Antibody Guided Exploration of V3 Exposure on Subtype C HIV

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Abstract

The limited neutralizing activity of V3 antibodies is typically attributed to V3 masking. While relatively much effort has been devoted to exploring V3 accessibility on subtype B viruses, V3 exposure and mechanism(s) that might restrict V3 exposure on non-subtype B viruses have yet to be understood. I have focused on exploring the significance of the conserved V3 tip motifs GPGR and GPGQ of subtype B and non-subtype B viruses for antibody recognition. Position 315 in representative subtype B and subtype C viruses was mutated to Gln and Arg respectively to assess the effect of the conserved Arg/Gln at position 315 on V3-specific neutralization. The Q315R subtype C viruses became sensitive to anti-V3 mAb B4e8 neutralization whereas the R315Q switched subtype B virus became resistant to V3 neutralization, even to V3 antibodies that do no contact the residue at position 315. These observations suggest that at least the tip of V3 is antibody accessible on the surface of some non-B viruses but it is the presence of Gln315 residue that modulates V3 antibody recognition. As such, engineering V3 tip antibodies to make high affinity interactions with a Gln315 residue could broaden V3 neutralization of non-subtype B viruses. To attempt to improve B4e8's neutralizing activity, targeted mutagenesis of B4e8 complementarity determining region residues was done to generate antibody libraries using yeast and phage display. The libraries were subjected to multiple rounds of selection on subtype C gp120s, however B4e8 variants with enhanced affinity for subtype C gp120 were not recovered after the final rounds of selection. This suggests that the residues targeted here were not sufficient for enhancing B4e8 affinity for non-subtype B HIV.

Keywords: Variable region 3 (V3), Epitope masking, Antibody affinity maturation

For Magic Johnson, if he'll have it

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List of Acronyms

AIDS	Acquired Immunodeficiency Syndrome
APC	Allophycocyanin
bnAb(s)	Broadly Neutralizing Antibody (Antibodies)
BSA	Bovine Serum Albumin
CCR5	C-C Chemokine Receptor type 5, co-receptor in HIV entry
CD4	Cluster of Differentiation 4, primary receptor for HIV entry
CD4bs	CD4-Binding Site
CoRbs	Co-receptor Binding Site
CTL	Cytotoxic T Lymphocyte
CRF	Circulating Recombinant Form
CXCR4	C-X-C Chemokine Receptor type 4, co-receptor in HIV entry
ELISA	Enzyme-linked Immunosorbent Assay
Env	Envelope spike
Fab/ $F(ab')_2$	Fragment, Antigen Binding Region
Fc	Fragment, Crystallizable Region
gp120	Glycoprotein 120 (of HIV-1)
gp41	Glycoprotein 41 (of HIV-1)
HCDR(1-3)	(First, Second, Third) Heavy Chain Complementarity Determining Region
LCDR(1-3)	(First, Second, Third) Light Chain Complementarity Determining Region
HIV-1	Human Immunodeficiency Virus type 1
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
MPER	Membrane Proximal External Region
nAb(s)	Neutralizing Antibody (Antibodies)
PBS	Phosphate Buffered Saline
scFv	Single-chain Variable Fragment
V3	Third Variable Region (on gp120)

1. Thesis Introduction

1.1. HIV and AIDS

The human immunodeficiency virus (HIV) was discovered to be the causative agent of acquired immunodeficiency syndrome (AIDS) in the early 1980's (3, 17). Since then, more than 25 million individuals have died of AIDS (http://www.unaids.org). Circa 34 million individuals worldwide are infected currently with the virus, with the greatest burden of infections in sub-Saharan Africa (http://www.unaids.org). The number of new infections as well as AIDS-related deaths have been gradually decreasing since the use of antiretroviral therapy as well as the implementation of preventative strategies such as male circumcision, sexual health education, and oral or topical pre-exposure prophylaxis (reviewed in (38)). However, despite these strategies, 2.5 million individuals were newly infected in 2011 (http://www.unaids.org). It is therefore generally agreed that a vaccine is ultimately needed to protect uninfected individuals from contracting the virus. Disappointingly, although candidate vaccine trials have been conducted, a broadly effective vaccine remains elusive.

1.2. Clinical Course of HIV Infection

The clinical course of HIV infection begins with the acute phase, which starts at the time of infection and is characterized by high viral loads and a drastic decline in $CD4^+$ T cell counts (8) (Fig 1.1). The immune response that ensues controls viral replication and largely restores $CD4^+$T cell numbers but does not eradicate the virus (8). A few months post infection the symptoms of acute viremia have usually subsided and the infected individual enters a period of clinical latency (34) (Fig 1.1). During this phase, which in some individuals lasts many years, there is persistent viral replication and a gradual loss of $CD4^+$T cell numbers and function (34). If left untreated, eventually, viral replication overwhelms the lagging immune response and $CD4^+$ T cell numbers decline to a level that leaves the infected individual susceptible to fatal opportunistic infections (34) (Fig1.1).

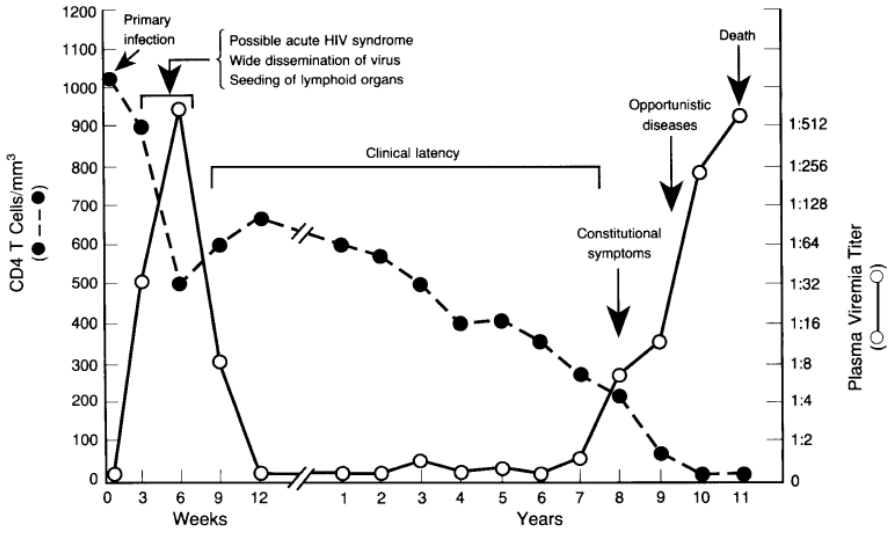

Figure 1.1. Typical clinical course of HIV infection in the absence of antiretroviral treatment. Initial viral dissemination results in depletion of $CD4^+$ T cells. The ensuing immune response decreases viral load, allowing $CD4^+$ T cell numbers to rebound. This period of clinical latency is typically maintained for a few years. Eventually, due to destruction and dysfunction of the immune system, $CD4^+$ T cells decline to where the immune system can no longer ward off opportunistic infections. (Figure adapted from (39) with permission from Dr. Anthony Fauci)

1.3. HIV Genetic Diversity

In this thesis the focus is on HIV-1, one of the two closely related genetic types into which HIV is divided (9). Phylogenetic analyses have resulted in the categorization of HIV-1 strains into 3 genetic groups: major (M), outlier (O) and non-M and non-O (N); the majority of global infections are caused by group M viruses with only a minority of infections, predominantly in central Africa, being caused by group O and N viruses (50). Group M viruses have been divided further into subtypes A-D, F-H, J and K that vary in sequence by 25-35% (20).

Of the aforementioned group M subtypes, subtype C viruses account for ~50% of global infections and are found predominantly in sub-Saharan Africa and India (20, 50). Subtype A viruses are responsible for ~12% of all global infections

and predominate in East and Central Africa as well as Eastern Europe whereas subtype B viruses account for ~10% of global infections and predominate in the Americas, Western Europe and Australia (20, 50). Advances in full-length genome sequencing have led to the discovery of circulating recombinant forms (CRFs) of HIV (50). CRFs are a result of genetic recombination of subtypes within an individual infected with viruses from more than one subtype (50). Of the identified CRFs, the most notable are CRF01_AE and CRF01_AG which dominate in Southeast Asia and West Africa, respectively, and together account for ~10% of global infections (20, 50). Viruses from the remaining subtypes and CRFs, are responsible for a minor fraction of infections worldwide (20, 50).

1.4. Antigenic Structure of HIV-1 Envelope Glycoprotein

HIV is a retrovirus that primarily infects $CD4^+$ T cells but that can also target other immune cells such as macrophages and dendritic cells (8). Infection is initiated upon host cell receptor engagement by the HIV envelope spike (Env) (15). Env is present on the virion surface as a trimeric complex between the gp120 surface unit and the non-covalently associated transmembrane gp41 unit (Fig. 2) (57). Full-length Env is synthesized as a polypeptide precursor which is post-translationally glycosylated and then proteolytically cleaved into gp120 and gp41 (13, 55, 58). The resulting gp120-gp41 trimeric complexes are displayed on the cell surface as spikes, where in the case of an infected cell they are picked up by budding virus particles (58). Env, because of its surface-exposed location,

is the principal target for so-called neutralizing antibodies (nAbs) (33) (see section 1.5).

Gp120, split traditionally into five sequence conserved (C1-C5) and five variable regions (V1-V5), binds to host cell receptor CD4 and co-receptor (typically CCR5 or CXCR4) (58) (57). Env structural changes, triggered by CD4 binding, expose the CD4 induced (CD4i) site which contains the conserved co-receptor binding site (CoRbs) (58). As such, the CoRbs is generally not presented on the unliganded Env trimer (26). Co-receptor binding initiates a second conformational change in Env that transiently exposes gp41 fusion intermediates (56, 57). Gp41, which mediates viral and host cell membrane fusion, is sandwiched between the larger gp120 proteins and the viral membrane (Fig 1.2) (56, 58).

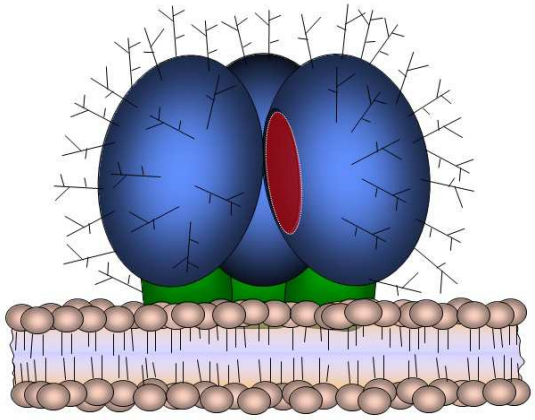

Figure 1.2. Schematic representation of the Env spike on the viral membrane. Blue spheres depict the gp120 surface unit while green cylinders represent the gp41 transmembrane unit. The CD4bs is shown in red and branched structures represent glycans that cover the surface of Env. (Figure adapted from (40) with permission from Dr. Ralph Pantophlet)

1.5. Immune Response to HIV-1

The initial adaptive immune response to HIV involves the cellular arm followed closely by the humoral arm (34). The cellular immune response controls

viral replication through the induction of effector $CD8^+$ T cells known as cytotoxic T lymphocytes (CTLs) that directly kill virus-infected cells mainly through the action of perforin and granzyme as well as antiviral cytokines (22, 53). Upon natural infection the host immune system reacts also with the production of antibodies to viral proteins such as Gag, which includes the matrix protein p24 and the capsid protein p17 (34).

The first antibodies to Env are anti-gp41 antibodies, with antibodies targeting gp120 following soon thereafter (Fig 1.3) (2, 37, 51). These initial antibodies bind to monomeric or non-functional forms of gp120 and gp41 but not Env on the surface of virions. After several weeks, antibodies that can neutralize the infecting virus, termed the autologous virus, are generated (Fig 1.3) (2, 37). These initial autologous nAbs exert selection pressure on the virus, resulting in rapid selection for neutralization escape variants to which the immune system then responds with a new wave of nAbs (Fig 1.3) (2, 16, 37). Thus, contemporaneous serum antibodies are normally able to neutralize virus from weeks or months previous but unable to neutralize virus from the same time period (10, 16, 18, 27, 35, 36, 41, 44, 54). Such nAbs typically do not neutralize heterologous viruses to a significant extent (i.e., those from other infected individuals). However, in recent years it has become apparent that 10-30% of individuals infected for >2 years develop broadly nAbs (bnAbs), which are capable of neutralizing autologous virus and a wide variety of heterologous viruses, in some cases from different subtypes (Fig 1.3) (7, 11, 12, 23, 28, 29,

43, 47-49). The goal of most prospective HIV vaccines is to design immunogens that are capable of eliciting such bnAbs (5).

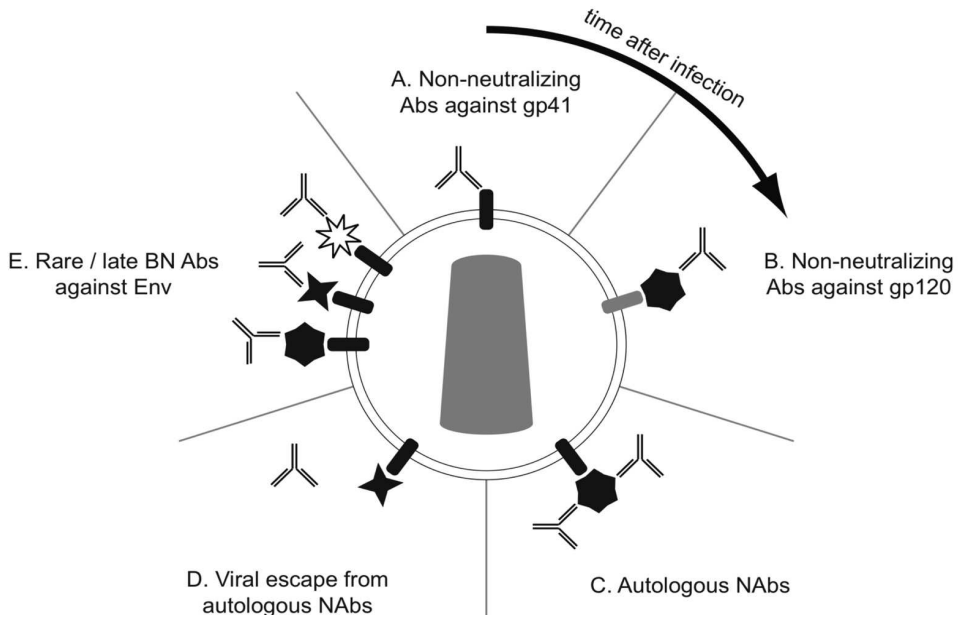

Figure 1.3. The stages of the antibody response to HIV-1. (A)Within the first week after infection non-neutralizing gp41 antibodies are elicited. (B) Soon thereafter non-neutralizing gp120 antibodies develop. (C) A few weeks or months post infection, autologous neutralizing antibodies (nAbs) arise that apply selection pressure on the virus. (D) Viral mutation results in neutralization escape, represented here by a change in the shape of gp120. (E) Some patients develop broadly neutralizing antibodies (bnAbs) that can neutralize a wide range of HIV strains, represented here by a variety of shapes of gp120. Mixing of envelope shapes on a single virus particle is shown for illustrative purposes only. (Figure adapted from (2) with permission from Dr. Galit Alter).

1.6. Antibody Recognition of the V3 region of HIV Gp120

The V1-V5 variable regions form exposed loops that mostly extend from the gp120 core. As such, the variable regions tend to mask underlying epitopes and act as decoy epitopes for nAb responses during natural infection. The V3 region is a 30-35 amino acid loop, characterized by a net positive charge, a turn at its center (the V3 tip) and a disulfide bond at its base (Fig 1.4) (19). Although

dubbed a sequence variable region, the V3 region sequence on most subtypes is relatively conserved, with the exception of subtypes B and D (32). Functionally, the V3 region interacts with co-receptor and as such plays a primary role in determining cellular tropism –the preference of HIV for infecting certain cell types (19). The obligatory role of co-receptor binding for viral entry suggests that the V3 region must be exposed at times during infection and that V3 structure or sequence motifs must be conserved across diverse strains (19).

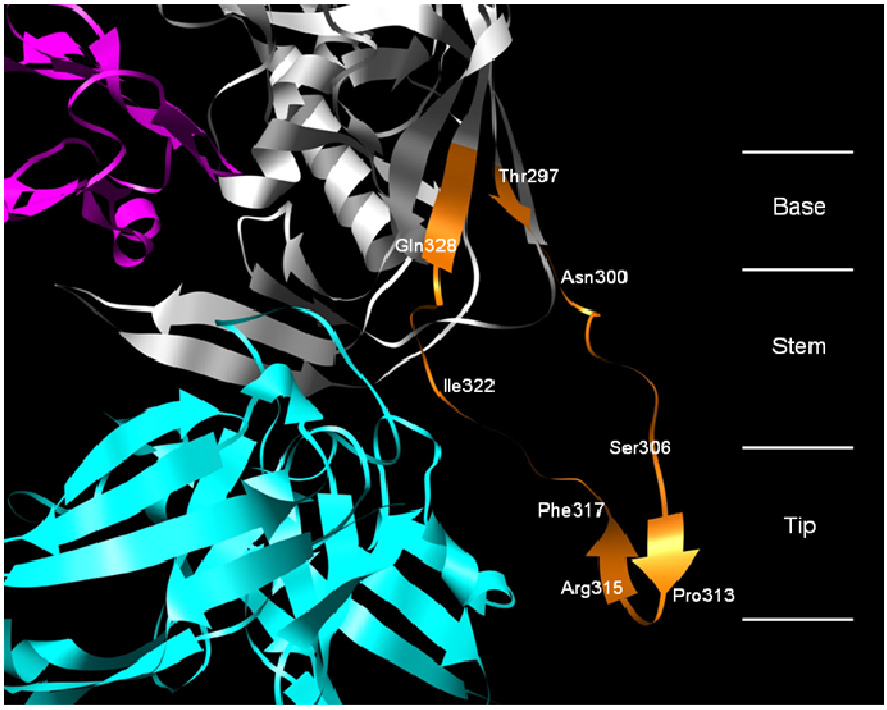

Figure 1.4. Structure of gp120 core with V3. Shown is the crystal structure of subtype B (JR-FL) gp120 (gray) with an intact V3 (orange) region, in complex with CD4 (magenta) and the antigen binding fragment (Fab) of CD4i mAb X5 (blue). The different segments of the V3 region (base, stem, and tip) are labeled. (Protein Data Bank accession number: 2B4C) (21).

High titers of V3 antibodies typically develop in an individual within a few weeks or months post infection (37). In subtype B, these initial V3 antibodies tend to exhibit autologous neutralizing activity while most individuals infected with subtype A and C viruses develop non-neutralizing V3 antibodies (24, 37). The majority of V3 antibodies isolated thus far tend to be strain specific or capable of neutralizing subtype B viruses but lacking neutralizing activity across subtypes (4, 6, 14, 59). This has led to current vaccine efforts that tend to disregard focusing on V3 immunogens. Both the absence of V3 nAbs in individuals infected with non-subtype B HIV strains and the current lack of broadly neutralizing V3 antibodies have been attributed to shielding of V3 on HIV by other variable loops, particularly the V1V2 variable regions and glycans (1, 19, 25, 30, 31, 42, 45, 46, 52).

1.7. Overview of this thesis

Although various features have been implicated in the masking of V3 on subtype B viruses, mechanisms that affect V3 exposure on non-subtype B viruses remain relatively undefined. Understanding HIV evasion of V3 antibody recognition could potentially provide clues into how sites outside of the V3 region could also be protected from antibody recognition.

The overall goal of this thesis was therefore to explore the accessibility of V3 on HIV non-subtype B strains. The results from this work are presented in two chapters. In **chapter 2**, I present the results of my investigation on characterizing

V3 exposure and mechanisms that modulate V3 exposure on non-subtype B viruses, with particular attention to subtype C. I focused on the conserved V3 tip sequence, to determine the significance of a residue located at position 315 at the center of V3 (Arg in subtype B and Gln in non-subtype B) for V3 antibody recognition. In **chapter 3**, I present the outcome of my research on the in vitro evolution of the anti-V3 mAb B4e8. B4e8 is a V3 specific mAb that is able to neutralize subtype B viruses but that in general neutralizes non-B viruses poorly. Yeast surface display and phage display were used to generate libraries comprised of complementarity determining region 3 (CDR3) mutants of the B4e8 light and heavy chains, respectively. These libraries were subsequently selected on subtype C gp120s to explore isolating B4e8 variants with improved affinity for and neutralizing activity against subtype C viruses. In **chapter 4**, I provide insights into the broader implications of the results from my thesis work on the understanding of HIV epitope exposure, mechanisms that modulate epitope exposure, and V3 antibody engineering.

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2. The tip of the V3 region in HIV-1 gp120 modulates V3 exposure and neutralizing antibody recognition

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(Submitted)

ABSTRACT: HIV-1 has evolved complex mechanisms to restrict recognition of antibody-accessible regions. Antibodies to the V3 region of the HIV-1 envelope spike (Env) tend to neutralize a narrow range of virus strains, whereby most antibodies neutralize HIV subtype B viruses better than non-subtype B viruses. The factors responsible for this resistance are still not entirely clear. One fairly unexplored factor is antibody affinity; subtype B V3's have a charged residue (mostly Arg) at position 315 that is important for the binding several V3 antibodies, whereas non-subtype B V3's generally have a charge-neutral residue (Gln) at this position. Here we have investigated the significance of these specific residues in the conserved V3 tip motifs GPGR (subtype B) and GPGQ (subtype C) for V3-targeted neutralizing activity. We found that whereas a representative subtype B virus (SS1196) was neutralized effectively by a panel of V3-specific mAbs (B4e8, 2219, 268-D, 2557, 3074 and HGN194) with varying fine specificities for the V3 tip, unexpectedly the same virus with an R315Q substitution was substantially resistant regardless of antibody dependence on Arg315 for binding. Conversely, two example neutralization-resistant subtype C viruses (ZM249M and CAP45) were sensitive to neutralization upon changing Gln315 to Arg, albeit only to mAb B4e8 and not mAbs 268-D (Arg315-dependent) or HGN194 (Arg315-independent). Additional mutagenesis analyses with viruses expressing Env heterotrimers suggest that dominant interactive forces between the N-terminal strand of V2 and V3 of neighboring Env protomers can mediate V3 resistance. Overall, these results provide new insight into how specific sequences in HIV limit antibody recognition of conserved target sites.

2.1. Introduction

The HIV-1 envelope spike (Env) is the sole target for neutralizing antibodies (nAbs) (41) but the extraordinary sequence diversity displayed by Env poses problems for the design of an effective vaccine (69). Dense glycosylation and a close interaction between the Env protomers (55, 72) also hinder broad nAb recognition and provide at least a partial explanation for why some antibodies may fail to neutralize a significant range of HIV strains (23).

The third variable (V3) region on gp120 is one of the initial antibody targets during subtype B infection (49). However, enthusiasm for targeting V3 in a vaccine formulation is weak because of the V3 region's sequence variability and the general inability of V3-specific monoclonal antibodies (mAbs) to neutralize viral isolates across many different subtypes (6, 7, 13, 76). The limited neutralization breadth of V3 mAbs is typically attributed to masking of the V3 region by other variable loops, particularly V1V2, and glycans (1, 22, 36, 62, 63, 65, 68).

There has been a tendency to assume that since the V3 region is more conserved in non-subtype B viruses than subtype B viruses that it is masked to a greater extent in the former (21). Greater occlusion of the V3 region has also been used to argue the lack of V3-specific nAbs in the sera of individuals infected with non-subtype B viruses (reviewed in (44)). While the notion of occlusion seems plausible, the factors that restrict V3 antibody

recognition of non-subtype B viruses, compared to subtype B viruses, remain poorly defined. Greater insight into these features could add to our understanding of how HIV protects itself from the neutralizing activity of antibodies and might reveal how these defenses can be overcome, as exemplified by mAbs such as PG9 and PG16 that interact with segments of both V2 and V3 (12, 42, 70).

We have been using the anti-V3 mAb B4e8 to gain a better understanding of V3 accessibility on HIV. MAb B4e8 is able to neutralize subtype B viruses but exhibits lesser neutralizing activity against non-subtype B virus strains (50). In a previous study (52) we suggested that a V3 antibody's fine specificity may result in a mode of interaction that limits epitope accessibility and thus neutralization breadth, a notion supported by meta-analyses of data obtained with other V3 mAbs (1).

Here, we sought to delve further into factors that might limit B4e8 and perhaps other V3 mAbs from binding to the V3 region of non-subtype B viruses. We focused here specifically on subtype C and on exploring the significance of the conserved V3 tip motifs ^{312}GPGR^{315} (subtype B) and ^{312}GPGQ^{315} (subtype C/non-subtype B) for antibody recognition. Position 315 in representative subtype B (SS1196) and subtype C viruses (ZM249M, CAP45) was switched to Gln and Arg, respectively, to assess the effect of these conserved residues on V3-specific neutralization. Increased neutralization sensitivity of the two subtype C viruses to mAb B4e8 but not two other anti-V3 mAbs upon replacing Gln315 with Arg suggests that V3 may be accessible to

antibody on subtype C viruses, and possibly others, though only to antibodies with select binding modes.

2.2. Materials and Methods

2.2.1. Antibodies

A panel of six V3-specific mAbs along with seven non-V3 mAbs were investigated in this study (Table 2.1). The detailed characterization of these mAbs has been described previously (3, 9, 16-18, 50, 54, 67, 70, 73).

Table 2.1. V3-specific and non-V3 MAbs used in this study

MAb	Specificity[a]
268-D	V3 (^{310}HIGPGR^{315})
B4e8	V3 (Ile^{309}, Arg^{315}, Phe^{317})
2219	V3 (Arg^{306}, Lys^{307}, [Ile, Val]^{309}, [Tyr, Phe]^{318})
2557	V3 (Lys^{307}, [Ile, Val]^{309}, Tyr^{318})
HGN194	V3 (^{306}RRSVRIGPGQ^{315})
3074	V3 ([Ile, Leu, Met]^{311}, Pro^{313}, [Phe, Trp] ^{317})
b12	CD4bs
b6	CD4bs
17b	CoRbs
412d	CoRbs
PG9	V2/V3
PG16	V2/V3
PGT128	Oligomannose/V3

[a]Residues in parentheses denote residues critical for antibody binding

2.2.2. Generation and purification of mAb B4e8

Synthetic genes encoding B4e8 heavy and light chains were purchased (DNA 2.0, Menlo Park, CA) and cloned into the bicistronic vector pBudCE4.1 (Invitrogen), modified so as to contain the CH2 and CH3 constant domains of IgG1 for expression of full-length IgG molecules. A stable CHO-K1 cell line expressing recombinant B4e8 was generated by transfection with FuGene 6 (Roche) and limiting dilution selection in the presence of zeocin (200 µg/ml; Invivogen). Clones producing the highest levels of protein from two limiting dilution rounds were sub-cultured to select the best-growing clone. To maximize production, the stably transfected clone was cultured in multi-level Cell Factories (Nunc). Supernatants were normally harvested at 10-day intervals, filtered to remove cell debris and then purified on a protein A column (Pierce) following standard protocols. The eluate was buffer-exchanged into Tris/HCl (0.1 M, pH 8) and protein purity then assessed by SDS-PAGE.

2.2.3. Pseudoviruses

One subtype B virus, a panel of 20 subtype C viruses and SIVmac239 were used in this study (Table 2.2). Env expressing plasmids were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (NIH ARRRP). Pseudoviruses were generated by transient co-transfection of 293T cells with Env expressing plasmid and the luciferase reporter plasmid pNL4-3.Luc.R−E− (at a 1:2 ratio as done previously (51)) using polyethylenimine (25 kDa; Polysciences) as the transfection reagent.

Pseudovirus containing supernatant was harvested at 3 d post-transfection, aliquoted and stored at -80°C until needed. Mutations in Env-expressing plasmids were generated using relevant primers by Quikchange XL II kit (Stratagene). Amino acid substitutions were verified by DNA sequencing.

Table 2.2. HIV-1 clones used in this study and their corresponding V3 sequence

Virus	Subtype	Accession No.	Tier[a]	V3 sequence[b]
SS1196	B	AY835442	1B	CTRPNNNTRKSIH**I**GPG**R**A**F**YATGGVIGDIRQAHC
HIV-00836-2	C	EF117265	1B	CTRPNNNTRKSIR**I**GPGQT**F**YATGEIIGNIRQAHC
HIV-001428-2	C	EF117266	2	CTRPNNNTRKSIR**I**GPGQT**F**YATGDIIGNIREAHC
HIV-0013095-2	C	EF117267	2	CTRPNENRRKSIR**I**GPGQA**F**YATGDIIGDIRQARC
HIV-16055-2	C	EF117268	2	CTRPNNNTRKSIR**I**GPGQT**F**YATGDIIGNIRQAYC
HIV-1 16845-2	C	EF117269	2	CTRPGNNTRKSIR**I**GPGQT**F**YATGDIIGDIRQAHC
HIV-1 16936-2	C	EF117270	2	CTRPNNNTRKSVR**I**GPGQT**F**YATGEIIGDIRQAHC
HIV-25710-2	C	EF117271	1B	CARPSNNTRTSIR**I**GPGQT**F**YATGAITGDIRQAHC
HIV-1 25711-2	C	EF117272	1B	CIRPNNNTRKSIR**I**GPGQT**F**YATGDIVGDIRQAYC
HIV-25925-2	C	EF117273	1B	CTRPNNNTRKSIR**I**GPGQT**F**YATGAIIGNIREAHC
HIV-26191-2	C	EF117274	2	CTRPGNNTRKSIR**I**GPGQT**F**YATGEIIGNIRQAHC
Du156	C	DQ411852	2	CTRPNNNTRKSVR**I**GPGQT**F**YATGDIIGDIRQAHC
Du172	C	DQ411853	2	CTRPSNNTRKSVR**I**GPGQT**F**FATGDIIGDIRQAHC
Du422	C	DQ411854	2	CTRPNNNTRKSVR**I**GPGQT**F**YATGEIIGDIREAHC
ZM197M	C	DQ388515	1B	CVRPNNNTRKSVR**I**GPGQT**F**FATGEIIGDIRQAHC
ZM249M	C	DQ388514	2	CTRPNNNTRKSIR**I**GPGQT**F**YATGEIIGKIREAHC
ZM53M	C	AY423984	2	CTRPGNNTRKSIR**I**GPGQA**F**FATTNIIGDIRQAYC
ZM109F	C	AY424138	1B	CIRPGNNTRKSIRLGPGQT**F**YATGDVIGDIRKAYC
ZM214M	C	DQ388516	2	CMRPGNNTRRSVR**I**GPGQT**F**YATGEIIGDIRQAHC
CAP45	C	DQ435682	2	CRRPNNNTRKSIR**I**GPGQA**F**YATNDIIGDIRQAHC
CAP210	C	DQ435683	2	CIRPGNNTRRSIR**I**GPGQA**F**YAMGDIIGNIREAHC

[a]Tier designation based on (67); [b]B4e8 epitope residues critical for high-affinity binding in bold

2.2.4. Neutralization assays

Luciferase-based pseudovirus neutralization assays were set up as previously described (51, 78). In general, mAbs were serially diluted, starting at 50 µg/ml and incubated for 1 h at 37°C with virus before adding to target cells (U87.$CD4^+$.$CCR5^+$ cells or, as indicated, TZM.bl cells; obtained from NIH ARRRP). TZM.bl cells were cultured in the presence of DEAE (20 µg/ml; Sigma). Luciferase activity was assayed immediately on a Viktor X5 luminometer (PerkinElmer). Percentage neutralization was calculated relative to cell-only and virus-only controls.

2.3. Results

2.3.1. V3 mAb B4e8 exhibits poor neutralizing activity against subtype C viruses

The V3 region is characterized by a length of 30-35 amino acids, a conserved turn at its tip, a disulfide bond at its base, and a net positive charge (27). The anti-V3 mAb B4e8 interacts primarily with the tip of V3, with particular affinity for residues Ile309, Arg315, and Phe317 (5) (Fig. A1). The requirement for an Arg residue at position 315 for binding potentially limits the neutralizing activity of B4e8 to mainly subtype B viruses (Fig. A1). However, B4e8 has been shown to neutralize a few non-subtype B viruses at high antibody concentrations, for example 93MW960 (subtype C) and 92UG046 (subtype D), both of which

have a Gln at position 315 (50), suggesting that the loss of binding affinity is a major but not the sole factor explaining the lack of B4e8 neutralizing activity against non-subtype B viruses. To further assess the ability of B4e8 to neutralize subtype C viruses we tested a panel of subtype C isolates (Table 2.2) for B4e8 neutralization. Consistent with previous observations (50), we found that B4e8 potently neutralized subtype B virus SS1196 but was unable to neutralize any of the 20 subtype C viral isolates tested here (Fig. 2.1). Various Env features, such as V1V2 length and number of glycosylation sites, have been proposed to explain resistance to V3 antibody mediated neutralization (37, 60, 61). We, however, did not observe a significant correlation between such features and resistance to B4e8 neutralization (Fig. A2).

To assess comparability of our assay results to those from previous reports, the neutralization sensitivities of SS1196 and the panel of 20 subtype C isolates to CD4 binding site (CD4bs) mAb b12 was also determined (Fig. 2.1). The results were in general agreement with those from previously published reports (32, 34); most viruses that previously were sensitive to b12 were also found to be sensitive here. However, contrary to the findings by Li et al (34) we did not observe neutralization of ZM197M and ZM53M by b12. This discrepancy may be due to inter-laboratory differences (14), especially given that both these viruses were previously reported as only marginally sensitive to b12 neutralization (34)

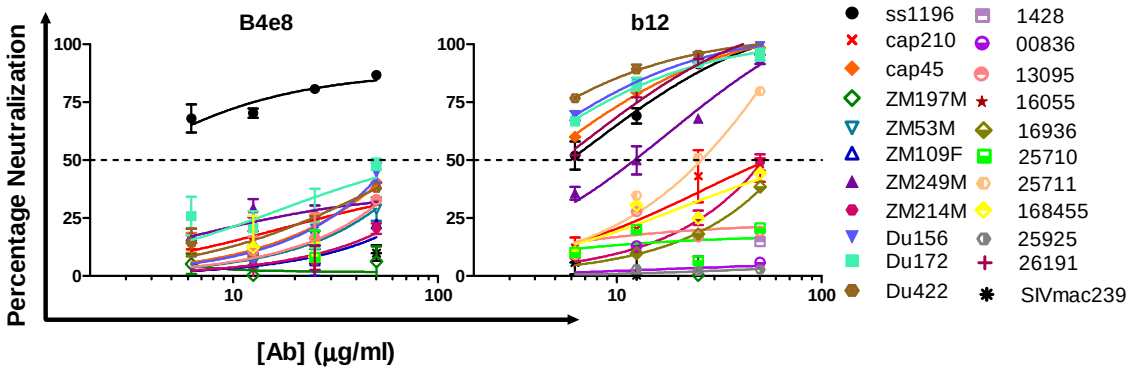

Figure 2.1. MAb B4e8 exhibits poor neutralizing activity against subtype C viruses. The neutralizing activity of B4e8 was assessed against a set of 20 subtype C viruses. The antibody only neutralized the subtype B virus SS1196, which was used as a positive control. In line with a previous report (34), mAb b12, used here as an internal control, neutralized 7 of the 20 subtype C viruses. SIVmac239 was used as a control for non-specific neutralizing activity. Dashed line indicates 50% neutralization. Error bars denote the signal range for replicate wells.

2.3.2. Introduction of an Arg residue at position 315 renders subtype C viruses sensitive to neutralization by anti-V3 mAb B4e8

Finding no correlation between V1V2 length or V1V2 glycosylation levels and resistance of subtype C viruses to mAb B4e8 neutralizing activity prompted us to consider the B4e8 target epitope itself. As noted above, B4e8 is dependent on the Arg at position 315 in the V3 region for high affinity binding (5, 50). We therefore generated mutants of two subtype C viruses (ZM249M and CAP45) with Gln-to-Arg substitutions at position 315 and tested them for sensitivity to B4e8 neutralization. ZM249M and CAP45 are representative tier 2 subtype C viruses (67) that are generally resistant to neutralization by V3 mAbs (Fig. 2.1 and (24)); this resistance has been attributed to masking of V3 by V1V2 and other elements (1). Based on previous structural data (8, 27) no major

conformational changes would be expected to occur in V3 by changing Gln to Arg.

Contrary to B4e8's inability to neutralize the parental viruses ZM249M and CAP45 at antibody concentrations as high as 50 µg/ml (Fig. 2.1), we found that B4e8 neutralized the ZM249M_Q315R and CAP45_Q315R mutant viruses at IC50's of ~0.07 and ~0.01 µg/ml, respectively (Fig. 2.2). We did note however that a fraction of the mutant viruses could not be neutralized even up to the highest antibody concentration used here (50 µg/ml), suggesting the presence of additional factors that might impede neutralization. Neutralization curves that plateau below complete neutralization have been previously reported for anti-HIV antibodies, including those with broad neutralizing activity (25, 57, 70). Env spike heterogeneity, observable by cryoEM tomography (15, 26), has been suggested as one possible explanation for this phenomenon (77), yet no specific mechanism has been identified to fully explain this occurrence. We also observed a reduction in infectivity with the Q315R mutation, suggesting a fitness cost associated with this mutation (Table A1). Compensatory mutations in Env could perhaps be necessary to compensate for this loss in infectivity.

We then tested the mutant viruses for sensitivity to the V3 mAb 268-D, which also makes contact with the Arg at position 315 as part of its core epitope (^{308}HIGPGR^{315}) (18, 75). Unlike B4e8, mAb 268-D was not able to neutralize the two Q315R mutant viruses (Fig. 2.2), suggesting that the Arg substitution had not simply rendered the V3 region more accessible to V3-

specific antibodies. Because mAb 268-D is highly strain-specific (27, 75), we also investigated sensitivity of the two subtype C mutant viruses to the more broadly reactive mAb HGN194, which contrary to B4e8 and 268-D does not interact with the residue at position 315 (9). Strikingly, mAb HGN194 also did not exhibit significant neutralizing activity against either of the mutant viruses (Fig. 2.2) despite the presence of all the required binding residues, supporting the notion that we did not simply render the V3 region more accessible. These results indicate that B4e8's limited neutralizing activity against at least some subtype C viruses is not due simply to V3 masking; rather, B4e8 at least requires position 315 to be an Arg residue in order to bind with sufficient affinity to neutralize virus. These results also provide further evidence that the angle or mode of antibody interaction with V3 is an important determinant of neutralization efficacy (52), as sensitivity of the two mutant viruses to neutralization was observed only with mAb B4e8.

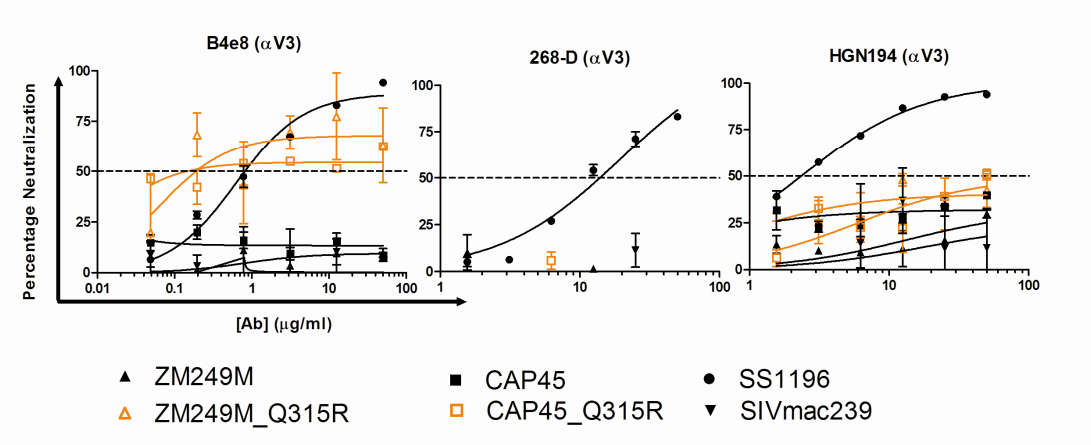

Figure 2.2. Mutating Gln315 to Arg in V3 renders neutralization-resistant subtype C viruses sensitive to neutralization by anti-V3 mAb B4e8. Parental (▲, ■) and Q315R mutant viruses (△,□) were tested for neutralization sensitivity to V3 mAbs B4e8, 268-D and HGN194. Because of low infectivity of the mutant viruses, assays were performed using TZM-bl cells as targets. Subtype B virus SS1196 was used as a positive control (●) and SIVmac239 was used as a control for non-specific neutralizing activity (▼). Dashed line indicates 50% neutralization.

To determine if mutating the residue at position 315 had resulted in significant changes in antigenic exposure of epitopes near the V3 region, the neutralization sensitivity of the two Q315R mutant subtype C viruses to a panel of CD4bs and coreceptor binding site (CoRbs) mAbs (b12, b6, 17b and 412d) was assessed. None of the mutant viruses were overly sensitive to the non-neutralizing CD4bs mAb b6 but the ZM249M_Q315R mutant was noticeably more sensitive to b12 neutralization than the parental virus (Fig. A3). This observation indicates that the Gln substitution causes some, albeit localized, allosteric changes to Env structure. Similarly localized changes appeared to occur also to the CoRbs; neither mutant virus was sensitive to mAb 412d but mutant virus CAP45_Q315R was significantly sensitive to mAb 17b (Fig. A3).

2.3.3. Switching Arg315 to Gln in a subtype B virus with an otherwise antibody accessible V3 region confers broad anti-V3 antibody resistance

As shown above, mutating the Gln at position 315 to an Arg in two subtype C viruses rendered both viruses sensitive to B4e8 neutralization. We interpreted this observation as suggesting that the V3 tip on both viruses may be at least somewhat accessible to antibody and that the lack of B4e8 neutralization of subtype C viruses might be due at least in part to poor binding affinity. To further characterize the effect that the residue at position 315 has on V3 exposure and antibody recognition, we used a subtype B virus (SS1196) with an antibody accessible V3 tip region as the backbone for creating a R315Q mutant virus. This mutant virus was tested for neutralization against the 3 previous mAbs (B4e8, 268-D, HGN194) and a selection of additional V3 mAbs (2557, 2219, 3074). These additional V3 mAbs were selected specifically because of detailed information of their epitopes; crystal structure and epitope mapping data indicate that whereas mAbs B4e8 and 268-D require the presence of an Arg at position 315 for binding, the residue at position 315 is not involved in the binding interaction for mAbs 2557, 2219, 3074 or HGN194 (5, 9, 27, 50). Indeed, it has been argued that the latter four mAbs are better able to neutralize subtype C viruses because they do not depend on Arg315 for binding (1, 9, 27).

We found, as expected, that mAbs B4e8 and 268-D were unable to neutralize the SS1196_R315Q mutant virus (Fig. 2.3). Unexpectedly however,

the neutralizing activity of the four Arg-independent V3 mAbs 2557, 2219, 3074 and HGN194 against the SS1196_R315Q mutant virus was also substantially, though not completely, reduced relative to the wild-type virus (Fig. 2.3). Thus, mutating the Arg at position 315 to a Gln on a subtype B virus with an accessible V3 region rendered this virus substantially resistant to V3 mAb neutralization and this resistance occurred seemingly regardless of the antibodies' dependence on the residue at position 315 for binding.

As with the mutated subtype C viruses, a panel of non-V3 mAbs were used to assess whether global changes in antigenic exposure had occurred upon substituting Arg315 for Gln in SS1196. As compared to the parent virus, SS1196_R315Q was slightly more resistant to neutralization by the CD4bs mAbs b12 and b6 (Fig. A4), suggesting that the R315Q mutation reduces somewhat antigenic exposure of the CD4bs. The remaining three mAbs (PG9, PG16, and PGT128), all of which bind epitopes involving V3, neutralized both the parental and mutant viruses similarly (Fig. A4), indicating that altering the residue at position 315 did not result in gross changes to the Env trimer.

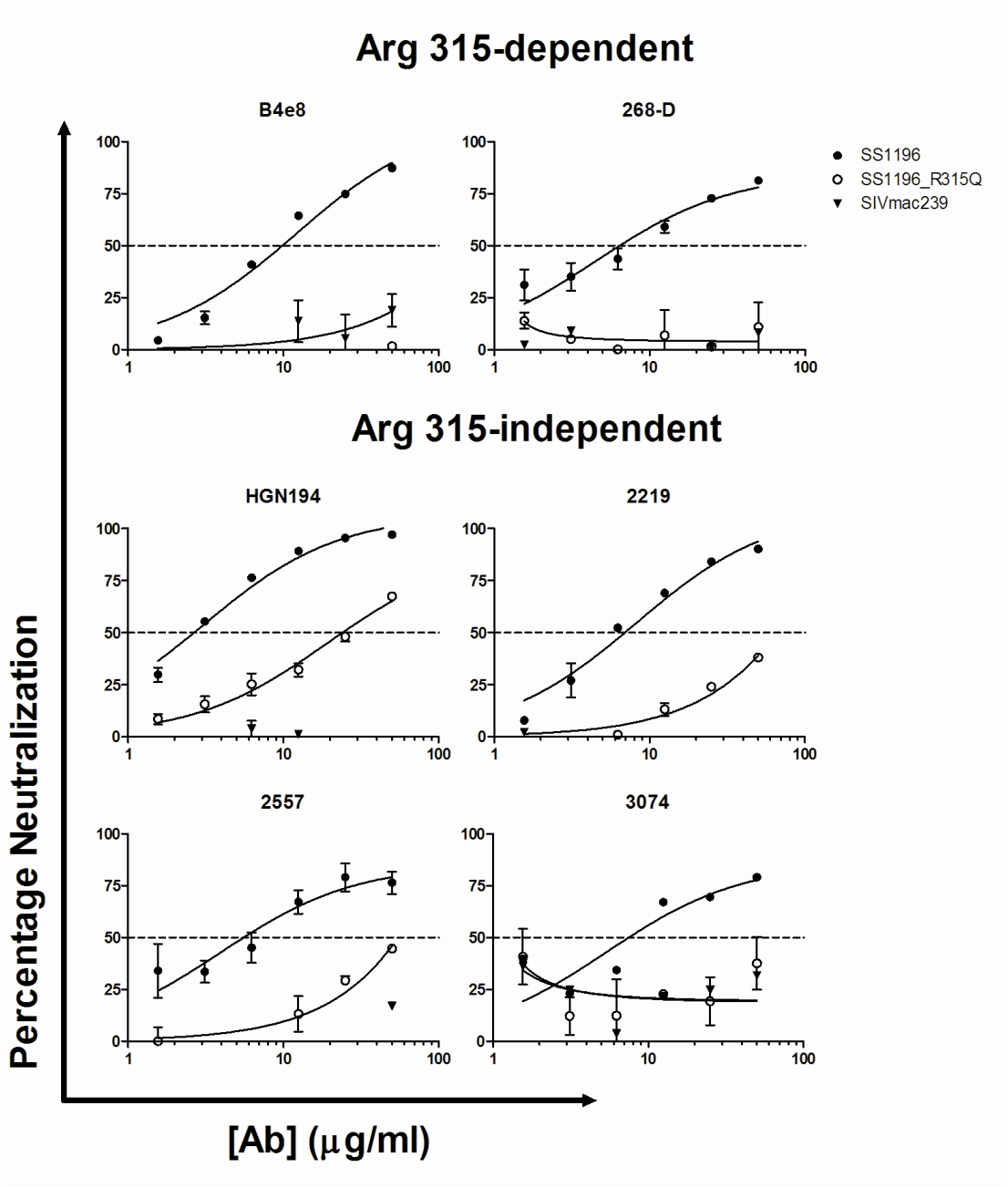

Figure 2.3. Arg315-to-Gln substitution in a neutralization-sensitive subtype B virus imparts resistance to neutralization by V3 mAbs. Replacing Arg315 with a Gln rendered the otherwise neutralization sensitive virus SS1196 resistant to the panel of V3 mAbs investigated here, independent of whether they require Arg315 for binding interaction. Non-specific neutralizing activity was controlled for with SIVmac239 (▼). The dashed line denotes 50% neutralization.

2.3.4. Anti-V3 antibody binding to a single gp120 protomer is sufficient for neutralization

We reasoned that the mechanism of neutralization resistance imparted onto SS1196 by the Arg-to-Gln switch may be due, at least in part, to changes in electrostatic forces resulting from removal of the positively charged Arg at position 315. Given that the V1V2 and V3 regions on each gp120 protomer are considered in fairly close proximity to their neighbors within the context of the Env trimer (35), we considered that an R315 repulsive effect in SS1196 leads to an exposed V3 region and a virus that is relatively sensitive to neutralization; changing the Arg to Gln allows the V3 regions to be relatively closer together and less exposed, resulting in a virus that is more resistant to neutralization (Fig. A5). To test whether this mechanism was controlled by repulsion/attraction of proximal V3 regions, SS1196-derived pseudoviruses displaying mixed R315/Q315 trimers on their surface were generated by co-transfection of the respective plasmids (66) and tested for neutralization sensitivity to B4e8 and HGN194. If valid, viruses with trimers comprised of 2 mutant (Q315) gp120 protomers and 1 wild-type protomer (R315) would be expected to be as resistant (or nearly so) as viruses expressing mutant homotrimers. However, none of the mixed trimer viruses were significantly resistant to mAbs B4e8 and HGN194 (Fig. 2.4), indicating that the presence of even one neutralization-sensitive gp120 protomer within an Env trimer is sufficient to achieve significant neutralization and that any molecular repulsion caused by the presence of Arg at position 315 was not due to opposing V3's.

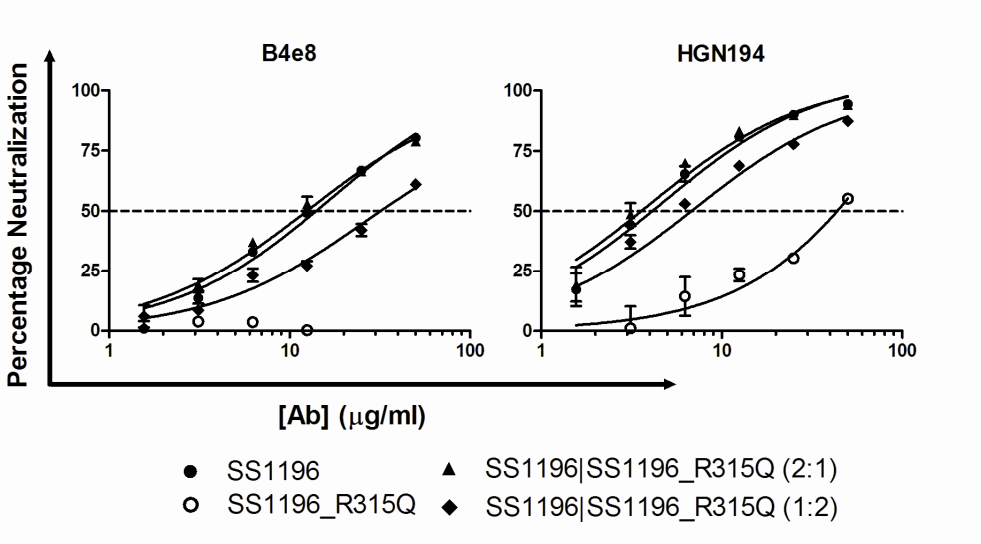

Figure 2.4. V3 antibodies require a single sensitive gp120 protomer for effective neutralization of virus. Neutralization-sensitive wild-type virus SS1196, neutralization-resistant mutant virus SS1196_R315Q and viruses displaying heterotrimers (wt/mut) on their surface, at indicated ratios, were tested for neutralization sensitivity to V3 mAbs B4e8 and HGN194. The dashed line marks 50% neutralization. Error bars denote range for replicate wells.

Transfecting cells with different ratios of SS1196 and SS1196_R315Q Env plasmids should produce virus expressing mixed trimers on their surface (64, 66, 74). However, there remains a possibility of generating virus particles with trimers comprised solely of wild-type or mutant trimers or trimers containing gp120 protomers at different ratios than transfected (40). This seemed unlikely given that transfections with the individual Env-expressing plasmids yielded equivalent levels of infectious pseudovirus. Nevertheless, to address this possible caveat we mixed supernatant containing SS1196 virus and supernatant containing SS1196_R315Q virus at different ratios and assessed the mixtures for B4e8 neutralizing activity. Contrary to the neutralization profile of the mixed trimer viruses, for the mixed supernatants we observed a decreasing trend

in neutralization sensitivity with the increasing fraction of neutralization-resistant mutant virus (Fig. A6), thus, confirming that the R315/Q315 co-transfections did likely yield viruses expressing heterotrimers on their surface.

2.3.5. Interactions between V3 and the N-terminal strand of V2 modulate V3 vulnerability

The V1V2 domain has been shown to play an important role in limiting antibody access to the V3 region (30, 31, 36, 58, 62). Based on the aforementioned premise of a potential role for electrostatic forces, we sought to identify residues in V2 that could potentially be responsible. V2 was chosen because of its apparent proximity to V3 (70). We compared the V2 region of SS1196 to that of the generally more neutralization resistant virus JR-FL, given their high degree of amino acid similarity and given that JR-FL has been used as a model to investigate HIV resistance to V3 antibodies (31, 58). The regions of the two viruses differ electrostatically most at position 168 in the N-terminal segment of V2, with SS1196 bearing a Lys at this position whereas in JR-FL it is occupied by a Glu. We therefore generated an SS1196 mutant in which Lys168 and the neighboring Gly169 were switched to Glu and Val, respectively, as occurs in JR-FL. These mutations resulted in a virus that is notably more resistant to B4e8 neutralization than the parental SS1196 virus (Fig. 2.5), thus suggesting that interactions between the N-terminal strand of V2 and the V3 tip modulate antibody access to V3.

We next sought to determine whether the resistance phenotype imparted by the aforementioned V2 mutations was occurring within the same protomer in the context of the functional spike (36) or between neighboring protomers (62). As above we therefore generated mixed heterotrimers by co-transfection of the respective plasmids at different ratios. We found, contrary to our observations with the Q315 heterotrimers above, that the introduction of a protomer with the V2 mutations (K168E_G169V) resulted in a rapid reduction in virus sensitivity to B4e8 neutralization (Fig. 2.5) to levels similar to the homotrimer mutant SS1196_K168E_G169V virus. Notably, the resistance phenotype due to the V2 mutation was dominant, with resistance measurable even at a 6-times higher ratio of sensitive protomer to resistant mutant protomer. Together this data supports the notion of a *trans* mechanism for V2 modulation of epitopes within V3 where changing the V2 on one protomer affects V3 accessibility across the trimer.

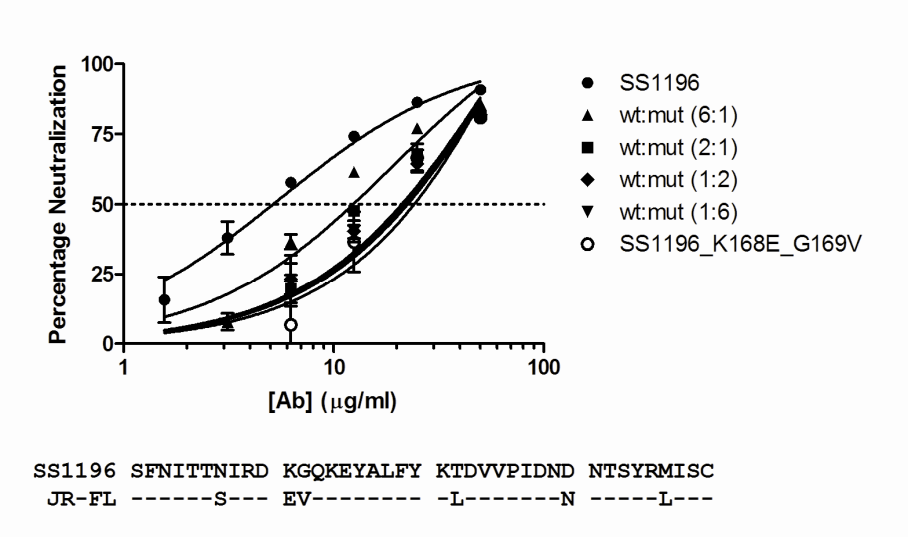

Figure 2.5. Electrostatic forces between the N-terminal strand of V2 and the tip of V3 as a mechanism of V3 masking. The neutralizing activity of B4e8 was tested against virus SS1196 and a derivative virus with amino acid substitutions at positions 168 and 169 in the V2 region. Viruses with mixed SS1196 (wt) and SS1196_K168E_G169V (mut) heterotrimers were tested for neutralization sensitivity to B4e8 as well. A sequence alignment of the V2 sequences of SS1196 and JR-FL is noted below the graphs with identical amino acid residues indicated by dashes. The sequences were aligned using Clustal (http://www.ebi.ac.uk/Tools/msa/clustalo/) and formatted for publication using SeqPublish (http://www.hiv.lanl.gov/content/sequence/SeqPublish/seqpublish.html).

2.4. Discussion

Our results indicate that, although seemingly resistant to antibody binding, the V3 tip of at least some subtype C viruses may not be as occluded as often assumed; mutating Gln315 in two tier 2 subtype C viruses to Arg resulted in these viruses becoming sensitive to the neutralizing activity of mAb B4e8. However, mutating Arg315 to Gln in an otherwise sensitive subtype B virus resulted in a substantial loss of neutralization sensitivity to antibodies that contact Arg315 as well as those that do not, suggesting that the presence of the Gln influences the manner in which V3 epitopes are presented. Extending previous

work by others (36, 57, 58, 62), we found also that V3 resistance is modulated by intersubunit interactions between the N-terminal portion of V2 and the V3 tip or region of neighboring Env protomers.

The V3 region is often suggested as occluded yet how this occurs is not entirely understood. While the V1V2 domain is considered a major determinant of V3-targeted resistance (58), others have proposed that structural or conformational differences between subtype B and non-subtype B viruses within certain segments of the V3 region might also play a role in virus resistance (2, 53). Using the V3 mAb B4e8 as an example, we observed no correlation here between B4e8 neutralization resistance and V1V2 length or the number of V1V2 N-glycosylation sites.

This is to our knowledge the first study to investigate how the presence of Arg instead of Gln at the tip of V3 in subtype C viruses might affect V3 antibody recognition. Little attention has been given to V3 exposure in subtype C viruses, presumably because of the perception that V3 antibodies play a limited role in neutralization of these viruses during infection. Indeed, individuals infected with subtype C HIV tend to mostly develop nAbs directed towards regions outside of V3 (19, 20, 38, 45); in those instances where V3 antibodies are detected they tend not to exhibit HIV neutralizing activity (10, 43). It is important to note however that, to our knowledge, V3 antibodies recovered from non-subtype B-infected individuals interact largely with residues in the N-terminal segment of V3 (9, 27), which may limit their neutralization breadth (52).

Epitope mapping of PG and PGT mAbs suggests that portions of the V3 region on non-subtype B viruses, including segments at the tip, are at least somewhat accessible to antibody (70) and the results presented here support the notion that the tip may be more accessible than might have been appreciated to date.

Two previous studies have reported on the influence of an Arg315 to Gln switch on the neutralizing activity of V3 antibodies, but in the subtype B virus SF162 (7, 31). These studies showed that SF162 harboring either the parental or a consensus subtype B V3 region with an R315Q substitution remained sensitive to neutralization by the V3 mAbs 2219, 2257 and 3074 (7, 31). Those results contrast with our findings here that SS1196 with a R315Q mutation is no longer sensitive to neutralization by these mAbs (Fig. 3). The specific reason for this discrepancy was not investigated here but we suspect that it may be due to the extreme sensitivity of SF162 to V3-targeted neutralization (67), which in turn may be due to the atypically few glycans in V1V2 (56) and other strain-specific features. We chose to introduce the R315Q mutation in the subtype B virus SS1196 which has features, including V1V2 length (60 residues) and glycosylation sites (n=5), that are similar to those of V3-antibody resistant subtype B viruses (33) and similar to the features of the subtype C viruses used in this study.

Observing that substitution of a charged residue (Arg) to a neutral one (Gln) could dramatically affect antibody neutralizing activity even for antibodies with core epitopes that do not involve the substituted residue led us to

a model of V3 access modulation based on the presence of a charged Arg or neutral Gln residue at position 315. We found that SS1196 expressing trimers with even just one gp120 protomer with an Arg residue was neutralized by V3 mAbs B4e8 and HGN194 similarly to SS1196 with trimers where all three gp120 protomers had an Arg315. This result indicated that the charge of the residue at position 315 does not substantially influence antibody accessibility of V3 via repulsion of neighboring V3 regions. We therefore focused on exploring charged residues in the V2 region as a potential mechanism affecting V3 accessibility. When we rendered the V2 of SS1196 more negatively charged by a K168E mutation, the virus was noticeably more resistant to B4e8-mediated neutralization, in support of our hypothesis.

One caveat of the co-transfection experiments above is the generation of mixed trimer pseudovirus populations that contain pseudoviruses expressing solely wild-type or mutant homotrimers. One previously used method to ensure that only mixed trimer pseudoviruses are contributing to infectivity is the complementation system (36). The complementation system relies on mutations in Env that separately result in non-infectious trimers; infectious Env results from co-expression of both mutations which results in mixed trimer psuedoviruses being the solely infectious psudovirus particles being generated (36). We were unable to use a similar method because additional mutations in Env could potentially affect the oligomeric configuration of Env, including V3 exposure.

We conclude therefore that the residue at position 315 and the attraction/repulsion of specific residues outside of V3, such as those in the N-terminal strand of the V2 region, are important factors that affect V3 exposure. It is worth noting that our observations do not signify that factors or residues other than those mentioned above cannot also contribute to differences in V3 exposure levels, for example the possible role of Ile309 in subtype C in also modulating V3 exposure (39). Results from characterization and comparison of nAb-sensitive and –resistant Env sequences from within viral quasispecies (47, 48) provide further evidence that the presence or absence of select residues can alter epitope exposure of regions in gp120.

To what extent is the V3 tip on non-subtype B viruses accessible to bona fide V3-specific antibodies? Failure to recover broadly neutralizing V3-specific mAbs from individuals infected with non-subtype B viruses may not be an adequate measure of the degree of accessibility of the V3 tip on these viruses. For example, the V1V2 region was long considered as highly variable and an unlikely target for broadly neutralizing antibodies, yet the discovery of the PG9/16 antibodies and related ones (e.g. CH01-04 and PGT141-145) has made clear that some segments of V1V2 are highly conserved and accessible (28). Of relevance to this study is that not all antibodies to the PG9/16 epitopes exhibit broad anti-viral activity because of critical differences in fine specificity (29, 71) or structural polymorphism that affects the manner of antibody interaction with the epitope (59).

One potential alternative avenue to assess V3 exposure on non-subtype B viruses is the in vitro evolution or mutagenesis of existing V3 mAbs, as has been done for other anti-HIV antibodies (4, 11, 46), and subsequent investigation of the neutralizing activity of the recovered variants relative to their affinity for non-subtype B V3 sequences. Information gained from such analyses should aid in understanding links between antibody specificity and functional activity.

2.5. Acknowledgments

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3. In vitro evolution of a V3 antibody to HIV-1 does not improve binding affinity for subtype C gp120

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(In progress)

ABSTRACT: The V3 region of HIV-1 gp120 is fairly conserved at its center, particularly among non-subtype B viruses. This segment, also termed the V3 tip, therefore represents a potential vaccine target for neutralizing antibodies. However, the extent to which the V3 tip on non-subtype B viruses is accessible to antibodies is unclear. MAb B4e8 is one of a few anti-V3 antibodies that binds an epitope located within the V3 tip. B4e8 exhibits modest neutralizing activity against subtype B viruses but is unable to neutralize most non-subtype B viruses. Here, libraries of B4e8 variants with 5-codon mutations in the third complementarity determining region (CDR) of the B4e8 light (LCDR3) and heavy chains (HCDR3) were generated as single-chain variable fragments (scFvs) and displayed on the surface of yeast and phage respectively. The libraries were subjected to 3-6 rounds of selection on subtype C gp120s to select for high affinity binders. Eleven mutant LCDR3 B4e8 variants were recovered by yeast display but none exhibited enhanced binding to subtype C gp120. Panning of the HCDR3 phage library also did not yield higher-affinity binders. These results suggest that simple mutation of the CDR residues targeted in this study is not sufficient to impart B4e8 with cross-neutralizing activity.

3.1. Introduction

Current V3 antibodies poorly neutralize non-subtype B viruses, which tend to have a conserved Gln at position 315 in their V3 region as opposed to an Arg, as occurs in most subtype B viruses (5, 10, 13, 34). The poor activity of anti-V3 antibodies is often attributed to the limited accessibility of V3 on the surface of non-subtype B viruses (1). However, as suggested by the results presented in Chapter 2, the binding of V3 antibodies to at least some non-subtype B viruses may not be as restricted as often assumed. The conserved presence of particular residues, such as the Gln at position 315, leads to a V3 presentation that existing V3 antibodies have difficulty recognizing. The isolation of bnAbs that bind to segments of V3 supports the notion that segments of V3 are at least somewhat accessible to antibody (19, 26, 29).

The V3 mAb B4e8 shows appreciable neutralizing activity against subtype B viruses, but the antibody neutralizes most non-subtype B viruses poorly (23). Structural data and scanning mutatgenesis of the B4e8 epitope indicate that B4e8 primarily interacts with residues in the V3 tip; particularly residues Ile309, Arg315 and Phe317 (4, 23, 25). One possible explanation for B4e8's weak neutralization of non-subtype B viruses is the loss of binding affinity because of the neutral Gln residue at position 315 in these viruses. Furthermore, B4e8 is less somatically mutated than most bNAbs, which tend to be extensively mutated (17, 29, 33). I have therefore hypothesized that generating B4e8 LCDR3

libraries and selecting these libraries on subtype C gp120 can yield B4e8 variants with improved neutralizing activity against non-subtype B viruses.

In vitro antibody affinity maturation using phage display has allowed for improvements in the neutralizing activity of select HIV antibodies, including those directed to the CD4bs and the membrane proximal external region of gp41 (2, 7, 22, 31). An NNK saturation mutagenesis was used here to generate yeast surface display and phage display libraries of B4e8 complementarity determining region 3 (CDR3) light and heavy chains respectively (12, 18). The NNK strategy (where, N presents A, G, C or T, K presents T or G) uses 32 triplet codons to produce all 20 naturally occurring amino acids, ensuring library diversity (21).

The α-agglutinin yeast adhesion receptor on the surface of the yeast *Saccharomyces cerevisiae* is composed of two proteins Aga1 and Aga2 (9, 27). The Aga2 protein associates with the cell wall bound Aga1 protein via two disulfide bonds (28). The yeast surface display of antibodies exploits the association of the Aga1 and Aga2 proteins to display a single-chain variable fragment (scFv) on the yeast cell (Fig 3.1) (6). Specifically, the gene of interest is cloned into an epitope tagged yeast display vector as an in frame fusion with the *aga2* gene (6). A recombinant yeast strain containing the *aga1* gene is subsequently transformed with the resulting construct (6). Expression of both the Aga2 fusion protein from the yeast display vector and the Aga1 protein in the yeast strain is regulated by the *gal1* promoter (6). As a result, galactose induction causes the Aga1 protein and the Aga2–scFv fusion protein to assemble within

the secretory pathway, and the epitope-tagged scFv antibody is displayed on the yeast cell surface (6). Flow cytometry can then be used to monitor both the scFv expression and scFv-antigen binding interactions. Yeast cells displaying scFvs that bind to antigen with higher affinity than the parental antibody can further be isolated by flow cytometry (6). Here, subtype B (JR-FL) and C (Du179 and consensus (Con) C) monomeric gp120s were used to attempt the isolation of B4e8 variants with improved binding affinity for HIV subtype C.

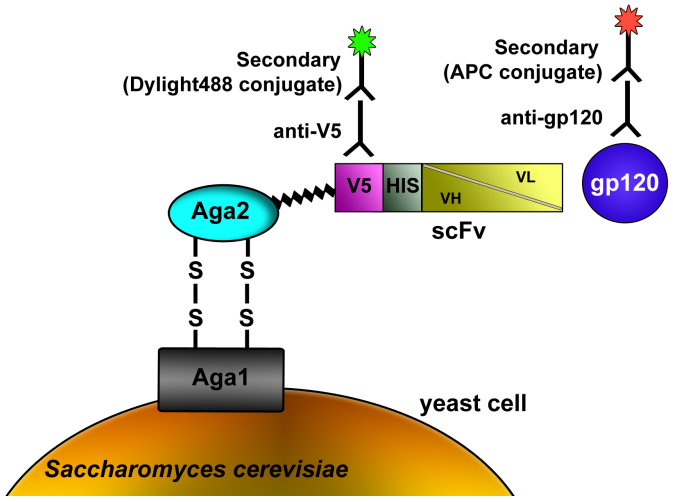

Figure 3.1. ScFv expression on the surface of *Saccharomyces cerevisiae.* V5 and HIS epitope tagged scFvs are displayed on the surface of yeast as Aga2 fusion proteins. ScFv expression can be detected by measuring the binding of an anti-V5 antibody through detection with a fluorescently labeled antibody detection with flow cytometry. Antigen (Ag) binding, in this case gp120, can be detected similarly.

Phage display is a technique in which proteins are displayed on the surface of filamentous phage while the genetic material encoding the displayed protein resides on the inside of the phage particle (Fig 3.2) (3). This creates a direct linkage between the protein being displayed and sequence of the DNA encoding for it (3). Bacteriophages are bacterial viruses that infect many Gram-negative bacteria (3). They possess a circular single stranded DNA (ssDNA) genome comprised of 11 genes, which can be divided into 3 groups: one group

(genes *II, V, X*) encode proteins required for replication of the phage genome (3); a second group encodes the capsid proteins (pVII, pIX, pVIII, and pVI); and the third group encodes proteins involved in the membrane-associated assemble of the bacteriophage (pI, pXI, and pIV) (3). The phage genome is encased in a cylindrical tube containing nearly 3000 copies of the major coat protein (pVIII) (3). One end of the phage particle contains roughly 5 copies each of the pVII and the pIX minor capsid proteins while the opposite end has 3-5 copies each of pIII and pVI minor capsid proteins (3). Phage display libraries of peptides and proteins have been made using pIII as the coat protein that displays the peptide or protein of interest (3). Here, we generated a B4e8 HCDR3 scFv phage display library and panned the library on a subtype C (Du179) gp120 to select for B4e8 variants.

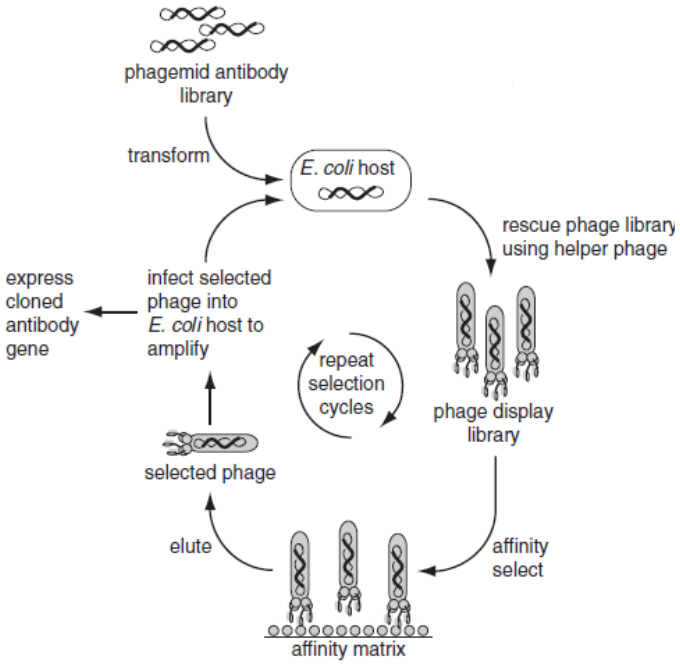

Figure 3.2. Phage library selection of an antibody library. Antibody library phagemid is transformed into *Escherichia coli*. Helper phage provide the assembly and replication proteins that are lacking in the phagemid in order to have antibody displaying phage. Affinity selection is carried out through so-called panning rounds, where the antibody displaying phage library is incubated with antigen (typically coated onto an ELISA plate well). Unbound phage are removed by washing, after which binding phage are eluted and reamplified (14).

3.2. Materials and Methods

3.2.1. Antigens and Antibodies

Three gp120 monomers were used in this study. JR-FL and Du179 gp120s were purified by Brenda Clark in the Pantophlet Lab from stably transfected CHO-K1 and *Drosophila* S2 cells, respectively, while purified ConC gp120 was provided by Penny Moore and Lynn Morris. The anti-gp120 mAb EH21, which binds a conserved linear epitope near the N-terminus of gp120 (16), was generously provided by James Robinson. Dylight^{TM}488-conjugated F(ab')$_2$ fragment goat-anti rabbit IgG Fc-specific and allophycocyanin (APC)-conjugated F(ab')$_2$ goat anti- human IgG Fc-specific (both from Jackson ImmunoResearch) were used to detect scFv expressed on the surface of yeast cells and antigen binding to scFv, respectively. The anti-HA mAb 16B12 (Covance) and lycopepti-conjugated goat anti-mouse IgG Fc-specific (Jackson ImmunoResearch) were used to test phage pools for binding to gp120 in ELISA.

3.2.2. Library Construction

B4e8 LCDR3 and CDRH3 libraries were constructed analogous to published protocols (3, 32) by Kate Auyeung using as template the yeast surface display plasmid pYD5 (30) (obtained from David Neville), modified to contain *Sfi*I restriction sites at the 5' and 3' ends of the gene insert (R. Pantophlet, unpublished), and the phagemid plasmid pCOMB3x (3) (obtained from Dennis Burton and Carlos Barbas III) into which a gene encoding the B4e8 scFv was cloned. Five codons were targeted in LCDR3 (^{89}QQ**YDNL**GDLS^{97}) and HCDR3

(^{95}**D**R**Y**YETSG**SN**AFDV^{102}) (double line indicates residues that were randomized and boldface indicates contact residues). Briefly, NNK site-saturation mutagenesis was performed with primers having partial complementary to the template DNA. After PCR, the reactions were enzymatically treated with *Dpn*I to digest parental methylated and hemi-methyated DNA. The resulting DNA reaction mixtures were pooled, precipitated with EtOH/NaAc following standard protocol, and then electroporated into EBY100 strain yeast cells for yeast display (1500 V; BioRad, GenePulser Xcell) or XL-1 Blue E. coli for phage display (2500 V; BioRad, GenePulser Xcell).

3.2.3. B4e8 LCDR3 Yeast Display Library Screening

The yeast B4e8 LCDR3 library was cultured following previously described procedures (11). Generally, a frozen aliquot (10 ml at a density of ~5x10^7 cells/ml) of the LCDR3 library was thawed at room temperature (RT) and grown in 1 L of selective growth media containing dextrose (SDCAA) and penicillin (Pen)/streptomycin (Strep) (Lonza) overnight at 30°C with shaking (250 rpm). An aliquot (1x10^{10} cells) from the overnight culture was transferred to fresh SDCAA (1 L) and grown at 30°C with shaking (250 rpm) overnight. The next day, 1x10^{10} cells were pelleted and resuspended in galactose induction media (SGCAA) supplemented with Pen/Strep and grown for 2 days at 25°C with shaking (250 rpm). From the induced yeast culture, aliquots of 10^8 cells per flow cytometry staining condition were centrifuged. The pellet was washed twice and resuspended in flow cytometry buffer (PBS supplemented with 0.5% BSA). The

cells were incubated with gp120 for 1 h at RT, washed twice with flow cytometry buffer and then incubated for 30 min at 4°C with the anti-gp120 mAb EH21 and an anti-V5 polyclonal antibody (Sigma). After pelleting, washing and resuspending the cells as before, the cells were incubated with appropriate fluorescently labeled secondary antibodies for 30 min in the dark (4°C). Samples were analyzed on either a FACS Aria (BD Biosciences; SFU) or Infux (BD Biosciences; UBC).

Yeast cells with both the highest level of scFv expression and the highest binding signal for antigen (as compared to unstained and single stain controls) were sorted into a conical tube with 2 ml SDCAA. An additional 2 ml of SDCAA supplemented with Pen/Strep was added to the sorted cells, which were then grown for 2 days at 30°C with shaking (250 rpm). Protein expression was then induced with SGCAA as above and the cells subjected to another round of antigen sorting. Concentrations of subtype C gp120 (Du179) for sorting were chosen based on wild-type B4e8 binding to titrated subtype B g120 (JR-FL) and Du179 (Fig. B1). The concentration of Du179 gp120 chosen was considered high enough to observe binding while sufficiently stringent to select for affinity enhanced variants. The sorting strategies used in this study are summarized in Table 3.1. After the final round of sorting the isolated cells were plated onto SDCAA agar and incubated for 2-3 days at 30°C until colonies were visible. Individual colonies were then picked, transferred to 5 ml SDCAA media with Pen/Strep and cultured overnight (30°C, 250 rpm). Plasmid DNA was isolated

with the Zymoprep Yeast Plasmid Miniprep Kit II (Zymo Research). PCR was performed to confirm that the isolated yeast clones contained an insert. Positive samples were then sent for sequencing (Operon).

Table 3.1. Overview of B4e8 LCDR3 yeast surface display library panning with gp120[a]

Panning Strategy	Round 1	Round 2	Round 3	Round 4	Round 5
1	Du179 (40)	Du179 (10)	Du179 (0.5)	-	-
2	Du179 (40)	ConC (10)	Du179 (4)	ConC (2)	-
3	ConC (40)	ConC (10)	ConC (4)	ConC (4)	ConC (4)
4	Du179 (1)	Du179 (1)	Du179 (1)	Du179 (1)	-
5	Du179 (0.2)	Du179 (0.2)	Du179 (0.2)	Du179 (0.2)	-
6	Du179 (1)	Du179 (0.1)	Du179 (0.05)	Du179 (0.01)	-

[a]Concentration of gp120 (μg/ml) in parentheses

3.2.4. B4e8 HCDR3 Phage Display Library Amplification and Panning

Panning of the phage B4e8 HCDR3 library was performed similarly as described previously (2, 3). Typically, XL-1 Blue *E. coli* were transformed with the HCDR3 library and cultured in Superbroth (SB) media, carbenicillin (carb) and tetracycline (tet) at 37°C with shaking (250 rpm). Helper phage was added and

the culture transferred to a 500 ml baffled flask with additional SB, carb and tet. The mixture was incubated for an additional 2 h at 37°C with shaking (300 rpm). Kanamycin (kan) was added and the culture incubated overnight at 37°C with shaking (300 rpm). An ELISA plate was coated with Du179 monomeric gp120 (Table 2) and incubated overnight (4°C). The overnight culture was centrifuged and the phage containing supernatant transferred to a centrifuge bottle with NaCl (BioShop) and polyethelene glycol (PEG)-8000 (Sigma). The suspension was placed on ice for 30 min, centrifuged, and the supernatant discarded. The phage pellet was resuspended in PBS supplemented with 3% BSA.

Phage library panning was performed as described previously (2, 3). Briefly, multiple antigen-coated wells (Table 3.2) were washed qnd the ELISA plate blocked with 3% BSA/PBS for 1 h at RT. Blocking solution was removed and 50 µl of prepared phage added to the wells and incubated for 1 h at RT. The plate was washed (5 washes in round 1, 10 washes in round 2, and 20 washes in subsequent rounds) and 100 µl of elution buffer (0.2 M glycine/HCl; pH 2.2) added to the wells. After a 10-min incubation at RT, the eluate was transferred to Eppendorf tubes containing 10 µl Tris buffer (1 M). The mixtures were pooled, added to an *E. coli* culture that was cultured simultaneously, and incubated for 15 min at 37°C. SB carb and tet were added to the mixture and an aliquot was used for output □lycopept while the remaining culture was incubated for 1 h at 37°C (250 rpm). Carb was added to the *E. coli* culture which was then incubated for an additional hour at 37°C. Helper phage was added along with SB, carb and tet

and the culture was incubated for 2 h at 37°C with shaking (300 rpm). Kan was added and the bacterial culture incubated overnight. An ELISA plate was prepared for the next round of panning. The phage panning strategies used here are summarized in Table 3.2.

Table 3.2. B4e8 HCDR3 Phage Display Library Panning on Gp120

Panning Strategy	gp120[a]
1 (4 rounds)	Du179 (0.2)
2 (6 rounds)	Du179 (20)

[a]Concentration of gp120 (µg/ml) in parentheses

3.2.5. Phage Antigen Binding ELISA

ELISAs were performed as described previously (24). 96-well ELISA plates were coated with subtype C Du179 gp120 (2 µg/ml) overnight at 4°C. Plates were washed and blocked with 3% BSA/PBS for 1 h at RT. After removing the blocking solution, undiluted phage was added to the plate and then serially diluted in 2-fold steps. The assay plate was incubated for 1 h at RT, washed 4-6 times (PBS with 0.05% Tween), and then incubated with anti-HA mAb 16B12 (2 µg/ml) for 1 h. After washing as before, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000 diluted in 1% PBS/BSA and 0.02% Tween) was added to the wells and incubated for 1 h. The plate was washed as before, and substrate (3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen

peroxide (H_2O_2) mixed 1:1 (Pierce/Thermo Scientific)) was added to all wells. The reaction was allowed to develop for ~10 min and then stopped with H_2SO_4 (2 M). The optical density (OD) was read at 450 nm (Thermo Scientific, Multiskan Ex).

3.3. Results

3.3.1. Gp120 antigen panning of a B4e8 LCDR3 yeast display library fails to yield variants with improved gp120 binding affinity

Residues in the LCDR3 region (notably AspL92) of B4e8 are important for the interaction with V3 (Fig. B2). Therefore, residues within this region were chosen for NNK saturation mutagenesis to generate a B4e8 LCDR3 yeast display library. Many different antigen selection strategies were used (Table 3.1) to attempt to isolate B4e8 variants with enhanced affinity for subtype C HIV. Flow cytometry dot plots from strategy 4 (Table 3.1) is shown below (Fig 3.3).

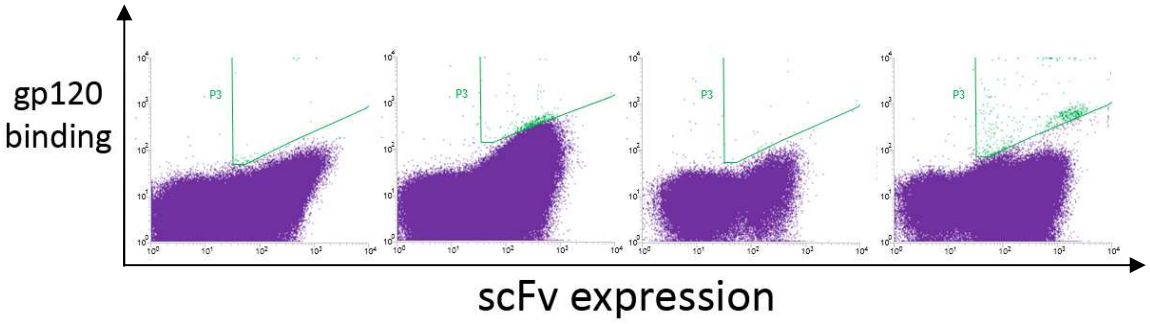

Figure 3.3. Sequential Rounds of antigen selection of a B4e8 LCDR3 yeast display library. Flow cytometry dot plots from rounds 1 to 4 are shown from left to right. In all 4 rounds the concentration of Du179 gp120 was kept constant at 1 µg/ml with high affinity binders (cells in the P3 gate; green) being enriched and used in subsequent rounds.

A total of 11 B4e8 LCDR3 variants (Table 3.3) were recovered from sorting strategies 1 and 2 (Table 3.1) based on a library size of 5×10^5. Sequencing of a representative number of clones (24-48) from each of the remaining sorting strategies resulted in no additional B4e8 LCDR3 variants; all were found to be the wild-type antibody. To assess gp120 binding affinity by flow cytometry, each of the 11 LCDR3 variants were individually transformed into EBY100 and the cells cultured to express scFv. Binding assessment of the 11 mutants to subtype B gp120 (JR-FL) and subtype C gp120s (Du179 and ConC) showed that the mutants had diminished binding to JR-FL and Du179 as compared to the parental antibody (Fig 3.4). Although, some of the LCDR3 clones bound to ConC better than wild type B4e8, this increased binding was not substantial (Fig 3.4). From these results it was concluded that affinity maturation of the LCDR3 region did not result in a B4e8 variants that can better bind to subtype C gp120.

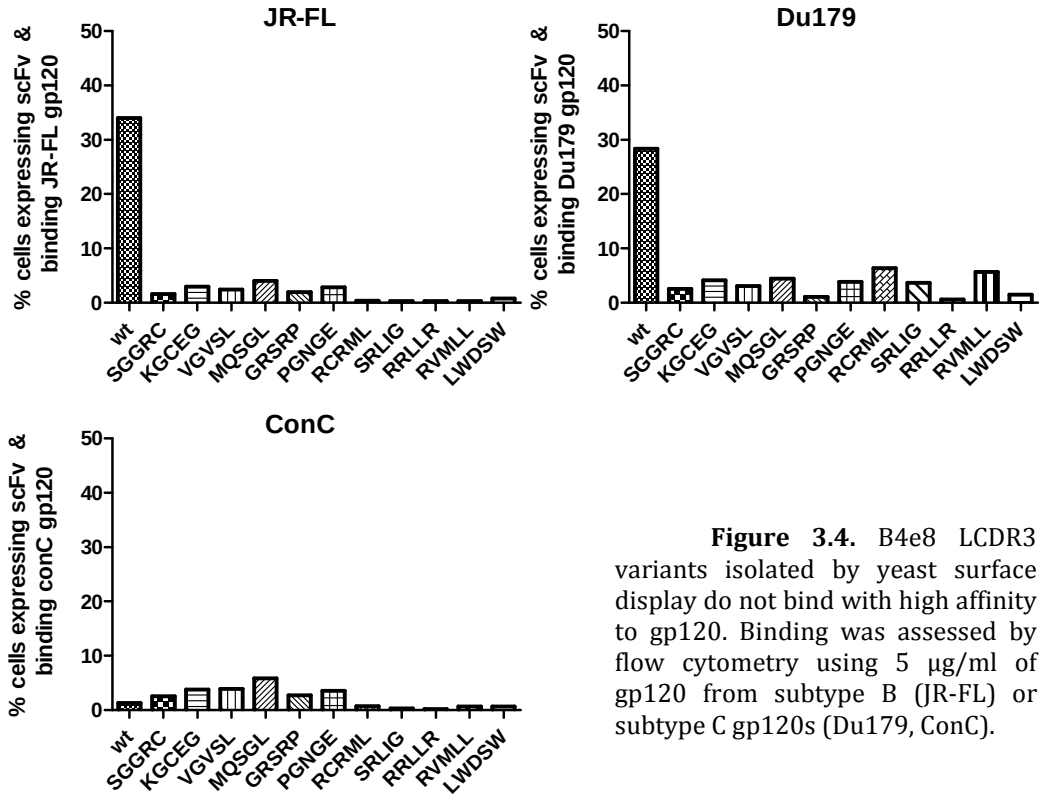

Figure 3.4. B4e8 LCDR3 variants isolated by yeast surface display do not bind with high affinity to gp120. Binding was assessed by flow cytometry using 5 μg/ml of gp120 from subtype B (JR-FL) or subtype C gp120s (Du179, ConC).

Table 3.3. Summary of B4e8 LCDR3 mutants isolated from sorting strategies outlined in Table 3.1.

Sorting strategy 1	Sorting strategy 2
RCRML	GRSRP
SRLIG	PGNGE
LWDSW	MQSGL
RVMLL	VGVSL
RRLLR	SGGRC
-	KGCEG

3.3.2. *Gp120 antigen panning of a B4e8 HCDR3 phage display library is unable to isolate variants with improved gp120 binding affinity as compared to the parental antibody*

Because B4e8 LCDR3 variants did not bind with higher affinity to subtype C gp120, the HCDR3 region of B4e8 was focused on. The B4e8 CDR H3 library used in this study targets two residues that interact with the Arg315 residue on V3, as well as neighboring residues. Two separate phage panning strategies were used (Table 3.2). The results from the second strategy in which the B4e8 HCDR3 library, at a size of 2.6 x 10^6, was subjected to 6 rounds of panning on subtype C (Du179) monomeric gp120 are shown below (Fig 3.5). Disappointingly, no improved binding was observed with any of the phage preps (Fig 3.5), suggesting that, similar to the LCDR3 library, mutations in the HCDR3 region of B4e8 did not enhance B4e8 binding to subtype C HIV.

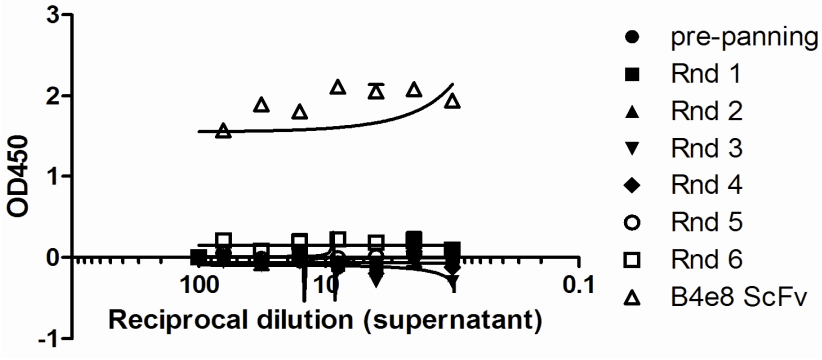

Figure 3.5. Gp120 binding ELISA of phage pools from the panning of a B4e8 HCDR3 phage display library. Phage preps from 6 rounds of library panning on Du179 gp120 were tested for binding to Du179 gp120. Parental scFv was also tested as a control and for comparison purposes.

3.4. Discussion

Targeted mutagenesis of CDRs has been used to improve antibody affinity to HIV (2, 7, 22, 31), yet here we were unable to isolate high affinity

variants of mAb B4e8 through targeted mutagenesis of segments of the LCDR3 and HCDR3 regions and affinity selection against monomeric subtype C gp120s. This suggests that altering the particular B4e8 CDR3 residues targeted in this study does not yield B4e8 variants capable of binding with better affinity to subtype C gp120. Engineering B4e8 to bind subtype C V3 may thus require alternate affinity enhancement strategies or the targeting of other CDRs. For example, mutagenesis of residues in HCDR2, which also makes contact with V3 (Fig. B2) may be beneficial.

The HCDR3 region of B4e8 is 14 residues long, whereas most, but not all, bNAbs have HCDR3 regions over 20 amino acids in length (8). Of relevance here is mAb PGT128, which has a 19-amino acid HCDR3 region and binds an epitope involving C-terminal V3 residues Ile323 and Asp325 (26). It has been postulated that extended HCDR3 regions, as found in PGT128, is required to reach more recessed sites on the trimer (20). In this study, the significance of the length of the HCDR3 region of B4e8 for B4e8 neutralization of non-subtype B viruses was not investigated. However, a recent report (15) shows that HCDR3 length can impact the binding and neutralization efficacy of anti-HIV antibodies, suggesting that similar studies with B4e8 may be of relevance. V3 antibodies that are able to bind non-subtype B virus gp120s could aid in shedding light on the extent to which the V3 region on non-subtype B viruses are exposed, as suggested by the results presented in chapter 2.

3.5. Acknowledgments

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4. Thesis Discussion

It is generally agreed that an effective HIV vaccine would have to elicit antibodies capable of blocking infection of antigenically diverse viral strains (28). To facilitate the design of such immunogens, a thorough understanding of epitope exposure on Env, the target for virus nAbs, is required. The objective of the work presented in this thesis was to characterize accessibility of the third variable region (V3) of gp120 and mechanisms used by HIV to evade V3 antibody recognition.

The V3 region is overall more conserved in sequence than the other gp120 variable regions (12, 22). Sequence conservation of V3 has been linked to the conserved functional role of V3 in binding co-receptor molecules on the host cell membrane, which is necessary for virus infection of the cell (10). However, much like other sequence conserved sites on Env, V3 seems protected from broad nAb recognition (1, 14-17, 23, 26, 27). That said, V3 seems more accessible to V3 antibodies on subtype B viruses as compared to non-subtype B viruses (7, 18, 20). The mechanism(s) that might control V3 exposure on non-subtype B viruses have yet to be fully characterized.

In this thesis, I used a panel of six V3 mAbs with known fine specificities to explore V3 access on subtype C viruses, with particular attention

to the V3 tip. Most non-subtype B viruses, including those from subtype C, have the sequence GPGQ at their V3 tip, whereas in subtype B viruses the Gln is typically replaced with an Arg. The V3 antibodies used here either require the Arg in the aforementioned motif for binding to V3 (mAbs B4e8, 268-D) or do not (mAbs HGN194, 3074, 2219, and 2557). I found that subtype C viruses with a Q-to-R mutation became sensitive to neutralization, although only to mAb B4e8, whereas an R-to-Q-switched subtype B virus became resistant to neutralization by all V3 mAbs, even those that do not contact the Arg.

It has been suggested that V3 mAbs that do not interact with the Arg/Gln residue are more broadly neutralizing than V3 mAbs that are Arg-315-dependent (6, 8, 9, 13). In a previous study, the V3 mAb HGN194 was indeed shown to exhibit fairly broad neutralization of subtype A and C viruses than the previously mentioned V3 antibodies (6). Although the V3 mAbs 2557, 2219, and 3074 have been dubbed ‘cross-neutralizing’, these V3 antibodies neutralize few subtype C isolates (9, 11).

It has been observed that several V3 neutralization resistant viruses have the same V3 amino acid sequence as sensitive viruses (1, 6), which has led to the idea of V3 epitope masking on the surface of HIV with the V1V2 length being strongly implicated (14, 16, 23, 25). However, I did not observe a correlation between V1V2 length and neutralization resistance of subtype C viruses to mAb B4e8, used in this thesis as a model to explore V3 access. This led me to focus on the sequence of the V3 tip itself which contains the B4e8

epitope. Although glycans and other variable loops have been shown to occlude V3 on the surface of HIV (1, 16, 21, 23-25, 27, 29), these mechanisms do not seem to be the only factors that explain resistance of non-subtype B viruses to V3 antibodies. Results presented in this thesis suggest that perhaps at least the tip of V3 is antibody accessible on the surface of some non-subtype B viruses but the presence of Gln315 residue prevents antibody recognition.

Observing that the V3 sequences on poorly neutralized subtype C viruses may be accessible to at least mAb B4e8, I attempted to alter the specificity of the B4e8 antibody by in vitro affinity maturation. Similar experimental approaches have been used to improve the affinity of HIV antibodies to the CD4 binding site (CD4bs) on gp120 and the membrane proximal external region (MPER) on gp41 (2, 4, 19, 30). Yeast and phage display were used here to make complementarity determining region 3 (CDR3) heavy and light chain mutant B4e8 libraries. Antibody sequence diversity was introduced at 5 consecutive positions for both the B4e8 LCDR3 and HCDR3 libraries, which were subjected to multiple rounds of antigen selection to enrich for high-affinity binders. Disappointingly, no B4e8 variants with improved binding affinity for subtype C gp120 were recovered, suggesting that changing the residues targeted here is insufficient to enhance B4e8 binding affinity for subtype C gp120.

The reason why I was unable to isolate B4e8 variants with higher binding affinity for subtype C gp120 is not entirely clear. In past studies, the

LCDR3 or HCDR3 were commonly targeted for in vitro maturation because these regions are hot spots for somatic hypermutation in vivo (3, 5). Another strategy to possibly affinity mature B4e8 could be to target residues other than the ones investigated here that might interact with Gln on non-subtype B V3. Of interest would be Tyr^{L32}, which was not targeted here, because the residue is located close to where the Gln residue on V3 is assumed to be located. The generation of a B4e8 variant antibody might aid in the understanding of structure-function aspects of V3 recognition by antibodies to the V3 tip.

Studies that address V3 exposure on non-subtype B viruses, which account for ~90% of circulating global strains, have been limited. The work in this thesis provides insights into mechanisms used by HIV to evade antibody recognition of a sequence conserved site. Although this thesis specifically focuses on how conservation of V3 sequences affects V3 antibody recognition, similar mechanisms could be protecting other epitopes on Env.

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Appendices

Appendix A: Supplementary Tables and Figures for Chapter 2

Table A1. Pseudovirus Infectivity[a]

Pseudovirus	Average Infectivity
CAP45	8.8×10^4
CAP45_Q315R	3.6×10^4
ZM249M	7.0×10^4
ZM249M_Q315R	2.4×10^4
SS1196	1.8×10^6
SS1196_R315Q	1.8×10^6

[a]Infectivity is measured as relative luciferase units averaged from triplicate values from a single experiment. Luciferase activity was measured after incubating a 1:2 dilution of pseudovirus on target cells for 3 d. U87.CD4$^+$.CCR5$^+$ target cells were used for SS1196 and SS1196_R315Q pseudoviruses and TZM.bl cells were used for ZM249M, CAP45 and the Q315R mutant pseudoviruses.

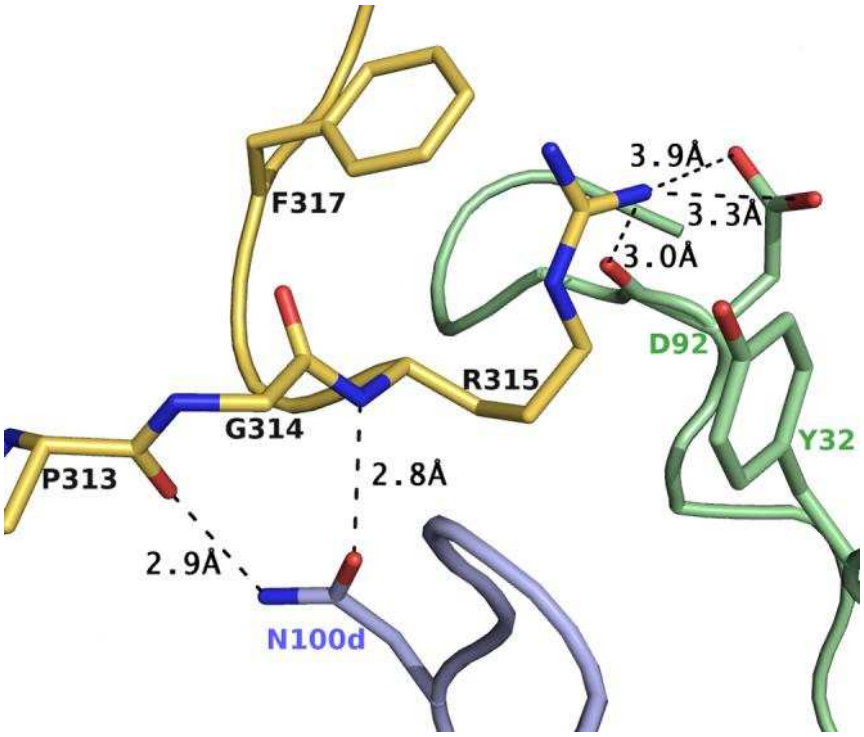

Figure A1. Interaction of Arg315 with the V3 mAb B4e8. The crystal structure of mAb B4e8 in complex with a V3 peptide was resolved by Bell et al. in 2008. The authors presented a focused view of the interaction between the Arg315 residue of a V3 peptide (yellow) and the B4e8 heavy and light chains (light blue and light green). Dashed lines depict hydrogen bonds and salt bridges. (Figure used from (5); PDB accession code 2QSC).

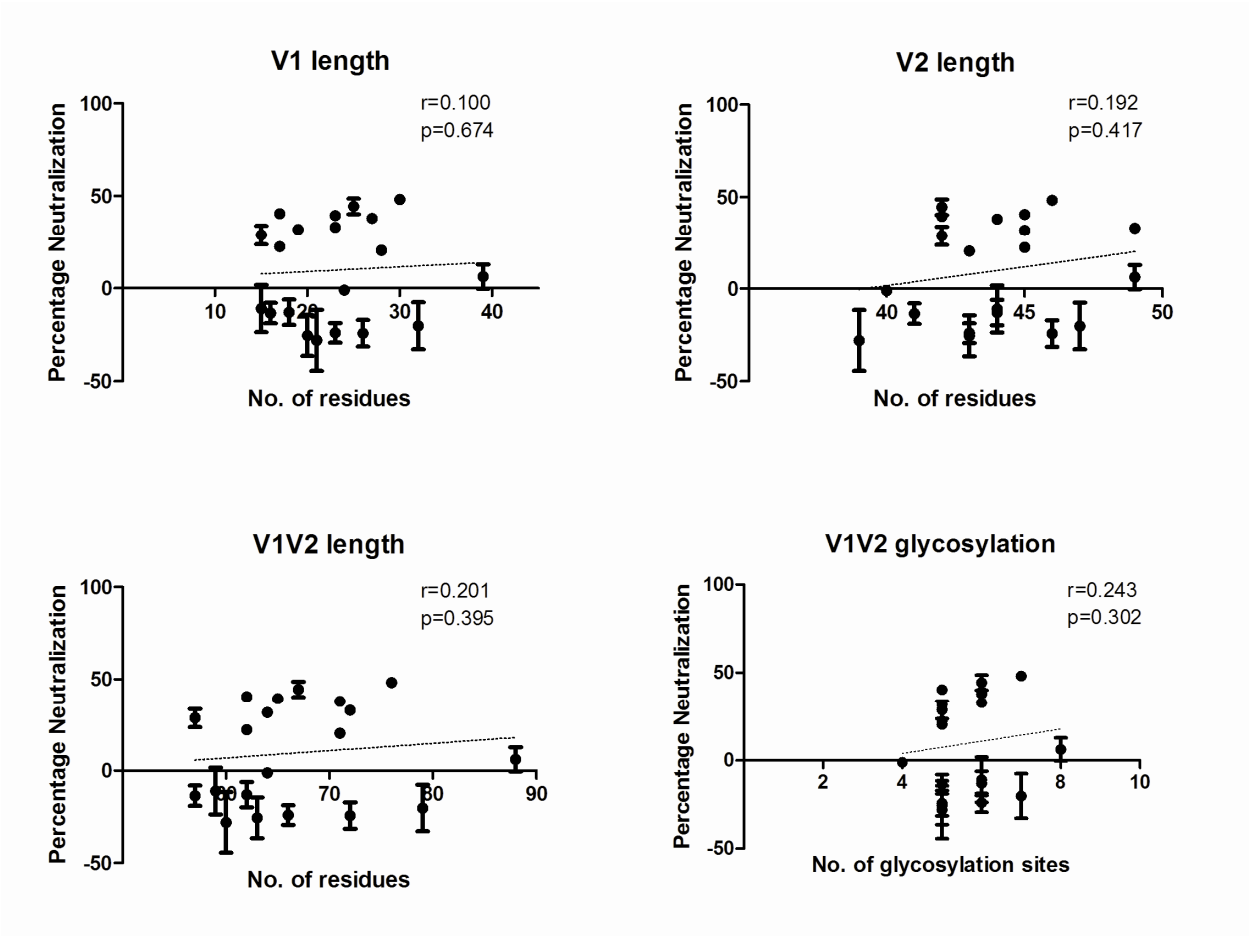

Figure A2. No significant correlation between Env features (V1, V2, and V1V2 lengths and number of N-glycosylation sites in V1V2) and resistance to B4e8-mediated neutralization against 20 subtype C viruses tested in this study. Each symbol represents the percentage neutralization of a subtype C virus from Fig. 1 with 50 μg/ml of B4e8. Spearman rank correlation coefficients (r values) are reported for each data set along with a dotted trend line. The N-Glycosite tool of the Los Alamos HIV sequence database was used to determine the number of N-glycan sites in the V1V2 sequences.

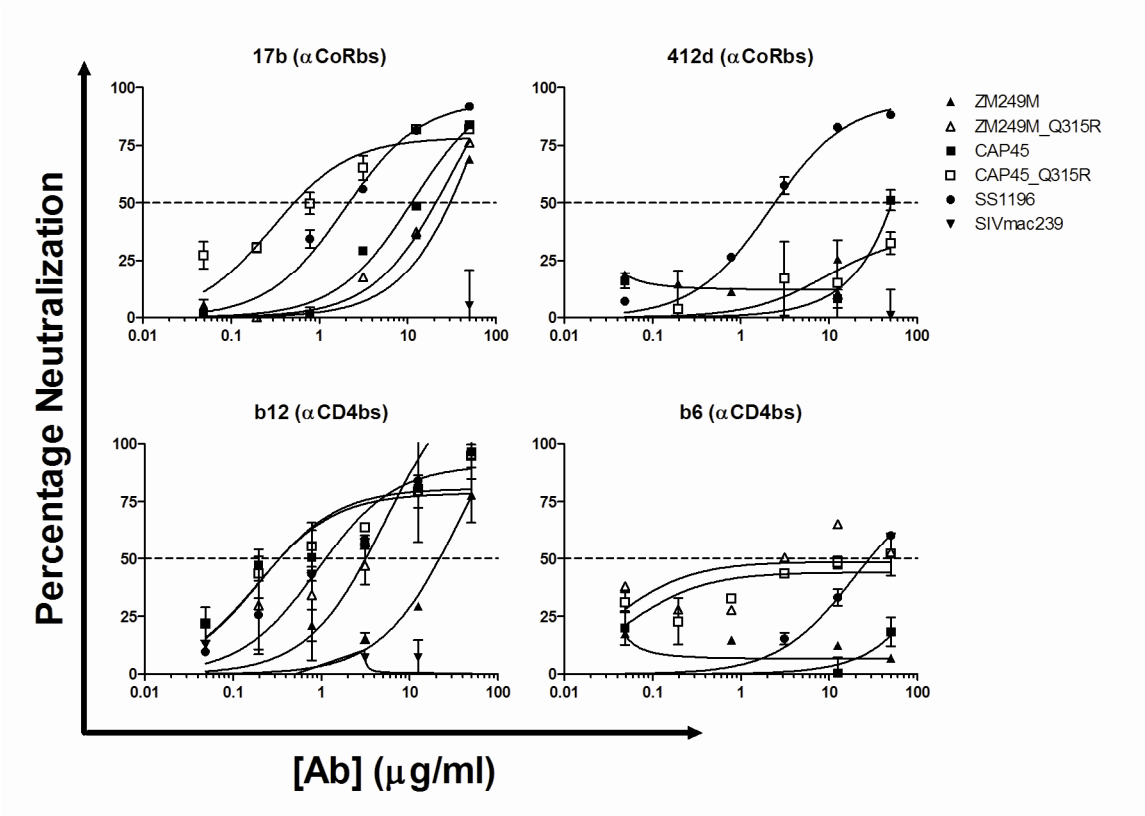

Figure A3. Neutralizing activity of non-V3 mAbs against subtype C viruses ZM249 and CAP45 with a Gln315-to-Arg substitutions in V3. Representative mAbs to the CD4bs and CoRbs were used to assess the level of epitope exposure at these V3-neighboroung sites. Subtype B virus SS1196 (●) and SIVmac239 (▼) were used as a positive control and negative control for neutralizing activity, respectively. Dashed line indicates 50% neutralization. TZM-bl cells were used as targets.

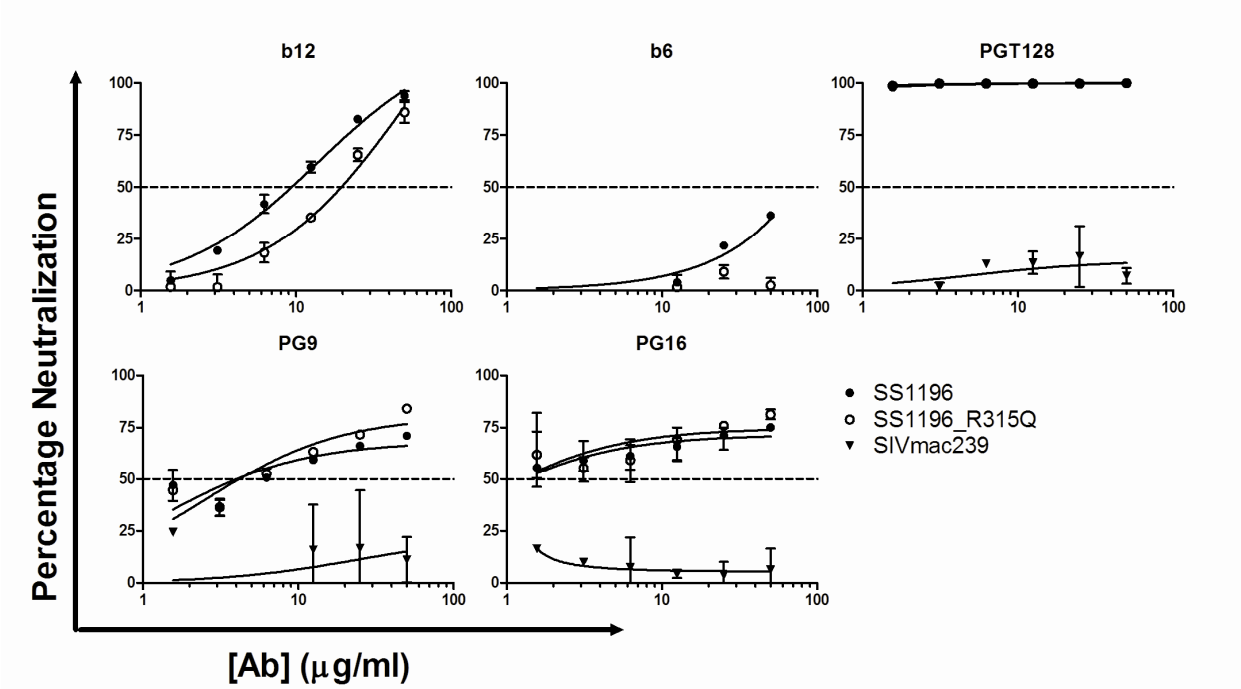

Figure A4. Sensitivity of virus mutant SS1196_R315Q to neutralization by non-V3 mAbs. MAbs b12 and b6 assessed changes in exposure of CD4bs epitopes caused by the R315Q mutation whereas PG9, PG16 and PGT128 were used to assess changes in the global positioning of the V2 and V3 loops. SIVmac239 was used as a control for non-specific neutralizing activity (▼). The dashed line denotes 50% neutralization.

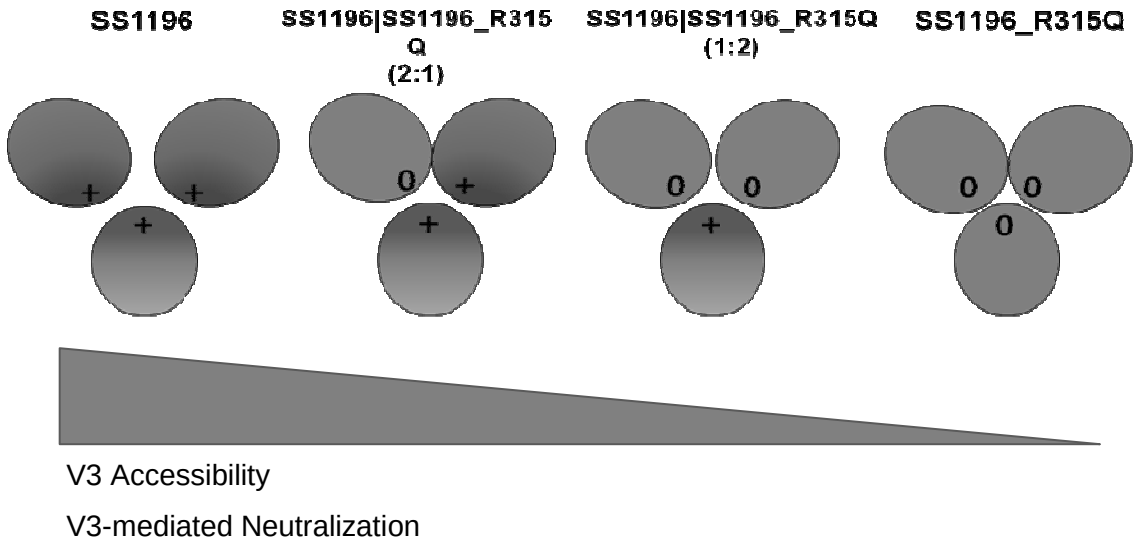

Figure A5. **R315 and Q315 Open and Closed Spike Model.** The Arg residue at position 315 carries a positive charge (+), whereas the Gln residue at position 315 is neutral (0). Based on the potential electrostatic interactions of V3 it is hypothesized that the R315 residue on the three V3 loops on the trimer repulse each other creating a more 'open' spike that is sensitive to antibody neutralization. With the Q315 residue the V3 regions are able to come closer together creating a 'closed' spike that is less sensitive to antibody neutralization.

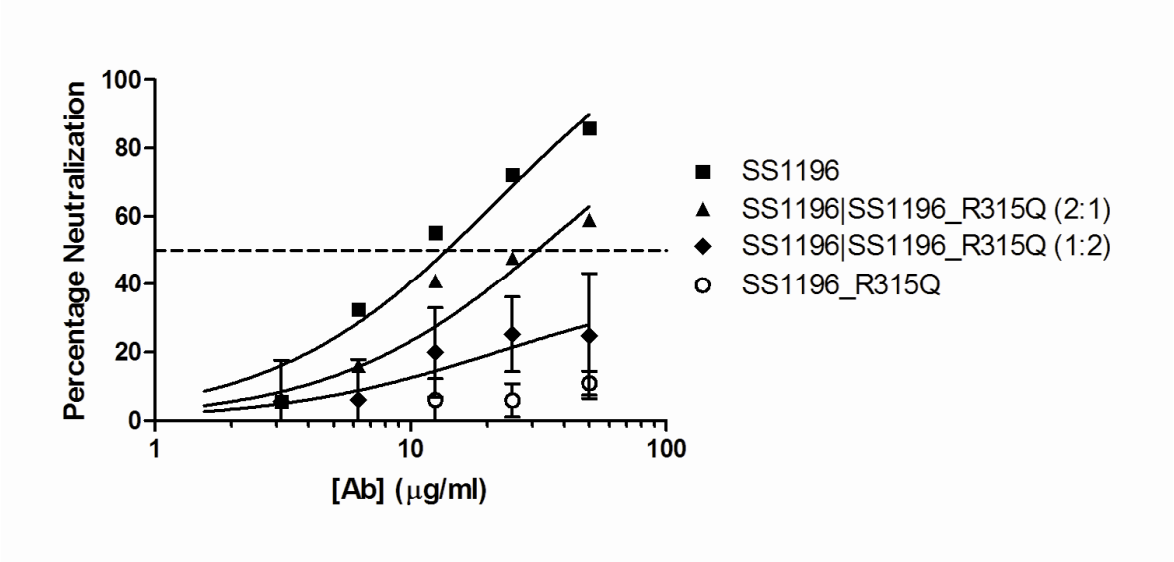

Figure A6. SS1196 wild-type and SS1196_R315Q mutant virus supernatants were mixed, at indicated ratios, and assessed for sensitivity to B4e8 neutralization. The graded decrease in sensitivity is consistent with the increasing fraction of neutralization-resistant mutant virus. This pattern differs from that observed with the heterotrimer viruses (Figure 4). Dashed line denotes 50% neutralization.

Appendix B: Supplementary Figures for Chapter 3

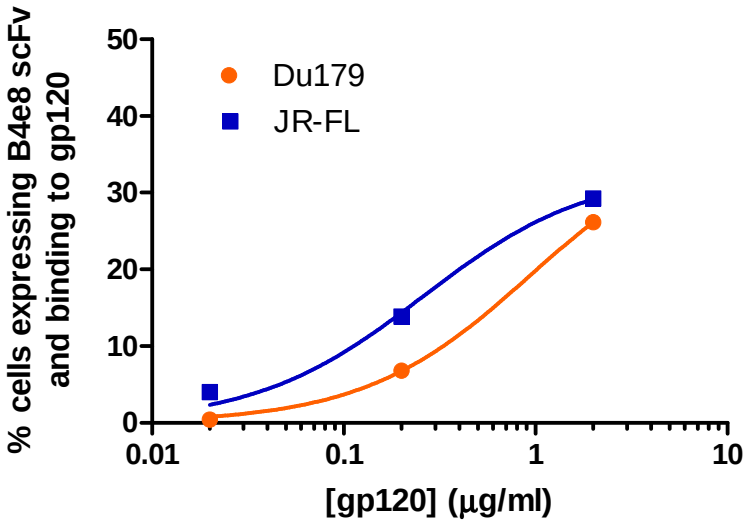

Figure B1. Titration of gp120 binding to wild-type B4e8 scFv expressed on the surface of yeast. Yeast cells expressing B4e8 scFv on their surface were analyzed by flow cytometry for scFv expression and binding to monomeric gp120 derived from HIV-1 JR-FL (subtype B) and Du179 (subtype C).

MAb B4e8 light chain (V_L)

	FR1	CDRL1
B4e8	DIQMTQSPSSLSASVGDRVTITC	QASQDISNY
IGKV1-33*01	DIQMTQSPSSLSASVGDRVTITC	QASQDISNY
	_____	_____

	FR2	CDRL2
B4e8	WYQHKPGKAPKLLIY	TASNLET
IGKV1-33*01	WYQQKPGKAPKLLIY	DASNLET
	___H_____	T_____

	FR3	CDRL3
B4e8	GVPSRFSGGGSGTHFSFTITSLQPEDAATYFC	QQYDNLGDLS
IGKV1-33*01	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP
	_____G____H_S___T_____A___F_	_____G

MAb B4e8 heavy chain (V_H)

	FR1	CDRH1
B4e8	EPQLVESGGGLVQPGGSLRLSCAAF	GFNFSSYVMH
IGHV3-64*01	EVQLVESGGGLVQPGGSLRLSCAAS	GFTFSSYAMH
	_____F	__N____V__

	FR2	CDRH2
B4e8	WVRQAPGQGLEYLS	AISSDGETTYHANSVKG
IGHV3-64*01	WVRQAPGQGLEYVS	AISSNGGSTYYANSVKG
	_____L_	____D_ET__H_____

	FR3	CDRH3
B4e8	RFTSSRDNSKNTLFLQMGSLRTEDVAVYYCAR	DRYYETSGSNAFDV
IGHV3-64*01	RFTISRDNSKNTLYLQMGSLRAEDMAVYYCAR	
	___S_____F_____T__V_____	

Figure B2. Residues in the light (L) and heavy (H) chain regions of mAb B4e8 as compared to germline sequence. Residues in boldface and green denote contact residues. Underlined residues are those that interact with Arg315 of V3. Sequence analysis was done using IMGT, the international ImMunoGeneTics information system. FR, Framework regions; CDR, complementarity determining region.