Evaluating the neutralizing antibody response to HIV-1 Membrane Proximal External Region; Implications for vaccine design

Submitted in fulfillment of the
Academic requirements for the degree of
Doctor of Philosophy

by

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Dedication

I dedicate this thesis to:

My loving parents, P.C Jacob and Valsamma Jacob
Acknowledgement

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List of abbreviations

6HB: 6 helix bundle
AIDS: Acquired Immune Deficiency Syndrome
ARRRP: AIDS Research and Reference Reagent Program
BNAB: Broadly neutralizing antibodies
CD: Cluster of differentiation
CDR: Complimentarity Determining Regions
CDRH3: Complimentarity Determining Region 3 (Heavy Chain)
CRF02_AG: Circulating Recombinant Form_AG
CTL: Cytotoxic T-Lymphocyte
DEAE-dextran: Diethyleminoethyl-dextran
DMEM: Dulbecco’s Modified Eagle’s Media
env: envelope gene
Env: envelope glycoprotein
gp120, gp41: envelope subunits, glycoprotein 120kda, 41kda
HAART: Highly Active Anti-Retroviral Therapy
HIV-1/HIV-2: Human Immunodeficiency Virus Type 1/2
IC50: 50% Inhibitory Concentration
ID50: 50% Inhibitory Dilution
IgG: Immunoglobulin
LLP-1, LLP-2, LLP-3: Lentivirus Lytic Peptide -1, -2, -3
mAb: Monoclonal antibody
mg: Milligram
MLV: Murine Leukemia Virus
MPER: Membrane Proximal External Region
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NMR: Nuclear Magnetic Resonance
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PNGS: Potential N-linked Glycosylation Site
RLU: Relative Light Units
RNA  
Ribonucleic Acid
sCD4:  
Soluble CD4
SGA:  
Single Genome Amplification
SIV:  
Simian Immune Deficiency Syndrome
V1, V2, V3, V4, V5  
Variable Regions 1-5

Amino acid abbreviations

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<thead>
<tr>
<th>Amino Acid</th>
<th>Three letter code</th>
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<tr>
<td>Alanine</td>
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Summary

Background:
Inducing broadly neutralizing antibodies targeting the HIV-1 envelope is thought to be crucial for developing an effective vaccine. The Membrane Proximal External Region (MPER) within the HIV-1 gp41 envelope is a promising vaccine target. The MPER is highly conserved, functionally constrained, facilitates virus fusion and is targeted by broadly neutralizing monoclonal antibodies. The objectives of this research were 1) To evaluate the neutralization breadth of antibodies induced by epitopes within the MPER compared to the PG9/16-site in chronically HIV-1-infected individuals, 2) to identify neutralization resistant HIV-1 isolates (using plasma samples infected with the same subtype) and to characterize their sensitivity to anti-MPER antibodies and 3) to determine the accessibility of the MPER to HIV-1 induced polyclonal anti-MPER antibodies in a highly neutralization resistant virus (253-11; CRF02_AG subtype).

Results:
Epitopes within the MPER and the PG9/16-site are two targets of broadly neutralizing monoclonal antibodies. Chapter two of this thesis describes the neutralization activity directed against the MPER and the PG9/16-site from serum samples of ARV-naïve chronically HIV-1 infected individuals (n=177) from South Africa. The neutralization breadth of the sera was measured on a diverse (n=24 viruses representing five different subtypes) and neutralization-resistant (tier 2 and tier 3) HIV-1 pseudovirus panel. Anti-MPER antibodies were measured using three HIV-2/HIV-1 MPER chimeric viruses and PG9/16 epitope mutants were used for detecting PG9/16-site specific neutralization. A substantial proportion of the sera had anti-MPER (19%, 33/177) and anti-PG9/16-site specific (34%; 37/108) neutralization activity. Twenty percent (35/177) of the cohort were identified as broad neutralizers (geometric mean ID$_{50}>200$ against the panel viruses). The neutralization breadth of the MPER recognizing sera was significantly higher than that of the non-MPER recognizing samples (median 102 vs 53, p<0.0001, Wilcoxon rank sum test). In contrast, samples with dominant anti-PG9/16 site antibodies did not elicit higher neutralization breadth than samples which lacked anti-PG9/16-site antibodies (median 114 vs 105, p=0.64, Wilcoxon rank sum test), and were less likely to be broadly neutralizing (p=0.04). The epitope variability of the MPER was substantially lower than that of the PG9/16-site and other antibody targets, measured by analyzing 3829 envelope sequences.

In chapter three, highly neutralization resistant CRF02_AG viruses were identified and their vulnerability to anti-MPER monoclonal antibodies was tested. The neutralization activities of ARV-naïve CRF02_AG subtype infected plasma samples from Cameroon were used to identify highly
neutralization resistant CRF02_AG viruses. The neutralization capacity of the CRF02_AG plasma samples was tested by assembling a virus panel (n=27) including several CRF02_AG viruses. CRF02_AG viruses have been previously reported to be highly neutralization resistant even to plasma pool infected with the same subtype. However, in contrast to previous reports, CRF02_AG plasma samples neutralized CRF02_AG panel viruses better than viruses from other subtypes. This included six of the eight CRF02_AG panel viruses previously designated as resistant (tier 2/3 or 3). Only two CRF02_AG viruses (253-11 and 278-50) remained highly resistant. Even the resistant CRF02_AG viruses were sensitive to MPER-specific monoclonal antibodies (2F5 and 4E10), revealing vulnerable neutralization targets.

Finally, (chapter four) we analyzed the sensitivity of the highly resistant CRF02_AG virus (253-11) to a set of polyclonal anti-MPER antibodies. An HIV-1 infected polyclonal serum panel (n=217) from the South Africa cohort rarely neutralized 253-11, even by recognition through the MPER. However, 19 other sera (9%) recognized 253-11’s MPER in an HIV-2 chimeric construct despite being unable to recognize the same MPER in the original 253-11 virus (ID_{50}<100). A similar resistant pattern was observed in a second virus (Du422.1, subtype C). Importantly, at least 13/19 of the 253 MPER recognizing sera neutralized other HIV-1 isolates via the MPER, indicating that these anti-MPER antibodies are not defective for neutralizing HIV-1 isolates. The MPER recognizing sera which failed to neutralize 253-11, targeted several distinct epitopes within the MPER as revealed by alanine scan mapping.

Conclusions:
For effective protection from a vaccine, antibodies induced by an immunogen targeting the HIV-1 surface envelope should be broadly neutralizing. Antibodies induced by MPER epitopes were broadly neutralizing in contrast to antibodies targeting the anti-PG9/16-site, suggesting that the conserved MPER might be more amenable to vaccine design than the more variable PG9/16-site. The anti-MPER monoclonal antibodies also neutralized highly resistant CRF02_AG isolates (253-11 and 278-50) suggesting that the MPER can be targeted to effectively neutralize CRF02_AG isolates.

However, the CRF02_AG isolate, 253-11 which we identified as highly resistant was rarely neutralized by anti-MPER antibodies from a polyclonal serum panel. We then directly tested for recognition of the 253-11 MPER in an HIV-2 chimeric construct. The anti-MPER neutralized HIV-1 isolates and recognized several distinct epitopes within the MPER suggesting that conformational differences of the MPER between the native 253-11 virus and the HIV-2/253-11 MPER chimera.
seems unlikely to explain the inability of so many independent antibodies (with distinct epitopes) to neutralize 253-11. Several possible explanations for our observations are discussed; but, we propose that the most parsimonious explanation for the rare anti-MPER-neutralization of 253-11 is obstruction of access of antibody to the MPER. Analysis of the natural immune response to the MPER by a virus such as 253-11 which is resistant to most anti-MPER antibodies will aid in the understanding needed to develop a vaccine that induces broadly neutralizing antibodies directed against the MPER.
Publications and conference papers from this thesis

This thesis has contributed to the following publications:
1. **Rajesh Abraham Jacob**, Fatima Abrahams, Marcel Tongo, Michael Schomaker, Paul Roux, Eitel Mpoudi Ngole, Wendy A. Burgers and Jeffrey R. Dorfman
Refined identification of neutralization-resistant HIV-1 CRF02_AG viruses.
PMID: 22573859
(Thesis chapter three)

This thesis has contributed to the following conference papers:
1. **RA Jacob**, F Abrahams, M Tongo, M Schomaker, P Roux, E Mpoudi Ngole, WA Burgers and JR Dorfman
Refined identification of neutralization-resistant CRF02_AG viruses and their sensitivity to anti-MPER neutralizing antibodies
Retrovirology; 2012, 9 (Suppl 2): P77

2. **RA Jacob**, F Abrahams, M Tongo, M Schomaker, P Roux, E Mpoudi Ngole, WA Burgers and JR Dorfman
Neutralization activity of HIV-1 subtype C- and CRF02_AG infected serum/plasma: some viruses are not as resistant as previously thought
AIDS Research and Human Retroviruses; 2011, 27(10), A-1-A-148

Generation of broadly neutralizing monoclonal antibodies to HIV-1 envelope protein and characterization of the epitopes
AIDS Research and Human Retroviruses; 2010, 26(10), A-1-A-184

Other publications
1. Maria Agthe, Elisa Nemes, **Rajesh A Jacob**, Fatima Abrahams, Nadine Fainguem, Suzie Moyo Ndiang Tetang, Giulia Cappelli, Vittorio Colizzi and Jeffrey R Dorfman
Preservation of CD4+ T cells positively correlates with anti-HIV neutralization capacity in HIVinfected children: the opposite effect from adults
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1.8. Study objectives
1.1. **Background:**

Acquired immunodeficiency syndrome, caused by the Human Immunodeficiency Virus (HIV), remains a global infectious disease threat with 2.5 million new infections and 34 million existing infections world-wide according to the UNAIDS 2011 report (www.unaids.org). Since the identification of HIV-1 three decades ago (Barre-Sinoussi et al., 1983), considerable progress has been made to understand HIV-1 pathogenesis (Swanstrom and Coffin, 2012), anti-HIV-1 drug development (Arts and Hazuda, 2012; Flexner, 2007) and vaccine design (Burton et al., 2012a). However, a cure for HIV-1 is very difficult (Yukl et al., 2013) and reported rarely (Persaud et al., 2013). Designing an effective vaccine which prevents the establishment of new viral infections is a high global priority.

Advances made in HIV therapeutics over the past years have made great progresses through multiple approaches to contain the virus. Highly Active Anti-Retroviral Therapy (HAART) have drastically reduced morbidity and mortality of HIV/AIDS (Coovadia, 2004; May et al., 2011; Moore et al., 1991) and have extended the survival of HIV-1 infected individuals (2008; May et al., 2011; Moore et al., 1991) by suppressing HIV-1 replication (to almost undetectable viral loads) and preventing disease progression (Graham et al., 1992). More importantly, at a population level, HAART can reduce new HIV-1 transmissions (Cohen, 2013; Montaner et al., 2010; Tanser et al., 2013). Most current HAART regimens consist of a combination of nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PI) and/or non-nucleoside reverse transcriptase inhibitor (NNRTI).

In South Africa, around 6.1 million people are estimated to be living with HIV-1 (UNAIDS, 2013b). As at 2012, an estimated 2.5 million HIV-1 infected people in South Africa were receiving primary health care (SANAC, 2012). Extensive antiretroviral drug programmes have substantially reduced AIDS related deaths and vertical transmission of HIV-1 from mother to child, with1.6 million (out of 8.6 million people having access to antiretroviral therapy in low- and middle-income countries globally) received antiretroviral treatment as at the end of 2011(SANAC, 2012; UNAIDS, 2013a). The initial HAART regimen uses fairly efficacious "first-line" drugs with low side-effect profiles. However, the high cost of HIV-1 drug management (approx. 13 billion South African Rand was spent on HIV/AIDS and TB for the financial year 2009-2010), need for lifelong treatment (with a lifelong need for clinical management and laboratory testing and risk of drug-associated toxicity) and the emergence of drug resistant virus mutants makes dependence on HAART alone difficult (Arts and Hazuda, 2012; Clavel and Hance, 2004; SANAC, 2012)
Latent virus reservoirs established early in acute HIV-1 infection pose an important challenge in eradicating the virus from the host (Finzi et al., 1999). They form a major barrier to curing HIV-1 infection; as the proviral DNA is integrated into the host genome, establishing a reservoir of virus variants within the host that is not susceptible to antiretroviral drugs (Noe et al., 2005) or immune surveillance (Richman et al., 2009). Upon receiving appropriate activation signals, replication competent viruses are generated from the latent reservoir. This makes cure difficult, if not impossible in most HIV-infected individuals.

A prophylactic vaccine which could prevent virus establishment within the host is highly essential to control the pandemic. One of the major vaccine approaches against HIV-1 (Walker and Burton, 2008) would be to generate a neutralizing antibody response which would prevent virus establishment within the host. The other major approach is to induce an effective T cell response which can attenuate HIV-1 replication soon after the initial infection event. The viral envelope, a trimer of hetero-dimers of gp120 and gp41 is the sole HIV-1 derived antigenic protein on the surface of HIV-1 (Corti and Lanzavecchia, 2013; Wyatt and Sodroski, 1998), and is thus an attractive target for antibody based vaccine design, while other HIV-1 proteins like Gag and Nef are targets for cell-mediated immune response (Barouch, 2008).

A critical component of most antibody-based vaccine approaches is neutralizing antibodies, which in some cases could prevent the establishment of infection or would at least limit the initial explosion of virus replication (Plotkin, 2008; Plotkin, 2013). There is convincing evidence for sterilizing protection provided by passively immunized neutralizing antibodies against viral infection in nonhuman primate animal models (Hessell et al., 2009; Mascola et al., 1999; Mascola et al., 2000; Moldt et al., 2012; Shibata et al., 1999). Once infection is established, neutralizing antibodies may contribute lesser towards disease progression (Doria-Rose, 2010; Euler et al., 2010; Piantadosi et al., 2009); largely due to the emerging escape variants (Moore et al., 2009; Rong et al., 2009) resulting from the humoral immune pressure (Frost et al., 2005; Richman et al., 2003), although this does not necessarily mean that antibodies cannot play a protective role (Huang et al., 2010).

In approximately 80% of heterosexual HIV-1 transmissions (Abrahams et al., 2009; Keele et al., 2008; Shaw and Hunter, 2012), 68% of mother to child transmissions (Russell et al., 2011) and 60% of homosexual transmissions (Li et al., 2010), virus dissemination results from a single virus variant referred to as the transmitted/founder (T/F) virus (Shaw and Hunter, 2012). If broadly neutralizing
antibodies targeting this T/F virus is pre-existing before establishment of infection (i.e. induced by a vaccine), they presumably can provide sterilizing protection (Haigwood and Hirsch, 2009). Designing an immunogen towards achieving this goal is a global health priority (Barouch, 2008; Walker and Burton, 2008).

1.2. **HIV-1 transmission:**

1.2.1. **Introduction:**

Around 2.5 million new HIV-1 transmissions were reported in 2011 by UNAIDS (http://www.unaids.org). The major routes of HIV-1 transmission can be attributed to three different means; transmission via genital mucosa (heterosexual transmission, transmission in men who have sex with men), mother to child transmission and transmission in injection drug users. However, HIV-1 is not very efficiently transmitted; for a productive clinical infection in heterosexual transmissions, approximately 200 or more coital acts are required (Powers et al., 2008). Various factors determine the probability of virus transmission; socioeconomic factors (Ajoge et al., 2013), higher viral loads (Shaw and Hunter, 2012) or sexually transmitted diseases in the partner (Galvin and Cohen, 2004) and type of sexual contact (Abrahams et al., 2009; Bar et al., 2010).

A rapid virus evolution occurs after transmission by multiple mechanisms; resulting in a quasi-species of virus variants in HIV-1 infected individuals (Taylor et al., 2008). Despite high degrees of viral diversity in the transmitting partner, there exists a “transmission bias” i.e. Some virus variants are preferentially transmitted from the transmitting partner to the recipient (Derdeyn et al., 2004; Wolinsky et al., 1992; Zhu et al., 1993). This “transmission bottleneck” would allow the carry-over of only a limited number of variants to the infecting partner while establishing a new infection. This bottleneck could be largely attributed to the selection of particular characteristics of the virus immediately following transmission. For example, the CCR5 co-receptor engaging virus variants are preferentially transmitted over the CXCR4 variants (Keele, 2010; Keele et al., 2008; Shaw and Hunter, 2012).

1.2.2. **Multiplicity of infection of Transmitted/Founder variants:**

A single genetic variant of virus (called the founder virus) is responsible for the establishment of most HIV-1 infections (Figure 1.1). The most convincing early evidence for this came from Derdeyn et al, who studied the envelope genotype and phenotype of viruses from eight heterosexual transmission pairs (Derdeyn et al., 2004). Full length envelope sequences from each of the donor and recipient were amplified and sequenced; Phylogenetic trees were constructed to understand the
similarities/differences between the viruses for each transmission pair. Viruses from the recipient were monophyletic with limited divergence after transmission in comparison to the viruses from the transmitting partner. Surprisingly, viruses within the recipient were sensitive to the antibodies from the transmitting partner indicating that even low titers of antibodies induced by a vaccine might be helpful in preventing transmission (Derdeyn et al., 2004).

A single genome amplification (SGA) technique was used later to precisely identify the transmitted virus and track their evolution in the recipient (Abrahams et al., 2009; Keele et al., 2008; Salazar-Gonzalez et al., 2008; Salazar-Gonzalez et al., 2011). SGA has several advantages (Keele, 2010) over the conventional PCR amplification techniques which were used widely before, because SGA eliminates errors induced by the PCR polymerase and viral genome recombination. Additionally any cloning bias for particular sequences over others is avoided as sequencing is performed on the PCR product before cloning. SGA was performed to amplify hundreds of HIV-1 envelope sequences from subtype B (n=102) and subtype C (n=69) HIV-1 infected individuals in the three studies (Abrahams et al., 2009; Keele et al., 2008; Salazar-Gonzalez et al., 2008). The studies concluded that approximately 75% of the heterosexual transmissions were by a single virus variant. The other infections were established by multiple variants; however these were generally limited to less than five virus variants. These studies established that there is severe reduction in viral diversity soon after heterosexual transmission of HIV-1. A later study on three acutely HIV-1 infected individuals with 454 sequence data on thousands of sequences also came to the conclusion that a single virus initiates acute HIV-1 infection (Fischer et al., 2010). The probability of single variant transmission in mother-to-child transmissions (Russell et al., 2011), men who have sex with men (Li et al., 2010), and injection drug users (Bar et al., 2010) is however lower than heterosexual transmissions (68%, 60% and 40% respectively in comparison to the 75% in heterosexual transmissions). Thus, most variants fail to make productive infections (considered defective or less fit) while other viruses have some attribute, making them efficient transmitters (Figure 1.1).
Figure 1.1: HIV-1 transmission (Shaw and Hunter, 2012):

In this model for HIV-1 transmission, the transmitting partner has a viral quasi-species as shown in the inoculums; however, only the most fit variant (T/F virus variant, coloured red) establishes infection in the recipient. The virus in the recipient eventually diversifies.

1.3. HIV-1 diversity:

The present day distinct HIV lineages (HIV-1 groups M, N, O, P and HIV-2) in humans are presumed to have resulted from independent zoonotic transmission events of Simian Immunodeficiency Virus (SIV) from non-human primates (Hemelaar, 2012; Hemelaar, 2013; Vallari et al., 2011). It have been estimated that SIV, which was previously thought to have evolved over millions of years, may have infected its natural hosts for only hundreds of years before giving rise to HIV in the twentieth century (Sharp and Hahn, 2011). This would make SIV and HIV the youngest and fastest evolving lentiviruses (Hemelaar, 2012; Wertheim and Worobey, 2009).

The worldwide spread of HIV is accompanied by its enormous genetic variability (Figure 1.2) and rapid evolution, which makes the virus highly adaptable to new hosts and selection pressures (Abecasis et al., 2009; Hemelaar et al., 2006; Kwong et al., 2012; Lynch et al., 2009). Consequently, within the HIV-1 group M alone, nine subtypes are recognized and designated by the letters A–D, F–H, J, and K (Aldrich and Hemelaar, 2012; Hemelaar, 2013). The high rates of viral replication and lack of proofreading mechanism of HIV’s reverse transcriptase enzyme is responsible for the high mutation and recombination rates (Taylor et al., 2008). As a result of high mutation rates, HIV
sequence diversity can differ by up to 10%, 30% and 40% respectively within a single individual, within a subtype and between subtypes (Lynch et al., 2009). High recombination rates led to early formation of recombinant forms; a few of these were widely transmitted and apparently gave rise to new circulating recombinant forms (CRFs), while others did not and remained what are termed unique recombinant forms (URFs) (Hemelaar et al., 2006). Recombination events earlier in the epidemics in Central Africa led to formation of some CRFs like CRF01_AE and CRF02_AG, although the sequence of events and which is the actual original and actual recombinant form is a matter of debate (Abecasis et al., 2007). Recombination between subtypes and CRFs is still ongoing in many places in the world and giving rise to new CRFs (Hemelaar, 2013; Ramirez et al., 2008).

**Figure 1.2: Phylogenetic tree of SIV and HIV-1** (Hemelaar, 2012): Phylogenetic tree based on polymerase sequences of reference sequences of SIV and HIV-1.

Two-thirds of global HIV infections occur in generalized epidemics and most of these epidemics occur in sub-Saharan Africa (Aldrich and Hemelaar, 2012). Only a few of the recognized subtypes and CRFs, subtype A-D, G, CRF01_AE and CRF02_AG, are responsible for the 95% of the global HIV-1 infections. Subtype C alone is responsible for nearly half (48%) of the global burden. Subtype C is nearly the exclusively subtype found in Southern Africa, Ethiopia and India. CRF02_AG, which is presently the predominant HIV-1 subtype circulating in West Africa (94% of global CRF02_AG), is the highest prevalent CRF and the fourth most prevalent subtype globally (Hemelaar, 2013; Hemelaar et al., 2006; Hemelaar et al., 2011b). HIV diversity is the critical driver of the HIV-1 epidemics and has important implications for pathogenesis, transmission, the immune response and
clinical management (Hemelaar, 2012; Hemelaar et al., 2011a; McBurney and Ross, 2008). Since the diversification and evolution of HIV is ongoing, the development of a preventative HIV vaccine will be a herculean task.

1.4. **HIV-1 envelope:**

1.4.1. **Introduction:**
HIV-1 like other primate lentiviruses has the common genomic structure LTR-gag-pol-vif-vpr-tat-rev-env-nef-LTR (Barre-Sinoussi et al., 1983; Ratner et al., 1985). Infection of the host cell by HIV is initiated by interactions between the gene products of env (“envelope spike”) located on HIV-1’s envelope lipid bilayer (which is derived from the membrane of the host cell) and cell-surface CD4 molecules (Julien et al., 2013a; Wyatt et al., 1998). Additional interactions with chemokine receptors trigger a conformational change that leads to fusion of the viral and cellular membranes (Blumenthal et al., 2012; Wyatt and Sodroski, 1998). This critical role of the envelope spike has made envelope the most important target for neutralizing antibody based vaccine development (Benjelloun et al., 2012).

The HIV-1 envelope spike is a heterodimeric trimer with three units of gp120 and three units of gp41 associated via non-covalent interactions (Wyatt and Sodroski, 1998). Overall, HIV-1 envelope adopts a mushroom-shaped structure with an overall mass of approximately 160 kDa and is highly glycosylated (Figure 1.3). The gp120 and gp41 complex are involved in different functions (Pantophlet and Burton, 2006); (1) The gp120 mediates attachment of the virus to the target cell membrane by interaction with the CD4 receptor and the coreceptor, CCR5 and CXCR4 and (2) the “stalk” gp41 helps attach the envelope spike to the virus membrane and in fusion of the virus and target cell membranes. The greatest amount of genetic diversity in HIV-1 genome is found in the envelope, whose amino acid sequences can differ as much as 15% between isolates within a single subtype and more than 35% between envelopes from different subtypes (Lynch et al., 2009). This diversity poses challenges towards the development of antibody-inducing AIDS vaccines. However, recently considerable advance has been made in the isolation of broadly neutralizing mAbs against the viral envelope, despite the variability (Kwong et al., 2012; Overbaugh and Morris, 2012). These broad antibodies are models for what an effective antibody-based vaccine might induce (Burton et al., 2012a; Burton et al., 2012b). Furthermore, a huge improvement in our understanding of the HIV-1 envelope architecture was possible at the atomic level by the recent cryo-electron microscopic study of a cleaved, soluble envelope trimer (BG505 SOSIP.664 gp140) in complex with two broadly neutralizing antibodies (Julien et al., 2013b; Lyumkis et al., 2013). The HIV-1 envelope structure which was visualized at 5-6 Å resolution gave us a glimpse on how each of the gp120 subunits hold
together in the trimer. Additionally, the studies elucidated the spatial arrangement of the envelope components and the stabilizing contacts made between gp120 and gp41.

**Figure 1.3: Schematic depiction of the HIV-1 gp120** (McBurney and Ross, 2008):
The top panel depicts the linear sequence of regions of gp160 and the bottom panel represents a simplified model of the gp120 conformation.

1.4.2. **Structure and function of gp120:**
For HIV-1 to infect the host cell, the envelope gp120 have to sequentially interact with the CD4 molecule (present on CD4 T cells) followed by an interaction with the co-receptors, CCR5/CXCR4 (Dalgleish *et al*., 1984; Feng *et al*., 1996; Klatzmann *et al*., 1984). The gp120 is highly glycosylated and contributes a “silent face”. The linear amino acid sequence of gp120 (Figure 1.3) comprises of five variable loops (V1-V5) and five constant regions (C1-C5) (Willey *et al*., 1986; Zolla-Pazner and Cardozo, 2010). The variable regions are generally more exposed and may occlude the conserved regions from Nabs (Krachmarov *et al*., 2005; Sagar *et al*., 2006; Wyatt *et al*., 1995).

- **CD4 binding site: Studies on the crystal structure of gp120:**
The first crystal structure of gp120 was deduced using a “gp120 core” bound to a two domain soluble CD4 (Kwong *et al*., 1998; Wyatt *et al*., 1998). The “gp120 core” used by Kwong *et al* was truncated (without V1, V2 and V3 variable loops) and highly de-glycosylated, still the gp120 core bound to CD4 and the CD4 binding site mAb, b12. Crystal structure revealed that the “gp120 core” comprised of an inner domain, an outer domain and a bridging sheet. Another study on the gp120 crystal structure (Figure 1.4) (Zhou *et al*., 2007) using a stabilized gp120 (stabilized such that the
gp120 maintains the CD4 bound conformation even in the absence of CD4) and b12 confirmed the observations made by Kwong et al (Kwong et al., 1998) and also gave insights into the structure of CD4 binding site (Julien et al., 2013a). The stabilized gp120 construct was important since flexibility in gp120 complicates antibody recognition analysis. The crystal structure revealed that the outer domain comprised of two barrels, stacked by a barrel-barrel juncture. The distal barrel comprised seven anti-parallel beta strands, while the proximal barrel had six beta strands.

The CD4 binding site in the gp120 is formed by a recess within the trimeric context of gp120. Many mAbs like b12, VRC-series, NIH45-46 and others target regions overlapping the CD4 binding site (Saphire et al., 2001; Scheid et al., 2011; Zhou et al., 2010; Zhou et al., 2007). There is also an overlap between the mAb binding sites and the CD4 binding site (Zhou et al., 2010; Zhou et al., 2007).

In principle, the CD4 binding surface forms an ideal vaccine target. The huge breadth and potency of CD4 binding site mAbs isolated to-date supports this (Scheid et al., 2011; Wu et al., 2010; Wu et al., 2011; Zhou et al., 2010). Binding of recombinant soluble CD4 to the CD4 binding surface results in gp120 shedding from virions, mimicking HIV-1 neutralization (Moore et al., 1990). Sensitivity of
viruses to soluble CD4 is also correlated with the CD4 binding site exposure of HIV-1 (Pugach et al., 2004). However, despite being constrained to bind CD4 to gain cell entry, the CD4 binding site within HIV-1 is differentially sensitive to soluble CD4, leading to the conclusion that the CD4 binding site is inaccessible/flexible in some viruses (Blish et al., 2009; Blish et al., 2007; Daar et al., 1990). Additionally, the CD4 binding site might be more flexible which might make them capable of evading even broadly neutralizing antibodies (Chen et al., 2009).

- **Chemokine receptor binding site in gp120:**

HIV requires the presence of co-receptor molecules on the surface of the target cells, in addition to CD4 receptors. HIV-1 uses either one of the two chemokine receptors CCR5 or CXCR4, for entry into the target cell after receptor activation (CD4 binding). Most transmitted/founder viruses use CCR5 as the coreceptor, but isolates arising later during infection may use CXCR4. This phenomenon is referred to as coreceptor switching and is more prominent in subtype B viruses. The chemokine binding surface in gp120 is formed by two separate components: the chemokine receptor surface within the bridging sheet (Pantophlet and Burton, 2006) and the V3 loop (Rizzuto et al., 1998). CD4 binding induces conformational changes, bringing the bridging sheet and the V3 loop close together to form a contiguous binding site. After receptor activation by CD4, in the closed conformation, the V3 protrudes into the envelope spike facilitating the co-receptor binding (Huang et al., 2007; Rizzuto and Sodroski, 2000).

1.4.3. **Structure and function of gp41:**

**Structure of gp41:**

HIV-1 gp41 catalyzes the fusion between the viral and target cell membrane. It is relatively more conserved in comparison to gp120. The membrane anchored gp41 comprises approximately 345 amino acid residues and is mostly occluded within the envelope spike. Structurally, it could be divided into three major domains (Figure 1.5): the ectodomain, the transmembrane domain and the cytoplasmic domain.
Figure 1.5: Schematic diagram of HIV-1 gp41 (Garg et al., 2011)
NHR: N-terminal Heptad Repeat, CHR: C-terminal Heptad Repeat, MPER: Membrane Proximal Ectodomain Region, MSD: Membrane Spanning Domain, LLP1 and LLP2 (Lentiviral Lytic Peptide 1 and 2).

1. **The ectodomain:**
The ectodomain (HXB2 numbering 512 to 683) mediates the major functions of gp41. It is further subdivided into four functionally distinct regions:

a. **Fusion Peptide:**
Fusion peptide (Bosch et al., 1989) comprises the N-terminal hydrophobic peptide of gp41 (Figure 1.5) and is approximately 23 amino acids long. The fusion peptide is believed to insert itself into the host cell membrane, following receptor activation by host CD4 and the co receptors. Mutational studies confirm the role of the fusion peptide region in the fusion process (Freed et al., 1992).

b. **N-terminal heptad region and C-terminal heptad region (NHR and CHR):**
The NHR (also known as HR1) is adjacent to the fusion peptide and together with the CHR (also known as HR2) forms a 6 helix bundle (6HB). Three NHR helices form a coiled-core loop which can be visualized as a trimer of hairpins (Buzon et al., 2010). The CHR helices bind into the hydrophobic NHR coiled-core loop. Thus each of the coiled-coil trimer associates together in an anti-parallel fashion to form the 6HB. The formation of the 6HB is thought to bring the viral and the host cell membrane together for fusion (Blumenthal et al., 2012). The NHR and the CHR are separated from each other by a loop (Figure 1.5).

c. **Membrane Proximal External Region (MPER):**
The MPER, a linear stretch of 24 amino acids located at the C terminus of the gp41 ectodomain, is detailed in section 1.7.
2. **The transmembrane domain/membrane spanning domain:**
As the name suggests, this region of approximately 25 amino acids, spans the virus membrane (Figure 1.5). The transmembrane domain anchors the envelope to the viral membrane. This region is highly hydrophobic and conserved in nature. Some studies point out that the transmembrane domain plays a role in virus fusion, as the substitution of the transmembrane domain by glycosyl-phosphatidylinositol (GPI) attachment results in reduced fusion (Salzwedel *et al.*, 1993). Another study by Miyauchi (Miyauchi *et al.*, 2005) demonstrated that the transmembrane domain replacement mutants have impaired fusion activity and further supports its role in fusion (Lin *et al.*, 2003).

3. **Cytoplasmic tail:**
HIV-1 gp41 contains an unusually long cytoplasmic tail (Postler and Desrosiers, 2013) typical of lentiviruses. The cytoplasmic tail can be approximately 150 amino acids long. It is divided into distinct domains (Figure 1.5): The N terminus is a richly hydrophilic while the C terminus contains three highly conserved Lentivirus Lytic Peptide (LLP) domains. LLP domains are amphipathic alphahelical domains (Montero *et al.*, 2008a). The cytoplasmic tail exerts multiple functions (Postler and Desrosiers, 2013) like viral replication and infection, clathrin mediated endocytosis of envelope (for maintaining a low but constant levels of envelope expression) and envelope incorporation into virions.

**Function of gp41 and the mechanism of viral fusion into the membrane:**
The genome of HIV-1 is wrapped with lipids, proteins and carbohydrates. Upon infection, the viral genome has to cross into the host cell membrane. In order to achieve this, penetrating a double barrier (viral membrane followed by the host membrane) is essential. This penetration is achieved by fusion, facilitated by the envelope gp41(Wyatt and Sodroski, 1998). Soon after host CD4-HIV-1 gp160 CD4 binding site interaction and the co-receptor binding (Figure 1.6 upper panel), large conformational changes occur which leads to the fusion of virus and cell membrane, resulting in the transfer of the viral genome into the host (Blumenthal *et al.*, 2012; Garg *et al.*, 2011).

The gp41 catalyzes the fusion process. Soon after CD4 binding (Kwong *et al.*, 1998), the inner and outer domains of gp120 gets rearranged. The gp120 domain turns outward (Liu *et al.*, 2008) and moves away from the gp41 stalk. This exposes the variable loops within gp120 for co-receptor binding, which in turn induces conformational changes within the gp41. The fusion peptide inserts into the target cell membrane and forms a highly stable intermediate known as the “pre-hairpin
intermediate” (Jacobs et al., 2007; Liu et al., 2008). The pre-hairpin then refolds into the 6 helix bundle conformation which collapses later and facilitates fusion (Figure 1.6, lower panel).

![Figure 1.6: Simplified diagram depicting the process of HIV entry (Garg et al., 2011):](image)

**Top panel:** gp120 associates with CD4 and causes a conformational change in gp120 which leads to co-receptor binding. **Bottom panel:** Binding of co-receptor induces further conformational changes, including formation of a gp41 extended intermediate, hemifusion and postfusion states.

1.5. **Neutralizing antibody response to HIV-1 infection:**

1.5.1. **Introduction:**

A critical component of many vaccines is that they induce an effective neutralizing antibody response. Many diseases have been controlled using a neutralizing antibody (NAb) inducing vaccine. Smallpox, diphtheria, tetanus and many other examples exist for control of infections by NAbs (Plotkin, 2008; Plotkin, 2013). Antibodies exert their function by multiple methods such as neutralization, antigenic binding, opsonisation or antibody mediated cellular cytotoxicity (ADCC) (Overbaugh and Morris, 2012). Neutralizing antibodies act by blocking viral entry into target cells. Pre-existing neutralizing antibodies before infection provide sterilizing protection from acquisition of HIV-1 in primate models (Mascola et al., 1999; Mascola et al., 2000; Shibata et al., 1999). A major focus of HIV-1 vaccine field is to design immunogens which could induce broadly neutralizing antibodies. Vaccine induced antibodies if present before infection could diminish viral acquisition (Doria-Rose, 2010; Haigwood and Hirsch, 2009; Stamatatos et al., 2009) (Figure 1.7; lower panel) and may contribute towards the control of HIV-1 replication. Key to this proposed approach is the fact that only one or a few viruses have to be neutralized for the vaccine to prevent an infection from an exposure. This is likely to be true because most (approximately 80%) heterosexual infections
results from the dissemination of a single virus variant (Abrahams et al., 2009; Keele et al., 2008; Salazar-Gonzalez et al., 2008).

![Diagram showing two scenarios:](image)

**Figure 1.7: Neutralizing antibodies before infection might be protective** (Haigwood and Hirsch, 2009):
Neutralizing antibodies that are induced by a vaccine before infection (lower panel) may be effective as the antibodies have to tackle one or only a few virus variants (mainly the T/F virus). However, once infection is established NAbs may not contribute much towards disease progression as viral diversity is established (upper panel).

### 1.5.2. **HIV-1 envelope: Target of neutralizing antibodies:**
The HIV-1 envelope is the major target for neutralizing antibodies (Mascola and Montefiori, 2010; Pantophlet and Burton, 2006), though antibody responses to HIV-1 proteins other than envelope are induced in natural infection (Wieland et al., 1990). HIV-1 envelope protrudes from the viral membrane in the form of spikes. Most HIV-1 infections induce host neutralizing antibody responses against their own virus envelope (autologous response) within few months after infection (Bar et al., 2012; Gray et al., 2007; Richman et al., 2003). Yet, only a small subset of HIV-1 infected individuals develop broadly neutralizing antibodies (capable of neutralizing diverse HIV-1 strains).

Multiple factors govern the generation of broadly neutralizing antibodies including time since infection (Gray et al., 2011; Mikell et al., 2011; Sather et al., 2009), higher viral load (Piantadosi et al., 2009), HIV-1 envelope diversity (Piantadosi et al., 2009), lower set point CD4 T cell count (Euler et al., 2010; Gray et al., 2011), greater CD4 count decline upon infection (Gray et al., 2011) and characteristics of the initially transmitted HIV-1 variant (Gnanakaran et al., 2011).
1.5.3. Initial autologous NAb response in HIV-1 infection:
Recently, there is a better understanding of the early immune response against the viral envelope. As early as a week after plasma virus detection, antigen-antibody complexes are detected followed by the development of envelope specific antibodies (Tomaras et al., 2008). This initial antibody response is directed against the transmembrane protein gp41 (Liao et al., 2011), conversely, the antibody response against gp120 appears later. The first antibody response is largely non-neutralizing and may have very little impact on the dynamics of early virus replication (Keele et al., 2008; Liao et al., 2011; Tomaras et al., 2008). However, a recent study appreciated even low titer neutralizing antibodies capable of exerting immune pressure on the virus as early as two weeks post sero-conversion (Bar et al., 2012).

The autologous NAb response developed during the early months of infection (Aasa-Chapman et al., 2004; Gray et al., 2007; Richman et al., 2003), are principally subtype specific (Moore et al., 2008; Moore et al., 2009). They may not confer wide protection against the virus quasi-species (Burton, 1997; Wyatt et al., 1998). However, autologous neutralizing antibodies can exert immune pressure. This results in the rapid selection of HIV-1 escape variants. Escape happens by insertions, deletions, substitutions, evolving glycan shield and other mechanisms (Mascola, 2009; Moore et al., 2009; Rong et al., 2009). Thus autologous neutralizing antibodies usually do not generally neutralize the contemporaneous viral variants and lags behind the viral variants at most time points (Mascola, 2009). However, de-novo immune responses capable of targeting the escape variants are generated eventually (Bar et al., 2012; Euler et al., 2012; Gray et al., 2007; Lynch et al., 2011; Mahalanabis et al., 2009; Moore et al., 2009; Rong et al., 2009; Wibmer et al., 2013).

The pressure exerted by the NAbs comes with a fitness cost on the escape variants (Bar et al., 2012; Huang et al., 2010; Moore et al., 2009), although some studies draw the opposite conclusion (van Gils et al., 2010a). Moore at al (Moore et al., 2009) demonstrated a seven fold drop in HIV-1 viral load in a study participant with autologous anti-C3 antibodies. Bar et al demonstrated that Nab escape variants had up to 24% low replication fitness (Bar et al., 2012). In summary, autologous neutralizing antibodies, though strain specific may contribute towards a partial containment of virus temporarily.
1.5.4. **Broadly neutralizing antibodies and disease progression:**

A desirable HIV-1 vaccine would have to generate antibodies capable of neutralizing multiple heterologous HIV-1 variants (Stamatatos et al., 2009). There has been a search for BNABs within infected individuals over the past years (Doria-Rose, 2010; Doria-Rose et al., 2010b; Euler et al., 2010; Gray et al., 2011; Mikell et al., 2011; Simek et al., 2009; Walker et al., 2010). The mechanism of neutralization and understanding the immunological conditions required for antibody maturation would improve our understanding on how to induce them using a vaccine (Euler et al., 2010; Gray et al., 2011; Mikell et al., 2011; Piantadosi et al., 2009; Sather et al., 2009). Approximately 10-30% of the individuals develop broadly neutralizing antibodies, indicating such antibodies are not rare and their generation at least in natural infections is not an insurmountable task (Binley et al., 2008; Doria-Rose et al., 2010a; Euler et al., 2010; Gray et al., 2011; Mikell et al., 2011; Simek et al., 2009). Broad neutralizing antibodies develop approximately after a year of infection and peaks at around three to four years post-infection (Euler et al., 2012; Gray et al., 2011; Mikell et al., 2011; Sather et al., 2009).

Multiple factors contribute towards the development of neutralization breadth. First, broadly neutralizing antibodies tend to develop later during infection (Piantadosi et al., 2009; Sather et al., 2009). In a longitudinal study, cross neutralizing antibodies developed at about an average of 2.5 years post infection, but as earlier as one year post infection in rare cases (Mikell et al., 2011). Second, the development of breadth is associated with higher viral loads (Gray et al., 2011; Piantadosi et al., 2009; Sather et al., 2009; Simek et al., 2009) and early HIV-1 envelope sequence diversity (Piantadosi et al., 2009). This implies that constant antigenic stimulation of the immune system is a criterion for generating breadth. Thirdly, breadth has been shown to be associated with lower CD4 T cell count (before infection) and with a greater drop in CD4 cell count soon after infection (Euler et al., 2010; Gray et al., 2011).

Having broad neutralizing antibodies may not mean a better clinical outcome (Doria-Rose, 2010) as shown by two studies (Euler et al., 2010; Piantadosi et al., 2009). In one study (Piantadosi et al., 2009), neutralization breadth was assessed by measuring the neutralizing antibody levels in 70 ARV-naive women from a Kenyan cohort. Breadth of neutralization at that time point did not correlate with slower disease progression (delayed time for CD4 T-cell decline, initiation of ART and death by AIDS). Another study by Euler et al (Euler et al., 2010) demonstrated the lack of association.
between neutralization breadth at three years’ post sero-conversion and time from sero-conversion to AIDS and AIDS related death in three groups of individuals (individuals with strong, moderate and weak neutralization breadth) with varying levels of neutralizing antibodies. To conclude, the breadth of neutralizing antibody response during an HIV-1 infection does not appear to be associated with protection from subsequent disease. At the very least, this suggests that any fitness cost associated with escape from broader antibodies is not sufficient to slow progression to disease.

1.5.5. **Viral defence mechanisms to neutralizing antibodies:**
Viruses evade neutralizing antibodies by multiple pathways (Dörrer and Radbruch, 2007; Pantophlet and Burton, 2006). Viral diversity is a major challenge to tackle HIV-1 by neutralizing antibodies (Mascola, 2009; Moore et al., 2009; Rong et al., 2009). The HIV-1 is highly diverse virus with >20% diversity in the envelope region within each subtype (Lynch et al., 2009). Nine subtypes and >35 circulating recombinant forms exist in group M: envelope sequences vary by >35% in their amino acid sequence (Hemelaar, 2012; Hemelaar, 2013; Hemelaar et al., 2006; Hemelaar et al., 2011a; Lynch et al., 2009). A broadly neutralizing antibody would have to target conserved regions to neutralize multiple variants (Burton et al., 2012b). Another concern is the glycan shielding (Pantophlet and Burton, 2006). Approximately 50% of the gp120 is covered with carbohydrates which frequently shield the inner conserved domains from NAbs. Additionally by an “evolving glycan shield”, HIV-1 evades neutralizing antibodies (Moore et al., 2012). However, the immune system also co-evolves to recognize the glycan moieties and generates glycan-specific neutralizing antibodies as demonstrated by the mAbs 2G12 and PGT128 (Scanlan et al., 2002; Walker et al., 2011). Mutation of target epitopes to escape neutralizing antibodies is another evasion strategy (Euler et al., 2012). In support of this, sera from infected individuals would neutralize viruses from earlier time points but not the contemporaneous virus variants (Frost et al., 2005; Mahalanabis et al., 2009; Richman et al., 2003).

1.6. **Major target sites for broadly neutralizing monoclonal antibodies:**
1.6.1. **Introduction:**
Though the viral envelope spike is highly secluded from NAbs, a handful of BNAbs have been isolated from HIV-1 infected individuals (Figure 1.8). These mAbs are useful reagents to understand the targets within HIV-1 envelope. Some mAbs are broad and neutralize viruses from different subtypes, implying they target conserved regions within gp160 (Mascola and Montefiori, 2010). However, autologous viruses within broad neutralizers from whom mAbs were isolated are generally resistant (Huang et al., 2012; Wu et al., 2012), as they acquire escape mutations.
Considerable advances have been made in the isolation of broadly neutralizing mAbs against the viral envelope in the recent years. Two key reasons for this advance are; novel techniques to isolate mAbs (Huang et al., 2012; Scheid et al., 2011; Walker et al., 2009a; Wu et al., 2010) and standardized neutralization assays for high throughput screening of HIV-1 infected sera/plasma and mAbs by neutralization (Montefiori, 2005). There exist multiple mAb target sites within the gp120 and one target site within the gp41 (Burton et al., 2012a) (Figure 1.8). mAbs targeting gp120 have been mapped to the CD4 binding site (Burton et al., 1994; Wu et al., 2010), V2 glycan (Bonsignori et al., 2011; Walker et al., 2011; Walker et al., 2009a) which recognize a quaternary virus envelope structure and glycan shield (primarily V3/V4) (Buchacher et al., 1994; Walker et al., 2011). However, within gp41, the MPER is the sole target of broadly neutralizing mAbs isolated to date (Buchacher et al., 1994; Huang et al., 2012).

**Figure 1.8: Major neutralization targets within HIV-1 envelope spike** (Burton et al., 2012a)
1.6.2. CD4 binding site:
The CD4 binding site (Figure 1.8) is an attractive mAb target (Burton, 1997; Burton et al., 2012a; Pantophlet and Burton, 2006; Wyatt et al., 1998; Wyatt and Sodroski, 1998). It is highly conserved and functionally essential to infect the target cell. The CD4 binds to a recessed region in the outer domain of HIV-1 gp120 (Wyatt and Sodroski, 1998) and this binding initiates infection (Pantophlet and Burton, 2006). Despite, being highly conserved, the CD4-binding site is protected from humoral recognition by glycan and conformational masking (Chen et al., 2009).

Monoclonal antibody b12:
The first broadly neutralizing CD4 binding site mAb b12 was isolated in 1994 by a phage display strategy (Burton et al., 1991; Burton et al., 1994). The mAb b12 neutralized approximately half of the virus panel tested by Binley et al (Binley et al., 2004) and is moderately broad. The epitope of b12 overlaps with the CD4 binding site of gp120 and mediates neutralization by occluding CD4 from binding to gp120 (Pantophlet and Burton, 2006). An early crystal structure of b12 (Saphire et al., 2001) revealed that the heavy chain CDR of b12 penetrates into the CD4 binding site of gp120. A later crystal structure (Zhou et al., 2007) of b12 with the gp120 stabilized in the CD4 bound state showed that the mAb binds to a conformationally invariant surface of gp120, and is thus a site of high vulnerability. However HIV-1 envelope diversity at even the b12 contact site is likely, explaining why b12 is not very broadly neutralizing (Chen et al., 2009; Wu et al., 2009).

VRC-series monoclonal antibodies:
Years after the isolation of b12, a novel technique was used to isolate several mAbs (VRC-series) of much greater breadth and potency compared to the previously isolated mAbs directed at the CD4 binding site (Wu et al., 2010). VRC01, for example, is a very broad and potent mAb. It neutralized over 90% of the isolates tested (Wu et al., 2010) and have extremely low geometric mean IC50 (0.25 ug/ml) titer (Huang et al., 2012). VRC01 antibodies are highly somatically hypermutated (Wu et al., 2010; Zhou et al., 2010). The CD4 binding site specific mAbs were isolated using a “bait method”, in which a resurfaced gp120 probe specific for the CD4 binding site was used to isolate and clone CD4 binding site specific memory B cells (Wu et al., 2010). Though the CD4 binding surface was retained in the probe, parts of the variable gp120 domains were not included to avoid isolating non-neutralizing antibodies. Around 90% of the binding surface of VRC01 was focused on the outer domain of gp120 (Zhou et al., 2010) which is very crucial for CD4 binding.
Monoclonal antibodies NIH45-46:
Another group of unusually broad and potent CD4 binding site antibodies were generated using a slightly different probe (Scheid et al., 2009). The probe (2CC) was a gp120 core stabilized in the CD4 bound conformation, but unlike the RSC3 probe (used to capture VRC-series antibodies) could also capture the co-receptor binding site antibodies in addition to the CD4 binding site antibodies (Scheid et al., 2011). NIH45-46 and 3BNC117 isolated with this method were very potent and neutralized 96% of the 118 viruses tested (Scheid et al., 2011). These antibodies are clonally related to VRC01 and mimic CD4 binding to gp120.

Conclusion:
In conclusion, all the CD4 agonist antibodies may act by destabilizing the envelope trimer. Such antibodies are not very rare, but are indeed made in some HIV-1 infected individuals (Scheid et al., 2011; Wu et al., 2010; Wu et al., 2011). However, some of the recently isolated mAbs are unusually potent and broad.

1.6.3. Quaternary sites:
Monoclonal antibody PG9/PG16:
The PG9/PG16 mAbs target another vulnerable site in the HIV-1 gp120 (Walker and Burton, 2010). They were isolated from a subtype A infected African donor (Walker et al., 2009a) by screening IgG culture supernatants from stimulated memory B cells distributed at clonal density. PG9/PG16 exhibit broad neutralization activity. 70-80% of the isolates tested were neutralized by PG9/PG16, which are somatically related variants. PG9 and PG16 tend to recognize the quaternary structure of the envelope in the context of the trimeric viral spike conformation, rather than binding to the monomer (Moore et al., 2011; Walker et al., 2009a). PG9/PG16 recognizes a glycosylated motif in the V2 domain. Additional residues in the apex of V1/V2, and V3 are also necessary (Walker et al., 2009a). However, PG16 shows a greater interaction with V3 domain than PG9 (Moore et al., 2011; Walker et al., 2009a). The V1/V2 regions in HIV-1 are thought to have evolved as a viral evasion strategy; however, PG9/PG16 mAbs show extensive neutralization breadth target the variable regions in gp120 via glycan recognition. Alanine scanning mutants revealed that Phe159, Asn160, Lys169, Lys171 and Lle181 within the V2 domain being the major amino acid residues recognized by PG9/PG16 (Moore et al., 2011; Walker et al., 2009a).

Crystal structure of the CDRH3 region of PG16 mAb revealed a hammerhead like conformation being critical for its neutralization property (Pejchal et al., 2010). Furthermore, the extended CDRH3
regions (28 amino acid residues long) were tyrosine sulphated and this sulfation was functionally
important in mediating neutralization. The crystal structure of PG9 (McLellan et al., 2011) with a
V1/V2 scaffold also revealed how PG9 interacts with the variable domains and also gave insight into
the V1/V2 domain structure. The CDRH3 loop of PG9 penetrates the glycan located at Asn160
Asn156. The CDR3 loops interact further with the V1/V2 region strand C. Besides, multiple
hydrogen bonds between the antibody and the glycans, supports and strengthens these interactions.

**Monoclonal antibody PGT141-145 series:**
Another clonally related set of mAbs also target a quaternary epitope (Walker et al., 2011). PGT141-
145 has specificities similar to PG9/PG16 antibodies. PGT145, the most potent in this series,
neutralized 78/162 viruses tested at IC50<50 ug/ml. PGT145 was extremely potent with a median
IC50 of 0.29ug/ml. Structural analysis indicated that PGT141-145 series mAbs penetrate the variable
loop glycans by extended anionic loops (McLellan et al., 2011). and the CDRH3 loop like
PG9/PG16 were tyrosine sulphated.

**Conclusion:**
PG9/PG16 and PGT-like antibodies with broad neutralization capacity are often inducible in natural
infections (Moore et al., 2011; Walker et al., 2011; Walker et al., 2009a). In a subtype C
superinfected individual (CAP256), antibodies targeted a quaternary V1V2 domain which
overlapped with the PG9/PG16 epitope (Moore et al., 2011). Thus regions within V2/V3 domain are
immunologic and conserved enough to induce broadly neutralizing antibodies. The quaternary
structure recognizing mAbs described above neutralize diverse strains of HIV-1 at low
concentrations which plausibly could be achieved by vaccination (Walker and Burton, 2010).
However, generating immunogens that mimic this surface and induce such potent antibodies are
likely to be challenging.

1.6.3. Glycan dependent monoclonal antibodies:

**Monoclonal antibody 2G12:**
The prototype glycan dependent mAb, 2G12 was isolated in 1994 by an EBV-electrofusion
technique (Buchacher et al., 1994). Having a neutralization breadth of 25-30% this mAb recognizes
a cluster of conserved high-mannose glycans (Figure 1.8) on gp120 (Scanlan et al., 2002). Alanine
scan mutants of the Asparagine residues at position 295, 332 and 392 resulted in a significant
reduction in binding to gp120. However, 2G12 has an unusual dimer conformation (Calarese et al.,
The mAb 2G12 is said to recognize the “silent face” of gp120, because the 2G12 epitope is highly shielded by other oligosaccharide molecules, thus protecting the conserved gp120 region.

**Monoclonal antibodies PGT 121-137 series:**
Recently, a series of mAbs have been isolated from a set of elite neutralizers (Simek et al., 2009). The epitopes of these mAbs (PGT121–PGT123, PGT125–PGT128, PGT130, PGT131 and PGT135–PGT137) are dependent upon the same glycan as 2G12 mAb (Walker et al., 2011). The PGT 121-137 series mAbs competed with 2G12, confirming that he epitopes overlap. However, the PGT series mAbs were much broader and potent than 2G12, with a median IC\textsubscript{50} of 0.02 ug/ml for PGT128 (Walker et al., 2011). Most mAbs also failed to bind to a V3 deleted gp120, suggesting their epitope overlaps V3 region. Mapping specific amino acid residues by alanine scanning suggested that N-glycans at position 301 and 302 to be critical for their neutralization. Crystal structures of PGT 128 revealed a glycan specific (mannose) binding site (Pejchal et al., 2011). Neutralization was attributable to its ability cross-link the envelope trimers by penetrating the glycan shield to reach the V3 loop of HIV-1 gp120 (Julien et al., 2013a; Pejchal et al., 2011).

**Conclusions:**
The PGT antibodies exhibit a multivalent targeting approach and recognize a combination of glycans and proteins on HIV-1 gp120 (Pejchal et al., 2011). Unlike the prototype glycan recognizing mAb 2G12, PGT 121 and 128 are broader and more potent (Walker et al., 2011).

1.6.4. **MPER:**
The MPER as a target site for existing monoclonal antibodies is detailed in section 1.7

1.7. **Membrane Proximal External Region (MPER) as a vaccine target:**
1.7.1. **Introduction:**
The membrane proximal external region of HIV-1 is also an important vaccine target. The MPER is a linear stretch of 24 amino acids (660-683 HXB2 numbering) and is located at the C terminus of the gp41 ectodomain. The MPER is considered an attractive vaccine target due to the following reasons (Table 1.1):
Table 1.1: Membrane Proximal External Region as a vaccine target

<table>
<thead>
<tr>
<th>Why MPER is an attractive vaccine target</th>
<th>Implication for vaccine design</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPER plays critical role in virus envelope fusion to the host cell membrane (Blumenthal et al., 2012; Wyatt and Sodroski, 1998)</td>
<td>Neutralizing antibodies targeting MPER before virus fusion may prevent virus entry into the host.</td>
</tr>
<tr>
<td>MPER is relatively less variable in comparison to other regions of HIV-1 gp160 (Sun et al., 2008; Zwick, 2005).</td>
<td>Antibodies targeting highly conserved HIV-1 epitopes may have greater neutralization breadth. But, steric occlusion of MPER epitopes in the native envelope spike seems to reduce accessibility to neutralizing antibodies (detailed in section 1.7.4)</td>
</tr>
<tr>
<td>Three human monoclonal antibodies (10E8, 2F5 and 4E10) target MPER and have exceptional neutralization breadth (Binley et al., 2004; Huang et al., 2012).</td>
<td>Proof-of-concept that broad anti-MPER neutralizing antibodies can be induced. However, most MPER directed broadly neutralizing mAbs tend to be polyreactive (detailed in section 1.7.3.3)</td>
</tr>
<tr>
<td>Many MPER epitopes are linear epitopes rather than complex tertiary structures (Gray et al., 2009a; Sun et al., 2008)</td>
<td>Short linear peptides may recapitulate MPER epitopes thus making vaccine design simpler. However inducing anti-MPER antibodies with MPER mimics has been largely unsuccessful (Dennison et al., 2011a; Guenaga et al., 2011; Wahome et al., 2012).</td>
</tr>
</tbody>
</table>

1.7.2. **Structure and function of MPER:**

The MPER comprises the last 24 amino acids of the gp41 ectodomain. At least three broadly neutralizing mAbs target different overlapping regions of the MPER (Huang et al., 2012; Zwick, 2005). The MPER region is highly conserved (Montero et al., 2008a), plausibly due to the functional constraints as the MPER is critical in viral fusion (Buzon et al., 2010). The amino acid sequence of MPER (HXB2, numbering 660-683) is “LLELDKWASLWNWFNITNWLWYIK” (Montero et al., 2008a). Fifteen of the twenty four amino acids within the MPER are hydrophobic in nature. The hydrophobic amino acids within the MPER tend to be the most conserved in comparison to the polar
amino acid residues (Sun et al., 2008) and many amino acid residues in the MPER are invariant across subtypes (see also Figure 2.8).

An early NMR study (Schibli et al., 2001) using a 19-residue peptide in the MPER solubilized using dodecylphosphocholine micelles revealed a helical structure for the MPER. The recent crystal structures of the MPER peptide with mAbs (Brunel et al., 2006; Buzon et al., 2010; Huang et al., 2012; Ofek et al., 2004) have given more clarity on the MPER structure. The 2F5 and 4E10 epitopes are partially embedded within the viral membrane (Cardoso et al., 2005; Sun et al., 2008). Crystal structure of 4E10 (Cardoso et al., 2005) with a 13-mer peptide containing the 4E10 epitope confirmed the helical nature of the MPER when bound to an anti-MPER antibody. Amino acid residues essential for 4E10 faced towards the same side of the helix. In another (Sun et al., 2008) NMR study carried on solubilized MPER peptide (HXB2), the MPER adopted a meta-stable L shaped structure. The N terminus (664-672) had a tilted alpha-helix and was joined to an almost horizontal C terminal helix (675-683) via a hinge (673-674). Parts of the C terminus were immersed into the membrane. The 10E8 mAb crystal structure with an MPER peptide (656-683) gave more insight into the structure of the MPER (Huang et al., 2012). Corroborating the previous studies (Cardoso et al., 2005; Liu et al., 2009; Sun et al., 2008), the MPER had an N-terminal helix (extending Asn 657-Ala 667) and a C terminal helix (Trp672-Arg683) separated by a 3_{10}-helix (Ser 668 and Leu 669). In sharp contrast to the above studies, a crystal structure of Z13e1 mAb with a 12 residue peptide (670-677), adopted a S-shaped structure (Pejchal et al., 2009) and differed from the alpha-helical structure adopted by 4E10 or 10E8 bound peptide (Cardoso et al., 2005; Huang et al., 2012; Sun et al., 2008).

The MPER plays an essential role in the fusion of viral membrane to the host cell membrane (Dimitrov et al., 2003; Munoz-Barroso et al., 1999; Salzwedel et al., 1999). Deletion of a hydrophobic amino acid stretch in the MPER (666-682) abrogated fusion. The Tryptophan residues were the most crucial, as alanine mutants of tryptophan residues in this region reduced fusion (Salzwedel et al., 1999). The role of the MPER in fusion was again confirmed by Dimitrov et al; deletion of amino anid residues (665-682), resulted in the loss of virus’s fusogenicity (Dimitrov et al., 2003). These studies confirm the role for at least the C terminal of the MPER in mediating fusion.
1.7.3. Antibody response to the MPER:

1.7.3.1. Introduction:
Exposure of gp41 to neutralizing antibodies is limited compared to gp120, because some regions of gp41 are occluded between gp120 trimer in the envelope spike (Pietzsch et al., 2010). The gp41 domain is only transiently exposed during fusion process and during the shedding of gp120. Due to the above reason, gp41-directed neutralizing antibodies tend to be rare (an exception is the monoclonal antibody D5 targeting the HR1 region of gp41 (Miller et al., 2005). However, gp41-targeting non-neutralizing antibodies are generated in HIV-1 infection and this may be due to stimulation of the immune system by free or poorly folded gp41 that may be released after shedding by dead HIV-1 viruses. Thus, gp41 may appear immunodominant with respect to total antibody production, but not with respect to neutralizing antibody production.

The MPER is the major neutralizing antibody target of HIV-1 gp41 and is well studied (Montero et al., 2008a; Zwick, 2005). MPER specific antibodies have been characterized in natural infections and MPER monoclonal antibodies have been isolated from broad neutralizers. Four mAbs exist against this region of which three are broadly neutralizing (Huang et al., 2012; Zwick, 2005) (see below). Binding to the MPER presumably blocks the viral entry to the host cell membrane by interfering with membrane fusion (Binley et al., 2003). Several studies on broad neutralizers have demonstrated the presence of MPER antibodies (Binley et al., 2008; Gray et al., 2011; Gray et al., 2009a; Tomaras et al., 2011). The most important studies are summarized below.

1.7.3.2. Natural immune response to the MPER:
Approximately a quarter of HIV-1 infected individuals develop anti-MPER antibodies (Gray et al., 2007; Huang et al., 2012) in the course of infection. In a cohort within USA, the prevalence of MPER antibodies was 27% (21/78) (Huang et al., 2012) with 18% having high titer anti (ID$_{50}$>1:1000) MPER antibodies. In a South African cohort, MPER antibodies were detectable in 15 of the 50 participants (30%), while only two (4%) individuals had high titer anti MPER antibodies (Gray et al., 2009b). As detailed in chapter two of this thesis, we find anti-MPER antibodies in 19% of the South African cohort (Figure 2.2).

Several studies have shown the existence of MPER antibodies within broad neutralizers in plasma samples (Gray et al., 2009a; Gray et al., 2009b; Li et al., 2009; Tomaras et al., 2011) and their direct contribution towards neutralization breadth has been explored (Gray et al., 2009a; Tomaras et al., 2011). One study (Tomaras et al., 2011) of nine broad neutralizers (Plot C, Figure 1.9) revealed anti-
MPER antibodies in four individuals, of which three directly mediated neutralization breadth via anti-MPER antibodies. This was shown by anti-MPER antibody depletion in these samples, which resulted in a drop of neutralization activity (2 fold or higher) of at least four viruses tested. Another confirmation of MPER mediated heterologous neutralization in three broad neutralizers was demonstrated by Gray et al (Gray et al., 2009a). In yet another study by the same group (Gray et al., 2011), a broad neutralizer (CAP206) was shown to mediate heterologous neutralization activity by the MPER antibodies (Plot B, Figure 1.9). The anti-MPER antibodies from the above studies primarily targeted the C-terminus of the MPER, but were distinct from the targets of existing mAbs. The antibodies were fine mapped to other amino acid residues (670, 673 and 674) within the MPER.

![Figure 1.9: Contribution of anti-MPER antibodies to neutralization breadth in broad neutralizers](plot)

Antibody targets in broad neutralizers were comprehensively mapped to understand the targets. Broad neutralizers (n=19) from Walker et al study (A) lacked MPER activity (Walker et al., 2010). Broad neutralizers (n=7 and 9 respectively) from the other two cohorts (Tomaras et al., 2011) had anti-MPER NAbs (B and C).

### 1.7.3.3. **Monoclonal antibodies targeting the MPER:**

Four broadly neutralizing mAbs targeting MPER have been isolated until now, of which three are broadly neutralizing. Their epitopes and neutralization mechanisms are different. Mutation of residues within the epitope resulted in the loss of neutralization activity (Brunel et al., 2006; Huang et al., 2012; Zwick et al., 2004).
**Monoclonal antibody 10E8:**

10E8 (Huang et al., 2012) is one among the most broad and potent MPER mAb and has a 6-9 fold lower geometric mean IC50 titre in comparison to 4E10 and 2F5. At an IC50 <50ug/ml, 10E8 neutralized 98% of the 180 viruses isolates tested. Additionally, the geometric mean IC50 of 10E8 for neutralizing a panel of 181 viruses was low (0.22ug/ml) and was comparable to the broad neutralizing antibodies like VRC01 and PG9/PG16. Anti-MPER mAbs have been shown to be associated with lipid binding and auto-reactivity (Haynes et al., 2005), but, 10E8 does not bind to phospholipids and is not auto-reactive in a manner similar to 4E10 and 2F5 (Haynes et al., 2005) however, a recent report suggests that its activity may depend upon association with membranes (Chen et al., 2013). 10E8 is highly somatically hypermutated indicating its extensive affinity maturation and possess long CDRH3 loops. In contrast to 4E10 and 2F5-like antibodies, 10E8-like antibodies are also more frequently induced (approximately 8%), although most 10E8-like antibodies appear not to be broad (Huang et al., 2012).

The minimal linear 10E8 epitope was recognized between residues 671-683 of the MPER. Though the 10E8 epitope had a considerable overlap with the 4E10 epitope; it differed from 4E10 epitope in the following aspects.

1. Both 10E8 and 4E10 require Trp672, Phe673 and Trp680 for neutralization. But additional residues like Asn671 and Trp683 are crucial for 10E8 recognition (which 4E10 doesn’t require).
2. 10E8 recognizes approximately 1/3rd of the C terminal helical face of the MPER, while 4E10 recognizes more than 50%.

Designing an immunogen which can induce 10E8-like broadly neutralizing antibodies would be presumably protective and is of high priority.

**Monoclonal antibody: 4E10:**

The mAb 4E10 was generated using EBV-electro-fusion (Buchacher et al., 1994). 4E10 has exceptional breadth and neutralized 98% of the 181 HIV-1 isolates at <50ug/ml (Huang et al., 2012). In a previous study, 4E10 neutralized all viruses tested (Binley et al., 2004). The epitope for 4E10 is located towards the C terminus of the MPER, with “NWFDIT” being considered as its core epitope (Zwick, 2005). Alanine scanning mutagenesis on HIV-1JR-FL confirmed that Trp672, Phe673 and Trp680 as the three most critical residues critical for 4E10 neutralization (Zwick et al., 2005) and this was confirmed by surface plasmon resonance (Brunel et al., 2006). Cardoso et al (Cardoso et al., 2005) identified the crystal structure of the 4E10 core epitope (NWFDIT) in a synthetlic peptide
bound with the mAb 4E10. 4E10 recognized a helical confirmation of 4E10 epitope with the key binding residues facing towards one side of the helix. 4E10 epitope has a L-shaped structure, with an alpha-helix at the N-terminus of the epitope followed by a helical C-terminal region (Sun et al., 2008) which is partially buried within the viral membrane. Both helices are connected by a hinge region. The mAb has the ability to extract the partially buried epitope after its initial binding to the surface protruding region of the epitope (Sun et al., 2008). This property of 4E10 mAb is unique and may explain its broad neutralization capacity.

Though broadly neutralizing, 4E10 has some undesired characteristics. 4E10 is an auto-reactive antibody (Haynes et al., 2005). 4E10 reacts with cardiolipin and systemic lupus erythematosus (SLE) auto-antigens. In addition, 4E10 reacts with some components of bio-membranes (e.g., Phosphatidyl serine and related members). This can readily explain why 4E10-like antibodies appear rarer during natural infections, as self reactive B cells may become anergic or get clonally depleted during B cell development. Although one report suggests that its autoreactivity is a minor part of its binding activity (Singh et al., 2011), B cells in mice expressing a transgenic 4E10 antibody are deleted (Doyle-Cooper et al., 2013).

**Monoclonal antibody: 2F5:**

The mAb 2F5 is less broad, but more potent than 4E10 (Binley et al., 2004). Also generated by electro-fusion, the 2F5 epitope is proximal to the 4E10 epitope. The linear epitope is designated as “ELDKWA”. In a comprehensive study by Binley et al, 2F5 neutralized 67% of the virus isolates tested; however all subtype C viruses tested remained resistant due to a substitution (DSW instead of DKW) in the 2F5 epitope (Binley et al., 2004). Alanine scanning mutagenesis of 2F5 epitope revealed Asp664, Lys665 and Trp666 as the critical target residues in the 2F5 epitope important for neutralization (Zwick et al., 2005). A crystal structure (Zwick et al., 2004) of 2F5 mAb with the linear 2F5 epitope (662-667) indicated that 2F5 binds at the base of the linear epitope using the extraordinarily long CDRH3 loops. 2F5 tends to bind to the charged face of the 2F5 epitope, while the hydrophobic unbound face of the peptide was occluded (Ofek et al., 2004). Like 4E10 mAb, 2F5 cross reacts with self antigens (Haynes et al., 2005) and is thus polyreactive. Possibly due to this reason, 2F5-like antibodies are rarely produced in natural infections (Shen et al., 2009).
1.7.4. **Changes in accessibility of the MPER to neutralizing antibodies during target cell binding:**

The MPER accessibility to neutralizing antibodies depends on the stage of virus fusion. The envelope trimer exist in three conformational states; the prefusion state when epitopes are less accessible (gp41 surrounded by the quaternary envelope spike), the metastable pre-hairpin intermediate state and the post-fusion state (Blumenthal et al., 2012; Garg et al., 2011; Montero et al., 2008a; Zwick, 2005). The MPER is most accessible at the pre-hairpin intermediate state as shown with 4E10 mAb (Frey et al., 2010; Frey et al., 2008). Additionally, parts of the MPER may get inserted into the viral membrane, reducing its accessibility to neutralizing antibodies (Montero et al., 2012; Sun et al., 2008). Dennison et al. (Dennison et al., 2009) suggested that the 4E10 epitope may be inserted into the membrane of synthesized liposome immunization constructs, reducing 4E10 binding. The MPER accessibility also varies at the time of viral invasion (Chakrabarti et al., 2011; Dimitrov et al., 2007). It is known that fusion is associated with a conformational change in gp41 following CD4 binding (Eckert and Kim, 2001; Gallo et al., 2003). Dimitrov et al. (Dimitrov et al., 2007) suggests that anti-MPER neutralizing antibodies target the fusion intermediate. Chakrabarti et al (Chakrabarti et al., 2011) present evidence that MPER is differentially exposed on the pre-attachment envelope spike. Those viruses that do not expose their MPER in the pre-attachment spike were more resistant to 4E10 and 2F5 antibodies overall. Thus MPER is partially accessible in the envelope spike of some viruses before the virus attaches to a cell, and viruses with an accessible MPER in the envelope spike are more sensitive to neutralization by MPER antibodies (Chakrabarti et al., 2011). Changes in the lentivirus lytic peptide-2 (LLP-2) domain of the cytoplasmic tail of gp41 also affect sensitivity to 4E10 (Gray et al., 2008).

1.8. **Study objectives:**

This thesis evaluated the MPER of HIV-1 envelope as a possible antibody based vaccine target. Additionally, this thesis aimed to identify highly neutralization resistant viruses and use them as a tool to characterize recognition of vulnerable epitopes (Jacob et al., 2012).

**Rationale of the study:**

The work in this thesis is an effort to gather the basic information needed to evaluate and potentially use the HIV-1 gp41 MPER as a neutralizing antibody based vaccine target. An approach to induce neutralizing antibodies against HIV-1 envelope might be fruitful for vaccine development (Mascola and Montefiori, 2010; Sattentau, 2008; Wyatt and Sodroski, 1998). The epitopes of broadly neutralizing monoclonal antibodies which target the HIV-1 gp120 and HIV-1 gp41 are candidates for
vaccine design (Burton et al., 2012a; Burton et al., 2012b; Walker and Burton, 2010). The MPER region of HIV-1 gp41 is an attractive vaccine target as it is highly conserved in nature (Montero et al., 2008a; Sun et al., 2008; Zwick, 2005). The MPER is also constrained by functions due to its involvement in viral fusion (Montero et al., 2008a; Sun et al., 2008; Zwick, 2005). Anti-MPER neutralizing antibodies in some broad neutralizers have been shown to mediate neutralization of viruses (Gray et al., 2011; Gray et al., 2009a; Tomaras et al., 2011) and three MPER monoclonal antibodies are broadly neutralizing (Binley et al., 2004; Huang et al., 2012). However, inducing neutralizing anti-MPER antibodies using an immunogen has proven largely unsuccessful (Dennison et al., 2011b; Guenaga et al., 2011; Kamdem Toukam et al., 2012; Wahome et al., 2012) although there have been some recent modest successes. Though conserved, epitopes within the MPER are reported to be only transiently available (Frey et al., 2010; Frey et al., 2008), difficult to access (Chakrabarti et al., 2011) and sometimes immersed in the viral membrane (Sun et al., 2008) in HIV-1 isolates. In this thesis, we investigated the ability of MPER epitopes to induce broadly neutralizing antibodies in natural HIV-1 infections. Additionally, we establish that although the MPER is a vulnerable target in highly neutralization resistant CRF02_AG viruses, it is inaccessible to most anti-MPER antibodies.

The specific objectives of my thesis are outlined below

1. **Specific objective 1 (Chapter 2):**

The Thai vaccine trial in Thailand showed modest efficacy (31%) (Rerks-Ngarm et al., 2009); with vaccinees showing a weak neutralization activity against highly sensitive (tier 1 (Seaman et al., 2010a)) viruses (Montefiori et al., 2012). Binding antibodies directed against the variable region 2 (V2) region of the envelope was a correlate for protection (Haynes et al., 2012). Yet, the antiviral mechanism behind V2 specific protection remains unclear. In the second chapter of this thesis, we asked the question: How likely does the MPER epitopes induce broadly neutralizing antibodies compared to the PG9/16 (a notable epitope within the V2) target site. Neutralization breadth was observed to be associated with the presence of anti-MPER antibodies. However, PG9/16-site specific antibodies were less likely to be broadly neutralizing in comparison to sera that lacked dominant anti-PG9/16-site neutralization activity suggesting that the MPER might be more amenable for vaccine design.
2. **Specific objective 2 (Chapter 3):**
The neutralization activity of CRF02_AG subtype infected plasma samples was examined and highly neutralization resistant CRF02_AG viruses were identified (Jacob *et al.*, 2012). The thesis further explored how such highly resistant viruses could be neutralized. Even highly resistant CRF02_AG viruses were sensitive to MPER specific monoclonal antibodies, suggesting that even the resistant viruses can be targeted by anti-MPER neutralizing antibodies (Jacob *et al.*, 2012).

3. **Specific objective 3 (Chapter 4):**
Finally, this thesis describes an HIV-1 isolate (253-11, CRF02_AG subtype) which is resistant to neutralization by most anti-MPER antibodies. This isolate remained resistant to anti-MPER antibodies in both the pre- and post-attachment conformation of gp41. The research provides evidence that MPER inaccessibility is a common but not universal phenomenon among HIV-1 isolates. The possible explanations for the MPER resistance against anti-MPER neutralizing antibodies are discussed.
Chapter Two:
HIV-1 neutralization breadth is associated with the presence of anti-Membrane Proximal External Region (MPER) antibodies and not of anti-PG9/16-site antibodies

2.1. Summary:

Background:
Broadly neutralizing antibodies in HIV-1 infected individuals target conserved regions in the viral envelope, and two of their targets include the Membrane Proximal External Region (MPER) and the PG9/16-site. In this report we measured the neutralization activity directed at the MPER and at epitopes overlapping the PG9/16-site in 177 serum samples from ARV-naïve chronically HIV-1 infected individuals, and correlated this with neutralization breadth.

Results:
Sera were screened for neutralization breadth on a diverse, neutralization-resistant HIV-1 pseudovirus panel (n=24). An ID₅₀ value for all serum/virus combinations was either directly measured or inferred from the percentage neutralization at 1/100 serum dilution using a statistical prediction model. Thirty-five sera (20%) were identified as broad neutralizers (geometric mean ID₅₀>200 against the panel viruses). Furthermore, antibodies directed to the MPER (measured using HIV-2/HIV-1 MPER chimeric viruses) and PG9/16-site (measured using N160A and K169E mutants) was measured. Anti-MPER activity (19%, 33/177) and anti-PG9/16-site antibodies (34%; 37/108) were frequent. The neutralization breadth of the MPER recognizing sera was significantly higher than that of the non-MPER recognizing samples (median 102 vs 53, \(p<0.0001\)); in contrast, samples with dominant anti-PG9/16 site antibodies did not elicit higher neutralization breadth than samples without dominant anti-PG9/16-site antibodies (median 114 vs 105, \(p=0.64\)), and were less likely to be broadly neutralizing. In accord with this, the epitope variability of MPER was substantially lower than that of the PG9/16-site and other antibody targets, measured from analyzing 3829 envelope sequences.

Conclusions:
This is the first study to systematically assess the correlation between neutralization breadth and anti-MPER and anti-PG9/16-site antibodies. Anti-MPER containing sera were more broadly neutralizing than non-anti-MPER-containing sera, and an equivalent association for sera containing anti-PG9/16 site antibodies was lacking. Although successes at inducing anti-MPER antibodies by vaccination have been limited to date, our data suggest that this may be more approachable, even if PG9/16-site immunogen models can be engineered.
2.2. **Introduction:**

A relatively small number of epitopes have been identified on the HIV-1 envelope glycoproteins, gp120 and gp41 that are targets of broadly cross-neutralizing antibodies (Kwong and Mascola, 2012). These targets are models for candidate vaccine antigens (Burton et al., 2012a). The membrane proximal external region (MPER), a linear stretch of 24 amino acids in the gp41 and the PG9/16 site, which is a quaternary epitope comprised of the gp120 V2 and V3 domains are two important vaccine targets; with different structural and antigenic properties. The MPER is the target of three (10E8, 4E10 and 2F5) broadly neutralizing monoclonal antibodies (mAb) (Binley et al., 2004; Huang et al., 2012), while the V2/V3 site is the target of the broadly neutralizing mAb PG9 and PG16 (Walker et al., 2009b) and the type specific mAb 2909 (Gorny et al., 2005). MPER appears to be a relatively simple antigen derived from a linear amino acid sequence (Zwick, 2005), but harbours substantial complexity (Liu et al., 2009; Montero et al., 2012; Montero et al., 2008b; Shen et al., 2010; Sun et al., 2008). Linear peptides can partially mimic MPER epitopes by binding to and hence depleting anti-MPER antibodies (Gray et al., 2009a; Li et al., 2009; Morris et al., 2011; Tomaras et al., 2011) and can be used to model the binding of antibodies to MPER (Huang et al., 2012; Ofek et al., 2004; Sun et al., 2008). However, inducing neutralizing anti-MPER antibodies with MPER mimics has proven largely unsuccessful (Dennison et al., 2011b; Guenaga et al., 2011; Kamdem Toukam et al., 2012; Wahome et al., 2012), although there have been some recent modest successes (Lutje Hulsik et al., 2013; Ye et al., 2011; Zhang et al., 2013; Zhou et al., 2012). On the other hand, the quaternary epitopes such as the PG9/16-site are presumably only formed in trimeric envelope structures, including the envelope spike. It is presumed that this will make immunogen design more difficult (McLellan et al., 2011).

Among broadly neutralizing sera, antibodies targeting the MPER (Gray et al., 2009a; Gray et al., 2009b; Li et al., 2009; Tomaras et al., 2011) and epitopes overlapping the PG9/16-site (Gray et al., 2011; Tomaras et al., 2011; Walker and Burton, 2010) are found. However, the prevalence of anti-MPER and anti-PG9/16 site antibodies and how frequently they are associated with neutralization breadth has not been systematically studied. In this chapter, the neutralization breadth, MPER-specific neutralization activity and the PG9/16-site-specific neutralization in a cohort of ART-naïve HIV-1 individuals (infected >1 year) was measured. Further, we investigated if these epitopes induce antibodies with broad neutralization activity. We noticed that the neutralization breadth of the MPER recognizing sera was significantly higher than that of the non-MPER recognizing samples; however this association was not observed for samples with anti-PG9/16-site specific neutralization activity.
2.3. **Methods:**

2.3.1. **Study participants and neutralization samples:**
Serum samples were collected upon recruitment for neutralization studies. Study participants were >18 years old, HIV-1 infected for >1 year and ART naive recruited from two clinics in Cape Town, South Africa. The median age was 33 (IQR 28, 37) with 17 (10%) males and 160 females (90%).

The median CD4 count was 407 (IQR 286, 533). For 85 participants from Groote Schuur Hospital, the median reported time since infection was 3 years (IQR 1, 7). For 92 participants from Khayelitsha Site B clinic, median time since the earliest HIV positive test or the earliest recorded CD4 count was 2.37 years (IQR 1.40, 4.60). Written informed consent was taken from study participants. This study was approved by the Human Ethics Committee, Faculty of Health Sciences at the University of Cape Town.

2.3.2. **Pseudoviruses and neutralization assay:**
Cloned envelope constructs (obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP, Germantown, USA and were co-transfected into 293T cells with a SG3Δenv HIV-1 genome plasmid (Montefiori, 2009). The envelope constructs were obtained through the ARRRP from Drs. D. Montefiori, F. Gao, C. Williamson, S. Abdool Karim, J. Overbaugh, B. Hahn, Y. Li, J Salazar-Gonzalez, D. Ellenberger, B. Li, M. Callahan S. Butera, R. Paranjape, S. Kulkarni, L. Morris, K. Mlisana, D. Montefiori, L. Stamatatos and C. Cheng-Mayer, except the constructs for Du151.2 (Li et al., 2006) and murine leukemia virus (MLV), which were provided by Dr. L. Morris, NICD, Johannesburg, South Africa. The HIV-2/HIV-1 MPER chimeras (C1, C1C, C6 and C7) were a kind gift from Dr George Shaw, while the HIV-2/253-11 MPER chimera was generated by site directed mutagenesis.

Supernatants were harvested at 48 and 72 hours post-transfection and filtered (0.45µm). Single-use aliquots of pseudoviruses were stored at -80°C. Each virus preparation was later titrated in TZM-bl cells to determine a standard dilution that resulted in approximately 50,000 Relative Light Units (RLU).

TZM-bl-based neutralization assays were preformed (Montefiori, 2009) by measuring the reduction in luciferase expression after pseudovirus infection. Briefly, dilutions of serum samples were pre-incubated with the pseudovirus for one hour and then added to 1x10^4 TZM-bl cells (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. John C. Kappes and Xiaoyun Wu) in flat bottom 96 well plates. DEAE-Dextran (Sigma Chemical Company, Schnelldorf, Germany) was used at 7.5µg/ml final concentration to enhance infection. The
serum ID_{50} values were generated by serial dilution of serum and the lowest dilution of serum used was 1:50. The percentage neutralization at single dilutions was analyzed using samples at 1:100 serum dilutions (performed in triplicate). After 48-72 hours of incubation at 37°C and 5% CO₂, cells were lysed with a detergent-containing buffer with Bright-Glo™ luciferase substrate (Promega, Madison, USA). Lysates were transferred to black 96 well plates and luciferase levels were measured on a VERITAS MicroPlate Luminometer (Turner BioSystems). Samples were tested against MLV as a negative control. ID_{50} titers were calculated using curve fit functions in Prism version 5 (GraphPad, La Jolla, USA). MLV was used as a negative control; detectable neutralization was rarely observed (<20% neutralization for all samples except for two with 20-30% neutralization at 1/100 serum dilution).

Percentage neutralizations were determined by the following calculation

\[
\frac{\text{Difference in average RLU between virus control and sample}}{\text{Difference in average RLU between virus control and cell control}} \times 100\%.
\]

### 2.3.3. Prediction of ID_{50} values from percentage neutralization at a single dilution:

A predicted ID_{50} value was generated for every sample/virus combination for which an ID_{50} value was not measured. This predicted ID_{50} value was derived from the % neutralization value at 1/100 serum dilution based upon a linear regression comparing the known ID_{50} values (n=240) to the % neutralization at 1/100. Sample/virus pairs measured as resistant were assigned an arbitrary value of ID_{50}=10 for the regression model and combinations with a predicted ID_{50}<10 were also assigned an ID_{50} value of 10. The prediction model was validated using a test set (n=234). There was no overlap in samples between the training and the test set.

### 2.3.4. Detection of anti-MPER and anti-PG9/16-site antibodies:

Chimeric HIV-2 virus (7312A) engrafted either with a consensus subtype C MPER (C1C; (Gray et al., 2007)) or a Yu2 MPER (C1; (Binley et al., 2008; Gray et al., 2007)) or the MPER sequence of a CRF02_AG virus (253-11; refer to chapter 4) was used to detect anti-MPER antibodies. Sera that neutralized any of these three chimeric viruses at ID_{50}>1000 were scored as containing anti-MPER neutralizing activity. Anti-PG9/16-site antibodies were detected using CAP45.2.00.G3 virus mutants (N160A and K169E) critical for PG9/16 recognition (Gray et al., 2011; Moore et al., 2011; Tomaras et al., 2011; Walker et al., 2009a). PG9/16-site specific neutralization activity was confirmed by a ≥3
fold drop in $\text{ID}_{50}$ for either one of the two PG9/16-site mutants compared to the parent virus. $\text{ID}_{50}$ values for this purpose were derived from titration curves and not from $\text{ID}_{50}$ predictions.

2.3.5. **Variability analysis:**
HIV-1 gp160 variability (Shannon’s entropy) in amino acid residue was assessed using the entropy one tool from the Los Alamos website (http://www.hiv.lanl.gov/content/indexml).

2.4. **Results:**
2.4.1. **Estimation of $\text{ID}_{50}$ for each serum/virus pair and its validation:**
A pseudovirus panel representative of the global HIV-1 pandemic was assembled to evaluate the neutralization breadth of the South Africa sera. The panel was selected based upon neutralization resistance, subtype and geographic diversity (Blish et al., 2009; Jacob et al., 2012; Seaman et al., 2010b). TZM-bl based neutralization assay (Montefiori, 2009) was used to assess the neutralization of each of the panel viruses (n=24) by each of the serum sample (n=177) screened at 1/100 dilution.

Of the 4248 serum/virus combinations, $\text{ID}_{50}$ values were measured from titration curves for 7.3% (312/4248) of serum/virus pairs. For the rest, an $\text{ID}_{50}$ was estimated from percentage neutralization (measured at 1/100 serum dilution) based on a linear regression analysis performed on a subpopulation (n=240; “training set”) of the sample/virus combinations with known percentage neutralization of serum (at 1:100 dilution) and measured $\text{ID}_{50}$ values. The effect of percentage neutralization was modelled both linearly and non-linearly. Based on $R^2$, we measured the goodness of fit of the corresponding models and chose a linear model (Figure 2.1a). The equation was used to estimate the $\text{ID}_{50}$ from % neutralization at 1/100 serum dilution (Figure 2.1a) (Jacob et al., 2012). The serum/virus pairs in the training set included some from this study (168/240) and the rest from a previous study [36] (72/240).
Figure 2.1: Estimation model to predict ID$_{50}$ value from percentage neutralization at 1/100 dilution and validation of the model. (a) Regression line comparing percentage neutralization at 1/100 dilution and ID$_{50}$ titers (n=240). The prediction equation, R$^2$ value, the corresponding p value and a line fit with 95% confidence interval are shown. (b) Regression line comparing estimated ID$_{50}$ values derived from the percentage neutralization at 1/100 serum dilution and the measured ID$_{50}$ values (n=234) for the test set. The R$^2$ value, the corresponding p value and a line fit with 95% confidence interval are shown.
The model was validated on a separate subset of sample/virus combinations (“test set”) with known percentage neutralization of serum (at 1:100 dilution) and measured ID$_{50}$ values (n=234). 122/234 serum/virus pairs used in the test set were from this study. The estimated ID$_{50}$ values derived from the percentage neutralization for the “test set” correlated well with the corresponding measured ID$_{50}$ values (linear regression R$^2$=0.6700, p < 0.0001, Figure 2.1b).

2.4.2. **Measuring the breadth of neutralization and its association with contemporaneous CD4 T cell counts**

Sera were evaluated for neutralization breadth by two definitions; either by measuring the serum geometric mean ID$_{50}$ titer or by considering the number of viruses neutralized (Figure 2.2). Twenty percent (35/177) of the cohort had a serum geometric mean ID$_{50}$ titer>200 against the 24 panel viruses and 18% (32/177) of the cohort neutralized ≥3/4$^{th}$ of the virus panel at ID$_{50}$>100 and were considered broad neutralizers. Geometric mean ID$_{50}$ titers correlated well with the number of viruses neutralized (Spearman’s correlation coefficient, ρ=0.97, p < 0.0001; data not shown). Whenever available measured ID$_{50}$ values determined by titration (n=312) were used; otherwise, ID$_{50}$ values predicted from % neutralization at 1/100 dilution (n=3936) was used for measuring neutralization breadth.

A previous study have found an association between neutralization breadth and contemporaneous CD4 T cell counts (Sather et al., 2009). We also noted a negative association (Spearman’s ρ=−0.21, p=0.0090) between CD4 counts and the log geometric mean titers; Figure 2.3.
Figure 2.2: Heterologous neutralization capacity of 177 HIV-1 infected (>1yr.) South Africa serum samples to a 24 pseudovirus panel. (a) ID$_{50}$ titers of the indicated pseudovirus for each of the serum samples are shown. The serum geometric mean titer and the number of viruses neutralized are shown. The MPER activity in the cohort was measured using three different (C1C, C1 and 253 MPER) HIV-2/HIV-1 chimeric constructs. The PG9/16-site directed antibodies were detected using CAP45.2.00.G3 N160A and K169E mutants. ≥3 fold drop in ID$_{50}$ in either the N160 or K169E mutants were considered a PG9/16-site specific hit. Colour codes: Red shading: ID$_{50}$$>$1000; Orange shading: ID$_{50}$ 300-1000; Yellow shading: ID$_{50}$ 100-300; Green shading: ID$_{50}$ 50-100. (b) the geometric mean ID50 titers of the South Africa sera against the 24 virus panel

Figure 2.3: Correlation of geometric mean ID$_{50}$ titer to contemporaneous CD4 T cell counts for South Africa donors: The corresponding $p$ value and spearman’s correlation coefficient are shown.
2.4.3. **Sensitivity of viruses to South Africa sera:**

The neutralization sensitivity of viruses was evaluated by ranking viruses using the geometric mean of the ID$_{50}$ values for all 177 serum samples neutralizing each virus (Figure 2.4). Within-subtype neutralization, i.e. better neutralization of viruses matched to the sera by subtype (Binley \textit{et al.}, 2004; Brown \textit{et al.}, 2008; Bures \textit{et al.}, 2002; Rademeyer \textit{et al.}, 2007; Seaman \textit{et al.}, 2010b; van Gils \textit{et al.}, 2010b) was notably observed with subtype C. Four subtype C pseudoviruses were among the six most sensitive viruses to the South Africa sera (~98% subtype C (Wilkinson and Engelbrecht, 2009); Figure 2.4).

<table>
<thead>
<tr>
<th>Virus</th>
<th>clade</th>
<th>Tier</th>
<th>Geometric mean ID$_{50}$</th>
<th>Sensitivity to PG9/16 Monoclonal antibody</th>
<th>Sensitivity to MPER Monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PG9</td>
<td>PG16</td>
<td>10E8</td>
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</tr>
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<td>0.50</td>
</tr>
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<td>5.00</td>
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</tr>
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<td>1.00</td>
<td>0.20</td>
</tr>
</tbody>
</table>

**Figure 2.4:** Relative sensitivity ranking of viruses to the South Africa serum samples and their sensitivity to monoclonal antibodies: Viruses are ranked by the geometric mean of the ID$_{50}$ values of all sera neutralizing that virus; four highly resistant viruses are shaded green. The largest percentage distance between any two viruses among the resistant viruses was taken as the clearest cutoff between the most resistant viruses and other viruses. The sensitivity of the 24 virus panel to PG9/16 and MPER monoclonal antibodies are also depicted (Huang \textit{et al.}, 2012).
2.4.4. **Clustering analysis of neutralization:**

We generated heat maps to study the clustering patterns of neutralization by serum samples (Figure 2.5). Serum samples clustered into three distinct groups. Cluster S1 (n=95) contained the most broadly neutralizing sera and cluster S2 (n=20) the least. Sera in clusters S1 and S3 (n=62) were generally similar, except that S1 were more potent and broadly neutralizing. S1 tended to neutralize the highly resistant viruses in comparison to S3. Viruses generally clustered by their overall neutralization sensitivities. Four most resistant viruses (253-11, Q461.e2 and QH343.21M.ENV.A10 and PVO.4) clustered together. Separately, the most sensitive viruses (Du151.2, 001428-2.42, CAP45.2.00.G3 and 255-34) clustered together. No obvious clustering by virus subtype was observed, except for the three highly sensitive subtype C viruses which clustered together.
Figure 2.5: Clustering map of serum samples and viruses. Serum samples and viruses clustered to four major groups based on their neutralization capacity and neutralization susceptibility respectively. Higher neutralization values are shown in darker colours while the lower values are shown in light shades. Log$_{10}$ of neutralization score was used to plot the dendogram.

2.4.5. Detection of antibodies overlapping the PG9/16 site within the cohort:
Next we investigated the prevalence of PG9/16-site specific antibodies in the cohort. Although previous studies have noted that some broadly neutralizing monoclonal antibodies (Walker et al., 2011; Walker et al., 2009a) or sera (Gray et al., 2011; Moore et al., 2011; Tomaras et al., 2011; Walker et al., 2010) target this site, their prevalence among chronically-HIV-1-infected individuals has not been reported. The V2 loop glycan at position 160 and the lysine residue at position 169 are two crucial residues essential for PG9/16 mAb recognition (Moore et al., 2011; Walker et al., 2009b); single amino acid substitutions in this region abolish PG9 and PG16 mAb recognition and neutralization (Moore et al., 2011; Walker et al., 2009b) and have been used to identify PG9/16-like antibodies from blood samples in various reports (Gray et al., 2011; Mikell et al., 2011; Moore et al., 2011; Walker et al., 2010).

Sera (n=108) which neutralized the HIV-1 subtype C virus CAP45.2.00.G3 (with an ID$_{50}$ titer >100) were further screened on the CAP45.2.00.G3 V2 region mutants N160A and K169E which disrupt the PG9/16 epitope (Figure 2.2a). Anti-PG9/16-site activity was captured from all available sera which neutralized the parent virus at an ID$_{50}$>100. Thirty four percent (37/108) of the CAP45.2.00.G3 neutralizers exhibited a diminished (≥3 fold drop) neutralization activity against either one of the two V2 region mutants in comparison to the parent virus, confirming anti-PG9/16-site specific neutralization activity in a substantial fraction of the cohort.

2.4.6. Lack of a positive association between presence of anti-PG9/16-site antibodies and neutralization breadth in sera:
Anti-PG9/16-site antibodies have been shown to mediate breadth in some broad neutralizers (Gray et al., 2011; Gray et al., 2009a; Tomaras et al., 2011; Walker et al., 2010). The association between the presence of anti-PG9/16-site specific antibodies and neutralization breadth was evaluated in this cohort. Anti-PG9/16-site neutralizing antibodies were present in approximately 1/5$^{th}$ of the broad neutralizers. 17% (6/35) of the broad neutralizers with serum geometric mean ID$_{50}$ titer >200 and seven of the thirty two (22%) serum samples which neutralized ≥3/4$^{th}$ of the panel viruses had
PG9/16-site specific antibodies. Surprisingly, the sample set (n=37) with anti-PG9/16-site neutralization activity did not elicit a higher neutralization breadth compared to the sample set (n=71) without any detectable anti-PG9/16-site activity. The median geometric mean ID$_{50}$ titer (114 vs 105; $p = 0.64$, Wilcoxon rank sum test) and the number of viruses neutralized (12 vs 12; $p = 0.84$) by samples with PG9/16-site specific neutralization activity was not significantly different from their negative counterparts (Figure 2.6). In fact, PG9/16 site neutralizers were less likely ($p=0.04$) than non-PG9/16 site neutralizers to be able to neutralize $\geq 18/24$ panel viruses; tested by a two-sample test for equality of proportions with continuity correction (Table 2.1).

Figure 2.6: Box-plot depicting the neutralization breadth of samples with and without PG9/16-site specific neutralization activity: A Wilcoxon-rank sum test was used to compare and calculate the median difference between the samples set with and without PG9/16-site activity. Neutralization breadth was defined either by the serum geometric mean ID$_{50}$ titer (left) or the number of viruses neutralized (right). Corresponding $p$ values and the number of samples analyzed are shown.
Table 2.1: The proportions of broad neutralizers in the sample set with and without PG9/16-site specific activity: Breadth is defined either by the geometric mean ID\textsubscript{50} titer (top) or the number of viruses neutralized (bottom). The \( p \) values are derived from 2-sample test for equality of proportions with continuity correction.

<table>
<thead>
<tr>
<th>PG9/16 category</th>
<th>Neutralization category: (serum geometric mean titer)</th>
<th>Proportions (%)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geometric mean titer&lt;200</td>
<td>Geometric mean titer&gt;200</td>
<td></td>
</tr>
<tr>
<td>&lt;3 fold (neg)</td>
<td>50</td>
<td>21</td>
<td>21/71 (29.5%)</td>
</tr>
<tr>
<td>&gt;3 fold (pos)</td>
<td>30</td>
<td>7</td>
<td>7/37 (18.9%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PG9/16 category</th>
<th>Neutralization category: (Num. of viruses neutralized with ID\textsubscript{50}&gt;100)</th>
<th>Proportions (%)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;18/24 viruses neutralized</td>
<td>≥18/24 viruses neutralized</td>
<td></td>
</tr>
<tr>
<td>&lt;3 fold (neg)</td>
<td>47</td>
<td>24</td>
<td>24/71 (33.8%)</td>
</tr>
<tr>
<td>&gt;3 fold (pos)</td>
<td>31</td>
<td>6</td>
<td>6/37 (16%)</td>
</tr>
</tbody>
</table>

2.4.7. **Anti-MPER antibodies within the cohort:**
To detect anti-MPER antibodies in the cohort, three different HIV-2 chimeric viruses with the native HIV-2 MPER replaced by an HIV-1 MPER was used (Gray \textit{et al.}, 2007). The HIV-2 MPER was replaced either with a consensus subtype C MPER (C1C; (Gray \textit{et al.}, 2007)) or Yu2 MPER (C1; (Binley \textit{et al.}, 2008; Gray \textit{et al.}, 2007)) or the MPER from a CRF02_AG virus (253-11 MPER chimera, refer to thesis chapter four). The parent HIV-2 virus (7312A) was screened alongside to detect cross-neutralization activity directed against the HIV-2 envelope. In order to be scored as containing anti-MPER antibodies, the serum had to exhibit high titers (ID\textsubscript{50}>1000) of neutralization against at least one of the three chimeric constructs. 19\% (33/177) of the cohort had anti-MPER antibodies by this definition (Figure 2.2a). Only one sample had high titer 4E10-like antibody (ID\textsubscript{50}>1000) while none had detectable 2F5-like antibodies measured by using HIV-2/HIV-1 MPER chimera containing the minimal 2F5 (C7) and 4E10 (C6) epitope. Antibodies directed against the chimeras in these samples were directed against the HIV-1 MPER: as none of these sera detectably neutralized the parent HIV-2 virus (data not shown).
2.4.8. **Thresholds for distinguishing sera with significant anti-MPER activity:**
A previous study established that high titers (C1C ID<sub>50</sub>&gt;1000) of anti-MPER antibodies are required to neutralize HIV-1 isolates via MPER (Gray et al., 2009a). The probability that samples with anti-MPER titers&lt;300 mediate HIV-1 neutralization via MPER has been reported to be negligible or low, as five samples tested with anti-MPER antibody titers &lt;300 failed to neutralize via MPER (Gray et al., 2011; Gray et al., 2009a).

Based on the above observations, the cohort was categorized into two groups; MPER<sub>pos</sub> (n=33) with high titer MPER activity (ID<sub>50</sub>&gt;1:1000 for at least one of the three MPER chimeras) and MPER<sub>neg</sub> (n=126) with anti-MPER titers&lt;300 for all the three HIV-2/HIV-1 MPER chimeric constructs.

Eighteen serum samples with which the highest tier among the three HIV-2/HIV-1MPER chimera constructs was between 300 and 1000 were dropped from the analysis as we were unable to score them as anti-MPER positive or anti-MPER negative with sufficient certainty (Gray et al., 2011; Gray et al., 2009a).

2.4.9. **Presence of anti-MPER antibodies is associated with neutralization breadth:**
Two previous reports concluded that MPER is the predominant neutralization target in samples with anti-MPER antibody titers&gt;1000 (against C1C/C1) (Gray et al., 2009a; Tomaras et al., 2011). Gray et al first reported that anti-MPER antibodies were the primary neutralizing antibody in 3/3 samples tested with a C1C ID<sub>50</sub>&gt;1000. This association was further confirmed on three broad neutralizers from the CHAVI study (Tomaras et al., 2011). Efficient depletion of anti-MPER antibodies resulted in a significant (≥3 fold) drop in neutralization of at least three viruses in six of the eight serum samples tested in our lab (Figure 4.5 in chapter 4 and data not shown). This suggests that the majority of the neutralization activity in samples with high titer anti-MPER antibodies (ID<sub>50</sub>&gt;1000) is attributable to this particular specificity.

We noticed a significant positive correlation between log anti-MPER antibody titer and neutralization breadth by number of viruses neutralized (data not shown). A similar association was observed in a previous report, in a set of 52 plasma samples (Gray et al., 2009b), although only 2/52 samples had an anti-C1C titer&gt;1000, a threshold subsequently found by the same group to be associated with dominant anti-MPER neutralization activity (Gray et al., 2011; Gray et al., 2009a)
(discussed above). Next we compared the neutralization breadth of samples with and without anti-MPER activity. MPER positive samples neutralized a significantly higher \( (p<0.0001) \) number of viruses (median=12) and were more potent \( (p<0.0001) \), serum geometric mean ID\(_{50}\) titer=102) than the sample set which lacked MPER activity (median number of viruses neutralized=6; serum geometric mean ID\(_{50}\)=53; Figure 2.7).

We wished to compare this effect directly to the same comparison made for anti-PG9/P16 site antibodies. Thus, this analysis was restricted to the 108 serum samples for which we measured PG9/16-site specific data. The association remained significant in this smaller sample set \( (p=0.04\) and 0.028; Figure 2.7). Furthermore, the MPER\(_{\text{pos}}\) samples were 2.25 (95% CI: 1.14-4.44, \( p=0.02 \)) times likely to neutralize \( \geq 18 \) viruses with an ID\(_{50}\)\( \geq 100 \) compared to the MPER\(_{\text{neg}}\) Samples (Table 2.2).

![Figure 2.7: Box-plot depicting the neutralization breadth of samples with and without anti-MPER activity: Neutralization breadth was defined either by the serum geometric mean ID\(_{50}\) titer (left) or the number of viruses neutralized (right). A Wilcoxon-rank sum test was used to compare and calculate the median difference between the samples set with and without anti-MPER activity. Neutralization breadth was defined either by the serum geometric mean ID\(_{50}\) titer (left) or the number of viruses neutralized (right). Corresponding \( p \) values and the number of samples analyzed are shown.](image)
Table 2.2: The proportions of broad neutralizers in the MPER positive and MPER negative category: Breadth is defined either by the geometric mean ID\textsubscript{50} titer (top) or the number of viruses neutralized (bottom). The relative risk ratios with the 95% confidence intervals (95% CI) and associated p value are depicted.

<table>
<thead>
<tr>
<th>MPER category</th>
<th>Neutralization category: (serum geometric mean titer)</th>
<th>Relative Risk ratio (95% CI)</th>
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<td>Geometric mean titer&lt;200 Geometric mean titer&gt;200</td>
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<th>Neutralization category: (Num. of viruses neutralized with ID\textsubscript{50}&gt;100)</th>
<th>Relative Risk ratio (95% CI)</th>
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<td>&lt;18/24 viruses neutralized ≥18/24 viruses neutralized</td>
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<tr>
<td>Anti-MPER activity (ID\textsubscript{50}&lt;300)</td>
<td>109 17</td>
<td>2.25 (1.14 - 4.44)</td>
<td>0.02</td>
</tr>
<tr>
<td>Anti-MPER activity (ID\textsubscript{50}&gt;1000)</td>
<td>23   10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.4.10. Variability analysis of neutralizing antibody epitopes within gp160:
We analyzed the epitope variability of neutralizing antibody target sites by calculating Shannon’s entropy score for all identified target sites for broadly neutralizing antibodies using 3829 HIV-1 envelope sequences chosen to represent the range of worldwide sequences (Figure 2.8b)
Figure 2.8: (a) Shannon’s entropy plot for HIV-1 gp160 from HIV-1 sequences downloaded from the Los Alamos database (n=3829): The curated HIV-1 gp160 alignment used to plot variability represented nine major subtypes, 51 circular recombinant forms and unique recombinant form. Of the 3960 sequences, 115 sequences with stop codons and 16 sequences whose amino acid residues were uncertain were removed from the alignment (b) Mean variability across the core epitopes of the existing monoclonal antibodies are depicted. For each mAb, the one letter amino acid residue and the corresponding position in HXB2 is indicated. Gray shading: Entropy <0.5; Yellow shading: Entropy 0.5-1.0; Light orange shading: Entropy 1.0-1.5; Dark orange shading: Entropy >1.5.

The gp120 had a higher average variability score (H=0.68) than that of gp41 (H = 0.41). The lower variability of gp41 compared to gp120 is possibly due to its occlusion from host neutralizing antibody pressure as the gp41 stalk is concealed within the quaternary envelope spikes (Dimitrov et al., 2003; Wyatt and Sodroski, 1998). Within the MPER, amino acid sequence variability was highly restricted (H=0.39). Interestingly, 13/24 amino acids in the MPER were highly conserved (H<0.1), in particular the hydrophobic residues. Importantly, the neutralizing antibody epitopes of the four mAbs within the MPER (2F5, 4E10 and 10E8; calculated singly or in aggregate) had a lower variability than the PG9/PG16 (H=0.65), glycan recognizing mAb epitopes (2G12, PGT128; 0.73) and the CD4 binding site antibodies (H=0.85) (Figure 2.8b).

2.5. Discussion:

HIV-1 is an antigenically diverse virus and good coverage by a neutralizing antibody-based vaccine is likely to entail targeting epitopes recognized by broadly neutralizing antibodies (Burton et al., 2012a; Kwong and Mascola, 2012). The MPER and the PG9/16-site are two such antibody targets. In
In this study, we measured the neutralization breadth of sera from a South Africa cohort and detected antibodies directed at the PG9/16-site and the MPER. In this chapter, the ability of PG9/16-site and the MPER epitopes to induce broadly neutralizing antibodies in natural infections was assessed by comparing samples with and without the above antibody specificities.

A predominantly neutralization resistant (Seaman et al., 2010b) and diverse virus panel (comprising five different subtypes) similar to our previously published panel (Jacob et al., 2012) was assembled to measure neutralization breadth. Approximately one in five samples of the cohort was broadly neutralizing depending on the definition of breadth: having a geometric mean ID₅₀ titer>200 (20%, 35/177) or the ability to neutralize ≥18 viruses (18%, 32/177) (Figure 2.2). This frequency of broad neutralizers appears similar to previously observed frequencies (Binley et al., 2008; Doria-Rose et al., 2010a; Doria-Rose et al., 2009; Euler et al., 2010; Gray et al., 2011; Sather et al., 2009; Simek et al., 2009; Tomaras et al., 2011) although differences in criteria for neutralization breadth, differences in panel viruses and cohort characteristics make precise comparisons difficult. We observed a significant inverse correlation between neutralization breadth and contemporaneous CD4 T cell counts (Figure 2.3).

A regression line was used to estimate the ID₅₀ values from percentage neutralization at 1/100 serum dilution (Figure 2.1). Our estimation model enabled us to analyze a large number of individual serum samples as against using pooled sera for determining neutralization breadth (Figure 2.2). This model was necessary especially as we had limited serum samples and other reagents necessary for conducting neutralization studies. The degree of error inherent in the model is likely very low the model was validated on a test set and the estimated ID₅₀ values correlated well with the corresponding measured ID₅₀ values (linear regression R²=0.6700, p < 0.0001, Figure 2.1b).

In this study, 19% (33/177) of the cohort had high-titers (ID₅₀>1000) of anti-MPER antibodies (Figure 2.2 a), comparable to a North American HIV-1 subtype B cohort (18%; 14/78; C1 ID₅₀ titer >1000) (Huang et al., 2012). This prevalence is much higher than a blood bank cohort from South Africa (4%; 2/50; C1C ID₅₀ titer >1000, (Gray et al., 2009b)) perhaps because the blood bank cohort participants may have been infected for shorter time periods on average than our study participants, and thus their sera would contain lower levels of neutralizing antibodies (Gray et al., 2011; Mikell et al., 2011; Sather et al., 2009).
Previous reports and our own data suggest that the ability to recognize an HIV-2/HIV-1 MPER chimeric virus is associated with the dominant neutralizing antibodies being MPER-specific: Combining data from previous reports (Gray et al., 2009a; Tomaras et al., 2011) and our data (not shown), of 14 samples with high (ID50>1000) titers against one or more MPER chimeric viruses, the anti-MPER antibody was the dominant neutralizing antibody for at least three HIV-1 isolates in 12 (86%) sera, as determined by anti-MPER depletion experiments. In contrast, five samples with low anti-C1C activity (ID50<300) did not neutralize any tested HIV-1 viruses primarily via MPER (Gray et al., 2011; Gray et al., 2009a). Thus, neutralization of the HIV-2/HIV-1 MPER chimeras (at titers>1000) is associated with the dominant neutralizing antibody activity being directed against MPER.

Three mAbs targeting the MPER are broadly neutralizing (Binley et al., 2004; Huang et al., 2012). We ascertained an association between the presence of anti-MPER antibodies and the ability to neutralize potently a wider range of HIV-1 isolates (Figure 2.7, Table 2.2). The highly conserved MPER (Figure 2.8) is presumably constrained by function because it plays a critical role in viral fusion and incorporation of envelope proteins into newly synthesized virions (Montero et al., 2008a; Sun et al., 2008; Zwick, 2005); this might explain why targeting MPER confers neutralization breadth.

This is the first study to document the prevalence of PG9/16-site specific neutralizing antibodies in a chronic HIV-1 infection cohort. Approximately 1/3rd (37/108) of the tested samples in our cohort targeted this site. Anti-PG9/16-site antibodies have previously been found to be frequent in broadly neutralizing sera (Gray et al., 2011; Tomaras et al., 2011; Walker et al., 2010); our study revealed that around 1/5th of the broad neutralizers in our cohort targeted this site, a frequency similar to that observed in broad neutralizers from other cohorts (Gray et al., 2011; Tomaras et al., 2011; Walker et al., 2010). However, we provide evidence that many antibodies directed to this site do not exhibit the broad neutralization activity of PG9/16 (Figure 2.6) even after considering ≥5 fold differences between the CAP45.2.00.G3 wild type virus and the corresponding PG9/16 mutant (Appendix 2). In fact, the opposite was true: samples with PG9/16-site specific activity were less likely to be broadly neutralizing (neutralize ≥18/24 panel viruses) compared to the set which lacked detectable antibodies against this site (p=0.04).

Different mAbs targeting the PG9/16-site elicits a range of neutralization activities. The mAb 2909’s neutralization is limited to a very few viruses such as SF162 (Gorny et al., 2005). The CH01-CH04
mAb (Bonsignori et al., 2011) series recognize PG9/16-like site but neutralize only 36-47% of tier 2 isolates while the PG9 and PG16 mAbs neutralize around 80% of the isolates (Walker et al., 2009a). We speculated that the lack of association between neutralization breadth and the presence of PG9/16-site specific activity was due to a higher variability at this site. The PG9/16 site indeed was more variable (H=0.66) than the MPER (H=0.39) which in turn remained more conserved than the CD4 binding site and the glycan epitopes (2G12, PGT128 (Figure 2.8).

In this study, we observed neutralization breadth to be associated with the presence of anti-MPER antibodies and not with the presence of PG9/16-site directed antibodies. However the study has some limitations. Some samples with anti-MPER antibody titer>1000 (detected using the HIV-2/HIV-1 chimeras) might be misclassified for their neutralization breadth. Specificity studies on some broad neutralizers have revealed antibodies targeting multiple epitopes, each contributing towards neutralization breadth (Gray et al., 2011; Tomaras et al., 2011). But, as argued above, the neutralization of HIV-2 / HIV-1 MPER chimeric virus at high-titers is strongly associated with anti-MPER antibody being the dominant antibody, suggesting that such misclassifications are few. A second limitation of this study is that screening for PG9/16-site antibodies are detected only when they contribute at least two-thirds of the neutralization activity against CAP45.2.00.G3. Therefore, we also did not test for the presence of PG9/16-site specific antibodies in the sera which did not neutralize the parent CAP45.2.00.G3 virus. However, the association between neutralization breadth and MPER neutralization remained significant (p=0.04) even for those samples that neutralized CAP45.2.00.G3 alone (Figure 2.7 b).

For effective protection from a vaccine, antibodies induced by an immunogen targeting the HIV-1 surface envelope should be broadly neutralizing. Designing immunogens which mimic the surface of the native, functional envelope complex which could induce broadly neutralizing antibodies is an important approach for vaccine development. Our results in natural HIV-1 infections suggest that raise the possibility that inducing broadly neutralizing antibodies against the PG9/16 epitope will be more challenging than against the MPER epitopes, suggesting that the MPER might be more amenable than the PG9/16-site for vaccine design.
Chapter Three
Identification and characterization of neutralization-resistant CRF02_AG subtype HIV-1 isolates

3.1. Summary:

**Background:** The first antibody-inducing HIV-1 vaccines are unlikely to protect against all HIV-1 isolates. There is thus a danger that a vaccine will select for HIV-1 viruses that are highly resistant to antibody-mediated neutralization. CRF02_AG viruses have been previously reported to be highly neutralization resistant.

**Results:** We sought to identify and characterize such neutralization resistant CRF02_AG viruses using plasma samples infected with the same subtype and monoclonal antibodies. In contrast to previous reports, CRF02_AG plasma samples neutralized CRF02_AG viruses better than other viruses. This included six of eight CRF02_AG viruses previously designated as resistant (tier 2/3 or 3). Only 253-11 and 278-50 remained highly resistant. All CRF02_AG viruses were sensitive to membrane proximal external region (MPER)-specific monoclonal antibodies.

**Conclusions:** MPER is a vulnerable neutralization target for even the resistant CRF02_AG viruses. We also propose using high-neutralizing-within-subtype samples for evaluation of neutralization resistance of viruses.

3.2. Introduction

Human Immunodeficiency virus-1 (HIV-1) is an inefficiently transmitted virus (Royce et al., 1997), and thus depends upon survival within the host for extended periods of time. To persist, it must survive in the face of intense and sustained immune responses (Mascola and Montefiori, 2010; McMichael, 2006). In part, this persistence is accomplished by its error-prone replication process and high recombination rate (Taylor et al., 2008) which generate substantial diversity early in infection (Keele, 2010; McMichael et al., 2010). This diversity, in turn, likely assures that viruses resistant to particular antibody responses are almost always present, even if at very low frequency (Loh et al., 2008), and that neutralizing antibody selects them (Bunnik et al., 2008; Burton et al., 2005; Frost et al., 2005; Gray et al., 2007; Li et al., 2006; Mahalanabis et al., 2008; Moore et al., 2009; Richman et al., 2003; Rong et al., 2009; Wei et al., 2003).

There is evidence that inducing neutralizing antibodies to HIV-1 may be a fruitful approach for vaccine development. Passive immunization with neutralizing antibodies can prevent infection in primate models (Johnson et al., 2009; Mascola et al., 1999; Shibata et al., 1999; Xu et al., 2002) and
also protects neonatal primates (Ruprecht et al., 2003), even at low doses of antibody (Hessell et al., 2009), all in cases in which the antibodies can neutralize the challenge virus. It thus appears likely that vaccine-induced antibodies will be able to protect a vaccinee from infection by viruses that they neutralize. The vaccine-induced prophylactic antibodies would have to be broadly neutralizing because of the great diversity of the pool of HIV against which vaccinees would have to be protected (Woodman and Williamson, 2009). Nonetheless, even a vaccine that gives rise to neutralizing antibodies with highly broad but less than 100% coverage of HIV-1 isolates may be able to prevent many infections. About three-quarters of heterosexual HIV-1 infections (Abrahams et al., 2009; Keele et al., 2008; Salazar-Gonzalez et al., 2008) can be traced back to a single virus. Neutralization by vaccine-induced antibody of one or a few infecting virus(es) will presumably be a protective event.

In the case of less than 100% strain coverage of a vaccine, a worrisome prospect is the possibility that such a vaccine might select for difficult-to-neutralize HIV-1 viruses. Viruses differ substantially in their neutralization resistance. A recent large study classified 107 viruses into 4 ordered categories, or tiers: tier 1A and 1B viruses were most sensitive and tier 3 viruses the most resistant (Seaman et al., 2010a). The relatively high neutralization resistance of CRF02_AG viruses was reported in the same study, with several fitting into tier 3 or tier 2/3 categories. CRF02_AG viruses were more likely to fit into the resistant category than other viruses. In addition, a CRF02_AG-infected plasma pool was unable to preferentially neutralize within-subtype viruses, including the viruses used in this study (Brown et al., 2008; Seaman et al., 2010a; Seaman et al., 2010b).

CRF02_AG viruses circulate primarily in West Africa and their neutralization sensitivity to antibodies is not well characterized. In this part of my thesis, we demonstrate that CRF02_AG viruses are not exceptional but are indeed vulnerable to within-subtype neutralization (ie. neutralization of viruses by serum/plasma samples infected with the same subtype). We also identified two highly resistant CRF02_AG viruses (even resistant to subtype specific neutralization). Last, we identified monoclonal antibodies (against the MPER region of gp41) that neutralize these highly resistant viruses, which will help understand how these viruses can be neutralized. This information will assist in designing the best possible HIV-1 vaccine.
3.3. Materials and methods

3.3.1. Study participants and reagents

Anonymous blood samples found to be HIV-1-infected were obtained from Yaoundé Central Hospital Blood Service, Yaoundé, Cameroon (n=64). Twenty-two samples confirmed to be CRF02_AG subtype infected (using information from gag and nef sequences) by Tongo et al. (Tongo et al., 2013) were screened and selected for longer duration of HIV-infection, as they tend to have a higher neutralization capacity. Twelve samples estimated to be HIV-infected for >5.5 months using the BED™ HIV-1 incidence test kit (Parekh et al., 2011) (CALYPTE Biomedical, Portland, Oregon, USA) were used for neutralization studies. This study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town and the National Ethics Committee of Cameroon.

Recombinant soluble human CD4 (sCD4) was obtained from Progenics, IgG1 b12 (Burton et al., 1994) from Dr. D. Burton, 2G12 (Trkola et al., 1996) 2F5 (Muster et al., 1993) and 4E10 (Buchacher et al., 1994) from Dr H. Katinger, all via the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (ARRRP).

3.3.2. Virus panel and pseudovirus preparation

A 27 virus panel comprising subtypes A, B, C, G and CRF02_AG were assembled to represent the global HIV-1 epidemic. Pseudoviruses were chosen based upon subtype diversity, neutralization resistance (Blish et al., 2009; Jacob et al., 2012; Seaman et al., 2010b) and geographic diversity of origin. All references to tier designations are according to (Seaman et al., 2010b). Viruses are described as “tier 2/3” if they were between the clusters of tiers 2 and 3. Molecularly cloned gp160 genes were co-transfected into 293T cells (obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH from Dr. Andrew Rice) with a SG3/env backbone (obtained through the ARRRP, Division of AIDS, NIAID, NIH from Drs. John C. Kappes and Xiaoyun Wu) using Fugene 6 transfection reagent (Roche, Basel, Switzerland) by standard protocols for HIV-1 pseudovirus production (Montefiori, 2009). Supernatants were harvested at 48 and 72 hours post-transfection and filtered (0.45µm). Single-use aliquots were stored at -80°C. Each virus preparation was later titrated in TZM-bl cells to determine a standard dilution that resulted in approximately 50,000 Relative Light Units (RLU).

The envelope constructs were obtained through the ARRRP from Drs. D. Montefiori, F. Gao, C. Williamson, S. Abdool Karim, J. Overbaugh, B. Hahn, Y. Li, J Salazar-Gonzalez, D. Ellenberger, B.
3.3.3. **Neutralization assay**

TZM-bl-based neutralization assays were performed as described (Montefiori, 2009). Briefly, dilutions of serum/plasma samples were pre-incubated with the pseudovirus for one hour and then added to 1x10^4 TZM-bl cells (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. John C. Kappes and Xiaoyun Wu) in flat bottom 96 well plates. DEAE-Dextran (Sigma Chemical Company, Schnelldorf, Germany) was used at 7.5ug/ml final concentration to enhance infection. The serum/plasma ID_{50} values were generated by serial dilution of serum/plasma and the lowest dilution of serum/plasma used was 1:50. The percentage neutralization at single dilutions was analyzed using samples at 1:100 serum/plasma dilutions (performed in triplicate). After 48 hours of incubation at 37°C and 5% CO2, cells were lysed with a detergent-containing buffer with Bright-Glo™ luciferase substrate (Promega, Madison, USA). Lysates were transferred to black 96 well plates and luciferase levels were measured on a VERITAS MicroPlate Luminometer (Turner BioSystems). Samples were tested against MLV as a negative control and against the highly neutralization-sensitive subtype B SF162.2 as a positive control.

Percentage neutralizations were determined by the following calculation

\[
\left( \frac{\text{Difference in average RLU between virus control and sample}}{\text{Difference in average RLU between virus control and cell control}} \right) \times 100\%.
\]

3.3.4. **Identification of highly resistant viruses**

To determine a cutoff for the highly resistant viruses, the percentage difference in neutralization score between any two adjacent viruses was calculated. The largest percentage distance between any two viruses among the more resistant 50% of viruses was taken as the clearest cutoff between the most resistant viruses and other viruses.

3.3.5. **Calculations and statistical analysis**

Twelve CRF02_AG plasma samples were screened at 1/100 serum dilution on the 27 virus panel. A predicted ID_{50} value was generated for every plasma/virus pairing for which an ID_{50} value was not measured from the percentage neutralization at 1/100 plasma dilution (refer figure 2.1 of chapter 2
for prediction model). The prediction model and its validation are described in detail in chapter 2. Geometric mean ID50 titers for the CRF02_AG plasma was measured by taking the geometric mean of all of the ID50 values for that plasma sample (measured where available; predicted where not). Separately, for each virus, a neutralization score, which was the geometric mean of all of the ID50 for all the plasma samples, was calculated. To measure the impact of each virus on sensitivities to neutralization we fitted a linear mixed model with ln(ID50) as the outcome and virus as co-variable. Serum/plasma samples were used to construct a random intercept and account for the correlation of within-plasma measurements, meaning that the model accounts for the fact that a serum/plasma sample that neutralizes one virus is therefore more likely to neutralize another virus. This model was used to calculate the 95% marginal prediction interval for each virus neutralization score.

To explore subtype-specific neutralization we fitted a linear mixed regression model with ln(ID50) as the outcome and subtypes as co-variable. Serum/plasma sample were again used to construct a random intercept. Subtype comparisons were then made using Wald tests. Comparisons among tier 3 CRF02_AG viruses are also based on a similar linear mixed model, but with virus as co-variable (except that 278-50 and 253-11 were treated as one virus).

3.4. Results
3.4.1. Plasma samples and study participants
Anonymous blood samples found to be HIV-1-infected were obtained from Yaoundé Central Hospital Blood Service, Yaoundé, Cameroon (n=64) between December 2006 and August 2007. Twenty-two samples CRF02_AG plasma samples confirmed to be infected with CRF02_AG subtype based on sequence information from gag and nef genes (Tongo et al., 2013) were screened for longer duration of HIV-1 infection. Of the 22, 12 samples likely to be HIV-infected for >5.5 months were selected, using the BED™ HIV-1 incidence test kit (Parekh et al., 2011) (CALLYPTE Biomedical, Portland, Oregon, USA, data not shown) because broad neutralizers are more frequent among individuals infected for longer time (Binley, 2009; Gray et al., 2011; Mikell et al., 2011; Sather et al., 2009). Median age of the donors of the 12 samples was 29 (27, 32); 33% (4/12) of donors were female; median viral load was 94200 copies/ml (53000, 231000), and median CD4 count was 464 cell/ul (316, 770).
3.4.2. **Evaluation of neutralization breadth**

### 3.4.2.1. Pseudovirus panel

A pseudovirus panel (n=27) representative of the global HIV-1 pandemic was assembled, CRF02_AG viruses were well represented in the panel (n=10). The panel viruses were screened for sensitivity to the CRF02_AG plasma samples at 1/100 serum dilution (Figure 3.1a). Pseudoviruses were chosen based upon subtype diversity, neutralization resistance ([Blish *et al.*, 2009; Seaman *et al.*, 2010b] and other unpublished data), within-subtype sequence diversity, and geographic diversity of origin. All references to tier designations are according to Seaman *et al* (Seaman *et al.*, 2010b). Viruses are described as “tier 2/3” if they were between the clusters of tiers 2 and 3.

We estimated the relative sensitivity of each virus (n=27) to neutralization by our CRF02_AG plasma samples (n=12). ID$_{50}$ values were measured for a subset of plasma/virus combinations (n=72). For the rest, an ID$_{50}$ value was predicted from the % neutralization at 1/100 plasma dilution using the prediction equation detailed in the chapter 2 of the thesis (Fig 2.1). Viruses were evaluated by a “virus neutralization score” that was the geometric mean of the ID$_{50}$ values for all samples neutralizing that virus. Sera were evaluated by a “serum neutralization score” that was the geometric mean of all of the ID$_{50}$ values for that serum against all panel viruses. Viruses and virus subtypes were ranked by sensitivity using this score (Figure 3.1b, c). The neutralization of CRF02_AG tier 3 viruses by CRF02_AG samples was based upon measured ID$_{50}$ values only (Figure 3.1d).

3.4.3. **Sensitivity of viruses to CRF02_AG plasma**

Neutralization of each of the panel viruses to CRF02_AG plasma (Figure 3.1a) was initially assessed using a single dilution (1/100) in the TZM-bi based neutralization assay (Montefiori, 2009). All plasma samples were screened for any non-HIV specific neutralization using the control Murine Leukemia Virus (MLV). SF162.2, a highly neutralization sensitive (tier 1a) virus was used as a positive control. The overall neutralization capacity was assessed by measuring the proportion of sample/virus combinations that neutralized ≥50% at 1/100 dilution. The overall neutralization capacity of the CRF02_AG infected plasma samples was 43.2% (140/324), indicating the existence of good neutralizing antibodies within the group. Two samples (BS47 and BS06) neutralized ≥23 of the 27 pseudovirus panel at ID$_{50}$≥100 and were deemed to be broad neutralizers. On the other hand, two samples (BS55 and BS43) had extremely low levels of neutralizing antibodies and elicited no neutralization for non-CRF02_AG viruses.
3.4.4. **CRF02_AG viruses are sensitive to CRF02_AG plasma**

Collectively, the CRF02_AG viruses as a subtype were 2-3 fold more sensitive to the CRF02_AG-infected plasma samples than the subtype A, B or C virus groups or the lone subtype G virus (Figure 3.1a, Table 3.1). All of the six most sensitive viruses to the CRF02_AG-infected plasma samples were CRF02_AG viruses, and two others were moderately sensitive (Figure 3.1a, c). Strikingly, three tier 3 CRF02_AG viruses (251-18, 33-7, 257-31) were moderately or highly sensitive (Figure 3.1a, c, d). These three were significantly and substantially (overall 3.33-fold) more sensitive to neutralization by our CRF02_AG-infected plasma than the two resistant CRF02_AG viruses (253-11 and 278-50, Table 3.1).
### 27 Virus panel

<table>
<thead>
<tr>
<th>Subtype A Varies</th>
<th>Subtype B Various locations</th>
<th>Subtype C Southern Africa</th>
<th>Subtype D India</th>
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<th>CRF02_AG</th>
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#### CRF02_AG plasma & tier 3 CRF02_AG viruses

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<td>279-50</td>
<td>300-999</td>
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<td>253-11</td>
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#### CRF02_AG plasma

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#### CRF02_AG plasma & tier 3 CRF02_AG viruses

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#### CRF02_AG plasma

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#### CRF02_AG plasma & tier 3 CRF02_AG viruses

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</thead>
<tbody>
<tr>
<td></td>
<td>&lt;50</td>
</tr>
<tr>
<td>33-7</td>
<td>50-99</td>
</tr>
<tr>
<td>251-16</td>
<td>100-299</td>
</tr>
<tr>
<td>279-50</td>
<td>300-999</td>
</tr>
<tr>
<td>253-11</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>
3.4.5. **Neutralization sensitivity of CRF02_AG viruses to monoclonal antibodies and sCD4**

Understanding the vulnerability of CRF02_AG viruses to characterized monoclonal antibodies (mAb) could provide information for vaccine design. Thus we assessed the neutralization of panel viruses to four commonly used mAbs and soluble CD4 (sCD4). Nine of ten CRF02_AG viruses were resistant to b12 (recognizes the CD4 binding site (Saphire *et al.*, 2001)) and 2G12 (cluster of α1→2-linked mannose residues on gp120 (Scanlan *et al.*, 2002)). Only one virus (278-50) had an intact N-glycosylation site (NxS/T) motif at positions N295, N301, N322, N386 and N397, which comprises the 2G12 epitope. However 278-50 was also resistant to 2G12. Five of the seven CRF02_AG viruses were also sensitive to sCD4. On the other hand, CRF02_AG viruses were sensitive to the anti-gp41 membrane proximal external region (MPER)-recognizing mAbs (Zwick *et al.*, 2001). All CRF02_AG viruses were sensitive to 4E10, and 8/10 were sensitive to 2F5 (Figure 3.2). Two (269-12 and 255-34) of the ten CRF02_AG isolates resistant to 2F5 had substitutions in their epitope.
While 269-12 had a K665N substitution, 255-34 had a K667E substitution, rendering them resistant to 2F5. A third virus (251-18) was moderately resistant due to the presence of E662S mutation.

Table 3.1: Differences in neutralization sensitivities between subtypes and groups of viruses

<table>
<thead>
<tr>
<th>Virus(es) or virus group</th>
<th>Fold difference (95% CI)</th>
<th>Wald χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF02_AG viruses</td>
<td>1.00</td>
<td>Reference</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Group A</td>
<td>2.84 (1.95–4.13)</td>
<td>29.92</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Group B</td>
<td>2.13 (1.51–3.02)</td>
<td>18.36</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Group C</td>
<td>3.14 (2.30–4.28)</td>
<td>51.68</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Group G</td>
<td>2.83 (1.46–5.48)</td>
<td>9.42</td>
<td>0.0021</td>
</tr>
<tr>
<td>278-50 and 253-11</td>
<td>1.00</td>
<td>Reference</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>33-7, 253-11, and 257-31</td>
<td>3.33 (1.95–5.67)</td>
<td>19.55</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>33-7</td>
<td>2.59 (1.34–5.00)</td>
<td>8.02</td>
<td>0.0046</td>
</tr>
<tr>
<td>251-18</td>
<td>2.05 (1.06–3.96)</td>
<td>4.57</td>
<td>0.0326</td>
</tr>
<tr>
<td>257-31</td>
<td>6.93 (3.59–13.39)</td>
<td>33.23</td>
<td>&lt;0.00005</td>
</tr>
</tbody>
</table>

a Statistical comparisons were made for the neutralization sensitivities of the indicated viruses or groups of viruses to the indicated reference group.
b Fold differences shown are ratios of geometric means of all measurements in each comparison group. The 95% confidence interval of the fold difference was obtained from a mixed regression model.
Figure 3.2: Fifty percent inhibitory concentration (IC$_{50}$) titers against the panel viruses for monoclonal antibodies and soluble CD4. The “REF” row refers to previously reported IC$_{50}$ values, as follows (reference indicated in parentheses): A (Blish et al., 2007); B (Blish et al., 2009); C (Li et al., 2005); D (Li et al., 2006); E (Kulkarni et al., 2009); F (this study). (unk, tier unknown; virus not analyzed by Seaman et al (Seaman et al., 2010a).

3.5. Discussion:

We studied neutralization responses of 12 CRF02_AG HIV-1-infected plasma samples from Cameroon. The samples were selected for greater time since infection by BED assay, as neutralization capacity increases with time since infection (Euler et al., 2010; Gray et al., 2011; Mikell et al., 2011; Sather et al., 2009). Two of the twelve (sixteen percent) CRF02_AG infected plasma samples neutralized ≥23/27 viruses at an ID$_{50}$>100 (Figure 1a) and were deemed broad neutralizers. The two broad plasma samples (BS06 and BS47) neutralized all the ten CRF02_AG viruses within the virus panel (Figure 3.1 a).

The major objective of the study was to characterize neutralization resistant CRF02_AG viruses to subtype-specific plasma and monoclonal antibodies. The relatively high neutralization resistance of CRF02_AG viruses has been reported previously with several classified as tier 3 and several others fitting between tier 2 and tier 3 groupings (Seaman et al., 2010a). CRF02_AG viruses were more likely to fit into the tier 3 category (8/17 vs. 20/90, $\chi^2=4.565$, p=0.033 ;) than other viruses (Seaman et al., 2010b). In addition, a CRF02_AG-infected plasma pool was unable to preferentially neutralize within-subtype viruses, including the viruses used in this study (Brown et al., 2008; Seaman et al., 2010b). In contrast, we observed substantial within-subtype neutralization with our CRF02_AG-infected samples (Figure 3.1). Not all tier 3 viruses were highly resistant to our CRF02_AG plasma samples: Four CRF02_AG viruses previously scored as highly resistant (tier 2/3 or tier 3) were highly sensitive to the CRF02_AG plasma samples (Figure 2.1c). Furthermore, four of the twelve CRF02_AG plasma samples neutralized all the five tier 3 viruses with an ID$_{50}$>50 (Figure 3.1 c). Two of the above five tier 3 viruses (251-18 and 33-7) were ranked among the three most resistant viruses of all 107 that were previously tier ranked (Seaman et al., 2010a). The most parsimonious explanation for this discrepancy may be that the CRF02_AG pools used in the previous reports did not contain high levels of heterologous neutralizing antibody, even specific for within-subtype CRF02_AG viruses. We demonstrate that CRF02_AG viruses are indeed sensitive to subtype specific neutralizing antibodies.
Our studies highlight two tier 3 CRF02_AG viruses to be highly neutralization-resistant (253-11 and 278-50), while the other three tier 3 CRF02_AG viruses were sensitive to CRF02_AG plasma (257-31, 33-7 & 251-18). The two resistant tier 3 viruses were significantly different from the three sensitive tier 3 viruses (Figure 3.1a, c and Table 3.1). Differential sensitivity to CRF02_AG plasma samples was also evident within the three sensitive tier 3 CRF02_AG viruses (Figure 3.1a, c and Table 3.1); with 257-31 being more sensitive than 33-7 or 251-18. Thus, our study demonstrates the utility of using pools or panels of within-subtype samples selected for good neutralizers to identify such viruses selectively. It is also striking that neutralizing antibody specific for highly resistant viruses such as 253-11 and 278-50 occur rarely, even among CRF02_AG-infected donors.

We further characterized the sensitivity of these CRF02_AG viruses to monoclonal antibodies and soluble CD4 (Figure 3.2). Sensitivity to sCD4 protein is thought to be associated with exposure of the CD4 binding site (Pugach et al., 2004), although the CRF02_AG viruses were mostly b12 resistant and thus apparently lack the b12 epitope or is not accessible to neutralizing antibodies. All the ten CRF02_AG viruses were sensitive to 4E10 and 8/10 CRF02_AG viruses were neutralized by 2F5. Although 253-11 and 278-50 were generally resistant to neutralization by plasma samples in our study, they were sensitive to anti-MPER mAbs (Figure 3.2). We conclude that it may be possible to neutralize even highly resistant CRF02_AG viruses such as 253-11 and 278-50 with antibodies directed at the MPER (eg: 4E10, 2F5) or the CD4 binding site (278-50 was sensitive to sCD4). This may be important for development of an HIV-1 vaccine effective for a wide variety of HIV-1 strains, including neutralization resistant strains such as 253-11.

It is important that viruses with high neutralization resistance be defined as rigorously as possible. Based upon our study, we propose that procedures for selection of highly neutralization-resistant viruses include within-subtype neutralization using samples selected for good neutralizers. Identification and study of these viruses is important because: (i) epitopes from resistant viruses may be desirable in a vaccine, and (ii) resistant viruses should be included in panels to evaluate candidate vaccines.
Chapter Four

Accessibility of the HIV-1 MPER to a set of polyclonal anti-MPER antibodies in a highly neutralization resistant virus

4.1. Summary:

Background:
The Membrane Proximal External Region (MPER) within HIV-1 gp41 envelope is an attractive vaccine target. The MPER is a highly conserved region of the viral spike and functionally constrained presumably because it mediates viral fusion to the target cell membrane and insertion of the envelope spike into the viral membrane. In this chapter, the anti-MPER neutralization activity is characterized in a highly neutralization resistant virus, 253-11 (CRF02_AG subtype).

Results:
We found that a neutralization-resistant virus, 253-11, is rarely neutralized by anti-MPER antibodies in a serum panel of individuals HIV-infected >1yr. Very few sera were able to neutralize 253-11 by recognizing its MPER; but, 19 of 217 sera (9%) tested recognized (as measured by neutralization) 253-11’s MPER in an HIV-2 chimeric construct even though they were unable to recognize the same MPER in the original 253-11 virus. At least 13/19 of these sera neutralized other HIV-1 isolates via MPER, indicating that these anti-MPER antibodies were not generally defective for neutralization of HIV-1. A similar resistant pattern was observed in a second virus (Du422.1, subtype C), suggesting that the resistant pattern is not uncommon. Alanine scan mapping revealed several overlapping but distinct epitopes; conformational differences of MPER between the native 253-11 virus and the HIV-2/253-11 MPER chimera seems unlikely to explain the inability of antibodies that recognize several distinct epitopes to neutralize 253-11.

Conclusion:
Our data suggests indicate that many sera are able to neutralize an HIV-2/HIV-1 MPER chimera displaying the 253-11 MPER, yet neutralize the native 253-11 isolate poorly or not at all. The anti-MPER antibodies in these sera are able to neutralize other HIV-1 isolates, indicating that they are not globally defective for neutralization of HIV-1 isolates. Strikingly, the only antibodies (sera or monoclonal antibodies) that are capable of neutralizing 253-11 virus via recognition of MPER are all highly broadly neutralizing. We considered conformational differences between the MPER displayed by the HIV-2 chimeric virus and the same MPER displayed by the 253-11 virus for the neutralization resistance to anti-MPER antibodies. However, for this to explain our results, the series of epitopes recognized by the different sera would all have to be present in the HIV-2/253-11 MPER chimera but
not in the native 253-11 virus. We thus consider this explanation unlikely. Several other possible explanations for our observations are discussed; but, we propose that the most parsimonious explanation for the rare anti-MPER-neutralization of 253-11 is obstruction of access of antibody to the MPER. If our proposed explanation is true, it would imply that the ability of rare antibodies to penetrate this shielding may be an important factor in determining their neutralization breadth and potency. In this case, only the limited proportion of anti-MPER antibodies that can penetrate this obstruction would be able to provide the protection against the large number of HIV-1 variants that would be desirable in a vaccine.

4.2. Introduction:
The membrane-proximal external region (MPER) of the gp41 subunit of HIV-1 envelope glycoprotein is one of very few attractive targets for vaccine-induced antibodies. It is relatively conserved, presumably because it plays critical roles in viral fusion with target cell membranes and in incorporation of envelope into new virions (Montero et al., 2008b). In addition, anti-MPER antibodies often bind to MPER peptides (Gray et al., 2009a), suggesting that peptides can recapitulate many MPER epitopes/surfaces. This raises the possibility that a vaccine that induces neutralizing antibodies directed at the MPER may be more feasible than for more complex targets of broadly neutralizing antibodies, such as the PG9/PG16 binding site, which is a relatively complex quaternary epitope (McLellan et al., 2011; Walker et al., 2009b). However, inducing neutralizing anti-MPER antibodies with MPER mimics has proven largely unsuccessful (Dennison et al., 2011b; Guenaga et al., 2011; Kamdem Toukam et al., 2012; Wahome et al., 2012), although there have been some recent modest successes (Lutje Hulsik et al., 2013; Ye et al., 2011; Zhang et al., 2013; Zhou et al., 2012).

There is substantial evidence that neutralization by anti-MPER monoclonal antibodies is affected by changes in sequences outside the MPER (Binley et al., 2004; Gray et al., 2008; Montero et al., 2012). There is speculation in these reports that differences in neutralization could be due to differences in the extent of MPER exposure. However, to our knowledge, there are few data confirming this hypothesis, particularly with respect to binding the MPER at the post-CD4 conformation, which is the primary target for anti-MPER antibodies (Frey et al., 2008; Liu et al., 2008). Most prior findings could also be explained by changes in the conformation of that monoclonal antibody’s epitope.
Here we describe an HIV-1 isolate that is resistant to neutralization by most but not all antibodies that recognize the MPER, despite the fact that its MPER is readily recognized by a substantial proportion (19/217) of sera in our cohort. This discrepancy (i.e. the ability to recognize 253-11’s MPER in an HIV-2/HIV-1 MPER chimera but not in the context of the native 253-11 virus), is very difficult to explain for such a large number of sera by changes in conformation that result in the loss of epitopes. Our data suggests that the resistance of MPER to anti-MPER neutralizing antibodies might be a common but not universal phenomenon among HIV-1 isolates, i.e. some viruses are easily neutralized through recognition of the MPER. Several explanations for the lack of neutralization of 253-11 through the MPER are considered in this chapter of the thesis.

4.3. Methods:
4.3.1. Study participants and neutralization samples:
Study participants attended the HIV wellness clinic at Khayelitsha Site B Clinic (n=125) or were caregivers of children attending the Paediatric HIV Clinic of Groote Schuur Hospital, n=92), in Cape Town, South Africa. All participants were >18 years old, known to be infected with HIV-1 for >1 year, and were ART naive, except for drugs to prevent mother to child transmission (PMTCT) >3 months prior. Median participant age was 33.0 years (IQR 27.6, 37.0), with 24 males (11%) and 193 females (89%), which largely reflected the sex ratio among the recruiting populations. Median CD4 count was 416 (IQR 308, 549). For 117 participants, including all from Groote Schuur Hospital, the median reported time since infection was 3 years (IQR 2, 7). For 99 participants, all at Khayelitsha Site B clinic, median time since the earliest CD4 count or positive HIV test recorded in the clinic folder was 2.37 years (IQR 1.40, 4.60). HIV-1 infection time data for 1 participant was not properly collected. Serum samples were collected upon recruitment for neutralization studies. Written informed consent was taken from participants involved in this study. This study was approved by the Human Research Ethics Committee of the University of Cape Town, Faculty of Health Sciences.

4.3.2. Reagents, envelope clones, pseudoviruses and chimeric viruses:
Monoclonal antibodies 2F5, 4E10 and 10E8 were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP, Germantown, USA) from Drs. H. Katinger and M. Connors. Cloned HIV-1 envelope constructs were obtained through ARRRP from Drs. D. Montefiori, F. Gao, C. Williamson, S. Abdool Karim, J. Overbaugh, B. Hahn, Y. Li, J Salazar-Gonzalez, D. Ellenberger, B. Li, M. Callahan S. Butera, R. Paranjape, S. Kulkarni, L. Morris, K. Mlisana, D. Montefiori, L.
Stamatatos and C. Cheng-Mayer. Du151.2, COT6.15, murine leukemia virus (MLV) and the alanine scan mutants of COT6.15 were provided by Dr. L. Morris, NICD, Johannesburg, South Africa.

The 7312A parent HIV-2 construct and all HIV-2/HIV-1 chimeric constructs containing HIV-1 MPER sequences (C1, C1C, C6 and C7) (Gray et al., 2007) (except the HIV-2/253-11 MPER) were provided by Dr. George Shaw, University of Pennsylvania, Philadelphia, USA. The HIV-2/253-11 MPER construct was produced by mutagenesis from HIV-2 C1 using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA). Mutagenesis was confirmed by sequencing.

The pseudoviruses were prepared from molecularly cloned gp160 genes by co-transfection with a SG3 Delta env backbone (obtained through the ARRRP, Division of AIDS, NIAID, NIH from Drs. John C. Kappes and Xiaoyun Wu) into 293T cells (obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH from Dr. Andrew Rice) using Fugene 6 transfection reagent (Roche, Basel, Switzerland) by standard protocols for HIV-1 pseudovirus production (Montefiori, 2009). Supernatants were harvested at 48 and 72 hours post-transfection and filtered (0.45µm). Single-use aliquots were stored at -80°C. Each virus preparation was later titrated in TZM-bl cells to determine a standard dilution that resulted in approximately 50,000 Relative Light Units (RLU).

4.3.3. Neutralization assay:
TZM-bl-based neutralization assays were preformed as described (Montefiori, 2009). Briefly, dilutions of serum samples were pre-incubated with the pseudovirus for one hour and then added to 1x10⁴ TZM-bl cells (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. John C. Kappes and Xiaoyun Wu) in flat bottom 96 well plates. DEAE-Dextran (Sigma Chemical Company, Schnelldorf, Germany) was used at 7.5µg/ml final concentration to enhance infection. The serum ID₅₀ values were generated by serial dilution of serum/plasma and the lowest dilution of serum used was 1:50. The percentage neutralization at single dilutions was analyzed using samples at 1:100 serum dilutions (performed in triplicate). After 48 hours of incubation at 37°C and 5% CO₂, cells were lysed with a detergent-containing buffer with Bright-Glo™ luciferase substrate (Promega, Madison, USA). Lysates were transferred to black 96 well plates and luciferase levels were measured on a VERITAS MicroPlate Luminometer (Turner BioSystems). Samples were tested against MLV as a negative control and against the highly neutralization-sensitive subtype B SF162.2 as a positive control.
Percentage neutralizations were determined by the following calculation

\[
\frac{\text{Difference in average RLU between virus control and sample}}{\text{Difference in average RLU between virus control and cell control}} \times 100\%.
\]

4.3.4. **Ranking of viruses for neutralization resistance and sera for neutralization breadth:**

The 24 virus panel were ranked for neutralization resistance based upon their sensitivity to a subset of the samples utilized in this study (n=177) tested at a screening dilution of 1/100. The 177 serum samples were also ranked for neutralization potency and breadth based upon this data. The 24 virus panel was comprised of 5 Tier 3 viruses, 4 tier 2/3 viruses, 13 tier 2 viruses and two viruses not analyzed for tier designation. All tier designations are as per Seaman *et al* (Seaman *et al.*, 2010b). Tier 3 viruses: PVO.4 (B); 278-50, 253-11,251-18 and 33-7 (all CRF02_AG), Tier 2/3 viruses: Q461.e2 (A); Du422.1 and 001428-2.42 (both C), and 928-28 (CRF02_AG); Tier 2 viruses: Q168.a2 (A); TRO11, RHPA4259.7, REJO 4541.67, SC422661.8 (all B); ZM249M.PL1, CAP45.2.00.G3; Du151.2, 26191-2.48, 16936-2.21 (all C); 252-7 (G); 269-12 and 255-34 (both CRF02_AG); and viruses not analyzed for tier: QG984.21M.ENV.A3 and QH343.21M.ENV.A10.

4.3.5. **Depletion of anti-MPER antibodies:**

A 10-virus panel was assembled to test sera for their capacity to neutralize HIV-1 isolates by recognition of the MPER. Five of the 10 panel viruses were previously shown to be sensitive to neutralization by recognition of MPER (Gray, *et al* 2009): COT6.15 (C, South Africa), Du151.2 (C, South Africa), CAP45.2.00.G3 (C, South Africa), TRO.11 (B, Italy) and REJO4541.67 (B, USA). Five additional moderately resistant viruses from the 24-virus panel were added to expand the geographic and subtype diversity of the viruses: RHPA4259.7 (B, USA), Du422.1 (C, South Africa), 001428-2.47 (C, India), 928-28 (CRF02_AG, Cote d'Ivoire) and 269-12 (CRF02_AG, Cameroon).

Antibodies were depleted in two rounds of depletion as described (Gray *et al.*, 2009a; Tomaras *et al.*, 2011) using a biotinylated MPER peptide (MPR.03 (Morris *et al.*, 2011); KKKNEQELLELDKWASLWNWFIDTNWLYIRKKK-biotin-NH2; Peptide Synthetics, Hampshire, UK). Streptavidin-magnetic Dynabeads (Invitrogen, Darmstadt, Germany) Control depletions were performed as above using a biotinylated control peptide with a scrambled sequence (KKKNEKSNNDWERLWLEWLYIWLQDWAFLIKKK-biotin-NH2). A threshold of a ≥3-fold
drop in ID\textsubscript{50} compared to control peptide depletion was accepted as positive for MPER-mediated neutralization, unless there was a corroborating ≥3-fold drop in ID\textsubscript{50} of any COT6.15 alanine scan MPER mutant, in which case a ≥2-fold drop was accepted.

MPER negative sera (ID\textsubscript{50} titer <50 for C1C, C1 and HIV-2/253 MPER) was used as an appropriate control. In no case, did we observed a >1.01 fold drop in neutralization of the MPER peptide depleted sera compared to the control peptide depletion (data not shown).

4.4. Results

4.4.1. A substantial proportion of cohort sera (19/217) recognize the 253-11 MPER but not in the context of the native 253-11 isolate:

In an attempt to better understand how antibodies mediated neutralization of the highly resistant virus 253-11, we screened sera for neutralizers of 253-11 (Kulkarni \textit{et al.}, 2009), which we previously identified as highly neutralization-resistant, even to within-subtype neutralization (Jacob \textit{et al.}, 2012). This virus was also highly resistant to serum samples from a South Africa cohort, which is mostly subtype C: HIV-1 infections in the Cape Town area are ~98% subtype C (Wilkinson and Engelbrecht, 2009). Virus 253-11 was the most resistant virus in a panel of 24 moderately to highly resistant viruses when screened against 177 sera from a South African cohort of ART-naive HIV-infected (>1yr) individuals (Figure 2.4 of thesis chapter 2).

We identified 19 sera (9\%) that neutralized 253-11 at ID\textsubscript{50}>1:100 from among 217 sera from this same South African cohort (Figure 4.1a). Sera that neutralized 253-11 at ID\textsubscript{50}>1:100 were frequently weakly neutralizing, with a median ID\textsubscript{50} of 297, i.e. half of the samples had ID\textsubscript{50} values against 253-11 between 100 and 297.

Despite being highly neutralization resistant, the CRF02_AG isolate 253-11 was demonstrated to be sensitive to the anti-MPER monoclonal antibodies 2F5 and 4E10 (see Figure 3.2 of chapter 3). In order to understand how 253-11 is neutralized via MPER, anti-MPER neutralizing antibodies and PG9/16 site directed antibodies were measured in the South Africa cohort. Samples with ID\textsubscript{50}>1:1000 against a chimeric HIV-2 virus construct in which the native MPER was replaced with a subtype C consensus MPER sequence (C1C) (Binley \textit{et al.}, 2008; Gray \textit{et al.}, 2007) were scored positive (Figure 4.1a). We use this cut-off because only sera that neutralized this C1C chimeric construct at ID\textsubscript{50}>~1000 also neutralized HIV-1 isolates by recognizing the MPER (Gray \textit{et al.}, 2011; Gray \textit{et al.}, 2009a). Anti-MPER activity was frequent in the cohort. Subtype C MPER
specificities at high titers (ID<sub>50</sub>&gt;1:1000) were detectable in 15% (33/217) of the sera (Figure 4.1a). Neutralization against the control HIV-2 parent, 7312A, was very low, with only two samples (1%) exhibiting detectable (ID<sub>50</sub>&gt;50) anti-7312A ID<sub>50</sub> values; neither of these two sera neutralized the C1C construct (data not shown).

To further understand the MPER-targeted neutralization of 253-11 we generated a chimeric HIV-2 virus displaying the MPER sequence of 253-11: referred to as HIV-2/253-11 MPER (Figure 4.1b). Of the analyzed sera that neutralize 253-11 potently (ID<sub>50</sub>&gt;100), only one recognized the HIV-2/253-11 MPER construct at ID<sub>50</sub>&gt;1000 (Figure 4.1b). In addition, one sample, BNAB0004, was no longer available to assess HIV-2/253-11 MPER neutralization but neutralized 253-11 by recognizing MPER (Figure 4.3, see below) and therefore would presumably have also recognized the 253-11 MPER chimeric construct. Of the remaining four sera that neutralized 253-11 potently and also neutralized the C1C consensus C MPER chimeric construct at ID<sub>50</sub>&gt;1000 (Figure 4.1, red triangles), three neutralize the HIV-2/253-11 MPER construct poorly compared to the C1C MPER construct (≥4 fold difference, Figure 4.1). This indicates an MPER sequence preference of their antibodies for sequences other than that of 253-11 (Figure 4.1b). There are 5 amino acid differences between the MPER sequences of 253-11 and C1C (Figure 4.1b). It is possible that these three sera neutralize 253-11 with non-MPER antibodies, which is directly tested below (Figure 4.4).

![Figure 4.1: Correlation between MPER antibodies and 253-11 neutralization.](image)

The neutralizing activity of serum samples against 253-11 and anti-MPER activity against the HIV-2 chimeric constructs (a) C1C, consensus subtype C MPER (n=217) or (b) 253-11 MPER (n=216).
were compared. A threshold titer of 1:1000 was used to define significant anti-MPER activity. A threshold titer of 1:100 was used to define substantial anti-253-11 activity. In all figures, resistant sera were displayed with an ID$_{50}$ set to 25. Red triangles represent sera which neutralized 253-11 (ID$_{50}$>100) and C1C (ID$_{50}$>1000), one of which was not analyzed in Figure 4.1b.

4.4.2. Nineteen sera recognize the MPER in the HIV-2/253 MPER chimera, but not in the 253-11 virus

Good recognition of the 253-11 MPER construct was nonetheless observed in several sera that did not neutralize 253-11: Nineteen of 216 sera (8.8%) recognized the 253-11 MPER construct well (ID$_{50}$>1000) yet neutralized the 253-11 virus poorly or not at all (ID$_{50}$<100) (Figure 4.1b). It appears that these 19 sera recognize the 253-11 MPER in the context of the HIV-2 chimeric construct but poorly or not at all in the context of the original 253-11 virus.

4.4.3. Several sera recognize Du422.1 MPER, but not in the Du422.1 virus:

This pattern of recognition did not appear to be unique to 253-11: The MPER sequence of virus Du422.1 (subtype C) matches that of the C1 HIV-2 chimeric construct derived from Yu2 MPER (Binley et al., 2008). We subjected Du422.1 to the same analysis as 253-11 (Figure 4.2). Of 97 sera analysed, 12 sera recognized the Du422.1 MPER construct at ID$_{50}$>1000; but, 8 of these 12 recognized the same MPER within the Du422.1 virus poorly or not at all (ID$_{50}$<100). Thus, the phenomenon of recognition of the MPER sequence of a virus by some sera but not in the context of the original virus is not restricted to the 253-11 virus and may be somewhat common.
4.4.4. **Anti-MPER monoclonal antibodies neutralize the HIV-2/HIV-1 MPER chimera more potently than the native virus:**

We compared the neutralization of 253-11, HIV-2/253-11 MPER chimera, Du422.1 and HIV-2/Du422.1 MPER chimera (C1) by anti-MPER monoclonal antibodies 2F5, 4E10 and 10E8 (Table 4.1). The MPER chimera virus was always neutralized substantially more potently than the native virus from which the MPER sequence came, with a ratio of greater than 15-fold in all cases except for 4E10.

**Table 4.1:** IC$_{50}$ values for anti-MPER monoclonal antibodies against 253-11, HIV-2/253-11 MPER chimera, Du422.1 and HIV-2/Du422.1 MPER chimera (C1)

<table>
<thead>
<tr>
<th>IC$_{50}$ (ug/ml)</th>
<th>10E8</th>
<th>4E10</th>
<th>2F5</th>
<th>Z13e1</th>
<th>CAP208-CH12</th>
</tr>
</thead>
<tbody>
<tr>
<td>253-11</td>
<td>0.55</td>
<td>0.59</td>
<td>2.17</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HIV-2/253-11 MPER</td>
<td>0.014</td>
<td>0.38</td>
<td>0.14</td>
<td>0.41</td>
<td>ND</td>
</tr>
<tr>
<td>Ratio</td>
<td>40.3</td>
<td>1.57</td>
<td>15.9</td>
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<td>&gt;20</td>
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<td>3.08</td>
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<td>&gt;32</td>
<td>&gt;17</td>
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</table>

4.4.5. **The nineteen sera recognize a series of overlapping but distinct epitopes:**

“Mapping of the epitopes by the 19 sera which recognize the HIV-2/HIV-1 253-11 MPER construct but not the native 253-11 virus revealed a minimum of six distinct epitope patterns, reflected by six different patterns of neutralization of MPER alanine scan mutants of COT6.15 (Gray et al., 2009a) and the C6 HIV-2/HIV-1 MPER chimera construct which contains the minimal 4E10 epitope (Gray
et al., 2007) (Figure 4.3). In order to err on the side of caution, samples with no alanine scan hits were not characterized into any group in case the anti-MPER antibody did not neutralize COT6.15 mutants and/or was diluted by a non-MPER antibody. In addition, increases in neutralization were not catalogued as part of the mapping profile because it has been proposed that such mutants can reflect increased MPER epitope exposure (Nelson et al., 2007; Zwick et al., 2005).

A seventh epitope pattern could be discerned when we noted that a few samples had strikingly high ratios between the ID₅₀ for the 253-11 HIV-2/HIV-1 MPER chimera and the C1C (Consensus C) HIV-2/HIV-1 MPER chimera: BNAB0025, BNAB0236, BNAB0211 and BNAB0083).
Figure 4.3: Mapping epitopes using COT6.15 MPER mutants.

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<td>546</td>
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<td>1.0</td>
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<td>18</td>
<td>BNABA0004</td>
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<td>44</td>
<td>186</td>
<td>&lt;50</td>
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[Neutralized 253-311 via MPER]
The 19 sera that recognize the HIV-2/HIV-1 253-11 MPER construct and one serum that neutralizes 253-11 by recognition of MPER were mapped by COT6.15 MPER mutants. Mutants for which there were no decreases ≥3-fold are not shown. ID50 values of 19 sera neutralizing COT6.15 and alanine scan mutants of COT6.15 within the MPER are shown. All decreases in ID50 of more than 3-fold are marked in red. A value of 25 was assigned when the ID50 was below the reliable detection threshold (<50) for the purpose of calculating ratios.

COT6.15 wild type ID50 values are colour coded: Red, >1000; Orange, 300-1000; Yellow 100-300; Green, 50-100; Gray, <50. The HIV-2/HIV-1 MPER chimera C6 contains the minimal 4E10 epitope and is also shown and used to distinguish epitope pattern groups 5 and 6. ID50 values against the HIV-2/HIV-1 MPER chimeras for 253-11 and C1C are also shown, along with the ratio between them, which was used to distinguish epitope pattern groups 6 and 7. ND: not determined; R: resistant.

**Some of the sera that neutralize both 253-11 and the HIV-2/253-11 MPER chimera appear not to neutralize 253-11 via recognition of MPER:**

We directly tested the five C1C-MPER-recognizing sera that neutralize 253-11 well (Figure 4.1a, red triangles) for their ability to recognize 253-11 via the MPER. We focused upon the sera that neutralized 253-11 at ID50 titers above 1:300 because, at this level, it is feasible for us to determine whether the antibodies in the sera neutralize 253-11 via recognition of the MPER. To do so, we depleted sera of anti-MPER antibodies using a biotinylated MPER peptide (MPR.03) (Morris et al., 2011), and compared to a control peptide with a scrambled MPER sequence. A highly sensitive virus (Seaman et al., 2010b), SF162.L.S was used as a negative control for depletion because anti-SF162 antibodies against multiple epitopes are expected in most sera; thus, depletion of the anti-MPER antibodies would normally be expected to have little effect because it would not affect antibodies to other epitopes in the same sample. Control depletions was set on serum samples with no detectable (ID50<50) MPER activity. Of the five samples, only BNAB0004 showed evidence for neutralization of 253-11 by recognizing the MPER (Figure 4.4), while the others appeared to neutralize 253-11 by targeting regions other than the MPER. One explanation for this could be incomplete depletion of anti-MPER antibodies. Depletion of anti-MPER antibodies was confirmed for all samples (>20-fold reduction in neutralization, Figure 4.4) except BNAB0179 (~2.8-fold depletion); however, BNAB0179 neutralized the 253-11 MPER HIV-2 chimeric construct poorly (Figure 4.4), suggesting that it is unlikely that the residual undepleted anti-MPER antibodies were able to neutralize 253-11. Generally, we cannot exclude that neutralization comes from both anti-MPER antibodies and non-
MPER neutralizing antibodies in the same sample (Gray et al., 2009a). Strikingly, BNAB0004 is very broad, neutralizing 22/24 panel viruses (>50% neutralization at 1/100 dilution) and is at the 90th percentile for neutralization breadth and potency among the 177 samples tested for breadth (data not shown).

<table>
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<tr>
<th>C1C (consensus C) HIV-2/HIV-1 MPER chimera</th>
<th>BNAB0004</th>
<th>BNAB0071</th>
<th>BNAB0174</th>
<th>BNAB0177</th>
<th>BNAB0179</th>
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<tr>
<td>Control depl</td>
<td>3892</td>
<td>1068</td>
<td>622</td>
<td>5709</td>
<td>572</td>
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<tr>
<td>MPER depl</td>
<td>&lt;50</td>
<td>40</td>
<td>&lt;50</td>
<td>169</td>
<td>157</td>
</tr>
<tr>
<td>Fold reduction</td>
<td>152.7</td>
<td>26.6</td>
<td>24.9</td>
<td>33.7</td>
<td>3.7</td>
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</tbody>
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<table>
<thead>
<tr>
<th>253-11 HIV-2/HIV-1 MPER chimera</th>
<th>BNAB0004</th>
<th>BNAB0071</th>
<th>BNAB0174</th>
<th>BNAB0177</th>
<th>BNAB0179</th>
</tr>
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<tbody>
<tr>
<td>Control depl</td>
<td></td>
<td>*</td>
<td></td>
<td>552</td>
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<td>45</td>
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<td>Fold reduction</td>
<td>ND</td>
<td>VR</td>
<td>22.1</td>
<td>137.1</td>
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<th>253-11</th>
<th>BNAB0004</th>
<th>BNAB0071</th>
<th>BNAB0174</th>
<th>BNAB0177</th>
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<tr>
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<td>330</td>
<td>1085</td>
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<td>1.2</td>
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<td>1.2</td>
<td>1.6</td>
</tr>
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**Figure 4.4: Effect of anti-MPER antibody adsorption on 253-11 neutralization.**

Five 253-11 neutralizing serum samples (ID_{50}>300) with high C1C and/or 253 MPER activity were used for MPER antibody depletion using the MPR.03 peptide as compared to a scrambled sequence control peptide. A value of 25 was assigned when the ID_{50} was below the reliable detection threshold (<50) for the purpose of calculating ratios. *: indicates a reference to the notation below; VR: Indicates that the virus is resistant to the indicated serum; ND, not determined; shading: Red, >10-fold; Orange, 3-10-fold; Gray, <3 fold/not neutralized via MPER.

4.4.6. **Anti-MPER antibodies that fail to neutralize 253-11 are nonetheless functional against other HIV-1 isolates:**

We considered the possibility that the anti-MPER antibodies in these 19 sera (Figure 4.1b, top left quadrant) recognized MPER epitopes/surfaces that appeared only in the HIV-2 chimeric constructs. To test this, we depleted anti-MPER antibodies from six of the 19 sera, and tested if this reduced
neutralization activity against HIV-1 isolates compared to a scrambled peptide depletion control (Figure 4.5). This would indicate that the MPER-specific antibodies in the particular serum were capable of neutralizing the MPER in the context of HIV-1 isolates. For each serum, anti-MPER antibodies were responsible for neutralization of at least three of the viruses tested (Figure 4.5); BNAB0149 neutralizing COT6.15 (2.1-fold) was scored as a positive despite exhibiting <3-fold drop because a D674A (inside the MPER) mutant of COT6.15 resulted in >3-fold lower neutralization (data not shown). An additional 7 sera also recognized COT6.15 by recognition of the MPER: these sera had reduced (≥3-fold) neutralization against one or more COT6.15 MPER alanine scan mutants compared to the parental COT6.15 virus (Figure 4.5).

In total, at least 13/19 sera neutralize HIV-1 isolate(s) by recognizing the MPER, indicating that the anti-MPER antibodies in these sera are not generally defective for neutralization of HIV-1 isolates. Notably, five of the ten HIV-1 isolates tested were neutralized by at least half of the six sera tested against them (Figure 4.5). They were TRO11, COT6.15, Du151.2, 928-28 and 269-12. Nonetheless, the sera do not recognize 253-11’s MPER in the context of 253-11.
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<th>BNAB0063</th>
<th>BNAB0075</th>
<th>BNAB0088</th>
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<th>BNAB0197</th>
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<td>337</td>
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<td>VR</td>
<td>26.4</td>
<td>2.8</td>
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<tr>
<td>RHPA 4259.7(B)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MPER depl</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Fold reduction</td>
<td>VR</td>
<td>VR</td>
<td>ND</td>
<td>VR</td>
<td>VR</td>
<td>ND</td>
</tr>
<tr>
<td>COT6.15(C)</td>
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<tr>
<td>MPER depl</td>
<td>&lt;50</td>
<td>191</td>
<td>302</td>
<td>373</td>
<td>1671</td>
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<td>8.4</td>
<td>9.2</td>
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<td>100</td>
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<td>*</td>
<td>1959</td>
<td>*</td>
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<td>Fold reduction</td>
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<td>VR</td>
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<td>ND</td>
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</tr>
<tr>
<td>MPER depl</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<td>Fold reduction</td>
<td>VR</td>
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<td>VR</td>
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<td>316</td>
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<td>VR</td>
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<tr>
<td>928-28(CRF02_AG)</td>
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<td>MPER depl</td>
<td>*</td>
<td>239</td>
<td>675</td>
<td>163</td>
<td>1410</td>
<td>*</td>
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<td>56.4</td>
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<tr>
<td>MPER depl</td>
<td>*</td>
<td>425</td>
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<td>*</td>
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<td>15.4</td>
<td>ND</td>
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<td>SFI62.1S (neg con for depletion)</td>
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<td>*</td>
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<td>1.4</td>
<td>1.5</td>
<td>1.6</td>
<td>1.1</td>
</tr>
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</table>

Figure 4.5: Anti-MPER antibodies are functional against other HIV-1 pseudoviruses.

Six serum samples which had high 253-11 MPER activity (ID<sub>50</sub>&gt;1000), but minimal or no 253-11 neutralization (ID<sub>50</sub>&lt;100) were used for MPER antibody depletion using the MPR.03 peptide as compared to a scrambled sequence control peptide. Controls to ascertain the extent of anti-MPER
depletion are shown on top. A value of 25 was assigned when the ID$_{50}$ was below the reliable detection threshold (<50) for the purpose of calculating ratios. *: indicates a reference to the notation below; VR: Indicates that the virus is resistant to the indicated serum; ND, not determined; shading: Red, $>$10-fold; Orange, 3-10-fold; Green, 2-3-fold with independent evidence from reduced sensitivity of one or more alanine substitution mutants in MPER; Gray, $<$3 fold/not neutralized via MPER. A value of 25 was assigned when the ID$_{50}$ was $<$50 for the purpose of calculating ratios.

4.5. Discussion:
Previously, a highly neutralization-resistant (Seaman et al., 2010b) CRF02_AG virus, 253-11 was characterized (Jacob et al., 2012). In this chapter, we examine the MPER region of 253-11 and characterize the accessibility of its MPER both in the native virus and in the HIV-2/HIV-1 MPER chimeric virus. The thesis demonstrates that 253-11 is resistant to neutralization by most but not all antibodies that recognize its MPER. The inaccessibility of MPER to antibodies was also observed in other HIV-1 isolates. Of the 12 serum samples that recognized the MPER of Du422.1, eight recognized the same MPER in the native Du422.1 context poorly or not at all (Figure 4.2). In addition, viruses 001428-2.42 and RHPA 4259.7 are poorly recognized by the tested anti-MPER antibodies (Figure 4.5), although we are unable to exclude MPER sequence preference as an explanation for this neutralization resistance. Nonetheless, five of the ten HIV-1 isolates tested were neutralized through MPER by at least 3/6 sera tested that was unable to neutralize the 253-11 MPER (Figure 4.5) indicating this pattern of resistance not to be universal.

2F5, 4E10 and 10E8 all neutralize 253-11 with substantial potency (Table 4.1), and thus apparently overcome the shielding of the MPER 253-11. We predicted that overcoming the MPER shielding would have a cost in potency. We measured the potency of each antibody against 253-11 and Du422.1 and the respective HIV-2/HIV-1 MPER chimeric viruses (Table 4.1). Each antibody neutralized the MPER chimera virus $\geq$15-fold more potently than the corresponding native virus, except in the case of 4E10. Although many other explanations are possible for this increased potency against the chimeric viruses, these data are in line with our prediction based upon shielding of the HIV-1 viruses but not the HIV-2/HIV-1 MPER viruses.

Only one (eg: BNAB0004, Figure 4.4) among the 217 sera analyzed, neutralized 253-11 detectably by recognizing its MPER (Figure 4.4). Nonetheless, a substantial number (19/217) of sera recognize
the 253-11 MPER sequence in the context of an HIV-2/253-11 MPER chimeric virus (Figure 4.1b, upper left quadrant). Most or perhaps all of these sera neutralize HIV-1 isolates other than 253-11 by recognition of the MPER (Figures 4.3, 4.5). A large number (19) and proportion (9%) of polyclonal sera neutralize the 253-11 MPER within the alternative chimeric construct but not the original virus. Various explanations for the lack of neutralization via MPER are outlined below and described (Figure 4.6) and discussed below.

Figure 4.6: Possible explanations for the lack of anti-MPER neutralization: Unlikely explanations are in pink backgrounds while likely explanations are in green backgrounds.

a) Sequence polymorphisms within and outside MPER determine neutralization in some HIV-1 isolates:
It has been established that various viruses with the same amino acid sequence in the 4E10 epitope are neutralized differentially (Binley et al., 2004). Rare polymorphisms within the MPER in some HIV-1 isolates have been reported to result in either the exposure or inaccessibility of MPER to neutralizing antibodies (Blish et al., 2008; Ringe et al., 2010; Shen et al., 2010). Blish et al (Blish et al., 2008) discusses two amino acid polymorphisms between two variant envelope protein sequences from the same donor, one which was unusually neutralization-resistant and one unusually
neutralization-sensitive. These two changes, when introduced into resistant viruses, conferred large increases in neutralization sensitivity to 4E10 and 2F5. The polymorphisms are I675V in the MPER and T569A in the N-terminal heptad repeat. Two other rare polymorphisms within MPER, Y681H (Ringe and Bhattacharya, 2012) and L669S (Shen et al., 2010) have a similar effect. Both COT6.15 (neutralized by anti-MPER antibodies) and 253-11 (rarely neutralized by anti-MPER antibodies) have the resistant polymorphism at all four positions. Thus, it is unlikely that the MPER sequence polymorphisms are controlling the inaccessibility of anti-MPER antibodies that we observe.

b) **Polymorphisms in the LLP2 domain (lentivirus lytic peptide-2 domain) affect MPER accessibility:**

Gray et al (Gray et al., 2008) show that changes in the LLP-2 domain of the cytoplasmic tail of gp41 affects sensitivity to 4E10. This could be an effect upon the accessibility or formation of the 4E10 epitope. The LLP-2 domain sequence of 253-11 (rarely neutralized by anti-MPER antibodies) and COT6.15 (easily neutralized by anti-MPER antibodies) are more similar to each other than they are to either the sensitive or resistant variants in Gray et al. Thus the polymorphisms in LLP2 domain observed by Gray et al are unlikely to be responsible for the inaccessibility of anti-MPER antibodies on 253-11, though we cannot rule out other residues in 253-11 virus.

c) **Immersion of MPER epitopes into the viral membrane:**

It has been reported that the MPER is partially inserted into the viral membrane of some viruses. Not only can viruses differ in the level of insertion, but antibodies can differ in their ability to extract the MPER from the membrane (Sun et al., 2008). Dennison et al (Dennison et al., 2009) suggest that the 4E10 epitope may be inserted into the membrane of synthesized liposome immunization constructs, reducing 4E10 binding. Montero et al (Montero et al., 2012) specifically suggest that the C-terminus of the MPER may be differentially inserted into the membrane, mediated by polymorphisms in amino acid residues 678 and 682-684. It thus appears possible that the ability to pull the MPER out from within the membrane and immobilize it may differ among anti-MPER antibodies (Sun et al., 2008). We note that 253-11 (resistant to anti- MPER antibodies) and COT6.15 (easily neutralized via MPER) have identical amino acid sequence in these potentially critical regions, residues 678-695. Thus, the potential of the MPER to sink into the membrane may not appear to play a role in the lack of MPER access we observe for 253-11.
d) **Conformational differences in MPER epitopes between 253-11 and HIV-2/253-11 MPER:**
Using COT6.15 MPER alanine scan mutants, we mapped the 19 sera targeting the 253-11 MPER in the HIV-2/253-11 MPER chimeric virus (Figure 4.1 b). Analysis of differential neutralization of the MPER alanine scan mutants of COT6.15 (Gray et al., 2009a) and HIV-2/HIV-1 MPER chimeric viruses, including C6, which expresses the minimal 4E10 epitope (Gray et al., 2007) indicate that at least seven distinct MPER epitopes are recognized among the 19 sera (Figure 4.3). Although the epitopes are largely overlapping, they are distinct making it unlikely that so many epitopes could be present in the HIV-2/HIV-1 253-11 MPER chimera and yet not in the native 253-11 envelope glycoprotein. Thus conformational change is highly unlikely to explain the inability of so many independent antibodies (with distinct epitopes) to neutralize 253-11.

e) **Limited MPER-accessibility in the pre-attachment spike:**
There are reports that detail variability in MPER accessibility over time during viral invasion (Chakrabarti et al., 2011; Dimitrov et al., 2007). It is known that fusion is associated with a conformational change in gp41 following CD4 binding (Eckert and Kim, 2001; Gallo et al., 2003). Dimitrov et al (Dimitrov et al., 2007) show evidence suggesting that anti-MPER neutralizing antibodies target this fusion intermediate. Chakrabarti et al (Chakrabarti et al., 2011) suggested that (i) the MPER is partially accessible in the envelope spike of some viruses before the virus attaches to a cell, and (ii) viruses with an MPER accessible in the envelope spike are more sensitive to neutralization by anti-MPER antibodies. In contrast, the neutralization resistance of the MPER to anti-MPER antibodies which we observe in 253-11 will have to occur in both the pre-attachment phase (envelope spike) and in the post-attachment phase (Frey et al., 2008; Liu et al., 2008) in order to result in the resistance we observe in a standard neutralization assay.

f) **Antibody maturation levels:**
Broadly neutralizing anti-MPER (Huang et al., 2012; Zwick, 2005) and other broadly neutralizing anti-HIV-1 (Scheid et al., 2011; Walker et al., 2011; Walker et al., 2009b; Zhou et al., 2010) antibodies have high levels of somatic hypermutation (Breden et al., 2011) (particularly in the framework regions (Klein et al., 2013)). Antibody affinity maturation is associated with breadth of neutralization of 2F5-like anti-MPER antibodies (Zhu et al., 2011). It is possible to speculate that a relatively low level of antibody maturation may contribute to the inability of these 19 sera to neutralize 253-11. But, 13 of the 19 sera which recognize the MPER of 253-11 in the chimera and does not neutralize the native 253-11 virus however neutralized other HIV-1 isolates via MPER (Figure 4.3, 4.5)
g) **Viral entry dynamics and MPER exposure:**

Single amino acid substitutions in the gp41 can mechanistically contribute to prolonged exposure of the MPER epitopes in HIV-1 isolates (Shen et al., 2010). This results in an enhanced neutralization via MPER. Most antibodies directed to MPER mediate neutralization at the pre-hairpin intermediate state (Frey et al., 2010; Frey et al., 2008). If the pre-hairpin intermediate state is extremely short lived, a MPER neutralization resistant phenotype is likely to develop. Thus, viral entry dynamics might be an explanation for the observed neutralization resistance to anti-MPER antibodies.

h) **Steric obstruction of MPER to antibodies in the pre- and post-attachment spike**

Another likely explanation for the lack of neutralization via MPER is steric obstruction of access to the MPER or shielding. The shielding phenomenon is in line with previous observations using engineered molecules each carrying one or two 4E10 (anti-MPER) binding site(s) and the 4E10 antibody itself: for a given number of binding sites per molecule, the smaller molecules neutralized more potently, suggesting that in order to bind to its epitope, 4E10 needed to fit into a sterically constrained space (Klein et al., 2009).

Crystal structures of anti-MPER antibody/antigen utilize peptide antigens (Huang et al., 2012; Ofek et al., 2004) and therefore do not address shielding of MPER by other parts of gp41 or gp120. A recently published cryo-electron microscopy analysis of an anti-MPER antibody complexed with an envelope trimer shows that the anti-MPER antibody inserts itself between gp120 and the viral membrane in order to bind to its target in the MPER (Harris et al., 2013). This gives ample opportunity for regions within gp120 to sterically hinder binding of most anti-MPER antibodies.

4.6. **Conclusion and vaccine implications:**

The high neutralization resistance of 253-11 to anti-MPER antibodies was studied in this chapter. We propose that the most parsimonious explanation for this observation is steric occlusion. Rare anti-MPER antibodies are capable of neutralizing 253-11 via MPER. In particular, monoclonal antibodies 4E10, 2F5 and 10E8 and serum BNAB0004 neutralize 253-11 by recognizing its MPER (Figure 4.4; (Huang et al., 2012; Jacob et al., 2012). Monoclonal antibodies 10E8 and 4E10 neutralized 98% of a panel of 181 viruses at IC_{50}<50ug/ml and 10E8 neutralized 72% at IC_{50}<1ug/ml (Huang et al., 2012); these antibodies therefore bind to the MPER despite any shielding exhibited by this wide range of viruses. Monoclonal antibody 2F5 is also broadly neutralizing although less so
(Huang et al., 2012), as is serum BNAB0004 (neutralizes 22/24 panel viruses and is at the 90th percentile for neutralization breadth and potency among our 177 samples, Fig 2.2 of chapter two).

Strategies to induce broadly neutralizing anti-HIV antibodies to any region of the envelope proteins with a vaccine are still in the very early stages of development. Analysis of the natural immune response to the MPER by virus such as 253-11 which is resistant to most anti-MPER antibodies will aid in the understanding needed to develop a vaccine that induces broadly neutralizing antibodies directed against the MPER.
Chapter 5: Conclusion and Recommendation:

Since HIV-1 was discovered three decades ago (Barre-Sinoussi et al., 1983), considerable efforts have been made to generate an effective vaccine (Barouch, 2008; Burton et al., 2012a; Walker and Burton, 2008). The targets of broadly neutralizing antibodies are models for candidate antibody based vaccines (Binley et al., 2008; Burton, 1997; Burton et al., 2012a; Corti and Lanzavecchia, 2013; Kwong et al., 2012; Mascola and Haynes, 2013; Mascola and Montefiori, 2010; Overbaugh and Morris, 2012). As such, characterizing epitopes in the HIV-1 envelope targeted by broadly neutralizing monoclonal antibodies will inform vaccine design (Binley, 2009; Binley et al., 2008; Dhillon et al., 2007; Gray et al., 2011; Gray et al., 2007; Gray et al., 2009b; Li et al., 2009; Mikell et al., 2011; Sather and Stamatos, 2010; Tomaras et al., 2011; Walker and Burton, 2010; Walker et al., 2010). The MPER within the gp41 is an attractive vaccine target (Gray et al., 2009a; Huang et al., 2008a; Zwick, 2005). This thesis evaluated 1) the association between the presence of anti-MPER and anti-PG9/16-site antibodies to neutralization breadth activity, 2) explored the neutralization sensitivity of CRF02_AG viruses to a set of plasma samples infected with the same subtype and to anti-MPER monoclonal antibodies (Jacob et al., 2012) and 3) observed that a highly neutralization resistant virus is rarely neutralized via MPER by a set of anti-MPER antibodies which targeted multiple distinct epitopes within the MPER.

Through the first project in this thesis, the ability of PG9/16-site and the MPER epitopes to induce broadly neutralizing antibodies in chronic HIV-1 infections was assessed by comparing samples with and without the above antibody specificities. Our results indicate an association between the presence of anti-MPER antibodies and the ability to neutralize potently a wider range of HIV-1 isolates (Figure 2.7, Table 2.2). In contrast, samples with dominant anti-PG9/16 site antibodies were less likely to be broadly neutralizing than samples without anti-PG9/16-site antibodies (Figure 2.6, Table 2.1). Our study confirms the observation made in a previous study that anti-MPER antibodies tend to be broadly neutralizing (Gray et al., 2009a; Gray et al., 2009b).

We developed an estimation model to predict ID$_{50}$ values from the percentage neutralization at 1/100 serum/plasma dilution using a linear regression prediction equation (Figure 2.1). The estimation model is useful for predicting the approximate serum/plasma ID$_{50}$ from a single dilution screening. The model was validated using a “test set”. The estimated ID$_{50}$ values derived from the percentage
neutralization for the “test set” correlated well with the corresponding measured ID$_{50}$ values (Figure 2.1).

In the third chapter (second project) of this thesis (Jacob et al., 2012), viruses with high neutralization resistance especially from the CRF02_AG subtype were defined rigorously. HIV-1 CRF02_AG subtype isolates circulate primarily in West Africa and have been reported to be fitting predominantly into the neutralization resistant category (tier 3 and tier 2/3) (Seaman et al., 2010a). Using the principle of within-subtype neutralization (Brown et al., 2008) (Gray et al., 2007; Oballah et al., 2011; Seaman et al., 2010a) we refine the identification of highly resistant CRF02_AG viruses by using a panel of CRF02_AG infected plasma samples. Two highly neutralization resistant CRF02_AG viruses were identified: 253-11 and 278-50 were resistant to subtype-specific neutralization (Jacob et al., 2012). Despite being highly resistant to polyclonal sera, we provide evidence that most CRF02_AG viruses (even the resistant strains) are sensitive to anti-MPER mAbs. This study revealed vulnerable neutralization targets (MPER) even within highly resistant CRF02_AG viruses and would contribute to the design of vaccines targeting MPER.

Further (chapter four), to understand the accessibility of MPER to anti-MPER neutralizing antibodies within highly neutralization resistant viruses, we used 253-11 (a CRF02_AG virus) as a model neutralization resistant virus. 253-11 was rarely neutralized by anti-MPER antibodies in a large serum panel of HIV-1 infected individuals. However, a substantial number of sera which hitherto were unable to neutralize 253-11 recognized the 253-11 MPER sequence in the context of an HIV-2 chimeric virus. The panel of anti-MPER antibodies which failed to neutralize 253-11 via MPER in the native virus were functional as they neutralized other viruses through MPER. We consider several explanations for the inaccessibility of anti-MPER antibodies in this highly neutralization resistant virus, 253-11. We propose that the most parsimonious explanation for this observation is steric occlusion. We also observe that the inaccessibility of MPER is a common but not a universal phenomenon among HIV-1 isolates.

Put together, the research in this thesis evaluates the neutralization breadth of anti-MPER antibodies and makes the observation that neutralization via MPER is a rare event in neutralization resistant isolates like 253-11. Based on the research recorded in this thesis, the following are recommended:

1. Data from chapter two suggests anti-MPER and not anti-PG9/16-site antibodies to be associated with neutralization breadth. Similar studies mapping antibodies to the 2G12/PGT128
epitope or the CD4 binding site and understanding which epitopes induce broadly neutralizing antibodies in chronic HIV-1 infections will inform vaccine design.

2. Based on data from chapter three, the thesis supports that procedures for studying and identifying highly neutralization-resistant viruses should preferentially include neutralization samples from the same subtype. Identification and characterization of these viruses is important because: (i) epitopes from resistant viruses may be desirable in a vaccine, and (ii) resistant viruses should be included in panels to evaluate candidate vaccines.

3. Construction of chimeric viruses with the MPER domains swapped between “MPER resistant” (eg: 253-11, chapter four) and “MPER sensitive” viruses (eg: COT6.15, TRO11, 928-28, Figure 4.5) will be helpful to understand MPER accessibility better.

4. Construction of appropriate gp120/gp41 envelope chimeric viruses will give insights into the mechanistic features behind the possibility of MPER occlusion. Chimeras generated with part of the envelope emanating from a virus with hidden MPER (eg: 253-11, chapter four) and another region originating from a virus with an exposed MPER (eg: COT6.15, TRO11 and 928-28, Figure 4.5) should shed light as to why anti-MPER antibodies are obstructed from MPER.

5. Isolation of mAbs from rare sera like BNAB0004 which mediate MPER-targeted neutralization of 253-11 (Figure 4.4) and understanding how they target MPER by structural studies will inform vaccine design. High levels of somatic hypermutations and long CDRH3 loops are hallmarks of broadly neutralizing anti-HIV-1 antibodies (Huang et al., 2012; Pejchal et al., 2011; Pejchal et al., 2010; Saphire et al., 2001; Sun et al., 2008).

6. Finally, it will be interesting to generate envelope clones from participants with significant MPER activity (anti-MPER antibody titer >1000) to analyze if immune pressure drives MPER escape mutants. Since, MPER is highly conserved (Figure 2.8, (Montero et al., 2008a)) and involved in fusion (Blumenthal et al., 2012; Garg et al., 2011; Munoz-Barroso et al., 1999), assessing the fitness cost of MPER escape mutants should guide antibody-based vaccine design in the control of HIV-1 infections.
Appendix

Appendix 1: Amino acid level map of MPER and the broadly neutralizing monoclonal antibody binding sites.

Appendix 2: Box-plot depicting the neutralization breadth of samples with and without PG9/16-site specific neutralization activity (using fivefold effect): A Wilcoxon-rank sum test was used to compare and calculate the median difference between the samples set with and without PG9/16-site activity. Neutralization breadth was defined either by the serum geometric mean ID\textsubscript{50} titer (left) or the number of viruses neutralized (right). Corresponding \( p \) values and the number of samples analyzed are shown.


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