Characterisation of functional properties of Envelopes of highly neutralisation resistant HIV-1 isolates

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Abstract

An effective antibody-based HIV-1 vaccine would ideally elicit potent antibodies capable of neutralising a wide range of HIV-1 isolates to better cover the human population. A primary concern is the virus' ability to rapidly escape an antibody response. A strong neutralising response elicited by a vaccine may, in principle, select for viruses that are highly antibody resistant thereby significantly reducing the benefit of a vaccine. It is therefore important to study and better understand highly neutralisation resistant viruses. To this effect, we characterized sets of subtype C and CRF02_AG viruses whose neutralisation phenotype were well defined using within subtype neutralisation (neutralisation by subtype matched sera). Our main aim was to determine if there exists a relationship between neutralisation resistance and entry efficiency. Very highly neutralisation resistant viruses appear under-represented in the population. We hypothesised that this may be at least partially explained by decreased entry efficiency as changes to Envelope (Env) during escape could affect the entry process and provide opposing selective pressure that discourages the appearance of very highly neutralisation-resistant viruses.

By comparing entry efficiencies of tier 3 viruses (highly resistant) to tier 2 (moderately resistant) and 1B (sensitive), we observed that the tier 3 viruses generally exhibited higher entry efficiency. This was the opposed of what we hypothesised at the outset of these experiments. We also measured characteristics of resistant HIV-1 Envs that can be inferred from the primary sequence such as the variable loop lengths, number of glycans and net charge. We found that the V2 net charge and the V5 loop length were associated with neutralisation resistance in subtype C viruses and the V2 loop length was associated with resistance in the CRF02_AG viruses. By analysing glycosylation patterns between the groups, we found that the presence of an N-linked glycan at position 413 and the lack of a glycan at N332 were predictors of neutralisation resistance in subtype C viruses. Tier 3 viruses were also more resistant than tier 2 and 1B viruses to the PGT121 (V3/glycan), 4E10 (MPER) and the CD4 binding site broadly neutralising antibodies VRC01 and 3BNC117; suggesting that the epitopes of these antibodies are important for driving resistance. Furthermore, we found no significant relationship between susceptibility to the entry inhibitors Maraviroc and PSC RANTES (CCR5 antagonists) and the fusion inhibitor T20 and resistance, indicating that neutralisation resistance did not alter inhibitor target sites.

Based on our findings, it is clear that reduced entry efficiency does not explain why highly resistant viruses are not more common. We may speculate that the evolutionary steps needed to reach very high neutralisation resistance may be difficult to go through and/or that other countervailing selective pressures may be involved. In the context of an antibody based intervention, highly resistant viruses with increased entry efficiency circulating in the population could be a set back in the control of the HIV-1 epidemic. Therefore, for any long-term antibody-based intervention to be globally relevant it must elicit responses that limit occurrence of resistance and also increase chances that escape would lead to severely impaired viral fitness.

Abbreviations

ABTS	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium
AIC	Akaike information criterion
AIDS	Acquired immune deficiency disease
ART	Antiretroviral therapy
bnAbs	Broadly neutralising antibodies
CCR5	Chemokine receptor 5
CD4	Cluster of differentiation 4
CD4bs	CD4 binding site
CDRH3	Complementarity-determining region in the heavy chain 3
CRF01-AE	Circulating recombinant form 01-AE
CRF02_AG	Circulating recombinant form 02_AG
CXCR4	C-X-Chemokine receptor 4
DEAE	Diethylaminoethyl
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's Phosphate-Buffered Saline
E. coli	Escherichia coli
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunoabsorbent assay
Env	Envelope glycoprotein
FBS	Fetal bovine serum
gp120	120kDa Envelope glycoprotein
gp160	160kDa Envelope glycoprotein
gp41	41kDa Envelope glycoprotein
HEK293T	Human embryonic kidney cells
HeLa	Henrietta Lacks cells
HIV-1	Human immunodeficiency virus-1
HRP	Horse radish peroxidase
IC_{50}	Inhibitory concentration 50
ID_{50}	Inhibitory dilution 50
IQR	Interquartile range
LB Broth	Luria bertani broth
LTR	Long terminal repeat
mAbs	Monoclonal antibodies
MPER	Membrane
MVC	Maraviroc
nAbs	Neutralising antibodies
NHPs	Non-human primates
NIH	National institute of health
NK cells	Natural killer cells
PEP	Post-exposure prophylaxis

PMTCT	Prevention of mother to child transmission
PNGs	Potential N-glycosylation sites
PreP	Pre-exposure prophylaxis
PSV	Pseudovirus
R5	Viruses that utilize CCR5 as the co-receptor
RLU	Relative light units
RLU/pg RT	Relative light units per pictogram of reverse transcriptase
RPM	Revolutions per minute
RT	Reverse transcriptase
SGA	Single genome amplification
SIV/SHIV	Simian immunodeficiency virus/simian human immunodeficiency virus
T20	Enfurvitide
V1-V5	Variable loop 1-variable loop 5
µ	Micro

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1 Introduction and literature review

1.1 Introduction

Human immunodeficiency virus-1 (HIV-1), the causative agent of Acquired Immunodeficiency Syndrome (AIDS), has claimed an estimated 35 million lives since its identification in 1981[1]. As of 2016, there were 36.7 million people living with HIV-1 worldwide. Majority of them in sub-Saharan Africa. South Africa alone contributes 19% of the global number of people living with HIV-1, 15% of new infections and 11% of AIDS related deaths[1].

A vaccine would be crucial in the fight against the HIV-1 virus[2]. Most of the licensed vaccines targeting other viruses function mainly by eliciting the production of protective antibodies[3]. In HIV-1 infection, the role of neutralising antibodies (nAbs) has been extensively studied[2]. Passive immunisation studies on non-human primates (NHPs) provide the most convincing evidence suggesting that such antibodies can prevent infection[4–6]. These studies demonstrated that passive immunisation with nAbs before HIV-1 challenge protected against acquisition of infection in NHPs and support the development of an antibody based HIV-1 vaccine. The target of these nAbs is the HIV-1 surface glycoprotein, Envelope (Env)[7]; a protein required for viral entry into target cells[8]. Thus, antibody-based vaccine immunogen development has been focused on Env.

One of the greatest challenges facing antibody-based vaccine development is the rapid escape of HIV-1 from the nAb response[9]. This escape could present a significant barrier to the efficacy of vaccine-induced nAbs in the population. Theoretically, escape from vaccine-mediated antibody responses could select for neutralisation-resistant viruses that could circulate in a vaccinated population, thus substantially reducing any benefits of a vaccine. Furthermore, several studies have reported that viruses are evolving towards a more neutralisation-resistant phenotype as the epidemic progresses[10–12]. For these reasons, it is important to study and characterise highly neutralisation resistant HIV-1. A study of these viruses will likely illustrate some of the unique Env features associated with neutralisation resistance and have potential implications for immunogen design. This thesis will focus on characterising a panel of highly neutralisation resistant viruses and investigating the effect of resistance on the function of Env.

1.2 Literature review

1.2.1 The global epidemiology of HIV-1

The HIV-1 epidemic is highly dynamic with different viral variants arising all over the world[13]. HIV-1 has been classified into four lineages; groups M, N, O and P, each of which is thought to have arisen from a separate transmission into the human population[13–15]. The vast majority of HIV-1 infections (and virtually all HIV-1 infections outside west and central Africa) belong to group M[13,16]. Within this group, nine subtypes are recognized; A, B, C, D, F, G, H, J and K along with recombinant forms[13,17]. Four subtypes (A, B, C and D) and two presumed circulating recombinant forms (CRF01-AE and CRF02_AG) account for most of HIV-1 infections (Fig 1.1)[13]. Within the Env glycoprotein on the surface of the virus alone, amino acid sequences can differ up to 20% within a particular subtype and over 35% between subtypes[18,19].

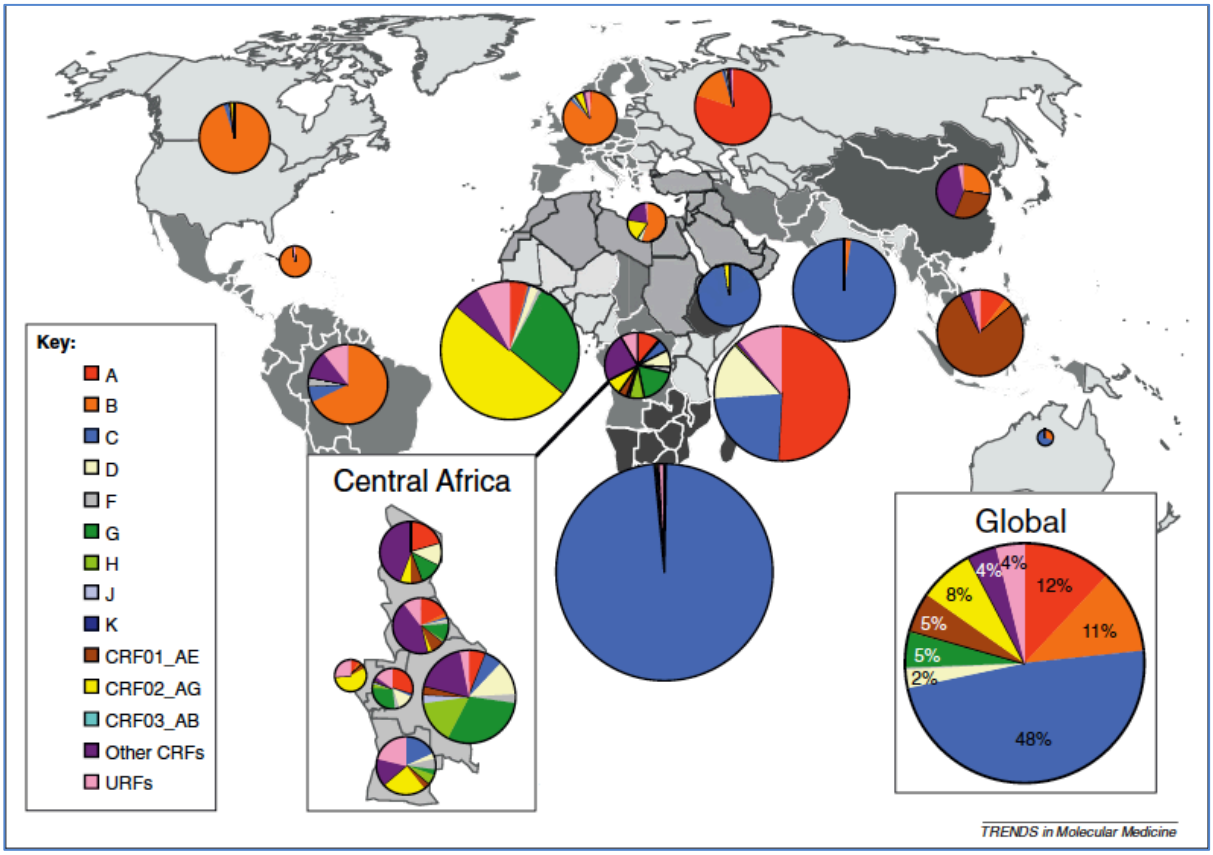


Figure 1.1 Global distribution of HIV-1. The distribution of HIV-1 group M subtypes and recombinant forms is indicated. Africa shows the greatest diversity with subtype C dominating Southern Africa and CRF02_AG prevalent in Western Africa[218].

1.2.2 Controlling the HIV-1 epidemic

Certain measures have been introduced in a bid to control the HIV-1 epidemic. The wide introduction of highly active anti-retroviral therapy (ART) particularly in high prevalence countries such as South Africa, has contributed to a 48% decline in AIDS-related deaths between 2005 and 2016[1]. Prevention strategies targeting transmission have also played a role in reducing HIV-1 incidence. Behavioural interventions aimed at preventing sexual transmission of HIV-1 include promotion of safe sex practices such as use of condoms, regular HIV-1 testing, promoting compliance to ART[20] and understanding noncompliance[21], male circumcision[22] and having one sexual partner[23]. Other interventions include the roll out of pre-exposure (PreP)[24] and post exposure prophylaxis (PEP)[25] for at-risk individuals and ART for prevention of mother to child transmission (PMTCT)[26].

1.2.3 An HIV-1 vaccine: challenges and possible solutions

While prevention strategies have played a key role[27] in reducing the burden of HIV-1, the number of new infections remains high. In 2016 alone, there were an estimated 1.8 million new infections worldwide[1]. Prevention strategies that are ART based such as PreP and PEP rely on strict adherence to be successful[28]. In particular, this requires continuous uninterrupted access to medication[29,30] which is difficult to achieve in some settings. Adverse reaction to ART use is also of concern[31,32]. Putting these factors into consideration, other strategies are needed to fill the prevention gaps that remain.

Vaccines have long been one of the most efficacious and cost effective methods of controlling and eradicating infectious diseases[33]. Consequently, one of the primary efforts of HIV-1 research remains the development of an effective vaccine. A recent study predicted an estimated 67% decline in HIV-1 incidence if a vaccine with even partial efficacy (30%) was introduced[34]. Considering the number of new infections in 2016 alone, this could have a substantial long term impact on the HIV-1 burden especially in Southern Africa, where HIV-1 incidence remains particularly high. Several vaccine strategies have been explored[35–37]. The RV144 trial in Thailand remains the only vaccine study to show some promise with 31% efficacy (95% CI, 1.1 to 52.1; p=0.04)[38]. Although this vaccine showed only modest efficacy, it was proof that a vaccine against HIV-1 was plausible.

One major challenge impeding the development of an effective vaccine is the enormous diversity of HIV-1. Diversity is driven by several factors including high viral replication rates coupled with high mutation rate by the viral reverse transcriptase that lacks proof reading activity[39]. In addition, genetic recombination that occurs when an individual cell is infected by more than one strain[13] generates new hybrid viral variants, some of which circulate widely. Selective pressure from the host also drives viral diversification[40] because immune responses in an infected individual lead to selection of escape mutants. Goulder *et al.* (2001) showed that due to cytotoxic T cell pressure, viruses developed mutations in the cytotoxic T cell epitopes and that these mutants could be transmitted[41]. Selection pressure by nAbs has also been well documented[42–44]. Escape from nAbs results in the accumulation of escape mutations in circulating viruses. These factors contribute to the continuous evolution of HIV-1 not only within the infected host but also within the population. Overcoming this diversity is crucial in developing a HIV-1 vaccine that is universally applicable.

One approach that is being explored is the development of an effective sterilising vaccine that targets the transmitted virus before it establishes a persistent infection. This approach is supported by two important findings. First, reports state that up to 80% of productive HIV-1 infections are a result of a single virus[45–48]. Secondly, as described above, outcomes from passive immunization studies in NHP show that presence of nAbs targeting the infecting virus before HIV-1 challenge protected the animals from infection[4,6]. Together, this means that even a vaccine with partial coverage can still be protective if it blocks the transmitted virus before the infection disseminates.

1.2.4 Design of a HIV-1 vaccine immunogen based on Env

The antibody-based HIV-1 vaccine candidates tested have targeted the surface envelope glycoprotein (Env). It is an attractive vaccine target as Env mediates viral entry[49] into target cells and is the target of broadly neutralising antibodies (bnAbs)[7]. These antibodies are capable of neutralising diverse HIV-1 isolates from different subtypes[50]. Thus, the goal of a vaccine would ideally be to induce the production of bnAbs in sufficient quantities. With the exception of the RV144 trial mentioned above, trials using recombinant gp120, a subunit of Env, have all failed[51]. For this reason, strategies have switched to using better approximations of the Env trimer spike as an immunogen[52]. The rationale behind this is that an immunogen

based on the trimer likely presents nAbs epitopes in a manner that best reflects the native Env spike. Although simpler forms of Env may present the same epitopes, they may not do so in a way that specifically mimics how they are presented on the native spike[53,54]. Most bnAbs recognize epitopes that are, to various extents, influenced by the quaternary structure of the trimer[55–57]. More specifically, the structure of the spike constrains how such epitopes are presented. While this strategy may not guarantee generation of bnAbs, it increases chances of inducing nAbs targeting conformational dependent epitopes.

bnAbs are useful in guiding immunogen design as they elucidate potential vaccine targets. However, bnAbs exhibit unusual characteristics such as long hypervariable loops, self-reactivity and extensive somatic hyper-mutation[58] and this suggests that eliciting these types of responses will be challenging. During antibody development in the course of an infection, the process of affinity maturation usually leads to accumulation of mutations (about 15%) on the variable antigen-binding region. However, bnAbs against HIV-1 gp120 exhibit a significantly increased number of mutations compared to the germline antibody (up to 46% in CD4 binding site antibodies)[59,60]. Deep sequencing and structural analysis suggested that there exists a number of affinity maturation intermediates in the bnAbs development pathway[61]. These intermediates of differing maturity may eventually evolve to give rise to bnAbs. This level of affinity maturation would have to be reproduced in the context of a vaccine and should be taken into consideration when designing an immunogen[62].

1.2.5 The structure and conformation of the HIV-1 Envelope

Due to the important role Env plays, it has been widely accepted that a thorough understanding of the structure and conformations of Env is important for vaccine design[63]. The surface of a newly produced HIV-1 virion contains approximately 70 spikes that consist of trimers of a heterodimer of a gp41 trans-membrane subunit non-covalently bound to a gp120 subunit[64]. Before expression on the surface of an infected cell, the envelope proteins undergo processing and trimerisation. The envelope first exists as a gp160 protein precursor that is cleaved enzymatically into gp120 and gp41 subunits[65]. The mature, processed oligomer is then attached to the membrane by the C-terminal end of gp41 and most of the gp41 and gp120 molecule is expressed extracellularly. The gp120 subunit contains five

variable regions (V1-V5) and five relatively conserved regions (C1-C5)[66]. The conserved regions form discontinuous structures that interact with the ectodomain of gp41 (Fig 1.2).

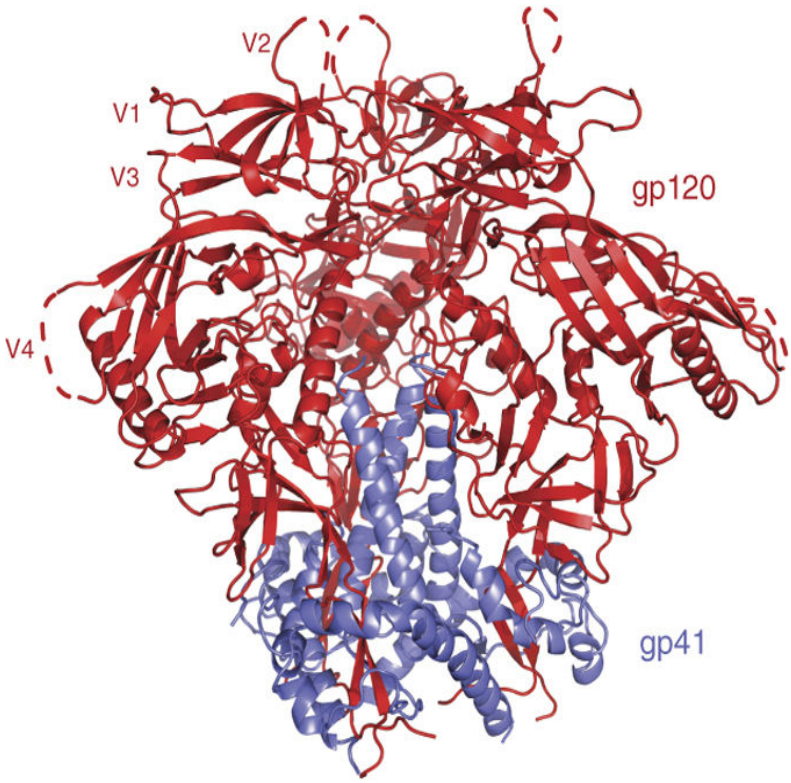


Figure 1.2 Model of the HIV-1 Env trimer. A ribbon representation of the Env trimer comprising gp120 (red) and gp41 (blue) based on the crystal structure of BG505 SOSIP.664. The variable loops V1, V2, V3 and V4 are shown. Dotted loops indicate that the region was not fully resolved on the crystal structures[7].

On the other hand, the variable regions, with the exception of the V5, contain disulphide bonds at their bases which form surface exposed loops[67]. The V1 and V2 loops contain great sequence and length variation amongst different isolates. The loop characteristics of the V1 and V2 have been associated with sensitivity to neutralisation and disease progression[68,69]. The epitopes on the V1/V2 and V3 are often the most exposed and some serve as targets for neutralising antibodies[70–73]. Additionally, the V3 region plays a key role in co-receptor usage[74]. These variable loops are also important for protecting the conserved epitopes from antibodies[75]. Following variable loop removal, several of the conserved epitopes spanning the CD4 binding site were found to be more accessible to antibodies[76]. The function of the V4 and V5 loops is not well as known, although their deletion led to poor Env expression on the surface of the virus[77].

The gp120 protein is highly glycosylated with up to 50% of its mass comprising glycans[78]. They are of host origin, added at different sites on the proteins (potential N-glycosylation sites; PNGs) and characterized by an N-X-T/S motif where X is any amino acid with the exception of proline[79]. These carbohydrates play numerous roles including correct folding of gp120[80] and antibody evasion[42] . The gp41 subunit is also glycosylated albeit at a much lower density. Interestingly, the isolation of bnAbs 2G12 and PGT121-130[81] showed that neutralising antibodies can target glycans.

Structural determinations on *in vitro* produced Env trimers have revealed that different conformational 'faces' of Env are exposed. Conformational changes are evident in the Env depending on whether or not it is bound to CD4. When trimeric Env is in the unliganded state, it is in a pre-fusion "spike" conformation with the V1/V2 loops located close to the apex of the spike[82–84]. However, this "spike" state breathes with some conformations within the equilibrium potentially available for binding to a wide range of antibodies, and others available only to a restricted set of (relatively broadly) neutralising antibodies[85]. Describing how neutralising antibodies bind to their epitopes on Env in its different conformational states can partially explain differences in sensitivity to neutralisation: Envs of viruses that are highly resistant are thought to spend more time in a low range of closed, pre-fusion conformations while sensitive viruses spend more time in open conformations[86]. With regards to the CD4 binding site at least, it was shown that bnAbs can generally bind Env in both open and closed conformations while poorly neutralising antibodies were shown to target epitopes that are only available in the open conformation[85].

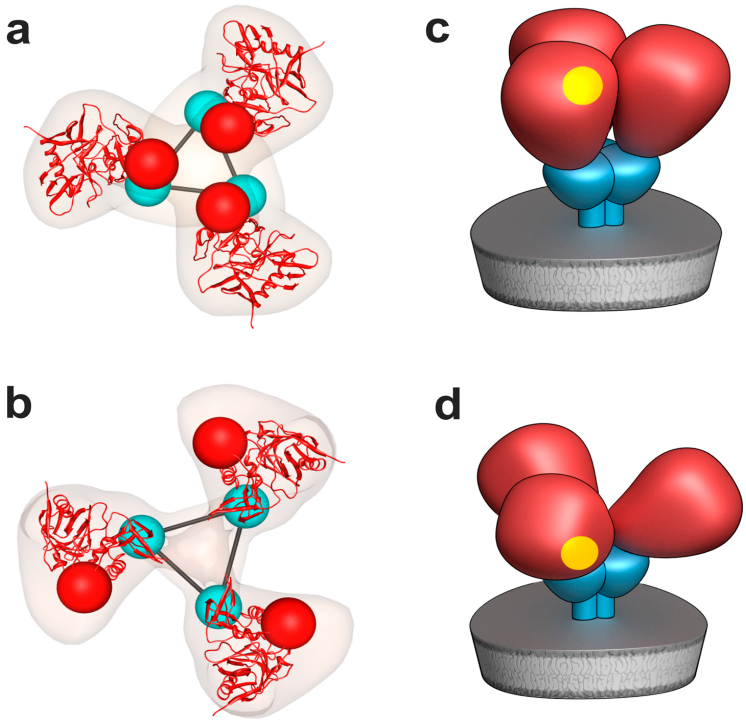


Figure 1.3 The model of the different conformations of Env. HIV-1 Env continually fluctuates between closed (**a, c**) and (**b, d**) open conformations. Coordinates for the gp120 core are shown in red ribbons in figures **a** and **b** while the predicted locations of the gp120/gp41 interface are indicated by linked cyan spheres, whereas V1/V2 loops are indicated by red spheres. In figure **c** and **d** gp120, gp41, and CD4-binding site are shown in red, cyan, and yellow, respectively[219].

1.2.6 The HIV-1 entry process

$CD4^+$ T lymphocytes and macrophages are the primary target of HIV-1. While the gp120 subunit is responsible for attachment to target cells via CD4, the gp41 region mediates fusion between viral and target cell membranes[87]. After binding of gp120 to the CD4 receptor, the trimer undergoes major conformational changes. The formation of a bridging sheet between the inner and outer gp120 domains exposes the co-receptor binding site on the virus to accommodate binding to the co-receptor on the surface of the target cell (either CCR5 or CXCR4). The fusion peptide located on the N-terminus of gp41 is then inserted into the target cell membrane and the six helical hairpin structure that is then formed facilitates fusion of the viral and target cell membrane (Fig 1.4)[8].

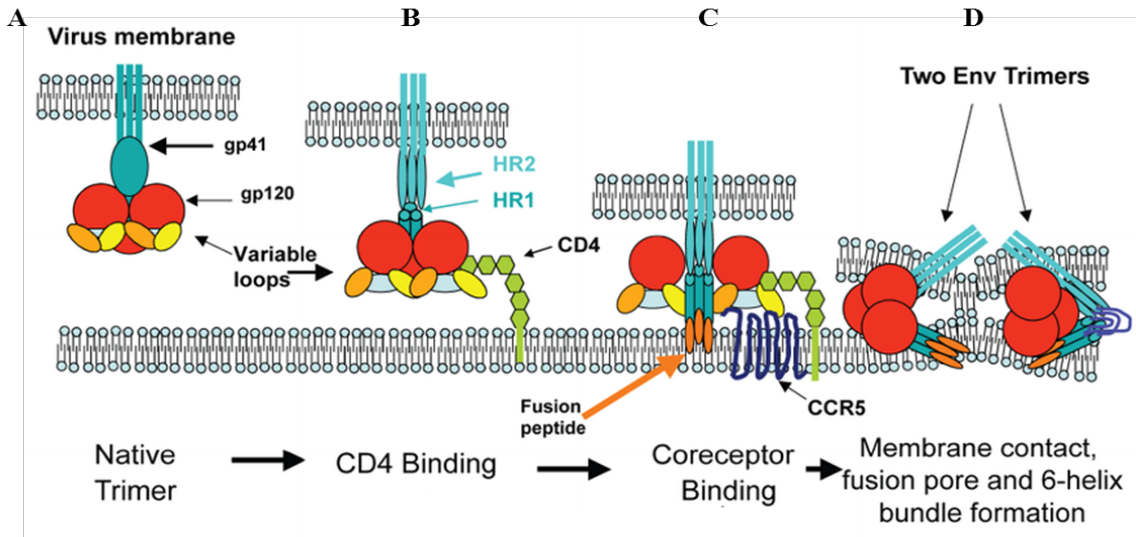


Figure 1.4 The HIV entry process. **(A)** The HIV-1 gp160 Env exists as a trimer made up of gp120 and gp41 heterodimers **(B)** The initial contact is through the viral CD4-binding site which binds to host cell CD4 receptor **(C)** Binding of CD4 leads to rearrangement of the trimer thus exposing the Co-receptor binding site (CoRbs) to allow binding to the co-receptor (CCR5 or CXCR4). The fusion peptide is formed and inserted into the host membrane (**D**) The viral and host membranes fuse, leading to the formation of a six-helix bundle and the virus completes entry into the host cell[220].

The number of trimers on the surface of Env can affect entry efficiency. Brandenberg *et al.* (2015) designed a study to define the number of trimers per virion required for viral entry (Env stoichiometry)[88]. They analyzed 11 HIV-1 isolates of differing subtypes, Env conformational states (open or closed) and co-receptor tropism using both experimental and modeling approaches. They reported that Env stoichiometry differed between isolates and ranged between 1-7 trimers. Higher stoichiometry was associated with reduced infectivity and entry kinetics[88]. However, for viruses with poor entry capacity, a high stoichiometry rescued its infectivity potential.

1.2.7 The Antibody response to HIV-1 in natural infection

Antibodies to HIV-1 can be broadly divided into three groups[7] based on their function. These include non-neutralising, narrowly neutralising and broadly neutralising antibodies (bnAbs). Non-neutralising antibodies are directed against Env but fail to neutralise the virus because they usually bind epitopes that are not presented on the functional spike. However, these

antibodies may still possess antiviral properties by activating effector cells such as natural killer cells (NK cells) through their Fc receptor[89]. The importance of the non-neutralising antibody response in preventing acquisition of HIV-1 was made evident in the RV144 vaccine trial[90].

The humoral response to HIV-1 infection develops within approximately a week of detectable viremia in the form of antibody-antigen complexes[91]. This is followed by production of antibodies targeting gp41. Anti-gp120 antibodies (targeting the V3 loop) can be detected a few weeks later[92]. However, these early antibodies are non-neutralising and it takes about 3 months for neutralising antibodies to be produced. In natural infection, the appearance of neutralising antibodies does not coincide with viral clearance[93]. This is possibly due to the constant viral escape from the antibody response[42]. Antibodies can block HIV-1 through multiple pathways. They can bind directly to the cell-free virus preventing viral entry into cells as well as work in conjunction with effector cells through the Fc receptor to block cell to cell spread of the virus[92].

1.2.7.1 Narrowly neutralising antibodies

Narrowly neutralising antibodies usually neutralise only the virus that induced them, suggesting that they target the variable regions of the viral envelope protein such as the V1/V2 loop[94]. Also known as autologous neutralising antibodies, they can only be detected about 3 months after infection. The production of these antibodies puts considerable pressure on the virus leading to rapid viral escape over time[40,42]. As such, the antibody specificities evolve over time as the virus changes. The host may gain additional specificities targeting different epitopes at conserved sites that are newly available due to changes in the Env driven by antibody pressure[94,95].

1.2.7.2 Broadly neutralising antibodies (bnAbs)

Broadly neutralising antibodies are relatively rare nAbs produced by a proportion of chronically infected individuals and are capable of neutralising a broad spectrum of HIV-1 strains[50]. These antibodies usually target regions that are relatively conserved. A number of

bnAbs have been isolated and characterised. They target numerous sites on Env with varying degrees of breadth and potency (Fig 1.5).

1.2.7.2.1 The CD4 binding site (CD4bs) bnAbs

The CD4bs is an attractive target in vaccine development as it is crucial for viral entry and therefore, is likely under functional constraints. Antibodies targeting the CD4bs are commonly elicited suggesting that these antibodies may be relatively easy to elicit upon vaccination [71]. The mAbs VRC01[96] and N6[97] that target the CD4 binding site are some of the broadest antibodies isolated to date. However, escape from VRC01-like antibodies has been demonstrated[98,99].

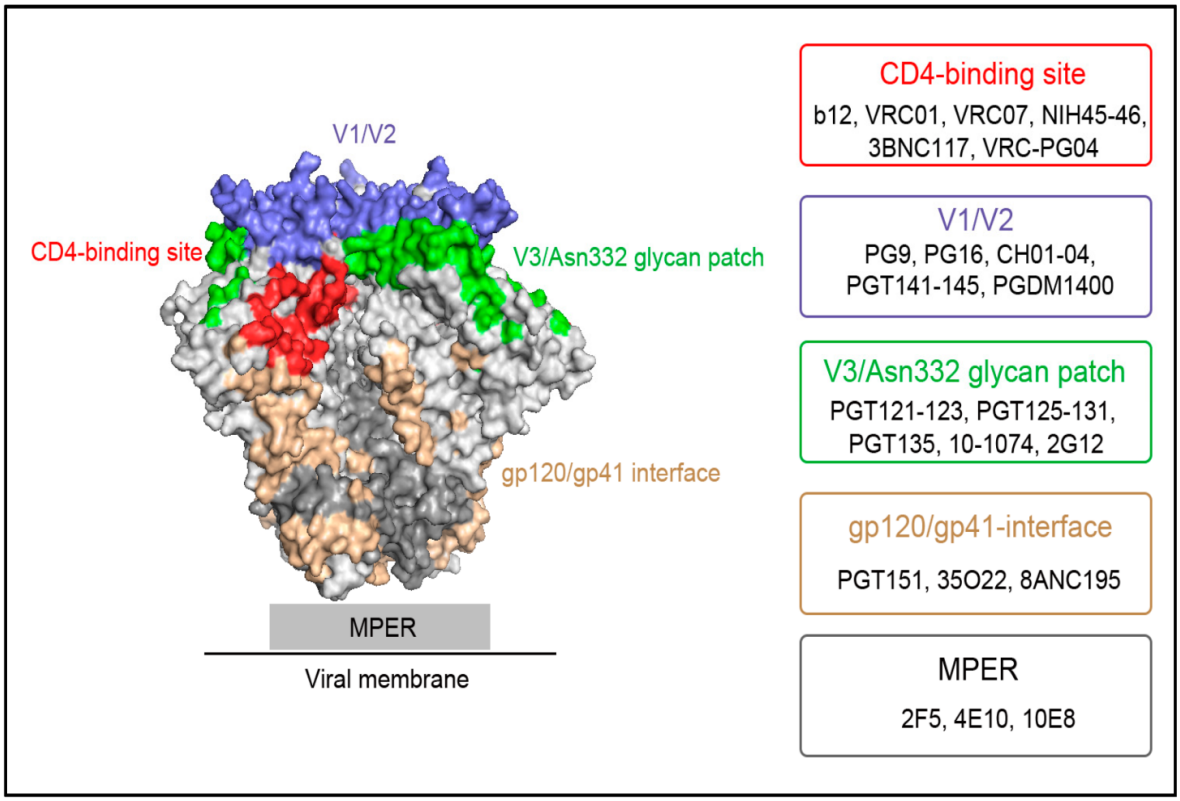


Figure 1.5: Targets of bnAbs on HIV-1 Env trimer. The colours highlighted represent the different epitopes targeted by some commonly studied bnAbs. CD4-binding site is shown in red, V1/V2 in purple, the V3/Asn 332 glycan patch in green, the gp120/gp41 interface in wheat and MPER in black. *Adapted from Zhang et al. 2016*

1.2.7.2.2 MPER

The membrane proximal external region (MPER) on gp41 is an attractive target as it plays an important role in fusion and the epitope is linear and conserved[100]. However, access to MPER is restricted in the 'closed' pre-fusion state which means immunization with closed trimer is not likely to induce anti-MPER antibodies[101,102]. 10E8[103] binds MPER and is capable of neutralising more than 90% of HIV-1 viruses tested with exceptional potency, whereas, 4E10[104] and 2F5[105] exhibit moderate breadth and potency. One of the challenges faced by inducing MPER antibodies is autoreactivity. The epitopes targeted by 4E10 and 2F5 mimic conserved self-antigens[106]. This indicates that responses targeting these epitopes are deleted due to immune tolerance. However, by characterising 177 sera samples from HIV+ individuals, Jacob *et al.* (2015) reported that MPER antibodies were major contributors to neutralisation breadth suggesting that autoreactivity was not critical to the elicitation of these antibodies[107].

1.2.7.2.3 The V3/glycans bnAbs

The V3 loop is a target for narrowly neutralising antibodies as viruses with exposed V3 loops are highly sensitive to neutralisation[108]. However, some of the most potent bnAbs are directed against the glycans at the base of the V3. This family includes PGT121-123, 125-128, 130, 131 and 135 and 10-1074[109,110]. bnAbs against the V3 glycans have shown remarkable efficacy in NHP models and human trials. Immunization with PGT121 protected macaques from infection[111] and the antibody 10-1074 was capable of controlling viremia in ART naïve HIV-1 infected individuals[112].

1.2.7.2.4 The V2/apex bnAbs

The V2 loop is highly variable. However, a conserved surface within this region forms the V2/apex epitope that is the target of some bnAbs. PG9 and PG16 are conformation dependent mAbs that bind a quaternary epitope on the V2 apex[70]. They are also dependent on surface glycans specifically at amino acid position 160 (N160)[81], but do not appear to bind directly to the glycan[113]. These two antibodies can neutralise up to 80% of HIV-1 isolates each[70]. Unfortunately, antibodies against this region are often deleted during antibody development

as they usually possess an unusually long and anionic complementarity-determining region in the heavy chain 3 (CDRH3)[114]. The long CDRH3 allows the antibody access to epitopes shielded by the dense glycosylation in this region[115].

1.2.7.2.5 The gp120-gp41 interphase

Antibodies targeting this site are dependent on distinct glycans that lie at the interphase of gp120-gp41. 8ANC195 recognizes glycans at positions 230, 234 and 276[116], PGT151 binding relies on glycans 611 and 637[117] while 35O22 targets 88[55]. Interestingly, escape from another interphase antibody CAP248 2B, resulted in a viral phenotype that was exceptionally sensitive to MPER antibodies. These escape mutations can be used in vaccine design to make the MPER more accessible and immunogenic[118].

1.2.8 Neutralising antibodies can protect against HIV-1 challenge

Several studies have been carried out to further explain the importance of antibodies. B cell depletion studies in NHPs suggested that antibodies had a role to play in reducing viral load and disease progression[119,120], although not in African green monkey hosts[121]. There is limited evidence that this may also be true in humans. Huang *et al.* (2010) reported an increase in viremia and the emergence of a neutralisation sensitive viral population after depletion of B cells in a chronically infected individual[122]. These findings tend to support the role of antibodies in controlling HIV-1 in natural infection.

The potential of neutralising antibodies in preventing HIV-1 was clearly illustrated by passive immunisation studies in NHPs and mice. Administration of neutralising antibodies before viral exposure was found to protect macaques against SIV/SHIV infection[123–125] and a combination of two mAbs (2F5 and 2G12) protected NHPs from SHIV challenge[4]. Immunisation with bnAbs such as PGT121[111] and PGT126[126] alone or bnAb combination of VRC01, VRC01-LS, 10-1074 and 3BNC117[6] also protected against infection. In a recent study, humanised mice were protected from repeated intravaginal HIV-1 challenge after immunisation with the CD4bs antibody b12. The authors also reported that treatment with PGT126 offered sterilising protection in these mice[127].

Although the antibody response to HIV-1 is complex, it is nevertheless an important functional response. nAbs have an enormous potential for therapy of HIV-1 as evidenced by

the studies briefly mentioned above. However, rapid and constant escape from antibodies poses a challenge that still needs to be overcome before these interventions can be introduced. Escape from bnAbs such as 2G12[128,129], 10-1074[112], VRC01[130] and 3BNC117[131] used as therapy in HIV+ individuals has been demonstrated.

1.2.9 HIV-1 escapes the neutralising antibody response

Although HIV-1 isolate specific neutralising antibodies could be detected quite early on in the course of an HIV-1 infection[132], this response is followed by clearance of sensitive viruses and the emergence of a resistant population[42]. The constant escape from nAbs any time partly explains the immune system's inability to control the infection. Additionally, antibody pressure contributes to the rapid evolution of the virus and the extensive variation observed in the *env* gene (see 1.2.1)[40]. This poses a problem for vaccine developers, as escape from vaccine-elicited responses would greatly reduce the benefit of the vaccine. A vaccine that targets multiple independent epitopes on the virus would ensure vaccine coverage. This may be achieved by using multivalent vaccines that exhibit breadth of coverage[133]. In the case of HIV-1 therapy, passive immunisation with combinations of bnAbs rather than single specificity bnAbs, would also limit the likelihood of escape[134].

1.2.9.1 Mechanisms of HIV-1 escape

HIV-1's tolerance for *env* sequence variability while retaining function makes it adept at escaping the neutralising antibody response[135]. The various mechanisms of viral escape have been described in a bid to overcome this challenge. Single point mutations on the *env* (even outside the antibody epitope) have been shown to affect susceptibility to neutralisation[136–138].

1.2.9.1.1 The glycan shield

Even though the glycans on the surface of gp120 are a critical target for nAbs, they also play a crucial role in the antibody evasion of HIV-1. They acts as a shield against nAbs by masking important epitopes on the envelope thereby rendering the virus less susceptible to neutralisation[42,44] Due to antibody pressure, the positions and numbers of PNGs varies thereby forming a continuously evolving 'glycan shield'[44]. More specifically, the removal, addition or shifts at key glycan positions have been shown to affect antibody neutralisation (as a single glycan occludes a volume roughly equivalent in size to the V3 loop)[5,11]. In a

longitudinal study, an important glycan site shifted from position N332 to N334 in two patients which abrogated sensitivity to neutralisation by broadly cross-neutralising antibodies[44]. This eloquently captured the evolution of the virus in response to the pressure exerted by neutralising antibodies. In another study, a loss of glycan at position N241 created a 'glycan hole' rendering the virus sensitive to neutralisation[139].

1.2.9.1.2 Conformational masking

Important antibody targets could also be 'hidden' from neutralising antibodies as the Envelope trimer retains a 'closed, pre-fusion' conformation that only becomes unmasked during entry after CD4 binding. This allows the virus to maintain receptor binding while avoiding antibody neutralisation[84,141]. Variation in variable loop length (V1/V2) has also been associated with neutralisation: Longer V1/V2 loops are thought to mask important epitopes targeted by CD4bs antibodies thereby making viruses more neutralisation resistant[142,143].

These evasion mechanisms contribute to the persistence of HIV-1 in the face of functional antibody responses that are often vigorous and sustained.

1.2.10 Evidence that HIV-1 is evolving to become more resistant to neutralisation

Several studies suggest that HIV-1 is evolving towards a more neutralisation resistant phenotype. Bunnik *et al.* (2010) compared neutralisation sensitivities of subtype B isolates from Dutch cohort studies carried out either early in the epidemic or more recently. They reported increased neutralisation resistance as the epidemic progressed especially towards CD4bs antibodies[144]. Another study validated this finding by comparing subtype B isolates from French individuals at 3 different time points spanning 20 years[11]. Rademeyer *et al.* (2016) compared subtype C viruses from three-time points and also reported enhanced resistance in the later isolates to the bnAbs 4E10, PG9 and VRC01. In another study, 34,000 HIV-1 sequences from samples collected at different time points were analysed and the researchers reported a drift towards increased resistance to a broad range of bnAbs[145]. This may pose an increasing problem in the design of antibody-based HIV-1 interventions. Therefore, it is critical to identify and study features of resistant viruses.

1.2.11 Categorising viruses based on neutralisation sensitivity: a tier approach

Viral isolates may be grouped into different tiers (1 to 3) based on their neutralisation sensitivity patterns. Tier 1A and 1B are described as highly sensitive, tier 2 moderately sensitive and tier 3 comprises of viruses with a highly resistant phenotype. Tier classification has been done by testing viruses against neutralising antibodies present in serum/plasma of HIV-1 positive individuals[146–148]. Seaman *et al.* (2010) tested 109 virus isolates representative of the circulating strains in the population against 7 plasma pools (also representative of the circulating subtypes). One or more of the pools could neutralise most of the viral isolates tested. Furthermore, they reported increased neutralisation if the virus was subtype matched to the plasma pool tested i.e. subtype C viruses were more sensitive to the subtype C plasma pool[146]. Viruses with similar neutralisation profiles were grouped using *k*-means clustering into subsets representative of distinct tiers of neutralisation sensitivity. However, it is important to note that tier definition is partially dependant on the sera or panels of sera used[146].

There is evidence suggesting that the increased susceptibility to neutralisation of tier 1 viruses could be due to their Env trimer sampling the open conformation more readily thus making epitope accessibility easier for antibodies[85]. So far, a number of the vaccine candidates tested have elicited antibodies that neutralise only Tier 1 isolates[149,150]. However, the majority of the circulating viruses tested belong to tier 2. Subsequently, vaccine efforts are likely to focus on eliciting tier 2 responses. Tier 3 viruses, although few in number are highly resistant and could offer valuable insight into vaccine design. This subset will be the focus of my study.

1.2.12 Does antibody resistance/escape affect Env function and fitness?

Despite Env's sequence plasticity to escape the immune response, it still needs to retain its function. We hypothesize that mutations away from wild type virus could incur some reduction in fitness of the virus. Rapid reversion to the wild type of some immune escape mutations upon transmission suggests fitness costs may be significant[9]. For instance, long variable loops and increased glycosylation density are features associated with resistance and chronic infection[68]. However, these features are reversed in transmitted viruses (shorter loop lengths and fewer glycosylation sites)[151] rendering the transmitted virus more sensitive to

neutralisation by one report[151], but not another report[152]. Together, this suggests that these changes in variable loop characteristics and glycosylation may have an impact on viral fitness. Therefore, the most effective immune responses may be those targeting the regions of the virus where escape mutation inflicts the largest fitness cost to the virus. Such knowledge could be used to rationally design more effective vaccines.

Due to the crucial role Env plays in viral entry and as a major target of the immune response, a number of studies have evaluated the effect of changes on *env* sequence and its impact on viral fitness. One study investigated the effect of mutations on antibody epitopes on HIV-1 entry efficiency (infectivity)[88]. This study reported that reconstitution of the N160 glycan in a virus that naturally lacks N160 led to a 4-fold increase in infectivity. The authors also created 3 variants of a tier 2 virus, JR-FL, with mutations mimicking escape mutants. A JR-FL D664N escape mutant that is resistant to the MPER antibody 2F5, a second JR-FL V549M N554D mutant which has highly increased resistance against the entry inhibitor T-20, and a third JR-FL mutant with point mutations at positions N332S, P369L, M373R and D664N which rendered it resistant to the bNabs PGT128, 2G12, b12 and 2F5. While the 2F5 mutant infected just as well as the wild-type (WT), the T-20 and multiple escape mutant exhibited reduced infectivity (9% and 19% respectively)[88]. These findings indicate that even a single amino acid change, potentially driven by antibody escape, has an appreciable effect on Env function. It is important to note, however, that mutations on other regions of the virus such as Gag contributes to much greater fitness impairment. Escape from cytotoxic T lymphocytes led to mutations on Gag p24 that incurred appreciable fitness costs compared to Env[153].

In addition, other studies have been conducted to investigate whether viral escape from the autologous antibodies targeting Env could lead to the selection of viral variants with overall reduced replication potential. While there is evidence to suggest that the constant escape due to immune pressure may sometimes lead to impaired viral fitness and replication, the reports have been inconclusive[154]. For instance, Sather and colleagues studied one donor whose sera contained broadly neutralising activity directed against the CD4bs. They demonstrated that viral escape from this response negatively impacted viral replication[155]. A later study demonstrated that while escape from VRC01 was associated with impaired fitness, compensatory mutations restored the ability of the virus to replicate[156]. However, it could be

argued that viral escape/resistance from a polyclonal response comprising multiple antibody specificities could have a much greater impact on viral replicative fitness compared to monoclonal antibody (such as VRC01 in the study above) that target a single site. Studying the effects of antibody resistance on Env function, viral fitness and eventually viral evolution may reveal Env properties that can be evaluated and exploited for vaccine and therapy development.

1.2.13 Do highly resistant viruses (tier 3) have decreased entry efficiency?

Tier 3 isolates are underrepresented even in viruses from chronically infected individuals[143,146]. In a sense this is surprising because viruses at the chronic phase are constantly escaping antibody, which should presumably lead to the emergence of highly neutralisation-resistant viruses. We hypothesize that these highly resistant strains could have diminished entry (due to changes on Env) and are out-competed by other less resistant but ultimately fitter viruses during an infection. In other words, a reduction in fitness reduces the opportunities for highly neutralisation-resistant viruses to emerge during chronic infection. As the neutralising antibody response provides positive selective pressure favoring viral escape, the envelope protein is not infinitely plastic and mutations can carry fitness costs. This is supported by the evidence that suggests that the transmission favours viruses that are more sensitive to the neutralising antibodies of the donor[151,157].

This thesis will determine if there is a relationship between resistance and viral entry, which in this study will be used as a component of viral fitness. While the virus' mechanism of resistance is not clear and may vary from virus to virus, we hypothesize that due to their resistance phenotype, they may exhibit decreased entry efficiency and this might explain their underrepresentation in the population.

1.3 Study rationale

The presence of circulating highly resistant viruses could greatly impede antibody based vaccine and therapy efforts. For this reason, it is important to study and characterise these highly resistant isolates. Neutralisation resistance may be explained by absence of key Env epitopes and in some instances by conformational changes that make these epitopes less accessible to nAbs. Envs of highly resistant viruses have been shown to exist in a low range of "closed" conformations which inhibit nAb binding[86,158]. This might coordinately inhibit key virus-target cell interactions such as co-receptor binding. Thus, it is important to understand if Env retains its full functionality in the context of such resistance. Differences in viral fitness between HIV-1 isolates have been sometimes mapped to Env[159–162]. As Env is the most diverse HIV-1 protein[163], these findings indicate that differences in viral fitness attributed to Env might influence viral evolution, transmission and disease progression[160,164–166]. The functional and genotypic changes of the *env* of resistant viruses as compared to less resistant viruses are explored in this study. We hypothesized that a highly resistant Env has impaired entry and that this mechanism that could explain why there are fewer tier 3 viruses in the population.

1.4 Study aims and objectives

The aim of this study is to characterize the viral Env protein of highly resistant viruses (tier 3) and determine whether there are functional differences between those viruses and more sensitive isolates. We seek to understand whether these differences, if present, could explain the resistant phenotype and the apparent underrepresentation of tier 3 viruses in the population.

1.4.1 Specific objectives

1. To describe the Env differences in Env characteristics between tier 3 viruses and more sensitive viruses.

We compared genotypic characteristics associated with resistance such as variable loop characteristics and glycosylation patterns between viruses with differing neutralisation sensitivities. The predicted sites of glycosylation, loop length and charge were determined using online prediction tools.

2. To determine whether differences in Env function exist between tiers by comparing entry efficiencies and susceptibility to entry inhibitors.

We hypothesized that resistant viruses have less favourable phenotypic characteristics affecting entry such as CD4 and CCR5 binding capacity. Pseudoviruses were generated and used for single-cycle entry assays to measure entry efficiency. As all the pseudoviruses shared the same backbone, any changes identified were assumed to be influenced by Env and not other viral proteins.

We also measured the susceptibility of these viruses to entry inhibitors that block binding to CCR5 in subtype C and CRF02_AG viruses and compared the differences between the two subtypes. This provided insight to the accessibility to the co-receptor binding site on Env.

2.0 Material and Methods

The main aim of this study was to investigate the genotypic and functional differences between Envs of differing neutralisation sensitivities. Resistance was defined using publicly available data of within-subtype neutralisation by a large panel of sera[143,147]. Within-subtype data was considered a better indicator of resistance as neutralisation is greater when a virus of a given clade is tested against plasma from an individual infected with that same clade[147,167–169]. This suggests that epitopes of viruses that belong within the same clade are likely to share similar epitopes compared to cross-clade serum/virus combinations. Thus, within-subtype neutralisation will likely give a better picture of the sensitivity of a virus to antibodies targeting wider range of epitopes than using subtype-mismatched serum/virus pairs. Importantly, using a large panel of sera/plasma likely increases the nAb specificities present increasing the chances of neutralising a large set of viruses.

- *Selection of viruses used in the study*

We evaluated genotypic and neutralisation properties of 89 Env clones belonging to two HIV-1 subtypes; C (n=81) and CRF02_AG (n=8). These were selected as the Env clones were readily available for use in the laboratory and their neutralisation sensitivity phenotypes have been well defined[143,147]. For the first analysis, variable loop (V1-V5) characteristics such as loop length, glycan density and net charge were compared to investigate the features associated with resistance.

For the second analysis, a panel of 16 subtype C Env clones were selected which represented a range of neutralistion resistances, and similar genotypic features (Table 2.1). All eight CRF01_AG viruses were used (Table 2.2). Pseudoviruses generated from these clones were used to evaluate if neutralisation resistance/tier 3 viruses had different functional Env characteristics as measured by entry efficiency and susceptibility to entry inhibitors assays.

2.1 Summary of tiered classification of subtype C and CRF02_AG viruses

The viral isolates used in this study had already been classified into tiers based on their neutralisation sensitivities in previous studies[143,146]. For tier classification, Rademeyer *et al.* (2016) tested clade C serum samples from 54 ART-naïve, chronically infected South African HIV-1+ individuals for breadth and potency against 3 subtype C PSVs, a consensus subtype

C and B virus and a subtype A PSV. A panel of 30 sera exhibiting varying breadth was then selected from this to be tested against subtype C Env clones. Viruses were grouped into tiers (1B, 2 or 3) based on their average Log_{10} ID_{50} (ID_{50} is defined as the serum dilution at which 50% of viruses are neutralised) titres using the k-means clustering method as described by Seaman *et al.* (2010) Tier 1B and tier 3 formed highly significant and robust clusters with every other virus in between classified as tier 2. This process was reproduced when neutralisation sensitivity of each virus against all 30 sera was measured as geometric mean ID_{50} titre[143]. The geometric mean ID50 values for the CRF02_AG viruses used in this study were determined by Jacob *et al*. (2012) using a pool of CRF02_AG plasma[147]. Seaman *et al*. (2010) categorised these CRF02_AG viruses into tiers however, there was a discrepancy in tier ranking as some of the viruses ranked as tier 3 by Seaman *et al*., (2010) were found to be sensitive by Jacob *et al.* (2012)[146,147]. This discrepancy might be because the plasma pools used in Seaman's study did not contain high levels of CRF02_AG specific antibodies. The CRF02_AG plasma pool in Seaman's study was not specifically selected for higher neutralisation activity. In contrast, in the Jacob *et al.*'s (2012) study, they selected for samples likely to have come from individuals with longer HIV-1 infection prior to sample collection[170], which selected for those with higher neutralisation activity. Jacob *et al* (2012) proposed that the low neutralising activity CRF02_AG blood plasma pool used by Seaman *et al.* (2010) artificially pushed some of the CRF02-AG viruses analysed into the tier 3 category. Due to the inconsistency in tiering of CRF02_AG viruses, I used the geometric mean ID_{50} values to reflect neutralisation sensitivity. In addition, all serum/plasma samples used in these three studies were from chronically infected individuals thereby increasing the chances of including good neutralisers thereby increasing our chances of identifying resistant viruses selectively.

Env clones used in the phenotypic assays were selected from these subtype C and CRF02_AG viruses. All subtype C tier 3 viruses that were available to me were included in the study. Thereafter, viruses were randomly selected to cover a range of neutralisation senstivities (tier 2 and 1B). All available CRF02_AG Env clones were used in the study.

Table 2.1 Description of subtype C subset used in entry efficiency and inhibition assays

Env Clone	Tier	Country/ origin[a]	Fiebig Stage/ minimal time from infection	Infection Stage[b]	Infection year	V1V2 loop length (aa)	V1V2 number of PNGs	V1V2 Net charge
CA146_H3_3	1B	SA	26 days	E	2009	68	5	4
CAP174.1.06_F3_1B	1B	SA	V	E	2005	65	4	2
CAP301.2.00_C3.20	1B	SA	4 weeks	E	2008	65	2	2
569-F1_37_10	2	TZ	V/VI	E	2001	69	5	-2
CAP307.2.00_24_1	2	SA	30 days	E	2008	68	8	0
CAP306.2.00_F9.1	2	SA	2 weeks	E	2008	75	9	0
CAP317.2.00_D4.10	2	SA	7 weeks	E	2008	66	5	-2
CAP382.2.00_D7.19	2	SA	7 weeks	E	2010	61	4	-3
CAP363.2.00_10_3	2	SA	37 days	E	2009	69	7	-1
CAP225.1.06_A2_18	3	SA	III	I	2005	75	6	1
CAP237.1.22_B2_2_39	3	SA	III	I	2007	70	5	2
CAP291.2.00_H2.15	3	SA	11 weeks	E	2008	74	6	-1
Ko243_H6.3	3	SA	58 days	E	2009	88	5	4
541-F1_A7_2	3	TZ	47 days	E	2001	68	5	1
98-F4_H5-13	3	TZ	V	E	2001	71	5	1
CAP260.2.00_TA1_1B	3	SA	V	E	2006	57	3	2

[a] Place of origin: Tanzania (TZ); South Africa (SA);
[b] Infection Staging: I (Intermediate) is Fiebig stage III/IV; and E (Early) is Fiebig stage V/VI. All samples were collected with 100 days of a previous HIV-1 negative diagnosis. Fiebig staging as defined by Fiebig *et al.* 2003

Table 2.2 Description of CRF02_AG viruses used in entry efficiency and inhibition assays

Env isolate	Geometric mean ID_{50}	Country/origin	Infection stage[a]	V1V2 length (aa)	V1V2 Number of PNGs	V1V2 Net charge
T278_50	23	Cameroon	E	75	7	2
T253_11	29	Cameroon	E	65	5	1
T251_18	53	Cameroon	E	72	6	0
T33_7	67	Cameroon	E			
T255_34	402	Cameroon	E	68	7	1
928_28	139	Cote D'Ivoire	E	70	6	1
T257_31	180	Cameroon	E	74	5	2
T250_4	314	Cameroon	E	61	3	5

[a] Infection Staging: E (Early) is Fiebig stage V/VI. Fiebig staging as defined by Fiebig *et al.* 2003. Geometric mean ID_{50} values of CRF02_AG viruses used here were generated previously[147].

2.2 Genotypic characterisation of viruses

2.2.1 Variable loop characterisation

Variable loop length, glycan density and net charge were determined using a web tool available on the Los Alamos HIV-1 sequence database (https://www.hiv.lanl.gov/content/sequence/VAR_REG_CHAR/index.html). An alignment of HIV-1 sequences is input and variable loop boundaries/postions are selected based on a refrence sequence (HXB2). The length of the V1 loop (position 131-157), V2 (position 158-196), V3 (position 296-331), V4 (position 385-418) and V5 (position 460-469) were measured. N-linked glycosylation sites were defined as the sequence motif N-X-T/S except when X is a proline. The net charge was determined as the sum of amino acid charges. The amino acids lysine (K), arginine (R) and histidine (H) were computed as positively charged and amino acids and aspartic acid (D) and glutamic acid (E) as negatively charged.

2.2.2 Glycan analysis

2.2.2.1 Univariate analysis

Potential N-glycosylation sites (PNGs) were tallied using the N-glycosite web tool (https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). Only subtype C viruses were included in this analysis. 200 subtype C sequences classified as either tier 1B, 2 or 3[143] were aligned separately and uploaded into the tool. Only sites that occurred in 20-90% of the sequences were tallied. This threshold was chosen to make sure that there would be enough viruses in the groups positive for the PNG and negative for the PNG to give reasonable statistical power in the analysis. In instances where a glycan site was conserved in one tier and not the other, the glycan sites were manually tallied for that tier in order for us to make comparisons. Once again, HXB2 reference sequence was used to number poistions.

2.2.2.2 Bayesian model averaging

To investigate which PNGs had the most influence on neutralisation resistance, linear regression models were fit using each PNGs selected above as a predictor. To determine the quality of each model relative to the other models and select the "best fit" ones, the Akaike information criterion (AIC) was used. Bayesian model averaging was then used to combine the estimates from the models selcted by AIC into one final model[171,172], with assitance from Dr. Michael Schomaker, CIDER, UCT). This method enables us to report the most stable

estimates while taking into account modelling uncertainty. The result of this analysis is presented as the posterior effect probability of each PNGs. This value is the probability that the regression model coefficients are not equal to zero which indicates that there is an effect. The higher the probability, the more evidence to support that a PNGs has an effect on neutralisation.

2.2.3 Monoclonal antibody analysis

We analysed sensitivity of subtype C viruses to commonly tested mAb. The epitopes of mAb have been well studied. Therefore, by carrying out this analysis, we may be able to determine the epitopes that drive resistance in tier 3 viruses. This analysis is important as mAbs are important for use as potential therapeutic and preventative interventions. All the data concerning the sensitivity of individual viruses to particular monoclonal antibodies used in this analysis was obtained from CATNAP, a web based tool available on the Los Alamos HIV-1 sequence database (https://www.hiv.lanl.gov/components/sequence/HIV/neutralization/index.html). To compare neutralisation sensitivities to bnAbs of tier 1B, 2 and 3 to the commonly studied bnAbs, 200 subtype C viruses that had bnAb data were used. Very few bnAbs were tested on tier 3 viruses. Therefore, only the nine bnAbs that had data for all tier 3 classified viruses were included in the analysis. Neutralisation sensitivity was represented as the concentration of antibody at which 50% of the viruses are neutralised (geometric mean IC_{50}) .

2.3 Production of pseudoviruses: Cell lines used

Pseudoviruses (PSVs) were generated by transfecting the HEK293T cell line obtained from the National Institute of Health (NIH) AIDS Reagent Program. HEK293T is a human cell line commonly used for the expression and production of proteins by transfection with plasmid DNA. Single cycle infectivity assays[173] were conducted using the TZM-bl cell line obtained through the National Institute of Health (NIH) AIDS Reagent Program- Division of AIDS, NIAID from Dr John C. Kappes and Dr Xiaoyun Wu. This is a HeLa cell line that has been engineered to express CD4 and CCR5 which allows for HIV-1 entry into the cell[174]. In addition, the cells have a firefly luciferase reporter gene under the control of a HIV-1 long terminal repeat (LTR)[175]. Transcription of the viral proteins is initiated when the viral Tat protein binds to the LTR. Viral entry, reverse transcription, integration and expression of the

viral Tat protein results in expression of the luciferase reporter which is quantified by measuring luminescence after the addition of a luciferase detection reagent (Promega, Madison USA) to the infected cells[176]. Both cell lines were maintained in culture media supplemented with 5% fetal bovine serum (FBS). However, assays were conducted in culture media supplemented with 10% FBS. Culture media contains Dulbecco's Modified Eagle Medium (DMEM) (Sigma chemical company, Schnelldorf, Germany) supplemented with 10 µg/mL of gentamicin, 25mM HEPES, 1% non-essential amino acids, 10 µg/mL pen-strep. All cultures were maintained in a humidified incubator at 37°C and 5% carbon dioxide. All cell culture incubation steps in this study were carried out in a humidified incubator at 37°C. Cells were grown in monolayers in tissue culture plates and passaged every 2 days. Cells were passaged by first aspirating out the media and then washed with 5mL Dulbecco's Phosphate-Buffered Saline (DPBS) without Calcium or Magnesium. Thereafter, 2mL room temperature 1X trypsin-EDTA (0.25% trypsin, 1mM EDTA) (Invitrogen, Dramstadt, Germany) solution was added to the cells and incubated at 37°C for 2 minutes in order to detach the adhered cells from the plate. The trypsin was inactivated by adding 8ml media with FBS to the plate and the cells carefully washed off and transferred to a 50ml conical tube for centrifugation at 1200rpm for 5 minutes. The pellet was then resuspended in 5ml of media and cells were counted using a haemocytometer. Cells were maintained at a density of 1 x 10^6 /ml until use in assays.

2.4 Env DNA preparation (transformation and plasmid purification)

Cloned HIV-1 envelope constructs for the subtype C and CRF02_AG viruses were used to generate PSVs. The constructs consists of the env gene cloned into a pcDNA expression vector obtained from the National Institute of Health (NIH) AIDS Reagent Program (http://www.aidsreagent.org/pdfs/11673_tab1.pdf) and Rademeyer *et al.*(2016). To generate more plasmid, the constructs were transformed and grown in Stbl 3 competent cells. 25 µL of Stbl 3 competent *E. coli* cells were thawed on ice and 200 ng of Env plasmid construct was added to the cells. The cells were incubated on ice for 30 minutes. Transformation occurred through the heat shock method. After the incubation on ice, the cells were incubated in a 42^0C water bath for 30 seconds and quickly returned on ice for 2 minutes. 200 µL of cell recovery media (S.O.C media) (Sigma chemical company, Schnelldorf, Germany) was added to the cells. The cells were then incubated in a shaking incubator (32^0C, 250rpm) for 1.5 hours. The cells were then plated on agar plates supplemented with 100 µg/ml ampicillin and

left overnight in a 32^0C incubator. The next day, single colonies were picked and grown in 5ml Luria-Bertani (LB) broth overnight at 32^0C. Cells were pelleted by centrifugation and plasmid extraction was carried out using the GeneJet™ Plasmid Miniprep Kit (Thermo Fischer Scientific, Massachusetts, USA). After successful extraction, the plasmid concentrations were determined using a nanodrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA).

2.5 Pseudovirus generation

Pseudoviruses (PSVs) lack a complete genome and are thus incapable of producing infectious progeny viruses. For this reason, they are suitable for single round infection assays. To generate PSVs, HEK293T cells were co-transfected with an *env* clone of interest and a plasmid containing a HIV-1 subtype B genome; pSG3Δ*env*[177]. pSG3Δ*env* contains all functional viral genes, except that the *env* gene contains a 4 base pair insertion which renders it defective. Using the same viral backbone for all PSVs meant that any differences in functional assays would be attributed to Env. The pSG3Δ*env* reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, and NIH from Drs. John C. Kappes and Xiaoyun Wu. HEK293T cells were seeded at 1 x 10^6 cells into a T25 culture flask in a final volume of 5ml 10% supplemented media. For each clone, 1334µg plasmid *env* DNA was added to 2667µg pSG3Δ*env* backbone DNA and the volume adjusted to 35µL with sterile plain DMEM. XtremeGENE 9 transfection reagent (Roche) was diluted in plain DMEM and added to the DNA mixture. This was mixed by vortexing for 5 seconds and then incubated at room temperature for 30 minutes. Each mix was subsequently added directly to the flasks containing HEK293T cells in 5ml media and the flasks were incubated for 48h in a 37°C, 5% $C0_2$ humidified incubator. Cell culture supernatants containing secreted PSV were collected after 48 hours.

2.6 Calculating entry efficiency

To determine the titre of the PSVs produced, TZM-bl cells were infected with PSVs at different concentrations to ascertain the dilution of supernatant at which there was 50,000 RLUs. Infection was carried out in the presence of DEAE dextran (Sigma chemical company, Schnelldorf, Germany). This is a poly-cation that enhances virus infection by stabilizing virion adsorption to the cell membrane. PSVs were diluted 2-fold to a final volume of 50ul

and topped up with 100ul of 10% DMEM in a 96 well microtitre tissue culture plate. 50ul of TZM-bl cells were added at a final density of 1 x 10^4 per well. Eight cell control wells were included in each plate by adding 10% DMEM to the cells instead of virus. The plates were incubated for 48 hours at 37°C, 5% CO_2 . After the 48 hours elapsed, 100µl of supernatant was removed from each well and replaced with 100µl Bright-Glo luciferase substrate, dissolved in Bright-Glo subtrate/lysis buffer (Promega, Madison, USA). The plates were incubated for 2 minutes in the dark and 100µl of the mixture was transferred into an opaque black plate to be read on a VERITAS MicroPlate luminometer (Promega, Madison, USA). Wells with RLUs greater than 2.5 times the average cell control value were considered positive. To determine entry efficiency, RLU measurements for each PSV were normalized to the amount of Reverse Transcriptase (RT, see section 2.6) activity detected in the sample and expressed as RLU/pg RT. Infectivity was determined from two independent experiments using two different PSV stocks for each isolate.

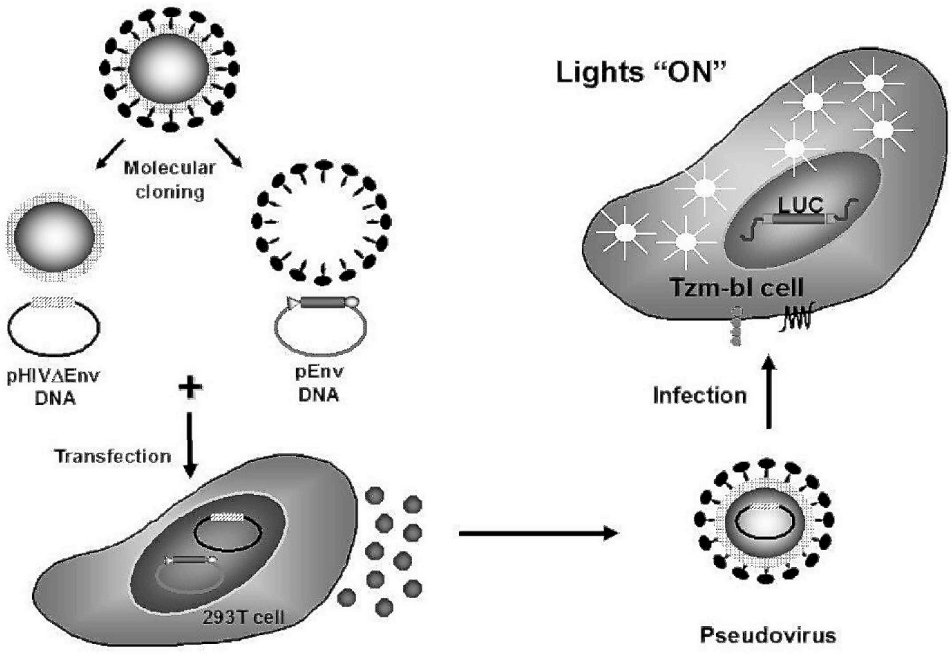


Figure 2.1 Schematic of pseudovirus (PSV) generation and TZM-bl infection assay. Co-transfection of a plasmid containing pSG3Δ*env* and pEnv into 293T cells allows for the generation of PSVs. Infectious PSVs infect TZM-bl and induce the expression of the luciferase reporter gene. Addition of a luciferase substrate leads to the emission of luminiescence. Adapted from Ozaki *et al* 2012

2.7 Quantifying viral Reverse Transcriptase activity for infectivity assays.

2.7.1 Virus isolation

Supernatant containing PSVs was harvested 48 hours post transfection. To quantify reverse transcriptase (RT) it was necessary to concentrate the virus particles. In this instance, we used ultra-centrifugation to concentrate virus particles. An initial centrifugation step was carried out to remove cellular debris (2000g for 30 minutes at 4^0C) from the supernatant. 1mL of the supernatant was then transferred to an ultracentrifugation tube (Beckman coulter, Indianapolis, United States) and centrifuged at 100,000g for 10 minutes in an ultracentrifuge (Beckman Coulter, Indianapolis, United States). The supernatant was then carefully decanted and the pellet was stored at -80^0C for RT quantification.

2.7.2 Reverse transcriptase colorimetric assay

To quantify the relative quantities of PSVs produced, viral RT activity was measured using a colorimetric immunoassay kit (Roche Holdings, Basel, Switzerland). This assay makes use of RT's ability to synthesize DNA from a template. The template/primer hybrid (poly (A) × oligo $(dT)_{15}$) required for the reaction were supplied with the kit. Digoxigenin (DIG) and biotin labelled nucleotides are then incorporated into the DNA molecule synthesized by the viral RT. The detection of the new DNA molecule follows a sandwich ELISA protocol.

Briefly, the stored pellets were lysed with a lysis buffer (50mM Tris, 80mM potassium chloride, 2.5mM DTT, 0.75mM EDTA and 0.5% Triton X-100, pH 7.8; provided with kit) and incubated at room temperature for 30 minutes to ensure complete resuspension and lysis. The template/primer hybrid and nucleotides mixture was then added to the viral lysate and incubated for 1 hour at 37^{o}C. The reaction mixture containing the newly synthesized DNA was transferred to a streptavidin coated 8-well microplate module, which will strongly bind to the biotin in the DNA, if reverse transcription has taken place, and incubated for 1 hour at 37^{o}C. The wells were then washed and an antibody to digoxigenin conjugated to peroxidase is added. This antibody binds to digoxigenin on the synthesized DNA molecule, if present. Following antibody binding, the wells were washed to remove unbound antibody and a peroxidase substrate, ABTS, was added. Peroxidase catalyses the cleavage of this substrate

into a coloured product, which was measured on an ELISA plate reader at 405nm and compared to a reference reading at 492nm. The absorbance at 492nm was subtracted from the 405nm reading and this is directly proportional to the amount of RT activity in the sample. To determine RT concentrations, an HIV-1 RT (supplied with kit) with known concentrations was used as a standard. This standard was serially diluted and used to draw linear calibration curves. The average absorbance for replicate readings were plotted on the y-axis and the concentration of HIV-1 RT standards were plotted on the x-axis. The concentrations of RT for unknown samples were then determined by plotting the sample absorbance values on the y-axis and the concentrations were extrapolated from the RT concentrations on the x-axis.

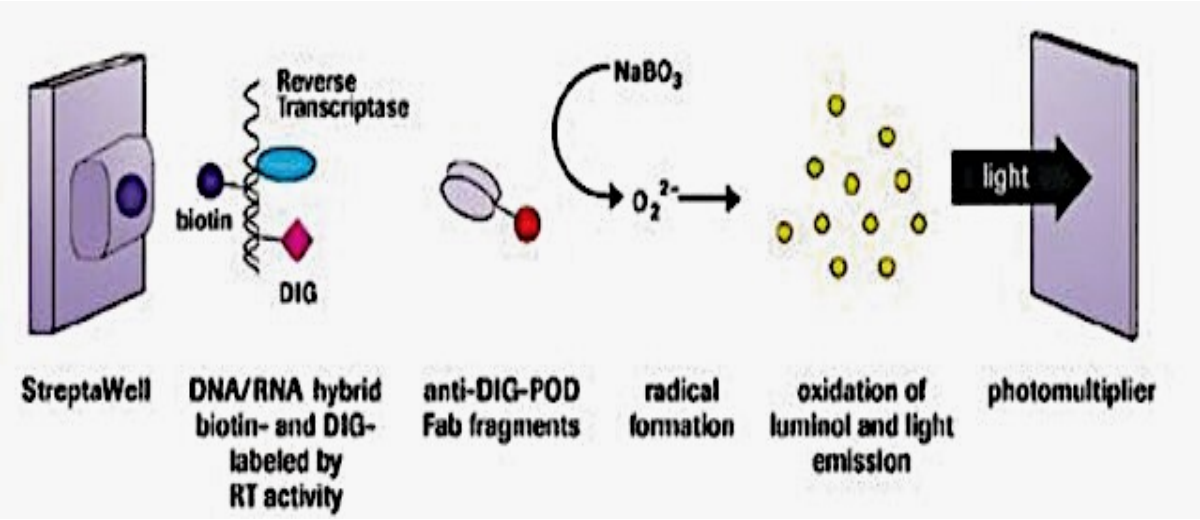


Figure 2.2 Schematic of the reverse transcriptase (RT) activity assay. Viral RT synthesizes DNA labelled with biotin and DIG which is added to a strptavidin coated well. Addition of a peroxidase conjugated anti-DIG antibody allows for the measurement of RT activity. Image from Sigma.

2.8 Entry inhibition assays

The entry inhibitors Maraviroc (CCR5 anatgonist), PSC RANTES (CCR5 competitive inhibitor) and T20 (fusion inhibitor) were used to determine a virus' dependence on aspects of the entry process. These inhibitors were selected as they showed a correlation to entry efficiency assays in preliminary studies. Five fold serial dilutions of the inhibitors were made and 50ul of the dilutions were added to a 96 well tissue culture plate in duplicate. 100µl of TZM-bl cells with DEAE dextran were added to the wells at a final density of 1 x 10^4 per well. The plates were incubated at 37^0C for 30 mins. Thereafter, PSVs were added at a concentration that would yield 50,000 RLUs. The plates were then incubated for 48 hours.

100µl of supernatant was removed and replaced with 100µl of luciferase substrate and incubated in the dark for 2 mins. The mixture was then transferred to an opaque plate and luminometer readings were obtained. Antiviral activity was determined as the percentage of viral inhibition in experiment wells compared to the control wells (no inhibitor). Viral inhibition was plotted against the concentration of the inhibitor compound tested and a curve fit function was used to calculate the concentration of inhibitor at which 50% of entry is inhibited represented as an EC_{50} (GraphPad Prism software, CA, USA).

2.9 Statistical analysis

Statistical anayses of all data were performed on STATA (STATACorp) and GraphPad Prism. Graphs were generated using GraphPad Prism. EC_{50} values were generated using the curve fit function on GraphPad Prism. Prior to carrying out any tests, the Shapiro Wilk's test was used to determine if data was normally distributed. Where data was normally distributed, a student t-test was used to calculate p-values. Otherwise, the Mann Whitney test was employed. In all analyses where the sample number was small, the Mann Whitney test was used. The median and interquartile range are shown on all dot plots. Correlations were performed using Pearson's correlation (CI 95%) or Spearman's correlation depending on the sample size and distribution. The p-value cut off for significance used in this study was 0.05.

3 Results

3.1 Neutralisation profile of subtype C and CRF02_AG viruses

The subtype C viruses used in this study had already been categorized by tier based upon their neutralisation sensitivity to a panel of subtype C-infected sera[143] (Fig 3.1). Selection of the 16 subtype C Envs to be used in the functional assays was primarily based on neutralisation phenotype. First, all tier 3 viruses were selected (red dots) for which I had access to clones for generating PSVs, thereafter; tier 2 (blue dots) and 1B (green dots) viruses were selected to represent a wide range of neutralisation sensitivities.

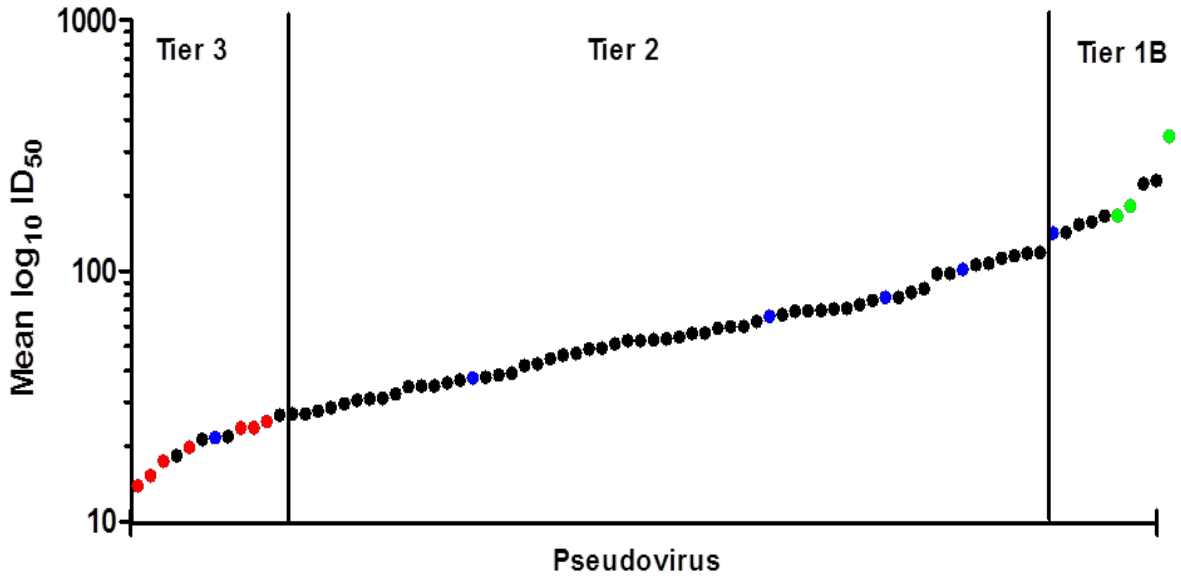
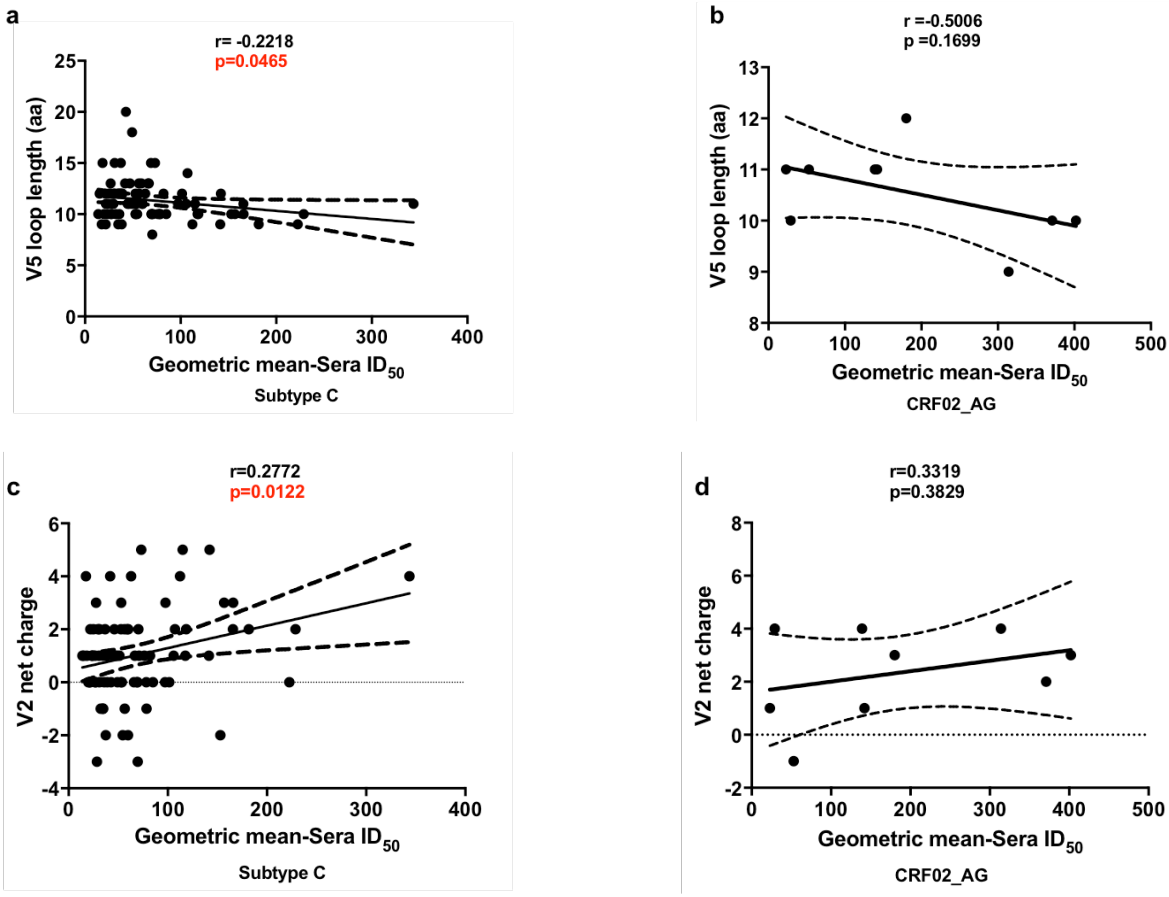


Figure 3.1 Geometric mean ID50 and tier classification of subtype C viruses used in this study. 81 subtype C Viruses were rank ordered according to their sensitivity to neutralisation to a panel of 30 sera from chronically infected HIV+ individuals[148]. Of the 81 viruses shown here, a subset of 16 viruses were chosen for analysis in pseudovirus-based infectivity assays. Of these 16 viruses, red dots represent tier 3, blue dots represent tier 2 and green dots represent tier 1B viruses. Clones that did not consistently cluster with tier 1 or tier 3 were categorized as tier 2. This is indicated by the single blue dot (clone 569-F1_37_10) that lies within the tier 3 range[143]. (ID_{50} values used were determined previously[143]

3.2 Variable loop characteristics associated with neutralisation resistance

Longer loops and increased glycan density has been associated with resistance to neutralisation with the V1V2 loop being a major driver of this association[68]. We analysed the V1, V2, V4 and V5 loop characteristics including length, glycan density and inferred net charge of 89 subtype C Envs in relation to their neutralisation phenotype. We found that the V2 loop net charge was negatively correlated with neutralisation resistance, and the V5 loop length was positively correlated with resistance in subtype C. In a similar analysis, with 8 CRF02_AG viruses, the V2 loop length was positively correlated with resistance (Fig 3.2). We observed no correlation between V1V2 loop length and glycan density and neutralisation in the subtype C panel as other studies have reported[68,148]. This subset of 81 subtype C viruses was selected from a bigger panel of 200 viruses in which V1V2 loop length and glycan density was associated with resistance[148]. Thus, the lack of an observed association in this subtype C data set is plausibly due to our smaller sample size, and thus smaller statistical power to detect associations.



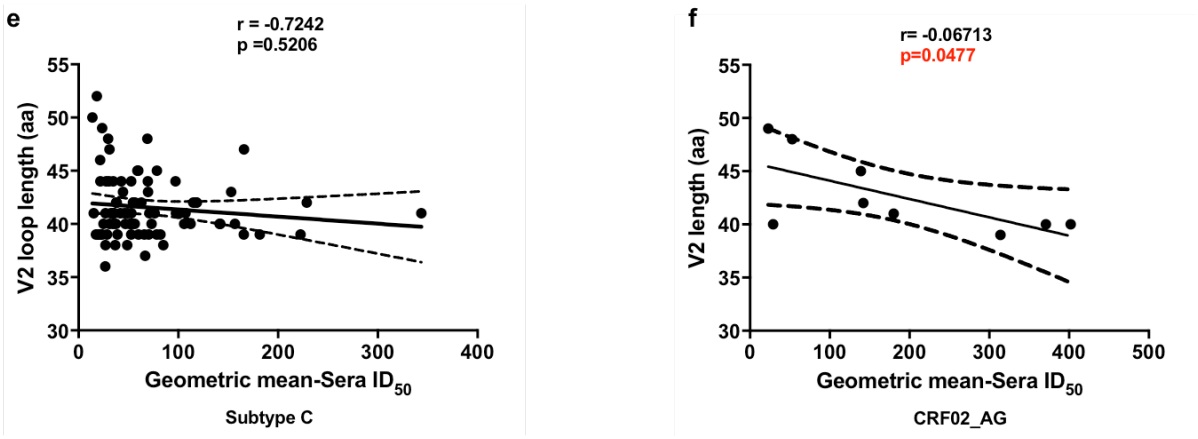


Figure 3.2. **Variable loop characteristics of *env*s that correlate with neutralisation resistance.** (**a**) V5 loop length is negatively correlated with neutralisation in subtype C but (**b**) is not significantly correlated in CRF02_AG. (**c**)V2 net charge is positively correlated with neutralisation in subtype C viruses but (**d**) is not significantly correlated in CRF02_AG. (**e**) V2 loop length is not associated with neutralisation in subtype C but is (**f**) negatively correlated with neutralisation in CRF02_AG. Loop length is measured in number of amino acids. 81 subtype C and 9 CRF02_AG sequences were used in the analysis**.** Sequences were downloaded from the LANL website and the variable region characteristics tool (on LANL) was used to calculate loop lengths and net charge. A Pearson's or Spearman rank correlation was applied to all data sets depending on the distribution of data and graphs generated by Graphpad Prism ($p<0.05$). Significant p values are shown in red text.

3.3 Relationship between neutralisation resistance and entry efficiency

Neutralisation escape leads to selection of changes in Env that confer resistance to nAbs[42]. As Env mediates viral entry into target cells, these changes might impact entry efficiency. We therefore hypothesized that highly resistant viruses (tier 3) have incurred fitness costs as measured by entry efficiency. To test this, we used the tier designation[143] (Fig 3.1) as a categorical designation of neutralisation resistance, and evaluated the selected panel of 16 subtype C viruses designated as tier 3 (highly resistant), 2 and 1B (highly sensitive) for Env function by measuring entry efficiency and sensitivity to entry inhibitors. The panel was restricted to 16 viruses as the entry efficiency assays could not be performed on all 89 Envs. (Both entry and reverse transcriptase activity must both be measured, and each value shown is the geometric mean of measurements from two separate preparations of PSVs.) A description of the subtype C (Table 2.1) and CRF02_AG viruses (Table 2.2) is shown in chapter 2.

Entry efficiency was calculated as the relative light units (RLU) generated in a PSV infectivity test per picogram of reverse transcriptase activity present in each viral stock (RLU/pg RT). We first investigated whether neutralisation resistance correlated with entry

efficiency and found no overall correlation between neutralisation resistance and entry efficiency in either subtypes (Fig 3.4). However, importantly, when we categorized the subtype C panel viruses into tiers, subtype C tier 3 viruses were significantly (p=0.014) more infectious that tier 2 viruses (Fig 3.3). Interestingly, these results were contrary to our original hypothesis; indicating that in these viruses, resistance did not result in decreased fitness as measured by entry efficiency. Within the tier 1B and tier 3 there were outliers with the highest entry efficiency; CAP174.1.06_F3_1B in the tier 1B and Ko243_H6.3 in the tier 3. With only three viruses in the Tier 1B group, it was not possible to make comparisons with other subtype C groups. This tiered comparison was not performed for the CRF02_AG viruses as they were too few to reliably tier categorize them by within-subtype neutralisation. For this reason, only the association with geometric mean ID_{50} is shown. 251-18 (105,423 RLU/pg RT) was the most infectious of the CRF02_AG viruses. The most neutralisation sensitive subtype C and CRF02_AG viruses tested (CA146_H3_3 and 255_34 respectively) were also the least infectious isolates tested. Nonetheless, contrary to our hypothesis, there seems to be no cost to entry efficiency associated with resistance.

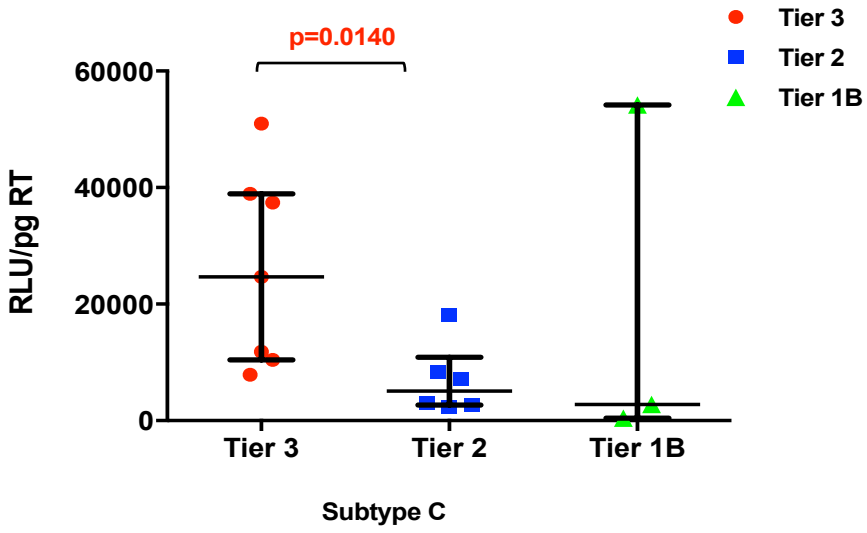


Figure 3.3 Entry efficiency is increased in subtype C tier 3 viruses. Entry efficiency of *env*-typed PSVs was assessed using a single cycle entry assay on TZM-bl cells. The figure shows the entry efficiency expressed as RLU per pg of RT in infection experiments with each PSV. Red dots represent tier 3, blue squares represent tier 2 and green triangles represent tier 1B viruses. Each symbol on the graph represents one PSV. The bars represent the median and interquartile range for all data sets. *P*-values were calculated by performing a Mann Whitney test and the graphs were generated using GraphPad Prism

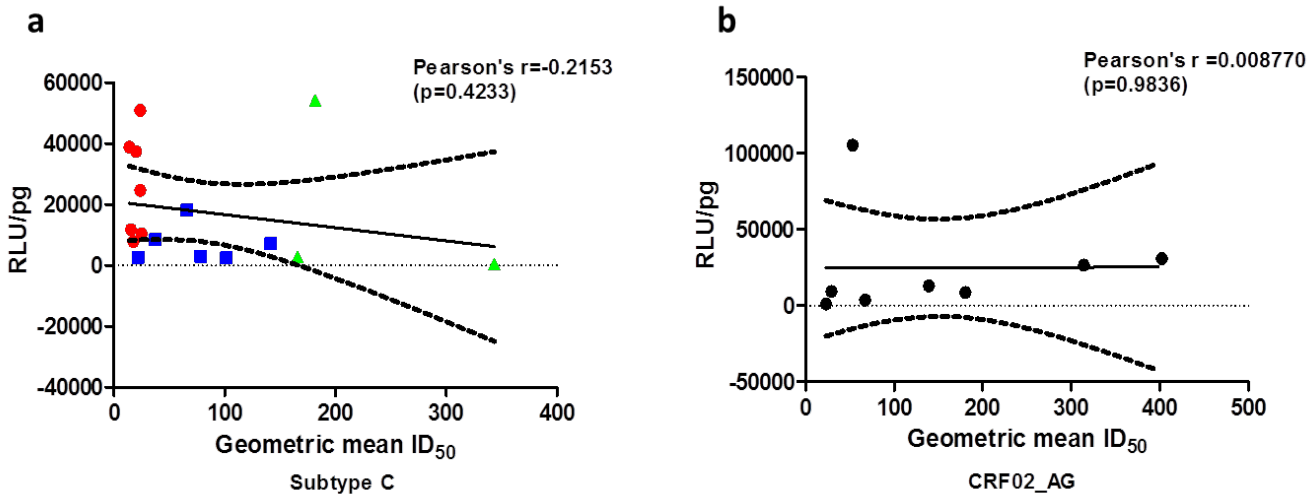


Figure 3.4 Neutralisation sensitivity does not correlate with entry efficiency. Entry efficiency for all PSVs is plotted on the y-axis while the neutralisation sensitivity is plotted on the x -axis. **(a)** Represents subtype C PSVs coloured according to tiers (red dots=tier 3, blue squares=tier 2 and green triangles=tier 1B viruses) while **(b)** represents CRF02_AG PSVs. A Pearson's rank correlation was applied to all data sets and graphs generated by Graphpad Prism.

3.4 Neutralisation resistance and susceptibility to entry inhibitors

Entry inhibition assays are a useful tool for uncovering functional differences between viruses in relation to one another. Hence, we investigated the effect of three inhibitors on virus entry. Inhibition sensitivities of the PSVs were determined using the 50% effective inhibitory concentration, that is, the concentration at which viral infectivity, measured as RLU, was inhibited by 50% in the presence of the drug (EC_{50}). Maraviroc (MRV) blocks entry by binding CCR5 in a non-competitive manner[178] while PSC RANTES competes with the virus for CCR5 binding and is an approximate test for CCR5 affinity[179]. Additionally, sensitivity to the fusion inhibitor T20 was measured.

All viruses tested were R5-tropic viruses. Tier 3 viruses exhibited a wide range of EC_{50} values for the various inhibitors (fig. 3.5). Overall, there was no significant association between neutralisation sensitivity and entry inhibition (Fig 3.5). A trend towards reduced sensitivity to Maraviroc in subtype C tier 3 viruses compared to tier 2 was observed (p=0.08) suggesting increased binding capacity to CCR5 of these viruses. We observed that the subtype C viruses that had reduced sensitivity to Maraviroc also had reduced sensitivity to T20. This is expected as T-20 can only inhibit fusion after CD4 attachment and before co-receptor binding[180]. Two subtype C viruses; CAP260.2.00_TA1_1B and CAP291.2.00_H2.15 were the least sensitive to Maraviroc (8.9 and 8.8nM respectively)

suggesting that these viruses can still infect cells despite blocking of CCR5 by Maraviroc. Ko243_H6.3 was the least susceptible to PSC Rantes within the subtype C clones indicating that it has a high affinity to CCR5 and this could partially explain its high entry efficiency (Fig 3.6).

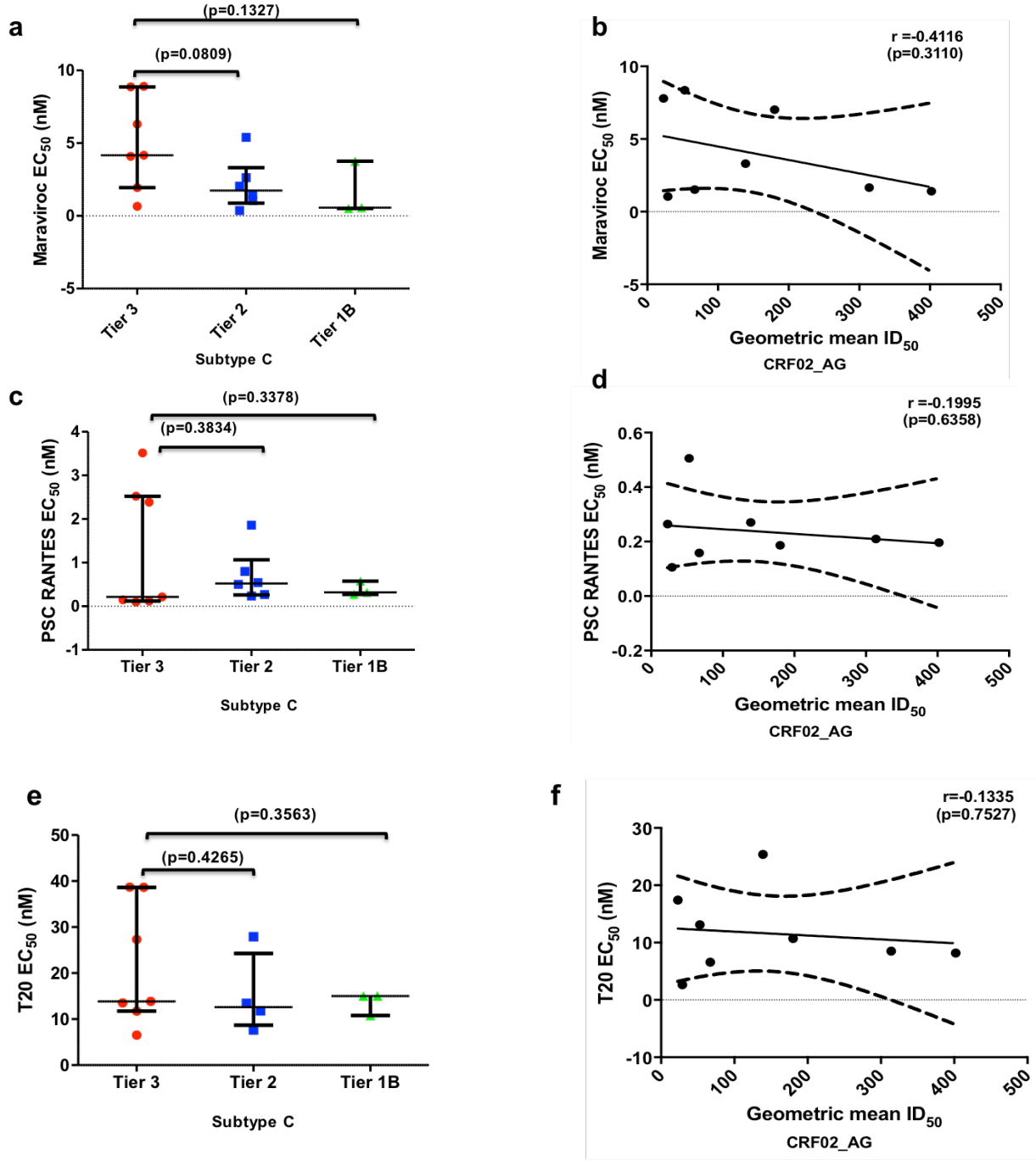
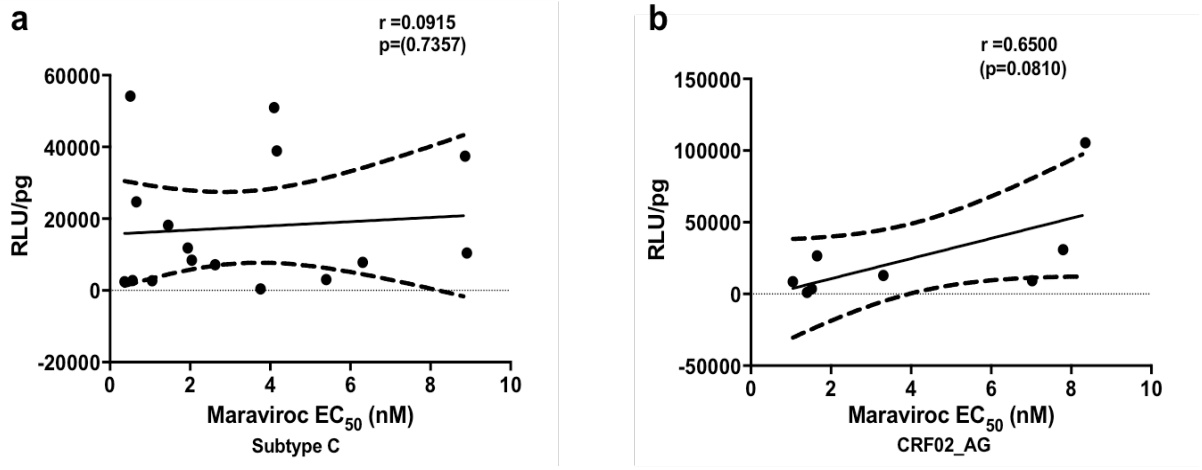


Figure 3.5 Inhibition sensitivities of viruses do not correlate with neutralisation phenotype. Inhibition sensitivities represented as effective inhibitory concentrations (EC_{50}) are represented on the y-axis for subtype C and CRF02_AG. (**a,c,e**) represents sensitivity of subtype C viruses to the inhibitors Maraviroc, PSC Rantes and T20 respectively. (**b,d,f**) represents sensitivity of CRF02_AG viruses to the inhibitors Maraviroc, PSC Rantes and T20 respectively. .Each point on the graph represents a PSV. The bars represent the median and

interquartile range for each group. The differences were not significant for any of the inhibitors, as indicated by the parentheses around the p values.

3.5 Susceptibility to entry inhibitors and entry efficiency

Next, we investigated which entry step correlated best with overall entry efficiency (Fig 3.7). We found that decreased susceptibility to PSC RANTES was associated with increased entry efficiency in CRF02_AG (r=0.9292, p=0.0008). However, this relationship is likely infulenced by the single point (100,000 RLU/pg/0.5 nM). This suggests that the relationship we are presenting could hinge entirely upon small errors in one data point. This might not have been true if we had a larger set of CRF02_AG viruses to analyse in this manner. Decreased susceptibility to PSC RANTES indicates that the virus out-competes the inhibitor for binding to CCR5. Therefore, this result is expected as increased affinity to CCR5 would, theoretically, make the virus more likely to infect a target cell (if all other conditions were equivalent). We observed no correlation between entry inhibitors and subtype C entry efficiency.



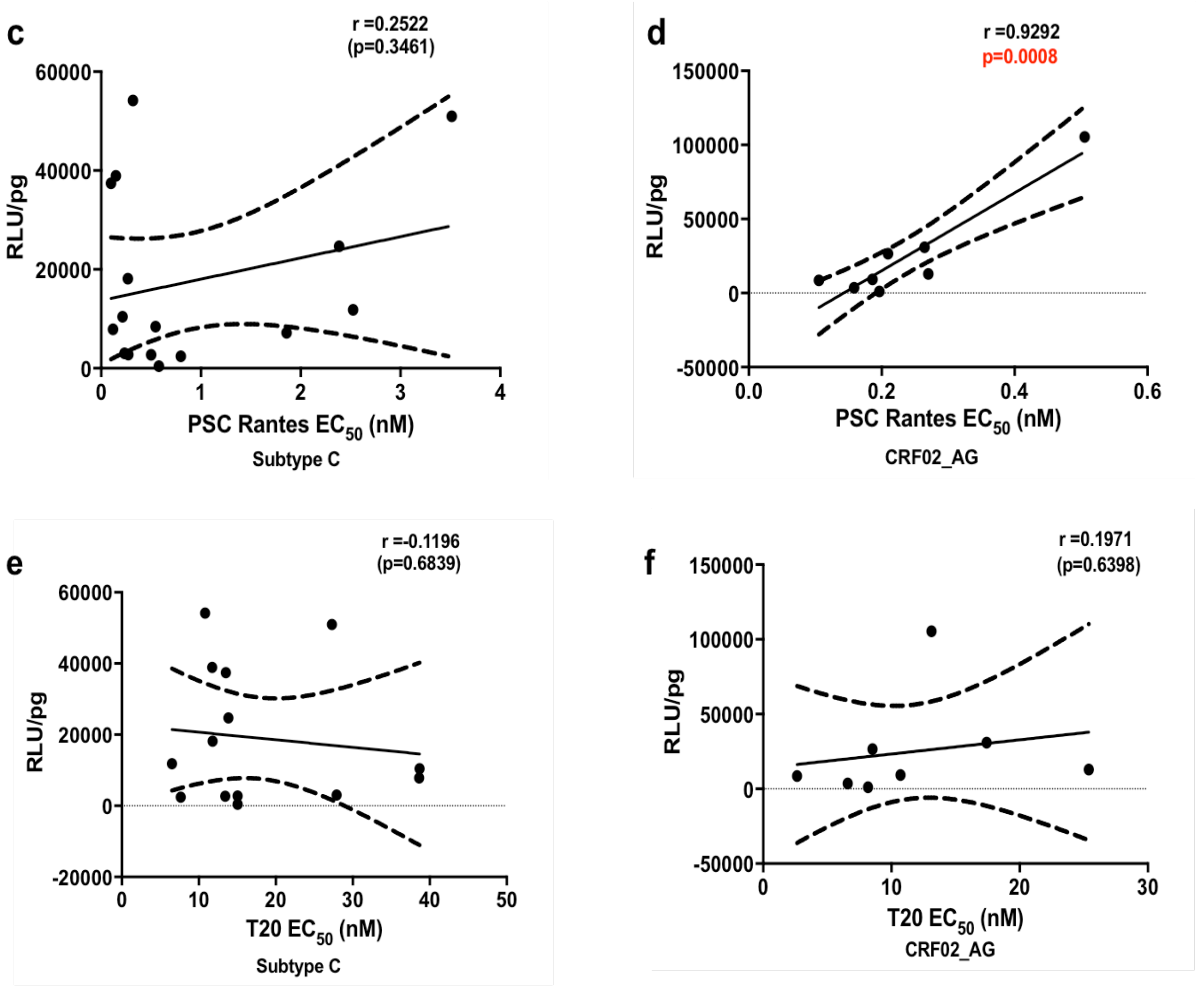


Figure 3.6 Correlation between entry efficiency and entry inhibitors. Inhibition sensitivities represented as effective inhibitory concentrations (EC_{50}) of the different inhibitors are represented on the x-axis, and entry efficiency on the y-axis. (**a,c,e**) represents subtype C viruses while (**b,d,f**) represents CRF02_AG viruses. A Pearson's rank correlation was applied to all data sets and graphs generated by Graphpad Prism.

3.6 V2 loop characteristics are associated with entry efficiency

The variable loops play an important role in viral entry[181]. We further investigated which variable loop characteristics were associated with entry efficiency for the viruses that we assayed. Of all the loop characteristics tested (V1-V5 loop length, PNGs density and net charge), we found that, in subtype C viruses, increased glycosylation in the V2 loop was positively correlated with entry efficiency but not in CRF02_AG. On the other hand, the overall V2 net charge was negatively correlated with entry efficiency in CRF02_AG viruses but not in subtype C (Fig. 3.7). The differences in association between the two subtypes observed here may be explained by the lack of statistical power as both subtypes show a

trend in the same direction. Of note, this data highlights the importance of the V2 loop in mediating viral entry, and that the attributes of the V2 loop that we detected as important are different for subtype C and subtype CRF02_AG viruses.

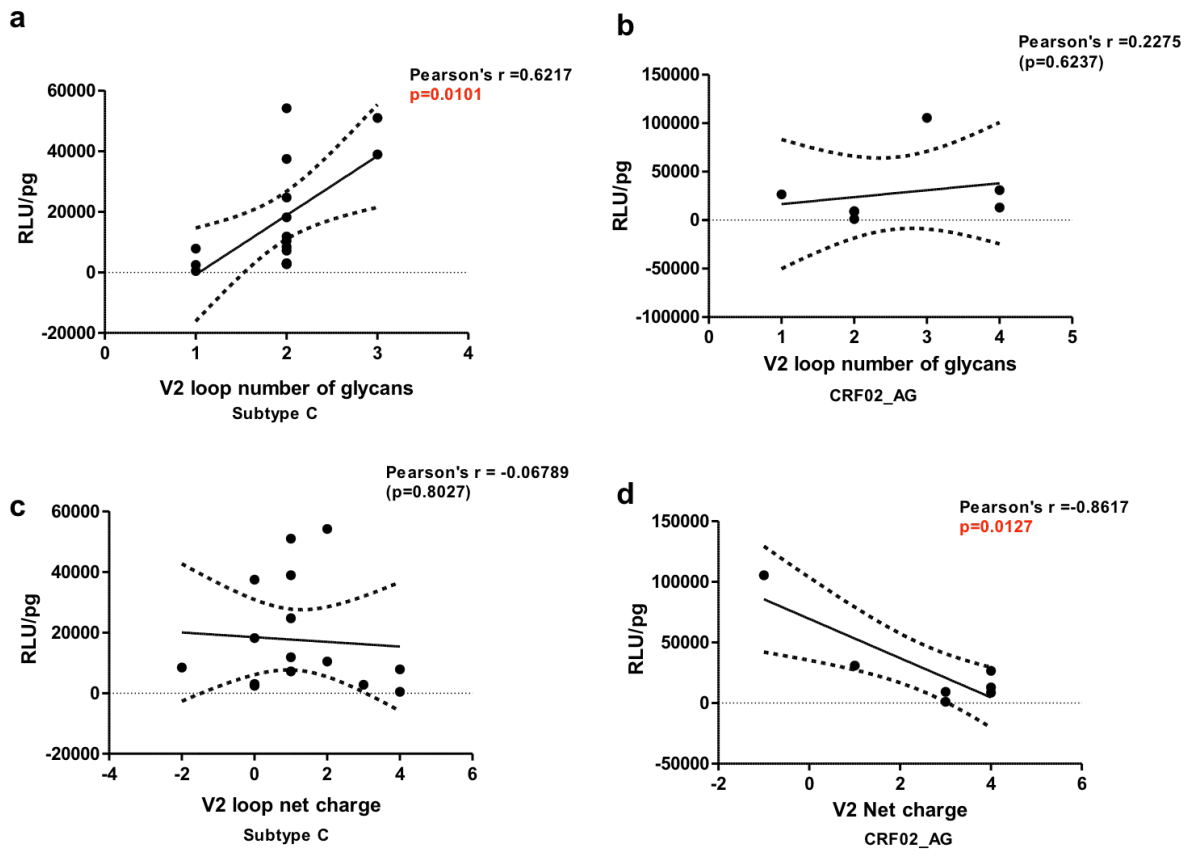


Figure 3.7 Entry efficiency correlates with V2 loop glycans and net charge. Entry efficiency for all PSVs is plotted on the y-axis while the neutralisation sensitivity is plotted on the x -axis. (**a**) Number of V2 loop glycans correlated with entry efficiency only in the subtype C viruses (**b**) but not in CRF02_AG; **(c)** V2 net charge did not correlate with entry efficiency in subtype C but (**d**) was negatively correlated in CRF02_AG viruses. A Pearson's rank correlation was applied to all data sets with significant p-values indicated in red. Graphs were generated using Graphpad Prism. Note that the y-axis scale is different for the subtype C vs the subtype CRF02_AG viruses. CRF02_AG virus 251-18 is the only virus tested with entry efficiency over 60,000 RLU/pg RT.

3.7 Differences in Env phenotypes between subtype C and CRF02_AG

Genetic variation between subtypes in the *env* gene is often 25 to 35%[182]. Differential characteristics of viral subtypes and their interactions with the human host may influence HIV transmission and disease progression. For example, a prior study showed that there were more maraviroc and vicriviroc-resistance mutations naturally occuring in subtype C viruses compared to subtype B strains[183]. Additionally, more T20 resistance mutations were present

in non-subtype B strains than in subtype B[184]. This might affect treatment with entry inhibitors. We investigated the differences in Env phenotype between subtype C and CRF02_AG viruses. We found no significant differences between the subtypes in entry efficiency and susceptibility to Maraviroc, PSC RANTES and T-20 (Fig 3.8). However, we did observe a trend of increased susceptibility to PSC RANTES in the CRF02_AG viruses compared to subtype C suggesting that these CRF02_AG viruses had lower affinity for CCR5 compared to the subtype C viruses. This might at least partially explain the difference in prevalence between these two subtypes. Maraviroc and T20 EC_{50} ranges were similar between the two subtypes. 251_18, a moderately to severely neutralisation resistant CRF02_AG virus, had the highest entry efficiency (105,423 RLU/pg RT) of all viruses tested, the only virus we tested with an entry efficiency over 60,000 RLU/pg RT.

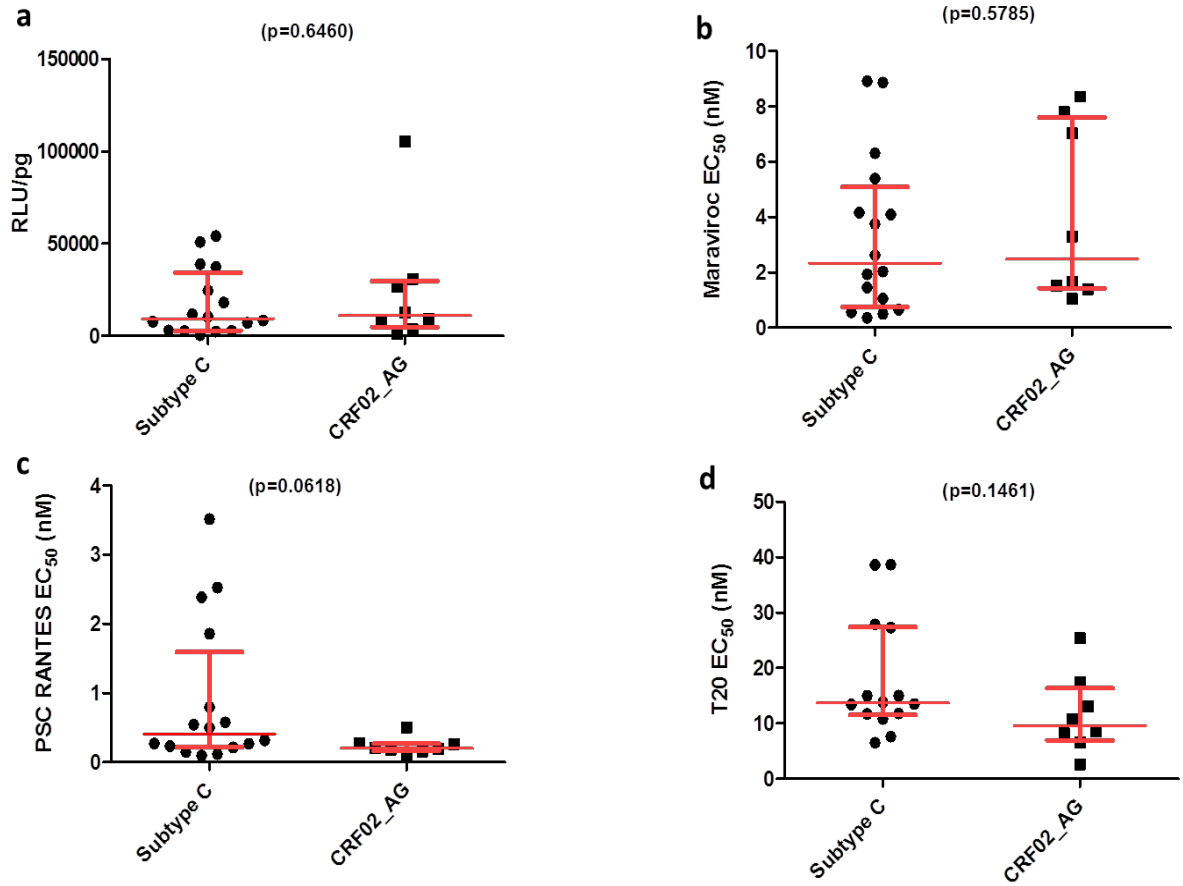


Figure 3.8 Phenotypic properties of subtype C and CRF02_AG do not significantly differ. Dot plots of **(a)** entry efficiencies (geometric mean RLU/pg RT) and sensitivity to (b) Maraviroc, (c) PSC Rantes and (d) T20 of both subtypes are shown. Each dot on the graph represents a PSV. The bars represent the median and interquartile range for each group. The differences were not significant for any of the inhibitors (p=0.05, unpaired t-test or Mann Whitney test depending on data distribution).

3.8 Glycan conservation analysis and neutralisation sensitivity

3.8.1 Univariate analysis

Glycans on Env play an important structural role and mediate evasion from nAbs[185]. An analysis of HIV-1 escape mutants showed that most of the mutations acquired during antibody escape involved changes at the N-linked glycosylation sites[42]. We investigated the inferred glycan profile of Env based on neutralisation phenotype to determine if there were differences in the glycosylation patterns between the tiers. We analyzed the 200 subtype C sequences that had been categorized as tier 3, 2 or 1 previously[143] based on within subtype neutralisation. This included 28 tier 3, 156 tier 2 and 16 tier 1B sequences. We included only subtype C in order to limit any confounding inter-subtype differences. This analysis was not done on CRF02_AG because of the small sample size (n=8). We used the N-glycosite tool on the LANL website which tallies all predicted N-glycan sites on the sequences. Sites that were conserved in 20-90% of the sequences were selected (Fig 3.9).

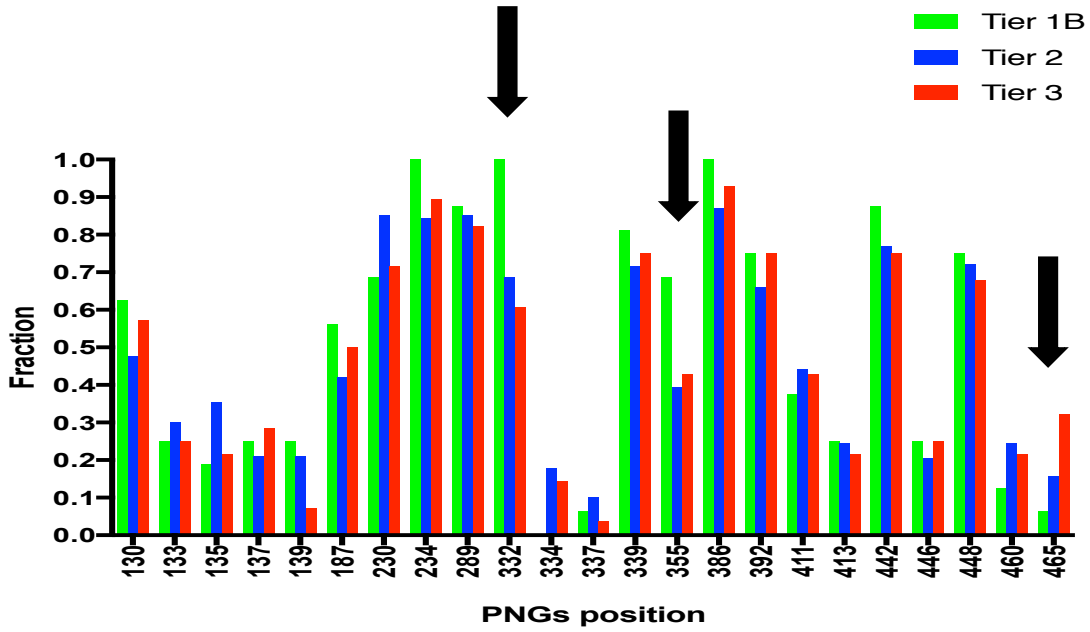


Figure 3.9 Comparing PNGS conservation in viral sequences in the different tiers. The x axis represents the position of PNGs while the y-axis represents the proportion of a particular PNGs in the total sequence per tier (fraction). Black arrows indicate the glycan positions at which a difference in prevalence was observed between tiers. We used the N-glycosite tool on the LANL website to tally PNGS that are conserved in tier 3 (n=28), 2 (n=156) or 1B (n=16) and compared the three groups.

N465 was more common in tier 3 (32%) and 2 (15%) viruses compared to tier 1B (6%) while N332 was enriched in tier 1B sequences (100%; 16/16) compared to tier 2 and 3 (68% and 60% respectively). About 16% of tier 2 and 17% of tier 3 sequences had a glycan at position

334 while some sequences lacked both N332 and N334 (6 tier 3 sequences and 29 tier 2 sequences). In addition, Tier 3 viruses were more enrinched with N355 (70%) compared to tier 2 and 1B and N139 was relatively more common (about 25%) compared to tier 3 (less than 10%).

3.8.2 Bayesian model averaging analysis

As some glycans can compensate for other glycans, this initial univariate analysis was not sufficient in informing us about which PNGs are strong predictors of resistance. Therefore, we used linear regression to fit the relationship between neutralisation sensitivity (geometric mean ID_{50}, data for which was available for 87 viruses of the original 200) and presence or absence of each of the PNGs selected above (Fig 3.9). 22 PNGs were identified from the initial analysis as being present in 20-90% of the sequences across tier 1B, 2 and 3 (Fig 3.9). Glycan 334 was added back because of its strong negative association with N332 (mutually exclusive due to motif overlap) and N674 was removed because glycosylation at 674 in the Membrane Proximal external Region would interfere with fusion. Model selection using the Akaike information criterion (AIC) favoured models that included N137, N332, N386, N413 and N446 as predictors, with the strongest likelihood of direct association with N413 and N332 (Table 3.1). To account for model selection uncertainty, we used Bayesian average modeling to combine all the models the AIC chose into a final model that could accurately predict neutralisation resistance. We report the posterior effect probability of this model for all 23 PNGs. The higher the probability (between 0-100%), the more evidence there is that the PNG has a direct effect on neutralisation, independent of the other glycans in the model. Based on this analysis, N413 and N332 were found to most likely influence neutralisation in these subtype C viruses independent of the presence or absence of PNGs at other sites. (Table 3.1). N355 which was also picked up by the univariate analysis, was the fourth most important PNG for neutralisation. Interestingly, N413 was not picked up by the univariate analysis. This maybe because of the influence of N411 or other PNGs which are accounted for in the bayesian analysis but not the univariate analysis.

Table 3.1 Bayesian average modeling for glycan sites important for neutralisation

PNGs[1]	Posterior effect probability[2]
N413	81.7
N332	38.2
N137	13.1
N355	12.8
N446	11.8
N386	7.3
N411	6.1
N442	5
N460	4.9
N130	4.4
N334	4.4
N234	3.7
N135	3.4
N339	3.3
N337	2.9
N448	2.8
N289	2.4
N465	2.2
N139	1.9
N187	1.9
N133	1.8
N392	1.8
N230	1.6

[1] PNGs numbering is based on the reference sequence HXB2

[2] The posterior effect probability indicates the calculated probability that the coefficient in the regression model used is not zero with model uncertainty taken into account.

The presence of N413 and the lack of N332 was associated with neutralisation resistance (Fig 3.10). This finding is consistent with previous reports[143,186] and once again highlights the influence of glycans on neutralisation sensitivity.

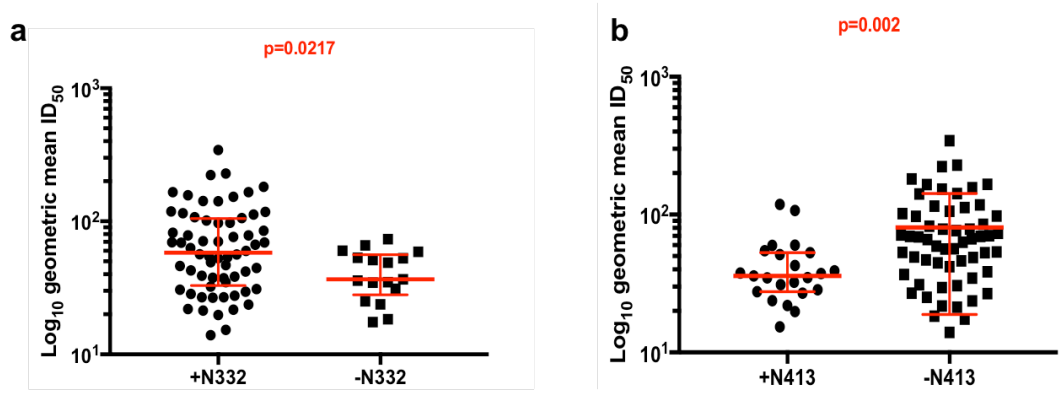


Figure 3.10 N332 and N413 are associated with neutralisation resistance in subtype C. The figure compares the neutralisation profile of 81 subtype C viruses that have or lack N332 and N413. (**a**) Absence of N332 was associated with neutralisation resistance while (**b**) Presence of N413 rendered viruses more resistant to neutralisation. The log geometric mean ID_{50} is plotted on the y axis and represents neutralisation sensitivity. Each symbol on the graph represents one virus. The bars represent the median and interquartile range for all data sets. P-values were calculated by performing a Mann whitney test and the graphs were generated using GraphPad Prism.

3.9 Tier 3 viruses are resistant to the commonly tested broadly neutralising antibodies

We wished to better understand which epitopes of Env are important for overall neutralisation resistance. As the commonly studied bnAbs have well defined epitopes; increased resistance to a particular bnAb in highly resistant viruses would indicate which epitopes play a role in driving resistance during escape. Therefore, we investigated if tier 3 viruses have differing sensitivity to the commonly studied bnAbs compared to tier 2 and 1B viruses. This analysis included only bnAbs with data for all tier 3 viruses were considered. Using the CATNAP tool on the LANL website, we compared IC_{50} values of 200 subtype C viruses categorized as tier 3, 2 or 1 previously[143] by within subtype neutralisation and had been tested against a number of bnAbs.

We found that tier 3 viruses were significantly more resistant to PGT121, 4E10 and the CD4bs antibodies (3BNC117 and VRC01) but not the V1/V2 bnAbs (PG9 and PGT145) compared to tier 2 and 1 (Fig 3.11). There were differences in sensitivity between tier 1 and tier 3 in 10-1074, PGT128 and 10E8, however, there were no differences between tier 2 and tier 3 viruses to these bnAbs. Together, this data suggests that the antibody selection pressure that drives resistance involves most of the major gp120 and gp41 epitopes rather than a single epitope.

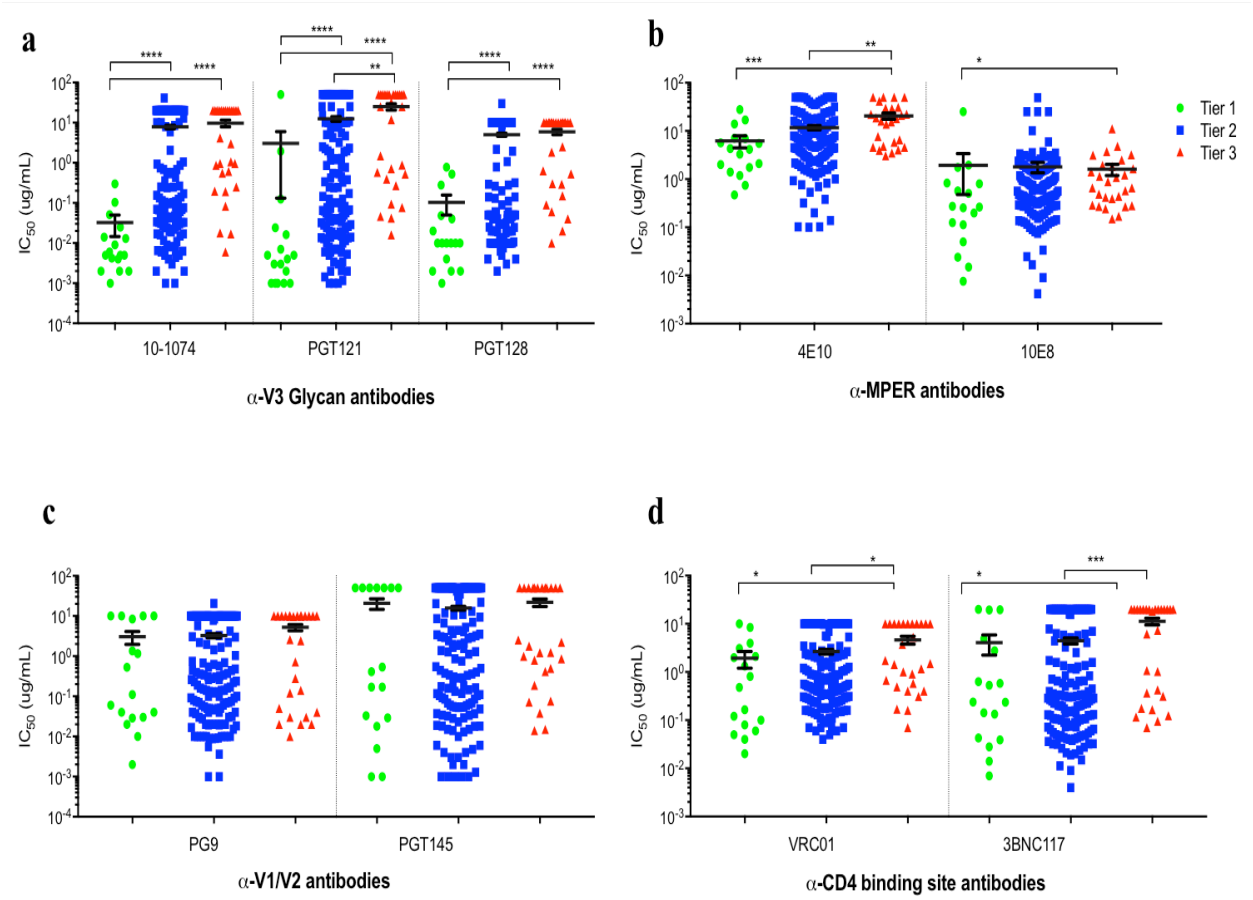


Figure 3.11 Tier 3 viruses are more resistant to bnAbs. Dot plots show the distribution of antibody titres represented by IC_{50} towards tier 3 (n=28), 2 (n=149) and 1 (16) viruses. The horizontal line and error bars indicates the mean and standard error of mean for each group. **(a)** V3 glycan antibodies 10-1074, PGT121 and PGT128. **(b)** MPER antibodies 4E10 and 10E8 **(c)** V1/V2 antibodies PG9 and PGT145 **(d)** CD4bs antibodies: VRC01 and 3BNC117. Tier 3 viruses were more resistant to the V3 glycan antibodies (10-1074, PGT121 and PGT128), the MPER antibodies 4E10 and 10E8 and the CD4bs antibodies VRC01 and 3BNC117. bnAb data was available on the LANL website. Each symbol on the graph represents one virus. Significant differences between medians are indicated by *P*-values for the Kruskal-Wallis test with multiple comparisons. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Graphs were generated using Graphpad prism.

4 Discussion

Induction of broad and potent neutralising antibodies (nAbs) by a vaccine remains a major goal of HIV-1 vaccine research[50]. In addition, broadly neutralising antibodies (bnAbs) offer an avenue for novel therapeutics and are considered a blueprint for vaccine immunogen design. In natural infection, HIV-1 co-evolves with the neutralising antibody response leading to viral escape and resistance[187–190]. In fact, several studies suggest that HIV-1 is evolving towards increased resistance to antibody neutralisation at a population level[10,11,143]. Transmission of neutralisation resistant viral variants would have significant implications for antibody-based interventions including vaccines and bnAb therapy. This study investigated the characteristics of a subset of highly neutralisation resistant viruses classified as 'tier 3'. Our aim was to characterise and identify genetic and functional differences between Envs of resistant viruses and sensitive viruses.

4.1 Genetic features associated with resistance

Several studies have shown that genetic characteristics of Env including increased length and number of PNGs in the V1V2 loop region are a feature of neutralisation resistant viruses[142]. Long loops and increased glycan density are thought to mediate resistance by occluding antibody epitopes. We assessed variable loop characteristics (length, number of glycans and net charge) in 81 subtype C viruses with differing neutralisation resistance profiles and found that only V2 net charge and V5 loop length were inversely associated with neutralisation resistance, however not V1V2 loop length nor the number of PNGs in V1V2 loop region (Fig 3.2). This finding in the subtype C panel was inconsistent with previous studies that reported associations between resistance and the V1V2 loop length and glycan density[68,148]. A small sample size may explain the lack of consensus. The 81 viruses analysed here were part of a larger panel of 200 subtype C viruses. Analysis of the 200 viruses showed an association between V1V2 loop length and neutralisation resistance[148]. The need for a large sample size to detect these associations indicates that the V1V2 length and glycan density may not be a strong predictor of neutralisation resistance in subtype C. Furthermore, neutralisation resistance can also be mediated by the structure of the V1V2 loop which may not be easily visible by analysing primary amino acid sequence variation[191]. Thus, while loop characteristics are associated with neutralisation resistance, many other factors mediate resistance and the important loop characteristics are not always easily discerned by analysis

of the amino acid sequence alone. Interestingly, in our panel of only eight CRF02_AG viruses, we found that only the V2 loop length was detectably negatively correlated with neutralisation resistance as found in previous reports (Fig 3.2). This also highlights potential inherent differences between subtypes for these properties.

4.2 Neutralisation resistance and Env function

Env mediated viral entry has been reported to have the greatest influence on replication fitness[164,192]. Reports from a number of studies suggest that the difference in neutralisation phenotypes could be explained by differences in Env structure[86,193,194]. Cai *et al.* (2017) report that Envs of resistant viruses exhibit a very narrow range of pre-fusion conformations with most epitopes occluded[86]. It is plausible that these changes to structure affect viral entry as such changes to Env that block antibody binding may also block key Env-target cell interactions during entry[85]. Therefore, a better understanding of the interplay between overall neutralisation resistance and viral fitness is important. In the present study, we evaluated the association between antibody resistance and viral entry efficiency (as a component of viral fitness). We expected to observe decreased entry capacity in highly resistant viruses. We hypothesized that this may explain why the most highly resistant isolates are generally underrepresented compared to moderately resistant isolates (tier 2), and that prolonged chronic infection under intense antibody pressure does not often result in selection of a tier 3 virus. However, in the subset of subtype C viruses we tested, tier 3 viruses had significantly *higher* entry efficiency compared to tier 2 (Fig 3.3), the opposite direction of what we had hypothesized. This finding is also not easily consistent with results from an HIV-1 infected individual who was treated with B cell ablative therapy for a malignancy. During the period of treatment in which this individual's antibody responses were suppressed, a neutralisation sensitive variant emerged and the viral load was higher, suggesting increased viral fitness compared to the neutralisation-resistant variant that dominated in the presence of antibody[122].

We propose two possible explanations for our observation. First, it is likely that the epitope specificities of the dominant nAb response in the serum panels used to define resistance may determine whether resistance will result in a fitness cost. In an instance where nAbs target regions of Env that are critical for function, then it is likely that escape will impart a fitness cost. For example, escape from an N332 response by shifting the glycan to position 334 had no significant impact on entry efficiency in CAP177 virus [195]. On the other hand, viruses that escape PG9/PG16 and VRC01 have been shown to have reduced entry efficiency[196]. This

may be evident in CA146, which lacks a glycan at position 160 making it resistant to PG9, and is also the least infectious subtype C virus in our subset. Overall, it is likely the tier 3 viruses tested here acquired changes on regions of Env (such as the V3 glycan patch) without affecting entry efficiency.

Second, selection of circulating viral variants is the result of an optimal balance between immune escape and fitness. Viruses with mutations that confer escape without a fitness cost will likely become dominant and maintained in the population. On the other hand, viruses with decreased fitness may be harder to detect as they are fewer in circulation. Thus it may be necessary to isolate more Env clones and substantially increase the sample size in order to detect viruses with decreased fitness.

Altogether, our results suggest that reduced entry efficiency is not the primary explanation for the apparent under representation of tier 3 viruses, even after years of chronic infection and antibody-mediated selection of HIV-1 populations. We were not able to shed light on why such viruses seem to appear less frequently among HIV-1 isolates taken from chronic infection (i.e. after substantial selection by antibody).

4.3 Susceptibility to entry inhibitors

The resistance pathway against nAbs may involves changes to the Env protein which is also a target for entry inhibitors. For this reason we investigated if there was any relationship between neutralisation resistance and susceptibility to entry inhibitors. Some studies have reported that greater sensitivity to neutralisation was associated with resistance to CCR5 and fusion inhibitors[197,198]. However, we observed no significant association between neutralisation resistance and susceptibility to entry inhibitors (Fig 3.5). The likely explanation for this discrepancy is that what is measured here are variations in resistance to Maraviroc and T20 occurring naturally in viruses. On the other hand, the studies that show an association between neutralisation and susceptibility to inhibitors used viruses that had been selected by their resistance to the inhibitors. These viruses contained known mutations on Env that also affected neutralisation[197,198].

Further, we defined the mechanisms involved in differential entry efficiency by using entry inhibitors that target different points of the entry process. Reduced viral fitness has been associated with increased susceptibility to CCR5 inhibitors and fusion inhibitors[199] presumably because the increased susceptibility to CCR5 inhibitors signals decreased CCR5

affinity/binding which in turn probably reduced entry efficiency. In the CRF02_AG viruses tested here, decreased susceptibility to PSC RANTES correlated with increased entry efficiency (Fig 3.6). This was not surprising as higher affinity to CCR5 (as measured by reduced inhibition with PSC RANTES) would presumably be a significant factor in influencing entry efficiency.

4.4 Glycan sites associated with resistance

Up to 50% of Env is covered in glycans and can block access of nAbs to neutralisation epitopes[42,44,113,185,200–202]. Wei *et al*. (2003) analysed escape mutants that arose in response to the autologous nAb response. They reported that changes in potential N-glycosylation sites (PNGS) were the most common mutations in these viruses[42]. Some glycans are evolutionarily conserved in HIV-1 sequences; however, the absence or presence of many glycans varies in different viruses. On average, Env matures from a less glycosylated state to the more fully glycosylated Env as the virus evolves in an individual[203] as transmitted/acute viruses have been shown to be less glycosylated relative to chronic viruses. We considered the role glycans play in overall neutralisation resistance in subtype C viruses and hypothesized that the resistant viruses would have a different glycosylation pattern compared to more sensitive viruses. The glycan at position 465 was enriched in tier 2 and 3 viruses compared to tier 1B (Fig 3.9); however, it did not show up as a likely independent correlate in the Bayesian model averaging analysis (Table 3.1), suggesting that it may not have been independently associated with neutralisation resistance. Similarly, The independent contribution of N355 to the best fit Bayesian averaged model is less than 1/6 that of N413 and about 1/3 that of N332. The glycans at position 413 (V4) and 332 (V3 base) were independently associated with neutralisation. Similar to previous reports, a glycan at position 413 was associated with decreased sensitivity to neutralisation in subtype B viruses[186]. N413 is also proximal to some CD4bs mAb epitopes and is associated with resistance to b12 (a CD4bs mAb)[186]. The glycan at position 332 is described as a supersite of vulnerability and for antibody recognition by the PGT and PGT-like bnAbs[44,109,110]. A shift from N332 to N334 has been shown to result in escape from strain specific responses[44,195]. The association of the absence of N332 with neutralisation resistance showed up more strongly than N334 in the bayesian association analysis, although it is difficult to rule out that this was due to a reduced statistical power because so few of the subtype C viruses tested (9/82) had a glycan at position 334.

4.5 Resistance to commonly tested broadly neutralising antibodies

We evaluated if there were differences in neutralisation sensitivity to bnAbs between sensitive and highly resistant viruses to investigate the epitopes that may drive resistance. The most consistent differences were evident in the V3/glycan bnAbs PGT121 (Fig 3.11). A number of studies have shown the effect of changes in N332/4 on neutralisation by V3/glycan mAbs[44,109,200]. Rademeyer *et al*. (2016) proposed that the resistance phenotype of tier 3 viruses in their clade C subset could be partially explained by lack of the N332 glycan[143]. However, PGT121 can neutralise both N332 and N334 viruses[109] and also uses the glycans at position 137,156 and 301[109] and so may not depend on 332 as much as 10-1074 does. Treatment of chronically HIV-1 infected individuals with 10-1074 resulted in escape mutants that had eliminated the glycan at position 332 making them resistant to 10-1074[112]. We also observed differences in sensitivity between tier 2 and tier 3 subtype C viruses to the CD4bs antibodies VRC01 and 3BNC117 and the MPER antibodies 4E10 (Fig 3.11). Interestingly, there were no detected changes in neutralisation sensitivity between tier 3, 2 and 1 viruses against PG9 and PGT145. One possible explanation for the differential sensitivity to these bnAbs observed here is that antibodies against the V3/glycan site, CD4bs and MPER are elicited in a substantial proportion of HIV-1+ individuals and viruses resistant to these antibodies are selected for in the population. Antibody based selection resulting from on going viral escape has been well documented[42,204,205].

Similarly, several studies have shown that contemporary HIV-1 isolates are more resistant to a number of bnAbs including those shown here compared to viruses isolated early in the epidemic[10,12,145]. Collectively, these data also suggest that resistance testing of viruses to bnAbs might be inevitable before introducing bnAbs as therapies.

4.6 Phenotypic differences between subtypes

The HIV-1 *env* gene can vary between subtypes by 35% of the amino acids with the gp120 bearing most of this diversity[206]. Such diversity can result in differences of Env function. Recent studies have revealed differences between subtypes in *in vitro* replication fitness[207,208] and rate of disease progression[209,210]. The relative replicative fitness of a virus likely influences the prevalence of viral subtypes pointing to the importance of within subtype comparisons. CRF02_AG is dominant in West and West Central Africa while subtype C

viruses are responsible for the vast majority of HIV-1 infections in southern Africa and India, and is responsible for half of all global infections[211]. Differences in replicative fitness have been shown between subtype C and B[164]. In our study, we found no significant phenotypic differences between subtype C and CRF02_AG as measured by entry efficiency and susceptibility to entry inhibitors (Fig 3.8). However, a growth competition assay could possibly yield more conclusive results as it is a more sensitive test of replication[212].

A higher number of glycans in the V2 region was associated with increased entry efficiency in subtype C while a high V2 net charge negatively associated with entry efficiency in CRF02_AG. These differences are not surprising as the loop regions are highly variable even within subtypes in addition to the inherent inter-subtype differences. There is evidence to suggest that adaptive pressures that shape Envs of different subtypes are distinct[213]. This could lead to subtle but significant sequence and structural differences between the subtypes and influence neutralisation sensitivity and fitness as seen here.

5 Conclusion

Overall, we report increased entry efficiency in a subset of subtype C tier 3 viruses compared to tier 2 viruses. Therefore, we conclude that decreased entry efficiency could not explain the low frequency of tier 3 viruses in chronic HIV-1 infection. Chronic HIV-1 infection is the stage in which antibody would presumably be capable of selecting for such highly neutralisation-resistant variants. Some studies do show that changes in Env associated with escape from neutralising antibody leads in a loss of Env fitness[214,215]. However, the association between entry efficiency and neutralisation resistance that we detected was in the opposite direction.

We also report the importance of two PNGs in neutralisation resistance in subtype C consistent with previous data. Presence of a glycan at position 413 is associated with resistance while lack of a glycan at position 332 was associated with resistance to neutralisation. Additionally, tier 3 viruses are significantly more resistant to neutralisation by the V3 glycan (PGT121), CD4bs (VRC01 and 3BNC117) and MPER (4E10) bnAbs. This may result from the fact that nAbs against these epitopes are elicited in a significant proportion of the population and contribute substantially to selection of the resistant variants in the population. As bnAbs are being explored for treatment, circulating resistant viruses would reduce the efficacy of these bnAbs. Furthermore, consistent with previous reports, we confirm that the V2 region (in our case, glycan density and net charge) is associated with entry efficiency[216,217]. Finally, our findings provide more evidence that for antibody based interventions to be globally relevant, they must target regions of the virus that are more prone to loss in fitness during escape.

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