Role of Envelope Compactness and Glycosylation in HIV-1 Resistance to Neutralising Antibody Responses

A dissertation submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Division of Immunology, Department of Pathology, Faculty of Health Sciences

By

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UNIVERSITY OF CAPE TOWN

August 2017
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Date: 28 August 2017
Acknowledgements

I would like to thank my supervisor, Jeffrey Dorfman. Thank you for aiding in my scientific development throughout my Masters and PhD. Thank you for the opportunities you have provided me with, for being so involved and interested in my project and for encouraging and pushing me to do better.

Thanks to the members of the Dorfman lab – past and present. You have all contributed to this PhD in one way or another. Special thanks to Rajesh Jacob for experimental guidance and 4th year BSc student, Reyaaz Davids, for performing a proportion of the CAP45.G3 and Du156.12 serum neutralisation assays under my co-supervision. Thanks to Fatima Abrahams and Zarinah Sonday for technical and administrative support. Thanks to the current members of the lab, Samuel Kariuki and Fatuma Guleid for the insightful conversations and many tea room laughs.

Thanks to Jean-Philippe Julien for giving me the opportunity to conduct research in your laboratory. Thanks to June Ereño-Orbea and Hong Cui for all the assistance you provided during my research visit. To the whole Julien lab, thanks for making me feel so welcome. Thanks to Michelle Letarte for putting me in contact with Jean-Philippe and taking such good care of me during my visit. I can proudly say I have survived Canadian winter!!

Thanks to Penny Moore, Lynn Morris and Carolyn Williamson for reagents and ICGEB, Cape town laboratories for equipment. I would like to thank the University of Cape Town, South African National Research Foundation and the Polio Research Foundation for providing me with funding throughout my PhD.

To my family, from the oldest Moyo right down to the youngest (currently!) and my partner. Thank you for supporting me in the decision to pursue a PhD and for believing that I could do it! Thank you, Buhle, for proofreading my thesis (perks of having a sister with a PhD in biochemistry!).

Thanks to Jesus Christ, my Saviour. Thank you for giving me the ability to pursue this degree. Thank you for the strength to finish what I started. Psalm 121.
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<tbody>
<tr>
<td>˚C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>6HB</td>
<td>Six-helix bundle</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ADCP</td>
<td>Antibody-dependent cellular phagocytosis</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AID</td>
<td>Activation-induced cytidine deaminase</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>BLI</td>
<td>Biolayer interferometry</td>
</tr>
<tr>
<td>BnAb</td>
<td>Broadly neutralising antibody</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CDRH3</td>
<td>Complementarity-determining region heavy chain 3</td>
</tr>
<tr>
<td>CD4, 8, 20</td>
<td>Cluster of differentiation 4, 8, 20</td>
</tr>
<tr>
<td>CD4-bs</td>
<td>CD4-binding site</td>
</tr>
<tr>
<td>CT</td>
<td>Cytoplasmic tail</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>Endo H</td>
<td>Endoglycosidase H</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding fragment</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>FP</td>
<td>Fusion peptide</td>
</tr>
<tr>
<td>GsS</td>
<td>GGGGS motif</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GNL</td>
<td><em>Galanthus nivalis</em> lectin</td>
</tr>
<tr>
<td>GNT I</td>
<td>N-acetylglicosaminyltransferase I</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>(His)$_{6x}$tag</td>
<td>Histidine-tag</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
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<tr>
<td>HIV-2</td>
<td>Human immunodeficiency virus type 2</td>
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Abbreviations

HR Heptad Repeat
ICAM-1 Intercellular adhesion molecule 1
IC50 50% inhibitory concentration
ID50 50% inhibitory dilution
IQR Interquartile range
Kd Dissociation constant
LFA-1 Lymphocyte function-associated antigen-1
M Molar
mAbs Monoclonal antibodies
Man Mannose
mM Millimolar
µM Micromolar
µl Microliter
MMP Methyl-α-D-mannopyranoside
NaCl Sodium Chloride
NHP Non-human primates
NK cells Natural Killer cells
nM Nanomolar
PDB Protein Data Bank
PBS Phosphate buffered saline
pI Isoelectric point
PNG Potential N-linked glycosylation site
RRRRRR R6 cleavage motif
SAXS Small-angle X-ray scattering
sCD4 Soluble Cluster of differentiation 4
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC Size-exclusion chromatography
SHIV Simian/Human Immunodeficiency Virus
SIV Simian Immunodeficiency Virus
STI Sexually transmitted infection
T/F Transmitted/founder
V1-5 Variable loops 1-5
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This thesis has contributed to the following publications:

  *these authors contributed equally to the work (Thesis Chapter 4)

  (Thesis Chapter 4)

  (Thesis Chapter 2 and 3)
Thesis Summary

Understanding the mechanisms used by HIV-1 to evade antibody neutralisation may contribute to the design of a high-coverage vaccine. This thesis explores the mechanism used by a Tier 3 virus leading to its high antibody neutralisation resistance phenotype. This thesis also describes how the glycans at the base of the V3 loop contribute to (i) breadth and potency in a cohort of unselected HIV-1-infected individuals and (ii) the selective pressures resulting from the V3/glycans shielding the virus from neutralisation and the glycans themselves being targets of broad antibody responses.

HIV-1 isolates that are highly resistant to broadly neutralising antibodies could limit the efficacy of an antibody-based vaccine. For this reason, it is important to understand the mechanisms behind high HIV-1 resistance to neutralising antibodies. Chapter 2 and Chapter 3 of this thesis describe virus 253-11, a highly neutralisation resistant virus, which is particularly resistant to commonly-elicited, anti-membrane proximal external region (MPER) antibodies in sera. To further understand its resistance, mutations in the MPER were introduced that are known to delay fusion following CD4-binding and thus increase the time the virus spends in the open conformation. Interestingly, we found that these mutations affect the 253-11 Envelope (Env) spike before CD4-binding by destabilising the closed trimer structure. From these data, we hypothesized that the neutralisation resistance of 253-11 was due to an unusually tight, compact pre-fusion Env trimer that resists transient changes to the open conformation. The open conformation frequently exposes narrowly-neutralising antibody epitopes. Because the unliganded 253-11 Env presumably transitions infrequently into the open conformation, it would be able to evade these responses.

253-11 was sensitive to most but not all of the most potent broadly neutralising antibodies (bnAbs) tested, most likely because those broadly neutralising antibodies can access their epitopes in the pre-fusion Env conformation. To gain further information about the structure of the 253-11 Env, we designed a recombinant 253-11 SOSIP trimer and found it to be stable and predominantly adopt a closed conformation. The crystal structure of the SOSIP trimer revealed structural elements likely responsible for 253-11 Env compactness including the inward disposition of the heptad repeat helices and gp120 protomers towards the trimer axis. Taken together, the data from Chapter 2 and Chapter 3 highlight an underappreciated Env
compactness mechanism of HIV-1 resistance to neutralising antibodies and these data may be useful in HIV-1 immunogen design research.

Previous candidate HIV vaccines have failed to induce wide-coverage neutralising antibodies capable of substantially protecting vaccinees. A key approach in HIV immunogen development has been to define and model epitopes recognised by anti-HIV bnAbs. Candidate immunogen models identified by bnAbs include the V3/glycans, the V2/apex and the MPER epitopes. Autoreactivity and polyreactivity of anti-V3/glycan and anti-MPER antibodies are thought to pose both direct and indirect barriers to achieving neutralisation breadth. Chapter 4 of this thesis explored which of these bnAb epitopes were associated with breadth and potency in a South African cohort of chronically HIV-infected individuals. The study found that antibodies targeting the V3/glycans were associated with breadth and potency. In contrast, antibodies to the V2/apex were not associated with neutralisation breadth/potency. This suggests that auto/polyreactivity are not critical factors in the development of breadth and potency and that the V3/glycans should remain a high-priority vaccine candidate.

Since targeting the V3/glycans was associated with breadth and potency in this cohort, the study continued to look at this epitope to investigate the role of these glycans in neutralisation resistance of Tier 2 viruses. The HIV-1 Env is surrounded by glycans that often prevent antibody neutralisation, leading to the term the “glycan shield”, however some bnAbs have evolved to recognise these carbohydrates. Chapter 4 of this thesis describes how the N-linked glycan at position N301 is critical for maintaining neutralisation resistance of one subtype C virus (Du156.12), but not for another subtype-matched virus (CAP45.2.00.G3). Thus, the loss of the N301 glycan may have a substantial antibody-related fitness cost for some viruses but not others. The N301 glycan, as well as glycans at positions 332 and 334, are the primary targets of the anti-V3/glycan class of neutralising antibodies, which may select for loss of the targeted glycan. The evidence presented in Chapter 4 suggests that in some viruses, loss of the N301 glycan may result in evasion of anti-V3/glycan antibody responses while maintaining overall neutralisation resistance. This phenomenon may impair efficacy of passively-infused anti-V3/glycan bnAbs or a therapeutic vaccine.
Chapter 1: Literature Review

1.1 Introduction

The human immunodeficiency virus type 1 (HIV-1) was found to be the cause of acquired immune deficiency syndrome (AIDS) in 1983 (Barre-Sinoussi et al., 1983, Chermann et al., 1983) and since that time it has become a major global health concern, with approximately 36 million people living with the virus worldwide as of 2015 (UNAIDS, 2017). Sub-Saharan Africa is home to the majority of HIV-1-infected individuals, with approximately 25 million people living with the virus in this region alone (UNAIDS, 2017, Vermund, 2014).

Upon introduction of anti-retroviral therapy (ART) in the 1990s (Sasson et al., 2005) and specifically highly active anti-retroviral therapy (HAART), there has been a tremendous reduction in the mortality and morbidity of the infection (Montaner et al., 2014, Crum et al., 2006, Casseb et al., 1999). Although HAART is effective, it does bear some challenges. Lifelong treatment comes at a high financial cost to governments and individuals (SANAC, 2016) and not all HIV-infected individuals have access to treatment as shown by statistics from 2014 which revealed that approximately 73% of HIV-positive adults living in South Africa were receiving ART (SANAC, 2016), leaving 27% without the life-saving therapy. Another challenge with ART is the need for infected individuals to adhere to the treatment to reduce the probability of the emergence of drug-resistant isolates (Viswanathan et al., 2015). Non-adherence predominantly occurs in sex workers, intravenous blood users, adolescents and individuals in low-resource areas due to factors including the difficulty in retaining them in care (Mtetwa et al., 2013, McGowan et al., 2011, Rudy et al., 2009, Hankins et al., 2002).

Another concern with relying on ART to control the HIV/AIDS pandemic is adverse drug reactions (Mouton et al., 2016). As more HIV-infected people are enrolled in therapy and live longer, the population risk of ART-related complications rises (Hima Bindu and Naga Anusha, 2011), leading to increased burdens on the healthcare system caused by HIV-1 infections.

There is still no cure for HIV-1 that completely eliminates the virus from the body and there are only two reported cases of a functional cure (Violari et al., 2017, Hutter et al., 2009), with
other reported cure cases experiencing viral rebound at a later date (Henrich et al., 2014, Ledford, 2014). As a result of the disadvantages of ART and the absence of an HIV-1 cure; there is a need for new strategies to fight the pandemic (Dangeti, 2014).

### 1.2 HIV-1 prevention

Current HIV-1 preventative strategies have shown varying success in preventing HIV-1 infection. These include microbicide vaginal gels (Abdool Karim et al., 2010), pre- and post-exposure prophylaxis (Vissers et al., 2008) and male circumcision (Wamai et al., 2011). These preventative measures are relatively inexpensive compared to developing potential HIV-1 cures or vaccines; however, it is highly unlikely that they will be adequately effective on their own in decreasing HIV-1 incidence enough to control and eventually eradicate the virus (Phillips and Pirkle, 2011, Burns et al., 2010).

Microbicide gels contain ART drugs such as tenofovir and are applied to the vagina or rectum prior to sexual intercourse (Abdool Karim et al., 2010). Before the tenofovir gel, six candidate gels were tested in trials but none were able to protect against HIV-1 acquisition (Abdool Karim et al., 2010). The tenofovir gel was able to prevent infection by up to 54% in women with high adherence, however, the women who adhered the most to gel application also had fewer coital acts (Abdool Karim et al., 2010). As the risk of HIV-1 infection increases with the number of coital acts (Hughes et al., 2012, Varghese et al., 2002) and the tenofovir gel requires adherence to work well, the gel may not be effective in reducing transmission in at-risk groups such as female sex workers.

Pre-exposure prophylaxis employs the use of ART such as Truvada in the prevention of HIV-1 before an exposure (Naswa and Marfatia, 2011); for example, for use by an HIV-negative individual participating in unprotected sexual intercourse with a person with an undisclosed HIV status. Post-exposure prophylaxis is taken after a suspected exposure (Moorhouse et al., 2015, Smith et al., 2005), such as a healthcare worker being exposed to the blood of an HIV-infected individual through a needlestick injury (Krakower et al., 2015). Although the use of pre- and post-exposure prophylaxis is highly effective in preventing HIV acquisition, there has been limited uptake of and benefit from this preventative method due to factors such as lack of
awareness and adherence, low perception of HIV acquisition risk and adverse side-effects (Cowan et al., 2016, Deutsch et al., 2015, Krakower et al., 2015).

Another HIV-1 prevention strategy that has shown moderate effectiveness, with up to 60% reduction in HIV-1 acquisition, is male circumcision (Bailey et al., 2007, Gray et al., 2007b, Auvert et al., 2005). Although the mechanism behind this is not fully understood, one explanation is that the foreskin is a conducive environment for HIV-1 replication as it harbours Langerhans cells and cluster of differentiation 4 (CD4)+ T cells that express C-C chemokine receptor type 5 (CCR5), a co-receptor used by HIV-1 to enter cells (Lemos et al., 2014). However, many men do not undergo the procedure because they do not believe it is effective in preventing HIV-1 (Wamai et al., 2011) and because of a lack of adequate healthcare services providing this option in some developing countries (Bulled and Green, 2016).

ART can be used as a preventative strategy as HIV-infected individuals on treatment are less likely to transmit when they have undetectable blood viral loads (Donnell et al., 2010). According to UNAIDS, 19.5 million out of the 36 million HIV-infected individuals worldwide were accessing ART in 2016 (UNAIDS, 2017). However, among the individuals on ART, a large proportion may not be adherent to the medication which usually diminishes the preventative effects (Donnell et al., 2010).

Due to the inability of these preventative measures to effectively control HIV-1 infections, there is still a need for an HIV-1 vaccine (Gray et al., 2016). Providing an HIV-1 vaccine to the population would be less expensive than maintaining ART because a vaccine is given once, with perhaps a few boosters, as compared to ART which is a life-long therapy (Harmon et al., 2016). Recent epidemiological models predict that an HIV-1 vaccine with 70% efficacy would reduce infections by as much as 44% within the first decade and 78% by 2070 (Harmon et al., 2016).

One of the leading research areas in HIV-1 vaccine design is the elicitation of broadly neutralising antibodies (bnAbs) by HIV-1 immunogens (Haynes and Mascola, 2017, Morris et al., 2017, Klaas et al., 2016, de Taeye et al., 2015, Sanders et al., 2015). BnAbs bind to diverse HIV-1 isolates and prevent entry into host cells and therefore are attractive in the development of a universal HIV-1 vaccine (Haynes and Burton, 2017, Burton et al., 2012).
1.3 HIV-1 diversity

Cohen et al., (2014) dates the transmission of HIV-1 from primates in Cameroon to the early 1900s, which led to the widespread dissemination of the virus, resulting in the AIDS pandemic (Cohen, 2014). Although there are currently 4 main groups of HIV-1: M, N, O and P, HIV-1 group M is the only group which has spread worldwide and is the leading cause of HIV-1 infections, while groups N, O and P remain in West Africa (Faria et al., 2014).

HIV-2 is another type of HIV which is thought to have originated from sooty mangabey monkeys in West Africa (McCutchan, 2006). It is less prevalent than HIV-1 and has predominantly remained in that region (McCutchan, 2006). HIV-2 is less transmissible than HIV-1 and progression to AIDS is much slower in HIV-2 infection (Nyamweya et al., 2013). HIV-1 and HIV-2 isolates differ at the nucleotide level by approximately 50% (Arien et al., 2007) but have similar gene arrangement, transmission and replication models as well as the eventual clinical outcome; AIDS (Nyamweya et al., 2013).

There is enormous HIV-1 diversity within group M itself (Fig 1.1), with at least 9 different “pure” subtypes (also known as clades). Subtype C is responsible for most HIV-1 infections and is the dominant subtype in sub-Saharan Africa and India (Hemelaar, 2012). There are numerous circulating and unique recombinant isolates (Hemelaar, 2012, Tongo et al., 2016). Circulating recombinant forms (CRFs) are HIV-1 isolates which contain sequences attributed to two different “pure” subtype lineages and are known to be circulating within human populations (McCutchan, 2006) . With the emergence of more sophisticated computational methods and more sequences available to analyse them, evidence has emerged that some “pure” subtypes and many presumed recombinants may have been incorrectly characterised (Tongo et al., 2016, Abecasis et al., 2007, Robertson et al., 2000). For example, one study suggests that the CRF02_AG recombinant is actually the parent of a recombinant subtype G rather than a CRF (Abecasis et al., 2007).
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Figure 1.1 Global distribution of HIV-1 group M. HIV-1 group M has disseminated around the world more than any other HIV-1 group. There are at least 9 “pure” subtypes and numerous recombinants of the virus. The largest percentage of infections is in sub-Saharan Africa and the largest diversity of HIV-1 isolates in one region is in West and Central Africa. Source: (Hemelaar, 2012).

Within HIV-1 Group M subtypes, the env gene has greater diversity than the rest of the HIV-1 genome (Lynch et al., 2009). The HIV-1 Envelope (Env) protein can differ up to 30% within a subtype and up to 36% between subtypes (Korber et al., 2001). HIV-1 diversity presents a challenge in vaccine development because an effective, global vaccine should protect against most, if not all, circulating variants (Burton et al., 2012). The Env glycoprotein is the sole target for bnAbs and therefore an important vaccine target (Wyatt et al., 1998). Understanding its diversity, structure and function is therefore essential for antibody-based immunogen design (Haynes and Burton, 2017, Wyatt and Sodroski, 1998, Freed and Martin, 1995).

1.4 HIV-1 Envelope structure and function

1.4.1 Role of HIV-1 Envelope in viral entry

The Env precursor, known as gp160, is made in the rough endoplasmic reticulum and eventually cleaved by furin in the Golgi apparatus, into the surface (gp120) and transmembrane (gp41) subunits forming the mature Env structure (Ward and Wilson, 2015). The mature HIV-1 Env exists as a trimer that is made up of 3 gp120 and 3 gp41 monomers (Fig 1.2a) (Freed...
and Martin, 1995). Env contains hypervariable loop regions resulting in increased HIV-1 Env diversity, as well as conserved regions (Didigu and Doms, 2012). Env is heavily glycosylated (Doores, 2015) with a high density of N-linked glycans (Zhu et al., 2000) and possibly O-linked glycans (Go et al., 2015); this glycosylation is found mainly in gp120 but also in gp41 (Doores, 2015, Freed and Martin, 1995).

Figure 1.2 Schematic of the HIV-1 Envelope and host cell entry. (a) HIV-1 Env is a trimer made up of gp120 and gp41 heterodimers (b) The initial contact of the HIV-1 trimer with the host cell is through the host cell CD4 receptor which binds to the viral CD4-binding site (c) Subsequently, the trimer binds to the co-receptor (CCR5 or CXCR4) and the fusion peptide enters the host membrane (d) The viral and host membranes fuse, leading to the formation of a six-helix bundle and the entry of the virus into the host cell. Adapted from: (Didigu and Doms, 2012).

The HIV-1 Env trimer initiates viral entry by binding to CD4 on the host cell through the CD4-binding site (CD4-bs) on Env (Fig. 1.2b), followed by the binding of the Env V3 loop-located co-receptor binding site to CCR5 or C-X-C chemokine receptor type 4 (CXCR4) on the host cell; a process inhibited by the anti-HIV drug maraviroc (Didigu and Doms, 2012) (Fig. 1.2c). While most chronic HIV-1 isolates can use one or both of the co-receptors to facilitate entry into cells (Vicenzi et al., 2013, Moore et al., 2004), transmitted/founder (T/F) viruses preferentially bind to CCR5 (Kariuki et al., 2017, Parrish et al., 2013). During co-receptor binding, the fusion peptide (FP) embeds itself into the membrane of the cell (Fig. 1.2c). The final step in viral entry is the formation of the six-helix bundle (6HB), which promotes fusion of the two membranes (Fig. 1.2d) (Gallo et al., 2003). The N- and C-terminal portions of gp41 – Heptad Repeat (HR) helices 1 and 2, respectively – coil together to form the stable 6HD structure (Gallo et al., 2003). Developing drugs which lock the trimer in the closed pre-fusion state (Fig. 1.2a) may be effective in inhibiting viral fusion and subsequent entry into the cell (Munro et al., 2014).
1.4.2 The different conformations of Env

Env changes conformation throughout the entry process (Munro et al., 2014). Initially, the Env trimer is in a closed, prefusion state (Bartesaghi et al., 2013) and upon CD4 and co-receptor binding, it changes to an open, bound state (Wang et al., 2016, Tran et al., 2012). Strikingly, free unliganded HIV-1 Env is constantly shifting between closed, partially open and open states (Fig 1.3) (Cai et al., 2017, Guttman et al., 2015, Munro et al., 2014).

Figure 1.3 Model of the dynamic conformations of unliganded Env. HIV-1 Env continually fluctuates between closed, partially open and open conformations. Epitopes of non- and poorly neutralising antibodies are often occluded in the closed conformation and become more exposed as the trimer structure opens. Adapted from: (Cai et al., 2017).

HIV-1 isolates are categorised into Tiers based on their antibody neutralisation resistance profiles (Seaman et al., 2010). As grouped by Seaman et al., Tier 1 viruses can be neutralised by most antibodies including narrowly neutralising antibodies. Tier 2 viruses are moderately neutralisation resistant and Tier 3 viruses are resistant to neutralisation by most antibodies (Seaman et al., 2010). The epitopes of common, poorly neutralising antibodies are most exposed in the open conformation (Fig 1.3), in essence, it seems that unliganded, neutralisation sensitive Tier 1 viruses tend to spend more time in the transient open conformation while neutralisation-resistant Tier 3 viruses have been found to favour the closed conformations with less fluctuations to an open state (Cai et al., 2017, Munro et al., 2014).

Researchers further explored this by using hydrogen/deuterium exchange coupled with mass spectrometry which is a sensitive technique used to monitor structural changes in Env upon ligand binding (Guttman et al., 2015). They confirmed that most poorly-neutralising antibodies access their epitope in the open, exposed trimer conformation and found that most potent bnAbs
can bind to both conformations (Guttman et al., 2015). The degree to which the Env conformation must change for an antibody to bind is usually correlated with the potency of that antibody (Haim et al., 2013). Both the Haim et al., and Guttman et al., studies concluded that the binding of poorly neutralising antibodies results in large, irreversible Env conformational changes in the open state whilst potent neutralising antibodies usually bind with smaller, localised conformational effects. BnAbs can therefore be used to lock the trimer in the closed conformation which is especially useful in X-ray crystallography of the closed trimer (Stewart-Jones et al., 2016, Julien et al., 2013a).

### 1.4.3 HIV-1 Env glycans

The HIV-1 Env trimer is a heavily glycosylated protein with up to 50% of its mass comprising of glycans (Doorens, 2015, Korber et al., 2001). Glycans are added to the protein at potential N-linked glycosylation sites (PNGs) which are characterised by an N-X-T/S motif; where X ≠ P (Marshall, 1972). There are approximately 25 (ranges between 18-33) PNGs on Env (Korber et al., 2001). Glycans serve various roles including protection from antibody neutralisation (Moore et al., 2012, Pantophlet and Burton, 2006, Wei et al., 2003) and correct folding of the Env protein which, in turn, affects Env antigenicity and immunogenicity (Li et al., 1993). Some studies have found that T/F isolates from subtypes A, C and D have fewer Env PNGs (Ping et al., 2013, Derdeyn et al., 2004) which helps the virus avoid glycan-specific mucosal immunity and improves the ability of the virus to attach to host cells (Moore et al., 2014). However, this phenotype seems to be subtype specific as it has not been observed in subtype B isolates (Liu et al., 2008, Frost et al., 2005a). Interestingly, glycans also serve a role in viral dissemination as dendritic cells attach to the gp120 glycans on virions through dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) lectins and transport the virions to the lymphoid tissues (Lama and Planelles, 2007).

HIV-1 Env glycan production (Fig 1.4) starts in the endoplasmic reticulum (Bonomelli et al., 2014). N-linked glycans are attached to the asparagine residue of the glycosylation motif through a N-glycosidic bond with the N-acetylglucosamine (GlcNAc) portion of the glycan (Corfield, 2017). This is followed by addition of other building blocks to produce a glycan precursor known as Glc$_3$Man$_9$GlcNAc$_2$ (Glc: glucose, Man: mannose) (Kornfeld and Kornfeld, 1985). This high mannose glycan (9 mannose sugars) is subsequently cleaved by other
endoplasmic reticulum enzymes known as α-glucosidase I and II, forming an intermediate which is monoglucosylated (Bonomelli et al., 2014).

α-glucosidase II further cleaves the intermediate into Man$_9$GlcNAc$_2$ - the high mannose glycan referred to as Man$_9$ (Fig 1.4), which is present on the mature Env trimer (Doores et al., 2010). Man$_9$ can be further trimmed in the endoplasmic reticulum or in the Golgi apparatus to Man$_{5-8}$GlcNAc$_2$ (Man$_{5-8}$) glycans (Fig 1.4) (Bonomelli et al., 2014). The types of glycans found at PNGs are dependent on multiple factors including the type of cell the virus was expressed in, post-translational processing in the ER and Golgi apparatus as well as the presence of nearby glycans (Hioe et al., 2014).

One unusual features of HIV-1 Env is the presence of mainly under-processed oligomannose-type glycans on gp120 which result from the numerous gp120 Env glycans restricting access of enzymes which further process the glycans in the endoplasmic reticulum (Behrens et al., 2016, Doores, 2015, Doores et al., 2010). The gp41 Env subunit consists mainly of highly processed, complex glycans as there is lower glycan density in this region so the enzymes can easily penetrate to process them further (Behrens et al., 2016, Pritchard et al., 2015).

Complex and hybrid glycans are formed from Man$_9$ in the Golgi apparatus by an enzyme known as N-acetylglucosaminytransferase I (GnT I) and other Golgi enzymes (Bonomelli et al., 2014, Freed and Martin, 1995). Cells which lack this enzyme such as human embryonic kidney (HEK) 293S cells (Chaudhary et al., 2012) are used widely in HIV-1 research to produce recombinant trimers enriched for high mannose glycans to control glycan heterogeneity which aids in the ease of protein crystallization (Sanders et al., 2015, Blattner et al., 2014, Julien et al., 2013a). (Fig 1.4). The reagents kifunensine and swainsonine inhibit the formation of both hybrid and complex glycans in the case of kifunensine and only complex glycans in the case of swainsonine (Hioe et al., 2014). This is achieved through the inhibition of mannosidase I in the endoplasmic reticulum or mannosidase II in Golgi apparatus, respectively (Hioe et al., 2014), resulting in predominantly high mannose glycans. These reagents can be used to manipulate the glycans that are created on Env to help elucidate glycan function (Bonomelli et al., 2014) and glycan dependency of antibodies (Doores and Burton, 2010).
Figure 1.4 Developmental pathway of HIV-1 Env N-linked glycans. Schematic of the production of HIV-1 Env glycans from the endoplasmic reticulum to the cis-, medial-, and trans-Golgi. The three types of glycans found on the HIV-1 Env are: high mannose (oligomannose), hybrid and complex glycans, with the latter two arising from the high mannose glycans. The high mannose glycans are produced in the endoplasmic reticulum and the Golgi apparatus, while the hybrid and complex glycans are solely produced in the Golgi apparatus. Reagents used to inhibit the formation of hybrid/complex glycans, kifunensine and swainsonine, are shown above the enzymes they inhibit. ER: endoplasmic reticulum Source: (Hioe et al., 2014)

Although glycans have a role in protecting the virus from antibody responses, and therefore have been called the “glycan shield” (Wei et al., 2003), some bnAbs have developed the ability to recognise certain oligomannose HIV-1 glycans on Env or use the glycans to facilitate recognition (Lee et al., 2015b, Falkowska et al., 2014, Huang et al., 2014, Scharf et al., 2014, Doores and Burton, 2010). Therefore, the HIV-1 glycan shield is dynamic and is continuously changing in response to neutralising antibody pressure (Wei et al., 2003).

1.5 HIV-1 Transmission

The most common route of transmission of HIV-1 is through sexual contact, with the virus entering the body through the genital or rectal mucosa (Hladik and Hope, 2009). The other common routes of transmission include direct blood contact through blood transfusions or use of HIV-1 contaminated needles (commonly during intravenous drug use) and mother-to-child transfer of the virus (Hladik and Hope, 2009).

HIV-1 sexual transmission is a very inefficient process, with some estimates placing the risk at 0.01% per coital act (Gray et al., 2001). Although Gray et al., did not find a significant difference in HIV-1 transmission between individuals who had a sexually transmitted infection
(STI) and those who did not, other studies have found that pre-existing STIs, such as herpes simplex virus II infection, increase the risk of HIV-1 acquisition (Freeman et al., 2006, Galvin and Cohen, 2004). This is most likely due to these infections increasing the proportion of HIV-1 target cells in the genital tract (Shaw and Hunter, 2012) and/or compromising the mucosal lining, thereby increasing the probability of viral entry (Sagar et al., 2004).

Although HIV-1 diversity in an infected individual is very high, transmission usually results in a single virion establishing the new infection in the recipient in approximately 75-90% of heterosexual cases (Abrahams et al., 2009, Haaland et al., 2009, Keele et al., 2008), 36-83% of men who have sex with men (Tully et al., 2016, Li et al., 2010), 80% of in utero mother to child transmissions, 56% mother to child transmissions during delivery (Russell et al., 2011) and 40-80% in intravenous drug users (Tully et al., 2016, Bar et al., 2010, Masharsky et al., 2010). This phenomenon is known as the transmission bottleneck (Kariuki et al., 2017, Joseph et al., 2015).

The bottleneck is influenced by the mucosal lining as damage to the genital lining as a result of STIs and ulceration is thought to facilitate the entry of multiple variants (Haaland et al., 2009). However, the fact that transmission through contaminated needles also results in a bottleneck effect implies that a physical barrier may not be the only factor facilitating this phenomenon (Kariuki et al., 2017). The transmitted variant is usually part of a minor population from the host viral community which suggests that transmission is not by chance but that there is a type of pressure selecting for a particular variant (Joseph et al., 2015), however the nature of this selective pressure is not yet fully understand (Kariuki et al., 2017).

1.5.1 Cell-free versus cell-to-cell transmission

The most studied transmission model of HIV-1 entry into cells is the cell-free route where free virions travel in the blood until they encounter a target cell to infect (Piguet and Sattentau, 2004). Cell-free HIV-1 virions predominantly infect CD4+ T cells but can also infect monocyte-derived dendritic cells (Dutartre et al., 2016) through the mechanism described in section 1.4.1. The risk of detection by the immune system when exposed in the blood is high for cell-free viruses (Piguet and Sattentau, 2004) so an alternative route of transmission could be beneficial to escape the immune response.
Cell-to-cell transmission is an alternate method of viral transmission where a virion passes directly from a host cell such as lymphocytes to a recipient cell such as another lymphocyte or macrophage (Malbec et al., 2013, Schiffner et al., 2013, Abela et al., 2012). The host and recipient cells form conjugates through the host intercellular adhesion molecule 1 (ICAM-1) binding to the recipient cell lymphocyte function-associated antigen-1 (LFA-1) (Schiffner et al., 2013) and the virion binding to the host cell CD4 (Malbec et al., 2013).

Studies have differing views on whether virus transmitted by cell-to-cell transmission is as susceptible to antibody neutralisation as cell-free virus. Studies have shown that cell-to-cell transmitted virions were more protected from neutralisation by some but not all neutralising antibodies, suggesting that neutralisation sensitive viruses perhaps could survive and replicate in the presence of antibody responses (Li et al., 2017, Abela et al., 2012). However, if that was the case, HIV-1 isolates which favour cell-to-cell transmission over cell-free transmission likely would have been selected for and dominate in the population.

Another study found that cell-to-cell transmitted viruses were susceptible to antibody neutralisation by some of the most broad and potent neutralising antibodies and therefore this method of transmission may not entirely escape the humoral response (Malbec et al., 2013). Cell-to-cell transmission-based antibody neutralisation assays are not as standardised as cell-free virus neutralisation assays (Montefiori, 2005) which could explain the different conclusions drawn by various studies. Reaching a consensus on which in vitro model corresponds more to cell-to-cell transmission in vivo would be important for a better understanding of this phenomenon and its role in immune escape.

1.6 Non-neutralising antibodies in HIV-1 infection

Antibodies have diverse functions apart from neutralisation which include, but are not limited to, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement activation (Forthal et al., 2013) (Fig 1.5).

The fragment crystallisable (Fc) domain of an antibody is responsible for these effector functions (Janeway Jr et al., 2001). Activation-induced cytidine deaminase (AID) drives class switching of the Fc domain leading to different antibody isotypes which results in different
immune responses (Janeway Jr et al., 2001). HIV-1-specific IgG3 responses are present weeks after infection, however they rapidly decline and a predominantly IgG1 response takes over in chronic infection (Forthal et al., 2013).

**Figure 1.5 Role of non-neutralising antibodies in the immune response against HIV-1 infection.** Non-neutralising antibodies have been implicated in antibody-dependent cellular cytotoxicity through the recruitment of natural killer (NK) cells, antibody-dependent cellular phagocytosis with the help of phagocytes, as well as complement activation. Non-neutralising antibodies also interact with mucins on the mucosal membranes and prevent virus from infecting cells. *Source: (Forthal et al., 2013).*

Individuals who are able to control their viremia have been found to have high levels of gp120-specific IgG1 and maintain their initial IgG3 response (Banerjee et al., 2010), while higher IgG4 production has been associated with progression to AIDS (Ljunggren et al., 1988). These data suggest that poorly functional non-neutralising antibodies may be associated with HIV-1 progression (Forthal et al., 2013). However, non-neutralising antibodies have also been shown to increase viral infectivity through complement-mediated antibody-dependent enhancement, which in one study resulted in over 350-fold enhancement of HIV-1 infection of SupT1/R5 T cells (Willey et al., 2011).

Most HIV-1 vaccine research focuses on neutralising antibodies because some studies have shown that non-neutralising antibodies do not protect nonhuman primate models against simian/human immunodeficiency virus (SHIV) infection (Santra et al., 2015, Walker and Burton, 2010, Barnett et al., 2008, Mascola et al., 1999) and because neutralising antibodies do not require additional immune cells to function as compared to non-neutralising antibodies (Forthal et al., 2013) – immune cells which may be more difficult to elicit or control through vaccination. However, in the Thai RV144 vaccine trial, non-neutralising IgG antibodies targeting the variable 1 and 2 (V1/V2) regions of the HIV-1 envelope correlated with protection.
from HIV-1 acquisition (Haynes et al., 2012). This has led to increased research on non-neutralising antibodies to better understand their role in HIV-1 prevention (Haynes et al., 2012).

1.7 Broadly neutralising antibodies against HIV-1

1.7.1 Immune response to HIV-1 natural infection – is there a role for antibodies?

B cell responses are initiated days after HIV-1 infection and can be detected in the form of antibody-antigen complexes (Tomaras et al., 2008). This is followed by the appearance of gp41-specific non-neutralising antibodies (Fig 1.6) (McMichael et al., 2010) and anti-gp120 antibodies targeting the V3 loop a few weeks later (Overbaugh and Morris, 2012). These responses, however, are unable to control viral replication (Tomaras et al., 2008). The emergence of autologous neutralising antibodies only occurs after approximately 3 months of infection, too late to control the initial viral infection (Fig 1.6) (Gray et al., 2011a, McMichael et al., 2010).

![Typical immune response to HIV](immunopaedia.org)

Figure 1.6 Immune response to HIV-1. (1) After transmission, the virus replicates rapidly followed by (2) dissemination from the site of infection. Viral replication is (3) controlled by cytotoxic CD8+ T cells which reduce the viral load to a set point. (4) Binding antibodies specific to gp41 slowly emerge during the CD8 T cell response and seroconversion occurs at approximately 3 months after infection. Neutralising antibodies only develop in chronic infection. Source: (Immunopaedia, 2016).

Autologous neutralising antibodies are antibodies that neutralise the host’s own virus (Gray et al., 2007a, Richman et al., 2003, Wei et al., 2003). These antibodies are detectable months after acute HIV-1 infection but are reduced considerably during the AIDS phase of the condition, most likely due to the compromised state of the immune system as a whole (Deeks et al., 2006).
In natural infection, studies have shown that autologous neutralising antibodies promote viral escape from neutralising responses (Bunik et al., 2008, Moore et al., 2008, Richman et al., 2003, Wei et al., 2003).

In the first year post-infection, autologous neutralising antibodies are frequently only able to bind to one or two variable regions on Env (Moore et al., 2009). The virus escapes autologous antibody neutralisation by several mechanisms including substitutions, insertions and deletions of single residues (Frost et al., 2005b) and through shifting glycans to block underlying epitopes (Moore et al., 2012). It is hypothesised that as escape mutants arise, autologous antibodies gain additional specificities towards new immunodominant epitopes and if these epitopes are within conserved regions, this may give rise to bnAbs (Moore et al., 2012, Moore et al., 2009). The specificities targeted by autologous neutralising antibodies include the V1/V2 regions across subtypes (Moore et al., 2009) and the C3 region and the α2-helix in subtype C infection (Gray et al., 2011b, Rong et al., 2009).

Although eliciting autologous neutralising antibodies is not the goal in vaccine development, an understanding of their structure, function and epitopes may be important in delineating how bnAbs develop because narrowly neutralising autologous antibodies are likely precursors to bnAbs (Doria-Rose et al., 2014, Liao et al., 2013).

1.7.2 Role of broadly neutralising antibodies in natural infection

BnAbs develop in chronic infection in approximately 25% of HIV-1-infected individuals (Gray et al., 2009b, Stamatatos et al., 2009, Binley et al., 2008), with approximately 1% developing highly potent cross-reactive response (Sather et al., 2012, Simek et al., 2009, Stamatatos et al., 2009), although the precise proportion depends upon the definition and cut-off used. One study has shown that up to 50% of HIV-1-infected individuals develop broadly neutralising responses (Hraber et al., 2014b). BnAbs typically only arise in chronic infection (Moore et al., 2015). Monoclonal antibodies (mAbs) have been isolated which neutralise more than 95% of HIV-1 isolates across subtypes (N6 and 10E8 mAbs) (Huang et al., 2016, Huang et al., 2012) and therefore elicitation of such antibodies with an HIV-1 immunogen may lead to effective protection from the virus.
There are opposing views on whether bnAbs are beneficial during natural infection in the control of the virus. Studies have shown that having broad anti-HIV-1 neutralising antibody responses is not associated with a better prognosis. In fact, neutralisation breadth at 3 years post-infection was found to be associated with lower CD4$^+$ T cell counts although this association is almost certainly indirect (Gray et al., 2011a, Euler et al., 2010, Piantadosi et al., 2009).

The study performed by Piantadosi et al., also revealed that the time to start of ART at a CD4$^+$ T cell count <200/µl and AIDS-related death were not influenced by the production of bnAbs in a cohort of women in Mombasa. Critically, this study found that the time to CD4$^+$ T cell count of <200 cells/µl was not associated with bnAb development (Piantadosi et al., 2009). Euler et al., presented similar results in their cohort as they showed that time to AIDS or death was not significantly different between individuals with strong, moderate or no bnAb responses 3 years post-seroconversion. This study found that CD4$^+$ T cell counts prior to and after infection were significantly lower in people with bnAbs (Euler et al., 2010). The explanation of the poor prognosis associated with the development of bnAbs in natural infection may be that the production of bnAbs is likely driven by a high antigen burden (high viral load) which stimulates B cells but subsequently leads to the reduction of CD4$^+$ T cells which leads to the development of AIDS in the absence of ART (Moore et al., 2015, Gray et al., 2011a, Doria-Rose et al., 2009, Sather et al., 2009).

Neutralising antibodies are unable to eliminate the virus in natural infection partly because the selective pressure exerted by these antibodies leads to neutralisation escape of the virus (Murphy et al., 2013). As soon as neutralising antibodies towards a viral variant develop, the virus mutates to evade the immune response (Liao et al., 2013, Moore et al., 2012, Richman et al., 2003, Wei et al., 2003). In most instances, the antibody pressure from bnAbs may be unable to efficiently select for less fit or less pathogenic isolates to delay disease progression (Bunnik et al., 2010b) leaving bnAbs unable to control viral replication in natural infection.

One way to determine the role of neutralising antibodies in HIV-1 infection is to deplete them from the host. B cell depletions have been performed in primate models and one case of B cell depletions has been reported in a human subject. Researchers used rhesus macaque models and depleted B cells using an anti-CD20 antibody prior to simian immunodeficiency virus (SIV) challenge (Gaufin et al., 2009a, Gaufin et al., 2009b, Miller et al., 2007, Schmitz et al., 2003).
Two of the studies mentioned showed that the presence of antibody pressure reduced viral load (Miller et al., 2007, Schmitz et al., 2003) and one study reported protection from disease progression by antibodies (Miller et al., 2007). Another study found that antibodies had limited to no influence on disease progression (Gaufin et al., 2009a). However, it is unclear how to interpret the lack of effect of the antibodies when comparing the B cell-depleted and control-depleted groups as neither of them made detectable responses to the challenge virus. Complicating the issue further, none of the studies were able to completely deplete B cells in the macaques, making interpretation more complex and the conclusions less clear.

A human B cell depletion study provides evidence that bnAbs may play a protective role in HIV-1 infection (Huang et al., 2010). Anti-CD20 was used to deplete B cells from a chronically HIV-1 infected individual which resulted in an increase in viremia and the emergence of an antibody neutralisation sensitive virus (Huang et al., 2010). These data suggest a role for antibody in controlling HIV-1 and therefore the field is still divided on the protective role of neutralising antibodies in natural infection.

1.7.3 Can individuals with low viral loads produce bnAbs?

Another key research question is whether people on ART and non-progressors – individuals who maintain low viral loads and high CD4+ T cell counts without ART – are able to produce bnAbs. In one study, only a small percentage of individuals on ART (1.3%) with low or undetectable viral loads produced bnAbs (Medina-Ramirez et al., 2011). In this small set of individuals who produced bnAbs, ART therapy likely improved functionality of the humoral immune response which increased the probability of production of neutralising responses (Medina-Ramirez et al., 2011) although the general trend is for antibody production to decrease after ART initiation as the viral load decreases (Morris et al., 1998).

Deeks and colleagues (2006) examined antibody responses against both autologous and heterologous viruses amongst 65 acute and chronically HIV-1-infected individuals. They found that in chronic infection, non-progressors possessed neutralising antibodies targeting contemporaneous autologous virus (Deeks et al., 2006). However, these antibody responses were detected at a very low level and therefore, it can be assumed they were not major contributors to HIV-1 control in those individuals. Studies have suggested that non-progressors
possess CD8+ T cells with superior functionality which suppress their viremia (Almeida et al., 2007, Deeks and Walker, 2007, Betts et al., 2006).

To better understand what determinants were involved in developing broad responses, Rusert and colleagues analysed over 4000 HIV-1-infected individuals. They concluded that there is an interplay between certain host and viral factors which are associated with the development of bnAbs (Rusert et al., 2016). They found that higher viral load, longer time of untreated HIV-1 infection, higher viral diversity and being of a certain race were associated with bnAb production (Rusert et al., 2016).

1.7.4 Can bnAbs protect from superinfection?

Since bnAbs do not necessarily have a protective role once HIV-1 infection is established, this raises the question of whether bnAbs can protect against HIV-1 acquisition. One method to determine whether bnAbs would protect individuals after vaccination is through the study of HIV-1 superinfection. Superinfection occurs when an HIV-1-infected individual gets re-infected with a different viral isolate (Redd et al., 2013).

Studies have found that individuals who had superinfecting viruses had lower anti-HIV-1 autologous and broad antibody responses as opposed to controls (Smith et al., 2006), with individuals with superinfections having higher IgA responses but lower IgG responses (Basu et al., 2012). Further evidence that neutralising antibodies aid in the prevention of superinfection is that most intra-subtype superinfections are observed in primary infection (Jost et al., 2002), presumably before a robust antibody response has formed. Superinfections also occur after treatment interruption (Altfeld et al., 2002) as the immune system becomes dysfunctional.

Some studies however, such as the one conducted by Blish and colleagues, show that the presence of neutralising antibodies does not prevent superinfection (Blish et al., 2008). In this study, the authors found that women who became superinfected possessed antibodies at the time of superinfection of similar breadth and potency to controls, however, when tested in vitro, the antibodies were able to neutralise the superinfecting virus, but with low ID50 values <100 (Blish et al., 2008). These data suggest that the antibody titres in superinfected individuals were not sufficient to suppress viral entry and a vaccine-induced antibody response would need to
be more robust (Blish et al., 2008). With the emergence of next generation sequencing (Chabria et al., 2014), superinfections which failed to establish in the host can, in principle, now be detected and therefore more knowledge on the titres required for antibodies to prevent superinfection can be investigated more robustly.

1.7.5 Can bnAbs protect from infection/challenge in a vaccine setting?

Non-human primate (NHP) models provide compelling data that bnAbs can prevent against HIV-1 infection (Overbaugh and Morris, 2012). BnAbs administered to NHPs have been shown to protect from SHIV challenge (Mascola, 2002). Early studies used 2F5 (MPER-specific), 2G12 (N332 glycan-dependent) mAbs and HIV immune globulin. Despite the two mAbs antibodies displaying moderate breadth and potency alone, the combination protected the NHPs from SHIV infection both vaginally (Mascola et al., 2000) and intravenously (Mascola et al., 1999).

The more recently isolated mAbs used alone such as PGT121 (Moldt et al., 2012) and PGT126 (Moldt et al., 2016) or in combination such as VRC01, VRC01-LS, 3BNC117, and 10-1074 (Gautam et al., 2016) have been found to prevent infection in NHP models. mAbs such as 3BNC117 (Scheid et al., 2016, Caskey et al., 2015), 10-1074 (Caskey et al., 2017), VRC01 (Bar et al., 2016) and N6 (Julg et al., 2017) have been shown to suppress viremia for a prolonged period of time in passive immunisation trials in HIV-1-infected individuals (Caskey et al., 2017, Bar et al., 2016, Scheid et al., 2016) and NHPs (Julg et al., 2017) but similar to previous studies (Mehandru et al., 2007, Trkola et al., 2005), viral escape mutants emerged during therapy.

Interestingly, Fc-mediated effector functions have been implicated in bnAb protection following passive immunisation. In vivo studies have found that FcγR-mediated effector functions are a substantial contributor to the effectiveness of some bnAbs and help in blocking viral entry and suppressing viremia (Bournazos et al., 2014). For example, Fc-mediated effector functions were important in achieving the full in vivo protective effect of mAb b12 (Hessell et al., 2009, Hessell et al., 2007).

HIV-1 viral escape from antibody pressure occurs early in acute infection and continues throughout infection with antibodies being major drivers of Env variation (Frost et al., 2005b).
Therefore, in principle, bnAbs can protect against HIV-1 infection but cannot protect once infection has already been established due to rapid and constant viral escape (Richman et al., 2003).

1.7.6 Broadly neutralising antibody epitopes as attractive vaccine targets

As of 2010 there has been a rapid increase in the number of isolated and characterised mAbs (Fig 1.7) due to the emergence of highly sophisticated isolation techniques including the use of recombinant gp120 and trimeric proteins to capture specific B cells (McCoy and Burton, 2017, van Gils et al., 2016, Mouquet, 2014, Burton et al., 2012, Wu et al., 2010). Identification and characterisation of bnAb epitopes has been performed by techniques such as alanine scan mutations (Jacob et al., 2015, Moore et al., 2011, Walker et al., 2011), HIV-1/HIV-2 chimeric viruses (Gray et al., 2009a, Binley et al., 2008) and co-crystallisation with HIV-1 proteins (Derking et al., 2015). Understanding the structure of bnAb epitopes and how these antibodies bind their targets may aid in the rational-based design of an effective, global HIV-1 vaccine (Derking et al., 2015).

1.7.6.1 V2/apex – the highly conformation-dependent epitope

The V2/apex is a quaternary, conformation-dependent bnAb epitope located on the V1/V2 loop and is usually stabilised by the glycan at position N160 (Fig 1.7) (Singh et al., 2011, Wu et al., 2011, Doores and Burton, 2010). The V1/V2 loop is a hypervariable region due to multiple polymorphisms, insertions and deletions, however, within this variable region lies a highly conserved surface which is targeted by bnAbs and makes up the V2/apex epitope (Moore et al., 2017).

The first identified bnAbs which target the V2/apex were PG9, which binds asymmetrically to the HIV-1 trimer at a 1:1 ratio (Julien et al., 2013b), and PG16 which binds to the target in a less conformation-dependent manner than PG9 but also at a 1:1 ratio (Pancera et al., 2013, McLellan et al., 2011). Separately, these two related antibodies neutralise 70-80% of HIV-1 isolates (Walker et al., 2009) but used together can achieve up to 90% breadth (McLellan et al., 2011). Currently, the only published bnAbs targeting this site that are not dependent on the presence of the N160 glycan and exhibit decreased potency if the glycan is present are some
of the members of the CAP256-VRC26.25 lineage (Doria-Rose et al., 2015). Eliciting such antibodies in a vaccine in conjunction with N160-dependent V2/apex antibodies would be advantageous in protecting from isolates with and without the glycan at this position. Another interesting characteristic of the CAP256 lineage is that these antibodies are able to develop breadth and potency very early in infection (Doria-Rose et al., 2014) which is rare for bnAbs. Understanding the pathway taken by such antibodies to achieve breadth may provide insight into designing an immunogen which can elicit bnAbs soon after vaccination.

In southern African individuals who do produce bnAbs, 1/3 of broadly neutralising sera target the V2/apex (Moore et al., 2017). Therefore, with the right stimulation, B cells may be able to produce these bnAbs at a high frequency.

![Figure 1.7 bnAb targets on HIV-1 Env](image)

**Figure 1.7 bnAb targets on HIV-1 Env.** The HIV-1 Env timer glycoprotein contains six identified bnAb epitopes. The V2/apex bound by antibodies such as PG9 and PG16, the V3/glycans bound by antibodies such as 10-1074 and PGT121, the CD4-binding site bound by antibodies such as VRC01 and b12, the membrane proximal external region bound by antibodies such as 10E8 and 4E10, the gp120-gp41 interface bound by antibodies such as PGT151 and 8ANC195 and the newly-identified fusion peptide (not depicted) bound by antibodies such as ACS202 and VRC34. Adapted from: (Mouquet, 2014).

1.7.6.2 V3/glycans – “supersite of vulnerability”

As mentioned, the glycans on Env can be recognised by bnAbs (Ward and Wilson, 2017) despite their role in shielding the virus from immune responses (Wei et al., 2003). The V3 loop
is a target of narrowly neutralising antibodies as evidenced by viruses with exposed V3 loops being exceptionally sensitive to antibody neutralisation, such as HIV-1 isolate SF162 (McCaffrey et al., 2004, Gorny et al., 2002). However, some of the most potent bnAbs isolated to date bind to the glycans at the base of the V3 loop, which form the “high mannose patch”, centred around the glycans at positions N301 and N332 (Fig 1.7) (Sok et al., 2014, Mouquet et al., 2012, Walker et al., 2011, Sanders et al., 2002). This site has been referred to as a “supersite of vulnerability” (Kong et al., 2013) due to the numerous bnAbs that have been isolated that target this site.

A crucial protein motif required for neutralisation by most N332-dependent V3/glycan-specific bnAbs is the $^{324}$GDIR$^{327}$ motif (Sok et al., 2016, Garces et al., 2015, Garces et al., 2014). N332-dependent antibodies including PGT126, 128, 130 and 131 also bind to the glycan at position N301 (Walker et al., 2011) which is adjacent to the N332 glycan at the base of the V3 loop (Garces et al., 2014). Intriguingly, V3/glycan-specific antibodies have been shown to have “promiscuous” binding as in the absence of a specific target glycan, some of these antibodies can bind to a substitute glycan. For example, in the absence of the glycan at N332, PGT128 can bind to the glycan at position N295 (Sok et al., 2014). This promiscuous binding may need to be considered when designing an immunogen based on the V3/glycans as multiple glycans, including ones in the V1/V2 loop, may need to be incorporated into an immunogen with the relevant glycans held in the correct orientation and position by scaffolding (Sok et al., 2014).

Antibodies towards V3/glycan epitope have demonstrated remarkable efficacy in NHP and human trials. 10-1074 transiently controlled viremia in HIV-1 infected individuals who were not on ART (Caskey et al., 2017) and PGT121 protected NHPs from viral acquisition at high doses of SHIV (Moldt et al., 2012).

1.7.6.3 CD4-binding site – the “common” bnAb target

The CD4-bs (Fig 1.7) is an attractive vaccine target because it is essential in viral fusion and entry into the host cell (Didigu and Doms, 2012) and therefore is presumably heavily constrained by function. Up to 15% of bnAbs that develop during natural infection target the CD4-bs (Walker et al., 2010). The fact that antibodies targeting the CD4-bs are so commonly elicited suggests that making these antibodies may be relatively easy upon vaccination (Walker
et al., 2010). One of the most potent and well-characterised antibodies that targets this site is VRC01 (Wu et al., 2010). This antibody binds to the CD4-bs partially by CD4 receptor mimicry (Zhou et al., 2010); however, like other bnAbs, it requires extensive somatic hypermutation which may make it difficult to elicit in a vaccine (Wu et al., 2015). Features such as mutations at key residues including those at positions 279, 363, 368 and 371 enable viruses to escape neutralisation by VRC01-like antibodies (Huang et al., 2016, Li et al., 2011). The most broadly neutralising antibody which targets the CD4-bs that has been identified to date is N6, which binds using a unique mode of recognition and overcomes the barriers which cause VRC01-resistance (Huang et al., 2016). Although N6 did not display autoreactivity, it also had undergone extensive somatic hypermutation (Huang et al., 2016). Again, this may limit ease of eliciting similar antibodies in a vaccine setting.

1.7.6.4 Membrane proximal external region – the linear epitope

The membrane proximal external region (MPER) is a bnAb epitope located solely in gp41 (Fig 1.7) (Zwick, 2005). It is an attractive vaccine target for several reasons including the fact that it is a conserved, linear epitope (Zwick, 2005). MPER plays a crucial role in fusion and entry of the virus into the host cell (Zwick, 2005). For this reason, targeting the MPER may prevent viral infection and its variability is heavily constrained by function (Montero et al., 2008). Therefore, it seems likely that the MPER would be an easier model to work with in immunogen design. The first-generation MPER-specific mAbs, 2F5 and 4E10, have moderate breadth and/or potency (Morris et al., 2011, Nelson et al., 2007, Stiegler et al., 2001, Purtscher et al., 1994) but the isolation of 10E8 mAb revealed that antibodies with greater than 90% breadth do target this epitope (Huang et al., 2012).

Access to MPER is restricted in the Env closed conformation (Chakrabarti et al., 2011, Dimitrov et al., 2007) and therefore immunisation with closed recombinant Env proteins may not efficiently elicit MPER antibodies. One strategy to improve anti-MPER antibody elicitation may be through de-glycosylation of Env, which has been shown to increase binding of unmutated ancestor antibodies from 2F5 and 4E10 (Ma et al., 2011) or by immunogen modelling of MPER scaffolds (Zhou et al., 2014).
1.7.6.5 gp120-gp41 interface – the inter-protomer epitope

The use of recombinant HIV-1 proteins to capture specific B cells by flow cytometry in selected donors facilitated the discovery of the gp120-gp41 interface epitope, another glycan dependent epitope which spans both gp120 and gp41 subunits (Fig 1.7) (Falkowska et al., 2014, Huang et al., 2014, Scharf et al., 2014). This site may be difficult to develop into an immunogen as the antibodies which have been isolated towards these sites all target distinct glycans. 8ANC195 is dependent on glycans at positions N234 and N276 (Scharf et al., 2015, Scharf et al., 2014) while glycans at N611 and N637 are required for PGT151 binding (Falkowska et al., 2014). 35O22 targets N88 (Huang et al., 2014) and CAP248-2B targets a distinct but overlapping epitope (Wibmer et al., 2017). However, all glycans targeted by these antibodies lie at the interface of the gp120-gp41 subunits and this is the underlying factor which groups them together (Mouquet, 2014). Strikingly, mutations that mediate viral escape from the CAP248-2B mAb led to increased MPER exposure of HIV-1 isolates (Wibmer et al., 2017). Exploiting the exposure of the MPER epitope by certain mutations could potentially be useful in the development of a vaccine to elicit anti-MPER antibodies.

1.7.6.6 Fusion peptide – the novel epitope

Until 2016, MPER was considered the only linear bnAb target in gp41 but now the FP has been implicated in bnAb recognition and neutralisation (Kong et al., 2016, van Gils et al., 2016). The FP is a glycine-rich, hydrophobic region at the Env N-terminus which is 15-20 amino acids long (Chan and Kim, 1998). Its conserved role in viral fusion and entry makes it an attractive vaccine target (Didigu and Doms, 2012).

Although the exposure of the FP in the closed spike is not well characterised, two FP-specific bnAbs have been isolated to date which bind to the closed trimer spike, ACS202 (van Gils et al., 2016) and VRC34 (Kong et al., 2016). Env recognition by both bnAbs is dependent upon the amino acids forming the FP and upon the glycan at position N88. Some antibodies targeting the gp120-gp41 interface also depend upon the glycan at N88 (Huang et al., 2014), however they do not require FP residues for neutralisation. More information on the FP epitope such as the frequency of bnAbs targeting this site and their characteristics are needed to determine whether an immunogen based on this peptide would elicit desirable responses.
1.7.7 Characteristics of broadly neutralising antibodies

BnAbs have several unusual characteristics that raise the question of whether they can be elicited easily and quickly after vaccination. One of these characteristics is extensive somatic hypermutation of up to 30% in the complementarity-determining regions and frame-work variable regions, which promotes specificity to their targets (Borrow and Moody, 2017, Georgiev et al., 2014, Klein et al., 2013). Somatic hypermutation is part of the affinity maturation process which selects for antibodies with high binding affinity (Allen et al., 2007). The fact that this process is so extensive in bnAbs may explain why they often take years to develop (Sok et al., 2017). For example, VRC01 is mutated about 30% from its inferred germline sequence and the lineage took approximately 15 years to gain breadth (Wu et al., 2015). Even bnAbs targeting the V2/apex that generally have lower levels of somatic hypermutation are 14-19% mutated (e.g. PG9 and PGT-145) (Sok et al., 2013).

A second characteristic of some bnAbs which is believed to limit the ability of individuals to produce bnAbs is autoreactivity (Borrow and Moody, 2017, Moore et al., 2017, Liu et al., 2015, Verkoczy and Diaz, 2014). Anti-MPER antibodies were the first antibodies to be identified as auto-reactive when 4E10 and 2F5 were found to bind to cardiolipin (Haynes et al., 2005). The authors hypothesised that HIV-1 may display epitopes such as MPER which mimic autoantibody epitopes to escape neutralisation because auto-reactive antibodies are often deleted from the B cell repertoire (Haynes et al., 2005).

Some V3/glycan-specific bnAbs including PGT125 and PGT128 are polyreactive, meaning that they can bind to various self-proteins with low affinity (Liu et al., 2015). Chuang et al., presented data suggesting that one way to prevent polyreactivity is through the addition of glycans on Env at positions proximal to sites that mimic self-epitopes (Chuang et al., 2015). Immunogens designed with these protective glycans may elicit bnAbs which are less likely to be self-reactive.

Despite antibodies such as 2F5, 4E10 and 3BNC117 being reported to be auto- and poly-reactive (Liu et al., 2015, Mascola and Haynes, 2013, Haynes et al., 2005), these antibodies have been used in clinical trials with no immediate adverse effects (Scheid et al., 2016, Mehandru et al., 2007, Trkola et al., 2005, Stiegler and Katinger, 2003). Therefore, self-reactivity seems unlikely to be a problem in terms of vaccine safety.
BnAbs are also reported to have unusually long complementarity-determining regions in the heavy chain 3 (CDRH3s) (Borrow and Moody, 2017). Long CDRH3s are common in bnAbs, especially those which target glycosylated regions as these recognition loops aid in entry of the antibody paratope through the dense glycan mesh to the residues below (Moore et al., 2017). V2/apex-specific antibodies possess long, anionic CDRH3 regions and this is due to aspartate and glutamate residues as well as tyrosine sulphation (Moore et al., 2017).

It was thought that possessing long CDRH3s was a rare phenomenon and therefore may be hard to reproduce in a vaccine-setting, however, studies suggest that although long loops are naturally found in low frequency, they are a normal feature of the antibody repertoire and should not be seen as a restriction to an antibody-based vaccine (Yu and Guan, 2014). Additionally, research has shown that long CDRH3s are commonly found in chronic infection in a variety of other viral infections (Breden et al., 2011). This study suggests that long CDRH3s may just be a consequence of chronic viral infection and that other processes are likely contributing to shaping the antibody repertoire during viral persistence and escape which leads to development of bnAbs (Breden et al., 2011).

Importantly, a recent study has revealed that V2/apex-specific, moderately broad antibodies which contain recognition loops of an average antibody length can develop (Cale et al., 2017). This type of antibody may be a more attainable vaccine response for the general population as they may develop relatively easily (Cale et al., 2017, Jacob et al., 2015).

Potent bnAbs which lack some of these unusual features have been identified. BnAbs such as those in the PGT121 lineage (Sok et al., 2013) and mAb BF520.1 (Simonich et al., 2016) developed breadth with relatively modest somatic hypermutation. Interestingly, BF520.1 was isolated from an infant at ~1 year of age, a much shorter time than usual for the development of bnAbs (Simonich et al., 2016). BF520.1 and the PGT121 lineage target the V3/glycans (Simonich et al., 2016) which suggests that antibodies with low somatic hypermutation can be elicited towards glycosylated epitopes.

Georgiev and colleagues (2014) found that versions of VRC01 and 10E8 with limited somatic hypermutation in their frame-work variable regions still possessed breadth and potency. These data reveal that only limited mutations may be needed to achieve breadth. A more detailed
understanding of this phenomenon may aid in design of an immunogen capable of eliciting bnAbs in a shorter period time than in natural infection (Georgiev et al., 2014).

1.7.8 HIV-1 antibody evasion strategies

HIV-1 has evolved multiple mechanisms to evade the immune response including the shifting of glycans (Wei et al., 2003), sequence polymorphism (Mascola and Montefiori, 2010) and conformational masking (Kwong et al., 2002). Viruses evade the immune response through the addition of Env glycans to form a “glycan shield” (Moore et al., 2012, Pantophlet and Burton, 2006, Wei et al., 2003, Wyatt et al., 1998). The removal of key glycans such as the glycan at position N301 has been shown to expose both underlying and distal epitopes such as the CD4-bs (Binley et al., 2010, Li et al., 2008, McCaffrey et al., 2004, Koch et al., 2003) highlighting the importance of glycans in protection from antibody neutralisation. Knowledge that viruses possess potential “holes” in the glycan shield which lead to neutralisation sensitivity has shed further light into the role of glycans in the evasion of antibody responses (McCoy et al., 2016).

The ability of the virus to mutate its Env sequence and remain functional is another important antibody evasion strategy (Mascola and Montefiori, 2010, Wei et al., 2003). A third mechanism used by HIV-1 to evade neutralisation is conformational masking where highly conserved bnAb targets such as the CD4-bs and MPER are hidden from antibody neutralisation in the pre-fusion Env conformation (Peachman et al., 2010, Kwong et al., 2002). Conformational masking is most common in highly neutralisation resistant viruses which preferentially remain in a relatively closed conformation shielding commonly-elicited neutralising antibody epitopes and infrequently fluctuating to an open conformation (Cai et al., 2017, Munro et al., 2014).

Most Env proteins on the surface of the virus are non-functional and do not aid in fusion and entry into the cells (Moore et al., 2006, Poignard et al., 2003). Some Env trimers are non-functional gp120/gp41 monomers or gp41 stumps lacking gp120 (Moore et al., 2006). This also serves as a viral escape mechanism from antibody-mediated neutralisation as antibodies bind to these non-functional spikes and stumps but cannot neutralise the virus through them (Moore et al., 2006, Poignard et al., 2003).
Longer V1/V2 and V4 lengths have been associated with HIV-1 resistance to antibody neutralisation (Rademeyer et al., 2016, van Gils et al., 2011, Bunnik et al., 2010a). Recently transmitted viruses gain neutralisation resistance by the development of longer V1 loops which contain more glycans (Bunnik et al., 2010a) and longer V1/V2 loops have been found to protect the CD4-bs from neutralisation (van Gils et al., 2011). There is also a relationship between V1/V2 net charge and neutralisation resistance, with lower net charge being associated with neutralisation resistance (Hraber et al., 2014a).

It has been found that certain bnAb classes demonstrate subtype-bias. For example, CRF01_AE viruses are resistant to a large proportion of antibodies targeting the V3/glycans (Walker et al., 2011). Subtype A and C viruses have been shown to be more sensitive than subtype B viruses to V2/apex-specific antibodies such as CAP256 (Moore et al., 2011).

1.8 Rational-design of an HIV-1 vaccine: is it really feasible?

Most HIV-1 vaccine studies focus on the rational-design method, which is the development of an immunogen based on a prediction of the type of antibodies that it will elicit (Burton, 2010, Ofek et al., 2010). However, previously-identified vaccines, such as the yellow fever vaccine, which is extremely successful and protects for 35 years to life (WHO, 2013), were identified using the empirical method, which relies on the isolation and attenuation of a pathogen, followed by injection into the recipient (Rueckert and Guzman, 2012).

In whole killed virus vaccines, the virus is grown in culture and chemically deactivated (Chiodi and Weiss, 2014). In this way, the structure of the virus is preserved to elicit a robust immune response upon immunisation (Chiodi and Weiss, 2014). Vaccines such as the Salk polio vaccine work on this principle (Chiodi and Weiss, 2014). A Phase I human clinical trial in HIV-1-infected individuals has shown that this type of immunogen may be safe and can enhance pre-existing anti-HIV-1 immune responses (Choi et al., 2016), however safety concerns have resulted in most research steering away from this method for a preventative vaccine. HIV-1 vaccine research now primarily focuses on delivering HIV-1 proteins instead of the whole virus (Rueckert and Guzman, 2012).
Recombinant gp120 vaccines have failed to elicit robust antibody responses (Esparza, 2013). The RV144 trial has been the only trial to show modest efficacy (31.2%) and was made up of a canarypox prime with a recombinant gp120 boost (Buchbinder et al., 2008). 31.2% efficacy is too low to license this vaccine and therefore more immunogens, building up on this moderate success, must be designed to produce sufficient vaccine efficacy (Esparza, 2013). One of the focuses of HIV-1 immunogen research has been the use of the HIV-1 trimer as an immunogen instead of the gp120 monomer alone (Sanders and Moore, 2017).

### 1.8.1 Env trimers as immunogens

One of the principles behind using a closed HIV-1 Env trimer as an immunogen is that the glycosylated HIV-1 trimer in its closed conformation displays all the known bnAb epitopes except the MPER whilst shielding multiple epitopes targeted by narrowly neutralising antibodies (Guttmann et al., 2015, Sattentau et al., 1995, Sattentau and Moore, 1995) and therefore using it as immunogen could bias the immune response towards bnAbs (Sanders and Moore, 2017). However, one of the challenges of HIV-1 trimer immunogen design is that the transmembrane domain of Env is highly hydrophobic (Freed and Martin, 1995) which decreases solubility (Sanders and Moore, 2017). To overcome this, researchers have modified the Env in several ways, with varying success (Sanders and Moore, 2017).

The most widely used method of modifying the trimer is through the generation of recombinant Env trimers known as SOSIP.664 trimers (Fig 1.8). The “SOS” refers to the addition of a disulphide bond between gp120 and gp41 to further stabilise these subunits (Sanders et al., 2013). The “IP” refers to an isoleucine to proline residue change at position 559 that strengthens intra-gp41 bonds (Sanders et al., 2013). SOSIP trimers contain gp120 and only the ectodomain of gp41 to eliminate hydrophobicity caused by the MPER and the transmembrane domain (Sanders et al., 2013), making them soluble trimers. SOSIP trimers also include the addition of a RRRRRR (R6) motif in place of the usual furin REKR cleavage motif to optimise the cleavage process (Fig 1.8) (Sanders et al., 2013).
Figure 1.8 Schematic of the original fully glycosylated BG505 SOSIP.664 gp140 trimer design and subsequent modified BG505 SOSIP trimers. The original BG505 SOSIP.664 gp140 trimer contains a single disulphide bond between the gp120 C5 and gp41 regions, an I559P mutation, a modified R6 furin cleavage site and is truncated before the transmembrane domain at position 664. The SOSIP.v4 trimer contains 4 additional stabilising mutations in the C1, V3 and HR1 regions. The DS-SOSIP contains an additional disulphide bond between gp120 C2 and C4 regions. The SOSIP.664 E49C-L555C trimer contains an additional disulphide bond between gp120 C1 and gp41 HR1 regions. The SC trimer is similar to the native flexibly linked trimer (Sharma et al., 2015). It contains an additional flexible GGGGS (G,S) linker between gp120 and gp41 to eliminate the need for furin-dependent cleavage.

The first virus to be successfully engineered into a SOSIP trimer was subtype A T/F virus BG505 (Fig 1.8) (Wu et al., 2006). BG505 SOSIP trimers are highly stable, soluble, fully cleaved and predominantly in a closed conformation (Sanders et al., 2013). The trimer can be bound by all bnAbs it has been tested with which neutralise the corresponding pseudovirus including conformation-dependent bnAbs such as V2/apex-specific (Julien et al., 2013b, Sanders et al., 2013) and gp120-gp41 interface antibodies (Scharf et al., 2015, Falkowska et al., 2014, Huang et al., 2014). This reveals that the trimer is a good mimic of the BG505 virus and has optimal antigenicity (Sanders et al., 2013).

The BG505 SOSIP trimer seemed to be the perfect immunogen to elicit bnAbs and it is still the gold-standard in terms of SOSIP trimers to date (Julien et al., 2015). The BG505 trimer elicited potent, autologous Tier 2 responses in immunogenicity studies using macaques, guinea pigs and rabbits but failed to produce broad responses (Pauthner et al., 2017, Cheng et al., 2015, Sanders et al., 2015). This was despite further modifications to increase trimer stability through
further mutations (Fig 1.8) (Chuang et al., 2017), reduction of non-neutralising epitope exposure (de Taeye et al., 2015) as well as trimer delivery through different methods such as nanoparticle presentation (Sliepen et al., 2015) and liposome display (Pauthner et al., 2017). The autologous antibodies elicited by the BG505 trimer were mainly directed at epitopes located under certain “glycan holes” on the trimer (McCoy et al., 2016).

Recently, the BG505 SOSIP trimer has been used in a cow immunisation study, where the trimer induced broad and potent responses in all four cows, with one cow developing bnAbs capable of neutralising 96% of isolates in a virus panel at 381 days post-immunisation (Sok et al., 2017). However, bovine antibody repertoires frequently contain long CDRH3s (Sok et al., 2017) and therefore eliciting bnAbs may be substantially easier in these animals as opposed to humans.

SOSIP trimers of other subtypes have been engineered including subtype B (Stewart-Jones et al., 2016, Verkerke et al., 2016, Pugach et al., 2015), subtype G (Stewart-Jones et al., 2016) and the most dominant subtype C (Julien et al., 2015) as well as other subtype A SOSIP trimers (Verkerke et al., 2016). However, similarly to BG505, the trimers tested for immunogenicity have failed to induce broad responses (de Taeye et al., 2015, Sanders et al., 2015).

SOSIP trimers do not contain the MPER epitope because the sequence does not include residues after position 664 to increase the solubility of the trimer (Sanders et al., 2013). This comes with the cost of not being able to elicit MPER antibodies or study the position of MPER in structures derived from SOSIP trimers. Notably, one of the most broadly and potently neutralising bnAbs, 10E8, binds to this epitope (Huang et al., 2012). The additional stabilising mutations and the exclusion of the MPER, transmembrane and cytoplasmic domains may alter the structure of these trimers which may limit their reliability in Env structural studies.

To overcome many of these limitations, researchers developed a method to obtain membrane-extracted Env trimers lacking only the cytoplasmic tail (EnvΔCT) (Blattner et al., 2014). These Env trimers do not have stabilisation mutations added (Blattner et al., 2014). One example is the Env trimer based on the subtype B virus, JR-FL, whose crystal structure has been solved in complex with 10E8, providing further insight into the binding of this bnAb to its epitope (Lee et al., 2016). These trimers may, therefore be more biologically related to the HIV-1 Env, although the exclusion of the cytoplasmic tail could modify the structure (Blattner et al., 2014).
Immunisation of rabbits with JR-FL EnvΔCT did not produce bnAb responses (Crooks et al., 2015), indicating that these improvements were not sufficient to generate broad responses.

Only one published recombinant Env trimer, the PVO.4 SOSIP trimer, has been engineered based on a neutralisation-resistant virus and there is no published data on its immunogenicity (Verkerke et al., 2016). However, Townsley et al., used a rabbit model to test a poxvirus prime-gp120 boost immunisation strategy which used the PVO.4 gp120. Cross-clade neutralising antibodies were elicited although their response was modestly potent and broad (Townsley et al., 2016). Although not Tier 3, Klasse and colleagues (2016) immunised rabbits with subtype A, B and C SOSIP trimers, either in combination or sequentially, but in all circumstances the trimers produced cross-neutralising antibodies inconsistently and with very little breadth (Klasse et al., 2016). These studies leave open the possibility that the use of one or a combination of Tier 3 neutralisation resistant Env trimers could lead to a vaccine which elicits potent and highly cross-reactive antibodies.

1.8.2 Epitope scaffolds as immunogens

Recombinant Env trimers pose the problem of displaying both non-neutralising and broadly neutralising epitopes. For example, the V3 loop of BG505 SOSIP.664 trimer is exposed more than that of the native virus (Ringe et al., 2017, de Taeye et al., 2015). One method to overcome this is the epitope-focused rational design of HIV-1 protein scaffolds (Morris et al., 2017). HIV-1 protein or epitope scaffolds consist of residues forming bnAb epitopes which are grafted onto heterologous protein structures based on computational design (Zhou et al., 2014, Ofek et al., 2010). A recent proof of principle study in respiratory syncytial virus showed that computationally designed protein scaffold immunogen based on a neutralisation epitope on this virus induced potent neutralising antibody responses in macaques (Correia et al., 2014) and therefore this method could possibly work in the context of HIV-1.

In early epitope-focused immunogen design, researchers designed scaffold-based immunogens based on partial MPER epitopes because the linear nature of the epitope made it easier to design as a scaffold (Ofek et al., 2010). The b12 epitope in the CD4 binding loop and the outer domain was also designed as a scaffold immunogen (Azoitei et al., 2011). A more recent study by Zhou et al., created epitope scaffolds which included the V1/V2 loop (based on the PG9 epitope),
the V3/glycan supersite (based on the PGT128 epitope) and the MPER (based on the 10E8 epitope) (Zhou et al., 2014), however, no in vivo validation was conducted and therefore no progress has been made with these particular immunogens (Morris et al., 2017).

Morris et al., constructed scaffolds containing the V3/glycan supersite and the MPER and tested their immunogenicity in mouse models using a variety of delivery systems (Morris et al., 2017). MPER scaffolds activated B cells and elicited an antibody lineage with long CDHR3s; a characteristic associated with bnAbs (Morris et al., 2017). Interestingly, a scaffolded gp140.681 trimer, which contained most of the MPER, elicited antibody responses targeting the trimer apex but the parent gp140.664 trimer could not elicit such responses which suggests scaffolding may result in a superior immunogen (Morris et al., 2017). These data suggest that using a combination of the principles of recombinant trimers and scaffolds may result in the design of a stabilised immunogen capable of eliciting broad antibody responses upon immunisation (Morris et al., 2017). Scaffolds and trimers may also be used at different stages of an HIV-1 immunisation regime to promote the elicitation of bnAbs (Morris et al., 2017) because the correct combination may drive the needed antibody evolution (Doria-Rose et al., 2014, Liao et al., 2013) towards neutralisation breadth.

### 1.9 Study Objectives

As studies have shown that HIV-1 has progressively become more resistant to neutralising responses over the HIV-1 pandemic (Bouvin-Pley et al., 2014, Bouvin-Pley et al., 2013, Bunnik et al., 2010a) an understanding of these highly neutralisation resistant viruses may be needed to develop a global HIV-1 vaccine capable of protecting against most, if not all, HIV-1 strains.

There were three main objectives of this thesis. The first objective was to identify and study the neutralisation characteristics of the Tier 3, CRF02_AG virus, 253-11 (Kulkarni et al., 2009). The second aim of this thesis was to delineate the mechanism of neutralisation resistance of 253-11 and better understand the structural implications of its neutralisation resistance phenotype using X-ray crystallography. The last aim was to better understand commonly-elicited antibodies to HIV-1 that might resemble those elicited by a vaccine and explore their
interplay with viral escape. This was achieved by first determining the contribution of antibodies targeting the V3/glycans to breadth and potency in a cohort of HIV-1-infected individuals and secondly, determining the effects on neutralisation resistance of the removal of the conserved V3/glycan at position N301.

**Objective 1: Identifying the neutralisation resistance characteristics of virus 253-11**

253-11 is a Tier 3, CRF02_AG virus which is poorly recognised by subtype-matched (Jacob et al., 2012) and subtype C sera (Jacob et al., 2015). This objective aimed to determine the mechanism of the resistance of this virus through investigating the presence of bnAb epitopes in the sequence, unusual PNGs and certain Env polymorphisms. Mutational analysis was also conducted on 253-11 and a subtype-matched, neutralisation sensitive virus, 928-28, to test key hypotheses to explain the resistance phenotype of 253-11. The effects of kinetic MPER mutations, L669S and Y681H, on 253-11 and 928-28 were also explored.

**Objective 2: Understanding the structural basis behind the resistance phenotype of virus 253-11**

To gain insight into the structure of the 253-11 Env trimer, a 253-11 SOSIP.664 trimer was engineered and used to determine the structural and antigenic properties of the Env trimer. The 253-11 SOSIP trimer was co-crystallised with the antigen-binding fragment (Fab) of the V3/glycan-specific antibody, 10-1074. The structure of 253-11 was compared with other solved trimer structures to delineate characteristics of the 253-11 structure that were unusual compared to other trimer structures, particularly the compactness of the closed Env trimer.

**Objective 3: Understanding the contribution of the V3/glycans to the breadth and potency of HIV-1 neutralising antibody responses and their potential interplay with antibody escape**

There is evidence that antibodies with moderately high neutralisation breadth are frequently attainable as compared to highly potent and broad antibodies which are found in only a small subset of HIV-1 infected individuals (Hraber et al., 2014b). These moderately neutralising antibodies may be a more realistic target for a vaccine. Therefore, this objective first examined whether the V3/glycans were preferentially targeted by moderately broadly neutralising sera.
from HIV-1 infected individuals. As the V3/glycans were found to be associated with breadth and potency, the second part of this objective investigated how the V3/glycans contribute to shielding the virus from antibody responses while also being bnAb targets themselves.
Chapter 2: Deciphering the Molecular Basis of Unusually High Neutralisation Resistance for Tier 3 Virus, 253-11

Summary

Understanding the mechanisms used by HIV-1 to evade antibody neutralisation may contribute to the design of a high-coverage vaccine. We studied 253-11, a highly neutralisation resistant (Tier 3) virus, to understand the molecular basis of its neutralisation profile. The 253-11 virus is poorly recognized by subtype-matched and subtype C sera, even when compared to other Tier 3 viruses. Although sequence polymorphisms in the V3 loop and N-linked glycosylation sites may contribute, they do not substantially explain the neutralisation resistance of 253-11. We also found that the 253-11 membrane proximal external region (MPER) is rarely recognized by sera in the context of the wild-type virus, but is commonly recognized in the context of an HIV-2/HIV-1 chimeric virus. Mutating the 253-11 MPER into a subtype-matched, MPER-sensitive virus, 928-28, revealed that the resistance of 253-11 to anti-MPER antibodies was primarily controlled by sequences outside MPER. Mutations in the 253-11 MPER – which were previously reported to increase the lifetime of the post-CD4 Envelope (Env) conformation – increased the sensitivity of 253-11 to antibodies targeting various epitopes on HIV-1 Env. Interestingly, PG9, a broadly neutralising antibody recognizing a quaternary epitope on the trimer spike, instead showed decreased neutralising ability towards the 253-11 MPER mutants. The same MPER mutations in virus 928-28 did not result in a decrease in PG9 neutralisation activity. This, along with the fact that PG9 targets the pre-fusion trimer spike, suggests that these MPER mutants specifically destabilise an otherwise unusually compact, closed 253-11 trimer structure, in addition to or in place of any other effects that the MPER mutants have upon the lifetime of the post-CD4-bound Env conformation. Together, these findings suggest that the 253-11 Env sequence confers an unusually compact trimer structure that contributes to its unusually high neutralisation resistance.
2.1 Introduction

The HIV-1 Envelope (Env) is the sole target of HIV-1-specific neutralising antibodies and therefore an attractive vaccine target (Wyatt et al., 1998). It has been proposed that HIV-1 has progressively become more resistant to neutralising antibodies over the course of the HIV-1 pandemic (Bouvin-Pley et al., 2014, Bouvin-Pley et al., 2013, Bunnik et al., 2010a). An understanding of these highly neutralisation resistant viruses may be needed to develop a global HIV-1 vaccine capable of protecting against most HIV-1 strains.

Extensive screening of sera from HIV-1-infected individuals has resulted in the identification of relatively rarely-elicited broadly neutralising antibodies (bnAbs) that primarily target six epitopes on the HIV-1 Env (Ward and Wilson, 2017, Wibmer et al., 2015): in gp120, the V2/apex region (Doores and Burton, 2010, Walker et al., 2009), the V3/glycan supersite (Kong et al., 2013, Walker et al., 2011, Sanders et al., 2002, Kunert et al., 1998) and the CD4 binding site (CD4-bs) (Wu et al., 2010, Burton et al., 1994); in gp41, the membrane proximal external regions (MPER) (Huang et al., 2012, Nelson et al., 2007, Zwick et al., 2001) and recently the fusion peptide (FP) (Kong et al., 2016, van Gils et al., 2016); and the gp120-gp41 interface (Falkowska et al., 2014, Huang et al., 2014, Scharf et al., 2014).

Neutralisation resistant viruses have evolved numerous mechanisms to evade antibody responses. One of the ways HIV-1 evades the immune response is through the formation of a “glycan shield”, protecting underlying Env epitopes (Moyo et al., 2017, Moore et al., 2012, Pantophlet and Burton, 2006, Wei et al., 2003, Wyatt et al., 1998). The ability of the virus to substantially mutate its sequence and remain functional is also a critical component of the viral antibody evasion strategy (Mascola and Montefiori, 2010, Wei et al., 2003). Additionally, HIV-1 expresses non-functional Env spikes (Moore et al., 2006) that may serve as decoys to divert the antibody response, and contribute to yet another immune evasion mechanism.

HIV-1 also evades neutralisation through conformational masking (Kwong et al., 2002). Conformational masking refers to the phenomenon in which the pre-fusion HIV-1 Env, which exists largely as closed trimer spikes, occludes key neutralising antibody targets (Kwong et al., 2002). As examples, the positioning of the V1/V2 loops aids in the occlusion of the V3 loop (Bartesaghi et al., 2013, Kwong et al., 1998, Stamatatos and Cheng-Mayer, 1998); neighboring protomers in the trimer spike restrict the angle of approach to the CD4-bs (Stamatatos and
Cheng-Mayer, 1998) and the MPER is often partially sunk into the membrane (Montero et al., 2012, Sun et al., 2008). In this closed state, narrowly neutralising antibodies are generally unable to bind and neutralise the virus (Guttman et al., 2015).

It is becoming increasingly clear that the HIV-1 Env spike is dynamic and shifts between closed and open conformations in the pre-fusion state (Guttman et al., 2015, Munro et al., 2014). During the transition of the Env trimer to the open state, the trimer re-arranges, facilitating a shift of the V1/V2 loops to the perimeter of the structure (Bartesaghi et al., 2013, Merk and Subramaniam, 2013, Shen et al., 2010). In this transient state in which Env is relatively open, narrowly neutralising antibodies generally bind more efficiently to Env because the V3 loop, CD4-bs and MPER are better exposed (Guttman et al., 2015, Ward and Wilson, 2015). It is thus possible that a virus could be relatively neutralisation resistant if this equilibrium was shifted in favor of the more closed state(s). Supporting this idea, only the broadest and most potent neutralising antibodies effectively bind to the virus in the closed spike conformation (Guttman et al., 2015, Kwong et al., 2002).

Here, we describe the molecular basis of antibody resistance of a highly neutralisation-resistant, Tier 3 virus, 253-11 (Kulkarni et al., 2009). In order to probe the resistance kinetics of 253-11 Env, we utilized changes in specific amino acid residues in Env that have been found to alter the lifetime of the CD4-bound open conformation. MPER exposure during viral entry can be prolonged by mutations in the MPER such as L669S (Shen et al., 2010) and Y681H (Ringe and Bhattacharya, 2012). These mutations have been shown to increase the sensitivity of viruses to antibodies targeting the V3 loop, CD4-bs and MPER. Their identified mechanism of increasing sensitivity is through delaying fusion of the virus and target cell, which increases the lifetime of the post-CD4-bound open conformation(s) (Ringe and Bhattacharya, 2012, Shen et al., 2010). However, other explanations cannot easily be ruled out.

Together with other experiments, we provide molecular evidence supporting a model in which the unusually high neutralisation resistance of 253-11 is substantially mediated by its unusually compact, pre-fusion Env structure. Knowledge of the mechanisms used by HIV-1 to escape antibody neutralisation has implications in immunogen design and in improving efficacy of candidate HIV-1 vaccines that induce neutralising antibodies.
2.2 Materials and Methods

2.2.1 Serum samples and monoclonal antibodies

Study participants were recruited from (i) caregivers of patients at the paediatric HIV clinic at Groote Schuur Hospital (n=92) and (ii) attendees of the HIV wellness clinic at the Khayelitsha Site B clinic (n=125). Both clinics are located in Cape Town, South Africa. In all, 217 blood samples were collected between December 2009 and July 2011 from donors who were >18 years old, chronically HIV-1 infected (>1 year) and were not exposed to antiretroviral therapy (ART), except for ART given for prevention of mother-to-child transmission (>3 months prior to sample collection). The median CD4 count of the donors was 425 (interquartile range (IQR) 305, 545). Viral loads were measured on 50 of the 217 samples; median viral load was 27,000 (IQR 8150, 100,000). Written informed consent was received from study participants. This study was approved by the Human Research Ethics Committee, Faculty of Health Sciences of the University of Cape Town. Monoclonal antibodies (mAbs) 2F5, PG9, PG16, Z13e1 were obtained from Polymun Scientific, Austria and the National Institute of Health, AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, USA. mAbs PGT128, PGT130, PGT151 and F105 were kind gifts from Dr. P. Moore, National Institute for Communicable Diseases, South Africa. All other mAbs were obtained through the National Institute of Health, AIDS Reagent Program.

2.2.2 Pseudovirus constructs and cell lines

The pSG3∆env HIV-1 backbone, TZM-bl cells and 293T cells were obtained from the National Institute of Health, AIDS Reagent Program. Cloned HIV-1 envelope constructs including 253-11 (Kulkarni et al., 2009) were obtained through the National Institute of Health, AIDS Reagent Program. The 7312A parent HIV-2 construct and the HIV-2 C1 and C1C chimeric constructs (Gray et al., 2007a) were kindly provided by Dr. George Shaw, University of Pennsylvania, USA. The HIV-2/253MPER chimeric construct was produced by mutagenesis from the HIV-2 chimeric construct C1 using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit 121 (Stratagene). L669S (Shen et al., 2009) and Y681H (Ringe and Bhattacharya, 2012) mutants of 253-11 and 928-28, as well as 253-11_928MPER and 928-28_253MPER swap mutants were generated using the GeneArt® Site-Directed Mutagenesis PLUS Kit.
(Invitrogen). All mutations and the absence of unrelated PCR errors were confirmed by sequencing both strands of the entire env open reading frame.

### 2.2.3 Generation of pseudoviruses and neutralisation assays

Pseudoviruses were prepared and tested as previously described (Montefiori, 2009). Briefly, env DNA was co-transfected with pSG3∆env (Wei et al., 2002) into human embryonic kidney 293T cells. Pseudovirus-containing supernatant was harvested 48h post-transfection and stored in single-use aliquots at -80°C. Neutralisation was tested using a standard TZM-bl pseudovirus-based neutralisation assay. Antibody and virus were incubated for one hour at 37°C. TZM-bl cells (10⁴ per well) were added to the antibody-virus combination and incubated at 37°C for 48 h. Titers (dilution of serum [ID₅₀] or concentration of mAb [IC₅₀] that inhibits 50% of infection) were calculated using curve fit functions in Prism (GraphPad). On log scale axes, undetectable neutralisation was plotted with the arbitrary values ID₅₀=25, ID₅₀=10 or IC₅₀=80 µg/ml. For clarity, scales on graphs of IC₅₀ values were inverted so that larger bars correspond to greater neutralisation activity.

### 2.2.4 Depletion of MPER-specific antibodies

Sera were tested for their capacity to neutralise HIV-1 isolates or chimeric HIV-2 viruses by recognition of the MPER. Antibodies were depleted in two rounds of depletion as previously described (Jacob et al., 2015, Tomaras et al., 2011, Gray et al., 2009a) using a biotinylated MPER peptide (MPR.03 (Morris et al., 2011): KKKNEQELLELDKWASNFWFDITNWLWYIRKKK-biotin-NH₂ (Peptide Synthetics). Completeness of depletion was tested by measuring the reduction of neutralising activity towards HIV-2/HIV-1 MPER chimeric viruses. Neutralisation sensitive HIV-1 virus SF162 was used as a negative control because we expect antibodies other than anti-MPER antibodies to dominate neutralisation of this virus (Pinter et al., 2004). Control depletions were performed as above using a biotinylated control peptide with a scrambled sequence: KKKNEKSNNDWERLWLEWLYIWLQDWAFTLKKK-biotin-NH₂. A threshold of a ≥3-fold drop in ID₅₀ compared to control peptide depletion was accepted as positive for MPER-mediated neutralisation.
2.3 Results

2.3.1 Sequence polymorphisms in the V3 loop and N-linked glycosylation only partially explain the neutralisation resistance of 253-11

253-11 is a Tier 3 (Seaman et al., 2010) CRF02_AG virus which is highly resistant to neutralisation by both subtype-matched blood plasma (Jacob et al., 2012) and subtype C sera (Jacob et al., 2015). The sensitivity of 253-11 to different bnAbs was measured in pseudovirus-based neutralisation assays. The virus was resistant to neutralisation by a range of bnAbs across several of the currently identified bnAb targets (Fig 2.1a), even though the 253-11 amino acid sequence possesses most of the key residues that make up the different bnAb epitopes (Fig 2.1b).

The virus is strikingly resistant to most antibodies tested which target the V3/glycans despite possessing key glycans at positions 301 and 332 (Sok et al., 2014, Walker et al., 2011) needed for recognition of this class of antibodies (Fig 2.1b). Notably, 253-11 lacks the $^{324}$GDIR$^{327}$ motif required for the binding of some N332-dependent antibodies (Garces et al., 2015, Garces et al., 2014, Walker et al., 2011) (Fig 2.1b), instead possessing a $^{324}$GNIR$^{327}$ sequence at that position. This could hinder binding of these antibodies, although PGT124 has been shown to bind in the presence of an asparagine at position 325 (Garces et al., 2014). The presence of an alanine at position 325 does not affect PGT121/128-like neutralisation either (Krumm et al., 2016, Sok et al., 2016, Walker et al., 2011). To analyse the effect of this polymorphism, we created an N325D mutant of 253-11 that restores the $^{324}$GDIR$^{327}$ motif, and tested its sensitivity to PGT121, PGT126, PGT128 and PGT130, to which the 253-11 wild-type (WT) is resistant. We found that the introduction of the $^{324}$GDIR$^{327}$ motif modestly increased the sensitivity of 253-11 to only one of the antibodies tested (PGT121) (Fig 2.1c), indicating that sequence polymorphism is not the main contributor to the resistance of the virus to our panel of V3/glycan-specific bnAbs tested (Fig 2.1a).

Because loop length has been associated with neutralisation resistance (Hraber et al., 2014a), we analysed the length of the 253-11 V1/V2 loops to determine whether it was unusually long. 253-11 has V1/V2 loops which are 65 residues long (Fig 2.1b). Loop lengths of Tier 3, Tier 2 and Tier 1 viruses are, on average, 72, 68 and 65 residues long, respectively (LANL, 2017). Therefore, 253-11 does not have an unusually long V1/V2 loop but in fact resembles an average
Tier 1 virus in this regard, which suggests that loop length is not a key factor in the neutralisation resistance phenotype of the virus.

Figure 2.1 253-11 neutralisation resistance is only partially due to sequence polymorphisms and N-linked glycosylation. (a) Depicts IC₅₀ values (µg/ml) obtained from pseudovirus-based neutralisation assays of 253-11 against mAbs with different specificities. (b) Amino acid sequence of 253-11, cytoplasmic domain not included (Accession number: ACC97453.1). N-linked glycosylation sites are depicted in blue boxes with key glycans involved in bnAb binding numbered: FP: N88, V2/apex: N156 and N160; CD4-binding site: N276, V3/glycans: N301 and N332, gp120-gp41 interface: N88, N611 and N637. Residues forming bnAb epitopes are annotated. Loop D (which forms part of the CD4-binding epitope): residues 273-283 (Li et al., 2011); CD4-binding loop: residues 364-373 (Li et al., 2011); MPER: residues 660-683 (Zwick, 2005); MPER: residues 512-525 (van Gils et al., 2016); V1 loop: residues 131-155 (Zolla-Pazner and Cardozo, 2010); V2 loop: residues 158-196 (Zolla-Pazner and Cardozo, 2010). All numbering shown is according to the HXB2 sequence. (c) A 253-11 N325D mutant was engineered to recreate the V3 loop GDIR motif. The sensitivity to V3/glycan-specific antibodies of the WT and N325D mutant was tested in pseudovirus-based neutralisation assays and the IC₅₀ values (µg/ml) are shown. (d) Two potential N-linked glycosylation sites, N293 and N363, present in 253-11 Env but not neutralisation sensitive viruses, 928-28 and Cot6.15, were removed by site-directed mutagenesis. Mutants were tested for sensitivity to serum samples from ART-naïve, HIV-infected individuals which neutralised 253-11 WT poorly or not at all. For calculations, all resistant serum/virus pairs in which the virus was resistant was considered to have an arbitrary ID₅₀ value of 25.
To determine whether glycans play a role in the neutralisation resistance of 253-11 by occluding key epitopes on the virus, we identified and mutated key potential N-linked glycosylation sites (PNGs) which are found in 253-11 but not in a subtype-matched neutralisation sensitive virus, 928-28, or in a well-characterized, neutralisation sensitive subtype C virus, COT6.15 (Gray et al., 2009a). We mutated PNGs at positions N293 and N363 and tested the sensitivity of these mutants to sera which poorly neutralised (ID$_{50}$<100) or did not neutralise the 253-11 WT virus. The removal of either of the two PNGs modestly increased the neutralisation sensitivity of 253-11 to a small proportion of tested sera (Fig 2.1d), probably by exposing underlying epitopes on Env (McCoy et al., 2016). Since the neutralisation resistance of 253-11 appears to be largely maintained in these glycosylation mutants, we propose that the neutralisation resistance phenotype of 253-11 is only minimally due to shielding by these glycans. We therefore, next hypothesized that other modes of escape could be critical for the resistance phenotype of the virus, such as conformational masking of epitopes.

### 2.3.2 253-11 resistance to MPER-specific neutralising antibodies is primarily controlled by sequences outside MPER

Pseudovirus-based neutralisation assays revealed that 253-11 was sensitive to the most broadly neutralising monoclonal antibodies (mAbs) targeting the MPER (Fig 2.1a). We further explored whether 253-11 was also sensitive to neutralisation by moderately neutralising anti-MPER antibodies commonly elicited in natural infection. The antibodies in these serum samples may be more representative of the responses that would be predominantly generated by an HIV-1 vaccine as compared to bnAbs which occur rarely in natural infection (Borrow and Moody, 2017, Simek et al., 2009). We screened 217 unselected serum samples from a South African cohort of chronically HIV-1-infected, ART-naïve participants and found that 253-11 WT was resistant to or poorly neutralised by 90% of the sera (Fig 2.2a).

To investigate the neutralisation susceptibility of the 253-11 MPER, we constructed a chimeric virus containing an HIV-2 backbone with the 253-11 MPER sequence (HIV-2/253MPER) (Fig. 2.2a). Interestingly, 19 sera (8.8%) recognized the 253-11 MPER in the HIV-2/253MPER chimeric construct, but poorly neutralised the 253-11 WT virus (ID$_{50}$<100, upper left quadrant, Fig 2.2a). A threshold of ID$_{50}$>1000 was chosen as an identifier of anti-MPER antibodies in sera (Jacob et al., 2015, Gray et al., 2009a, Li et al., 2009, Binley et al., 2008, Gray et al., 2007a). Six of these 19 sera were tested and shown to neutralise other Tier 2/3 HIV-1 viruses.
by specifically targeting the MPER (Jacob et al., 2015). This strongly suggests that the MPER antibodies in these sera are not defective in recognising the MPER in HIV-1 pseudoviruses and further emphasises that 253-11 is unusually resistant to anti-MPER antibodies in unselected sera compared to other HIV-1 isolates.

Figure 2.2 Sensitivity of 253-11 to MPER-directed antibodies. (a) 217 serum samples were tested for sensitivity to 253-11 and an HIV-2 virus containing the 253-11 MPER sequence. The HIV-2 backbone is derived from the 7312A construct. A threshold titer of 1:1000 was used to define significant anti-MPER neutralisation (Jacob et al., 2015, Gray et al., 2009a). A threshold titer of 1:100 was used to define substantial anti-253-11 neutralisation. Resistant sera were displayed on the graph with an arbitrary value of ID$_{50}$=10. The top panel is a schematic representing how the HIV-2/253MPER was constructed; adapted from (Dimitrov et al., 2007). (b) MPER sequences between subtype-matched viruses 253-11 and 928-28 were swapped between the viruses by site-directed mutagenesis. 10/19 sera from the upper left quadrant of Fig 2.2a were tested for neutralisation sensitivity to 253-11 and 928-28 MPER-swap mutants. Neutralisation was compared to the wild-type viruses of each. In this panel, undetectable neutralisation was displayed with the arbitrary value of ID$_{50}$=25 (Note: Data in Fig 2.2a from (Jacob, 2014)).

To determine whether the resistance of 253-11 WT to anti-MPER antibodies in sera was induced by polymorphisms in the MPER itself, we exchanged MPER amino acid sequences between 253-11 and 928-28, a subtype-matched virus that is substantially less neutralisation resistant (Jacob et al., 2015, Seaman et al., 2010). We tested these MPER swaps for sensitivity to 10/19 sera from the upper left quadrant (Fig 2.2a), and observed that the pattern of neutralisation generally followed the Env “backbone” and not the MPER sequence (Fig 2.2b) suggesting that sequence differences outside of the MPER itself were the cause of 253-11
MPER neutralisation resistance. The specificity of 5 of these 10 sera to the 928-28 MPER were previously confirmed in anti-MPER-depletion experiments (Jacob et al., 2015).

Two sera were tested in anti-MPER-depletions with the 928_28 chimera containing the 253 MPER sequence (928-28_253MPER). These two sera were found to contain MPER antibodies and neutralised the viruses primarily via recognition of MPER as shown by a lack of neutralising ability (ID$_{50}$<50) after depletion of MPER antibodies from the samples (Fig 2.3). Combined, our data thus strongly suggest that 253-11 likely evades neutralisation by common MPER-specific antibodies through factors outside the MPER such as conformational masking and not sequence polymorphism within the MPER.

<table>
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<td>&lt;50</td>
</tr>
<tr>
<td>C1C (consensus C)</td>
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<td></td>
</tr>
<tr>
<td>Control depl</td>
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</tr>
<tr>
<td>MPER depl</td>
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<tr>
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<tr>
<td>C1 (YU2)</td>
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</tr>
<tr>
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<td>*</td>
</tr>
<tr>
<td>MPER depl</td>
<td>&lt;50</td>
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<tr>
<td>MPER depl</td>
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<tr>
<td>Fold reduction</td>
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Fold change in ID$_{50}$
- $>$10
- 3-10
- $<$3

ND= not determined
Control depl: control depletion with scrambled peptide
MPER depl: sera depletion with MPER peptide

Figure 2.3 Sera neutralise 928-28_253MPER mutant through recognition of its MPER. Two samples which did not neutralise 253-11 WT but exhibited high 253MPER neutralising activity (Fig 2.2a) were used for MPER antibody depletion experiments using the MPR.03 peptide as compared to a control scrambled sequence peptide to determine whether MPER specific antibodies were the major contributors towards neutralisation of 928-28_253MPER mutants. SF162 was a negative control and the HIV-2/HIV-1 chimeras using MPER sequences from 253-11, C1C and C1 were the positive controls for depletion.
2.3.3 MPER mutations alter the exposure of epitopes on the 253-11 trimer to sera

We studied the effect of MPER mutations, L669S (Shen et al., 2010) and Y681H (Ringe and Bhattacharya, 2012), on 253-11 neutralisation resistance. These mutations have been described as increasing the lifetime of the HIV-1 Env conformation after CD4 has bound (Ringe and Bhattacharya, 2012, Shen et al., 2010). We tested the 253-11 MPER mutants for sensitivity to 12/19 sera which neutralise the 253-11 MPER in the chimeric virus but not in the WT. In all cases, the L669S and Y681H mutations increased the sensitivity of 253-11 to these sera, with L669S frequently having a greater effect than Y681H (Fig 2.4).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>ID_{50} 253-11 WT</th>
<th>ID_{50} 253-11 Y681H</th>
<th>ID_{50} 253-11 L669S</th>
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Figure 2.4 253-11 L669S and Y681H mutants generally have increased sensitivity compared to 253-11 WT. Neutralisation of the 253-11 WT and L669S/Y681H mutants was assessed by 12/19 of the serum samples which recognize the 253-11 MPER in the HIV-2/HIV-1MPER chimera but not in the 253-11 WT virus. For calculations, all resistant serum/virus pairs in which the virus was resistant was considered to have an ID_{50} value of 25.

To test if the L669S and Y681H mutations were not only directly affecting recognition of the 253-11 MPER but had a more global effect on the structure of 253-11, we depleted MPER antibodies from 2/19 sera that have dominant anti-MPER neutralising antibodies. When the 253-11 L669S and Y681H mutants were tested for sensitivity to MPER-depleted samples, we observed substantial residual neutralisation (Fig 2.5), suggesting that antibodies targeting epitopes other than the MPER were also responsible for the increase in neutralisation sensitivity for the L669S and Y681H mutants. Depletion of MPER-specific antibodies was confirmed by testing capacity to neutralise three HIV-2/HIV-1 MPER chimeric viruses, with reductions of 41-416-fold observed; therefore, it was unlikely that the results were due to incomplete...
depletion. These data indicate that recognition of HIV-1 Env epitopes other than the MPER are affected by these mutations.

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*ND* = not determined

Control depl: control depletion with scrambled peptide

MPER depl: sera depletion with MPER peptide

**Figure 2.5 Neutralisation revealed by L669S and Y681H mutations is only partially due to anti-MPER antibodies.** Two serum samples which neutralised 253-11 poorly (ID₅₀<100) but had exhibited high 253MPER neutralising activity (Fig 2a) were used for MPER antibody depletion using the MPR.03 peptide as compared to a control scrambled sequence peptide to determine whether MPER specific antibodies were the major contributors towards the increase in neutralisation observed with the L669S and Y681H mutant viruses. SF162 was a negative control and the HIV-2/HIV-1 chimeras using MPER sequences from 253-11, C1C and C1 HIV-2/HIV-1 were the positive controls.

We then investigated whether neutralising antibodies in sera which preferentially neutralise the L669S and Y681H mutants over the WT also target the V2/apex and V3/glycan regions. We introduced N160A and N301A.N332A mutations to the L669S and/or Y681H mutants to map antibodies responsible for increased neutralising activity to the V2/apex or V3/glycans, respectively (Jacob et al., 2015, Moore et al., 2011, Tomaras et al., 2011). We found that, in some sera, V2/apex and V3/glycan-specific antibodies were responsible for the increases in neutralisation activity (**Fig 2.6**). These data suggest that the MPER mutations affect distal epitopes that are normally occluded in 253-11 WT, possibly by increasing their exposure to antibodies.
2.3.4 MPER mutations increase 253-11 sensitivity to some broadly neutralising monoclonal antibodies, but decrease sensitivity to mAb PG9

We further explored how bnAb epitopes across Env are affected by the 253-11 MPER mutants by testing the 253-11 L669S and Y681H mutants for sensitivity to mAbs that target five of the major sites of vulnerability on the HIV-1 Env (Fig 2.7).

Figure 2.6 V2/apex and V3/glycan epitopes are affected by 253-11 L669S and Y681H mutations. Serum samples whose increased neutralising activity towards the L669S and Y681H mutants was mapped to the V2/apex (L669S.N160A and Y681H.N160A mutants) or V3/glycans (Y681H.N301A.N332A mutant only; L669S.N301A.N332A did not produce infective virus). Three sera (displayed) out of seven tested exhibited dominant V2/apex-directed (N160-dependent) neutralisation that was revealed by L669S and/or Y681H. Two sera (displayed) of five tested exhibited dominant V3/glycan-dependent neutralisation that was revealed by Y681H. For calculations, all resistant serum/virus pairs in which the virus was resistant was considered to have an ID50 value of 25.

Figure 2.7 Impact of 253-11 L669S and Y681H mutants on bnAb recognition. Neutralisation of the 253-11 WT and L669S/Y681H mutants by mAbs targeting most of the broadly neutralising antibody epitopes. On the log scale axes, undetectable neutralisation was plotted with the arbitrary values IC50=80 µg/ml. For clarity, scales of IC50 values were inverted so that higher bars always correspond to greater neutralisation activity.
Importantly, and in contrast to their effect upon V2-targeted antibodies in sera which neutralise the L669S and Y681H mutants better than the WT (Fig 2.4), the MPER mutants 
*decreased* sensitivity of 253-11 to mAb PG9 (Fig 2.7). PG9 binds to a conformation-dependent epitope in the V2/apex that preferentially exists in the pre-CD4-bound Env spike (Walker et al., 2009). We hypothesize that the decrease in neutralisation may be due to a destabilization of the PG9 quaternary epitope at the apex of the pre-fusion trimer. This effect was only seen in 253-11; no change in sensitivity to PG9 was observed when the same mutations were introduced into 928-28 (Fig 2.8a).

L669S and Y681H increased 253-11 sensitivity to V2-specific (N160-dependent) antibodies in some sera (Fig 2.6), yet decreased sensitivity to the V2/apex-specific antibody PG9 (Fig 2.7) in a manner apparently specific for 253-11 (Fig 2.8b). We propose that the V2/apex-specific antibodies in sera bind to a different and plausibly less conformationally-dependent epitope than PG9.

**Figure 2.8** Effect of L669S and Y681H mutants on sensitivity of 928-28 to bnAbs. (a) 928-28 and its corresponding L669s and Y681H mutants were tested for sensitivity to a subset of mAbs. For clarity, scales of IC$_{50}$ values were inverted so that larger bars always correspond to greater neutralisation activity. (b) Comparison of IC$_{50}$ (µg/ml) between 253-11 WT, 928-28 WT and their L669S and Y681H mutants.
Unsurprisingly, compared to 253-11 WT, the MPER mutants were more sensitive to anti-MPER mAbs 4E10, 2F5, Z13e1 and 10E8 (Fig 2.7). Strikingly, the MPER mutants were also more sensitive to neutralisation by V3/glycan mAbs 10-1074 and PGT128 but both WT and mutants were resistant to PGT121, PGT126 and PGT130 (Fig 2.7). Several bnAbs that target other epitopes were also not affected by these mutants, including PGT151 targeting the gp120-gp41 interface and VRC01, NIH45-46 and 3BNC117 targeting the CD4-bs (Fig 2.7).

As expected, 928-28 and its MPER mutants displayed similar changes in antibody sensitivity as 253-11 and its mutants when tested with anti-MPER antibodies 2F5 and 10E8 (Fig 2.8b). sCD4 also neutralised the 928-28 mutants better than WT, while the neutralisation of 10-1074 was not substantially affected by the mutations as it was for 253-11 (Fig 2.8b). Our data indicate that L669S and Y681H mutations drastically affect the neutralisation sensitivity of 253-11 to V2/apex (PG9) and V3/glycan (10-1074) bnAbs compared to subtype-matched virus, 928-28. We propose that the mutants are likely disrupting the pre-fusion conformation of 253-11 substantially more than that of the more neutralisation sensitive virus, 928-28. We therefore, hypothesize that the mutants disrupt an otherwise unusually compact, closed 253-11 trimer structure.
2.4 Discussion

A global HIV-1 vaccine will likely need to protect against neutralisation resistant viruses to effectively reduce HIV-1 incidence. In this report, we studied 253-11, a highly neutralisation resistant, Tier 3 (Seaman et al., 2010), CRF02_AG virus whose *env* gene was obtained from Cameroon (Kulkarni et al., 2009). We noted that 253-11 was resistant to bnAbs and sera targeting different epitopes on the HIV-1 Envelope (Env). The virus was resistant to V3/glycan-specific broadly neutralising antibodies (bnAbs) despite the presence of potential N-linked glycosylation sites (PNGs) at positions 301 and 332 which are the primary targets of these antibodies (Walker et al., 2011). We considered that this resistance may be explained by a polymorphism in the \(^{324}\)GDIR\(^{327}\) motif. However, restoration of this motif by mutagenesis did not restore neutralisation sensitivity to the bnAbs. We also tested the role of glycans in the neutralisation resistance of 253-11 and found that the deletion of key glycans only marginally affected neutralisation. Thus, sequence polymorphisms and N-linked glycans glycosylation of Env do not substantially explain the neutralisation resistance of 253-11. The high neutralisation resistance of this virus may be more clearly explained by its highly compact trimer spike.

The introduction of L669S (Shen et al., 2010) and Y681H (Ringe and Bhattacharya, 2012) mutations in the MPER of viruses have previously been shown to shift viral entry kinetics by delaying fusion and thus increase the neutralisation sensitivity of viruses. These mutations increased the sensitivity of the 253-11 virus to serum antibodies targeting the V2/apex (N160-dependent antibodies) and the V3/glycans, soluble CD4 (sCD4) and monoclonal antibodies (mAbs), including 2F5, 10E8, 4E10, Z13e1, 10-1074, PGT128 and VRC03. These results suggest a change in exposure of a range of epitopes across Env due to these mutations.

Strikingly, the mutations decreased neutralisation sensitivity to PG9, a conformation-dependent antibody targeting the V2/apex in the Env trimer spike. The mutations did not alter the sensitivity of subtype-matched virus 928-28 to the same antibody. Additional Tier 2 and Tier 3 viruses should be tested in this manner to determine to what extent compactness is an effect that is specific to 253-11. This is the first report to show that the L669S and Y681H mutations can affect neutralisation of HIV-1 isolates by antibodies that target the closed, pre-fusion conformation such as those which bind the V2/apex, mAb PG9 (Julien et al., 2013b) and V3/glycans, mAbs 10-1074 (Mouquet et al., 2012) and PGT128 (Lee et al., 2015a). Trimer
structures solved in complex with V3/glycan and V2/apex bnAbs are locked in a closed conformation (Lee et al., 2017, Gristick et al., 2016, Garces et al., 2015, Lee et al., 2015a, Julien et al., 2013a), strongly suggesting that their epitopes are properly displayed in the trimer spike.

Nonetheless, many of the bnAbs tested showed no change in neutralising activity between the WT and MPER mutants. This may be because many of the most broadly neutralising antibodies are broad and potent because they have evolved to bind to both the closed and open conformations of Env (Guttman et al., 2015). Together, these data led us to hypothesize that the 253-11 trimer is in a stable, unusually compact conformation in its native, pre-fusion form and that the L669S and Y681H mutants destabilize and open this structure, leading to a more neutralisation sensitive phenotype. We cannot rule out contributions to neutralisation sensitivity from other mechanisms, including the delay of fusion (Shen et al., 2010); however, the change in sensitivity to PG9 clearly suggests a mechanism associated with the Env spike before CD4 binding. One caveat of our conclusions concerning the increased PG9 sensitivity of the 253-11 mutants is that we only studied the 253-11 virus. This makes it difficult to generalise the findings to other neutralisation-resistant viruses. We postulate that some but not all highly neutralisation-resistant viruses will likely exhibit similar characteristics. Future studies should include a larger panel of Tier 3 viruses to determine whether this is a global effect.

More generally, it seems plausible that the equilibrium of compact Env spikes, such as those of 253-11, strongly favors a closed conformation that is less likely to enter transient open conformations as compared to spikes of most other HIV-1 isolates (Cai et al., 2017, Guttman et al., 2015). It has been suggested that Tier 3 viruses preferentially remain in their closed conformation while Tier 1 viruses tend to remain in a relatively open conformation (Cai et al., 2017, Guttman et al., 2015). Our data lean towards the mechanism of neutralisation-resistance of 253-11 mainly being due to having an unusually compact, closed Env trimer structure. An understanding of the mechanisms employed by viruses to gain neutralisation resistance is critical because it may aid in the design of immunogens capable of eliciting antibodies that have high strain coverage.
Chapter 3: Understanding the structural basis behind the neutralisation resistance phenotype of CRF02_AG virus, 253-11

Summary

253-11 is a CRF02_AG virus that is highly resistant to antibody neutralisation as determined by several antibody panels. Sequence polymorphisms in the V3 loop and N-linked glycosylation sites play a role in viral escape, but only marginally explain its neutralisation resistance phenotype. We found that the 253-11 membrane proximal external region (MPER) is rarely recognised by sera in the context of the wild-type virus, but is commonly recognised in the context of an HIV-2 chimeric virus. Mutations L669S and Y681H in the 253-11 MPER – which were previously reported to increase the lifetime of the pre-fusion Envelope (Env) conformation – increased the sensitivity of 253-11 to antibodies targeting various epitopes on HIV-1 Env. Remarkably, PG9, a broadly neutralising antibody recognising a quaternary epitope on the trimer spike, instead showed decreased neutralising ability towards the 253-11 MPER mutants. This suggests that the MPER mutants destabilise the pre-fusion Env trimer spike. Together, these data led us to hypothesize that 253-11 preferentially stays in an unusually compact, closed Env conformation that makes it difficult for antibodies to penetrate and neutralise the virus. To gain information about the structure of the 253-11 Env trimer, a SOSIP.664 trimer based on the 253-11 virus was engineered. We found that the 253-11 SOSIP trimer has a high melting temperature, is a good antigenic mimic of the functional Env spikes expressed by the corresponding pseudovirus and exhibits a predominantly closed conformation. We solved the crystal structure of the 253-11 SOSIP trimer in complex with V3/glycan-specific antibody, 10-1074, at a resolution of 6.5 Å. The crystal structure of the 253-11 trimer revealed that the heptad repeat helices in gp41 are drawn closer to the trimer axis as compared to other SOSIP trimer structures, suggesting local compactness in gp41. 253-11 gp120 protomers also showed a relatively compact disposition around the trimer axis. Together, these data suggest that the 253-11 Env sequence confers an unusually compact trimer structure, supporting the hypothesis that a robust form of conformational masking is used by this virus, and plausibly other Tier 3 viruses, to achieve high neutralisation resistance.
3.1 Introduction

In Chapter 2 of this thesis, we hypothesised that the neutralisation resistant phenotype of the 253-11 virus was mainly a result of the unusually compact and closed conformation of its Envelope (Env) protein that masks neutralising antibody (nAb) epitopes. To further investigate the compactness of the 253-11 Env and to probe the ability of the 253-11 sequence to preferentially adopt a pre-fusion Env structure, we engineered a 253-11 SOSIP.664 Env trimer as previously described (Sanders et al., 2013) and we solved its structure by X-ray crystallography.

Determining how broadly neutralising antibodies (bnAbs) recognise the HIV-1 Env trimer is an important element of rational-vaccine design based on immunogens that elicit bnAb responses (Dimitrov et al., 2011, Kwong et al., 2011, Zhou et al., 2007). X-ray crystallography has long been the dominant method for deducing high-resolution protein structures (Green et al., 1954, Kendrew et al., 1958, Blake et al., 1965). Prior to the design of recombinant HIV-1 Env trimers such as SOSIP.664 trimers, most antibodies were crystallized with fragments of HIV-1 Env (McLellan et al., 2011, Zhou et al., 2007). For example, Zhou and colleagues crystallized the CD4-bs antibody b12 complexed with an HIV-1 gp120 monomer and PG16 was co-crystallized with a V1/V2 gp120 domain (Pancera et al., 2013). Another study used monomeric V1/V2 loop Env protein scaffolds in complex with antibodies such as PG9 to delineate their epitope binding (McLellan et al., 2011). However, these methods are not able to address trimeric structural changes which may be more immunologically relevant, such as the compactness that our study suggests may explain the high neutralisation resistance of 253-11. The development of SOSIP.664 trimers paved the way to a better understanding of Env immunogenicity (Sanders et al., 2013) and eventually led to the first high resolution crystal structure of HIV-1 Env (Julien et al., 2013a).

The first structure of the HIV-1 Envelope (Env) trimer was solved at 4.7 Å from crystals of the BG505 SOSIP.664 trimer (Julien et al., 2013a). The crystal structure of the BG505 trimer shed light on the architecture of the HIV-1 trimer at atomic resolution. However, crystallization of Env trimers has been a challenge and only four other Env SOSIP.664 trimers have been solved by crystallography or cryo-electron microscopy to date: JR-FL (Subtype B), X1193.c1 (Subtype G) (Stewart-Jones et al., 2016), 16055 (Subtype C) (Guenaga et al., 2017) and most recently, B41 (Subtype B) (Ozorowski et al., 2017). Some of the difficulties in crystallizing
HIV-1 trimers include instability, protein flexibility especially at the variable loop regions and dense gp120 glycosylation (Ward and Wilson, 2017). To overcome this, most Env trimers have been crystallized without their native glycans and in complex with the antigen-binding fragment (Fab) of an HIV-1-specific antibody (Ward and Wilson, 2017).

As very few HIV-1 strains are amenable to SOSIP design (Sanders and Moore, 2017, Julien et al., 2015), little is known about how Tier 3 viruses from chronic infection would perform as SOSIP trimer immunogens and if their structures differ from more neutralisation sensitive viruses. It is plausible that their structures differ from Tier 1 and 2 viruses as it has been shown that unliganded Tier 3 viruses preferentially remain in a closed conformation with fewer conformational fluctuations compared to neutralisation sensitive viruses (Cai et al., 2017, Munro et al., 2014). Only one neutralisation resistant SOSIP trimer has been described in the literature, based on the PVO.4 virus but it is only moderately neutralisation resistant (Tier 2/3) (Verkerke et al., 2016, Seaman et al., 2010) and its structure has not been solved to date. Therefore, there is a need for more Tier 3 SOSIP trimers because they may induce a robust broadly neutralising response when used as immunogens as preferentially remaining in a closed conformation may display bnAb epitopes and occlude narrowly-neutralising ones more efficiently (Cai et al., 2017).

We engineered a SOSIP trimer based on the 253-11 Env sequence and in preliminary studies by our collaborators Ereño-Orbea and Julien, small-angle X-ray scattering (SAXS) measurements coupled to size-exclusion chromatography (SEC) (SEC-SAXS) revealed that the 253-11 SOSIP.664 trimers were almost entirely free of aggregation and properly folded, and the calculated diameter ($D_{\text{max}}$) for the 253-11 trimer (152.4 Å) was comparable to BG505 (146.2 Å) (Fig 3.1). Although the 253-11 and BG505 structures analysed by SAXS contained a (His)$_{6x}$ tag at their C-termini, the $D_{\text{max}}$ of BG505 was almost identical to BG505 without the (His)$_{6x}$ tag (147 Å) (Liang et al., 2016). This suggests that the (His)$_{6x}$ tag had little to no effect on the measurements. The low-resolution three-dimensional volume obtained from SAXS measurements demonstrated that the 253-11 trimer adopts a closed conformation that was of similar dimensions to that of the BG505 trimer (Fig 3.1). This led us to hypothesize that the compact phenotype of 253-11 may be as a result of discrete features in its secondary structure that cannot be observed at this low resolution.
Figure 3.1 Low-resolution characterisation of 253-11 SOSIP.664 Env trimers. Three views (side, top and bottom) of the SAXS envelope reconstruction obtained for 253-11 (grey) and BG505 (yellow) trimers by SEC-SAXS. The crystal structure of the fully glycosylated BG505 SOSIP trimer (PDB ID:5FYL) was fitted into the SAXS 3D volume and is shown as a secondary structure cartoon.

In this chapter, the 253-11 SOSIP.664 trimer, initially designed by our collaborators Ereño-Orbea and Julien, was further analysed. We first investigated its biophysical properties including antigenicity and thermostability. Then, to gain further understanding of the discrete structural elements of 253-11 resulting in its neutralisation resistant phenotype, we co-crystallized the 253-11 trimer with 10-1074, a V3/glycan-specific antibody which is highly potent and broad (Mouquet et al., 2012), even against the 253-11 virus. Thus, the 253-11 SOSIP trimer we designed increases the number of recombinant Env trimers currently available, shedding light on the structure of Tier 3 Env trimers and adding to the potential HIV-1 immunogens to test in immunogenicity studies.
3.2 Materials and Methods

3.2.1 253-11 SOSIP, BG505 SOSIP, VRC01, PG9 and 10-1074 Fab construct design

All sequences were codon-optimized for expression in human cells and synthesized by GeneArt (Life Technologies). 253-11 and BG505 SOSIP.664 constructs were subcloned into the pHLsec vector (Aricescu et al., 2006) using restriction enzymes AgeI and KpnI, such that a (His)$_{6x}$ tag was at the C terminus of the constructs to facilitate affinity purification. Addition of a stop codon before the (His)$_{6x}$ tag allowed for the removal of the tag and production of an untagged 253-11 trimer for crystallization. The heavy chain and light chains of the 10-1074, PG9 and VRC01 Fabs were subcloned into the pHLsec vector (Aricescu et al., 2006) using restriction enzymes AgeI and KpnI.

3.2.2 Trimer expression and purification

253-11 SOSIP.664 and BG505 SOSIP.664 plasmids were transiently co-transfected with a furin protease plasmid into HEK293F or HEK293 suspension cells (ATCC). Cells were passaged in 200 ml cultures at 0.8 x 10$^6$ cells per ml. 50 µg of DNA was filtered and mixed in a 1:1 ratio with transfection reagent FectoPRO (Polyplus Transfections). The DNA:FectoPro solution was incubated with the cells at 37 °C for 6-7 days. Cells were harvested by centrifugation and supernatants filtered using a 0.22 µm Steritop filter (EMD Millipore). Supernatant containing protein with the (His)$_{6x}$ tag was passed through a HisTrap Ni-NTA column (GE Healthcare). The column was washed with 1x Phosphate buffered saline (PBS) buffer pH 7.4 with 5 mM imidazole prior to elution with an increasing gradient of imidazole up to 500 mM. The untagged 253-11 trimer protein was purified through a Galanthus nivalis lectin (GNL) column. The trimer was washed with 1x PBS, then 0.5 M sodium chloride (NaCl) in 1x PBS followed by an additional 1x PBS wash. The trimer was eluted with 1 M methyl-α-D-mannopyranoside (MMP) in 1x PBS. In both cases, the affinity-purified proteins were further purified to size homogeneity using two runs of Superdex 200 10/300 increase size-exclusion chromatography (SEC) (GE Healthcare) in 20 mM Tris pH 9.0, 150 mM NaCl buffer. Purity of the SOSIP.664 trimers was assessed by non-reducing and reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) gels stained with Quick Coomassie Blue (Regeneron) for visualisation. The Precision Plus Protein™ Dual Color Standards (Bio-rad) molecular weight marker was used to determine the apparent size of the
proteins. Protein concentrations were measured using the Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).

3.2.3 Fab expression and purification

HEK293F or HEK293S cells were transfected with heavy and light chain plasmids (2:1 ratio) in a total DNA amount of 90 ug for each 200 ml culture. FectoPRO reagent was used as a transfection reagent in a 1:1 ratio of DNA:FectoPRO. Cells were transfected at a cell density of 0.8 x 10^6 cells per ml and incubated at 37°C for 6-7 days. Cells were harvested and supernatants and filtered through a 0.22 µM membrane. Supernatants were passed through an anti-Kappa affinity column (GE Healthcare) or anti-Lambda affinity column (GE Healthcare) using an AKTA Start chromatography system (GE Healthcare) and eluted with 100 mM glycine pH 2.2-2.7. Eluted fractions were immediately neutralised with 1 M Tris-HCl pH 9.0. Fractions containing protein were buffer exchanged into 20 mM sodium acetate, pH 5.6. Ion exchange chromatography was performed using a cation MonoS column (GE Healthcare) and eluted with a potassium chloride gradient. Fractions were pooled, concentrated and flowed through a Superdex 200 Increase gel filtration column (GE Healthcare) in 20 mM sodium acetate pH 5.6 to obtain purified samples. Purity of protein samples was assessed by non-reducing and reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE gels stained with Quick Coomassie Blue (Regeneron) for visualization. The Precision Plus Protein™ Dual Color Standards (Bio-rad) molecular weight marker was used to determine the apparent size of the proteins. Protein concentrations were measured using the Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).

3.2.4 Determination of the SOSIP trimer melting temperatures (T_m)

The conformational stability of the 253-11 trimer and 253-11 (His)₆-tag trimer was assessed by measuring the melting temperature (T_m) determined by an intrinsic fluorescence intensity ratio (350/330 nm) using the UNit system (Unchained Labs). SEC-purified SOSIP trimer samples were concentrated to 1 mg/ml and heated from 20 to 90 °C using 1°C increments, with an equilibration time of 60 s before each point measurement. Trimer thermostability was measured in three different buffers; 50 mM Tris pH 9, 50 mM HEPES pH 7 and 50 mM sodium acetate. All buffers contained 150 mM NaCl. Data was processed using the standard UNit
analysis software. Sample measurements were taken in triplicate; values were averaged and standard errors were calculated using GraphPad Prism v5.

3.2.5 Fab and antibody binding assay using biolayer interferometry

Binding affinities of VRC01, PG9 and 10-1074 Fabs, and 17b and F240 antibodies to 253-11 SOSIP trimers were measured by biolayer interferometry (BLI) (Rich and Myszka, 2007) using the Octet Red system (Pall FortéBio) (Shah and Duncan, 2014). Anti-human Fab-CH1 or anti-human IgG biosensors were hydrated in 1x kinetics buffer (1X PBS, pH 7.4, 0.002% Tween, 0.01% BSA) and loaded with 10 μg/ml Fab for 60 s. Biosensors were then transferred into wells containing 1x kinetics buffer to baseline for 60 s before being transferred into wells containing serial dilutions of 253-11 SOSIP.664 trimer starting at 1500 nM and decreasing to 187.5 nM. The 300 s association phase was subsequently followed by a 600 s dissociation step in 1x kinetics. For antibody F240, the association phase was 150 s and the dissociation phase was 350 s. Analysis was performed using the Octet software, with a 1:1 fit model. Experiments were repeated in triplicates. 17b and F240 antibodies were obtained from the National Institute of Health, AIDS Reagent Program.

3.2.6 Crystallization of the 253-11 SOSIP trimer in complex with 10-1074 Fab

Molar excess of 10-1074 Fab was added to purified 253-11 SOSIP trimer expressed in HEK293 GnT I−/−cells. To obtain deglycosylated samples, the complex was then treated with the enzyme Endoglycosidase H (Endo H) (New England Biolabs) for 1 h at 37 °C. Deglycosylated 253-11 SOSIP trimer in complex with 10-1074 Fab was purified by SEC using a Superose 6 SEC column (GE Healthcare). The purified 253-11 SOSIP trimer with 10-1074 Fab complex was concentrated to 5 mg/ml in a buffer containing 20 mM Tris pH 9.0 and 150 mM NaCl. Crystals were obtained by hanging drop vapor-diffusion in 0.5 M sodium chloride, 1.9 M ammonium sulphate and 0.1 M sodium cacodylate, pH 6.5. Crystals were improved by micro-seeding after mixing 0.6 μl of protein, 0.4 μl solution and 0.2 μl crystal seeds. Crystals were cryo-protected by soaking in mother liquor solution containing 20% glycerol and flash cooled in liquid nitrogen. X-ray diffraction data were collected at the 23-ID-D beamline at the Argonne Photon Source, USA. A unique dataset for the 253-11 SOSIP trimer + 10-1074 Fab complex was processed using XDS (Kabsch, 2010). Based on the R3:H space group and Matthews volume calculation (Weichenberger and Rupp, 2014, Matthews, 1968, Kantardjieff and Rupp, 2003)
we estimated that there were two molecules of the 253-11 SOSIP protomer + 10-1074 Fab complex in the asymmetric unit. The crystal structure was solved by molecular replacement using one protomer of BG505 SOSIP in complex with 10-1074 Fab (PDB ID: 5T3X) (Gristick et al., 2016) as a search model in Phaser (McCoy et al., 2007). The structure was refined by manual building in Coot (Emsley et al., 2010) and using phenix.refine (Adams et al., 2002) with strict reference model and secondary structure restraints given the resolution. The structure of BG505 SOSIP in complex with a PGT121-family precursor (PDB ID: 5CEZ) (Garces et al., 2015) was used to manually build the fusion peptide of 253-11 in Coot. Protein structure superimpositions and center of mass analyses were conducted using Pymol v1.8.6.0 (Schrodinger, 2015). The centre of mass of individual gp120 protomers (residues 32-504) and distances between the three gp120 centers of mass were calculated using the “centreofmass” and “measurement” commands in Pymol, respectively. Software were accessed through SBGrid (Morin et al., 2013).
3.3 Results

3.3.1 SOSIP.664 trimer expression and purification

The 253-11 SOSIP.664 was designed as described previously (Sanders et al., 2013) with a disulphide bond linking gp120 and gp41 subunits and an isoleucine to proline mutation at position 559 to increase trimer stability (Fig 1A). The sequence was truncated at position 664 before the membrane proximal external region (MPER) to increase the solubility of the trimer (Fig 1A).

The 253-11 Env is a heavily glycosylated glycoprotein (Fig 1A) (Ward and Wilson, 2017). For this reason, the 253-11 trimer constructs were expressed in HEK293F or 293S mammalian cell lines. A (His)$_{6x}$-tag was added to 253-11 and BG505 trimers to allow affinity purification using nickel chromatography (Bornhorst and Falke, 2000). However, because of the flexibility and charge caused by the C-terminal (His)$_{6x}$-tag, it was removed from the 253-11 trimer for crystallization.

HEK293F cells are fast growing, highly transfectable cells that contain a mutant version of the SV40 large T antigen (Rio et al., 1985). These cells express proteins with wild-type (WT) N-linked glycans and are therefore useful in biophysical analysis of proteins as they are representative of HIV-1 Env in its native state. However, complex and hybrid N-linked glycans increase the chemical and conformational heterogeneity of proteins, which dampens the chances of getting good diffracting crystals. This is one of the major challenges in crystallization of the HIV-1 trimer as it is densely covered by glycans (Ward and Wilson, 2017). To overcome this, for crystallization trials, the 253-11 trimer and glycosylated Fabs were expressed in HEK293S cells. HEK293S cells are also highly transfectable but are deficient in the GnT I enzyme which limits the heterogeneity of N-linked glycans as they enrich for high mannose glycans only (Chaudhary et al., 2012).

After 6-7 days of expression, protein was harvested from the supernatant and purified until homogeneity using two chromatography steps; a first step of affinity chromatography followed by size-exclusion chromatography (SEC). While 253-11 trimers (253-11_histag trimer) and BG505 trimers containing the (His)$_{6x}$-tag were affinity purified using nickel-coated columns (Fig. 3.2b), the 253-11 trimers lacking the (His)$_{6x}$ tag were purified by a lectin column through binding of the HIV-1 Env glycans to D-mannose subunits (Barre et al., 2001) (Fig. 3.2c).
Figure 3.2 Representative affinity chromatography purification profiles and SDS-PAGE gels of 253-11 trimers. (a) Schematic linear representation of the glycosylated 253-11 SOSIP.664 trimer design highlighting the features which constitute a SOSIP trimer. (b) After expression in either HEK293F or HEK293S cells, the proteins first underwent affinity chromatography as an initial purification step. 400 ml 253-11_histag trimer was purified through a nickel column and eluted with 500 mM imidazole in 1x PBS. (c) 600 ml 253-11 trimer was purified through a GNL column and eluted with 1M MMP in 1x PBS. The proteins were run on a SDS-PAGE either with β-mercaptoethanol (reducing) or without (non-reducing). Before: protein before affinity purification; After: protein after affinity purification. FT: flow-through from affinity column. Blue line: UV absorbance corresponding to protein elution; red line: elution buffer conductivity.

Protein purity was assessed by running the eluted peak on an SDS-PAGE in either reducing (by adding 5% β-mercaptoethanol) or non-reducing conditions. Under non-reducing conditions trimers of above 250 kDa molecular weight and gp120-gp41 protomers of approximately ~140 kDa were obtained. Addition of β -mercaptoethanol reduced the disulphide linkage between the gp120 (band at 120 kDa) and gp41 (band around 37 kDa). Multiple bands for gp41 are shown in reducing conditions representing gp41 glycoforms containing different glycan compositions.
To separate 253-11 SOSIP.664 trimers to size homogeneity from aggregates, monomers and dimers, we further purified the protein by SEC using a Superdex 200 Increase column (GE Healthcare) (Fig 3.3). To further polish purification, a second SEC was conducted. 253-11 SOSIP trimers eluted as a single peak at the same volume (~10 ml) as BG505 SOSIP trimers and expressed in slightly higher yields compared to BG505 SOSIP trimers. The trimers were efficiently cleaved into gp120-gp41 subunits during processing in the Golgi as observed in SDS-PAGE under reducing conditions (Fig 3.3). In summary, we successfully expressed the 253-11 SOSIP.664 trimer and produced the yields and purity needed for subsequent biophysical and structural experimentation.

### 3.3.2 Evaluation of trimer stability reveals (His)6x-tag on 253-11 trimer affects thermostability

Thermostability is an important characteristic of SOSIP trimers (Sanders et al., 2013, Ward and Wilson, 2017) and may give some insight into their overall stability. We analysed the thermostability of 253-11 trimers with and without a His_{6x}-tag using the UNit system (Unchained Labs) and compared them to BG505 trimers with a (His)_{6x} tag.
Figure 3.4 Comparison of thermostability between 253-11 trimers, 253-11_His-tag trimers and BG505 trimers at different pH values. The conformational thermostability of the 253-11 trimers was measured using an intrinsic fluorescence measurement carried out using the UNit (Avacta). The trimers were in buffers of different pH values and their $T_m$ were measured by exposing the proteins to increasing temperatures and measuring the changes in fluorescence over time. Measurements were conducted in triplicate. BG505 trimers were used as a comparison of trimer stability. Thermostability was measured in different pH conditions: 50 mM Tris pH 9.0, 50 mM HEPES pH 7.0 and 50 mM sodium acetate pH 5.6. All buffers contained 150 mM NaCl.

The UNit system can simultaneously measure intrinsic fluorescence spectroscopy and static light scattering to provide information on changes in tertiary structure and aggregation, respectively (He et al., 2013). The trimer contains aromatic residues that are folded into the centre of the protein structure. These aromatic residues fluoresce when excited with UV light. As the protein denatures when exposed to increasing temperature from 20 to 90 °C, these core aromatic residues are increasingly exposed to UV light. The changes in fluorescence emission intensity (350/330 nm) of the protein are measured and a $T_m$ is determined. (He et al., 2013).

The 253-11 trimers appear to be more stable at higher pH values, with the greatest stability at pH 9 (Fig 3.4). Interestingly, the 253-11_histag trimer had higher thermostability overall compared to the 253-11 trimer. In comparison, the BG505 trimer (with a (His)$_{6x}$ tag) was consistently more thermostable than both 253-11 trimers and BG505 thermostability appears to be largely independent of pH. Despite the 253-11 trimer having a slightly lower $T_m$ compared to the gold-standard BG505 trimer, it is one of the most thermostable SOSIP trimers that has been described to date (Julien et al., 2015).
3.3.3 Expression and purification of 10-1074, VRC01 and PG9 Fabs

For our studies, we chose PG9, 10-1074 and VRC01 Fabs because the corresponding antibodies neutralised the 253-11 trimer potently (Fig 2.1) and therefore would be good markers for testing trimer antigenicity. These antibodies are also known to bind to the closed, pre-fusion conformation of the HIV-1 Env (Guttman et al., 2015, Julien et al., 2013b, Mouquet et al., 2012, Li et al., 2011) and therefore were good candidates for use in 253-11 trimer co-crystallization. Fabs were initially purified using anti-lambda or anti-kappa affinity columns depending on their light chains (Fig 3.5). PG9 and 10-1074 have lambda light chains while VRC01 has kappa light chains. PG9 and VRC01 both expressed very well and only 200 ml of culture was required to produced high yields of protein. 10-1074 expressed poorly and to obtain approximately the same amount of protein as the other Fabs, 800 ml of culture supernatant was required (Fig 3.5).

![Figure 3.5](image_url)

Figure 3.5 Representative affinity chromatography purification profiles and SDS-PAGE gels of 10-1074, VRC01 and PG9 Fabs. After expression in either 293F or 293S cells, the proteins underwent affinity chromatography as an initial purification step. Blue line: UV absorbance corresponding to protein elution; red line: elution buffer conductivity (a) 800 ml of 10-1074 Fab was purified through an anti-lambda column and eluted with 100 mM glycine, pH 2.7 (b) 200 ml VRC01 Fab was purified through an anti-kappa column and eluted with 100 mM glycine, pH 2.2 (c) 200 ml PG9 was purified through an anti-lambda column and eluted with 100 mM glycine, pH 2.7. The proteins were run on a SDS-PAGE either with β-mercaptoethanol (Reducing) or without (Non-reducing). Before: protein before affinity purification; After: protein after affinity purification. FT: flow-through from affinity column.
All the Fabs ran to a distinct band at ~50 kDa as expected in non-reducing conditions and reduced to two bands ~25 kDa as the light and heavy chain separated. Fabs then underwent ion-exchange chromatography to purify the proteins based on charge (Fig 3.6). The theoretical isoelectric point (pI) values of the Fabs are 8.4 for 10-1074, 8.0 for VRC01 and 7.9 for PG9. The pI is the pH value at which the protein carries no charge (Bjellqvist et al., 1993). Because of their pI values, we buffer exchanged the Fabs into a 20 mM sodium acetate buffer, pH 5.6 to ensure they had a positive charge to purify them on a cation exchange resin. A cation exchange resin is negatively charged and therefore binds positively charged proteins (Jungbauer and Hahn, 2009).

**Figure 3.6** Representative ion exchange and size-exclusion chromatograms for 10-1074, VRC01 and PG9 Fabs. (a) 10-1074, (b) VRC01 and (c) PG9 were run through a cation ion exchange resin to separate Fabs from impurities based on charge, followed by a Superdex 200 Increase 10/300 resin as a final purification step based on size. The purity throughout the steps was validated by SDS-PAGE in both reducing and non-reducing conditions. Numbers correspond to individual peaks and fractions of each of these peaks were tested on SDS-PAGE. **Blue line**: UV absorbance corresponding to protein elution; **red line in ion exchange chromatograms**: elution salt conductivity.

Fabs eluted out in multiple peaks with 10-1074 and VRC01 Fabs having separable peaks, likely as a result of glycan heterogeneity. This is likely because both Fabs contain one potential N-linked glycosylation site (PNG). Differences in the peaks were difficult to distinguish on the gels as the molecular weight of a glycan is only ~2 kD, which may be too small to differentiate
on SDS-PAGE. PG9 had multiple, overlapping peaks that were difficult to separate. The reason for the multiple peaks is likely caused by the presence of a variable number of sulphated tyrosine residues (McLellan et al., 2011). Tyrosine sulphation is a post-translational modification which occurs in the Golgi apparatus (Huttner, 1982). Since the process is variable, distinct PG9 proteins will have different charges which likely led to closely overlapping peaks on the chromatogram.

Fabs then underwent SEC for purification based on their molecular weight (Fig 3.6). For VRC01, both peaks from the ion-exchange were run separately through the SEC (chromatogram for peak 1 shown in Fig 3.6b) yielding one well separated peak. For PG9, however, pooled fractions from peaks 1-3 were run on SEC which produced 4 distinct peaks. On the PG9 SDS-PAGE gel after SEC, all four peaks corresponding to the expected size of the Fab were observed, highlighting micro-heterogeneity. The 10-1074 peak 3 from ion-exchange chromatography yielded a single pure peak after SEC. Once purified, Fabs were used for Fab-trimer binding studies. 10-1074 Fab was also used in crystallization trials.

3.3.4 The 253-11 trimer is a good antigenic mimic of its corresponding pseudovirus

To probe whether the 253-11 SOSIP trimer is a good antigenic mimic of the corresponding 253-11 pseudovirus, we tested binding of the three Fabs and two non-neutralising antibodies, 17b and F240, to the 253-11 trimer by biolayer interferometry (BLI) using the Octet RED96 system (Pall FortéBio). BLI is a label-free technology which measures intermolecular interactions through the interference of white light that is reflected from two surfaces: (i) a layer of immobilized protein (Fab or antibody) on an anti-Fab/antibody biosensor tip, and (ii) an internal reference layer (Shah and Duncan, 2014). Any change in the number of molecules bound to the sensors will cause an optical interference between the light reflected (Shah and Duncan, 2014). Binding between the immobilized Fab/antibody on the biosensor and the Env trimer in solution produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift (Shah and Duncan, 2014). Dissociation of the two proteins when the biosensor is moved from the wells with trimer to wells containing buffer will lead to a further shift (decrease) in the interference pattern (Shah and Duncan, 2014) (Fig 3.7a) This information is then processed and the binding kinetics analysed (Shah and Duncan, 2014). Using BLI, we tested the binding of 10-1074, VRC01, PG9, 17b and F240 with four 2-fold dilutions of the 253-11 trimer (Fig 3.7b).
Figure 3.7 Determination of Fab-trimer binding by biolayer interferometry. a) Principle of biolayer interferometry. Biosensors are dipped into kinetics buffer, followed by loading with Protein 1 (Fab/antibody). The Fab/antibody-covered biosensor is then dipped into a second baseline of buffer then transferred to Protein 2 (trimer) where association is measured. The biosensors are then moved to the buffer from the second baseline to measure the dissociation. (b) Representative plots for the association and dissociation between the VRC01, PG9 and 10-1074 Fabs and 17b and F240 antibodies. The dissociation constant (K_D) values are shown in nM and compared to the IC_{50} values from neutralisation assays shown in µg/ml. (c) On-rate (K_{on}) (1/Ms) and off-rate (K_{off}) (1/s) values for each of the three Fabs.

We found that the VRC01, 10-1074 and PG9 Fabs bound well to the trimer with K_D values of 11.8, 25 and 137 nM, respectively (Fig 3.7b), suggesting that the 253-11 trimers are well folded and correctly display broadly neutralising antibody (bnAb) epitopes including the V2/apex conformational epitope bound by PG9. The 253-11 trimer was not bound by antibodies 17b
and F240, which do not neutralise the 253-11 pseudovirus (Fig 3.7b). This strongly suggests that the trimer efficiently occludes the corresponding epitopes that are occluded on the pseudovirus.

The Fabs associated (K\text{on}) and dissociated (K\text{off}) very slowly to the Env trimers (Fig 3.7c). The on-rate (K\text{on}) refers to the rate at which the Fab binds to the trimer (association) and the off-rate (K\text{off}) refers to the rate of dissociation. The slow on-rates are likely partly attributable to the dense glycan shield of the HIV-1 Env which they need to penetrate to reach their epitopes. Once bound, the Fabs dissociated slowly and this is a characteristic which makes bnAbs more effective in neutralising HIV-1 as they remain bound to their target for longer periods of time (Klasse, 2014, Yasmeen et al., 2014)

3.3.5 Crystallization of the 253-11 SOSIP trimer in complex with 10-1074

To gain a further understanding of how Env compactness contributes to the neutralisation resistant phenotype of the 253-11 virus, we crystallized the 253-11 SOSIP trimer. HIV-1 trimers are generally extremely dynamic proteins (Guttman et al., 2015) which makes crystallization difficult and therefore we co-crystallized the 253-11 trimer with Fabs to stabilise it in its closed conformation as others have done previously (Stewart-Jones et al., 2016, Scharf et al., 2014, Julien et al., 2013a).

Although we set up crystallization trials with all three Fabs, we focused our efforts mainly on 10-1074. This was because the PGT121-family Fabs (which include 10-1074) have been the most successful in obtaining crystals for other SOSIP trimers (Gristick et al., 2016, Garces et al., 2015).

To increase our chances of crystallization we expressed 253-11 SOSIP.664 and 10-1074 in HEK293S cells, which enrich for more homogeneous, high-mannose type of N-linked glycans (Chaudhary et al., 2012). 10-1074 contains one PNG (glycosylation motif: N-P-S) in the heavy chain region of the sequence as predicted by NetNGlyc 1.0 Server (Gupta and Brunak, 2002). The presence of a proline between the asparagine and serine residues makes the probability of the addition of an N-linked glycan very low at this site (Kornfeld and Kornfeld, 1985), however, we still decided to treat the Fab with Endo H glycosidase to truncate the potential high mannose glycan (Trimble and Maley, 1984). We treated the Fab with Endo H prior to ion
exchange chromatography because separation by SEC would be difficult as deglycosylated Fab (~50 kDa) and glycosylated Fab (~52 kDa) are similar in size.

The purified 253-11 SOSIP.664 trimer was complexed with 10-1074 by adding a 6-fold molar excess of purified Fab. The complex was Endo H-treated to cleave the 253-11 SOSIP N-linked glycans unprotected by Fab binding to single N-acetylglucosamine (GlcNAc) moieties. Trimer deglycosylation was performed after 10-1074 bound to the trimer because 10-1074 binding is dependent on glycans at the base of the V3 loop, particularly the glycan at position N332 (Mouquet et al., 2012). Once deglycosylated, the trimer-Fab complex was run through a Superose 6 Increase (GE Healthcare) SEC column. This column was specifically chosen to efficiently separate a complex of larger size (~400 kDa) from unliganded Fab (~50 kDa). Once purified, there was a clear peak corresponding to the purified trimer-Fab complex and a peak corresponding to excess Fab (Fig 3.8). The trimer-Fab complex was extremely pure as shown by SDS-PAGE (Fig 3.8). Fractions corresponding to the trimer-Fab were pooled together and concentrated to ~5 mg/ml in a buffer containing 20 mM Tris pH 9.0 and 150 mM NaCl.

Figure 3.8 Representative chromatogram and SDS-PAGE for purification of 253-11 SOSIP trimer-10-1074 Fab complex. The 253-11-Fab complex was run through a Superose 6 Increase SEC resin as a final purification step. The trimer-Fab complex eluted out first followed by the excess unbound Fab. The purity of the proteins was assessed throughout the purification process by SDS-PAGE in both reducing and non-reducing conditions.

Crystallization screens, JCSG Top96 (Rigaku), Top96 Cryo (Rigaku) and MCSG 1, 2, 3 and 4 (Anatrace), were initially used, which are designed for high-throughput initial screening of crystallization and solubility conditions for biological macromolecules. These screens are formulated to maximize the coverage of crystallization parameters (buffers, salts, precipitant and pH) and eliminate redundancy. The crystallization method used was the vapour-diffusion method (Rhodes, 2006) where 0.1 µl 253-11 trimer-Fab complex was added to 0.1 µl precipitant from various crystallization screens in 96-well plates using an Oryx4 crystallization
robot (Douglas Instruments). In the vapour-diffusion method, the trimer and precipitant equilibrate with a larger volume of the same precipitant conditions (Rhodes, 2006). As equilibration occurs, the protein concentration increases and solutes move randomly in solution until they interact and form intermolecular forces (Rhodes, 2006). Under the right conditions, nucleation occurs. Nucleation is the process whereby solutes in a saturated solution transition into a crystal lattice (Rhodes, 2006).

Seven small and poorly diffracting crystals - best diffraction at 12 Å resolution - were obtained using synchrotron radiation at the Canadian Light Source. To optimise diffraction, we employed the microseeding method to increase the quality of initial crystals. The microseeding technique consists of transferring crushed crystal fragments (crystal seeds) generated from initial small crystals from one condition to a similar condition (Rhodes, 2006). As crystal seeds are already arranged in an ordered lattice, their presence eliminates the process of random collisions to form bonds as the solute will be drawn to the ordered lattice and start forming crystals around it (Rhodes, 2006). The newly formed crystals will then detach from the seeds after they reach an adequate size (Allahyarov et al., 2015). In our experiments, we performed three rounds of microseeding optimisation; each time using crystal seeds obtained from the previous crystallization trial. Upon microseeding, crystals grew within 3 days and much more crystals were obtained compared to without seeding.

To further optimize crystal diffraction, we dehydrated some of the crystals we obtained to remove excess solution, thereby improving crystal packing and diffraction (Russo Krauss et al., 2012). Prior to freezing, some crystals were dehydrated by the addition of 1 M NaCl for 4-6 hours or 48 hours or by air-drying for 2 minutes immediately prior to cryo-protection. X-ray diffraction data were collected at the 23-ID-D beamline at the Argonne Photon Source and the best crystal (out of 125 crystals screened) diffracted at 6.5 Å. (Table 3.1 and Fig 3.9).
Table 3.1. Crystallographic data collection and refinement statistics.

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Values in parentheses correspond to the highest resolution shell.
Data for the 253-11 trimer in complex with 10-1074 Fab was processed in the space group R3:H (Table 3.1). The space group describes the symmetry of the structure (Rhodes, 2006) and an R3:H space group means that the structure has a 3-fold symmetry across the axis. Based on the space group and unit cell parameters (a=237.3 Å, b=237.3 Å, c=284.3 Å; alpha=90°, beta=90° and gamma=120°), the Matthews Coefficient (Matthews, 1968) estimated that the asymmetric unit consisted of two molecules of the 253-11 trimer + 10-1074 Fab complex (Fig 3.9). The asymmetric unit is the smallest unit of a crystal that can be used to build the complete protein structure by application of symmetry (Rhodes, 2006). A unit cell is the smallest unit which makes up the complete structure of the protein (Rhodes, 2006). As expected, the biological assembly obtained after applying crystallographic symmetry consists of the 253-11 trimer bound by three 10-1074 Fabs (Fig 3.9). BG505 SOSIP.664 complexed with 10-1074 and IOMA (PDB ID: 53TX) was used to build the 253-11 structure by molecular replacement, as it is the only deposited trimer crystal structure in complex with 10-1074 (Gristick et al., 2016).

**3.3.5.1 Local gp41 compactness identified in heptad repeat helices of 253-11**

As expected, the 253-11 SOSIP.664 trimer adopted a closed, pre-fusion conformation with three 10-1074 Fabs protruding from the membrane distal gp120 subunits, contacting glycans at the base of the V3 loop (Fig 3.9 and Fig 3.10a). Prior to refinement, electron density maps revealed distinct density for gp41 heptad repeat (HR) 1 and 2 helices (Fig 3.10b and c). Compared to the BG505 SOSIP.664 trimer that was used as the search model in molecular replacement (PDB ID: 5T3X), the HR1 helices are slightly shifted inwards towards the trimer axis in 253-11 (Fig 3.10b). We note that the HR2 helices in gp41 are also displaced towards...
the trimer axis in 253-11 (Fig 3.10c), however, as has previously been reported (Guttman et al., 2014, Julien et al., 2013a, Lyumkis et al., 2013), this region is flexible in SOSIP constructs terminating at position 664 and is involved in crystal packing here. As such, we cannot conclude whether this observation highlights a specific attribute of the 253-11 Env or more likely, is induced by the arrangement of 253-11 SOSIP.664 trimers in the crystal lattice. As an internal reference, such differences in the disposition of helices between 253-11 and the BG505 trimer were not observed in gp120; all structural elements aligned well in this subunit (e.g. gp120 α1, Fig 3.10d).

Figure 3.10 Crystal structure of 253-11 SOSIP trimer in complex with 10-1074 Fab. (a) Side view of the crystal structure of 253-11 SOSIP trimer in complex with 10-1074 Fab. One of the protomers is highlighted. gp120 and gp41 are colored in yellow and cyan, respectively, and represented as surface and cartoon. The heavy chain (in black) and the light chain (in grey) of the 10-1074 Fab are represented as cartoon and are binding to the V3 base, specifically to the N332 glycan (represented as maroon spheres). Glycans for gp120 and gp41 are represented as spheres in yellow and cyan, respectively. (b and c) Bottom view of the 253-11 SOSIP.664 trimer crystal structure showing the Fo-Fc electron density map (green mesh) obtained after molecular replacement for (b) HR1 and (c) HR2. (d) Top view of the 253-11 SOSIP trimer showing the 2Fo-Fc electron density map (blue mesh) for the gp120 α1 helices. (e) A single 253-11 SOSIP.664 protomer represented as cartoon with gp120 (yellow) and gp41 (cyan) represented along the trimer axis (dashed black line). 253-11 is superimposed with the HR1 and HR2 helices of BG505 (PDB IDs: PDBs: 5T3X, 5CEZ, 4ZMJ, 5ACO, 5C7K, 5FYL, 5I8H, 5D9Q, 5THR, 5U1F, 5UTY, 5UTF, 5V8L and 5V8M); JR-FL (PDB: 5FYK); X1193.c1 (PDB: 5FYJ) and 16055 (PDB:5UM8) trimers (all shown as cartoon in grey color).

Comparison of the refined crystal structure of 253-11 with other Env trimer structures (PDBs: 5T3X, 5CEZ, 5FYK, 5FYJ, 4ZMJ, 5ACO, 5C7K, 5FYL, 5I8H, 5D9Q, 5THR, 5U1F, 5UTY, 5UTF, 5V8L, 5V8M and 5UM8) revealed that the 253-11 trimer complexed with 10-1074 Fab had HR helices that are more tightly positioned towards the trimer axis than all other solved HIV-1 trimer structures, except for BG505 SOSIP complexed with PGT128 and 8ANC195 Fabs (PDB: 5C7K) (Fig. 3.10e).
3.3.5.2 253-11 had smaller gp120 inter-protomer distances than other trimers

After molecular replacement, we also noted a clear difference in the disposition of the 253-11 gp120 protomers along the trimer axis. Rigid body refinement slightly re-positioned the gp120 protomers in closer proximity to the trimer axis. To investigate how compact gp120 protomers are within the 253-11 SOSIP.664 trimer compared to trimers of other sequence backbones, we calculated distances between the centre of masses of each of the three 253-11 gp120 protomers and compared the inter-protomer distances with other trimers for which the structures have been solved to date by X-ray crystallography and cryo-EM (Fig 3.11). Specifically, we compared gp120 inter-protomer distances for 253-11 and ligand-free BG505 trimer (PDB: 4ZMJ), BG505 complexed with Fabs (PDBs: 5T3X, 5CEZ, 4NCO, 3J5M, 4TVP, 5ACO, 5C7K, 5FYL, 5I8H, 5D9Q, 5THR, 5U1F, 5JS9, 5UTY, 5UTF 5V8L, 5V8M and 5UM8) and trimers derived from JR-FL (clade B) (PDB: 5FYK), X1193.c1 (clade G) (PDB: 5FYJ) and 16055 (clade C) (PDB: 5UM8) isolates (Fig 3.11).

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**Figure 3.11 gp120 compactness of 253-11 SOSIP.664 trimer.** (a) Top view of 253-11 SOSIP trimer showing the three gp120 protomers (yellow). The centre of mass (blue spheres) for each gp120 protomer was calculated using Pymol v1.8.6.0. The calculated distances between the protomers are represented as a blue line with the corresponding values. (b) Superposition of the centre of mass of each of the gp120 protomers for the 253-11 trimer (blue spheres) with 19 other SOSIP trimers. Yellow: X1193.c1; green: BG505 open trimer (PDB: 5THR); orange: JR-FL (PDB: 5FYK); pink: 16055 (PDB: 5UM8); grey: BG505 trimers (PDB IDs: 4ZMJ, 5T3X, 5CEZ, 4NCO, 3J5M, 4TVP, 5ACO, 5C7K, 5FYL, 5I8H, 5D9Q, 5U1F, 5JS9, 5UTY, 5UTF, 5V8L and 5V8M) (c) Individual gp120 inter-protomer
distances are shown for the different trimers from the smallest to the largest. 253-11 trimer is highlighted in blue. The 253-11 trimer PDB ID will be deposited into the Protein Data Bank upon publication.

253-11 had smaller gp120 inter-protomer distances compared to the clade B, C and G SOSIP trimers and had smaller or comparable gp120 inter-protomer distances as 13 of the BG505 structures (PDB IDs: 5ACO, 5CEZ, 5JSA, 5JS9, 5C7K, 3J5M, 5U1F, 4TVP, 4NCO, 5T3X, 5V8L, 5V8M). As an internal validation of our analysis, the CD4/17b/8ANC195-bound open structure of BG505 (PDB: 5THR) had the highest inter-protomer distances out of the trimers analysed (Fig 3.11c). Interestingly, engineered stabilisations in BG505 trimers (PDB: 5U1F, 5UTY, 5UTF) (Chuang et al., 2017, Liu et al., 2017) did not substantially affect distances between the three gp120 protomers. Taken together, our structural data support the hypothesis that the 253-11 Env sequence encodes for a generally compact structure that is linked to a highly neutralisation resistant phenotype for this virus.
3.4 Discussion

A global HIV-1 vaccine will likely need to protect against antibody neutralisation resistant viruses to effectively reduce HIV-1 incidence. Therefore, an understanding of the mechanisms employed by viruses to gain neutralisation resistance is critical. Most HIV-1 immunogen research focuses on closed, pre-fusion Env trimers to elicit bnAbs, however, immunisation studies with BG505 and other Tier 2 SOSIP trimers have largely yielded autologous responses (Sanders et al., 2015, de Taeye et al., 2015, Klasse et al., 2016) despite further modifications to increase trimer stability (Chuang et al., 2017, de Taeye et al., 2015) and trimer delivery through different methods such as nanoparticle presentation (Sliepen et al., 2015). Therefore, a further understanding of the mechanisms used by Tier 3 HIV-1 isolates to evade neutralising responses and the development of trimers based on these Tier 3 viruses may aid in the design of immunogens capable of eliciting bnAbs.

To gain insight into the structure of 253-11 Env, we constructed a 253-11 SOSIP.664 recombinant trimer. 253-11 SOSIP trimers predominantly adopt a closed, pre-fusion conformation that is comparable to BG505 SOSIP trimers. This indicates that the compactness phenotype we hypothesise for 253-11 is not a global structural characteristic but likely caused by local, discrete changes in secondary structure elements.

253-11 SOSIP trimers were thermostable and strikingly, addition of a (His)$_6$-tag at the C terminus increased the thermostability of the trimer. The (His)$_6$-tag has been used to incorporate trimers into liposomes and resulted in well-ordered, highly stable trimers which remain in a tight trimeric conformation over extended periods of time (Ingale et al., 2016). More studies focusing on the role of the (His)$_6$-tag in trimer stability should be performed because its addition to SOSIP trimers is likely useful in increasing thermostability.

The 253-11 SOSIP trimers were bound well by Fabs derived from antibodies which potently neutralise the virus and not by antibodies that don’t neutralise the virus. This indicates that the trimers likely resemble functional trimer spikes on the 253-11 pseudovirus. More antibodies may need to be tested to further ascertain that the 253-11 SOSIP trimer correctly displays the gp120-gp41 interface bnAb quaternary epitope and the fusion peptide bnAb epitope while occluding other narrowly- and non-neutralising epitopes.
Unlike the crystallization of small, homogenous and less complex proteins, HIV-1 Env trimer crystallization is generally challenging (Ward and Wilson, 2017). This is because of dense glycosylation, protein flexibility and the overall size of the trimer (~250 kDa) (Ward and Wilson, 2017). Therefore, 253-11 trimer crystallization required numerous preparatory and optimisation steps. The trimer needed to be expressed in HEK293S cells to ensure it contained high-mannose glycans, which were subsequently cleaved by the Endo H glycosidase. Crystallization of unliganded trimers is also challenging and therefore the 253-11 trimer was complexed with a Fab in the hope that it would increase the stability of the closed, pre-fusion conformation. To further improve crystal diffraction, microseeding and dehydration techniques were also used.

We crystallized the trimer in complex with 10-1074 Fab. This antibody is of particular interest in HIV-1 research because in a recent human trial to determine whether it can therapeutically control the virus in HIV-1-infected individuals, 10-1074 suppressed viremia in participants for an extended period of time (Caskey et al., 2017) and therefore is likely a good therapeutic vaccine candidate. The crystal structure of the 253-11 trimer in complex with 10-1074 Fab indicated that the gp41 HR1 and HR2 helices are in a more compact disposition relative to the trimer axis than most trimer structures solved to date. In addition, our data support a molecular mechanism of interconnectedness between inward movements in gp41 HR helices and relatively small distances between gp120 protomers for 253-11. Together, this suggests a compact phenotype that co-exists with the high neutralisation resistance of this virus. We hypothesize that the 253-11 MPER may be less accessible because of the inward disposition of the gp41 HR helices and may in part explain why 253-11 is so resistant to anti-MPER antibodies prevalent in sera. We cannot address this point directly because the MPER is not present in SOSIP constructs.

One of the limitations of the study was the medium-resolution obtained from the 253-11 SOSIP trimer crystals. A high-resolution structure would have allowed us to observe the trimer at an atomic level. Since we were limited to secondary structure analysis, we may be missing key atomic information which could be contributing to the neutralisation resistance phenotype of the 253-11 virus.

To date, few published SOSIP trimers based on neutralisation resistant viruses have been engineered. One example is PVO.4 (Verkerke et al., 2016), which is more neutralisation
sensitive than 253-11 when tested for sensitivity to a panel of Subtype C sera (Jacob et al., 2015). The development of a SOSIP trimer which contains the neutralisation resistance and Env compactness properties of the 253-11 virus thus enables future studies of Tier 3 Env structure and immunogenicity. The use of trimers has so far proven ineffective in producing broad responses (Klasse et al., 2016) but a compact Tier 3 trimer may overcome this problem (Cai et al., 2017).

Most of the SOSIP trimers which have been engineered are derived from transmitter/founder (TF) viruses (Julien et al., 2015, Verkerke et al., 2016, deCamp et al., 2014, Sanders et al., 2013). The 253-11 virus was isolated in chronic infection at Fiebig Stage VI (Seaman et al., 2010). SOSIP trimers based on chronic viruses, CAP45 and CH505, failed to produce good quality trimers (Julien et al., 2015) which further illustrates that producing SOSIPs from chronic infection is challenging. The 253-11 SOSIP trimer, being one of a few based on a virus from chronic infection, can therefore also shed light on the structure of chronic viruses and may be an invaluable tool in HIV-1 pathogenesis studies.

In summary, we present evidence that 253-11 is particularly resistant to antibody neutralisation, which coincides with an unusually compact, closed Env structure. The ability of a virus to escape from all but the most broad antibody responses through relatively discrete structural characteristic has strong implications for understanding how viruses might circulate in a population of individuals who have moderate antibody responses induced by a future vaccine.
Chapter 4: Role of HIV-1 V3/glycans in breadth, potency and resistance to broadly neutralising antibody responses

Summary

The HIV-1 Envelope (Env) V3/glycans form a protective “glycan shield, protecting the V3 loop and CD4-binding site (CD4-bs) from antibody neutralisation. However, some broadly neutralising antibodies (bnAbs) have evolved to target this region making it an attractive vaccine target. Understanding the role of the V3/glycans in shielding antibody epitopes in natural infection and how some viruses have evolved to maintain their neutralisation resistant phenotypes in the absence of certain glycans may provide insight into HIV-1 evolution and pathogenesis and may be important in the design of a glycan-based immunogen.

We, therefore, investigated two aspects of the V3/glycan epitope. The first aspect explored whether there is an association between antibody breadth and potency and the presence of V3/glycan-specific antibodies in sera. There were no previously published systematic studies of specific epitopes being preferentially targeted by commonly-occurring, moderately neutralising antibodies found in sera. Therefore, we measured the relationship between the presence of V3/glycan-specific antibodies and neutralisation breadth and potency, and compared this to V2/apex-specific antibodies. We screened 177 sera samples from chronically HIV-1-infected individuals to assess their breadth and potency. We then explored which sera recognised the V3/glycan-site using N301A.N332A mutations and the V2/apex using N160A/K and I/K169E mutants in three viral isolates. We found that recognition of the V3/glycans was independently associated with breadth and potency, while recognition of the V2/apex was not.

Secondly, upon observing this important association, we investigated how the V3/glycans may be contributing to antibody neutralisation resistance in HIV-1 isolates. We examined the role of key glycans at the base of the V3 loop (N301, N332 and N334) in maintaining the integrity of the glycan shields of two subtype C viruses, CAP45.2.00.G3 (CAP45.G3) and Du156.12. We considered that these glycans are themselves targets of neutralising antibodies and that may select for their loss, while simultaneously antibodies targeting epitopes blocked by the V3/glycans may select for their retention. The removal of the N301 glycan from Du156.12
exposed epitopes recognised by neutralising antibodies in 28% of sera tested, whilst removal of the same glycan from CAP45.G3 resulted in exposure of epitopes recognised by neutralising antibodies in only 5% of the sera tested. This suggests that removal of the N301 glycan did not substantially affect the integrity of the glycan shield in CAP45.G3 but did in Du156.12. The different roles of the N301 glycan in the preservation of the glycan shields of CAP45.G3 and Du156.12 were further observed with differences in susceptibility to anti-CD4-binding site monoclonal antibodies upon removal of the glycan in Du156.12 compared to CAP45.G3.

Together, these data show that differences in the contribution of the glycan at N301 in overall resistance to antibodies commonly elicited in HIV-1-infected individuals differs between viruses. While V3/glycan-specific antibodies may select for loss of the N301 glycan, the cost in neutralisation sensitivity of this selection may be very low in some viruses, as we observed for CAP45.G3, yet substantially higher in others, as we observed for Du156.12. The efficacy of passively-infused antibody therapies or a vaccine based on the V3/glycan target may be affected by this phenomenon.
4.1 Introduction

The V3/glycans and the V2/apex are amongst the targets of broadly neutralising antibodies (bnAbs) (Moore et al., 2017, Doores, 2015, Mouquet, 2014, Burton et al., 2012). These sites are attractive vaccine targets and efforts to elicit antibodies towards them is ongoing (Moore et al., 2017, Sanders and Moore, 2017, Zhou et al., 2014, Alam et al., 2017). The V2/apex is a quaternary epitope which usually (Moore et al., 2017, Doores and Burton, 2010) but not always (Doria-Rose et al., 2015) relies heavily on stabilisation by the glycan at N160. Antibodies which target the V3/glycan epitope depend heavily on the presence of the N301 and/or N332 glycans (Walker et al., 2011, Doores, 2015), although some of this class of antibodies are able to recognise other glycans, particularly PGT121 (Sok et al., 2014). Most N332-dependent antibodies also rely on the underlying V3 loop $^{324}$GDIR$^{327}$ motif for binding (Garces et al., 2015, Garces et al., 2014, Sok et al., 2016).

Some bnAbs targeting the V3/glycans and membrane proximal external region (MPER) – another bnAb target - have been found to be self-reactive (Haynes et al., 2005, Liu et al., 2015, Verkoczy and Diaz, 2014). V3/glycan-specific antibodies such as PGT125 and PGT128 were shown to be polyreactive – recognising multiple self-proteins with low affinity (Liu et al., 2015) whilst MPER antibodies such as 2F5, 4E10 and 10E8 are autoreactive – binding to a discrete number of self-proteins with high affinity (Liu et al., 2015, Haynes et al., 2005). Liu et al. characterised 4E10 as polyreactive although Haynes et al., characterised it as autoreactive. Very few bnAbs which target the V2/apex have been shown to be autoreactive. In the study by Liu et al., only 1 out of 5 V2/apex-specific antibodies tested showed evidence of polyreactivity (Liu et al., 2015).

Self-reactivity may be a barrier in the production of bnAbs, as self-reactive B cells are thought to be frequently deleted from the repertoire (Haynes et al., 2005, Verkoczy and Diaz, 2014). However, despite the autoreactivity of particular MPER-specific antibodies, the presence of antibodies recognising the MPER in natural infection is associated with higher neutralisation breadth and potency (Jacob et al., 2015). Therefore, an investigation of an association between breadth and potency and targeting other sites such as the V3/glycans (with known polyreactivity) and the V2/apex (with very little polyreactivity) is of interest.
Although the glycans at the base of the V3 loop (N301, N332, N334) are targets of bnAbs (Walker et al., 2011), they also block underlying and distal epitopes, contributing to the HIV-1 Env “glycan shield” (Wei et al., 2003, Pantophlet and Burton, 2006, Zolla-Pazner et al., 2015). The N301 glycan at the base of the V3 loop protects distal epitopes from antibody-mediated neutralisation such as the CD4-binding site (CD4-bs) and protects underlying, highly neutralisation sensitive, V3 loop epitopes (Binley et al., 2010, Koch et al., 2003, Zolla-Pazner et al., 2015).

Glycans at positions N332 and N334 are mutually exclusive due to the overlapping N-X-T/S (where X ≠ P) motif (Marshall, 1972). The N332 glycan is recognised by anti-V3/glycan bnAbs including the PGT-family of antibodies (Walker et al., 2011), 10-1074 (Mouquet et al., 2012) and 2G12 which binds this site distinctly from the rest and is almost exclusively dependent upon direct binding to the glycan (Trkola et al., 1996, Sanders et al., 2002). Some moderately-neutralising precursors of PGT121-123 bnAb lineages depend on both glycans at positions N301 and N332 for binding, while the mature bnAbs gain higher affinity for the N332 glycan (Sok et al., 2013). This suggests that antibody dependence on glycans is a dynamic process, changing with the evolution of those antibodies. N332-dependent antibodies are sometimes able to bind to other glycans in the absence on the N332 glycan, including the N334 glycan (Sok et al., 2014).

Antibodies which recognise epitopes shielded by the V3/glycans may select for viruses which express the shielding glycans whilst V3/glycan-specific bnAbs may select for viruses which lack these glycans. There is little known about how glycan shield construction affects contemporaneous antibody-mediated selection against or for the glycans on the V3 loop. Therefore, in this chapter we first investigated whether there is an association between breadth and potency and the targeting of the V3/glycans and the V2/apex in sera from a cohort of antiretroviral therapy (ART)-naïve, chronically HIV-1-infected individuals. This study sheds light on whether polyreactivity would limit the breadth and potency of antibody responses towards the V3/glycans as opposed to the V2/apex. Previously, targeting the V2/apex was not found to be associated with breadth and potency, however, that study only mapped V2/apex responses using a single virus (Jacob, 2014) and therefore we aimed to perform a more robust analysis by using 3 viruses altogether to map V2/apex-specific responses. Using 3 viruses...
increased the proportion of sera that could be mapped. Sera can only be mapped if they neutralise the parent virus because a reduction in neutralisation after mutating out the epitope is what must be observed to map sera to the epitope. We, then investigated the role of the V3/glycans at positions N301, N332 and N334 on antibody neutralisation resistance to understand the balance between the role of glycans in shielding antibody responses and being bnAb targets.
4.2 Materials and Methods

4.2.1 Sera samples

Sera samples from chronically (>1-year) HIV-1-infected participants, >18 years of age from Cape Town were collected from December 2009 to July 2011. Participants were ART naive, except for ART given transiently to participants for prevention of mother-to-child transmission >3 months before recruitment. Participants who were recruited were either (i) caregivers at the paediatric HIV Clinic in Groote Schuur Hospital, Cape Town or (ii) patients at the HIV Wellness Clinic in Site B clinic, Khayelitsha, Cape Town. Written, informed consent was obtained from the participants and the study was approved by the Human Research Ethics Committee, University of Cape Town.

4.2.2 Pseudovirus constructs, monoclonal antibodies and cell lines

Cloned HIV-1 envelope constructs were obtained through the National Institute of Health, AIDS Reagent Program, except for Du156.12 wild type (WT), N301A, N160K, K169A and CAP45.2.00.G3 WT (herein CAP45.G3), N160A and K169E, which were provided by Drs. E. Gray, P.L. Moore and L. Morris, South Africa. Mutants of QH343.21M.ENV.A10 (herein QH343.A10) (N301A, N301A.N332A, N160A, I169E), of CAP45.2 (N301A), of Du156.12 (N301A.N332A) and of 253-11 (N301A) were generated using the GeneArt® Site-Directed Mutagenesis PLUS Kit (Invitrogen). V3/glycan mutants of CAP45.G3 and Du156.12 expressing different combinations of potential N-linked glycosylation sites (PNGs) at N301, N332 and N334 were similarly produced by site-directed mutagenesis. Amino acids were mutated as follows: PNG 301 removal: N301A; PNG 332 removal, N332A; PNG 334 removal: N334A; PNG 334 shift to PNG 332 for CAP45.G3: N334T; PNG 332 shift to PNG 334 for Du156.12: S334N.N336T. Successful mutagenesis and the absence of unintended mutations during PCR were confirmed by sequencing both strands of the entire env open reading frame. Monoclonal antibodies (mAbs) PG9 and 10E8 were obtained from Polymun Scientific, Austria and from the National Institute of Health, AIDS Reagent Program. mAbs NIH45-46G54W (herein NIH45-46), 3BNC117, VRC01, b12, soluble CD4 (sCD4), 2219, 3869 and 3074, as
well as the pSG3Δenv HIV-1 backbone plasmid, human embryonic kidney (HEK) 293T and TZM-bl cells were obtained from the National Institute of Health, AIDS Reagent Program.

**4.2.3 Pseudovirus generation and antibody-based neutralisation assays**

HIV-1 pseudoviruses were made by co-transfection of HIV-1 env and pSG3Δenv plasmids in HEK 293T cells as previously described (Montefiori, 2005). After 48 hours, supernatant containing pseudovirus was harvested and frozen at -80°C in single use aliquots. Antibody neutralisation assays were performed as previously described (Montefiori, 2005). Briefly, antibody and virus were incubated for one hour at 37°C, and then 10⁴ TZM-bl cells were added per well to the antibody-virus combination and incubated at 37°C for 48 h. Titers (dilution of serum [ID₅₀] or concentration of mAb [IC₅₀] that inhibits 50% of infection) were calculated using curve fit functions in Prism 5 (GraphPad). The Chi-square (χ²) test was used to test the statistical significance of differences in proportions between sera in particular groups.

**4.2.3 Neutralisation breadth and potency comparison between sera targeting HIV-1 Envelope epitopes**

Breadth and potency of 177 sera from our cohort was determined using a 24-virus panel (Jacob et al., 2015) consisting of Tier 2 and neutralisation resistant Tier 3 viruses (Seaman et al., 2010, Blish et al., 2009). A broadly neutralising sample was defined as such if it neutralised ≥18/24 viruses at ID₅₀≥100 and a potently neutralising sample was defined by a geometric mean ID₅₀≥220 for the 24-virus panel (Jacob et al., 2015). Aggregated breadth and potency values for sera which targeted the three epitopes (V3/glycans, V2/apex or MPER) was calculated and summarised by means of ratios (for potency) and differences (for breadth) and was compared to sera that did not map to those sites. Wilcoxon rank sum analysis was used to determine distribution differences between breadth and potency. Bootstrap estimation (Hastie et al., 2002) was used for estimation of the confidence intervals between the differences and ratios calculated; an estimation of the error in the determination of each serum’s neutralisation breadth and potency was included. Multivariate analysis was conducted using a linear regression model on Stata/IC v13.1 (StataCorp).
4.3 Results

4.3.1 Determination of the frequency of sera targeting the V3/glycans and V2/apex epitopes

In a previous study from our research group, the frequency of anti-MPER antibodies in our cohort was determined by mapping 177 sera with HIV-2/HIV-1 chimeric viruses (Jacob et al., 2015). The constructs used were made with a 7312A HIV-2 backbone and an HIV-1 MPER from either the consensus C sequence, C1C (Gray et al., 2007a), Yu2 MPER sequence, C1 (Gray et al., 2007a, Binley et al., 2008) or the MPER from a CRF02_AG virus, 253-11 (Jacob, 2014) (Fig 4.1a). Previous studies have shown that an ID$_{50}$>1000 obtained when sera are tested with these constructs is associated with the presence of anti-MPER activity in the samples (Gray et al., 2007a, Binley et al., 2008, Gray et al., 2009a, Jacob et al., 2015). Nineteen percent (33/177) of serum samples recognised one or more of the 3 chimeric viruses (Jacob et al., 2015) (Fig 4.1b, Table 4.1).

We investigated the frequency of antibodies which recognise the V2/apex and the V3/glycans. To map antibodies targeting the V3/glycans and V2/apex, we introduced mutations to residues critical for formation of these epitopes. These mutations abrogate antibody binding to these sites. Mutational mapping determines which epitope is targeted by the dominant antibodies present in a serum sample by detecting a substantial drop in neutralisation in the mutant virus compared to its unmutated parent. For the V2/apex, we introduced N160A/K mutations to prevent addition of the key, stabilising glycan at N160 (Doores and Burton, 2010) and also introduced I/K169E mutations (Tomaras et al., 2008, Moore et al., 2011) (Fig 4.1a). These mutations were introduced into three viruses: subtype C, Tier 2 viruses CAP45.G3 (tested in (Jacob, 2014), Du156.12 and subtype A virus, QH343.A10. QH343.A10 has not been categorised into a tier, however, studies from our laboratory (Moyo, 2014) and others (Blish et al., 2009) have shown that it is highly neutralisation resistant to subtype C and other serum panels.

The effect of mutations in one site upon neutralising epitopes in distal sites has been somewhat well studied for anti-HIV neutralisation. For example, an N160A mutation in the V2/apex marginally reduces neutralisation (2-3-fold difference) of some anti-V3/glycan monoclonal antibodies (Walker et al., 2011). In another report, Wang and colleagues found that an N301Q
mutation in the V3/glycan region induced only marginal drops (up to 2-fold) in neutralisation by a series of bnAbs, including PG9 and PG16 which target the V2/apex (Wang et al., 2013). Another study found that antibodies that recognise the V2/apex were unaffected by N332A mutants (Tomaras et al., 2011). Because drops in neutralisation due to mutations outside of the antibody’s epitope were mostly <3-fold in these studies, we chose a >3-fold threshold in our study.

Figure 4.1 Mapping responses to the MPER, V2/apex and V3/glycans. (a) Schematic of approach used to map sera to the three bnAb sites. C1, C2, C3: constant region 1, 2, 3; V1, V2, V3: Variable loop regions 1, 2, 3; HR2: Heptad Repeat 2; TM, transmembrane domain; CT, cytoplasmic tail. (b) Distribution of MPER-specific responses towards the three MPER chimeric viruses. Gray shading indicates MPER-positive samples (log scale). (c, d, e) Distribution of fold drops in neutralisation from WT to (c) N160A/K mutants (d) K/I169E mutants (e) N301A or N301A.N332 mutants. Gray shading indicates (c, d) V2/apex-specific and (e) V3/glycan-specific hits. (Note: MPER data from (Jacob et al., 2015)).
Twenty-nine percent (34/118) of sera were mapped to the V2/apex epitope (recognized ≥1 mapping virus) (**Fig 4.1c and d, Table 4.1**). Interestingly, 10 samples neutralised the samples better on removal of the N160 PNG (i.e. fold drop ≤ 0.33) (**Fig 4.1c**). It is likely that the removal of the N160 glycan exposed underlying epitopes recognised by antibodies present in these samples.

To map for V3/glycan-specific antibodies, we constructed double mutants of N301A.N332A to prevent the addition of these two key glycans; one or both of which is necessary for neutralisation by every characterised V3/glycan-specific mAb (Walker et al., 2011, Mouquet et al., 2012, Sanders et al., 2002) (**Fig 4.1a**). These mutations were introduced into the same WT viruses as for V2/apex mapping. CAP45.G3 does not possess a PNG at N332 and therefore a single N301A mutant was engineered. Nineteen percent (21/113) of sera samples from our cohort recognised at least one of the three viruses through targeting the V3/glycans (**Fig 4.1e, Table 4.1**).

Table 4.1 Comparison of the likelihood of being a broadly or potently neutralising antibody depending upon target recognition of neutralising antibodies

<table>
<thead>
<tr>
<th>Category</th>
<th>Less potent a</th>
<th>Potently neutralizing b</th>
<th>Relative Risk (95% CI)</th>
<th>p value ($\chi^2$)</th>
<th>Less broad c</th>
<th>Broadly neutralizing d</th>
<th>Relative Risk (95% CI)</th>
<th>p value ($\chi^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti –MPER neg</td>
<td>124</td>
<td>20</td>
<td>1.00 (reference)</td>
<td>122</td>
<td>22</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Anti-MPER pos</td>
<td>24</td>
<td>9</td>
<td>1.96 (0.99, 3.91)</td>
<td>0.061</td>
<td>23</td>
<td>10</td>
<td>1.98 (1.04, 3.78)</td>
<td><strong>0.043</strong></td>
</tr>
<tr>
<td>Anti-V2/glycan site neg</td>
<td>63</td>
<td>21</td>
<td>1.00 (reference)</td>
<td>62</td>
<td>22</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Anti-V2/glycan site pos</td>
<td>29</td>
<td>5</td>
<td>0.59 (0.24, 1.43)</td>
<td>0.222</td>
<td>27</td>
<td>7</td>
<td>0.79 (0.37, 1.67)</td>
<td>0.522</td>
</tr>
<tr>
<td>Anti-V3/glycan neg</td>
<td>75</td>
<td>17</td>
<td>1.00 (reference)</td>
<td>73</td>
<td>19</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Anti-V3/glycan pos</td>
<td>12</td>
<td>9</td>
<td>2.32 (1.21, 4.46)</td>
<td><strong>0.017</strong></td>
<td>12</td>
<td>9</td>
<td>2.08 (1.10, 3.92)</td>
<td><strong>0.033</strong></td>
</tr>
</tbody>
</table>

a Geometric mean ID$_{50}$<220  
b Geometric mean ID$_{50}$>220  
c <18/24 panel viruses neutralized  
d ≥18/24 panel viruses neutralized  

(Note: MPER data from (Jacob et al., 2015)).

A ≥3-fold drop in neutralisation from WT to mapping mutant was used as the threshold to determine whether a serum sample contained dominant antibodies towards that site, similar to the threshold used by others (Gray et al., 2011a). Therefore, since the sera had to initially
neutralise the WT virus to be mapped, only the subset of the 177 samples which neutralised at least one of the 3 WT viruses with ID\textsubscript{50} \geq 100 were analysed.

### 4.3.2 Assessment of the association between breadth and potency and targeting the V3/glycans and V2/apex

The 177 sera samples from our cohort were tested for breadth and potency. 18% were found to be broad (neutralised ≥18/24 viruses) and 16% were potent (geometric mean ID\textsubscript{50} ≥ 220) with a strong correlation between breadth and potency (Jacob et al., 2015). Using these samples, the presence of anti-MPER antibodies was found to be associated with breadth and potency (Jacob et al., 2015, Jacob, 2014). We therefore, investigated whether there was any association between the presence of anti-V3/glycan or anti-V2/apex antibodies and breadth and potency (Fig 4.2, Table 4.1).

**Figure 4.2 Neutralisation breadth and potency differences between sera targeting particular epitopes.** Comparisons of the distribution of neutralisation (a, c, e) breadth and (b, d, f) potency are shown for (a, b) MPER positive and negative sera, (c, d) anti-V3/glycan positive and negative sera and (e, f) V2/apex positive and negative sera. \(p\) values obtained from Wilcoxon rank sum tests. (Note: MPER data from (Jacob et al., 2015)).
Sera with dominant V3/glycan-specific antibodies were broader and more potent than sera which were not demonstrably V3/glycan-specific (Fig 4.2 c, d). Using a bootstrap-based method to include an estimate of the error in determining the breadth and potency of individual sera, we found that sera with dominant anti-V3/glycan antibodies on average neutralised 3.24 more viruses and were on average 1.68-fold more potent (Fig 4.3) than sera without V3/glycan antibodies. They were twice as likely to be highly broadly neutralising and 2.32 times more likely to be highly potent than V3/glycan antibody-negative sera (Table 4.1).

![Figure 4.3](image-url) Neutralisation breadth and potency changes between the three epitopes. Changes in (a) breadth and (b) potency were investigated for positive and negative samples within one epitope and between different epitopes. Differences in breadth were calculated by subtraction of viruses neutralised while differences in potency were calculated by ratios of geometric mean ID_{50} values. No difference between number of viruses neutralised (breadth) = 0; no difference between geometric mean ID_{50} values (potency) =1. A 95% prediction interval was calculated for all the values by bootstrap-based error estimation. (Note: MPER data from (Jacob et al., 2015)).

In contrast, we did not observe an association between the presence of V2/apex-specific antibodies in sera and neutralisation breadth and potency. Sera containing dominant V2/apex antibodies were not more broad or potent than sera which did not contain these antibodies (Fig
4.2e, f). V2/apex-positive samples only neutralised a negligible number of panel viruses (0.38 more viruses) as compared to V2/apex negative samples (Fig 4.3). V2/apex-specific samples were not more potent that negative samples either (0.98-fold difference) (Fig 4.3).

A multivariate analysis was conducted to investigate which of the three epitopes was independently associated with breadth and potency (Table 4.4). For breadth, the regression coefficients represent how many more viruses are neutralised by antibodies targeting either the V3/glycans, V2/apex or MPER compared to antibodies that do not target these sites (without the bootstrapping or error estimation that was included in the values in Fig 4.3). For potency, the regression coefficients represent how much more potent (defined by geometric mean ID$_{50}$ values for neutralisation of the 24-virus panel) a sample is if it targets one of the 3 epitopes as compared to antibodies that do not. If the 95% confidence interval crosses over zero, it means there is no significant difference in breadth and potency between the antibodies targeting that site and those which do not. Strikingly, of the three targets tested, only the V3/glycans were independently associated with neutralisation breadth and potency in the multivariate analysis (Table 4.2).

### Table 4.2 Multivariate analysis of association of breadth and potency with targeting the V3/glycans, V2/apex and MPER

<table>
<thead>
<tr>
<th></th>
<th>Regression Coefficient (95% confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V3/glycans</td>
<td></td>
</tr>
<tr>
<td>Breadth</td>
<td>3.13 (0.46 - 5.79)</td>
<td><strong>0.022</strong></td>
</tr>
<tr>
<td>Potency</td>
<td>70.1 (1.01 - 139)</td>
<td><strong>0.047</strong></td>
</tr>
<tr>
<td></td>
<td>V2/apex</td>
<td></td>
</tr>
<tr>
<td>Breadth</td>
<td>0.05 (-2.22 - 2.32)</td>
<td>0.965</td>
</tr>
<tr>
<td>Potency</td>
<td>-27.9 (-86.6 - 30.8)</td>
<td>0.349</td>
</tr>
<tr>
<td></td>
<td>MPER</td>
<td></td>
</tr>
<tr>
<td>Breadth</td>
<td>1.18 (-1.32 - 3.68)</td>
<td>0.35</td>
</tr>
<tr>
<td>Potency</td>
<td>15.4 (-49.3 - 80.2)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

As our results show, targeting the V3/glycans, and not the V2/apex, is associated with breadth and potency in natural infection. In addition to being targeted by bnAbs (Walker et al., 2011) and being amenable to scaffold immunogen design (Zhou et al., 2014), our data further suggests that the V3/glycans are an achievable vaccine target. The fact that targeting the V3/glycans is associated with breadth and potency, suggests that these antibodies are commonly broad and potent in unselected sera and that it may be easier to elicit broad and potent antibodies to the V3/glycans after vaccination.
However, this epitope is not only recognised by bnAbs but also serves to protect HIV-1 Env from neutralisation, forming a “glycan shield” (Ward and Wilson, 2017, Wei et al., 2003). Therefore, we investigated the interplay between the selection of glycans which block antibody epitopes and those which are recognised by bnAbs to better understand how this may drive V3/glycan evolution in viral escape variants.

4.3.3 N301 glycan plays a key role in glycan shield integrity of Du156.12 and not in CAP45.G3

We investigated the role of the N301 glycan in maintaining glycan shield integrity in two viruses, CAP45.G3 and Du156.12. These two viruses were chosen because they are similar in many ways. They are both subtype C, CCR5-dependent viruses obtained from acute infection in female donors from KwaZulu-Natal, South Africa (Li et al., 2006, Williamson et al., 2003). They are both Tier 2 viruses (Seaman et al., 2010) but differ in their glycan shield composition. CAP45.G3 has PNGs at positions N301 and N334 whilst Du156.12 has PNGs at positions N301 and N332. To understand whether these differences affect the maintenance and protective function of their glycans shields we made viral mutants with all three glycan combinations (N301, N332 and N334) (Table 4.3).

Table 4.3. Nomenclature used for pseudovirus mutants containing different combinations of glycans at positions 301, 332 and 334

<table>
<thead>
<tr>
<th>Virus Name</th>
<th>PNG 301</th>
<th>PNG 332</th>
<th>PNG 334</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP45\textsuperscript{301,332}</td>
<td>Du156\textsuperscript{301,332*}</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>CAP45\textsuperscript{301,334*}</td>
<td>Du156\textsuperscript{301,334}</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>CAP45\textsuperscript{301}</td>
<td>Du156\textsuperscript{301}</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>CAP45\textsuperscript{332}</td>
<td>Du156\textsuperscript{332}</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>CAP45\textsuperscript{334}</td>
<td>Du156\textsuperscript{334}</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\*wild-type virus

We tested these viral mutants with 64 randomly-selected sera from our cohort of chronically HIV-1-infected, ART-naïve participants to see how viral sensitivity shifts on removal or substitution of glycans. An increase in neutralisation sensitivity from one glycan variant to another was defined as a $\geq 3$-fold increase in ID\textsubscript{50}, while a decrease in neutralisation was defined as a decrease in ID\textsubscript{50} of $\geq 3$ fold, a threshold others have previously used (Gray et al., 2011a). An increase in neutralisation sensitivity of a glycan-deleted variant was interpreted to be a...
result of exposure of epitopes upon the removal of the glycan whilst a decrease in neutralisation sensitivity was interpreted to indicate that the removed glycan formed a key part of the epitope recognised by dominant antibodies in that serum sample.

Strikingly, on removal of the N301 glycan from CAP45.G3 and Du156.12, Du156.12 was significantly more sensitive to neutralisation by a larger proportion of sera \( (p<0.0005, \chi^2 \text{ test}) \) as compared to CAP45.G3 (Fig 4.4). Du156.12 became more sensitive to neutralisation by 18/64 (28%) of sera upon removal of the glycan at position N301 whilst CAP45.G3 was only sensitive to 3/64 sera samples tested upon removal of the N301 glycan suggesting that the dependence on the glycan at N301 for neutralisation resistance differed between the two viruses.

Figure 4.4 Removal of the N301 glycan increase neutralisation sensitivity of Du156.12 WT but not CAP45.G3 WT. Neutralisation of Du156.12*301,332 and CAP45.301,334 WT viruses and mutants without the N301 glycan was tested with 64 sera samples from HIV-1-infected individuals. Individual lines of the graph correspond to the change in sensitivity of one serum sample. Increases in neutralisation are shown in red lines and decreases in neutralisation are shown in blue lines. No change in neutralisation is shown in grey lines. The \( p \) value represents the difference in proportion of sera which Du156.12*301,332 and CAP45.301,334 had increased sensitivity to upon mutating out the N301 glycan.

4.3.4 Removal of the N301 glycan increases Du156.12 neutralisation sensitivity irrespective of the presence of an N334 or N332 glycan

To investigate whether the naturally-occurring N334 glycan in CAP45.G3 was compensating for the loss of the N301 glycan, we shifted the glycan from position N332 to N334 in Du156.12
to create Du156$^{301,334}$. The N301 glycan was removed from both viruses containing N334 glycans and the changes in sensitivity to sera were found to be similar as those of the WT viruses (Fig 4.5). Du156$^{301,334}$ was still significantly more sensitive than CAP45$^{301,334*}$ WT to antibody neutralisation upon removal of the N301 glycan, with increased sensitivity to 19/51 sera samples tested, despite the presence of the N334 glycan (Fig 4.5).

Figure 4.5 N332 to N334 shift does not restore integrity of glycan shield upon removal of N301 glycan in Du156.12. Differences in neutralisation sensitivity between Du156$^{301,334}$ and CAP45$^{301,334*}$ (both viruses possessing glycans at position N334) with and without the N301 glycan were investigated by testing the viruses with 51 (Du156$^{301,334}$) and 64 (CAP45$^{301,334*}$) sera. Each line of the graph corresponds with the change in sensitivity of one serum sample. Individual lines of the graph correspond to the change in sensitivity of one serum sample. No change in neutralisation is shown in grey lines. The $p$ value represents the difference in proportion of sera which Du156$^{301,334}$ and CAP45$^{301,334*}$ had increased sensitivity to upon mutating out the N301 glycan.

To determine whether the glycan at position N332, which is naturally found in Du156.12, was affecting the role of the N301 glycan in protecting Du156.12 from neutralisation but not CAP45.G3, we shifted the N334 glycan in CAP45.G3 to N332 (CAP45$^{301,332}$) and tested this mutant for sensitivity to our sera samples. The removal of the N301 glycan on the CAP45$^{301,332}$ mutant result in increased sensitivity to 8 sera samples (as opposed to 3 with the naturally-occurring N334 glycan (Fig 4.5)). Nonetheless, CAP45$^{301,332}$ still exhibited N301 glycan-dependent sensitivity to significantly fewer sera ($p=0.028$) than Du156$^{301,332*}$ (Fig 4.6).
Figure 4.6 Shifting the glycan at N334 to N332 in CAP45.G3 does not substantially affect its neutralisation sensitivity upon removal of the N301 glycan. Differences in neutralisation sensitivity of Du156$^{301.332^*}$ and CAP45$^{301.332}$ (both viruses possessing glycans at position N332) with and without the N301 glycan were investigated by testing the viruses with 64 sera samples. Each line of the graph corresponds with the change in sensitivity of one serum sample. Individual lines of the graph correspond to the change in sensitivity of one serum sample. No change in neutralisation is shown in grey lines. The $p$ value represents the difference in proportion of sera which Du156$^{301.332^*}$ and CAP45$^{301.332}$ had increased sensitivity to upon mutating out the N301 glycan.

Altogether, these data suggest that irrespective of the neighbouring glycan, the removal of the N301 glycan in Du156.12 creates a greater glycan shield “hole” resulting in more exposed epitopes as opposed to CAP45.G3 which maintains its glycan shield integrity in the absence of the N301 glycan.

Although not the focus of the study, it should be noted that as expected, removal of the N301 glycan resulted in a decrease in neutralising activity of some of the sera samples (blue lines, Figs 4.4-4.6). This is presumably because the dominant antibodies in those sera are dependent on the N301 glycan and therefore, its removal results in a decrease in neutralising activity. N301 glycan-dependent neutralisation by V3/glycan-specific antibodies has been observed in many studies (Walker et al., 2011, Mouquet et al., 2012, Garces et al., 2014, Sok et al., 2013). Differences between the proportions of sera with dominant antibodies targeting the N301 glycan was relatively minor between Du156.12 and CAP45.G3.
4.3.5 Glycans at positions N332 and N334 play in a role in blocking neutralising antibodies in sera

We next assessed the role of the glycans at positions N332 and N334 on maintaining the integrity of the glycan shield and blocking Env neutralisation by antibodies in sera. To do this, we mutated out the N332 and N334 PNGs but retained the PNG at N301. The removal of the glycans at positions N332 and N334 had a smaller effect on Du156.12 sensitivity than removing the N301 glycan alone (Table 4.4). This again highlights that the N301 glycan is particularly essential in the maintenance of the Du156.12 glycan shield.

Table 4.4 Effect of removal of either the 332 or 334 PNG on neutralisation sensitivity for both Du156.12 and CAP45.G3, while retaining the 301 PNG.

<table>
<thead>
<tr>
<th>BASE VIRUS:</th>
<th>Change:</th>
<th>% of sera showing increased neutralization:</th>
<th>p (γ2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Du156.12</td>
<td>Remove N332</td>
<td>11.8% (6/51) Du156.12 vs CAP45.G3</td>
<td>0.16</td>
</tr>
<tr>
<td>CAP45.G3</td>
<td>Remove N334</td>
<td>4.7% (3/64) CAP45.G3 vs Du156.12</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Removal of the N332 and N334 glycans in CAP45.G3 hardly increased neutralisation sensitivity of the virus in the same way that the removal of the N301 glycan had little effect on CAP45.G3 sensitivity (Table 4.4). Therefore, we can conclude that the individual V3/glycans in CAP45.G3 are not playing dominant roles in the maintenance of the glycan shield of this virus on their own.

4.3.6 Distinct differences between CAP45.G3 and Du156.12 sensitivity to mAbs after removal of N301 glycan

We examined the impact of the removal of the N301 glycan on the neutralising activity of mAbs which target different bnAb epitopes on Env. First, we tested the Du156.12 and CAP45.G3 WT viruses and mutants lacking the N301 glycan with V3 loop specific mAbs. Both viruses were neutralised better in the absence of the N301 glycan (Table 4.5), which is expected as this glycan has been implicated in blocking underlying V3 loop epitopes as shown previously (Zolla-Pazner et al., 2015). Also, as expected, soluble CD4 (sCD4) was able to access the CD4-bs better upon removal of the N301 glycan, neutralising the mutants more potently as others have also shown (Zolla-Pazner et al., 2015, Koch et al., 2003, Binley et al., 2008).
2010) (Table 4.5). Interesting, b12 neutralised Du156.12 better than CAP45.G3 upon removal of the N301 glycan (Table 4.5). This again alludes to differences in the glycan shield maintenance of these two viruses. As an internal validation, controls of 10E8 (targets the MPER) and PG9 (targets the V2/apex) revealed that these two sites were not exposed by the removal of the N301 glycan, confirming that the shielding effect of this glycan is not the result of global changes in Env structure.

Most striking in our mAb data was the result of the broad and potent neutralising antibodies which target the CD4-bs. We tested sensitivity of the WT viruses and mutants to VRC01, 3BNC117 and NIH45-46. There was no difference in neutralisation between Du156301.332* and its N301A mutant to these bnAbs, consistent with a previous report that VRC01 neutralisation is not impeded by glycan-based shielding (Li et al., 2011). However, strikingly, neutralisation of CAP45301.334* by these three mAbs was substantially enhanced by the presence of the N301 glycan, as CAP45301.334* was more resistant to these antibodies upon removal of the N301 glycan (Table 4.5). To investigate the extent to which this effect was CAP45.G3-specific, we introduced N301A mutations into two neutralisation resistant viruses, 253-11 and QH343.A10. We found that the activity of 3BNC117 was dependent on the N301 glycan for QH343.A10 whilst 253-11 was neutralised better without the N301 glycan by VRC01, 3BNC117 and sCD4, which suggests that the glycan is blocking the CD4-bs of this virus (Table 4.5).

In summary, the mAb data confirm that there are differences in N301 glycan reliance by Du156.12 and CAP45.G3. The data also highlight that for some viral isolates (including CAP45.G3 and QH343.A10), the N301 glycan may be aiding in the binding and neutralisation by the potent VRC01, 3BNC117 and NIH45-46 bnAbs, suggesting a substantially different relationship between the CD4-bs and the N301 glycan in virus CAP45.G3 as compared to Du156.12.
### Table 4.5 Effects of the removal of the N301 glycan on viral neutralisation by HIV-1-specific monoclonal antibodies and soluble CD4

<table>
<thead>
<tr>
<th>mAb/reagent</th>
<th>Target</th>
<th>CAP45&lt;sub&gt;301-334&lt;/sub&gt;</th>
<th>CAP46 &lt;sub&gt;334&lt;/sub&gt;</th>
<th>Fold inc neut</th>
<th>Du156&lt;sub&gt;301-332&lt;/sub&gt;</th>
<th>Du156&lt;sub&gt;332&lt;/sub&gt;</th>
<th>Fold inc neut</th>
</tr>
</thead>
<tbody>
<tr>
<td>b12</td>
<td>CD4bs</td>
<td>0.6</td>
<td>0.4</td>
<td>1.5</td>
<td>0.5</td>
<td>0.042</td>
<td>11.9</td>
</tr>
<tr>
<td>sCD4</td>
<td>CD4bs</td>
<td>10.9</td>
<td>1.1</td>
<td>9.9</td>
<td>12.1</td>
<td>0.907</td>
<td>13.3</td>
</tr>
<tr>
<td>NIH45-46</td>
<td>CD4bs</td>
<td>0.22</td>
<td>3.89</td>
<td>0.06</td>
<td>0.012</td>
<td>0.008</td>
<td>1.5</td>
</tr>
<tr>
<td>VRC01</td>
<td>CD4bs</td>
<td>0.31</td>
<td>18.9</td>
<td>0.02</td>
<td>0.072</td>
<td>0.039</td>
<td>1.8</td>
</tr>
<tr>
<td>3BNC117</td>
<td>CD4bs</td>
<td>0.25</td>
<td>9.56</td>
<td>0.03</td>
<td>0.043</td>
<td>0.023</td>
<td>1.9</td>
</tr>
<tr>
<td>2219</td>
<td>V3 loop</td>
<td>&gt;20</td>
<td>5.06</td>
<td>&gt;4.0</td>
<td>&gt;20</td>
<td>17.4</td>
<td>&gt;1.1</td>
</tr>
<tr>
<td>3869</td>
<td>V3 loop</td>
<td>&gt;20</td>
<td>2.35</td>
<td>&gt;8.5</td>
<td>&gt;20</td>
<td>2.44</td>
<td>&gt;8.2</td>
</tr>
<tr>
<td>3074</td>
<td>V3 loop</td>
<td>&gt;20</td>
<td>1.51</td>
<td>&gt;13.2</td>
<td>&gt;20</td>
<td>1.12</td>
<td>&gt;17.9</td>
</tr>
<tr>
<td>10E8</td>
<td>MPER</td>
<td>0.078</td>
<td>0.064</td>
<td>1.2</td>
<td>0.017</td>
<td>0.009</td>
<td>1.9</td>
</tr>
<tr>
<td>PG9</td>
<td>V2 apex</td>
<td>0.005</td>
<td>0.002</td>
<td>2.5</td>
<td>0.076</td>
<td>0.039</td>
<td>1.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>mAb/reagent</th>
<th>Target</th>
<th>253-11</th>
<th>253-11 N301A</th>
<th>Fold inc neut</th>
<th>OH343.A10</th>
<th>OH343.A10 N301A</th>
<th>Fold inc neut</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCD4</td>
<td>CD4bs</td>
<td>35.5</td>
<td>5.5</td>
<td>6.5</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>-</td>
</tr>
<tr>
<td>VRC01</td>
<td>CD4bs</td>
<td>0.10</td>
<td>0.01</td>
<td>10</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>-</td>
</tr>
<tr>
<td>3BNC117</td>
<td>CD4bs</td>
<td>0.09</td>
<td>0.02</td>
<td>4.5</td>
<td>5.7</td>
<td>&gt;20</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>
4.4 Discussion

One of the challenges of an antibody-based HIV-1 vaccine based on bnAbs is that these antibodies are rare, only developing in a subset of HIV-1-infected individuals (Borrow and Moody, 2017). Understanding which broadly neutralising antibody (bnAb) targets on Envelope (Env) are associated with breadth and potency in natural infection may shift vaccine focus as it may be easier to elicit broadly neutralising responses towards these sites. Therefore, we used sera from unselected chronically HIV-1-infected individuals to investigate whether there was an association between breadth and potency and the targeting of the V3/glycans and V2/apex by moderately broad antibodies. We used sera instead of mAbs as these samples are a more representative model of the type of responses the vaccinated population will elicit unlike bnAbs which are relatively rarely produced and therefore, may be more challenging to elicit (Borrow and Moody, 2017).

Despite reported polyreactivity of some anti-V3/glycan bnAbs (Liu et al., 2015), targeting this site in our cohort was associated with breadth and potency whilst we did not find an association with anti-V2/apex antibodies, despite their relative lack of self-reactivity (Liu et al., 2015) and their frequency in broadly neutralising sera (Walker et al., 2010). The Walker et al., study differs from ours as they only characterised broadly neutralising serum samples while we used unselected serum samples which had varying levels of breadth and potency. Self-reactivity has been considered an obstacle which limits the production of bnAbs (Verkoczy and Diaz, 2014), however, this study suggests that it is not generally a barrier in the human population for the production of antibodies which are moderate to broadly neutralising.

As stated, no association was found with targeting the V2/apex epitope and breadth and potency. Antibodies targeting the V2/apex were less broad than antibodies which did not target this site (Fig 4.3). Although there are isolated bnAbs which target the V2/apex including PG9, PG16 (Walker et al., 2009), PGT 145 (Walker et al., 2011) and the CAP256-VR26 lineage (Doria-Rose et al., 2015), antibodies of such high breadth are rare (Moore et al., 2017) and it seems that commonly-elicited V2/apex antibodies in sera from unselected HIV-1-infected individuals fail to gain breadth and potency presumably due to the complexity needed by these antibodies to gain these characteristics (Doria-Rose et al., 2014). Most bnAbs which target the V2/apex have rare, long and anionic complementarity-determining regions in the heavy chain
3 (CDRH3) which are needed to penetrate the dense glycan shield of the HIV-1 Env (Moore et al., 2017). To date, only one broad antibody lineage has been discovered which targets this site and has CDRH3s of more normal, shorter length (Cale et al., 2017), however, the antibody with the highest breadth in this lineage could only neutralise 42% of panel viruses and therefore cannot be considered exceptionally broad. In our study, our threshold for breadth was neutralisation of 75% (18/24) of viruses in a highly neutralisation resistant virus panel which is plausibly a better goal for vaccine coverage.

One limitation of our study is that we did not evaluate other bnAb Env targets including the CD4-bs (Burton et al., 1994), gp120-gp41 interface (Scharf et al., 2014, Falkowska et al., 2014, Huang et al., 2014) and the newly identified bnAb target, the fusion peptide (FP) (Kong et al., 2016). The gp120-gp41 interface and FP epitopes are not well-characterised and therefore, mutational mapping based on current epitope knowledge would presumably not detect most antibodies targeting these sites. The CD4-bs is difficult to map through mutational mapping as mutating key residues which form this epitope inhibits viral entry (Platt et al., 1997), a process needed for the read-out of our assays (Montefiori, 2005).

Altogether, our study shows that broad and potent responses are independently associated with the recognition of the V3/glycans and developing this target into an immunogen may aid in the ease of eliciting vaccine-grade bnAb responses. However, before designing an immunogen based on this target it is also important to understand the role of the V3/glycans in shielding antibody epitopes on Env. Commonly occurring neutralising antibodies in HIV-1-infected sera may play a role in the selection of glycans in viral populations. Therefore, we investigated the role of the V3/glycans N301, N332 and N334 on the maintenance of the glycan shield of two similar subtype C viruses, Du156.12 and CAP45.G3.

Interestingly, the glycan at position N301 plays a more crucial role in maintaining the integrity of the glycan shield in Du156.12 than in CAP45.G3. It appears that the removal of the N301 glycan forms a larger “hole” in the glycan shield of Du156.12, exposing both underlying and distal epitopes regardless of neighbouring glycans at positions N332 or N334. A recent study by McCoy et al. has shown that removal of specific glycans results in the formation of “glycan holes”, allowing antibodies which would not usually bind to neutralise the virus (McCoy et al., 2016). Commonly-occurring neutralising antibodies in sera from natural infection which target epitopes shielded by the N301 glycan may be major contributors to selective pressure to
maintain the glycan in viral populations. This could be the reason the glycan is so prevalent in HIV-1 isolates across different subtypes (Travers, 2012).

CAP45.G3, on the other hand, largely maintains its neutralisation resistance upon removal of the N301 glycan, regardless of whether an N332 or N334 glycan was present on the Env. Of note, CAP45.G3 contains a rare PNG at position N335. This glycan may be glycosylated instead of the N334 PNG on the virus. Little is known about the role of the N335 glycan in maintenance of the glycan shield and this should be explored further. However, this PNG is only present in <1% of viral isolates and individual mutation of this PNG did not result in increased sensitivity to sera in one study (MacLeod et al., 2016).

The 301 PNG is found in 93% of viral isolates (Moyo et al., 2017) and therefore is likely playing a substantial role in the formation and maintenance of a glycan shield of high integrity. It would be interesting to determine the neutralisation resistance profiles of viruses naturally lacking the 301 PNG and those like CAP45.G3 who can afford to lose the glycan, presumably with limited neutralisation fitness cost. It may be worthwhile to investigate what alternative evasion strategy they may have evolved.

The removal of the N301 glycan has been shown to increase neutralisation sensitivity to sCD4 and CD4-bs targeting mAbs such as b12 (Binley et al., 2010, Zolla-Pazner et al., 2015, Koch et al., 2003), however only one prior published study has looked at the effect of the removal on this glycan on the new-generation, broad and potent antibodies targeting the CD4-bs. Li et al. 2011 found that the neutralisation sensitivity of virus PVO.4 to VRC01 did not change upon removal of the N301 glycan (Li et al., 2011) and concluded that neutralisation by this antibody was not affected by the N301 glycan.

We show that the removal of the N301 has no effect on the neutralisation of Du156.12 by VRC01, 3BNC117 and NIH45-46, as expected based upon the results published by Li et al. However, these antibodies partially depended upon the presence of the N301 glycan to neutralise CAP45.G3 and QH343.A10 (for 3BNC117). This strongly suggests that the glycan shield in the region of the CD4-bs is constructed differently in CAP45.G3 (and likely QH343.A10) compared to Du156.12. This is further evidence suggesting that the N301 glycan is positioned differently in CAP45.G3 compared to Du156.12.
Although glycans such as N276 have been shown to play a role in neutralisation by CD4-bs-specific antibodies (Wibmer et al., 2016, Balla-Jhagjhoorsingh et al., 2013), this is the first study to implicate the N301 glycan in CD4-bs antibody binding. Based on these data, it is plausible that some of the decreases in neutralisation upon removal of the N301 glycan by the sera we tested were due to the dominant VRC01-like antibodies in the sera. However, VRC01-like antibodies are rarely found in unselected sera (Georgiev et al., 2013) and therefore, this is unlikely to explain our results.

Our collaborators performed molecular dynamic simulations to predict what surface of the HIV-1 Env was occluded by the N301 glycan and how this differed between Du156.12 and CAP45.G3 (Moyo et al., 2017). In this model, removal of the N301 glycan resulted in increased solvent accessible surface area for Du156.12 compared to CAP45.G3, irrespective of the presence of an N332 or N334 neighbouring glycan (Moyo et al., 2017). Residues 323-325 in the V3 loop and residues 440 and 441 in the C4 region were more exposed in Du156.12 upon removal of the N301 glycan (Moyo et al., 2017), supporting our hypothesis of a larger glycan “hole” created by the removal of the N301 glycan from Du156.12 compared to CAP45.G3. The models did not identify increased exposure of CD4-bs residues which could be due to limitations in the modelling technique (Moyo et al., 2017).

In summary, we show that in natural infection, antibodies targeting the V3/glycans are independently associated with neutralisation breadth and potency, presumably because they themselves are more likely to be broad and potent. Moderately broadly neutralising antibodies have been found to occur frequently in natural infection (Hraber et al., 2014b). In aggregate, these responses can produce highly broad and potent responses (Scheid et al., 2009). Vaccine design strategies may need to shift to focus on the V3/glycan epitope as it may be easier to elicit broad responses to the V3/glycans than other epitopes such as the V2/apex. The challenge will be to design an immunogen that more closely resembles the V3/glycans high-mannose patch than generic, relatively unstructured glycans that are found on many human proteins. Our data also reveal that the contribution of the N301 glycan to neutralisation resistance differs between HIV-1 isolates. Some viral isolates such as CAP45.G3 may more easily escape antibody responses targeting the N301 glycan by losing the glycan whilst maintaining resistance towards antibodies which target underlying or distal epitopes. This immune evasion strategy has important implications in the efficacy of V3/glycan-based antibody infusion therapies as well as vaccines.
Chapter 5: Conclusions and Perspectives

HIV-1 escapes antibody neutralisation by a variety of methods including the addition of glycans (Moore et al., 2012), Envelope (Env) diversity (Korber et al., 2001), increasing loop lengths (Hraber et al., 2014a) and conformational masking (Kwong et al., 2002). However, these features do not easily explain the neutralisation resistance phenotypes of all viruses. As shown in this thesis, virus 253-11 is neutralisation resistant despite having loop lengths comparable to highly neutralisation sensitive (Tier 1) isolates (LANL, 2017) and despite having most of the amino acids normally required to form epitopes of key broadly neutralising antibody (bnAb) epitopes. Additionally, two key Env glycans, that 253-11 possesses but two neutralisation sensitive viruses lack, only marginally affect the resistance phenotype of 253-11. Chapter 2 and Chapter 3 of this thesis describe data supporting the hypothesis that the neutralisation resistance profile of 253-11 is primarily a result of an unusually tightly closed, compact Env trimer as compared to other Env trimers. The resistance mechanism of compactness described in this thesis can be viewed as unusually robust conformational masking in which the pre-fusion Env resists transient shifts to the open conformation and results in more effective shielding of neutralising epitopes.

Moderately-neutralisation resistant, Tier 2, isolates contribute to the majority of viruses in circulation (Rademeyer et al., 2016, Seaman et al., 2010) and most HIV-1 antibody-based immunogenicity studies focus on eliciting responses towards Tier 2 viruses (Pauthner et al., 2017). Although forming a small proportion of the HIV-1 population, there are neutralisation resistant, Tier 3 isolates in circulation (Seaman et al., 2010) and a protective global vaccine most likely would need to protect against such isolates too. If an HIV-1 vaccine were only able to neutralise Tier 1 or Tier 2 viruses it is plausible that some Tier 3 isolates would be preferentially capable of infecting and circulating in a vaccinated population. Therefore, the mechanisms used by Tier 3 isolates to evade neutralisation more efficiently than Tier 2 isolates may need to be further understood to build an immunogen capable of eliciting a response towards all viruses.

Chapter 3 of this thesis describes the design of a recombinant SOSIP.664 Env trimer, based on the Tier 3 virus 253-11, in order to study what structural characteristics may be causing the high neutralisation resistance of the virus. The gp120 of 253-11 was smaller than those of most
of the crystal structures it was compared to and its heptad repeat helices were closer to the trimer axis as compared to other trimers. These findings further suggest that 253-11 Env is compact. A future recommendation would be to test the 253-11 SOSIP trimer in immunogenicity studies, preferably in non-human primates (NHPs) as they closely resemble humans. No published work has tested any Tier 3 SOSIP.664 trimers in immunogenicity studies and this platform could lead to the elicitation of broadly neutralising responses.

253-11 is not completely unique in its compactness phenotype. Tier 3 virus, CH120.6 (Cai et al., 2017) has been described as preferentially remaining in a tight, closed structure which occludes antibodies. An important future step for this project would be to further delineate neutralisation resistance through the compactness mechanism by identifying and characterising more viruses with compact Envs. One potential method of identifying these viruses would be to test the effect of soluble CD4 (sCD4) on opening the trimer and exposing epitopes of non-neutralising antibodies such as 17b. More compact viruses will likely need more sCD4 to open up the trimer while Tier 1 and Tier 2 viruses would need much less. High resolution structures and site-directed mutagenesis may further help identify the regions and specific residues responsible for the compactness phenotype. Another future recommendation for this project would be to determine whether compactness has a replicative fitness cost. This could be performed using Growth Competition Assays as previously described (Manocheewa et al., 2015). This may give insight into why most HIV-1 isolates from chronic infection do not resemble 253-11.

Chapter 4 of this thesis describes another key factor in neutralisation resistance: the glycans at the base of the V3 loop, usually referred to as the V3/glycans. Only a limited percentage of individuals develop bnAbs (Gray et al., 2009b, Stamatatos et al., 2009, Binley et al., 2008) and only 1% of HIV-1-infected individuals, “elite neutralisers” (Simek et al., 2009), develop the extremely broad and potent bnAbs (Sather et al., 2012, Simek et al., 2009, Stamatatos et al., 2009). The rarity of these antibodies in natural infection makes the development of such highly broad responses appear to be unlikely in the majority of vaccinated individuals. An understanding of bnAb epitopes that are preferentially targeted by moderately broad antibodies may focus immunogen development on sites that could potentially increase the probability of bnAb production. Chapter 4 of this thesis showed that the presence of antibodies targeting the V3/glycans and not the V2/apex was independently associated with antibody breadth and potency in our cohort of HIV-1-infected individuals in South Africa.
Using sera from unselected chronically infected individuals has the advantage that they may better represent antibody responses that can be elicited from vaccinated individuals. From the conclusions of this study and work from others who have created V3/glycan-based scaffolds (Zhou et al., 2014), the V3/glycans seem to be an achievable vaccine target.

Although vaccine efforts should include protection from Tier 3 viruses, Tier 2 viruses still make up the majority of HIV-1 isolates in circulation (Rademeyer et al., 2016, Seaman et al., 2010). Therefore, understanding the mechanisms used by Tier 2 viruses to gain their moderately neutralisation resistant profiles may be important in immunogen design to ensure the highest coverage possible for this Tier. A major role of the Env glycans is to protect the virus from neutralisation (Wei et al., 2003), even though some bnAbs have evolved to target these carbohydrates (Doores, 2015). This leads to the possibility that antibody-associated pressure may select for or against the presence of particular glycans. Antibodies whose epitopes contain or depend upon particular glycans may select for the absence of those glycans while antibodies whose epitopes are blocked by the same glycan may select for their presence. In Chapter 4 of this thesis, the effect of the removal of the glycan at position N301 on two subtype C Tier 2 viruses, CAP45.G3 and Du156.12, was investigated. CAP45.G3 maintained its resistant phenotype upon removal of the glycan at position N301 whilst Du156.12 become remarkably more sensitive to neutralisation. These data suggest that some viruses, such as CAP45.G3, can maintain neutralisation resistance in the absence of this glycan, which may aid in their ability to escape glycan-specific antibody responses.

The antibody neutralisation resistance of some viruses, like Du156.12, is more affected by the loss of the N301 glycan, which presumably creates a hole in the glycan shield leading to increased neutralisation susceptibility. Loss of the N301 glycan in PVO.4 (Li et al., 2011), loss of the N241 glycan in BG505 (McCoy et al., 2016) and loss of the N197 glycan in JR-FL (Crooks et al., 2015) made these viruses more susceptible to antibody neutralisation. These studies support our findings that the loss of a single glycan can dramatically increase neutralisation sensitivity.

As shown in a recent study by Zhou et al., Env trimers which are deglycosylated at specific sites have the potential to be used as priming immunogens to elicit anti-CD4 binding site (CD4-bs) antibodies (Zhou et al., 2017) and therefore future studies on the removal of the N301
glycan may also be useful in the development of priming immunogens to elicit pre-cursor antibodies toward the CD4-bs. “Natural lineage vaccines” would use multiple recombinant Env trimers based on longitudinal env sequences usually from individuals who have developed bnAbs (Sanders and Moore, 2017). However, not all the longitudinal env sequences may be amenable to recombinant trimer design (Sanders and Moore, 2017). The method of glycan removal may be advantageous due to it being an approach that does not depend upon the details of a chosen lineage and therefore eliminates the difficulty of developing Env precursor recombinant trimers as immunogens to elicit germline antibodies.

In summary, this thesis describes important advances in the understanding of the mechanisms used by HIV-1 to escape from antibody neutralisation. Compactness is the key mechanism that 253-11 and plausibly additional Tier 3 viruses may use to prevent neutralisation and evade the immune response. Although it was already known that glycans play an important role in shielding Env from neutralisation, this thesis highlights that the dependency upon key glycans for protection from antibodies varies between HIV-1 isolates. Interestingly, some viruses likely have compensatory mechanisms to ensure that even in the absence of certain glycans, they are still able to retain the neutralisation resistance that glycans afford them. Altogether, the knowledge gained from this thesis may aid in the design of an HIV-1 vaccine able to protect against neutralisation resistant viruses. The data also has implications on passive immunisation strategies as immune evasion by the loss of glycans while retaining resistance must be considered to avoid antibody therapy failure.
References


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