalently linked dimers of gp120 which is the receptor-binding surface (SU) component, and gp41 which is the membrane-spanning trans-membrane (TM) component forming the functional Env spikes that extend from the surface of the HIV virion (Moore *et al.*, 1990; Helseth *et al.*, 1991; Wyatt & Sodroski, 1998) (Figures 2-3 and 2-4). Each virion has about 7–14 envelope spikes on its surface making approximately 21–42 gp120/gp41 molecules (Mak & Saunders, 2006) and each Env spike is made up of about 845-870 amino acids (Earl *et al.*, 1990). The diversity in Env sequences is vast such that amino acid sequences of Env can differ up to 20% within the same subtype and over 35% between different subtypes (Gaschen *et al.*, 2002). This diversity presents a huge challenge in the development of an effective HIV vaccine that can protect against different HIV-1 subtypes and recombinants (Barouch & Korber, 2010).

2.2.2 Gp120 of the HIV-1 envelope

The gp120 is made up of two domains, an inner and an outer domain, and a beta-sheet or the "bridging sheet" that does not form part of either domain (Figure 2-5). The names of the domains reflect how the gp120 is positioned in the assembled envelope glycoprotein trimer wherein the inner domain faces the trimer axis and, presumably, gp41, whereas the outer domain is exposed on the surface of the trimer. The elements of both domains and the bridging sheet contribute to CD4 binding and co-receptor interactions during viral infection (Wyatt & Sodroski, 1998).

The gp120 sequence reveals the existence of five variable regions (V1 through V5) distributed with five conserved regions (C1 to C5), (Figure 2-5) (Starcich *et al.*, 1986; Burton *et al.*, 2012). Each of the variable loops, excluding V5 is made of a loop structure formed by a disulfide bond at its base (Wilen *et al.*, 2012).

The five conserved regions which have a critical contribution to the folding and function of the gp120 are mainly located in the gp120 inner domain, while the variable loops are predominantly sited at the protein surface and play a role in immune evasion by presenting a constantly moving target for the host immune system. Additionally, variable loop 3 plays an important part in receptor binding (Poignard *et al.*, 2001; Hartley *et al.*, 2005; Wilen *et al.*, 2012). The binding of the CD4 receptor to the Env protein results in rearrangements of V1/V2 loops and subsequently V3 loop. The CD4 binding also leads to the formation of the bridging sheet, a four-stranded β sheet comprised of two double-stranded β sheets that are spatially separated in the unliganded state (Figure 2-5) (Kwong *et al.*, 1998; Chen *et al.*, 2005). The bridging sheet and repositioned V3 loop play a critical role in the next step of virus entry, the coreceptor engagement (Wyatt & Sodroski, 1998; Wang & Zhang, 2020).

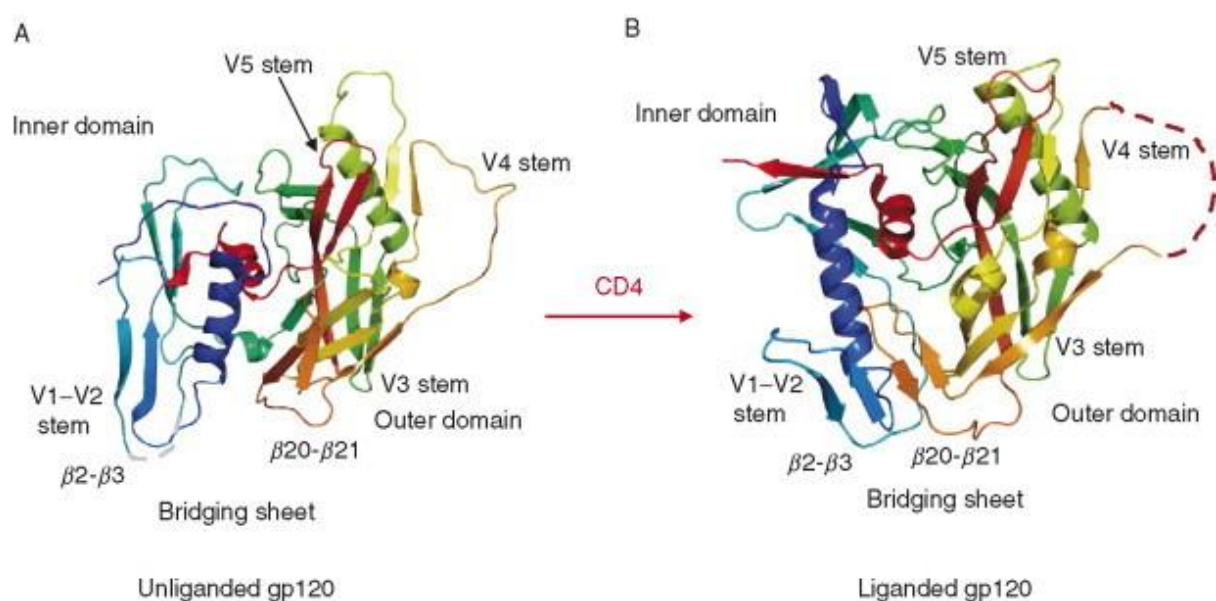


Figure 2-5: Structure of gp120 core in unliganded and liganded states. The diagram indicates the V1V2, V3, V4 and V5 stems (A) Diagram of the unliganded SIV gp120 core. (B) Diagram showing the 3D-structure of HIV-1 liganded gp120 core. The outer domains (in green and yellow) of liganded and unliganded gp120 are relatively conserved while a dramatic change in the inner domain (blue and cyan) occurs. β -strands making up the bridging sheet are proximal to each other in the liganded gp120 core, while in the unliganded structure they are displaced. Adapted from Prabakaran *et al.* (2007).

The V1-V2, V4 and V5 regions of the gp120 all display remarkable differences in length, and V1-V2, and V4 are quite different in glycosylation. However, the differences found in the V1-V2 region are significantly associated with variability in viremia, disease progression and the course of infection (Curlin *et al.*, 2010). For example, the length of the V5 loop tends to be highly variable during acute infection but the length decreases over the course of infection (Curlin *et al.*, 2010). Moreover, the variable loops have an accumulation of mutations which cause extensive structural variability by changing the composition and length of the loops as well as changing variable loop glycosylation patterns. This in turn prevents them

from interacting with antibodies, thus offering virus protection (Wyatt & Sodroski, 1998; Wood *et al.*, 2009).

The gp120 is more glycosylated than the gp41 and accounts for the overall heavy glycosylation of the Env protein (Doores, 2015). The gp120 has a higher density of N-linked glycans (Zhu *et al.*, 2000) and possibly O-linked glycans (Go *et al.*, 2015) than gp41 (Doores, 2015). Glycosylation in the Env region is essential for the proper conformation of the gp120 protein (Li *et al.*, 1993). Although it was reported that glycans are not directly involved in CD4 binding (Li *et al.*, 1993), they contribute to the infectivity of HIV (Wang *et al.*, 2013). This was shown when mutations were introduced to glycosylation sites on or near the V1-V2 domain, and the mutants had reduced infectivity or no infectivity (Wang *et al.*, 2013). Moreover, the virus uses mechanisms such as deleting, adding or moving glycans from antibody epitopes to escape the humoral immune system, mostly in highly variable regions with less conserved glycans. Several glycosylated sites that are highly conserved are associated with bNAb epitopes (Behrens & Crispin, 2017).

2.2.3 Gp41 of the HIV-1 envelope

During infection, gp120 sheds the gp41 protein upon binding to the receptor and this initiates viral and cellular membrane fusion, a process that results in the release of viral contents into the host (Weissenhorn *et al.*, 1997; Harrison, 2008). The gp41 connects the infectious spike to the viral membrane and contributes a major role to viral entry into the cell. It is made up of approximately 345 amino acids with a molecular mass of 41 kDa with no clearly defined variable regions and is more conserved compared to gp120 (Montero *et al.*, 2008; Checkley *et al.*, 2011). It is divided into three major domains: an extracellular domain (or ectodomain), a transmembrane domain (TMD), and a C-terminal cytoplasmic tail (CT) (Figure 2-6) (Helseth *et al.*, 1991; Checkley *et al.*, 2011).

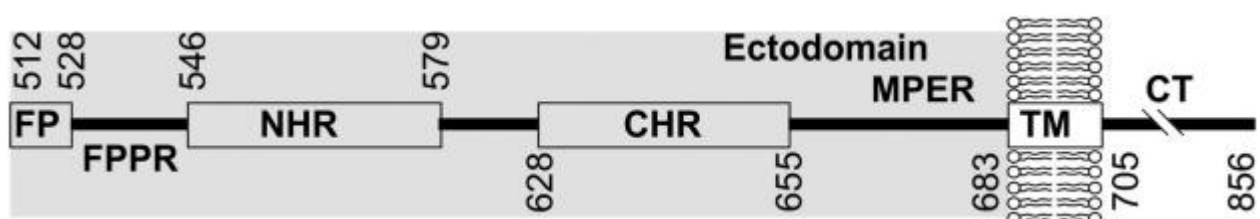


Figure 2-6: Diagram that shows the different regions of gp41. FP is the fusion peptide, FPPR: is the fusion peptide proximal region, NHR is the N-terminal heptad repeat, CHR is the C-terminal heptad repeat, MPER is the membrane proximal external region, TM is the trans-membrane domain, and CT is a cytoplasmic tail. Amino acid numbering is based on the HIV-1 strain HXB2. Adapted from Aisenbrey and Bechinger (2020).

The extracellular domain consists of regions that are major determinants of HIV-1 fusion: an N-terminal hydrophobic region known as the fusion peptide, a polar region, two hydrophobic regions that form α -helical coiled-coil structures referred to as the heptad-repeat regions HR1 (N-helix) and HR2 (C-helix), and a Trp-rich domain named the membrane-proximal external region (MPER) (Checkley *et al.*, 2011). The fusion peptide (FP) is generally buried in the gp120/gp41 quaternary complex and is an amino acid terminal hydrophobic region that forms part of the major fusion determinants of the extracellular domain (Freed *et al.*, 1990), together with the HR1 and HR2 regions (Gallaher *et al.*, 1989; Delwart *et al.*, 1990) and the MPER. HR1 folds into a central triple-stranded coiled coil of α -helices, and the three C-terminal segments (HR2) packed, antiparallel, as α -helices into the three grooves of the coiled-coil forming a six-helix bundle (6HB) (Chan *et al.*, 1997; White *et al.*, 2008). Conformational changes that take place after the formation of 6HB cause the formation of a pore that permits passage of the viral nucleocapsid into the host cell (Gallaher *et al.*, 1989; Delwart *et al.*, 1990; Markosyan *et al.*, 2003). Moreover, MPER residues contribute by stabilizing the membrane-interactive end of the 6-helix bundle conformation to initiate membrane fusion (Lay *et al.*, 2011).

Overall, the involvement of the envelope glycoprotein during the initial entry of HIV-1 into the host cells makes it an ideal target for vaccine development as it is one of the processes that ensure viral infection of target cells.

2.3 HIV prevention strategies

Various strategies are successful in preventing HIV-1 transmission. The use of condoms plays a big role in preventing the spread of HIV-1. There is also substantial evidence that supports male circumcision as a means to greatly reduce the risk of HIV-1 transmission (Weiss *et al.*, 2010; Cork *et al.*, 2020). The removal of the foreskin during circumcision is believed to reduce HIV target cells and also reduce the bacterial load, ultimately reducing bacteria linked to HIV transmission (Dinh *et al.*, 2011; Liu *et al.*, 2013; Prodger & Kaul, 2017). Genital bacteria infect the Langerhans cells (LC) of the foreskin which play a role in mediating HIV infection. However, these cells generally degrade HIV particles, that is, the presence of active bacterium-associated inflammatory mediators, active sexually transmitted infection (STI) and HIV load, activates the LCs to bind and present HIV particles to CD4⁺ T cells (de Witte *et al.*, 2008; De Jong & Geijtenbeek, 2009). Thus, male circumcision has been shown to reduce HIV-1 acquisition and protect males by up to 60% (Auvert *et al.*, 2005; Bailey *et al.*, 2007; Gray *et al.*, 2007; Cork *et al.*, 2020). Despite the doubt expressed in a meta-analysis by Millett *et al.* (2008) towards male circumcision in reducing HIV-1 acquisition in men who have sex with other men, the World Health Organization (WHO) and The Joint United Nations Programme on HIV/AIDS (UNAIDS) recommended that voluntary medical male circumcision be added to HIV prevention methods in high prevalence regions (World Health Organization, 2007). However, the challenge is that some men do not believe it is an effective method of HIV prevention

(Wamai *et al.*, 2011), and in other countries, the procedure is conducted in ways that may result in serious complications (Lawal & Olapade-Olaopa, 2017). Moreover, in some developing countries barriers such as fear of undertaking the procedure, assumed surgical complications and cost implications, have been reported (Nxumalo & Mchunu, 2020; Masese *et al.*, 2021).

HIV-1 treatment has also gained the attention of researchers and healthcare workers over the years as a method to prevent transmission. One of the interventions of treatment that has shown success is the use of antiretroviral therapy (ART) to prevent mother-to-child transmission (Connor *et al.*, 1994; Shaffer *et al.*, 1999; Barron *et al.*, 2013). The mother-to-child prevention strategy works successfully mostly by providing protection during pregnancy and child labour, where most transmission events occur (Paintsil & Andiman, 2009). Also, the use of ART has been shown to reduce transmission of HIV-1 in heterosexual relationships where one partner is HIV-1 positive and the other is negative (Attia *et al.*, 2009; Donnell *et al.*, 2010). In a clinical trial, HIV Prevention Trials Network 052 (HPTN 052), where two study groups (therapy administered immediately or after CD4 cell count declined) received ART, there was a 96% overall reduction in HIV-1 transmission (Cohen *et al.*, 2011). Moreover, the provision of ART before or after HIV exposure known as pre-exposure prophylaxis (PrEP) or post-exposure prophylaxis (PEP), respectively, is currently the most effective method of HIV suppression. The use of Tenofovir disoproxil fumarate and emtricitabine (TDF–FTC) as PrEP by HIV-negative individuals protects against HIV-1 acquisition (Peterson *et al.*, 2007; Baeten *et al.*, 2012; Murchu *et al.*, 2022). These methods of prevention have thus far helped in the control of HIV-1, however they demand adherence and are more prone to discontinuation (Abdool Karim *et al.*, 2010). Evidence has shown that these methods are effective when tested at an individual level, thus there is a need to introduce these methods in larger populations (Krishnaratne *et al.*, 2016).

Despite the number of existing prevention methods, there is a need for an HIV-1 vaccine (Gray *et al.*, 2016). Considering that a vaccine is given once with a few possible boosters, the administration of an HIV-1 vaccine would provide a more affordable prevention plan for the population compared to the life-long administration of ARV therapy (Harmon *et al.*, 2016). HIV vaccine researchers worldwide have tested many different approaches but a vaccine that offers significant protection is yet to be discovered (Kim *et al.*, 2021). Recent vaccine-based studies are aimed at inducing broadly neutralising antibodies (bNAbs), which have the advantage of neutralising a wide spectrum of HIV-1 subtypes (del Moral-Sánchez & Sliepen, 2019). Moreover, research on passive immunization using bNAbs has been greatly explored in vaccine development as a potential method to prevent and treat HIV-1 infections in humans (Klein *et al.*, 2012; Caskey *et al.*, 2015; Lynch *et al.*, 2015a; Corey *et al.*, 2021).

2.4 Broadly neutralising antibody response to HIV infection

During the first 2-4 years of infection, about 10-30% of HIV-1 infected individuals develop antibodies cross-reactive with viruses isolated from other infected individuals (Gray *et al.*, 2009) and of different subtypes (Walker *et al.*, 2009; Walker *et al.*, 2011). Generally, antibodies are produced because of activation of B cells. The activation of B cells and their differentiation into antibody-secreting plasma cells is triggered by the antigen and usually requires helper T cells (Charles *et al.*, 2001). Development of bNAbs is associated with a number of factors, such as the duration of infection, high viral load and low CD4+ count (Sather *et al.*, 2009; Gray *et al.*, 2011). Early infection generally does not trigger broadly neutralising capabilities, however, persistent infection with multiple rounds of viral escape and the development of antibody somatic hypermutation are essential for the development of bNAbs that recognize more conserved epitopes (Landais & Moore, 2018). One other key factor is the high level of circulating T follicular helper cells and germinal center (GC) function, which then supports the antibody somatic hypermutation essential for continued maturation (Cohen *et al.*, 2014). On the other hand, low levels of T regulatory cells, possibly enabling survival of B-cell intermediates with potential for autoreactivity, are also associated with development of bNAbs (Moody *et al.*, 2016). All bNAbs have at least one of the following characteristics : a) self-/polyreactivity, b) elongated, and highly hydrophobic (and/or charged) heavy chain third complementarity determining regions (HCDR3), and c) high numbers of Ig somatic mutations (Verkoczy *et al.*, 2011). Early infection events shape the development of neutralisation breadth of bNAbs in chronic infections (Piantadosi *et al.*, 2009). Furthermore, subtype-specific envelope features have been shown to influence the nature of bNAbs. Specifically, CD4bs-directed bNAbs were associated with subtype B infection, while V2 glycan-specific bNAbs developed in individuals with non-subtype B infection (Rusert *et al.*, 2016).

Just like most strain-specific antibodies, bNAbs can inhibit cell-free and cell-to-cell viral entry, induce cell phagocytosis and macrophages or antibody-dependent cellular cytotoxicity (ADCC) by Natural killer (NK) cells and stimulate dendritic cells (DC) to mature by binding to HIV envelope sites through Fc receptor interactions (Stephenson & Barouch, 2016). However, bNAbs can neutralise a wide range of globally circulating viral strains, and as such these antibodies are a major focus for HIV vaccine design (Klein *et al.*, 2013). The bNAbs show a wide range of breadth and potency when tested against global viral isolates (Walker *et al.*, 2009; Wu *et al.*, 2010; Moore, Penny L. *et al.*, 2011; Walker *et al.*, 2011; Doria-Rose *et al.*, 2016).

There is great progress made with advanced methods of isolation and characterization of antibodies after the hybridoma technology, which was the first technology developed for the isolation of monoclonal antibodies (mAbs) (Köhler & Milstein, 1975). The hybridoma technology produced antibodies by fusing short-lived antibody-secreting cells (ASCs) with myeloma cells to immortalize them and screen the

supernatant of the immortal clones for the presence of antigen-specific antibodies (Parray *et al.*, 2020). Other technologies are currently used to isolate a wider range of mAbs and this has also improved the understanding of their development (Sun *et al.*, 2018; Pedrioli & Oxenius, 2021). The advancement of antibody isolation by B cell culturing systems has led to retrieving the sequences of significant bNAbs against HIV-1 (Wilson & Andrews, 2012; Pedrioli & Oxenius, 2021) and have promoted passive immunization studies by isolating panels of potent bNAbs that led to new insights into the fundamentals of antibody-mediated neutralisation of HIV (Sun *et al.*, 2018). Scheid *et al.* (2016) as well as Tiller *et al.* (2008) used the antigen-specific memory B cell sorting method combined with single-cell polymerase chain reaction (PCR), to isolate antibodies from individual memory B cells. The HIV Env trimer such as BG505 SOSIP664 trimer which mimic the HIV-1 envelope trimer, have been used for antibody studies (Kong *et al.*, 2013; Sanders *et al.*, 2013; Julien *et al.*, 2015). Such a trimer presents epitopes which allow the binding of various bNAbs, while excluding non-neutralising antibodies (non-NAbs) (Sanders *et al.*, 2013; Julien *et al.*, 2015). Other methods that contributed to bNAb isolation are the identification of CD4bs bNAb N49P7 from plasma using proteomics and antibody lineage (Sajadi *et al.*, 2018) and next-generation sequencing (Wu *et al.*, 2011; Zhu *et al.*, 2013; Wu *et al.*, 2015). Thus, efforts have been invested in studying and isolating bNAbs in search of effective immunogens against HIV.

2.4.1 Targets of bNAbs on HIV envelope

Analysis of structure and sequence mapping of antibody-antigen complexes have allowed the identification of numerous epitopes targeted by bNAbs such as PG9 (Priddy *et al.*, 2019) and CAP256-VRC26.25 LS (Mahomed *et al.*, 2020) that binds to the V1-V2 region, 10E8, 2F5 and 4E10 binding to gp41 MPER (Figure 2-8) (Burton *et al.*, 1994; Zwick *et al.*, 2001; Kwon *et al.*, 2016), 10-1074 (Caskey *et al.*, 2017) and PGT121 (Moldt *et al.*, 2012) binding to the V3-glycan supersite and N6LS (Walsh & Seaman, 2021), b12 and VRC01 (Wu *et al.*, 2010; Lynch *et al.*, 2015a; Lynch *et al.*, 2015b) are CD4bs mAbs. The gp120-gp41 interface is targeted by antibodies that are immune to the conformational change of Env triggered by CD4 molecules (35O22 and 8ANC195) (Wang & Zhang, 2020), gp41 fusion peptide located at the N terminus of gp41 (Kong *et al.*, 2016) (Figure 2-6) and the silent face targeted by VRC-PG05 and SF12 (Zhou & Xu, 2018; Schoofs *et al.*, 2019; Wang & Zhang, 2020).

For example, the cross-neutralising activity of antibodies has been observed in some plasma samples that do not match the binding specificities of any of the known epitopes (Wibmer *et al.*, 2013; Ditse *et al.*, 2018), demonstrating the possibility of other significant specificities yet to be characterized (Ndlovu *et al.*, 2020).

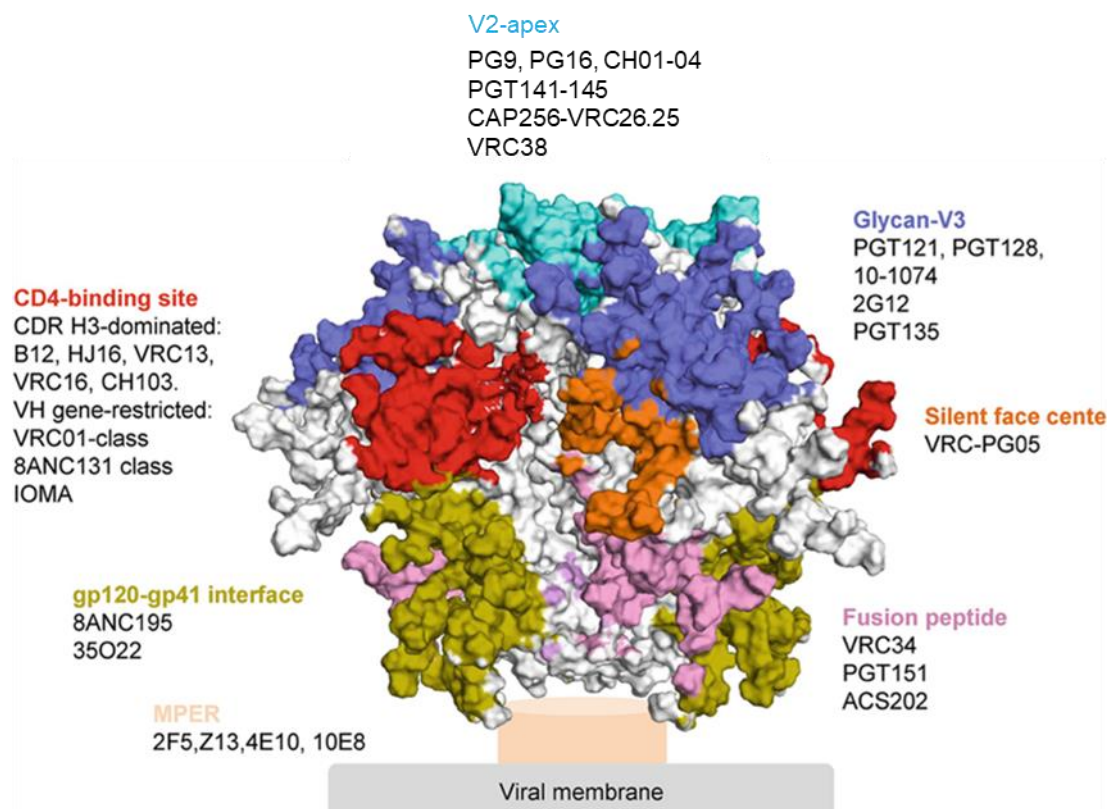


Figure 2-7: Major sites of vulnerability to neutralising antibodies on the pre-fusion closed HIV-1 Env trimer. The target sites for CD4-binding site antibodies, the V1V2 apex antibodies, the glycan V3 antibodies, the fusion peptide-targeting antibodies, the gp120-gp41 interface antibodies, and the MPER antibodies are coloured on the trimeric HIV-1 Env. The epitope for VRC-PG05 targeting the centre of the silent face is also shown in orange. Adapted from Zhou and Xu (2018).

Although antibodies directed against the CD4bs and MPER display greater breadth and potency to diverse HIV strains (Sajadi *et al.*, 2018), according to Conti *et al.* (2021), MPER binds fewer bNAbs and binds antibodies in a mode that is more diversified compared to CD4bs or the V1-V2 and V3 mAb binding sites. The CD4bs is of particular interest because antibodies against this site can prevent interaction with CD4, the primary receptor for viral entry (Zhou *et al.*, 2007; Conti *et al.*, 2021) and because they demonstrate outstanding potency and breadth (Huang *et al.*, 2016; Schommers *et al.*, 2020).

CD4bs bNAbs are divided into two distinct classes based on their development and mode of recognition: CDRH3 (complementarity-determining region-3 of the heavy chain) dominated or VH-gene-restricted. According to Zhou *et al.* (2015), CD4bs antibodies belonging to the VH-gene-restricted class share similar orientations, in reference to the angle of approach, and has similar orientation between the heavy chain and light chain with respect to the Env spike (Figure 2-8). The CDRH3-dependent bNAbs interact with the CD4bs by the CDRH3 loop, and the VH-gene-restricted are CD4 mimics (Zhou *et al.*, 2015). The CDRH3-dominated bNAbs are further subdivided into four classes: the CH103, HJ16, VRC13, and VRC16 classes,

meanwhile the VH-gene restricted are divided into two classes: the VRC01-class antibodies which are derived from VH1-2, and the 8ANC131-class antibodies which are derived from VH1-46 (Scheid *et al.*, 2011; Zhou *et al.*, 2015; Stamatatos *et al.*, 2017). The naming of the subclasses is based on the first antibody isolated from that class (Stamatatos *et al.*, 2017). There are, however, antibodies classified under one subclass but with structural features similar to another subclass. For example, a new anti-CD4-BS bNAb (IOMA) is derived from VH1-2, but shares structural features of both VRC01-class and 8ANC131-class antibodies (Stamatatos *et al.*, 2017; van Schooten *et al.*, 2022). This study focused on the CD4bs bNAb VRC01 which falls under the VRC01-class antibodies.

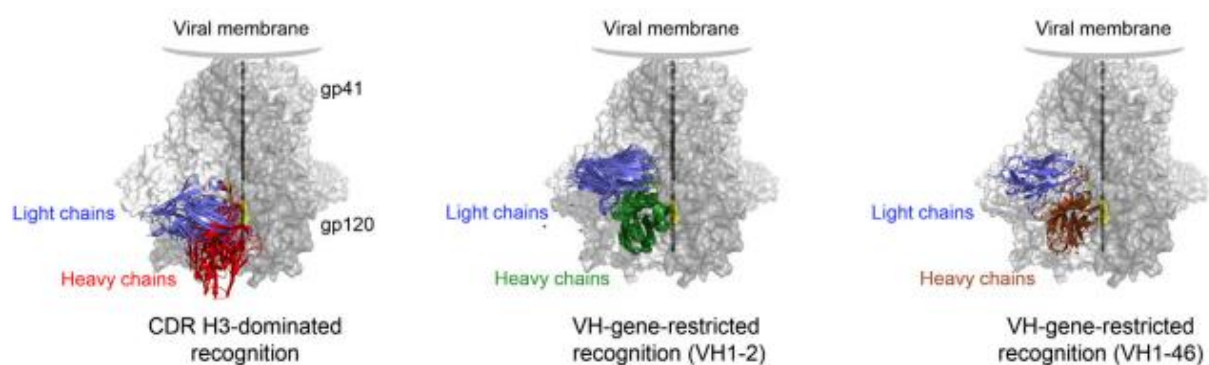


Figure 2-8: Different broadly neutralising CD4bs antibody binding orientations shown as CDR H3-Dominated and VH-Gene-restricted recognitions. A blue colour indicates the light chains, and heavy chains are indicated by: red for chains CDR H3-dominated antibodies, green for VH1-2-gene-restricted antibodies, and brown for VH1-46-restricted antibodies. Adapted from Zhou *et al.* (2015).

2.4.1.1 The broadly neutralising antibody VRC01

The VRC01 bNAb was isolated from a 65-year-old African American homosexual man infected with HIV-1 subtype B for 20 years without symptoms of infection and antiretroviral therapy (ART) (Wu *et al.*, 2010). The discovery of this bNAb was the result of RSC3 protein probing which also identified two other antibodies of the same class (VRC02 and VRC03) (Wu *et al.*, 2010). More antibodies (VRC06, VRC07, VRC08, NIH45-46, NIH45-177, NIH45-243, VRC06, and VRC06b) were also isolated from the same individual (Scheid *et al.*, 2011; Li *et al.*, 2012; Wu *et al.*, 2015). Although VRC01 was isolated from a subtype B infected individual, it neutralised about 90% of diverse viral strains when a panel of 190 HIV strains of major circulating subtypes, were assessed for potency and neutralisation breadth compared to other mAbs (Wu *et al.*, 2010; Lynch *et al.*, 2015b). Similarly, about 78% of virus isolates from Zambian infants (Nakamura *et al.*, 2013) and five of clade C founder viruses from Malawian-infected infants were neutralised by VRC01 (Russell *et al.*, 2013). VRC01 not only demonstrate the breadth of neutralisation of clinical HIV-1 isolates (Wu *et al.*, 2010; Sok & Burton, 2018) but also prevents simian HIV infection in nonhuman primates (Moldt *et al.*, 2012; Ko *et al.*, 2014; Pegu *et al.*, 2017; Hessel *et al.*, 2018).

Although antibodies of the VRC01-class share common structural features, such as the use of VH1-2 allele, 5AA CDRL3 and a short/ flexible LCDR1(Wu *et al.*, 2010; Zhou *et al.*, 2015; Huang *et al.*, 2016; Sajadi *et al.*, 2018), VRC01 is characterized by an unusually short five amino-acid light chain complementary-determining region (CDR) L3 loop (Zhou *et al.*, 2015) and higher levels of somatic hyper-mutation, than antibodies targeting other pathogens (Wu *et al.*, 2015). The antibody targets a conserved region of the CD4bs on the HIV envelope gp120 (Wu *et al.*, 2010; Lynch *et al.*, 2015b). VRC01 mimic the CD4-gp120 interaction, and this interaction was used to explain its broad activity (Wu *et al.*, 2010). VRC01 focuses its binding onto a conformationally invariant domain, that is, the site of initial CD4 attachment, which allows the antibody to overcome the glycan and conformational masking that diminishes the neutralisation potency of most CD4bs antibodies (Wu *et al.*, 2010). In a study by Watkins and colleagues, it was found that VRC01 avoids conformational masking by the V2 loop which increases the efficacy of VRC01 towards HIV-1. This was shown in an R5-tropic clade C SHIV (SHIV-C) model (Watkins *et al.*, 2011). They noted that although CD4bs is an attractive epitope for VRC01, avoiding conformational masking by the V2 loop may retain optimal exposure of the conserved CD4bs giving VRC01 an advantage of increased neutralisation potency. Additionally, instead of exactly mimicking the CD4 receptor binding, it was further found that VRC01 binds gp120 at a 45° angle relative to the orientation of CD4 binding to Env (Wu *et al.*, 2010). This angle is preferred for the binding of bNAbs to the CD4bs by a family of VRC01 variants and in genetically diverse bNAbs (Zhou *et al.*, 2015; Lynch *et al.*, 2015b). One of the significant ways that VRC01 binds to gp120 is by the amino acids on the VRC01 light chain, tyrosine and serine at position 28 and 30, respectively, making contact with the protein-proximal N-acetylglucosamine from the N-linked glycan at residue 276 of the gp120 (Zhou *et al.*, 2010).

2.4.1.2 VRC01 bNAb in human trials

VRC01 clinical studies began in 2013 and were conducted to examine the safety, tolerability, dose and pharmacokinetics of the antibody in HIV-1 infected adults [VRC 601 (NCT01950325)] (Lynch *et al.*, 2015a), and uninfected adults [VRC 602 (NCT01993706) (Ledgerwood *et al.*, 2015) and HVTN 104 (NCT02165267) (Mayer *et al.*, 2017)]. This was supported by models such as popPK model, which was designed to identify VRC01 serum concentration levels sufficient for protection against HIV infection at a population level (Huang *et al.*, 2017). Furthermore, the safety, tolerability and favourable pharmacokinetic properties of VRC01 have been reported in infants born to women with HIV-1 (Cunningham *et al.*, 2020). The Fc mutated version of VRC01, termed VRC01-LS displayed a higher affinity for neonatal Fc receptor

(FcRn), with a half-life that was threefold more than VRC01 (Ko *et al.*, 2014; Zhang *et al.*, 2016). VRC01-LS is identical to VRC01, but contains two amino acid changes (M428L and N434S). This is one of the modifications incorporated to enhance bNAbs with more stability and offer more protection *in vivo* for therapy and potentially passive immunization (Pace *et al.*, 2013). Another study focused on HIV-exposed infants and assessed the safety and pharmacokinetics of VRC01, its Fc mutated version VRC01-LS, and the related antibody VRC07-523LS (NCT02256631) (ClinicalTrials.gov). It was further tested in two similar human clinical trials, ACTG A5340 [NCT02463227] and NIH 15-I-0140 [NCT02471326], in which fully suppressed patients on stable ART underwent an analytic treatment interruption (ATI) after receiving 40 mg/kg of VRC01 (Bar *et al.*, 2016). Thus, VRC01 moved through four phase 1 human clinical trials (VRC601, VRC602, A5340, and HVTN 104) and advanced to testing for HIV-1 prevention efficacy in humans in the Antibody Mediated Prevention (AMP) phase 2b trials.

2.4.2 Passive immunotherapy for HIV-1 prevention using bNAbs

The administration of antibodies by intramuscular or intravenous infusions has proven over the years to protect against different types of infections (Pollard & Bijker, 2021). Passive immunization has been used in diseases such as diphtheria, tetanus, hepatitis B and botulism; however, licensed immunoglobulins are only available for anthrax, respiratory syncytial virus in premature infants and rabies (Marston *et al.*, 2018; Sparrow *et al.*, 2019; Mahomed *et al.*, 2021). The use of antibodies for the treatment of HIV-1 infection was first tested in 1992 using infusions of pooled polyclonal antibodies (Vittecoq *et al.*, 1992; Stephenson & Barouch, 2016), and in 1998 the first monoclonal antibody was tested in HIV-1 infected individuals (Cavacini *et al.*, 1998). There are a few human pre-clinical trials that have been conducted using bNAbs that showed the potential to protect animals from simian-human immunodeficiency virus (SHIV) infection and treat nonhuman primates (NHPs) (Pegu *et al.*, 2017; McCoy, 2018). Several potent bNAbs have progressed to human clinical trials since the year 2010 (Mahomed *et al.*, 2021). Mathematical modelling has further shown that combinations of three or four bNAbs are more effective in therapy compared to a single or double approach, an option that is likely to be successful for vaccination (Wagh *et al.*, 2016). One of the initial studies to assess the combination therapy was the trial that tested mAbs 2F5, 2G12, and 4E10 that were used in MABGEL 1 for topical application in the genital tract of healthy females (Morris *et al.*, 2014). The study assessed the pharmacokinetics and safety of these mAbs in combination. Other studies include a combination of PGT121 + PGDM1400, PGT121 + VRC07-523LS + PGDM1400 as well as VRC01 + 10-1074 (clinicalinfo.hiv.gov, 2021). Safety and tolerability was reported when PGDM1400 antibody was administered intravenously, alone or in combination, with the bNAbs PGT121 and VRC07-523LS in participants with and without HIV (Julg *et al.*, 2022). Although extensive neutralization breadth of PGT121 + PGDM1400 combination was observed in participants, viral rebound in the presence of selected resistance mutations occurred. For PGT121 + VRC07-523LS + PGDM1400, viral rebound was observed in all participants after a single intravenous infusion of each antibody (Julg *et al.*, 2022). VRC01

+ 10-1074 combination study was terminated due to inability to recruit because of the COVID-19 pandemic (ClinicalTrials.gov, 2021). More studies showed that when 3BNC117 was used in combination with 10-1074 or alone, it was safe when administered intravenously to HIV-uninfected individuals (Schoofs *et al.*, 2016; Caskey *et al.*, 2017). The combination of the two antibodies was also effective in suppressing the virus and delaying viral rebound in HIV-infected participants who discontinued ART (Mendoza *et al.*, 2018; Caskey, 2020). These bNAbs progressed to subcutaneous administration, and their improved candidates with an enhanced half-life, have been tested in phase 1 human clinical trials (Mahomed *et al.*, 2021).

Although bNAbs have been generally safe and well tolerated, there are still antibodies and virus/host interaction factors that challenge their use in passive immunization (Figure 2-9). For example, the administration of 10E8VLS bNAb that targets the MPER caused a reaction on the injection site with grade 3 erythema, which was associated with fever and malaise and as a result, the clinical trial (NCT03565315) was terminated (ClinicalTrials.gov). Likewise, an increased resistance was observed with 3BNC117 which indicated selection for viral escape variants towards the bNAb (Caskey *et al.*, 2015). Similarly, HIV-1 demonstrated a high rate of resistance against VRC01, a bNAb which had a progressive trial and demonstrated safety and tolerability with stable pharmacokinetics (Lynch *et al.*, 2015b; Mayer *et al.*, 2017; Otsuka *et al.*, 2018; Corey *et al.*, 2021). Therefore, the observations from these studies emphasize the need for more studies that focus on resistance mutations that compromise the efficacy of bNAbs in humans.

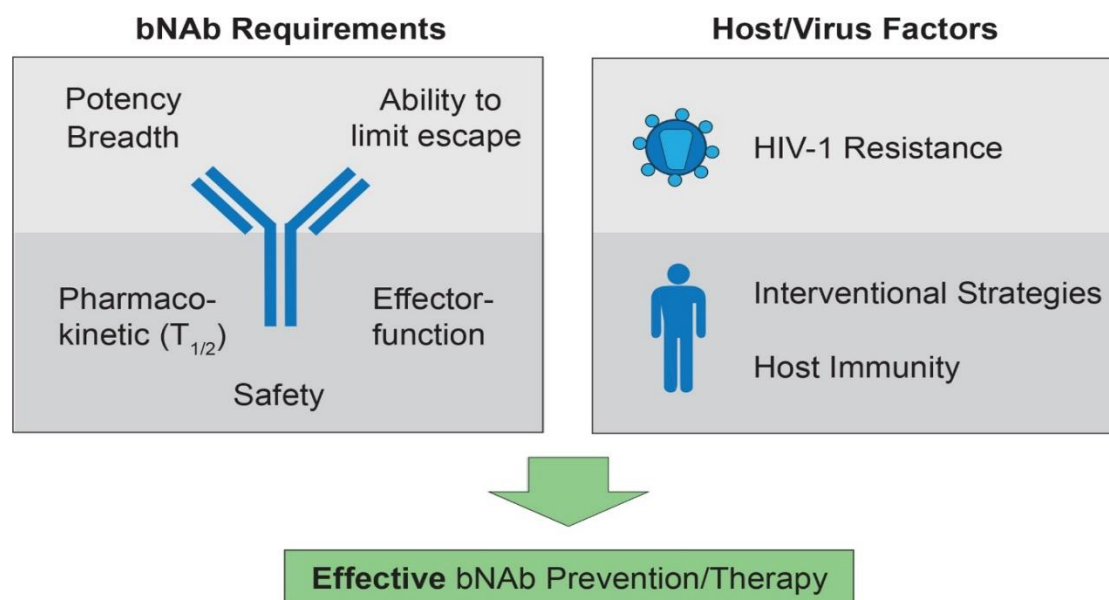


Figure 2-9: Schematic representation characteristics of the bNAb and virus/host factors. Characteristics of the bNAb and virus/host factors determine the efficacy of passive immunotherapy and the prevention of HIV-1. Adapted from Caskey *et al.* (2019).

2.4.2.1 Antibody Mediated Prevention (AMP) trial

The AMP trial evaluated the safety and efficacy of the 8-week intravenous infusions of VRC01 in two study populations at risk of being infected with HIV-1, that is, adult women in sub-Saharan Africa (HVTN 703/HPTN 081; ClinicalTrials.gov #NCT02568215) and men and transgender persons in Brazil, Peru, Switzerland, and the United States who have sex with men (HVTN 704/HPTN 085; ClinicalTrials.gov #NCT02716675). Participants received infusions of VRC01 at a dose of either 10 or 30 mg/kg (low-dose group and high-dose group, respectively) or placebo, every 8 weeks for 10 infusions in total. The participants were tested for HIV-1 every 4 weeks (Ledgerwood *et al.*, 2015; Corey *et al.*, 2021). For both trials, the prevention efficacy was the same for each dose. Although this bNAb was reported to neutralise a large percentage of HIV-1 in many studies (Wu *et al.*, 2010; Nakamura *et al.*, 2013; Russell *et al.*, 2013; Lynch *et al.*, 2015b), the results from the AMP trial were different. VRC01 did not prevent HIV-1 infection by resistant viruses (Corey *et al.*, 2021). It however showed 75% protective efficacy to the VRC01 sensitive viruses which accounted for 30% of the isolates (Walker, 2021).

It was suggested that at the early stages of infection, VRC01 applied pressure on the virus and may have suppressed replication, and gradually resistant isolates emerged over time (Corey *et al.*, 2021). Although the AMP results did not produce the anticipated outcome, the analysis of VRC01-sensitive HIV-1 isolates provided proof of concept that bNAb prophylaxis can be effective in VRC01-sensitive isolates (Corey *et al.*, 2021). The virus resistance is therefore presenting itself as a formidable challenge in the use of VRC01 as an HIV prevention method. The results of the AMP study demonstrated that most circulating HIV-1 strains are resistant to VRC01 and most studies suggest that bNAbs that target different epitopes should be used in combination.

2.4.3 HIV-1 resistant mutations to VRC01 bNAb

Although VRC01 is not yet used for vaccination, it provides a good model of CD4bs bNAb to study how HIV-1 escapes inhibition by such antibodies. VRC01 still fails to neutralise a significant portion of virus panels despite its superior potency and breadth. For example, about 10% of tested viruses were not neutralised by VRC01 in a study by Wu *et al.* (2010). In another study, a group of individuals who discontinued ART and had repeated infusions of VRC01 sustained viral suppression for a median of about 4 weeks. However, a viral rebound occurred despite VRC01 serum concentrations above 50 µg/ml and it was concluded that the effects of the antibody were limited by extensive pre-existing resistance to VRC01 (Bar *et al.*, 2016). Furthermore, VRC01, 3BNC117 and N6-resistant strains were observed in both animal models and human clinical trials (Lynch *et al.*, 2015b; Schoofs *et al.*, 2016), indicating that resistant virus strains occur naturally (Zhou *et al.*, 2019). It is therefore vital to understand how HIV-1 escapes immunization by VRC01 bNAb.

The ability of the virus to mutate its Env sequence and remain functional is a critical antibody evasion strategy (Wei *et al.*, 2003; Mascola & Montefiori, 2010). However, HIV-1 Env develops multiple mechanisms of immune evasion, including conformational masking, glycosylation, and sequence variation (Kwong *et al.*, 2002; Chen *et al.*, 2009; Burton & Mascola, 2015; Cuevas *et al.*, 2015; Zhou *et al.*, 2015; Zhou *et al.*, 2017). Evasion by conformational masking involves the covering of highly conserved bNAb targets, such as the CD4bs, from antibody neutralisation in the prefusion Env conformation (Kwong *et al.*, 2002).

Glycosylation directly impacts protein folding, enhances stability, and prevents the aggregation of proteins (Helenius *et al.*, 2001). The molecular mass of gp120 of the HIV-1 Env is made up of about 50% glycans (Leonard *et al.*, 1990), and as a result, a large number of conserved epitopes on gp120 are often shielded as the outer domain is heavily glycosylated (Leonard *et al.*, 1990). This shielding of conserved epitopes is one of the various factors that has hindered the development of an Env-derived immunogen capable of eliciting bNAbs against HIV-1 (Rathore *et al.*, 2014). N-linked glycosylation sites play a critical role in protecting the virus from neutralisation by monoclonal antibodies (Wang *et al.*, 2013). In a molecular dynamics simulation of the BG505 SOSIP.664 Env trimer with all N-linked sites modelled with Man5GlcNAc2 (the smallest high mannose N-linked glycans commonly found), the addition of N-linked glycans at sites N197, N276, N362, and N462 blocked access of VRC01 (Stewart-Jones *et al.*, 2016). Another N-linked glycosylation, particularly in the β 23/loop V5/ β 24 region (N461) substitution was reported to confer resistance to VRC01 (Guo *et al.*, 2012; Zhou *et al.*, 2017; Zhou *et al.*, 2019). Even though N-linked glycosylation sites that surround the CD4bs sterically limit recognition by bNAbs (Zhou *et al.*, 2017), particularly those present at position Asn276 in Loop D and along the V5 loop, mature VRC01 bNAbs overcome this barrier and potently neutralise numerous HIV-1 viral clades (Lynch *et al.*, 2015a; Huang *et al.*, 2016; Stewart-Jones *et al.*, 2016; Zhou *et al.*, 2017). Although there is enough evidence of VRC01 overcoming binding limitations by glycosylation, there are other sites that would still impose an obstruction for VRC01 access. For example, the high level of sensitivity of Clade 07_BC HIV-1 strain with combined mutations (N197D and N463Q) to VRC01 neutralisation indicate that the glycans at these sites play a critical role in shielding the virus from being recognized by the antibody (Wang *et al.*, 2013). This is an indication that HIV-1 may still develop either one or multiple glycosylation sites that work alone or in synergy to avoid recognition by VRC01.

The VRC01 antibody has 35 contact sites on Env, 22 which are in the highly conserved CD4 binding domain (Zhou *et al.*, 2010). The ability of the virus to mutate its Env sequence and remain functional is another important antibody evasion strategy (Wei *et al.*, 2003; Mascola & Montefiori, 2010). In some HIV-1 strains, Env mutates the residues to escape an antibody response and these residues also have an impact on viral infectivity. In several studies, when PNGS did not account for the lack of VRC01 susceptibility, variability of the sequences of the HIV-1 clones accounted for VRC01 resistance. In one study, amino acid

deletion at the V5 region of the Env was the cause of VRC01 resistance (Tachibana *et al.*, 2017). Otsuka and colleagues also identified a subset of the escape mutations identified using soft randomization *in vivo* studies and in natural isolates. They detected five residues that were frequently substituted in their study (N276, D279, A281, N461 and N465) (Otsuka *et al.*, 2018) and others that were previously reported (K276, L277, S278, R282, S460, S/D at residue 461, N/T at residue 462, and R463) in studies evaluating CD4bs bNAbs, VRC01 and 3BNC117 (Caskey *et al.*, 2015; Lynch *et al.*, 2015a; Bar *et al.*, 2016; Scheid *et al.*, 2016).

There are diverse pathways through which HIV-1 can escape CD4bs antibodies. A model that was used for predicting envelope features that contribute to VRC01 sensitivity helped to identify a vast number of amino acid features that confer VRC01 resistance in HIV-1 sequences (Magaret *et al.*, 2019). These features include N665, 279E, I471, M181, W456, H456, D459, S280, R425, D279, S456, Q455, M428 and T280 (Magaret *et al.*, 2019). This model further identified features such as the length of the Env, the length of gp120 and the total cysteines in the Env to be associated with VRC01 resistance. Although some of the features are novel to their study, most have been identified in previous studies. Four of the top-ranked amino acids reported in their study (D279, N280, R456, and G459) have been reported previously as sites of common interactions with potent VRC01-like antibodies (West *et al.*, 2012), and D279 and E459 have been identified as making critical interactions with VRC01 (Li *et al.*, 2011; Shang *et al.*, 2011). Furthermore, the mutation of residue D279 to E279 (D279E) was shown to be part of the VRC01 escape pathway in the donor from whom VRC01 was isolated (Lynch *et al.*, 2015b). In addition to the D279E mutation, an insertion of a glycan at position 276 made the virus completely resistant to VRC01 (Lynch *et al.*, 2015b). Several previous studies indicated that specific amino acid residues in loop D and β 23-loop V5 were strongly associated with resistance to the VRC01 class of antibodies (Klein *et al.*, 2012; West *et al.*, 2012). For example, the double mutation of loop D and β 23-loop V5 (N279K-R456W) resulted in complete resistance to the VRC01 class of antibodies (Lynch *et al.*, 2015b).

HIV-1 mutates and survives to evade VRC01 and enhance its replication fitness. Lynch *et al.* (2015b) reported a diminution of replicative capacity in envelope clones with certain mutations. Single mutations in β 23 (R456W or G458D) resulted in decreased viral replication. However, the double mutant (N279K-R456W) exhibited increased levels of replication, suggesting that the N279K mutation restored the replication capacity of the R456W mutant. Furthermore, the addition of glycan 276 alone or glycan 276 with D279E resulted in a marked attenuation of replicative capacity. This indicates that replication capacity is one of the features that are affected by mutations developed by HIV-1 to escape bNAb neutralisation.

2.4.3.1 VRC01 resistance in HIV-1 subtype C

It has been shown that certain bNAb classes demonstrate subtype resistance bias. For instance, CRF01_AE viruses are resistant to a large proportion of antibodies targeting the V3/glycans (Walker *et al.*, 2011).

Subtype A and C viruses are more sensitive than subtype B viruses to V2/apex-specific antibodies such as CAP256-VRC26.25 (Moore, P. L. *et al.*, 2011).

However, it has been shown that 20% of subtype C viruses are naturally more resistant to CD4bs bNAb VRC01 compared to subtype B viruses, with resistance increasing as the epidemic matures (Rademeyer *et al.*, 2016). CD4bs antibodies have enhanced potency against clade A viruses, and resistance signatures are relatively rare in clade A, in contrast, 3BNC117 and VRC01 have reduced breadth and potency against clade C viruses (Bricault *et al.*, 2019). There is therefore a need for studies to investigate and evaluate resistance mutations of the VRC01 bNAb to understand how HIV-1 mutates, particularly HIV-1 subtype C as it is more resistant towards VRC01 compared to other subtypes, also because this subtype is more prevalent in regions burdened with the HIV-1 pandemic (Hemelaar *et al.*, 2019; Gartner *et al.*, 2020) and accounts for most of the current HIV-1 infections (Elangovan *et al.*, 2021). Reports suggest that subtype C-HIV viruses demonstrate a reduced replication capacity compared to other group M viruses and may cause a slower disease progression, which in turn may increase the opportunity for new transmission events (Gartner *et al.*, 2020).

Therefore, this study identified mutations in the Env sequences of breakthrough HIV-1 subtype C viruses of the AMP trial and validated their ability to cause resistance towards VRC01. The IC₅₀ titres were compared to other CD4bs and non-CD4bs antibodies to assess mAb cross-resistance in relation to VRC01.

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CHAPTER 3 MATERIALS AND METHODS

3.1 HIV envelope plasmids, sequences, antibodies and cells

Ethics approval for this study was granted by the North-West University Health Research Ethics Committee (NWU-HREC). Ethics number: NWU-00171-21-A1.

In this study, we used HIV-1 subtype C envelope (Env) plasmids and sequences that were resistant to VRC01 bNAb, from the three groups (placebo, 10 mg/kg and 30 mg/kg) of the HVTN 703/HPTN 081 AMP trial. Seven VRC01 resistant Env plasmids of HIV-1 subtype C and the pSG3ΔEnvbackbone plasmid were obtained from the National Institute of Communicable Disease (NICD), Johannesburg, South Africa. Three Env plasmids/sequences were from the 30 mg/kg group (V703_1255WT, V703_0132 WT and H703_0132 WT), two of which are quasi-species (V703_0132 WT and H703_0132 WT). Two Env clones (H703_0902 WT and H703_1798 WT) were from the 10 mg/kg group while two (V703_0217 WT and V703_1298 WT) were from the placebo group. Five broadly neutralizing monoclonal antibodies and HEK293T and TZM-bl cells were also received from the NICD. The antibodies were CD4bs bNABs (VRC01, VRC07-523LS and 3BNC117), and 10E8 and PG9 mAbs (targeting the MPER and V2 epitopes, respectively). The cell lines (HEK293T and TZM-bl cells [JC53BL-13]) were maintained in complete Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Massachusetts, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Thermo Fisher Scientific) and 50 µg/ml gentamicin (Thermo Fisher Scientific) and incubated at 37°C, 5% CO₂. Cell monolayers were split at 70-80% confluency or after 48 hours of incubation.

3.2 Sequence analysis of VRC01-resistant viruses

The HIV LANL database (Los Alamos National Laboratory, 2021) was used to obtain previously identified VRC01-resistant mutations from the latest features described in the CATNAP (Compile, Analyze and Tally Nab panels). Amino acid changes in the breakthrough sequences were identified by aligning to the HXB2 HIV-1 reference sequence using Aliview software (Larsson, 2014). HXB2 is a subtype B sequence derived from the first HIV-1 isolate (Hahn *et al.*, 1984) that is used as a reference strain for the HIV genome. All the identified putative-resistant residues were reverted towards the sensitive residues in order to construct VRC01-sensitive Env sequences to generate pseudoviruses from the sequences. The putative-resistant residues were identified in four regions: C1, loop D, β23 and β24 of the Env sequences.

3.3 Prediction of VRC01 sensitivity of the identified mutations

Following sequence analysis and identification of the mutations, FASTA files of aligned mutant Env sequences together with their corresponding wild type sequences were sent for machine learning analysis at Fred Hutchinson Cancer Research Center (FHCRC), Seattle, Washington, USA. The analysis was used to predict the sensitivity of the reverted mutant Env sequences towards the VRC01 antibody. The machine learning software measures the predicted probability that a virus will be sensitive to VRC01. It predicts the IC_{80} and the probability of sensitivity for any given Env, however, it does not predict which mutation leads to a change in VRC01 sensitivity. According to the software, a higher predicted IC_{80} indicates a more resistant virus than a lower predicted IC_{80} .

3.4 Generating mutations by site-directed mutagenesis

Site-directed mutagenesis was then used to substitute the putative-resistant mutations with sensitive residues in order to convert the VRC01-resistant Envs to VRC01-sensitive Envs. Site-directed mutagenesis was performed using QuickChange Lightning multi-site directed mutagenesis kit (Agilent Technologies, California, USA) to introduce individual site mutations of interest by annealing mutagenic primers to the denatured DNA template as shown in Figure 3-1 (Hogrefe *et al.*, 2002).

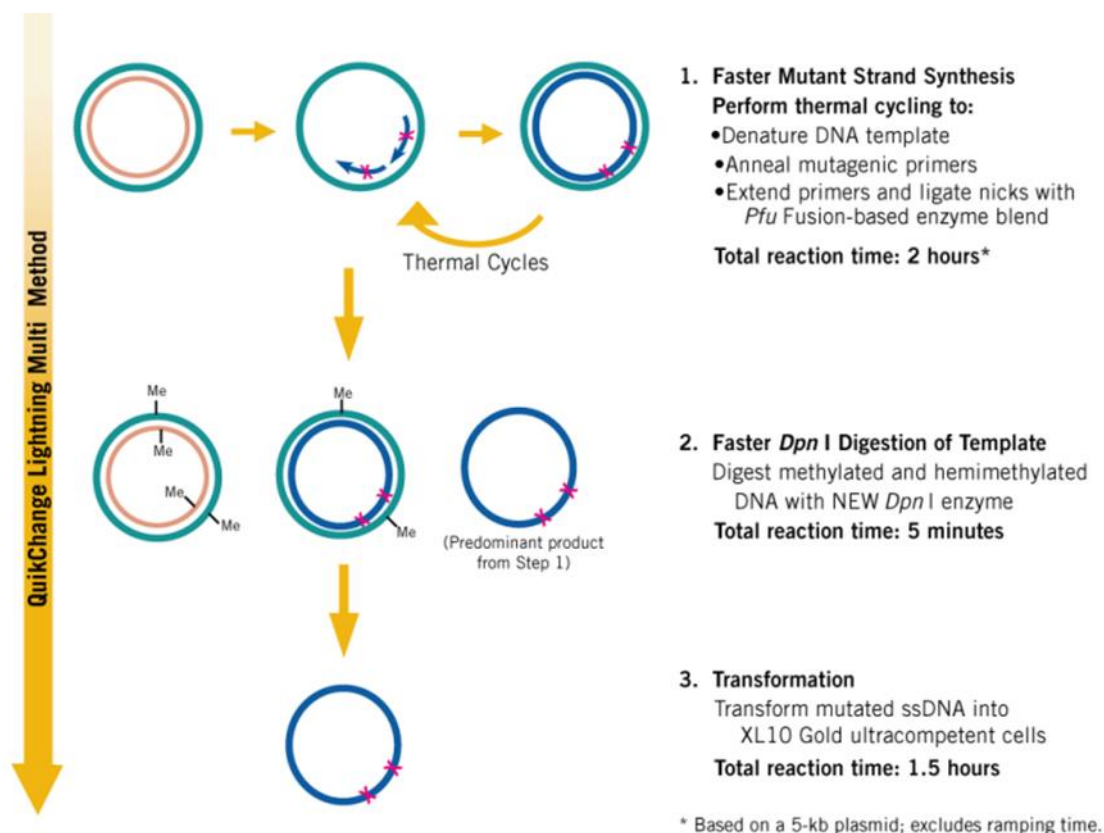


Figure 3-1: Diagram of an overview of the QuickChange Lightning multi-site directed mutagenesis system. Adapted from Agilent Technologies

The template DNA that was used was the HIV-1 Env of the wild type in the pcDNA3.1/V5-His-TOPO plasmid (Genewiz, South Plainfield, NJ). The mutagenesis process involved the synthesis of the mutant strands by PCR using a proofreading DNA polymerase (Hemsley *et al.*, 1989; Hogrefe *et al.*, 2002). Primers containing the mutations of interest (Table 3-1) were designed using the OligoCalc website which calculates the primers' melting temperatures and prevents potential internal secondary structures (Kibbe, 2007). The procedure used the reagents outlined in Table 3-2 following the thermocycling conditions in Table 3-2.

Table 3-1: Sequences of primers used in site-directed mutagenesis.

Primer name	Sequence
V703_1298 E455T	5'- GGA CTA TTA TTA ACA CGT GAT GGA GGA AAA GAT AAT AAC ACA GAG -3'
V703_0132 W456R	5'- ATC ACA GGA CTA CTA TTG ACA CGG GAT GGA AAT -3'
H703_0132 W456R	5'- ATC ACA GGA CTA CTA TTG ACA CGG GAT GGA AAT - 3'
V703_1255 Q471G	5'- AGA AGT AAT GAG ACA TTC AGG CCT GGA GGA GGA AAT ATG AAG GAC -3'
V703_0217 E279D	5'- GAG ATA ATA ATT AGA TCT GAA AAT CTG ACA GAC AAT ACT AAA ACA - 3'
V703_1255 D99N	5'- ATG TGG AAA AAT AAC ATG GCA GAT CAG ATG CAT GAG -3'
V703_1298 D99N	5'- ATG TGG GAA AAT AAC ATG GTG GAT CAG ATG CAT GAG -3'
H703_0902 T471G	5'- ACA GGG GAG ATA TTC AGA CCT GGA GGA GGA AAT ATG AAA GAC -3'
H703_0902 K279D	5'- AGA TCT GAA AAT TTG ACA GAC AAT ATC AAA ACA ATA ATA GTC CAC - 3'
H703_1798 T471G	5'- ATA AAC GAG ACA TTC AGA CCT GGA GGA GGA GAT ATG AGG AAC -3'
H703_1798 E455T	5'- TCA AAC ATC ACA GGA CTA CTA TTA ACA CGT GAT GGA GGC ACC -3'

Table 3-2: Site-directed mutagenesis reaction.

Reagents	Volume
10× QuikChange Lightning Multi reaction buffer	2.5 µl
QuikSolution	1 µl
ds-DNA template	1 µl (100 ng)
QuikChange Multi enzyme blend	1 µl
Mutagenic primer	1 µl (100 ng each primer)

Reagents	Volume
dNTP mix	1 μ l
Distilled water	17.5 μ l

Table 3-3: Thermocycling conditions for mutagenesis reactions.

Cycle step	Number of cycles	Temperature	Time
Initial denaturation	1	95°C	20 sec
Denaturation	30	95°C	2 min
Annealing		55°C	30 sec
Extension		65°C	4 min
Final extension	1	65°C	5 min
Hold	-	4°C	Until use

The mutagenesis reactions were digested using the Dpn I restriction endonuclease enzyme (Agilent Technologies) which specifically digests methylated or hemimethylated DNA. The dsDNA vectors are propagated in *Escherichia coli* (*E. coli*) strains which are dam⁺, that is, with methylated (5'-Gm6ATC-3') DNA. Briefly, 1 μ l of Dpn I enzyme was added to the mutagenesis reaction mixture and incubated at 37°C for 1h30 min. This ensured that the methylated wildtype plasmid was digested leaving the newly synthesized and non-methylated DNA strand with mutations to be transformed.

3.5 Transformation

The mutated non-methylated single-stranded DNAs were absorbed by the *E. coli* cells where they are converted into double-stranded DNAs, through the transformation process. XL10-Gold ultracompetent cells (Agilent Technologies) were thawed on ice and incubated together with β -mercaptoethanol (BME) (Agilent Technologies) to destroy nucleases that may have reduced transformation efficiency. About 2 μ l of each mutagenesis reaction was added to pre-chilled tubes containing 45 μ l of the ultracompetent cells and 2 μ l of BME. Tubes were heat shocked at 42 °C for 30 sec to increase the fluidity of bacterial membranes for the penetration of DNA into cells, followed by incubation on ice for 2 minutes. Some 500 μ l of SOC (super optimization broth with catabolite repression) media (Thermo Fisher Scientific) were added to each tube and incubated at 37°C with shaking at 225 rpm. After 1 hour, the cells were plated on LB agar (Merck) containing carbenicillin (Merck) at 50 μ g/ml and cultured at 37°C for 16-20 hrs. Four colonies were then picked from each agar plate and cultured in 10 ml LB broth (Merck) containing ampicillin at 37°C for 16-20 hrs.

3.6 Plasmid DNA purification

The plasmids were purified using QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). Briefly, the Luria broth culture prepared as described in Section 3.5 was pelleted at 3 500 g for 15 min and the bacterial pellet was then re-suspended in 250 µl buffer P1. The RNase enzyme was added to buffer P1 prior to its use to efficiently degrade the liberated RNA during the alkaline lysis. About 250 µl of lysis buffer P2 was added to the resuspended cells and mixed gently (without vortexing which can cause shearing of DNA) in order to lyse the cells. The P2 buffer contains sodium dodecyl sulphate (SDS) detergent which solubilizes the phospholipids and proteins of the cell membrane resulting in cell lysis and the release of the cell contents. The mixture was then precipitated upon the addition of 350 µl of buffer N3. This buffer causes the large chromosomal DNA to be precipitated while the small plasmid DNA remains in solution. The supernatant which formed after centrifugation at 13 000 rpm for 10 min, was then transferred to a spin column to purify the plasmid DNA using the PE wash buffer. The plasmid DNA was eluted by adding 50 µl of elution buffer to the spin column and centrifuging for 1 min. The DNA was quantified using the NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific).

For quantification, the spectrophotometer was blanked using 2 µl of the elution buffer, and 2 µl of the DNA solution was used to measure the DNA concentration. The plasmid DNAs were later sequenced to confirm the presence of the mutations of interest.

3.7 Sanger sequencing

Sequencing was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) with the reaction mixture outlined in Table 3-4. The forward and reverse primers listed in Table 3-5 were added in separate wells for each mutant plasmid. Thermocycling conditions were followed as summarised in Table 3-6.

Table 3-4: Sequencing reaction reagents.

Reagents	Volume
BigDye™ Terminator v3.1 Ready Reaction Mix	1 µl
5X Sequencing Buffer	2 µl
Sequencing primers (2 pmol/µl)	3 µl
Mutant plasmid DNA (300 ng)	1 µl (300 ng)
Distilled water	3 µl

Table 3-5: Primers used for sequencing.

Primer name	Sequence (5'-3')
A589Re	CAGAGTGGGGTTAACTTTACAC
E220	TATCAAAATGGCTGTGGTATATAA
A589	GTGTAAAGTTAACCCCACTCTG
A590	AATCGCGAAACCAGCCGGCGCACAAT
EnvARev	TGCTGCTCCCAAGAACCCAA
EnvARev F	TTGGGTCTTGGGAGCAGCA
Mf	GGAGGAGATATGAGGGACAATTGG
EnvB	AGAAAGAGCAGAAGACAGTGGCAATGA
E85	GTCCCTCATATCTCCTCCTCCAGGTCT
Kr	CTTATAGCAGGCCATCC
Nr	GGTGAGTATCCCTGCCTAACTCTA
Nf	TGACCTGGATGCAGTGG
E220	TATCAAAATGGCTGTGGTATATAA
gp41Fo	GCCAGTGGTATCAACTCAAC

Table 3-6: Thermocycling conditions for sequencing reactions using BigDye™ Terminator v3.1 Cycle Sequencing kit.

Cycle step	Number of cycles	Temperature	Time
Initial denaturation	1	96°C	1 min
Denaturation	25	96°C	10 sec
Annealing		50°C	5 sec
Extension		60°C	4 min
Hold	1	4°C	Until use

Sequencing reactions were run on the Genetic Analyser and sequences were analysed using Sequencher 5.4 software (Gene Codes Corporation, Michigan, USA). Briefly, the analysis was as follows: the sequences of the wild-type Env gene, the generated Sanger sequences and the primer sequences (with mutations) were aligned and compared using the Sequencher software. Mutagenesis was considered successful when the generated Sanger sequences match the primer sequences rather than the wild type sequences. Subsequently, each mutated Env gene was sequenced completely using primers listed in Table 3-5 to ensure no off-site mutations were inserted.

3.8 Production of Env pseudoviruses

Env plasmids containing the desired mutations were co-transfected in HEK293T cells with the pSG3ΔEnv backbone plasmid that consists of the entire HIV-1 genome except for a functional Env gene. Co-transfection with this plasmid produces Env pseudoviruses that can infect cells but are incapable of producing progeny virions as they only undergo one cycle of replication. Briefly, HEK293T cells were trypsinized and a 5-fold dilution trypan blue (Merck) cell count was performed to determine cell viability and to prepare a concentration of 2×10^6 which was seeded in complete DMEM and incubated at 37°C, 5% CO₂ for 24 hrs. The cells were seeded in 10 cm² sterile cell culture dishes (Thermo Fisher Scientific). Transfection reactions were prepared by incubating 500 µl of serum-free DMEM with 24 µl of transfection reagent PEI max (made up to 1mg/ml with distilled water) followed by the addition of 4 µg of both the mutant Env plasmid and pSG3ΔEnv plasmid. This was prepared for each Env mutant to be transfected. The mixture was gently mixed and incubated for 30-45 min before being added dropwise into the 293T/17 cells. The transfected cells were incubated at 37°C in a 5% CO₂ environment for 48 hrs, after which the virus-containing supernatants were harvested. The supernatants were filtered through a 0.45 µm filter (Sartorius AG, Göttingen, Germany) to eliminate cell debris and 10% FBS was added to the viral stocks as a cryopreservant. The viral stocks were stored as 1 ml aliquots in sterile 1.5 ml screw cap cryogenic vials (Merck) and frozen at -80 °C until use.

3.9 TCID₅₀ assay

For each pseudovirus stock, a 50% tissue culture infectious dose (TCID₅₀) was measured. This identified a viral dilution at which the generated pseudovirus can infect 50% of the cell monolayers and was performed using TZM-bl cells. This cell line is derived from HeLa cells and expresses the CD4, CCR5 and CXCR4 receptors that are found in human cells. The cells also contain the firefly luciferase gene, under the control of the HIV-1 long-terminal repeat (LTR) which permits the measurement of viral infection by luminescence (Wei *et al.*, 2003). Infection of the cells induces the reporter luciferase gene that is regulated by HIV-1 tat protein (Platt *et al.*, 1998; Wei *et al.*, 2002; Wei *et al.*, 2003). The TCID₅₀ was measured by performing a five-fold serial dilution in duplicates, in complete DMEM, using sterile, flat bottom low evaporation 96-well culture plates (Merck). TCID₅₀ is a viral dilution that is able to cause infection and subsequently kill 50% of the cells.

The wells in the first two columns of the 96-well plate were used as cell control and virus control wells, respectively. About 100 µl of complete DMEM was added in all the wells. Pseudoviruses were added at 25 µl in the virus control wells, and sample wells (in duplicate for each virus to be tested) and serially diluted five-fold. The serial dilution was done by transferring and mixing 25 µl such that the end of the plate (row H) had highly diluted virus concentrations. Thereafter, the cells were prepared as described below.

TZM-bl cells at a confluency of 50-80% were rinsed in PBS, incubated with Trypsin-EDTA (0.25% trypsin, 1mM EDTA) (Thermo Fisher Scientific) for 4 min at 37°C, 5% CO₂, to detach them from the flask, and suspended in 10 ml of complete DMEM. A 5-fold dilution trypan blue (Merck) cell count was performed to determine cell viability and concentration to use for the assay. Thereafter 40 µg/µl of diethyl aminoethyl (DEAE)-dextran (Thermo Fisher Scientific) was added to the cell suspension to enhance viral infectivity (Manning *et al.*, 1971; Lopata *et al.*, 1984). Thereafter, 100 µl of the cells (1x10⁶ cells/ml) was added to all the wells of the 96-well plate (10 000 cells/well) with pseudoviruses. The culture plates were incubated at 37°C, 5% CO₂ for 48 hrs and monitored for virus-induced syncytium formation and cell killing by microscopic examination.

After the incubation, 100 µl of the culture supernatant was removed from each well and replaced with Bright-Glo Luciferase substrate (Promega, Wisconsin, USA) prepared as per the manufacturer's recommendation. The plates were incubated for 2 min at room temperature to allow cell lysis after which 150 µl of cell lysate was transferred to 96-well black solid plates (Merck). The luminescence was measured using either Victor 2 luminometer or Victor 3 1420 Multilabel Counter (Perkin-Elmer, Massachusetts, USA) and reported as RLUs of the pseudovirus dilution to use in the neutralisation assay.

3.10 Neutralisation Assay

The neutralisation assay was used to measure the ability of monoclonal antibodies (mAbs) to inhibit infection in TZM-bl cells. The first two columns of the 96-well plate were used as cell control and virus control wells, respectively (Figure 3-2).

	1	2	3	4	5	6	7	8	9	10	11	12	Increase Dilution
	Cell Control	Virus Control	Antibody 1		Antibody 2		Antibody 3		Antibody 4		Antibody 5		
A	50 µl complete DMEM + 20 µl Cells	25 µl complete DMEM + 25 µl PSV + 20 µl Cells	25 µl complete DMEM + 25 µl PSV + 20 µl Cells + serially diluted Antibody		25 µl complete DMEM + 25 µl PSV + 20 µl Cells + serially diluted Antibody		25 µl complete DMEM + 25 µl PSV + 20 µl Cells + serially diluted Antibody		25 µl complete DMEM + 25 µl PSV + 20 µl Cells + serially diluted Antibody		25 µl complete DMEM + 25 µl PSV + 20 µl Cells + serially diluted Antibody		↑
B													
C													
D													
E													
F													
G													
H													

Figure 3-2: Schematic layout of a typical neutralisation assay. Column 1 represents the cell control wells consisting of complete DMEM and cells. Column 2 represents the virus control wells consisting of complete DMEM, pseudovirus (PSV) and cells. Columns 3 to 12 represent wells with serial antibody dilutions incubated with PSV and cells in complete DMEM.

About 50 µl of complete DMEM were added to the cell control wells while 25 µl of complete DMEM, together with 25 µl of pseudovirus were added to the virus control wells. To the sample wells (columns 3-12) 25 µl of complete DMEM was added. Five monoclonal antibodies (mAbs) were also added in duplicates as shown in Figure 3-2, at a starting concentration of 25 µg/ml in row H with a total volume of 12.5 µl. The mAbs were titrated at 3-fold serial dilutions from row H to row A, resulting in row A having the most diluted antibody (Figure 3-2). Thereafter, 25 µl of the pseudovirus was added to all sample wells. The pseudoviruses were used at TCID₅₀ dilutions that yielded 50 000-150 000 RLUs. The serially diluted mAbs were incubated with the pseudoviruses in a total volume of 50 µl for 1 hr at 37°C, 5% CO₂.

The TZM-bl cells were added to the serially diluted wells at 2x10⁵ cells/ml (10 000 cells/well) with 14 µg/µl DEAE-dextran. The plates were incubated at 37°C, 5% CO₂ for 24 hrs. After the incubation, 130 µl of complete DMEM was added to all wells to replace fluid lost to evaporation, and the plate was incubated for a further 24 hrs. After the 48 hrs incubation, 100 µl of the culture was removed from each well before adding 100 µl of Bright-Glo Luciferase substrate as described above (Section 3.9). Luminescence was measured in RLUs as mentioned above. The measured luminescence is directly proportional to the number of infectious viral particles and inversely proportional to the ability of mAbs to block infection (Wei *et al.*, 2002; Montefiori, 2004). The antibody titres were reported as 50% inhibitory concentrations (IC₅₀) of the

mAbs needed to inhibit half of the viruses from infecting the cells. The neutralisation assays were repeated twice.

3.11 References

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CHAPTER 4 RESULTS

4.1 Background

VRC01 resistance is complex and has been associated with a number of amino acids in multiple regions of the envelope protein. VRC01 contact residues are found on the C1, D loop, the CD4 binding loop, β 20/ β 21 loop, the β 23-loop, V5 and β 24 regions of gp120, and several studies have identified a number of mutations associated with VRC01 resistance in HIV-1 isolates in these regions (Wang et al., 2015; Lynch et al., 2015b; Otsuka et al., 2018; Magaret et al., 2019). This study utilized functional env clones and sequences generated from the AMP trial (HVTN 703/HPTN 081) to identify features that correlate with VRC01 neutralisation resistance (clinicalinfo.hiv.gov, 2021). The viral sequences were generated and analysed from both the placebo and the experimental groups. Two viral sequences belonged to the placebo group (V703_0217 WT & V703_1298 WT) and five were from the experimental group: two (H703_0902 WT & H703_1798 WT) belonged to the 10 mg/kg and three (V703_1255 WT, H703_0132 & V703_0132 WT) belonged to the 30 mg/kg. The wild-type VRC01-resistant viruses and their mutated partners (putatively towards a sensitive phenotype) were tested in order to find out whether these reversions restored neutralisation sensitivity to VRC01 and other CD4bs-directed antibodies. Complete sensitivity is described in this study as IC_{50} values of ≤ 0.01 to $1.0 \mu\text{g/ml}$, partial sensitivity as IC_{50} of ≥ 1.0 to $24.9 \mu\text{g/ml}$, and complete resistance as IC_{50} values of $\geq 25 \mu\text{g/ml}$ (mAbs starting concentration). The mutations that were identified to cause VRC01 resistance were assessed *in silico* through machine learning, in addition to the experimental data that was generated *in vitro*.

4.2 Analysis of VRC01 resistant sequence Envs

HIV-1 subtype C Env sequences were available for the breakthrough viruses that were discovered in the African trial (HVTN703/HPTN 081) (www.clinicaltrials.gov). Compile, Analyze and Tally NAb Panels (CATNAP) database (Yoon *et al.*, 2015) was used to access the latest VRC01-related features, which allowed the identification of all the amino acids and sites that relate to either sensitivity or resistance of VRC01. The amino acid changes in the breakthrough sequences were identified with the use of Aliview software (Larson, 2014), by aligning the breakthrough Env sequences to the HXB2 reference strain retrieved from the Los Alamos HIV Sequence Database. The alignment in Figure 4-1 below highlights the mutations identified in the Env regions; C1, Loop D, β 23 and β 24 sheets.

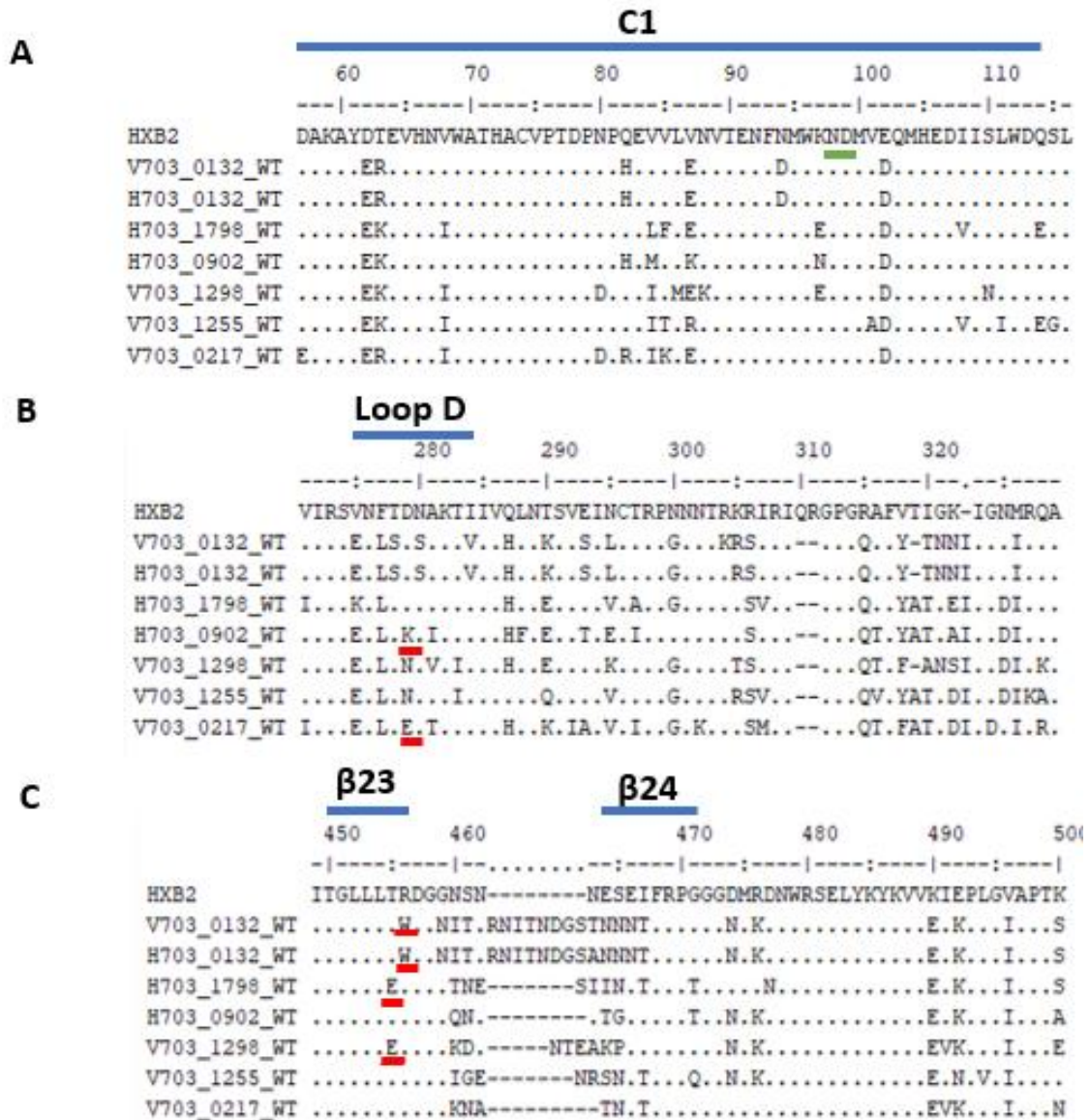


Figure 4-1: Sequence alignment of resistant wildtype (WT) viruses with HXB2 reference strain in the a) C1 region, b) Loop D region and c) β23 and β24 regions. Mutations identified and associated with VRC01 are underlined with a red line and their respective regions are depicted with a blue line and the region name. Residue 99 (underlined in green) is an exception as it did not have an amino acid change in the selected WT, however, it is listed on CATNAP as an Env feature associated with VRC01 resistance. Numbering is based on the HXB2 strain.

According to the CATNAP database, the Aspartic acid (D) in position 99 confers resistance towards VRC01 and other CD4bs mAbs such as 3BNC117, 8ANC131 and CH31, hence it was included in this study, however it was evaluated in two Env clones, V703_1255 WT and V703_1298 WT (Figure 4-1). In the D loop region, two mutations K279 and E279 are also listed in the CATNAP database and this study identified them in wild-type viruses, H703_0902 WT and V703_0217 WT, respectively (Figure 4-1). Another mutation, arginine (R) to tryptophan (W) at position 456 in the β23 loop captured in the database to cause

VRC01 resistance, was also identified in both V703_0132_WT and H703_0132_WT (Figure 4-1). Four other wild-type viruses that were included in this study had mutations at sites 455 and 471 in β 23 and β 24 sheets, respectively and the sites are also listed in the CATNAP database to cause VRC01 resistance. They are H703_1798_WT which has both E455 and T471 mutations, V703_1298_WT with E455 mutation, H703_0902 WT with T471, and V703_1255 WT with Q471 mutation (Figure 4-1).

VRC01 contact residues are found in regions such as loop D, the CD4 binding loop, and the V5 regions of gp120. The study identified VRC01-resistant sites in C1, Loop D, β 23 and β 24 regions as shown in Figure 4-1 the VRC01 resistant. The sites were therefore assessed and validated for their role in VRC01 resistance and a possible newly emerged VRC01-resistant mutation was also identified.

4.3 Confirmation of mutations in gp120

The study reverted the identified sites to residues commonly found in VRC01-sensitive viruses. Following site-directed mutagenesis (SDM), which was performed to introduce the putative sensitive amino acids into the wildtype resistant Env plasmids, the region of mutation was sequenced, followed by sequencing of the full Env gene as described in Chapter 3, Section 3.8, to ensure that the rest of the Env remained unchanged after SDM. Below is the figure that shows the sequencing chromatograms of each mutation introduced at different sites.

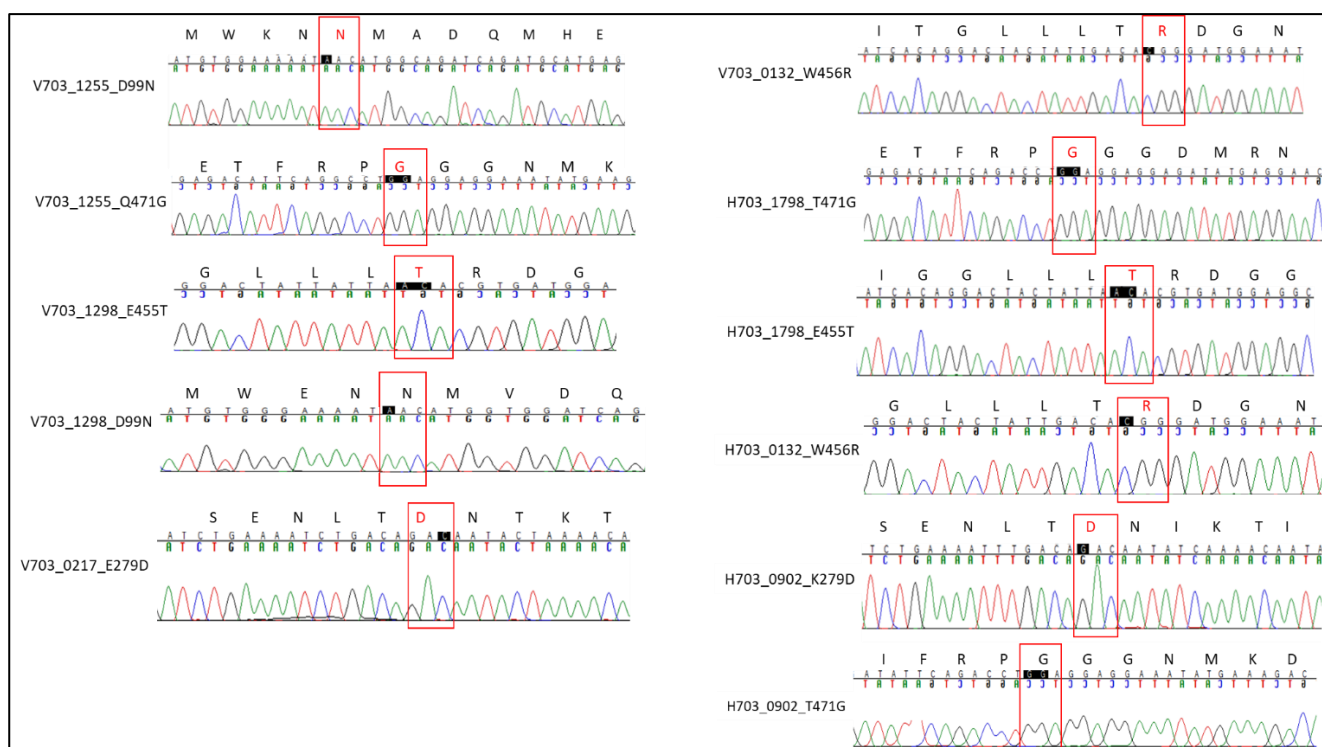


Figure 4-2: Sanger sequencing chromatograms for each mutation that was introduced. The mutated residues are highlighted in a red box. The peaks are colour coded for each DNA base; red represents thymine (T), blue, cytosine (C), green, adenine (A) and black, guanine (G). Amino acid sequences are shown above each chromatogram.

4.4 Assessment of the functionality of mutant pseudoviruses

Env-pseudotyped viruses were produced by co-transfecting HIV-1 mutant Env plasmids together with the pSG3ΔEnv backbone plasmid into 293T/17 cells. The same was performed using HIV-1 wildtype Env plasmids to serve as controls. The co-transfection generates pseudoviruses capable only of a single-entry event, which simplifies measurements of pseudovirus infectivity and neutralisation. It generates pseudoviruses that have functional envelope genes that can infect susceptible cells; however, they are unable to produce infectious progeny virions as a result of an incomplete HIV-1 genome. Viral transfections were unsuccessful in some of the plasmids including H703_1798_T471G, V703_1255_Q471G, H703_0132_W456R and V703_0132_W456R mutant thus excluded from downstream *in vitro* assays.

The Env-pseudotyped viruses were tested for entry capacity using TZM-bl cells (Chapter 3, Section 3.10), which express the luciferase gene under a Tat responsive promoter, to confirm the functionality of each mutated virus. The luciferase gene expression is measured in Relative Light Units (RLU), and higher RLU values indicate a greater chance of a successful generation of functional pseudoviruses. The results in Table 4-1 confirmed that the mutations did not affect viral infectivity in some of the mutant Env plasmids while others lost their infectivity and were incapable of mediating viral entry into TZM-bl cells.

Table 4-1: Confirmation of the functional nature of wildtype (WT) and mutant viruses. The relative light unit (RLU) values obtained for each mutant are a measure of maximum infectivity in comparison with 100% infectivity of the WT strains.

Virus name	Maximum infectivity in RLUs
H703_0132 WT	943,523 (100%)
H703_0132_W456R	1,848 (0.2%)
V703_0132 WT	670,459 (100%)
V703_0132_W456R	2,096 (0.3%)
H703_1798 WT	304,693 (100%)
H703_1798_E455T	250,181 (82%)
H703_1798_T471G	1,640 (0.5%)
H703_0902 WT	1,064,996 (100%)
H703_0902_K279D	1,369,085 (128%)
H703_0902_T471G	810,112 (76%)
V703_1255 WT	412,772 (100%)
V703_1255_D99N	122,694 (27.7%)
V703_1255_Q471G	1,989 (0.5%)
V703_0217 WT	172,423 (100%)
V703_0217_E279D	85,557 (49.6%)
V703_1298 WT	152,695 (100%)
V703_1298_D99N	23,416 (15%)
V703_1298_E455T	16,293 (10.7%)

Mutants with arginine mutation at residue 456 on the β 23 region (H703_0132_W456R and V703_0132_W456R) were completely non-infectious with a cut-off of less than three-fold when compared with the wildtype strains. Similarly, glutamine (Q) and threonine (T) in residue 471 on the β 24 region caused a complete loss of infectivity in mutants V703_1255_Q471G and H703_1798_T471G. However, mutant H703_0902_T471G was infectious and equivalent to its wild type and higher than the other two 471 mutants. H703_0902_T471G mutant had an original K279 residue (not reverted to a sensitive residue) known for its compensatory effect on viral fitness, which may explain its ability to be more functional with higher RLU values compared to clone H703_1798 with the same mutation (T471G). Other mutants such as V703_1298_E455T, V703_0217_E279D and both D99N mutants had lower infectivity compared to wild-type strains. Mutant H703_0902_K279D had a maximum infectivity of 28% more compared to the wild-type strain. Interestingly, mutant V703_0217_E279D had a reduced infectivity of 50% when compared with the wild-type strain. Although H703_0902 and V703_0217 had E279 and K279,

respectively, mutated to aspartic acid which is common in globally circulating viruses, their infectivity profile was different. Other mutations that occurring in other regions of the Env gene may affect the viral fitness.

4.5 Assessment of neutralisation sensitivity of the mutants to VRC01 and other CD4bs bNAbs

Neutralisation assays were conducted using the mutated pseudoviruses to test their sensitivity to the VRC01 antibody and other CD4bs bNAbs (VRC07-523LS and 3BNC117), as well as 10E8 and PG9 mAbs that were used as controls as described in Chapter 3, Section 3.11. The reversion of resistant viruses to VRC01-sensitive residues increased the neutralisation sensitivity of some of the tested antibodies while others remained resistant as shown in Table 4-2. As expected, VRC01 bNAb did not neutralize the wild-type resistant viruses as shown with IC₅₀ values of ≥ 25 $\mu\text{g/ml}$ (Table 4-2). However, the wild type and mutant pseudoviruses reserved their sensitivity towards 10E8 and PG9 mAb controls.

Table 4-2: Neutralisation data represented as IC₅₀ values of the wildtype and mutant viruses against VRC01 and four other mAbs (VRC07_523LS, 3BNC117, 10E8 and PG9).

Viruses	mAbs				
	VRC01	VRC07_523LS	3BNC117	10E8	PG9
V703_1255 WT	>25	0.17	>25	0.26	0.11
V703_1255_D99N	17.21	0.26	>25	0.43	0.09
V703_0217 WT	>25	0.04	20.39	0.09	0.03
V703_0217_E279D	0.14	0.08	0.05	0.14	0.05
H703_1798 WT	>25	0.37	>25	0.14	0.08
H703_1798_E455T	>25	0.15	1.00	0.05	0.06
V703_1298 WT	>25	0.73	>25	0.46	0.05
V703_1298_D99N	>25	7.47	>25	0.42	0.05
V703_1298_E455T	0.47	0.15	0.18	0.42	0.04
H703_0902 WT	>25	8.00	>25	1.15	0.02
H703_0902_K279D	0.02	<0.01	0.08	1.96	0.02
H703_0902_T471G	>25	>25	>25	1.02	0.02

Red denotes IC₅₀ values of ≤ 0.01 to 1.0 $\mu\text{g/ml}$, orange, ≥ 1.0 to 10 $\mu\text{g/ml}$, yellow, ≥ 10 $\mu\text{g/ml}$ to 25 $\mu\text{g/ml}$, which represents moderate neutralisation and blue, ≥ 25 $\mu\text{g/ml}$ which represents neutralisation resistance.

4.5.1 Neutralisation sensitivity of the C1 region mutation

According to the CATNAP database, residue 99D causes resistance to VRC01 and to CD4bs mAb 3BNC117 which has a similar mode of interaction as VRC01 and VRC07_523LS. This study reverted the aspartic acid to asparagine (N) to investigate whether the reversion would cause the virus envelope to become sensitive to VRC01. Indeed VRC01 neutralised the V703_1255_D99N clone with an IC_{50} value of 17.21 $\mu\text{g/ml}$ (indicating moderate (neutralisation)) (Table 4-2). However, no impact on sensitivity was observed for 3BNC117 as the clone remained resistant while VRC07_523LS mAb remained effective against the clone (Figure 4-3). In contrast, mutant V703_1298_D99N did not exhibit sensitivity to VRC01 (Figure 4-3b) and it caused a 24-fold decrease in sensitivity towards VRC07_523LS mAb (Figure 4-3b) with an IC_{50} decrease of 0.73 for wildtype to 7.47 $\mu\text{g/ml}$ (Table 4-2).

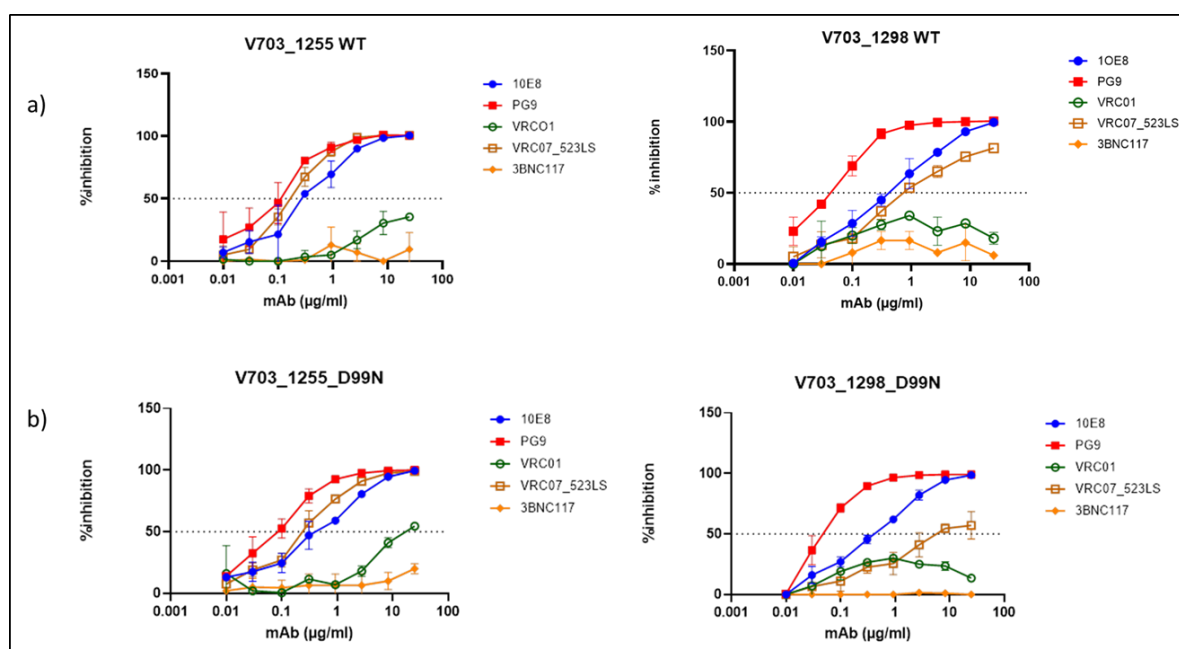


Figure 4-3: Neutralisation sensitivity of a) wildtype viruses V703_1255 and V703_1298 and b) their respective C1 region mutants, V703_1255_D99N and V703_1298_D99N, to CD4 binding bNAbs (VRC01, VRC07-523LS and 3BNC117), MPER (10E8) and V2 (PG9) bNAbs. The x-axis represents mAb concentration in $\mu\text{g/ml}$, and the y-axis represents the neutralisation percentage.

4.5.2 Neutralisation sensitivity of the loop D mutations

The loop D mutations that were performed in this study showed that when 279E and 279K mutations in wild-type clones V703_0217 WT and H703_090 WT (Figure 4-4a) were reverted to a putative sensitive residue 279D, the mutant clones V703_0217_E279D and H703_0902_K279D had increased sensitivity towards VRC01 (Figure 4-4b) with IC_{50} values of 0.14 $\mu\text{g/ml}$ and 0.02 $\mu\text{g/ml}$, respectively (Table 4-2). A similar finding was observed for 3BNC117 mAb where the wild-type clone with 279K was resistant (Table

4-2, Figure 4-4b), however, reversion to a putative sensitive residue in mutant H703_0902_K279D, resulted in sensitivity towards 3BNC117 (Figure 4-4b) with an IC_{50} of 0.08 $\mu\text{g/ml}$. Also V703_0217_E279D exhibited increased sensitivity towards the mAb with an IC_{50} value change from 20.39 $\mu\text{g/ml}$ to 0.05 $\mu\text{g/ml}$ resulting in a 408-fold increased sensitivity (Table 4-2). Interestingly, the mutant H703_0902_K279D also showed enhanced sensitivity towards VRC07_523LS mAb from an IC_{50} of 8.00 $\mu\text{g/ml}$ (H703_0902 WT) to <0.01 $\mu\text{g/ml}$ (800-fold increased sensitivity) (Table 4.2).

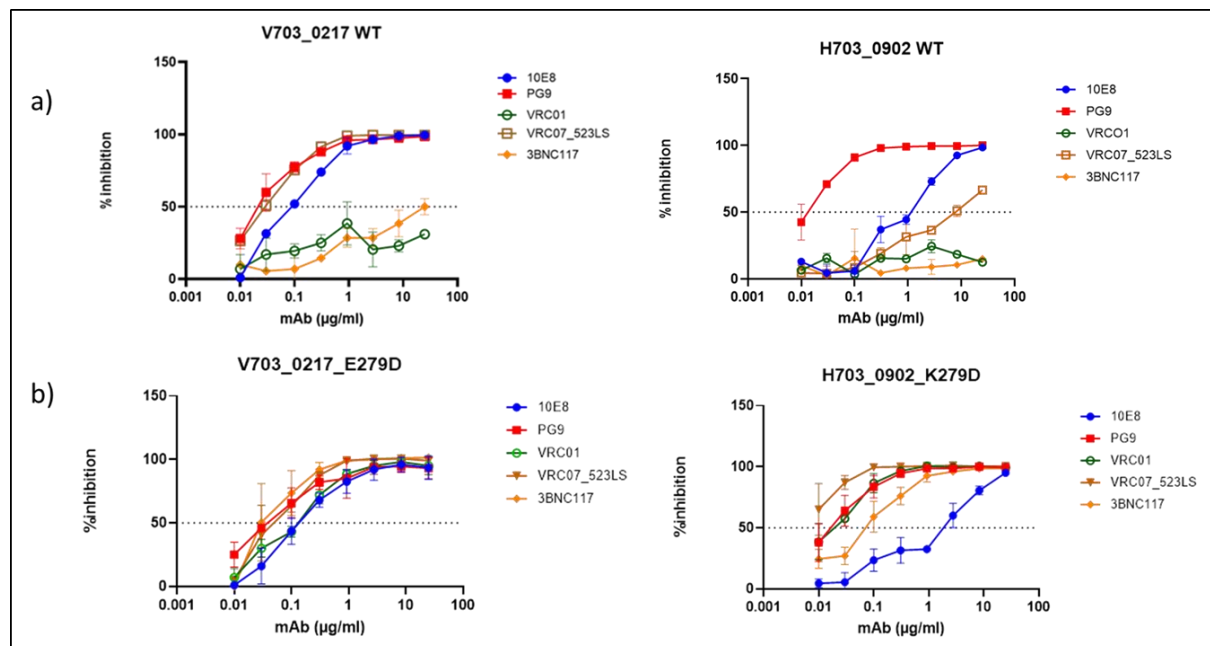


Figure 4-4: Neutralisation sensitivity of a) wildtype viruses V703_0217 and H703_0902 and b) their respective mutants, V703_0217_E279D and H703_0902_K279D, to CD4 binding bNAbs (VRC01, VRC07-523LS and 3BNC117), MPER (10E8) and V2 (PG9) bNAbs. The x-axis represents mAb concentration in $\mu\text{g/ml}$, and the y-axis represents the neutralisation percentage.

4.5.3 Neutralisation sensitivity of the mutations in the $\beta 23$ region

To determine the impact of mutations on other sites that may contribute to VRC01 activity, the study further investigated the $\beta 23$ region. The data revealed varied sensitivity of the amino acid residues at position 455 towards VRC01. H703_1798_E455T remained resistant while V703_1298_E455T was sensitive to VRC01 (Figure 4-5b) with an IC_{50} value of 0.47 $\mu\text{g/ml}$ (Table 4-2). Although E455T mutation did not cause sensitivity to VRC01 in H703_1798 clone, this reversion caused sensitivity to 3BNC117 mAb (Figure 4-5b). A change from resistance (IC_{50} of >25 $\mu\text{g/ml}$) to IC_{50} of 1.00 $\mu\text{g/ml}$ was observed (Table 4-2). The same was observed for V703_1298_E455T with an increase in 3BNC117 sensitivity from resistance (>25 $\mu\text{g/ml}$) to IC_{50} of 0.18 $\mu\text{g/ml}$ (Table 4-2). The E455 mutation also had a positive impact on VRC07_523LS as V703_1298_E455T mutant had a 5-fold increase in sensitivity to this mAb with an IC_{50} change from 0.73 $\mu\text{g/ml}$ to <0.15 $\mu\text{g/ml}$.

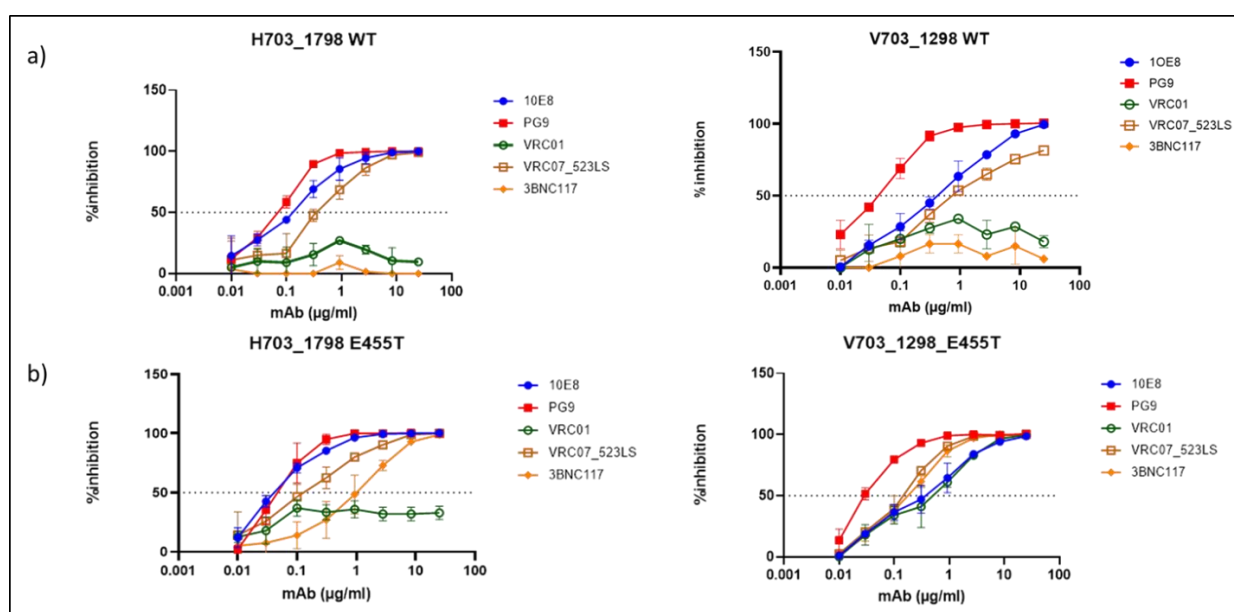


Figure 4-5: Neutralisation sensitivity of a) wildtype viruses H703_1798 and V703_1298, and b) their respective mutants H703_1798_E455T and V703_1298_E455T to CD4 binding bNAbs (VRC01, VRC07-523LS and 3BNC117), MPER (10E8) and V2 (PG9) bNAbs. The x-axis represents mAb concentration in $\mu\text{g/ml}$, and the y-axis represents the neutralisation percentage.

4.5.4 Neutralisation sensitivity of mutations in the $\beta 24$ region

The study identified 471T in the $\beta 24$ region of the VRC01-resistant clone H703_0902. Although the 471 site is not a direct contact for VRC01, it is a CD4 contact site and according to the CATNAP database isoleucine (I) and threonine in this residue can cause resistance towards VRC01 (www.hiv.lanl.gov/). When the 471T mutation was reverted to a putative-sensitive residue 471G (Figure 4-6b), there was no neutralisation by VRC01. The mutant remained resistant with an IC_{50} value of $>25 \mu\text{g/ml}$ equivalent to the wild type, H703_0902 WT. The same was observed for 3BNC117 mAb. Interestingly, the same mutation caused resistance to VRC07_523LS mAb, with a change from the sensitivity of the wild-type virus, H703_0902 WT, (Figure 4-6a) at $8.0 \mu\text{g/ml}$ IC_{50} to resistance ($>25 \mu\text{g/ml}$) (Table 4-2).

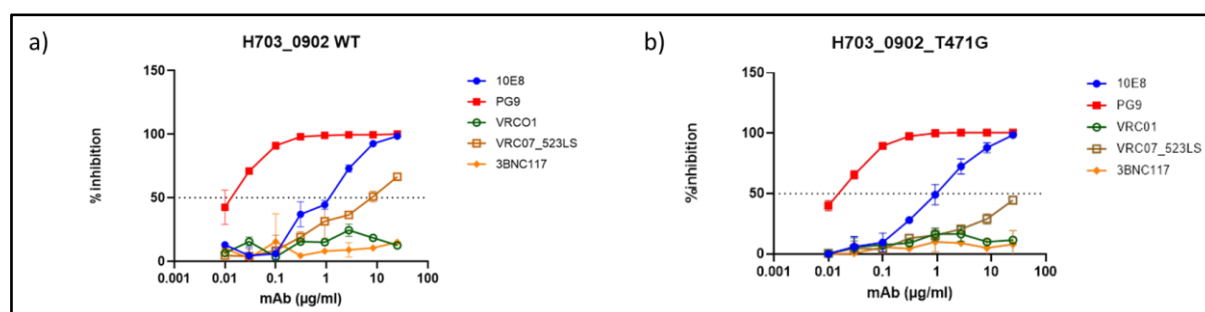


Figure 4-6: Neutralisation sensitivity of a) wildtype virus H703_0902 and b) its respective mutant H703_0902_T471G to CD4 binding bNAbs (VRC01, VRC07-523LS and 3BNC117), MPER (10E8) and V2 (PG9) bNAbs.

(10E8) and V2 (PG9) bNAbs. The x-axis represents mAb concentration in $\mu\text{g/ml}$, and the y-axis represents the neutralisation percentage.

All the mutant clones that were tested maintained sensitivity to the non-CD4bs antibodies, PG9 and 10E8, equivalent to the wild types (≤ 3 -fold differences) (Table 4-2).

4.6 Prediction of VRC01 neutralisation sensitivity *in silico*

The study further applied *in silico* analysis where machine learning software was used to predict the impact that the mutated sequences might have on VRC01 sensitivity as described in Chapter 3, Section 3.3 in comparison to the *in vitro* neutralisation assay. The 7 wild-type sequences and their respective mutants were used in this analysis, and these included the seven mutants that were used in the neutralisation assay and four non-functional mutations (as mentioned in Section 4.3 above). The study further explored other sites of the wild-type sequences that may have an impact on the VRC01 resistance as shown in Appendix . The predictions were reported as IC_{80} values (Table 4-3) and were analysed by comparing the values of the predicted probability of sensitivity and the predicted IC_{80} of the wildtypes and their respective mutants (Table 4-3). Since the wild types are already identified as resistant to VRC01, ranges were created based on the wildtype values. The predicted values of the mutants were classified as resistance or sensitive to VRC01 based on whether predicted values are low or higher than their respective wildtype only. The colour coding in Table 4-3 was used to categorize the ranges used for analysis.

Table 4-3: *In silico* and invitro analysis of VRC01 neutralisation sensitivity of the wildtype sequences and their respective mutated sequences. For *in silico* analysis: blue colour represents a low predicted probability of sensitivity and orange, a high predicted probability of sensitivity. For the predicted IC₈₀ values, yellow presents high IC₈₀ (VRC01 resistance), and red, low IC₈₀ (VRC01 sensitivity). *n vitro* VRC01 analysis, red denotes IC₅₀ values of ≤0.01 to 1.0 µg/ml, green, ≥10 µg/ml to 25 µg/ml, which represents moderate neutralisation and blue ≥25 µg/ml which represents neutralisation resistance.

High predicted probability of sensitivity		High predicted IC ₈₀	
Low predicted probability of sensitivity		Low predicted IC ₈₀	
Sequence name	Predicted probability of sensitivity to VRC01	Predicted IC ₈₀	In vitro VRC01 IC ₅₀ titers
H703_1798 WT	0.114563198190253	23.8921454938796	>25
H703_1798_T471G	0.135828859497665	18.6611399748979	N/A
H703_1798_E455T	0.114150875219547	21.6496398653033	>25
V703_1255_WT	0.274892521402269	9.08089196838291	>25
V703_1255_Q471G	0.334140879103367	7.30202863038325	N/A
V703_1255_D99N	0.277117275584662	9.03962521290106	17.21
V703_1298WT	0.472991563638535	2.9644784997838	>25
V703_1298_E455T	0.473425514497667	2.68582290229372	0.47
V703_1298_D99N	0.481180263044197	2.96534685871561	>25
V703_0217 WT	0.398168807070049	1.17849525838891	>25
V703_0217_E279D	0.411357097419816	0.774855196282794	0.14
H703_0902 WT	0.462916911155694	3.55718371247594	>25
H703_0902_K279D	0.446829117157587	1.76071568845005	0.02
H703_0902_T471G	0.573653820227449	2.63564966583951	>25
H703_0132 WT	0.161977461954073	30.9414184671987	>25
H703_0132_W456R	0.183062256083025	14.5342542396192	N/A
V703_0132 WT	0.144892291795115	40.8780487203073	>25
V703_0132_W456R	0.164189984312526	19.2023431768027	N/A

According to the analysis, the software predicted 9/11 total mutants to be sensitive towards VRC01 neutralisation and 2/11 were resistant. Based on the functional mutant clones H703_0132_W456R, V703_0132_W456R, V703_1255_Q471G, V703_1298_E455T, V703_0217_E279D, H703_0902_K279D, H703_1798_E455T, H703_1798_T471G and H703_0902_T471G sequences were predicted to have low IC₈₀ values when compared with their respective wild types, representing sensitivity to VRC01. The sequences were also predicted to have a high probability of sensitivity except for H703_0902_K279D. Sequences V703_1255_D99N and V703_1298_D99N were predicted to have high IC₈₀ values when compared with their respective wildtypes, thus representing resistance to VRC01.

Only two differences were noted between the *in silico* and the *in vitro* analyses. That is, V703_1255_D99 was predicted to be resistant to VRC01 while the neutralisation assay showed the clone to be moderately sensitive (IC₅₀ of 17 µg/ml). Furthermore, H703_0902_T471G mutated sequence was predicted to have sensitivity towards VRC01 while the neutralisation assay revealed it to be resistant with IC₈₀ of >25 µg/ml.

The non-functional mutated sequences, H703_1798_T471G, V703_1255_Q471G, H703_0132_W456R and V703_0132_W456R, were also predicted to have a high probability of sensitivity with low IC₈₀ values in respect to their wildtype sequences, thus representing sensitivity to VRC01 (Table 4-3). However, their comparison with the *in silico* data was not feasible due to having no neutralisation assay data.

4.7 References

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CHAPTER 5 DISCUSSION AND CONCLUSION

Passive immunization using broadly neutralising antibodies (bNAbs) is an attractive concept in medicine for the prevention of HIV-1. The bNAbs have been studied and shown to prevent SHIV in animal models (Shingai *et al.*, 2014; Julg *et al.*, 2017). Although bNAbs can offer some level of protection, they do not exhibit the ability to completely protect humans from HIV infection. Considering that HIV-1 Subtype C is the most predominant subtype in Africa and India, a broader understanding of emerging mutations contributing to its neutralisation diversity will allow a better understanding of prevention and treatment using VRC01 alone or in combination with other bNAbs. This study utilized HIV-1 subtype C envelope (Env) sequences that were resistant to VRC01, from the three groups of the AMP trial (placebo, 10 mg/kg and 30 mg/kg). The sequences were mutated to generate VRC01-sensitive Env proteins which were tested for infectivity and neutralisation sensitivity towards VRC01 and other related CD4bs (VRC07_532LS and 3BNC11), and non-CD4bs bNAbs (10E8 and PG9).

This study identified mutations in various sites of the Env gene of VRC01-resistant HIV-1 subtype C sequences. The sites included the C1, Loop D, β 23, and β 24 which have been reported previously to consist of amino acid residues that contribute to VRC01 sensitivity (Lynch *et al.*, 2015b; Otsuka *et al.*, 2018; Magaret *et al.*, 2019). The resistant amino acid residues identified in these sites were reverted to sensitive residues and the impact on the viral infectivity was assessed. The comparison of the maximum infectivity values (RLU) of the wild type viruses to those of their mutant counterparts showed that there was a substantial reduction in viral infectivity in some of the mutants, with some having lost infectivity completely while others had infectivity that was equivalent to that of the wild-types.

Following the reversion of resistant amino acid residues to putative-sensitive residues, 4/11 single mutants had lost their infectivity 4 had reduced infectivity, while 3 maintained infectivity that was equivalent to that of the wild-types. Amongst the residues that caused loss of infectivity is residue 456 (VRC01 contact site), and the loss was observed when tryptophan was reverted to arginine (W456R). Arginine is common in global viruses, therefore the loss of infectivity in these clones may be explained by other existing mutation in other regions. For example, the insertion in the V5 loop and other mutation on other V5: the N460A and N460T in H703_0132_W456 and V703_0132_W456R, respectively. The study by Lynch *et al.* (2015b) also identified a loss of infectivity when they introduced tryptophan at this position (R456W) within the β 23 region. The study also observed low infectivity in mutants with E455T, D99N and E279D mutations. Although some of these sites (455 and 279) are fairly conserved with T (455T) and D (279D) or N (279N), site 99 is not. This was contrary to the finding in the Lynch *et al.* (2015b) study that identified the D279E to cause loss of infectivity. They attributed the reduced replicative capacity to Env clones with single mutations. Interestingly, mutant H0902_K279D had maximum infectivity that was 22% more, compared

to the wild-type clone. There are also differences in amino acids in the V5 region. Loss of fitness in mutants has been observed in many of the CD4bs bNAb escape variants (Zhou *et al.*, 2013; Zhou *et al.*, 2015). This is due to changes in the antibody epitopes on the virus that may alter virus binding to the CD4 receptors (Otsuka *et al.*, 2018). Evidence shows that replication capacity can be affected by antibody escape mutations found on the viral envelope (Bar *et al.*, 2012). In other studies, it was shown that mutations in CD4bs residues at the end of the V5 loop that abolished binding to anti-core antibodies, also reduced viral fusion and viral infectivity (Pietzsch *et al.*, 2010; Sather *et al.*, 2012).

Mutation E455 is rare among circulating viruses (0.9% prevalence) and had a reduction in infectivity (80.3%) in V703_1298_E455T mutant compared with the wild-type. This site mutation was observed also with mutant H703_1798_E455T (10.7%) to cause a significant loss of infection. Moreover, there are differences between the two Env clones, V703_1298_E455T and H703_1798_E455T which may contribute to the loss of infectivity. Differences such as insertions of two amino acids at position 507 in H703_1798_E455T clone, The positively charged lysine (K) at position 279 is relatively rare among circulating viruses (8%).

In a study by Gruppung *et al.* (2012), mutations introduced to make viruses resistant to CD4bs resulted in a severe reduction in infectivity or complete loss of infection. All mutated positions in their study were part of, or near the CD4bs; most were highly conserved, and all had an impact on the entry efficiency, which suggested their importance for optimal virus infectivity. In this study, the mutated sites were part of, or close to the CD4bs and although less conserved sites such as 99 were noted, most mutations were at conserved sites and had a major impact on viral infectivity. Other studies have shown that the infectivity may be impacted by other factors, such as the presence of N-glycans on the Env (Wang *et al.*, 2013; Wang *et al.*, 2015), accumulation of mutations in other loops which may cause structural changes by changing composition and length of the loops of the Env (Wyatt & Sodroski, 1998; Wood *et al.*, 2009) or the charge of the amino acid introduced in the mutant sequences. This study confirms that the accumulation of mutations in loops which cause structural changes of the Env loops is associated with loss in viral replication.

The study assessed the neutralisation sensitivity of 7 putative VRC01-sensitive Env pseudoviruses and their corresponding VRC01-resistant wild-type pseudoviruses. These were tested against five antibodies, CD4bs mAbs: VRC01, 3BNC117, VRCO7_523 LS and antibodies binding to MPER (10E8) and V2 region (PG9). The study found that 4/7 mutants were sensitive to VRC01, one being less sensitive than the other three that were completely sensitive while 3/7 remained resistant. The highly sensitive pseudoviruses V703_0217_E279D and H703_0902_K279D had mutations in the Loop D region and V703_1298_E455T in the β 23 region. Both E279D and K279D reversion mutations resulted to an increased sensitivity to all VRC01-class bNAbs. These findings correlate with findings from other studies where the reversion in these

regions restored neutralisation sensitivity to VRC01 (Lynch *et al.*, 2015b; Otsuka *et al.*, 2018). Interestingly, the V703_0217 wild-type clone is from the placebo group of the trial and the H703_0902 wildtype is from the VRC01 10 mg/kg group. This may indicate that the V703_0217_E279 resistant mutation is not the result of exposure to VRC01.

The V703_1298_E455T mutant clone exhibited increased sensitivity to VRC01 while H703_1798_E455T remained resistant. The same mutation did not have the same impact on the sensitivity of the two pseudoviruses. Resistance phenotype also depends on other mutations that exist in other regions within the mutant Env clone. This may have caused the varied VRC01 sensitivity (De Feo & Weiss, 2012). Nowhere in the literature was 455E reported to cause VRC01 sensitivity. This is interesting as this is a VRC01 contact site and is in close proximity to the CD4 binding site (Magaret *et al.*, 2019). Moreover, the partial sensitivity to VRC01 demonstrated by V703_1255_D99N was not observed with the V703_1298_D99N mutant clone. The partial sensitivity is also an indication that 99D has an impact on VRC01 resistance but probably requires other residues to cause complete resistance. The synergistic impact on VRC01 sensitivity between mutations at multiple sites of the Env has been reported in other studies (Utachee *et al.*, 2010; Wang *et al.*, 2015).

The varying VRC01 sensitivity in the mutants with the same mutations in the same amino acid residues indicates that VRC01 resistance depends on other sites or features of the sequence which may be unique with each sequence. Although 3/7 mutant clones (V703_1298_D99N, V703_1298_E455T and H703_0902_T471G) had mutations that had been reported by other studies to cause VRC01 sensitivity (www.hiv.lanl.gov/; Magaret *et al.*, 2019), they remained resistant to VRC01, and some with the same mutations showed sensitivity to VRC01 (V703_1298_E455T and V703_1298_D99N).

This study further assessed the impact of the mutations on sensitivity to other VRC01 class antibodies that bind to the CD4bs. Previous studies have shown that viruses that escape VRC01 neutralisation also show resistance to other bNAbs of the same class, and reverting residues to VRC01 sensitive residues also increases sensitivity to the VRC01 class antibodies (Lynch *et al.*, 2015b; Otsuka *et al.*, 2018). This study used two CD4bs antibodies, 3BNC117 and VRC07_532 LS, in comparison with VRC01. CD4bs antibodies bind to the Env spike with a similar orientation between the heavy chain and light chain, to the Env spike (Zhou *et al.*, 2015). All wild-type viruses were resistant to 3BNC117 except for one (V703_0217), which showed sensitivity to this mAb. Also, the effect of the mutations on 3BNC117 was similar to that of VRC01 except for two mutants V703_1255_D99N and H703_1798_E455T which showed varied sensitivity where V703_1255_D99N was partially sensitive to VRC01 but resistant to 3BNC117, and H703_1798_E455T which was resistant to VRC01 showed enhanced sensitivity to 3BNC117.

For VRC07_532LS, all the wild-type Env clones were sensitive except for H703_0902. The mutated Envs maintained sensitivity to this bNAb, except V703_1298_D99N which had increased resistance when

compared to its wild-type but was sensitive compared to VRC01 and 3BNC117. H703_0902_T471G mutant on the other hand was completely resistant to VRC07_532LS compared to its sensitive wild-type and showed resistance to all other CD4bs bNAbs tested. Our study show similar findings to the study by Scheid *et al.* (2011) in which they reported that CD4bs-directed 3BNC117 show similar or even higher potency and breadth compared to VRC01. Moreover, it has been reported that CD4bs antibodies VRC01 and 3BNC117 neutralise some subtypes more potently and with more breadth than other subtypes. For example, VRC01 and 3BNC117 demonstrate more neutralisation breadth and potency against subtype A than subtype C viruses (Bricault *et al.*, 2019). Although this study did not have subtype A viruses to confirm the above statement, the reduced breadth and potency of VRC01 and 3BNC117 were noted when compared with VRC07_532LS. VRC01-resistant clones and most reversion mutants remained sensitive to VRC07-523LS. This is a common observation even for other studies where VRC07_532LS would neutralise HIV-1 viruses more potently and with greater breadth compared to VRC01 and 3BNC117. For example, some studies have reported that this mAb neutralised $\approx 95\%$ of tested subtype B and C viruses more potently than VRC01 (Rudicell *et al.*, 2014; Wagh *et al.*, 2016; Hraber *et al.*, 2017). When tested in non-human primates, the antibody again demonstrated the ability to provide protection more potently than VRC01 (Rudicell *et al.*, 2014). This is because VRC07_532LS has been engineered with a modification of a set of two amino acid mutations (M428L/N434S; referred to as LS) (Ko *et al.*, 2014; Rudicell *et al.*, 2014), incorporated by site-directed mutagenesis to increase its binding affinity for neonatal Fc receptor (FcRn) to enhance bNAbs, and to offer more protection *in vivo* for therapy and potential passive immunization (Ko *et al.*, 2014). To improve the plasma half-life of VRC07, we incorporated a previously described that increase half-life by increasing affinity for the neonatal Fc-receptor (FcRn),

The slight variability in the sensitivity of the two mAbs observed in this study indicates that although VRC01 and 3BNC117 bind to the same epitope in a similar manner, there are finer differences that play a role when binding the epitope (Falkowska *et al.*, 2012; Lynch *et al.*, 2015b). For example, the CD4bs bNAbs VRC01 and NIH45-46 were reported to induce a conformation of the bridging sheet similar to that induced by CD4 and therefore compatible with 17b and X5 recognition which are typical coreceptor binding site antibodies that can broadly neutralise HIV (Falkowska *et al.*, 2012). However, other CD4bs bNAbs, PGV04 and VRC03, were shown to induce a different conformation between residues 428 and 431 in the β 20/21 strands (Falkowska *et al.*, 2012). Moreover, there are clashes observed between interacting residues with CDHR of the CD4bs while others maintain a non-clashing conformation (Falkowska *et al.*, 2012).

There are two possible impacts that the mutations impose to cause the escape from CD4bs, either the mutation directly reduces the affinity of the mAb for gp120 or they directly affect events after mAb binding (McKeating *et al.*, 1993). This study however would require cocrystal structure (Zhou *et al.*, 2010; Guo *et al.*, 2012) to demonstrate mAb binding affinities and the impact after mAb binding to HIV-1 gp120 with mutations, to identify which mechanism each virus uses to escape VRC01 and the other mAbs. As expected,

all the mutations had no impact on non-CD4bs antibodies PG9 and 10E8, confirming that their epitopes do not covary with those of CD4bs antibodies or alter the viral conformation.

The study further tested the effect of the mutants on VRC01 sensitivity using machine learning. The machine learning software measured the predicted probability of sensitivity for the given Env sequences however, it does not predict which mutation leads to a change in VRC01 sensitivity. The predictions were reported as IC₈₀ values where a higher predicted IC₈₀ indicates VRC01 resistance. This was an advantage to the study since the data from the software comprised all the mutants including those that could not be confirmed by laboratory testing due to loss of infectivity. Thus, 9/11 mutants were predicted to be sensitive to VRC01 neutralisation and the remaining 3 were shown to be resistant. Four of the eight sensitive mutants are those that lost infectivity during laboratory testing and they consisted of T471G, Q471G, and W456R mutations. Mutation T471G has also been reported to cause sensitivity to VRC01 in the CATNAP database however, the H703_0902_T471G mutant clone remained resistant to VRC01 when tested for neutralisation. Also, the prediction that mutating Q to G in site 471 of clone V703_1255 (V703_1255_Q471G) would cause sensitivity to VRC01 is in agreement with Magaret *et al.* (2019) who predicted that 471Q would cause resistance to VRC01 and was ranked number 37 of the amino acid sites that contribute to VRC01 sensitivity or resistance. Similarly, W456R was predicted to cause sensitivity to VRC01 because the IC₈₀ was lower compared to the wild-type. The impact of R456W mutation has been reported by many studies to contribute to VRC01 resistance and as one of the top features that are strongly associated with resistance to VRC01 (Wibmer *et al.*, 2013; Lynch *et al.*, 2015b; Magaret *et al.*, 2019).

For some mutants, there was no correlation between the predicted sensitivity and the predicted IC₈₀ value. For example, clone V703_1298_D99N had a higher probability of sensitivity with a high IC₈₀ value (VRC01 resistance). These non-correlations were observed in other mutations that were explored and generated *in-silico* (**Appendix 1**), rendering the software complex to analyse. Therefore, the study attests that values of the predicted IC₈₀ can be more useful in analysis than the predicted probability of sensitivity values, however, the predictions showed partial concordance with the neutralisation data in this study. Nevertheless, the tool has the potential to be useful in the future.

In conclusion, this study successfully identified mutations at sites that are important in VRC01 resistance in HIV-1 subtype C as identified or predicted previously by other studies. Some of these features may result in cross-resistance to other antibodies that bind to CD4bs which is clinically significant for the testing of other CD4bs antibodies. The weakened viral replication observed in mutants with sensitive residues indicates that escape mutations to VRC01 can compromise viral infectivity (Lynch *et al.*, 2015b). Although not all the reverted mutants were sensitive, the data show that complete neutralisation sensitivity to VRC01 can be regained by reverting escape mutations to sensitive mutations in some resistant viral envelope clones.

This is because each mutation confers different levels of resistance to VRC01, mediated by a different pathway and dependent upon the Env background sequence. It was also shown that machine learning is an important tool in screening sequence features that may impact antibody resistance in escape variants. This can be useful in identifying mAb resistance in variants prior to laboratory confirmation.

The study could be improved by mutating multiple sites of interest in combination, in addition to single mutations. Changes to multiple sites in the gp120 may improve VRC01 sensitivity (Li *et al.*, 2011) and may allow the identification of more sites that cause VRC01 resistance. Nevertheless, the study identified the E455T mutation, which to our knowledge, is the first study to report this mutation to cause neutralisation sensitivity to VRC01 in HIV-1 subtype C. This warrants further investigation of the E455T mutation and its contribution to VRC01 escape.

Overall, our data support the concept that some HIV-1 subtype C isolates develop natural resistance towards VRC01 by changing critical features used by CD4 binding antibodies. The data also promote continued efforts of characterizing VRC01-resistant sites in the pursuit of generating a prophylactic HIV vaccine.

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APPENDICES

APPENDIX 1: HIV-1 Env mutations and their predicted probability of sensitivity and IC₈₀ values.

Sequence name	Predicted probability of sensitivity to VRC01	Predicted IC ₈₀
H703_1798WT	0.114563198190253	23.8921454938796
H703_1798_T471G_V371I	0.136956564044118	18.5967232323781
H703_1798_T471G	0.135828859497665	18.6611399748979
H703_1798_A316T	0.124148897485314	19.8718198864605
H703_1798_H364S_V371I	0.123416094791094	8.15440204366146
H703_1798_H364S	0.122613372006902	8.15124650195217
H703_1798_E455T	0.114150875219547	21.6496398653033
V703_1255_260WT	0.274892521402269	9.08089196838291
V703_1255_Q471G	0.334140879103367	7.30202863038325
V703_1255_Q471G_H364S	0.335980239378858	3.29344771512983
V703_1255_H364S	0.276548697369054	3.97890704640223
V703_1255_E429R	0.277099839401754	9.11671456017407
V703_1255_E429K	0.276437775809446	9.16389012849118
V703_1255_N618S	0.275336104564661	9.16360602348256
V703_1255_N197D	0.276809476091184	9.13854710784869
V703_1255_D99N	0.277117275584662	9.03962521290106
V703_1298WT	0.472991563638535	2.9644784997838
V703_1298_N130K	0.477604640369832	2.79060980989877
V703_1298_E455T	0.473425514497667	2.68582290229372
V703_1298_G429K	0.473418425416554	2.92143290197005
V703_1298_D99N	0.481180263044197	2.96534685871561
V703_1298_V371I	0.474763594560057	2.86189610813499
V703_1298_R683K	0.487717847143323	2.94007804531127
V703_0217 WT	0.398168807070049	1.17849525838891
V703_0217_E279D	0.411357097419816	0.774855196282794
V703_0217_G429K_Y353F	0.371115635479934	0.967320152823334
V703_0217_Y353F	0.367956513099562	0.964408051550634
V703_0217_E279N	0.395039909448923	0.818782509186943
V703_0217_R683K	0.379763731928409	0.954871015231582
H703_0902 WT	0.462916911155694	3.55718371247594
H703_0902_K279D	0.446829117157587	1.76071568845005
H703_0902_K279N	0.467782819949175	3.58960414942878
H703_0902_T471G	0.573653820227449	2.63564966583951
H703_0902_T471G_K279N	0.578430089364478	2.65967123464088
H703_0132 WT	0.161977461954073	30.9414184671987
H703_0132_W456R	0.183062256083025	14.5342542396192
H703_0132_S280N_W456R	0.183062256083025	14.5155944797173

Key	
Low predicted probability of sensitivity	High predicted IC ₈₀
High predicted probability of sensitivity	Low predicted IC ₈₀

H703_0132_S278T	0.175098091556173	30.9327687776512
H703_0132_S278T/S280N	0.175098091556173	30.9010572427318
H703_0132_S280N	0.161977461954073	30.9096980648244
H703_0132_V284I	0.161977461954073	30.9414184671987
H703_0132_A316T	0.182229448193773	29.6613775406341
H703_0132_Y353F	0.161977461954073	30.9302121773244
H703_0132_GP120_SHORT	0.18464957859906	31.5781735887504
H703_0132_s_short_V5	0.313022676962498	13.7785076426064
V703_0132WT	0.144892291795115	40.8780487203073
V703_0132_W456R	0.164189984312526	19.2023431768027
V703_0132_S280N_W456R	0.164189984312526	19.1776903045379
V703_0132_S278T	0.156888631435829	40.8666212406294
V703_0132_Y353F	0.144892291795115	40.8632436051527
V703_0132_S278T_S280N	0.156888631435829	40.8247257576927
V703_0132_S280N_Y353F	0.144892291795115	40.8213515848871
V703_0132_S280N	0.144892291795115	40.8361415221916
V703_0132_I284V	0.144892291795115	40.8780487203073
V703_0132_A316T	0.163425285242888	39.1869311842588
V703_0132_G429K	0.147989724894999	39.9179862705586
V703_0132__short V5	0.284660973112236	18.2002541740818