The screening of neutralising antibodies against a resistant HIV-1 strain to identify novel epitopes

By

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DECLARATION

I, **Thandeka Moyo,** hereby declare that the work on which this dissertation/thesis is based on my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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List of Abbreviations

%:	Percentage
˚C:	Degrees Celsius
β-ME:	beta mercaptoethanol
AAV:	Adeno-associated virus
ADCC:	Antibody-dependent cell-mediated cytotoxicity
AIDS:	Acquired Immune Deficiency Syndrome
ARRP:	AIDS Research and Reference Reagent Program
ART:	Antiretroviral therapy
BnAb:	Broadly neutralising antibody
CCR5:	C-C chemokine receptor type 5
CD:	Cluster of differentiation
CD4-bs:	Cluster of differentiation 4 binding site
CD4i:	Cluster of differentiation 4 inducible site
CRF:	Circulating Recombinant Form
CRF02_AG:	Circulating Recombinant Form AG
CTL:	Cytotoxic T-Lymphocyte
CXCR4:	C-X-C chemokine receptor type 4
DC:	Dendritic cell
DEAE-Dextran:	Diethylaminoethyl dextran
DMEM:	Dulbecco's Modified Eagle's Media
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
ELISA:	Enzyme-linked immunosorbent assay
env:	envelope gene

FBS:	Fetal Bovine Serum
gp120, gp41:	Envelope subunits glycoprotein 120kda, 41kda
HEK:	Human embryonic kidney
HIV:	Human Immunodeficiency Virus
ICAM-1:	Intercellular Adhesion Molecule 1
IC_{50}:	50% Inhibitory Concentration
ID_{50}:	50% Inhibitory Dilution
IgG:	Immunoglobulin
IQR:	Interquartile range
LB:	Luria-Bertani
LFA-1:	Lymphocyte function-associated antigen 1
LTR:	Long terminal repeat
mAb:	Monoclonal antibody
MHC I:	Major histocompatibility complex I
MLV:	Murine Leukemia Virus
MPER:	Membrane Proximal External Region
mRNA:	Messenger ribonucleic acid
nAb:	Neutralising antibody
NIAID:	National Institute of Allergy and Infectious Diseases
NIH:	National Institutes of Health
NCBI:	National Center for Biotechnology Information
NK:	Natural killer
PAMP:	Pathogen-associated molecular pattern
PCR:	Polymerase Chain Reaction
PRR:	Pattern recognition receptor
RLU:	Relative light units

RNA:	Ribonucleic acid
RPM:	Revolutions per minute
RT:	Reverse transcriptase
sCD4:	Soluble cluster of differentiation 4
SGA:	Single genome amplification
SHIV:	Simian/Human Immune Deficiency Virus
SIV:	Simian Immune Deficiency Virus
STI:	Sexually transmitted infection
T_m:	Melting temperature
URF:	Unique recombinant form
V1, V2, V3, V4, V5:	Variable Regions 1-5
VIP	Vectored Immunoprophylaxis
WT:	Wild-type

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Summary

Since the start of the HIV/AIDS pandemic in the 1980s, over 75 million individuals have been infected with the virus and it has been the cause of approximately 36 million deaths worldwide. With such a high morbidity and mortality in HIV-1 infected individuals, there is a need to find ways of controlling the disease. Development of an HIV-1 vaccine would help in the fight against HIV/AIDS. It is clear that other prevention strategies poorly reach vulnerable groups such as intravenous drug users and people living in war zones. More importantly, they generally provide very transient protection and do not provide the durable and affordable protection that could be expected from a vaccine. Antiretroviral therapy (ART) may be effective in reducing death and morbidity; but, treatment is life-long and ART is not a cure. However, producing immunogens that elicit neutralising antibodies that are protective against HIV-1 acquisition has proven difficult. This is not only because of the genetic diversity of the viruses circulating in the human population but is also as a result of an incomplete understanding of how to design effective immunogens based on the known targets for broadly neutralising antibodies (BnAbs). The availability of more potential targets for BnAbs is also an important goal.

In this project, we designed a system to help identify novel targets of BnAbs if one or more does exist. We selected the QH343.A10 virus as the basis for this system. We found this virus to be moderately resistant to sera and resistant to all the BnAbs we initially tested against it (which target various epitopes on the HIV-1 envelope). QH343.A10 is resistant to monoclonal antibodies (mAbs) b12 (anti-CD4-binding site), 2G12 (anti-V3/glycan), 2F5 and 4E10 (both anti-membrane proximal external region). Of note, the virus is resistant to the extremely broad and potent mAb VRC01 (anti-CD4-bs). QH343.A10 was also found to be resistant to neutralisation by soluble CD4 (sCD4). This made the virus attractive to use in our system as antibodies that recognise QH343.A10 in the same manner as these mAbs are also unlikely to neutralise the virus.

Therefore, we tested the ability of 474 serum samples, from ART-naïve chronically HIV-1-infected individuals from a Cape Town cohort, to neutralise QH343.A10. Sixty-six sera (14%) were able to neutralise the virus by an ID_{50} value of 150 or higher and were retained for further analysis. The sera which recognise the MPER, CD4-bs and V3/glycan regions in a similar way to the mAbs that are unable to neutralise QH343.A10 would presumably be similarly unable to neutralise the virus. Thus, just by identifying sera able to neutralise QH343.A10, we propose

that we are already partially enriched against sera that recognise these three targets. Because we expected this enrichment to be only partially effective, we then systematically tested for and removed QH343.A10-recoginising sera that recognised the MPER, the V2/glycan-site and V3/glycan region. For technical reasons, we have not yet attempted to remove sera that recognise QH343.A10 through the CD4-binding site and CD4-inducible site (3BC176 mAb site), which are both targets for BnAbs.

After exclusion of sera recognising the MPER, V2/glycan-site and V3/glycan region, we were left with 19 samples. We analysed neutralisation breadth and potency of these remaining 19 serum samples as we wanted to retain sera containing potent BnAbs. We remained with 12 sera samples which were broad and potent and did not detectably neutralise QH343.A10 through the MPER, V2/glycan -site or V3/glycan region. In this manner, we believe we have selected heavily for sera that could plausibly neutralise QH343.A10 through the recognition of a novel target of BnAbs.

We propose that further study of this very select set of sera taken from a large serum cohort may allow identification of a novel target of broadly neutralising anti-HIV-1 antibodies, if such a target does exist. Our unique system can be used to screen a large panel of serum samples and allows the scientist to focus on those few samples that are broadly neutralising but do not detectably neutralise most of the already identified targets of broadly neutralising anti-HIV-1 antibodies.

Chapter 1: Literature Review

1.1 Introduction

The human immunodeficiency virus type 1 (HIV-1) is the causative agent leading to the acquired immune deficiency syndrome (AIDS) in the absence of antiretroviral therapy (ART) and has become a major global health concern, with over 30 million people worldwide living with the virus (UNAIDS, 2013, Euler and Schuitemaker, 2012, McCutchan, 2006). Sub-Saharan Africa has a large majority of HIV-1-infected individuals, with approximately 25 million people living with the virus in this region (Vermund, 2014). With such a large number of people infected and the devastating socio-economic impact caused by the disease (Bachmann and Booysen, 2003, Piot et al., 2001), there is a need for an HIV-1 cure, vaccine and more effective prevention strategies (Burton et al., 2012, Burns et al., 2010).

Curing HIV-1 has proven difficult with only two reported cases of a functional cure (Persaud et al., 2013, Hutter et al., 2009). There are HIV-1 preventative strategies which have been shown to be effective against HIV-1 acquisition such as microbicide vaginal gels (Abdool Karim et al., 2010), pre-exposure prophylaxis (Vissers et al., 2008) and male circumcision (Wamai et al., 2011). Although these preventative measures are much cheaper than cure or vaccine development, it is highly unlikely that they will be effective enough on their own to decrease HIV-1 incidence enough to control the disease (Phillips and Pirkle, 2011, Burns et al., 2010). Although there is no effective HIV-1 vaccine available, the development of a highly efficacious vaccine that would prevent HIV-1 infection and/or progression to AIDS is one of the important tools that may contribute to the control of the disease and seems likely to be easier than implementing expensive attempts at a cure (Dangeti, 2014).

Although various treatment options are available to prolong an HIV-1-positive individual's life (Kohli et al., 2006, Wu, 2000) the pandemic still has high morbidity, most particularly but not only in many of the at-risk subpopulations (Vermund, 2014). ART first emerged in the early 1990s (Sasson et al., 2005) and since then has been widely used in the clinical treatment of HIV/AIDS (Sigal and Baltimore, 2012). Nonetheless, despite substantial efforts, particularly recently in South Africa, of the 28 million HIV-1-positive individuals eligible for treatment in 2012, only 10 million HIV-1-positive individuals were on ART (SANAC, 2013, UNAIDS, 2013).

Not only has ART increased the lifespan of HIV-1-infected individuals but it has also decreased the risk of acquisition of the virus (Tanser et al., 2013). Unfortunately, the sole dependence on ART to fight the pandemic becomes increasingly difficult because of the need for lifelong

patient treatment and the high cost of HIV-1 drug management, which cost the South African government approximately R13 billion for the 2009/10 financial year (SANAC, 2013). There are also populations that are at higher risk of HIV-1 acquisition who are harder to retain in care such as sex workers, intravenous drug-users and individuals in war zones (Vermund, 2014, Hankins et al., 2002).

Although ART has decreased new diagnoses of HIV-1-associated complications such as HIV-1 dementia, other ART-related complications have emerged (Palella et al., 1998). Individuals on ART are more vulnerable to conditions such as diabetes and chronic kidney disease (Phair and Palella, 2011) and there are other substantial drug-associated adverse events that must be tolerated and/or medically managed. For example, HIV-1-infected individuals are also more likely to have a lower bone mineral density and ART can amplify this problem resulting in a greater frequency of fractures (Mallon, 2010). As ART also poses health risks because of drug-associated toxicity, it is therefore beneficial for patients to undergo regular clinic visits, which also adds to the burden of taking the drugs (Arts and Hazuda, 2012, Clavel and Hance, 2004).

The emergence of HIV drug resistant strains also poses challenges in ART as viral mutants rapidly emerge if the treatment regime is not correctly adhered to (Clavel and Hance, 2005). As a result of such disadvantages of ART, the limited current preventative strategies and the absence of an HIV-1 cure; there is a need for new therapies and strategies to fight the pandemic (Dangeti, 2014). One of these strategies would be the development of an efficacious, global HIV-1 vaccine (Burton et al., 2012). Vaccines have been used to eradicate smallpox and substantially reduce the burden of a series of other infectious diseases such as polio and measles, and it is hoped that an effective HIV-1 vaccine could have similar effects (Burton et al., 2012).

One of the vaccine approaches against HIV-1 would be to generate a neutralising antibody (nAb) response which would prevent virus establishment within the host (Burton et al., 2012a, Walker and Burton, 2010). A second approach would be to induce an effective T cell response which can attenuate HIV-1 replication soon after the initial infection event (Barouch, 2008, McMichael and Rowland-Jones, 2001). The viral envelope spike is the sole HIV-derived antigenic protein on the surface of HIV-1 (Corti and Lanzavecchia, 2013, Wyatt et al., 1998, 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). The focus of this thesis study was to develop a system to identify sera that could be used to map novel epitopes bound by broadly neutralising antibodies (BnAbs).). This

is important as the discovery of unknown epitopes on the HIV-1 envelope, if any, may lead to the development of an effective immunogen which could elicit BnAbs protecting against the acquisition of HIV-1 (Stamatatos et al., 2009, Burton et al., 2005).

1.2 HIV Diversity

HIV strains fall into distinct lineages; HIV-1 M, N O and P and HIV-2 which are all believed to represent separate transmission from primates in central Africa (Vallari et al., 2011, McCutchan, 2006). HIV-2 is thought to originate from sooty mangabey monkeys in West Africa and is less common than HIV-1 both in prevalence and geographic spread (McCutchan, 2006). HIV-2 differs from HIV-1 by being less transmissible and they also differ substantially in the magnitude of risk of progression to AIDS over unit time (Nyamweya et al., 2013). HIV-1 and HIV-2 have similarities in gene arrangement, their modes of transmission, mode of replication as well as the eventual clinical outcome, that is, progressing to AIDS; although they differ in terms of the magnitude of the time to disease (Nyamweya et al., 2013). HIV-1 and HIV-2 variants may differ by approximately 50% at the nucleotide level (Arien et al., 2007).

This report will focus on immune responses to HIV-1 type M, which is responsible for the vast majority of HIV infections worldwide and causes the highest morbidity and mortality (McCutchan, 2006). Over decades the HIV-1 group M viruses are thought to have evolved in the Congo Basin where they have diverged into at least 9 subtypes that have spread worldwide and continue to evolve. The subtypes are: A-D, F-H, J and K (Figure 1.1)(Yebra et al., 2013, Abecasis et al., 2007, Arien et al., 2007). Subtypes share an average of 70-90% identity (Arien et al., 2007). Initially subtypes were characterized by partial sequences, but as evidence for recombinant strains began to emerge and sequencing became easier and cheaper, sequences of the entire 9.2 kb genome of viruses were sequenced which improved the accuracy of characterisation of subtypes (McCutchan, 2006, Robertson et al., 2000).

Recombinant forms are classified as either circulating recombinant forms (CRFs) which are viruses containing three or more different subtype lineages and which are epidemic in populations or known as unique recombinant forms (URFs) which are viruses with unique subtype compositions and are found only in linked individuals (McCutchan, 2006).

Also with the increase in the number of available sequences, computational methods have yielded substantial evidence that “pure” subtypes and recombinant forms may sometimes have been mis-categorised as CRFs as well as CRFs being described as their own subtype in error (Abecasis et al., 2007, Robertson et al., 2000) . For example, when HIV-1 subtypes were being

named, a "subtype E" was described which was later discovered to actually be a CRF (Robertson et al., 2000, Louwagie et al., 1993). Abecasis et al. (2007), suggest that the CRF, CRF02_AG is not in fact a CRF but actually is the parent of a recombinant subtype G. Re-ordering and naming of subtypes and CRFs may be warranted with our increasing understanding of the evolution of HIV-1 (Abecasis et al., 2007).

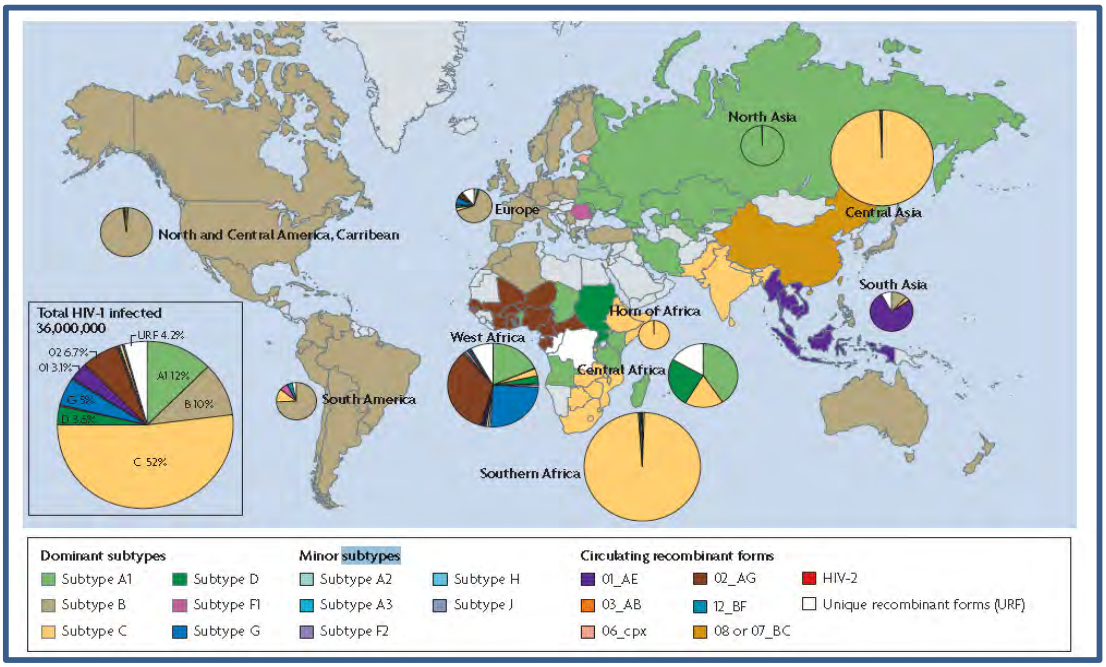

Figure 1.1: Global prevalence of the various subtypes of HIV-1. This map shows the prevalence of the different subtype of HIV-1, group M worldwide. The map also illustrated the proportions and geographic distribution of the various subtypes. Source: (Arien et al., 2007).

HIV-1 is one of the most variable pathogens known to man and this diversity can be attributed to three main factors (McCutchan, 2006). The viral reverse transcriptase (RT) which converts viral RNA into DNA is extremely error-prone leading to approximately one substitution per genome per round of replication (Ho, 1997). In addition, viral replication is extremely rapid, producing approximately 10^{10} new virions per day (Ho et al., 1995) which increases the chances of replication errors. Furthermore, recombination occurs when RT copies the two viral RNA molecules and packages them separately into virions which can lead to a mosaic DNA genome – the frequency is very high: between 7 to 30 crossovers per replication round (McCutchan, 2006). To further emphasize the diversity of HIV-1, one analysis suggests that the variation in one isolate from a single HIV-1-infected individual sampled years after infection is greater than the worldwide variation of an influenza epidemic strain during an influenza season (Burton et al., 2005). This variability is one of the greatest challenges in designing a global vaccine

against HIV-1 as a successful vaccine must protect against most, if not all, possible HIV-1 variants. (Burton et al., 2005).

1.3 HIV-1 Structure

1.3.1 HIV-1 Genome

HIV-1 belongs to the lentivirus family, a subfamily of retroviruses (Frankel and Young, 1998). The HIV-1 genome consists of nine genes that code for 15 proteins (Figure 1.2) (Frankel and Young, 1998). Like other retroviruses, it has three major genes that code for the structural proteins, *env*, *gag* and *pol*, and in contrast to other retroviruses, HIV encodes six genes that express regulatory and accessory proteins, involved in the regulation of the viral life cycle or virion infectivity (Lu et al., 2011). The outer covering of the virus is encoded by the *env* gene and consists of Env proteins embedded in a bilayer of lipids derived from the human cell membrane that the virus previously budded off from (Frankel and Young, 1998). Env is essential for the binding of virus particles to host cells and entry into these cells (Frankel and Young, 1998). The virus contains two strands of viral RNA each with a complete copy of all the viral genes (Frankel and Young, 1998).

The Tat protein is required to increase the amount of viral mRNA and possibly assists in regulating the translational efficiency of the viral mRNA (Garcia et al., 1988). The Rev protein is responsible for upregulating *gag* and *env* mRNA levels and achieves this by affecting the mRNA splicing process and overall stability of the mRNA (Garcia et al., 1988). The function of the Vif protein is to deactivate antiviral host proteins as well as enhance HIV-1 replication (Frankel and Young, 1998). The Vpu protein functions in the release of new virus particles from host cells while the Nef protein has been shown to aid in viral replication (Mwimanzi et al., 2013).

The HIV-1 Gag proteins, the matrix, capsid and nucleocapsid, are responsible for the assembly of the virus particle as well as maturation and particle release from the host cell (Freed, 1998). The Gag proteins are also involved in early viral replication steps once the virus has entered a host cell (Freed, 1998). The Vpr regulatory protein is important for viral entry into host cells (Kogan and Rappaport, 2011).

The HIV-1 *pol* gene encodes for three proteins: protease, reverse transcriptase and integrase with reverse transcriptase aiding in converting the viral RNA into DNA and integrase responsible for the incorporation of viral DNA into the host's genome (Frankel and Young, 1998). The protease is involved in the cleavage of the viral polyprotein into the active proteins needed for viral function and replication (Frankel and Young, 1998). At the end of each of the RNA strands of the viral genome are the long terminal repeats (LTRs) which are regions involved in the control of production of new virus particles (Garcia et al., 1988).

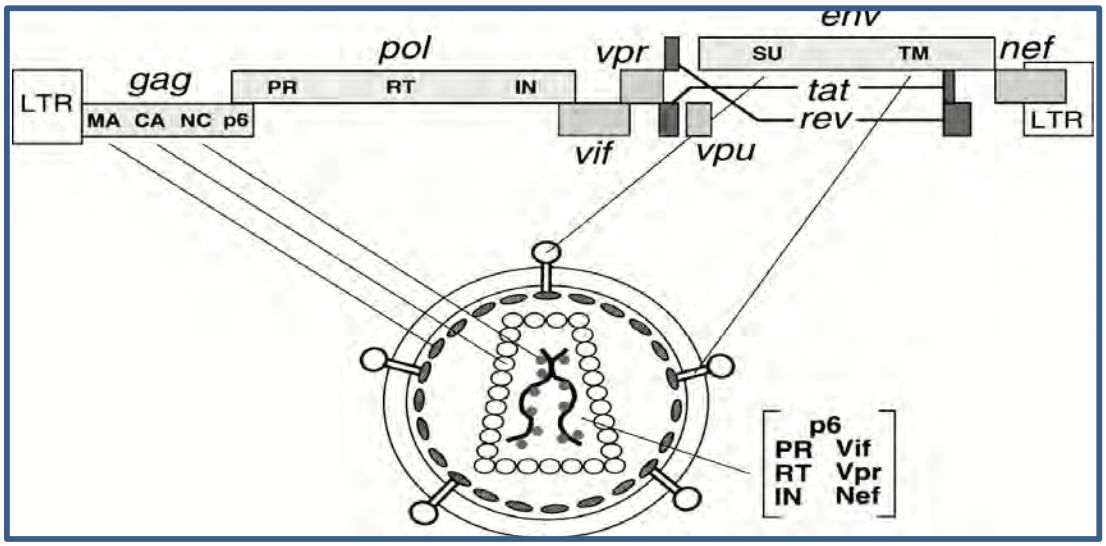

Figure 1.2: HIV particle structure and genome organisation. The diagram highlights the main features of a fully formed HIV-1 particle with the relative locations of the different proteins as well as a schematic of the genome.SU: surface protein, TM: transmembrane protein, LTR: long terminal repeat, PR: protease, RT: reverse transcriptase, IN: integrase, MA: matix, CA: capsid, NC: nucleocapsid Source: Frankel and Young, 1998.

1.3.2 HIV-1 Envelope

The HIV-1 Envelope is the key component that assists the virus in entering host cells (Frankel and Young, 1998). The *env* gene encodes for the viral envelope which consists of a trimer of heterodimers and these heterodimers are known as gp120 (Figure 1) (Hunt, 2010). gp160 is cleaved into gp120, a surface protein, and gp41, a transmembrane fusion protein, by proteases such as furin (Gu et al., 1995). The proteins that make up the envelope spikes are known as glycoproteins because they are heavily glycosylated, with approximately half of envelope mass being N-linked carbohydrates (Burton et al., 2012a). Most Env proteins on the surface of a virion are non-functional and do not aid in fusion with and entry into the cells (Moore et al., 2006, Poignard et al., 2003). These non-functional Env proteins can be made up of gp120/gp41 monomers or gp41 stumps lacking gp120 (Moore et al., 2006).

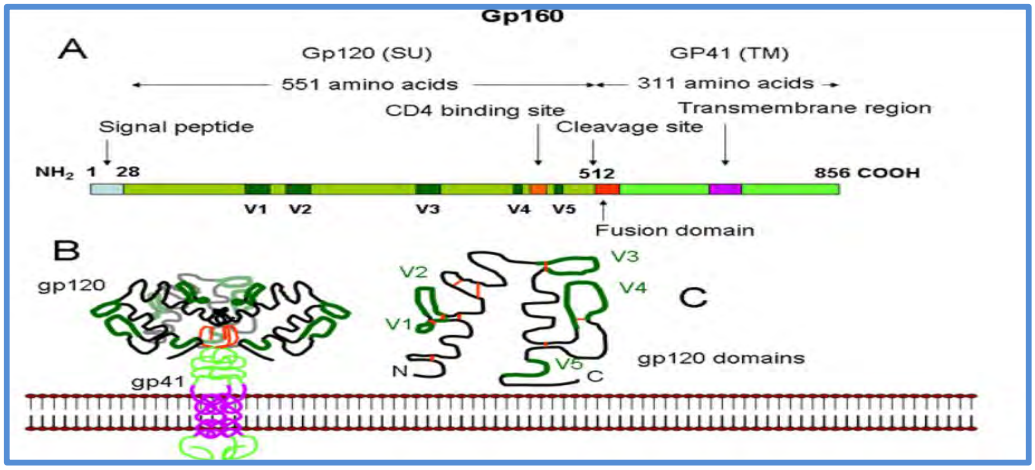

Figure 1.3: Schematic structure of gp160 glycoprotein. (a) The linear domain of the gp160 glycoprotein showing the surface protein gp120 and the transmembrane fusion proteingp41. **(b).** gp120/gp41 trimer shown embedded in the viral membrane. The CD4 binding site is shown in orange. This site is much more complex than shown in this diagram as it is not linear but forms a cavity in the gp120 subunit **(c)** gp120 domains in relation to the viral membrane. The gp120 is made up of conserved regions and variable loop regions. Disulfide bridges shown in red, variable loop regions highlighted in green. Source: (Hunt, 2010).

The HIV-1 envelope has the greatest amount of genetic diversity in the HIV-1 genome (Lynch et al., 2011). Envelopes can differ as much as 15-30% within a subtype and up to 35-42% between different subtypes (Lynch et al., 2009, Korber et al., 2001). Although there is vast variation between the envelopes of different isolates, it does contain conserved regions, some of which can be bound by neutralising antibody (nAb) (Burton et al., 2012). The viral envelope is the only region of the virus that can be accessed by neutralising antibodies, i.e. those that inhibit viral entry into cells (Burton et al., 2012a, Euler and Schuitemaker, 2012). The envelope is also involved in viral fusion and entry into the cell (Zwick et al., 2005). Because of these characteristics, the HIV-1 envelope is the only target for antibody-based vaccines (Burton et al., 2012).

1.4 HIV-1 Transmission

HIV-1 is predominantly transmitted through sexual intercourse, gaining entry into the body either from the genital tract or rectal mucosa (Hladik and Hope, 2009). The virus can also enter the body through blood transfusions, needles from intravenous drug use and mother-to-child transmission (Hladik and Hope, 2009).

HIV-1 is very inefficient in its transmission, with a key early report estimating the risk as 0.01% per sexual exposure (Gray et al., 2001). Gray et al. (2001) report that in their cohort of over 15 000 participants in rural Uganda, transmission risk did not significantly differ by HIV-1

subtype or sexually transmitted infections (STIs). However, other reports contradict the latter conclusion. It has been reported that both men and woman with a pre-existing herpes simplex virus II infection have a three-fold increase in their risk of HIV-1 acquisition (Freeman et al., 2006). Another study with over 12 000 participants showed that improved STI treatment in a rural region of Tanzania reduced HIV-1 acquisition by 40% (Grosskurth et al., 1995). STIs may compromise the mucosal barrier by introducing breaks in the epithelial lining and therefore allow virions to pass through more easily and initiate infection (Sagar et al., 2004). Alternatively, there could be an increased availability of activated $CD4^+$ T-cells which were fighting the STI and therefore the virus spreads more rapidly as target cells are readily available at the site of infection (Shaw and Hunter, 2012).

The efficiency of HIV-1 transmission is also related to the donor's viral load. In particular, there is evidence that when an infected individual's viral load is approximately <1500 copies of viral RNA per ml of blood, transmission is not observed (Pope and Haase, 2003).

1.4.1 HIV-1 Transmission Bottleneck

During heterosexual transmission, a single virion will establish the disseminated infection in the newly infected recipient in about 75-80% of cases (Abrahams et al., 2009, Keele et al., 2008). Keele et al. (2008) analysed over 3000 complete *env* sequences from 102 acutely Subtype B-infected participants derived from single genome amplification (SGA) and found that in 76% of cases, there was evidence of a single virus establishing infection. Infection in the remaining 24% of participants was established by between 2-5 viruses (Keele et al., 2008). Abrahams et al. (2009) reported similar results in a study conducted on over 1500 *env* sequences from Subtype C-infected male and female donors. The sequences were obtained by SGA and it was found that 78% of HIV-1 infections were from a single virus and 22% involved multiple viruses establishing the infection, with a median of 3. Both studies were conducted with heterosexual participants who were infected through sexual intercourse in very different settings, one in South Africa (Abrahams et al., 2009) the other from HIV-1 infected individuals from Trinidad and USA (Keele et al., 2008).

During infection, it has been proposed that not only one virus that penetrates the mucosal wall but it is the virus(es) that are the most fit which establish infection (Figure 1.4) (Shaw and Hunter, 2012). A study by Boeras et al., 2011 shows that the virus(es) that are transmitted and establish infection are not always the most prevalent virus(es) in the genital tract of the donor. This study suggests that the transmission bottleneck is therefore, not driven by low viral

diversity in the donor's genital tract but rather that selection plays a key role in the process (Boeras et al., 2011).

The transmission bottleneck is presumably subtype-specific as some subtypes exhibit the bottleneck and others do not (Haaland et al., 2009). It is also influenced by the absence/presence of STIs (Haaland et al., 2009). The transmission bottleneck has been mostly observed in Subtype B and C infected individuals and inflammation of the genital region of the newly infected individual was shown to be positively associated with multiple genetic variants (Haaland et al., 2009). In contrast, in a small cohort of 26 subtype A infected individuals, infection was found to be established by multiple viral variants (Ritola et al., 2004). The main difference between the study by Ritola et al. (2004) and the studies from Abrahams et al. (2008) and Keele et al. (2008) is that Ritola et al. used a heteroduplex tracking assay rather than SGA. SGA is a more precise technique to identify transmitted virus and when the 26 samples from Ritola et al.'s study were re-analysed by SGA, they found that 80% of subjects had in fact been infected by a single virus (Keele et al., 2008).

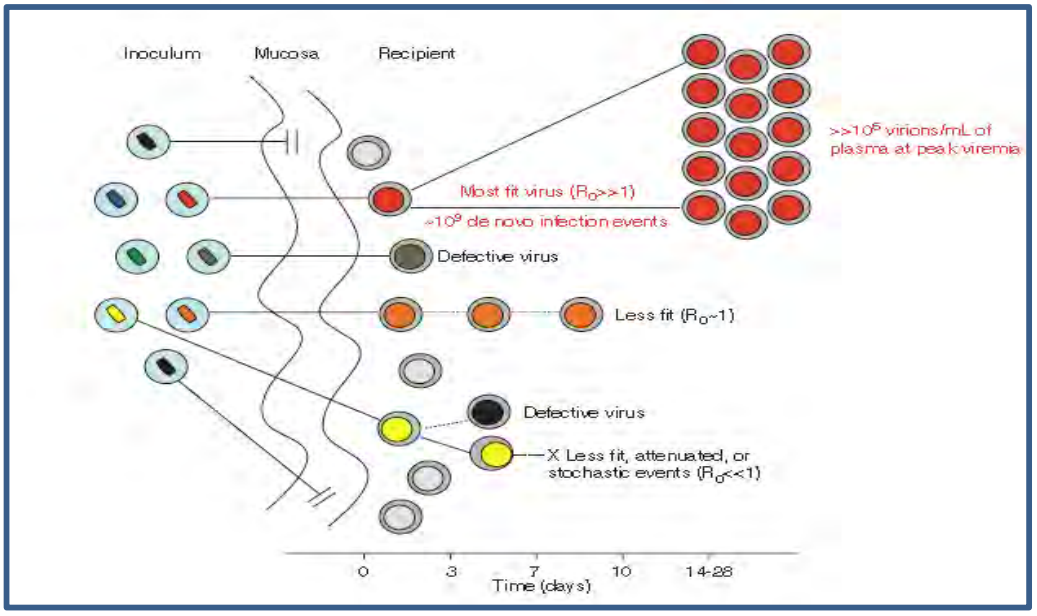

Figure 1.4 Schematic diagram of the HIV-1 bottleneck leading to established infection during heterosexual transmission. Genetically diverse virus populations derived from the genital tract attempt to pass the mucosal barrier. The virions able to pass through have different fates. Some will not be able to establish infection as they are defective while others are generally less fit. Only the most fit virus(es) will establish infection (shown in red). Source: (Shaw and Hunter, 2012).

Identifying the HIV-1 clones responsible for clinical infection and understanding the molecular fundamentals of HIV-1 transmission could possibly aid in designing an effective anti-HIV vaccine or prophylactic microbiocide (Abrahams et al., 2009, Keele et al., 2008).

1.4.2 HIV-1 entry into host cells–Cell-free Transmission

Viral entry from the extracellular environment is mediated through the CD4 receptor as well as co-receptors known as C-C chemokine receptor type 5 (CCR5) and CXCR4 C-X-C chemokine receptor type 4 (CXCR4) (Knysz et al., 2007). The CD4 receptor is a glycoprotein that is found on T cells, macrophages, monocytes, eosinophils as well as microglia cells of the central nervous system (Knysz et al., 2007). This is the reason why HIV-1 infects the cells of the immune system as these are the cells which have the relevant receptors needed for viral fusion and entry (Knysz et al., 2007).

The viral glycoprotein gp120 is responsible for binding with the CD4 receptor at its CD4 binding site causing conformational changes leading to the V3 loop region of gp120 binding to the co-receptors which subsequently leads to viral fusion to the cell membrane (Figure 1.5) (Kwong et al., 1998). The fusion of the cell is mediated by the gp41 portion of the *env* glycoprotein (De Clercq, 2007). It is important to note that gp120 can also bind to other targets to induce viral entry such as heparin sulphate proteoglycans as well as mannose-C-type lectin receptors (Shattock and Moore, 2003). The different cell types susceptible to HIV-1 infection do not express every attachment receptor; for example, mannose-C-type lectin receptors are expressed on dendritic cells, CD4 is expressed on Langerhans' cells and CD4, CCR5 and CXCR4 are expressed on $CD4^+$ T lymphocytes (Shattock and Moore, 2003).

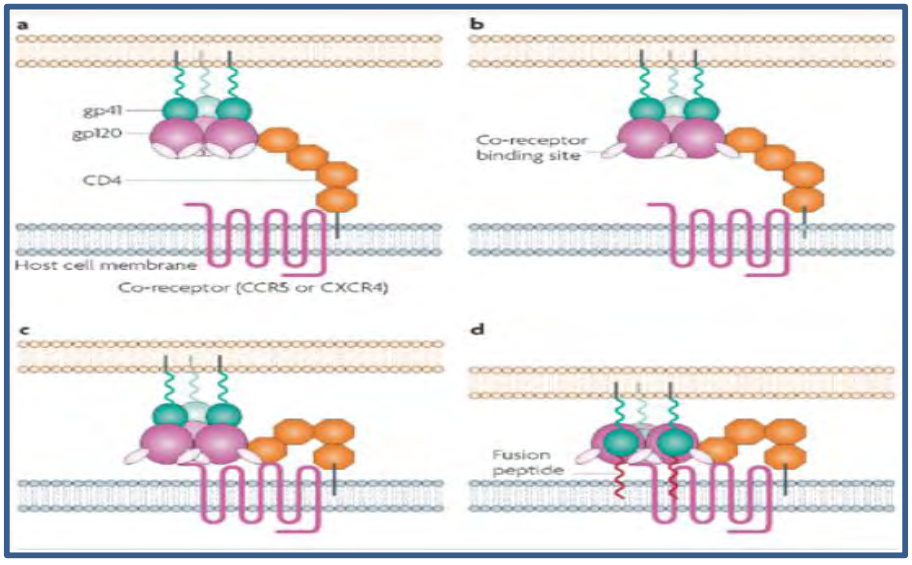

Figure 1.5. Schematic representation of the process of viral fusion with host membrane. (a) As gp120 CD4-binding site binds to the CD4 receptor, a conformational change is induced with exposes co-receptor binding sites. **(b)** The co-receptor binding site is found in the V3 loop of gp120 and also consists of amino-acids in CD4 and is collectively known as the "bridging sheet". **(c)** Co-receptor and "bridging sheet" bind inducing conformational changes in gp41. **(d)** Fusion of the viral envelope with the host membrane mediated by gp41 occurs. Source: (De Clercq, 2007).

1.4.3 HIV-1 entry into host cells – Cell-to-Cell Transmission

HIV-1 cell-to-cell transmission is an extremely efficient mode of virus transfer (Malbec et al., 2013, Schiffner et al., 2013, Abela et al., 2012) and occurs even during ART (Sigal et al., 2011). In this process, a virus particle is directly transferred between lymphocytes or between lymphocytes and either macrophages and dendritic cells (Malbec et al., 2013). The HIV-1-infected donor cells form conjugates with uninfected cells through the binding of their adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) from the donor cells and lymphocyte function-associated antigen-1 (LFA-1) from the target cell (Schiffner et al., 2013). The HIV-1 envelope, which is expressed on the surface of the infected cell also binds to the CD4 ligand of target cell (Malbec et al., 2013). The order of events during cell-to-cell transmission between T lymphocytes are conflicted with some studies suggesting the formation of conjugates occurs first, independent of the envelope (Puigdomenech et al., 2009) while others believe that the CD4 binding to the target cell is the first event (Schiffner et al., 2013). The conflicting results may be because of the different experimental models used to determine cell-to-cell transmission. Various assays, cell types and readouts have been used and with each, different results have been obtained (Malbec et al., 2013, Abela et al., 2012, Puigdomenech et al., 2009)

The small, 500 nm, space between the donor and target cells is known as the virological synapse and this is where the virus is transferred between cells (Schiffner et al., 2013). As a virion is approximately 120 nm in size (Grgacic and Anderson, 2006), there is limited space for the virus to manoeuvre in the virological synapse and therefore it immediately enters the target cell and that is what makes this mechanism of transmission distinct from cell-free HIV-1 transmission. Cell-to-cell transmission can also occur through transient cell-to-cell contacts (without conjugate formation) as well as through longer-range intercellular interactions which involve nanotubes and filopodia (Abela et al., 2012).

Cell-to-cell transmission may be the dominant form of HIV-1 transmission as it is a highly efficient process, transferring more than one virus particle at a time into target cells and may aid the virus in evading immune responses as the virus does not enter the extracellular environment (Schiffner et al., 2013). The implication of multiple virions entering a single target cell is the facilitation of more recombination events which may lead to the increased frequency of viral mutants capable of escaping immune responses (Schiffner et al., 2013, Malbec et al., 2012, Abela et al., 2012).

1.5 Immune Responses to HIV-1

The ability to evade the immune system is one of the factors that contributes to the success of HIV-1 as a virus (Burton et al., 2005, McMichael and Rowland-Jones, 2001). Understanding the mechanisms involved in interacting and fighting the virus can aid in developing an effective vaccine that induces the most effective immune response (McMichael et al., 2010, Burton et al., 2005, McMichael and Rowland-Jones, 2001).

1.5.1 HIV-1 and the innate immune system

The innate immune system is the first line of defence against HIV once the virus enters the body (Carrington and Alter, 2012). As the virus enters the body, the first cells recruited to fight it are dendritic cells (DCs) and macrophages which are resident in the mucosa, engulfing the virus to process it for antigen presentation (Carrington and Alter, 2012, Knysz et al., 2007). DCs cause immune stimulation after taking up the virus by secreting non-specific and specific cytokines and chemokines such as interferon-α (IFN-α), interleukins 1, 6-10, 12, 18 and tumour necrosis factor-α (TNF-α) (Carrington and Alter, 2012, Knysz et al., 2007). Interestingly, Meier et al. (2009) show that female DCs produce more IFN-α than men which could possibly be part of the explanation for why females show an overall lower viral set point as compared to men since this area of the innate immune response is stronger in women (Meier et al., 2009). HIV-1 enters immature DCs that then migrate to the lymphoid organs where they mature and this is where they would have presented viral proteins to naïve $CD4^+$ T-cells but instead the virus infects these cells (Knysz et al., 2007).

Macrophages recognise pathogen-associated molecular patterns (PAMPs) of the virus through their pattern recognition receptors (PRRs) and as a result produce type 1 interferons that suppress viral replication (Rasaiyaah et al., 2013). However, the virus can also replicate within these cells which makes them another cell target and reservoir for the virus (Rasaiyaah et al., 2013, Knysz et al., 2007). The virus has also found ways to evade the antiviral activity of macrophages as Rasaiyaah et al. (2013) show that in the early stages of infection, the viral capsid is able to hide viral nucleic acids as well as recruit host factors which allow the virus to escape any detection from the innate immune system, thus preventing macrophages from producing type 1 interferons and therefore enabling virus replication in this cell type.

The cytokine storm caused by the macrophages and dendritic cells leads to the activation of natural killer (NK) cells (Alter and Carrington, 2012). However, HIV-1 has developed ways of evading the antiviral activity of NK cells (Alter and Carrington, 2012). For example, NK cells

express both CCR5 and CXCR4 through which HIV-1 may initiate intracellular signalling and dysregulate the activation of NK cells (Fauci et al., 2005).

1.5.2 HIV-1 and the adaptive immune system

The innate immune response is active approximately 5-7 days before the HIV-carrying immune cells reach the lymphoid organs and adaptive immune response of HIV is initiated (Knysz et al., 2007). Both cellular and humoral responses are activated during acute and chronic infection (Knysz et al., 2007) and each arm of the adaptive immune system has a role to play.

There is evidence that $CD4^+$T-cells specific to HIV may enhance control of viraemia by providing help to either $CD8^+$ T-cells or B-cells or by direct antiviral effects (Ranasinghe et al., 2012). However, $CD4^+$ T-cells are the subtype of adaptive immune cells that are depleted the most by the virus and as $CD4^+$T-cells are depleted during late stage infection., the other arms of the immune system in turn become less efficient (Ranasinghe et al., 2012, Roederer et al., 1995). In acute infection, memory $CD4^+$ T-cells are depleted from circulation and as the disease reaches chronic phase its affects both the naïve and memory phenotypes of $CD4^+$ T-cells that are in circulation as well as at the lymphoid sites (Roederer et al., 1995). Individuals who are not on ART and are able to control viral replication have been found to have a higher number of HIV-specific $CD4^+$ T-cells and highly HIV-exposed sero-negative individuals have been found to possess HIV-specific $CD4^+$ T-cells which suggests a protective role for these cells (Ranasinghe et al., 2012).

$CD4^+$ T-cell depletion has adverse effects for the other arms of the innate immune system as these cells play a role in helping both $CD8^+$ T-cells and B-cells (McMichael and Rowland-Jones, 2001). In acute infection, the number of HIV-specific $CD8^+$ T-cells increases (Knysz et al., 2007). $CD8^+$ T-cells are a subtype of T-cells controlling HIV infection by cytotoxic effects such as secreting perforins to destroy and stimulate apoptosis of antigen-presenting cells (Knysz et al., 2007). There is a positive correlation between time of $CD8^+$ T-cell appearance and viral load decrease (Knysz et al., 2007). Betts et al. (2006) compared HIV-specific $CD8^+$ T cells from HIV-infected individuals and known HIV-positive non-progressors. By using polychromatic flow cytometry they were able to simultaneously test five $CD8^+$ T-cell functions including degranulation and chemokine and cytokine production (Betts et al., 2006). They found that it was not the quantity of HIV-specific $CD8^+$ T cells which correlated with viral load decrease but rather the quality of the cells (Betts et al., 2006). The non-progressors had

qualitatively superior functionality in their $CD8^+$ T-cells and therefore suppressed viral loads (Betts et al., 2006).

The protective role of $CD8^+$ T-cells has been shown *in vivo* in animal models which show that elimination of $CD8^+$ T-cells results in an increase of viral load (Jin et al., 1999, Schmitz et al., 1999). However, in chronic infection $CD8^+$ T-cells have been shown to become dysfunctional, with diminished cytolytic effects, phenotypic maturation and proliferation (Benito et al., 2004). This could be caused by a number of factors such as reduced help from $CD4^+$ T-cells, T cell exhaustion as a result of the chronic inflammatory state as well as an increased expression of programmed death 1 (PD-1) receptor on both $CD4^+$ and $CD8^+$ lymphocytes which has been shown to disrupt $CD8^+$ T-cell function (Porichis et al., 2011). $CD8^+$ T-cells have been investigated in vaccine models (McMichael and Rowland-Jones, 2001). Studies have shown that $CD8^+$ T-cells cannot prevent cells from being infected with the virus but instead can eliminate infection before it establishes itself or can control the virus at a very low viraemia such that it cannot cause disease (Gallimore et al., 1995, McMichael and Rowland-Jones, 2001, Allen et al., 2000).

$CD8^+$ T-cells have been shown to reduce viral replication in primary infection, however the role of the humoral response is not as clear (Schmitz et al., 2003). B-cells produce antibodies that can prevent virus establishment and replication, coming in the form of nAbs as well as non-neutralising antibodies which perform functions such as antibody-dependent cellular cytotoxicity (Moir and Fauci, 2009, Walker and Burton, 2010). Antibodies can also contribute to protection by interacting with and recruiting complement (Parren and Burton, 2001).

Like the other components of the immune system, an effective antibody response against the virus in natural infection is hindered by the numerous B-cell abnormalities that set in during the progression to AIDS (Moir and Fauci, 2009). It is believed that HIV cannot replicate inside B-cells *in vivo* (Moir and Fauci, 2009), however, the B-cells are indirectly affected by the virus (Shen and Tomaras, 2011). HIV-1 infection has been found to cause hypergammaglobulinemia – increased level of gamma globulins, most commonly IgM, which causes chronic inflammation and lymphomas (Shen and Tomaras, 2011). The virus also causes loss of memory B-cells, B-cell exhaustion because of chronic inflammation as well as atypical B-cell surface markers (Shen and Tomaras, 2011).

The HIV-1 Nef protein has been found to inhibit class switching which prevents antibodies from developing higher specificity and efficiency (Qiao et al., 2006). HIV-1 also causes

destruction of organized lymphoid tissues in the intestinal mucosa which reduces the number of B-cells (Moir and Fauci, 2009).

Early ART has been shown to preserve the early B-cell response as the therapy minimizes viral replication and therefore suggests a correlation between HIV-1 replication and B-cell stimulation (Shen and Tomaras, 2011). ART has also been shown to

1.6 Non-neutralising and Neutralising Antibodies in HIV-1 Infection

Since the discovery of HIV-1, scientists have been attempting to make an effective vaccine against the virus; however despite numerous attempts, no effective vaccine has been developed to date (Burton et al., 2012). One area scientists are investigating is the elicitation of BnAbs by an immunogen (Burton et al., 2012). Focus for vaccine development has been drawn to nAbs because many existing licensed vaccines seem to work by eliciting nAbs (Plotkin, 2008). Some studies have shown that non-neutralising antibodies do not provide protection against simian/human immunodeficiency virus (SHIV- a chimeric virus with the components of both simian immunodeficiency virus and HIV-1 genes) in nonhuman primate models (Walker and Burton, 2010, Barnett et al., 2008, Mascola et al., 1999). Non-neutralising antibodies are the first to arise after HIV-1 infection at approximately day 12 post-infection and target the gp41 glycoprotein (Euler and Schuitemaker, 2012, Doria-Rose, 2010, Tomaras et al., 2008). Non-neutralising antibodies were previously thought to have no effect on reducing viral replication; however, the recent RV144 HIV-1 vaccine trial showed that the binding of non-neutralising IgG antibodies to the V1/V2 region of the HIV-1 envelope correlated with protection from HIV-1 acquisition (Haynes et al., 2012). Therefore, more research must be done to better understand the role of non-neutralising antibodies in HIV-1 prevention (Haynes et al., 2012).

1.6.1 Neutralising antibodies in natural infection

In natural infection, HIV-1-infected individuals develop nAbs, known as autologous nAbs, against the virus they are infected with (Richman et al., 2003, Wei et al., 2003). These autologous antibodies only develop approximately 2-3 months post-seroconversion, long after HIV-1 has established itself (Figure 1.6) (Tomaras et al., 2008).

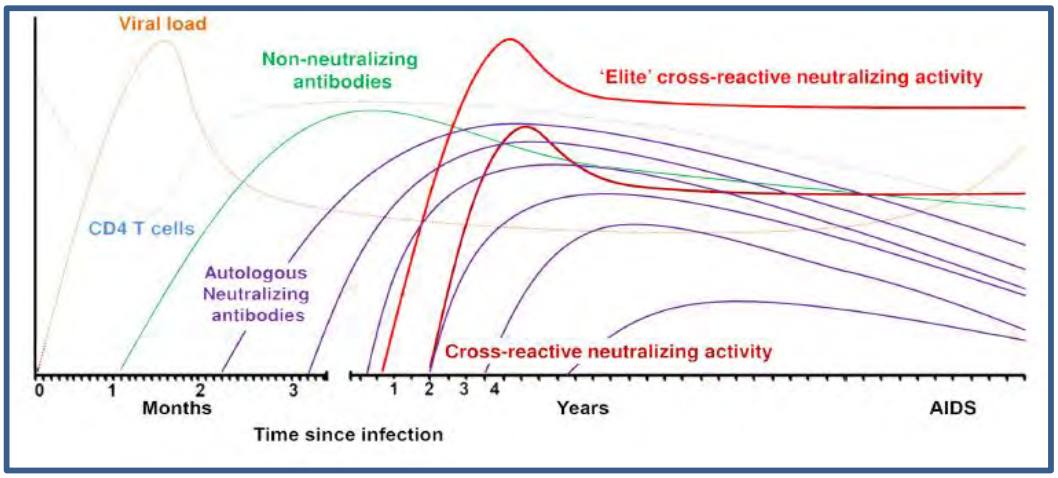

Figure 1.6: Timeline of the emergence of neutralising antibodies in HIV-1 infection. Non-neutralising antibodies (green) develop within a month of infection but do not prevent virus replication. Autologous neutralising antibodies (purple) that bind specifically to the individual's virus only develop within 2-3 months of infection. Cross-reactive or broadly neutralising antibodies (red) begin to develop after more than one year post-infection. New antibody responses are constantly appearing as the virus attempts to escape neutralisation. Source: (Euler and Schuitemaker, 2012).

The antibodies that many scientists are interested in eliciting by an anti-HIV-1 immunogen are the broadly neutralising antibodies (BnAbs) which are cross-reactive, heterologous antibodies (Euler and Schuitemaker, 2012). These antibodies are of interest because they target conserved regions of the viral envelope spike and therefore, if elicited by a vaccine, could theoretically protect against a range of different viruses (Euler and Schuitmaker, 2012). Approximately 15-25% of HIV-1-infected individuals develop BnAbs and these antibodies usually only appear more than 1 year after seroconversion (Sather et al., 2012, Gray et al., 2011, Doria-Rose et al., 2009).

Within the HIV-1-infected group who develop BnAbs only 1-5% will produce the potent BnAbs presumably required in a vaccine (Streeck et al., 2013, Euler and Schuitemaker, 2012, Sather et al., 2012, Simek et al., 2009) The reason for their delay in appearance is because of the complexity of affinity maturation that takes place in these antibodies to equip them with the relevant breadth and potency needed to effectively prevent viral entry into cells (Burton et al., 2012b, Kwong and Mascola, 2012).

1.6.2 Do neutralising antibodies help in HIV-1 natural infection?

Some of the questions that can be raised by current research on nAbs and prevention of HIV include: why do these nAbs not completely eliminate the virus already in the body? Does possessing potent and broad antibodies aid in slowing down the progression to AIDS?

Early evidence made it clear that nAbs are not sufficient to eliminate the virus from the body because of viral diversity (Richman et al., 2003, Wei et al., 2003). . The range of viral diversity, even in a single person ~3 months post-infection is such that once an autologous nAb response arises, it is virtually certain that there already exist a set of viral variants that are resistant to that nAb and presumably, all they have to do is grow out as their competitor viruses are selected against by the nAb response (Moore et al., 2013, Learn et al., 2002, Nowak and May, 1991).

nAbs do not eliminate the virus in an individual because of the selective pressure exerted by nAbs which cause the virus to mutate to evade neutralisation; the process known as viral escape (Doria-Rose et al., 2014, Moore et al., 2013, Murphy et al., 2013, Overbaugh and Morris, 2012). Longitudinal studies in humans have revealed that nAbs can respond and act against the dominant virus in an individual until a threshold is reached and an escape variant is selected for (Burton et al., 2005).

Moore et al. (2013) suggest that HIV-1 is constantly escaping antibody responses by mutating the sites recognised by the antibodies present and describe how new antibodies begin to emerge in a response to epitopes found on the escaping virus (Moore et al., 2013). The same group followed a patient's antibody response to autologous virus as it escaped neutralisation and found that the mechanism by which the virus escaped was by the addition of a glycosylation to mask the targeted epitope (Moore et al., 2012). Virus escape can occur as early as 6 months after infection (Bar et al., 2012) or later in chronic infection as it was found that even 10 years after initial infection virus was still escaping from antibody (Chaillon et al., 2012). An interesting research question to address on this topic would be why the appearance of antibody in natural infection drives the virus to escape rather than to switch to the exclusive use of cell-to-cell transmission which is known to be a transmission method that evades neutralisation relatively well. It may be possible that cell-to-cell transmission may allow a small number of viruses that are sensitive to autologous neutralisation to survive and replicate at very low frequency (Malbec et al., 2013).

HIV-1 escape causes changes to the viral envelope such as the addition of glycosylations mentioned earlier (Moore et al., 2012). There has been much debate in the scientific world about whether viral escape from antibody lead to a loss of viral fitness. It has been shown that viruses whose envelope genes were cloned later in infection did not exhibit a loss of replication fitness as compared to envelope genes cloned from the same individual but earlier in infection (van Gils et al., 2010, Bunnik et al., 2010). On the contrary, Sather et al. (2012) documented a case of viral escape leading to a fitness cost. The virus in this individual was sensitive to an

anti-CD4 binding site broadly neutralising antibody and escaped neutralisation through mutations in the CD4 binding site (Sather et al., 2012). The acquisition of resistance to this autologous antibody resulted in the virus having lower entry potential into host cells as well as a reduced replicative fitness (Sather et al., 2012).

Another key question that comes out of nAb research is whether possessing these antibodies helps slow down the progression to AIDS.

It has been shown previously that there is no relationship between having BnAbs and a subsequent better prognosis (Euler et al., 2010, Piantadosi et al., 2009, Gray et al., 2011). Piantadosi et al. (2009) showed in their cohort of women in Mombasa that the time for CD4 cell count decline to <200 cells/ul, the initiation of ART or death was not influenced by neutralisation breadth. Euler et al. (2010) confirm the above findings as they showed that the time to AIDS disease or AIDS-related death was not significantly different between three groups of their cohort; individuals with either strong, moderate or no cross-reactive nAb responses 3 years post-seroconversion (Euler et al., 2010).

Interestingly, however, a study by Huang et al. (2010) show that depletion of B cells of a patient by anti-CD20 therapy resulted in an increase in viral load set point as well as the re-emergence of virus that was sensitive to neutralisation by the antibody present before treatment. Although this study was conducted on one individual, the findings are very striking and suggest that nAbs may in fact have a role in control of viral load (Huang et al., 2010).

Gray et al. (2011) studied a cohort of 40 HIV-1 infected women and found 7/40 to have BnAbs. They found that amongst their cohort, having a higher viral load and lower CD4 count in acute infection correlated with greater neutralisation breadth (Gray et al., 2011). However, another study conducted by Bonsignori et al. (2014) showed contradictory results. The authors suggest that development of BnAbs can also occur while controlling viraemia, and that stimulation with high viraemia may not be required to acquire breadth (Bonsignori et al., 2014).

Bonsignori et al. (2014) identified an individual who was infected with HIV-1 and also had systemic lupus erythematosus (SLE), an autoimmune disease. They found that this individual had BnAbs against HIV-1 which possibly originated from a similar pool of B cells as SLE-associated autoantibodies (Bonsignori et al., 2014). This individual had a controlled viral load of 4,150 HIV RNA copies/ml and a relatively normal CD4 count of 568 cells/mm^3 (Bonsignori et al., 2014). The individual had no HIV-1-controlling HLA phenotypes, no defective *nef* genes in the HIV-1 strain the individual was infected with and was not on ART at this time; so these

factors could not have been the cause of the HIV-1 control (Bonsignori et al., 2014). HIV-1 control could be a result of BnAbs although there is no direct evidence for this.

1.7 Identified Targets of Broadly Neutralising Antibodies

BnAbs are attractive for immunogen elicitation as they have been found to target conserved regions of the HIV-1 envelope (Figure 1.7) (Burton et al., 2012), regions found in all HIV-1 viruses because of their essential function in HIV-1 fusion and entry into host cells (Burton et al., 2012). Until recently, very few broad and potent monoclonal antibodies (mAbs) had been isolated and few epitopes had been characterised, however, new approaches in the isolation of mAbs have been developed leading to the identification of new broadly and exceptionally potent mAbs (Thenin et al., 2012).

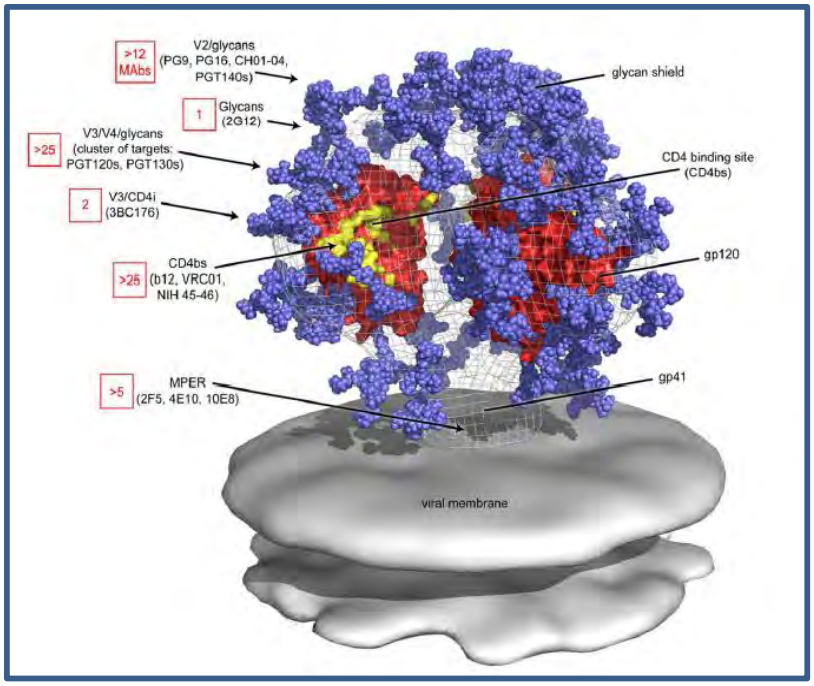

Figure 1.7: HIV-1 envelope targets for BnAbs. The HIV-1 envelope spike has a mushroom like appearance with gp41 subunits making up the stalk and gp120 subunits creating a cage-like structure. BnAbs bind conserved regions of the trimer of heterodimers made up of gp120 and gp41. The CD4-binding site is shown in yellow; the glycans (carbohydrates) surrounding the proteins are shown in blue. The approximate regions of the known BnAbs epitopes are pointed to by arrows and number of mAbs targeting the regions is shown in the red boxes (more have been discovered since this publication). Source: Burton et al., 2012 (a).

1.7.1 V2/glycan Epitope

The V2/glycan epitope is a conformation epitope found in the V2/V3 loop of the gp120 glycoprotein (Moore et al., 2011, Tomaras et al., 2011). This region is one of the most variable

and glycosylated regions of the HIV-1 envelope and therefore makes it challenging to mimic in immunogen design (Thenin et al., 2012). The mAbs PG9 and PG16 which have marked potency and cross-reactivity bind to this epitope (Doores and Burton, 2010, Walker et al., 2009).

PG9 and PG16 antibodies were first isolated by Walker et al. (2009) who showed that that the binding of PG9 and PG16 to their epitope was dependent on the presence of an N-linked glycosylation that was in the position 160 (HXB2-numbering) as well as other glycosylations such as at position 156 (Doores and Burton, 2010, Walker et al., 2009). Glycosylations are characterized by an N-X-S/T motif (Johnson et al., 2001). After mapping the epitope they discovered that it was conformational, formed by certain amino acids of the envelope trimer being in close proximity as a result of the folding of the envelope structure (Doores and Burton, 2010). PG16 is relatively trimer-specific but PG9 can bind to a single gp120 subunit in several viral isolates although it binds to the trimer preferentially (Hoffenberg et al., 2013). The frequency of PG9/16-like antibodies in natural infection is approximately 21% (Walker et al., 2010). To date there has been no successful immunogen made that elicits PG9/16-like antibodies (Burton et al., 2012).

1.7.2 V3/Glycan Region

Broad mAbs have been found to bind to the glycan region of the V3 loop of gp120 (Thenin et al., 2012, Walker et al., 2011). These include 2G12, and PGT121-128, 130, 133, 135, 136 and 137 (Pejchal et al., 2011). The main amino acid responsible involved in binding of antibodies to the target is a glycan at position N332 but other glycans have been shown to mediate binding of antibodies to this region, especially N301 (Pejchal et al., 2011, Walker et al., 2011). Precursor antibodies seem to require both N332A and N301A glycans to bind, perhaps because of their low affinity and therefore need both arms of the antibody to neutralise the virus (Julien et al., 2013).There are exceptions however, such as PGT-121, which also recognises glycans outside the V3 loop such as N137 in the V2/glycan region (Sok et al., 2014).

Glycans have been known to shield the virus against nAb activity, however, a small fraction of antibodies have evolved which are glycan-dependent (Mouquet et al., 2012). This is an advantageous adaptation which aids neutralisation of some escape mutants (Mouquet et al., 2012). The frequency of nAbs that bind to this epitope is very low, with anti-glycan antibodies making up approximately 5% of the BnAbs naturally induced in infection (Walker et al., 2010).

1.7.3 CD4-binding Site

The CD4-binding site (CD4-bs) is a target for BnAbs such as VRC01 and b12 (Thenin et al., 2012). This is an attractive vaccine target as it is a highly conserved region because of its key role in viral fusion with the cell membrane (Knysz et al., 2007). However, the CD4-bs is well hidden and therefore, it is hard for antibodies to access it (Burton et al., 2005). VRC01 binds to the CD4-bs by mimicking the interaction of the primary ligand, CD4, to this receptor (Li et al., 2011). b12 is another antibody that targets this region but instead of mimicking the CD4 ligand it binds to an epitope that overlaps the CD4-bs (Pantophlet and Burton, 2006). Interestingly, deleting the V1/V2 and V3 loops of the HIV-1 envelope generally increases the affinity of antibodies which have epitopes overlapping the CD4-bs which suggests that these variable loops may be responsible for shielding these epitopes from antibody recognition (Pantophlet and Burton, 2006). CD4-bs-targeting antibodies make up between 11-15% of the nAbs in natural infection (Walker et al., 2010).

1.7.4 Membrane Proximal External Region (MPER)

Although most of gp41 appears to be occluded from antibody, there is a region proximal to the membrane known as the membrane proximal external region (MPER) which is located on the transmembrane region of gp41 where broad antibodies such as 2F5, 4E10 and Z13 as well as the broad and extremely potent mAb 10E8 bind (Huang et al., 2012). MPER is a linear epitope, spanning 24 amino acids from 660-683 (HXB2 numbering) and even though it is linear and therefore, suggestively an easier target to bind, very few broad antibodies are directed towards this epitope (Thenin et al., 2012, Zwick, 2005).

MPER is an attractive vaccine target for a number of reasons. It is a highly conserved linear epitope which is less variable than other regions of the gp120/gp41 trimer (Zwick et al., 2005). mAbs (although very few) with high levels of breadth and potency have been isolated against this region (Huang et al., 2012). MPER plays a critical role in viral fusion and entry into host cells and therefore an immunogen eliciting anti-MPER antibodies would possibly be effective in neutralising the virus before it invades a cell (Zwick et al., 2005).

1.7.5 CD4-inducible Site (CD4i)

HIV-1 requires binding to the CD4 receptor for entry into host cells and this binding has been found to change the conformation of the gp120 trimer such that a gp120 BnAb target is exposed which is known as the CD4-inducible site (CD4i) (Klein et al., 2012a, Xiang et al., 2003, Xiang et al., 2002). Xiang et al. (2002) identified three novel CD4i-site mAbs that inhibit the binding of the virus to the chemokine co-receptor CCR5 and therefore inhibiting entry of the virus into the cell. The mAb 17b also binds to this region and since the crystal structure of this antibody bound to gp120 has been determined, the 17b epitope has been precisely defined (Xiang et al., 2002). The epitope for 17b is believed to be partially hidden by the V2 loop as variants which lack the V1/V2 loops are more sensitive to neutralisation by this mAb (Xiang et al., 2002). Another broad antibody that binds to the CD4i-site is known at 3BC176 (Klein et al., 2012a). Although this specific antibody binds to a novel epitope encompassing both the V3 loop and the CD4i-site, its neutralisation spectrum is comparable with potent BnAbs against the CD4i-site (Klein et al., 2012a).

1.7.6 Mapping of Broadly Neutralising Targets

Identifying novel targets for BnAbs is a major component in the understanding of antigen-resistance mutations, designing probes for antibody isolation and importantly in epitope-based vaccine design (Chuang et al., 2013). One of the most common ways of mapping for novel epitopes is by mutating key amino acids on the suspected target site on the HIV-1 envelope (Moore et al., 2011, Tomaras et al., 2011, Doores and Burton, 2010). By introducing point mutations of the HIV-1 envelope and comparing the neutralisation of the parent virus against the mutated virus, BnAb targets can be efficiently mapped (Moore et al., 2011, Tomaras et al., 2011, Doores and Burton, 2010).

For the mapping of sera with anti-MPER activity, Li et al. (2007) describe the used of an HIV-1/HIV-2 chimeric virus, known as C1C. This virus contains an HIV-2 backbone but an HIV-1 consensus C MPER (Li et al., 2007). They found that the high threshold of ID_{50} >1000 was associated with a serum sample that neutralised the native HIV-1 MPER in this chimeric pseudovirus and that an ID_{50}<300 corresponded with not being an MPER-recogniser (Lie et al., 2007). There is ambiguity with samples falling between 300-1000 but Walker et al. (2010) found that samples with ID_{50} values between 300-500 tend not to contain antibodies which can neutralise the MPER region.

Zwick et al. (2005) also used the technique of alanine scanning to determine the epitopes for 4E10 and 2F5, the broad anti-MPER mAbs. They made alanine-scan mutants of residues 660-680 (HXB2 numbering) which are amino acids forming the MPER region of gp41 and tested the ability of the mAbs to neutralise these mutant viruses (Zwick et al., 2005). Their results revealed the linear epitopes for both mAbs with the core epitope of 2F5 being LELDKWANL, with the greatest effects in residues D, K and W while 4E10's epitope consisted of NWFDISNWLW with loss of residues W and F conferring resistance to the mAb (Zwick et al., 2005).

For mapping sera against the V2/glycan region – the same region bound by PG9 and PG16 – Moore et al. (2011) and Tomaras et al. (2011) both made point mutations throughout the V1/V2/V3 loops of the HIV-1 envelope and compared mutant neutralisation to the parent virus. In both cases they were able to map out the amino acids that were most important for binding of these V2/glycan recognising sera and found again that it was mostly N-linked glycosylation-dependent (Moore et al., 2011, Tomaras et al., 2011).

To identify the specific amino acids which form the V2/glyan epitope recognised by PG9 and PG16 mAbs, Walker et al. (2009) performed alanine scanning where they point mutated amino acids into alanine residues and tested whether there would be a drop in sensitivity of the alanine-scan mutants to PG9 and/or PG16 (Walker et al., 2009). From this epitope mapping procedure, it was identified that the PG9/16 epitope is a conformational epitope with a glycosylation at N160 and a residue at 169 playing critical roles in antibody binding (Walker et al., 2009).

Antibodies targeting the V3 glycan region can be identified in the same way with making mutations in key residues N332 and N301 (Walker et al., 2011). Although other glycans have been shown to be important in anti-V3/glycan neutralisation (Sok et al., 2014), mutating either N332 or N301 to alanines has been shown to cause drastic drops in neutralisation for most of the V3/glycan antibodies (Walker et al., 2011).

CD4-binding site mapping has proven more difficult as the key point mutations to inhibit nAb binding to this site make the virus unable to invade cells and therefore neutralisation cannot be measured (Wu et al., 2010). The use of a recombinant gp120 protein is now commonly used to determine anti-CD4-bs nAbs (Wu et al., 2010). In this method, sera are run through a column with either a wild-type or mutated recombinant gp120 protein (Tomaras et al., 2011, Wu et al., 2010). Anti-CD4-bs neutralising antibodies will be absorbed onto the column. The sera's

neutralising activity are then tested and the changes in neutralisation between the CD4-bs wild-type protein and mutated protein are compared (Tomaras et al., 2011, Wu et al., 2010).

Recently, Chuang et al. (2013) described a computational method for the identification of BnAb epitopes. Their method predicts antibody binding sites to HIV-1 at the residue level using information based on neutralisation panels of diverse viral strains (Chuang et al., 2013). The method uses neutralisation potencies for a range of viruses as well as information of the antibody bound/unbound virus to calculate a score for a given residue, indicating the probability the residue forms part of the epitope (Chuang et al., 2013).

To map out an epitope targeted by a specific antibody or serum sample, the technique of domain swapping can be used. In domain swapping, two viruses are chosen, one which is sensitive and one which is resistant to the particular antibody (Moore et al., 2008, Rong et al., 2007). Certain domains are swapped between them, for example, the gp120 or gp41 regions, and the changes in sensitivity and resistance of these swapped viruses to the antibody help narrow down the region the epitope lies in (Moore et al., 2008, Rong et al., 2007). Once the larger domains that the epitope is found in have been identified, domain swaps of smaller regions can be made to further narrow down the epitope (Moore et al., 2008, Rong et al., 2007).

1.8 HIV-1 Vaccine Strategies

1.8.1 Why do we still not have an effective HIV-1 vaccine?

It has been over 30 years since HIV-1 and AIDS were first described and still there is no anti-HIV-1 vaccine in licensure (Burton et al., 2012). The discovery of ART has dramatically increased HIV-1 infected individual's life span offering hope in the field however; an effective preventative measure is yet to be found (Dangeti, 2014). Scientists are still battling to produce an effective vaccine, firstly, because most of the classical vaccine techniques do not work in the context of HIV-1 (Burton et al., 2012). For example, infections with smallpox and polio renders one immune to reinfection however, with HIV-1 the infection persists until the development of AIDS (Burton et al., 2012). In persistent viruses, such as HPV, the elicitation of IgG antibody from a vaccine is effective to prevent infection but this strategy has failed to work in the context of HIV-1 as current immunogens have failed to elicit effective antibody responses (Day et al., 2010).

In approaching the task of making an HIV-1 vaccine, the capacity of the immunogen to protect against the diverse viral strains circulating globally must also be taken into serious consideration (Palesch and Kirchhoff, 2013). Potential strategies to overcome this are under exploration and include developing multiple clade-specific HIV-1 vaccines or inducing an immune response against the conserved targets of the viral envelope (Palesch and Kirchhoff, 2013). The first option is not ideal as it would mean that multiple vaccines with high efficacy must be developed and approved (Palesch and Kirchhoff, 2013, Burton et al., 2012a). The second option, although theoretically easier, has not yielded fruitful results as currently no vaccine has succeeded in eliciting BnAbs against HIV-1 envelope targets (Palesch and Kirchhoff, 2013). Many challenges have prevented the development of an effective HV-1 vaccine but past failures have added to the knowledge needed to develop one in the future.

1.8.2 Past HIV-1 Vaccine Attempts

The first vaccine trial to prevent HIV-1 acquisition started in the late 1980s and since then only six trials have been approved for phase IIb/III efficacy trial (Figure 1.8)(Temchura and Tenbusch, 2014). The first HIV vaccine trial was conducted by Zaguryet al. (1988)in the Democratic Republic of Congo and used a vaccinia vector prime expressing the HIV-1 uncleaved envelope protein gp160 and as well as a gp160 boost in the hope of inducing nAbs and cytotoxic T cells (Zagury et al., 1988). Interestingly, the poxvirus-prime/protein-boost protocol they used is strikingly similar to the modestly successful Thai RV144 trial conducted 16 years later (Esparza, 2013). Zagury et al. (1988) reported high levels of nAbs as well as cell-mediated cytotoxicity however, this study was widely criticised by the scientific community as a result of questionable ethical practises (Esparza, 2013, Zagury et al., 1988).

A well-known trial, the STEP or Phambili trial, commenced in 2004 and used a trivalent adenoviral vector vaccine which encoded for HIV-1 *gag, pol* and *nef* and aimed to induce high titres T-cell responses (Buchbinder et al., 2008). Although this vaccine candidate had previously been found to control SHIV infections in non-human primates, the vaccine not only had low efficacy in humans but also increased the risk of acquiring HIV-1 in vaccinated volunteers who had pre-existing antibodies to adenovirus and/or volunteers who were uncircumcised (Duerr et al., 2012, Buchbinder et al., 2008). The increased risk of HIV-1 acquisition was thought to be caused by the presence of adenovirus-specific $CD4^+$ lymphocytes in the mucosa but this hypothesis has been dismissed by subsequent studies (Masek-

Hammerman et al., 2010, O'Brien et al., 2009). There is still debate in the HIV-1 vaccine field about whether general immune activation in the mucosa increases the risk of HIV-1 acquisition although studies *in vitro* have found that it does (Temchura and Tenbusch, 2014). The STEP/Phambili trial also highlights the fact that animal testing is not completely reliable since different results were seen for SHIV infection in primates and HIV in humans. Although a treatment may work in an animal model, in humans the same treatment may be ineffective and even detrimental (Duerr et al., 2012).

The most efficacious vaccine made to date was the RV144 vaccine run in a trial in Thailand – with 31.2% efficacy (Burton et al., 2012, Doria-Rose, 2010, Dangeti, 2014). Although the trail failed; like other failed vaccine trials, it provides knowledge for continued research towards making a more effective vaccine (Palesch and Kirchhoff, 2013, Dangeti, 2014).

The RV144 vaccine consisted of a canarypox prime with a recombinant gp120 boost (Buchbinder et al., 2008). Studies on the immunology of the vaccine revealed that there was no $CD8^+$ T-cell response (Doria-Rose, 2010). The $CD4^+$ T-cell response, although modest, was targeted against peptides derived from the V2 loop of the HIV-1 envelope (de Souza et al., 2012). The biggest immunologic force exerted in this vaccine was the non-neutralising IgG antibody response targeted at the V1/V2 loop of the HIV-1 envelope (Haynes et al., 2012). The binding of IgA antibodies to envelope positively correlated with HIV infection (Haynes et al., 2012). Development of a vaccine which elicits higher levels of V1/V2-specific IgG antibodies and lower levels of envelope-specific IgA antibodies may improve the efficacy of future HIV-1 vaccines (Haynes et al., 2012). This finding was surprising as primate models have previously shown non-neutralising antibodies to be ineffective in preventing SHIV transmission (Barnett et al., 2008, Mascola et al., 1999). The results of this study reveal new possibilities for non-neutralising antibodies playing an important role in the immune response to prevent HIV transmission by the process of antibody-dependent cell mediated cytotoxicity (ADCC) (Bonsignori et al., 2012). A modified version of the RV144 trial is being initiated in South Africa with the hope of building on the modest efficacy achieved (Esparaza, 2013).

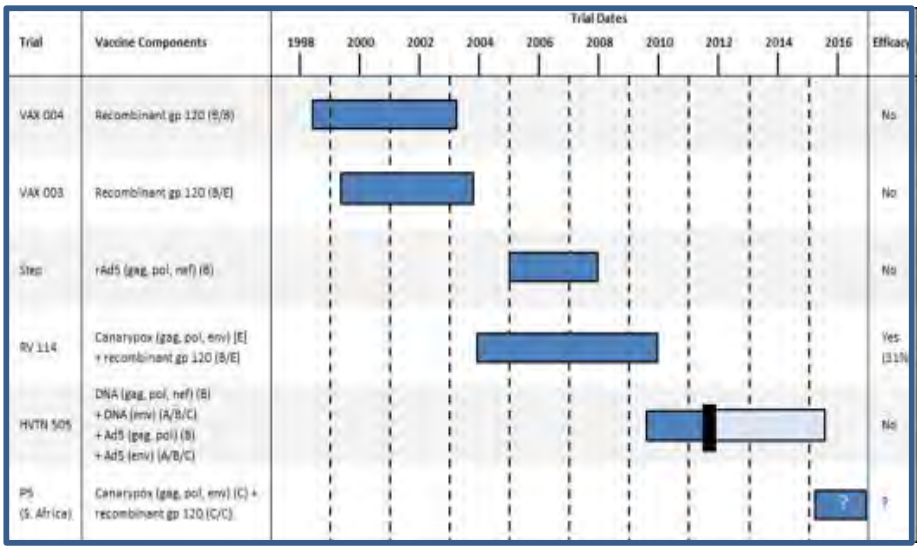

Figure 1.8: HIV efficacy trials since the late 1990s. Of the six immunogens approved for efficacy trials, presently, only one of them, RV144 trial conducted in Thailand, had modest efficacy. Soure: Esparaza, 2013.

Even though no successful HIV-1 vaccine has been made to date, HIV vaccine research is moving in the right direction. Lessons must be learnt from past failures and successes to ensure an effective vaccine is discovered in the near future.

1.8.3 Neutralising Antibodies in HIV-1 Vaccines: proof of concept

Eliciting a broadly neutralising response to antibodies is an attractive prospect to scientists and therefore *in vitro* and *in vivo* studies have commenced in this field of vaccine research (Doria-Rose, 2010). There is compelling experimental data from animal models that nAbs can prevent the acquisition of HIV-1 but no similar data in humans because of ethical reasons (Overbaugh and Morris, 2012)

As early as 1999, Mascola et al. found that administration of mAbs by passive infusion was able to protect the macaques from SHIV challenge. Recently, scientists have administered a cocktail of BnAbs to SHIV-chronically infected rhesus monkeys to determine whether the antibodies were able to protect against infection and they found that the mixture of antibodies was able to reduce plasma viraemia to undetectable levels (Barouch et al., 2013a).

Barnett et al. (2008) immunized rhesus macaques with an HIV-1_{SF162} envelope protein vaccine either intramuscularly or intranasally and then were challenged introvaginally with the SHIV expressing the same envelope protein as the vaccine. They found that the macaques which received intramuscular immunizations, alone or combined with intranasal immunizations, were protected against SHIV infection and had no detectable plasma viral RNA and did not

seroconvert (Barnett et al., 2008). It must be noted that in this study they used the virus SHIV-SF162P3, which in HIV-1 would be SF162 – an extremely neutralisation-sensitive virus – and this could have biased their results. Additionally, using one virus to observe a protective effect is not sufficient and they should use more than one virus in subsequent experiments to further validate their findings.

To attempt to deal with the diversity of viruses in circulation, Barouch et al. (2013) used HIV-1 mosaic antigens which are immunogens optimised using bioinformatics tools to make them represent the diversity of HIV-1 strains circulating worldwide. They found that rhesus macaques were protected against SHIV challenge after administration of these immunogens with 90% efficiency and this protection correlated with the elicitation of binding, neutralising and functional non-neutralising antibodies (Barouch et al, 2013). This finding, therefore, suggests that multiple antibody functions may be necessary to protect against the divergent strains of HIV-1 (Barouch et al., 2013). Again, in this study they used the easy-to-neutralise SF162 virus for challenge so it would be interesting to see further studies using harder-to-neutralise viruses or a range of diverse viruses.

Balazs et al. (2014) also proved the principle that neutralising antibodies were protective against HIV-1 acquisition by administering an adeno-associated virus (AAV) vector containing a gene encoding the modified mAb VRC07. They showed that this vectored immunoprophylaxis (VIP) was able to protect humanized mice from both intravenous and vaginal challenge from diverse HIV-1 strains even after repeated exposure to the virus (Balazs et al., 2014). The advantage of using VIP is the direct provision of the BnAbs through transgene expression which eliminates the dependence on the immune system to mount the humoral response (Yang and Wang, 2014). Development of VIP seems promising and currently recruitment of participants for a phase I clinical trial using an AAV vector that encodes for the mAb PG9 has commenced (IAVI, 2014).

1.8.4 Monoclonal therapy for HIV-1 therapeutic vaccine

There have been numerous efforts, both in animal models and humans, to test the efficacy of administering purified mAbs as a therapeutic measure to reduce HIV viraemia to undetectable levels at least temporarily and to use this method for a therapeutic vaccine (Klein et al., 2012b, Trkola et al., 2005, Mehandru et al., 2007).

Studies have been conducted in humans where HIV-1-infected individuals (eight chronically infected and six acutely infected) were treated with three mAbs (2G12, 2F5, and 4E10) in combination with ART (Trkola et al., 2005). Only two out of the eight chronically infected patients showed a delay in viral rebound once ART had been stopped; while the acutely infected patients showed significantly slower viral rebound as compared to the control group (Trkola et al., 2005).Mehandru et al. (2007) also performed a study in which 10 acutely-infected individuals were administered 2G12, 2F5, and 4E10 monoclonal antibodies. They did not observe substantial benefits to this therapy as viral rebound occurred post-ART interruption in 8/10 subjects (Mehandru et al., 2007). So therefore, although the administration of monoclonals is safe, it has not shown sufficient efficacy in humans to warrant further phase clinical trials to be conducted (Mehandru et al., 2007).

Klein et al. (2012) studied antibody passive transfer as a therapeutic vaccine against HIV-1 infected humanized mice. They administered a cocktail of 5 mAbs (3BC176, PG16, 45-46^{G54W}, PGT128 and 10-1074) to mice with already established infection and found that viraemia in all fourteen mice treated with this mix was controlled for an average of 60 days after cessation of therapy (Klein et al., 2012). As much as this study gives optimism that mAbs can be used in HIV-1 therapy, it does have its flaws. Humanised mice are a good model for human disease but have limitations. For example, the dynamics of HIV-1 replication may differ in humanised mice as compared to humans so viraemic suppression by mAbs may be easier to achieve in mice than it would be in nonhuman primates or humans. For example, the tissue in humanised mice may not completely replicate that of humans and therefore these mice most likely have less viral reservoirs than human which could influence the results. Another limitation in the Klein et al. (2012) paper is that they administered mAbs which are very broad and potent. The chances of these developing after vaccination in a human population is very low as only 20% of individuals develop BnAbs (Sather et al., 2012, Gray et al., 2011, Doria-Rose et al., 2009).

Studies using passive immunisation strategies have also been performed on non-human primates (Barouch et al., 2013b, Shingai et al., 2013). Barouch et al. (2013b) found that on giving rhesus monkeys are cocktail of BnAbs, their viraemia was reduced for a median of 56 days and their gag-specific T-cells had improved functionality. In another study by Shingai et al. (2013), they found that on administration of two extraordinarily potent mAbs, 3BNC117 and 10-1074, viraemia was controlled in both recently infected and chronically infected macaques. This viraemic control was observed even when only one of these mAbs was

administered (Shingai et al., 2013). These two studies show potential for the use of mAbs to control the virus in HIV-1 infected individuals but more investigation needs to be done in this field.

A prophylactic vaccine with incomplete coverage may be able to prevent infection in some individuals while a therapeutic vaccine with the same coverage would not be able to control viraemia due to viral escape (Letin, 1998). In addition, development of a therapeutic vaccine may be more challenging that developing a prophylactic vaccine because a therapeutic vaccine would need to suppress the viraemia of a more diverse population of HIV-1 in an individual. A prophylactic vaccine must just be able to protect the individual from a limited number of viruses that specific individual is exposed to (Abrahams et al., 2009, Keele et al., 2008, Letvin, 1998).

1.8.5 Challenges in producing neutralising antibody-inducing vaccines

As attractive as BnAbs are in vaccine research, there still are no vaccines able to efficaciously produce nAbs to block HIV-1 establishment (Palesch and Kirchhoff, 2013, Dangeti, 2014, Burton et al., 2005). This can be explained by a number of reasons.

Before the discovery of new techniques to isolate mAbs and map their epitopes, HIV-1 researchers had been attempting to design immunogens from the limited mAbs available for example, b12 mAb against the CD4-bs; for example all immunogens focused on the CD4-bs were all based on the knowledge of one mAb, b12 (Burton et al., 2012). Over the past four years there has been a substantial increase in the number of mAbs isolated and with them more epitopes have been discovered and therefore, the field of immunogen design is moving at a faster, more efficient rate now than ever before (Burton et al., 2012).

To aid in the identification of immunogens with an enhanced capacity of eliciting broad and potent BnAbs, a tiered system was proposed (Seaman et al., 2010). Clustering analysis of 109 viruses was used to define sensitivity patterns of HIV-1 viruses and four subgroups were identified; highly sensitive (tier 1A), above-average sensitivity (tier 1B), moderate sensitivity (tier 2) and low sensitivity (tier 3) viruses (Seaman et al., 2010). mAbs able to neutralise a range of viruses of lower sensitivity (tier 2/3) generally have increased breadth and potency and are the types of antibodies that need to be induced by an effective vaccine (Seaman et al., 2010).

The linear epitopes on the MPER of gp41 appear to be attractive vaccine targets (Zwick et al., 2005). Modest success has been observed in eliciting nAb responses to MPER peptides in one

or more viral conformations (Burton et al., 2012). However, they come with two main challenges. Firstly, MPER is extremely close to the viral membrane and many antibodies struggle to manoeuvre past gp41 and bind the MPER epitopes (Burton et al., 2012). Secondly, MPER being so close to the viral membrane leads to many anti-MPER antibodies being autoreactive; 2F5 and 4E10 both being shown to cross-react with human phospholipid cardiolipin (Haynes et al., 2005). Recently however, Huang et al. (2012) isolated an extremely broad and potent antibody, 10E8, which neutralised approximately 98% of tested viruses which implies it has no difficulty accessing the MPER region. mAb 10E8 has, however, been shown to also be lipid-membrane reactive (Chen et al., 2014). For an HIV-1 vaccine, a linear epitope like MPER may be easier to recapitulate in an immunogen, in a short peptide form, and this could potentially be easier than using more complex, conformational epitopes such as PG9 and PG16 (Zwick et al., 2005). However, the autoreactivity of these anti-MPER antibodies (Chen et al., 2014) and their transient accessibility (Harris et al., 2013) pose a challenge which must be addressed before these antibodies are considered for an HIV-1 vaccine (Chen et al., 2014).

Another major challenge in the inducing of an effective antibody-based immunogen is designing an immunogen which mimics the Env trimer sufficiently enough to elicit a BnAb response (Dangeti, 2014, Burton et al., 2012, Burton et al., 2005). There have been strides in the field to overcome this challenge as shown by a study conducted by Jardine et al., 2013). In this study, they created an immunogen, eOD-GT6, a gp120 outer domain, that showed the rare ability to produce an effective immune response and acted against HIV-1 subtypes (Jardine et al., 2013). This study was performed solely *in vitro* and now the challenge will be replicating these results in an animal model (Dangeti, 2014, Burton et al., 2012, Burton et al., 2005).

Another problem in inducing nAbs in a vaccine is that most mAbs that neutralise diverse viral strains can only prevent cell-free virus transmission which is problematic as the virus can also be transmitted directly from cell-to-cell (Monel et al., 2012). Cell-to-cell transmission of HIV-1 has been found to be more potent, faster and more efficient as compared to cell-free transmission and therefore this mode of transmission should not be neglected (Monel et al, 2012). nAbs capable of preventing cell-to-cell transmission of HIV-1 by accumulating at virological synapses, hindering fusion of infected and target cells and inhibiting the transfer of virus particles between host cells have been described (Malbec et al., 2013). Inhibition of cell-to-cell transmission has been found to require a larger BnAb response compared to cell-free virus transmission, with up to 20 fold higher concentrations needed for inhibition of cell-to-cell transmission (Malbec et al., 2013). Abela et al. (2012) have similar data except they find

that the difference in required potency between the two transmission methods is small for anti-MPER mAb 4E10 as well as anti-CD4 and anti-CCR5 inhibitors. This has serious vaccine implications as an immunogen must elicit a large enough antibody response to protect against both modes of transmission (Malbec et al., 2013).

BnAbs tend to have unusual features such as long complementary determining regions, high levels of somatic hypermutation, rare structural motifs and post-translational modifications which take years to arise (Burton et al., 2012). Creating a vaccine which will elicit this type of complex antibody is a challenge and a successful vaccine will require incredible ingenuity and resources (Burton et al., 2012).

The difficulty with producing immunogens based on nAbs is not only as a result of the genetic diversity of the viruses circulating in the human population but is also because of the incomplete knowledge of all the epitopes found on HIV-1 envelope protein. Vaccines which have been developed to elicit nAbs have not been successful partly as a result of the knowledge gap of the epitopes that bind these antibodies and therefore there is a need for the discovery of novel epitopes (Stamatatos et al., 2009, Burton et al., 2005).

1.9 Study Objectives

In this study, we designed a novel system for identifying broad and potent sera to be mapped to find novel HIV-1 epitopes. The discovery of novel epitopes targeted by BnAbs may aid in the effort towards finding an efficacious HIV-1 vaccine.

We selected an HIV-1, Subtype A virus known as QH343.21M.ENV.A10 (Blish et al., 2009). This virus will be referred to as QH343.A10 in this thesis for simplicity. We found this virus to be moderately resistant to sera and resistant to mAbs 2F5, 4E10, 2G12, VRC01 and b12. Combined, these mAbs bind to at least 3 of 5 previously identified BnAb targets. This made the QH343.A10 virus attractive for us to use in our mapping system in part because sera with dominant nAbs that target envelope structure in the same way as these antibodies presumably are unable to target QH343.A10 and will be screened out early in our scheme because of this.

We tested QH343.A10 against PG9 and PG16 mAbs which target the V2/glycan epitope. We found the virus to be sensitive to these mAbs. Therefore, we engineered several QH343.A10 mutant viruses to inhibit V2/glycan -site sensitivity by mutating key amino acids which form

this epitope. This was done to identify sera that targeted QH343.A10 via recognition of this epitope.

We first screened 474 serum samples against QH343.A10. From the QH343.A10-recognising sera we then excluded sera which recognised the virus through the MPER, V2/glycan -site and V3-dependent glycan region by various mapping methods. The sera that recognised via MPER and V3/ dependent glycan region presumably did so by recognising in a manner different that 4E10, 2F5 and 2G12. We also presumably excluded sera that recognise the CD4bs similarly to b12 or VRC01 because such antibodies presumably cannot recognise QH343.A10.

After exclusion of sera whose main antibody recognition was against these previously identified targets, we identified the broadly reactive and potent sera. These are the candidate sera to map out a novel epitope.

Specific Objectives:

- **Screen 474 serum samples against QH343.A10**. We screened 474 serum samples from chronically HIV-1-infected, ART-naive individuals in Cape Town against QH343.A10 to identify sera that were able to neutralise the virus. QH343.A10 is resistant to all the mAbs available in the laboratory except for PG9 and PG16. This suggested that antibodies in our serum cohort that recognise QH343.A10 similarly to the mAbs 4E10, 2F5, b12, VRC01 and 2G12 were unable to recognise QH343.A10 and would be screened out in this step.
- **Narrow down the candidate sera by identifying and excluding sera that neutralise QH343.A10 through previously defined BnAb targets**. Although we expected to have selected against sera that recognise QH343.A10 through known epitopes such as MPER, CD4 binding site and V3/glycan epitope, we expected that some sera would still be able to recognise these sites in a different way to the mAbs which QH343.A10 has been found to be resistant to. We thus excluded sera that neutralise QH343.A10 through MPER, V2/glycan -site and the V3/dependent glycan region. Various viral constructs were used to identify such sera. Because of technical reasons, we did not analyse the CD4-bs and the CD4i-site.
- **Identify the broad and potent neutralisers from among the serum candidates.** Breadth and potency were measured on the remaining serum samples. This was achieved by using a 24-virus panel consisting of relatively hard-to-neutralise viruses.

The broad and potent samples are the sera which can now be mapped to potentially find a novel HIV-1 target.

Chapter 2: Materials and Methods

2.1 Study participants

In this study there were 474 study participants in our cohort from two HIV Wellness Clinics in Cape Town, South Africa. 99 were from Groote Schuur Hospital and 375 were from Khayelitsha Site B clinic. They were all older than 18 years old, ART-naïve and reported infected for more than 1 year at the time of recruitment. Study participants included 380 females, 65 males and 29 individuals for whom we did not collect sex data. The median CD4 count for the 387 participants we have CD4 count data on was 435 cells/mm^3 (IQR 322, 566). All participants gave written informed consent and the study was approved by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town.

2.2 Blood collection and preparation of sera

Blood was taken from HIV-positive participants at the respective clinics and delivered the same day for processing. Blood was centrifuged at 2000 rpm for 15 minutes. Sera were pipetted into polypropylene tubes and heat-inactivated in a water bath at 56 ˚C for 1 hour. Heat-inactivation is important to remove complement activity which can interfere with the neutralisation assays performed with the sera. Once heat-inactivated the sera were aliquoted into tubes and stored at -80 ˚C until further use.

2.3 DNA preparation

Cloned HIV-1 envelope constructs for QH343.A10 and Q23.7 were obtained from Dr Julie Overbaugh (Fred Hutchinson Cancer Research Centre, Seattle, USA through the NIH AIDS Reagent Reference Program, Rockville, USA) and Du156.12 was obtained from Professor Lynn Morris (National Institute for Communicable Diseases, Johannesburg, South Africa). Constructs were transformed and grown in DH5α™ *E.coli* cells (Invitrogen, Darmstadt, Germany). Single colonies were subsequently grown in Luria-Bertani (LB) broth (Sigma Chemical Company, Schnelldorf, Germany) and extracted using Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany) or GeneJet™ Plasmid Miniprep Kit (Thermo Fisher Scientific, Massachusetts, USA). Concentrations of the DNA were determined using a Nanodrop 2000 spectrophotometer (ThermoScientific, Massachusetts, USA). The presence of DNA was confirmed by using agarose gel electrophoresis when necessary. The DNA was run on 1% agarose gel (Lonza, Basel Switzerland) at 100 V, using GelRed Nucleic Acid Stain (Phenix Research Products, North Carolina, USA) as the DNA staining agent.

2.4 Site-directed mutagenesis for the generation of viral mutant DNA

2.4.1 Primer design

The viral envelope sequence for QH343.A10 was retrieved from the National Center for Biotechnology's (NCBI) Genbank (https://www.ncbi.nlm.nih.gov/genbank/) (QH343.A10, accession number: FJ866119) and aligned with the HXB2 envelope (accession number K03455.1) using the BioEdit Sequence Alignment Editor (Ibis Bioscience, California, USA). The positions to mutate were identified and the primers designed accordingly. Primer sequences were generated using the GeneArt® Primer and Construct Design Tool (Life Technologies, http://www.lifetechnologies.com/order/oligoDesigner/).

2.4.2 Mutagenesis Reaction

For all the QH343.A10 mutants generated, point mutations were introduced at positions: N156A, F159A, N160A, R166A, I169E, Q171A using either the GeneArt® Site-Directed Mutagenesis PLUS Kit (N156A, F159A, N160A, I169E and Q171A) (Figure 2.1) (Invitrogen, Darmstadt, Germany) or the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit (R166A) (Figure 2.2) (Strategene, La Jolla, USA). Mutagenesis was performed according to the manufacturer's instructions.

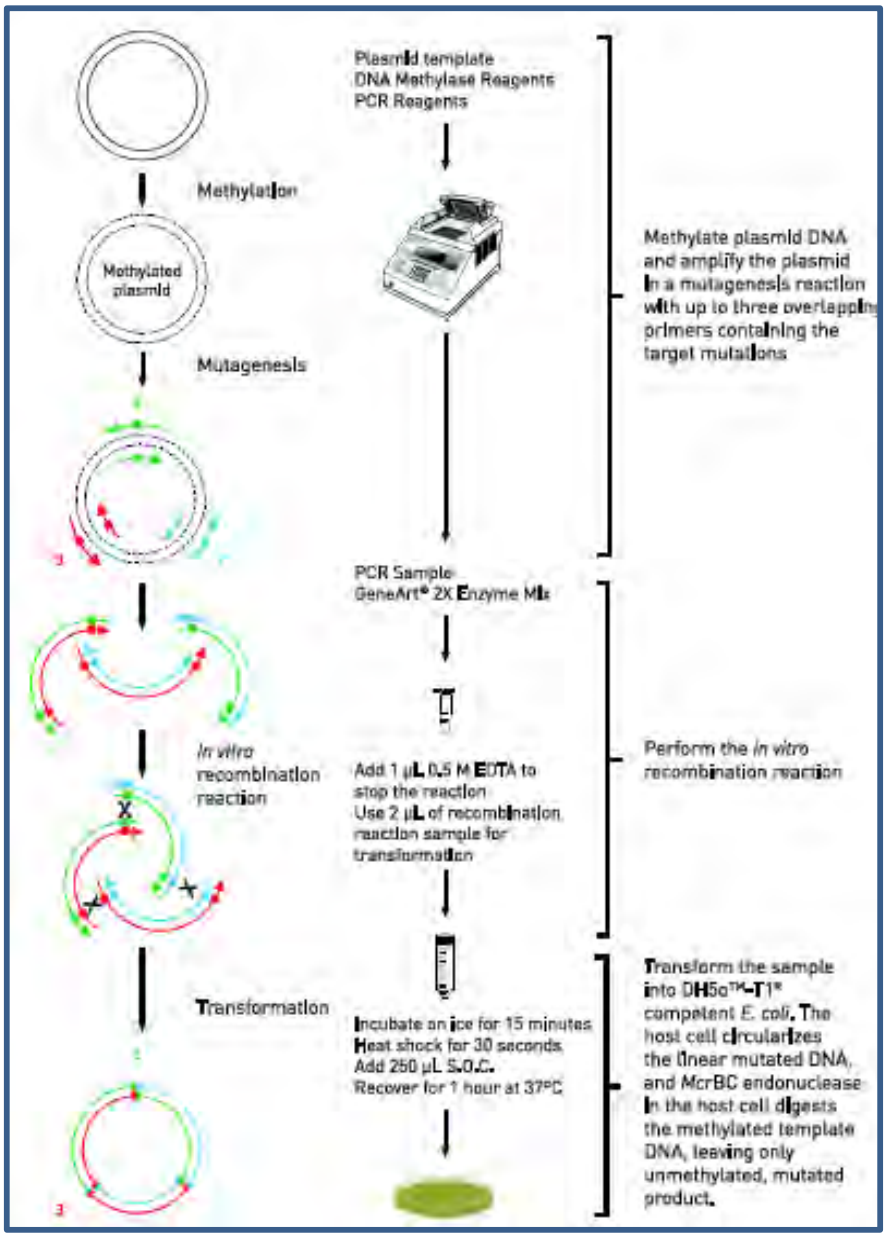

Figure 2.1: Protocol for GeneArt® Site-Directed Mutagenesis PLUS Kit. Briefly, in this system the parental DNA, primers, reaction buffers and methylase were combined and then the polymerase chain reaction (PCR) was performed. Methylase is added to the PCR reaction because its denatures the parental strand ensuring the PCR product only contains mutant strands with the appropriate mutation. After verification of a PCR product by agarose gel electrophoresis, the PCR product is combined with the GeneArt® 2X Enzyme Mix. This Enzyme Mix catalyses a recombination reaction, whereby parts of the mutant strand are recombined to form one strand. The product of the recombination reaction is then transformed into DH5α-T1® cells and the DNA from the bacteria is subsequently extracted (GeneArt® Site-Directed Mutagenesis PLUS Kit Manual).

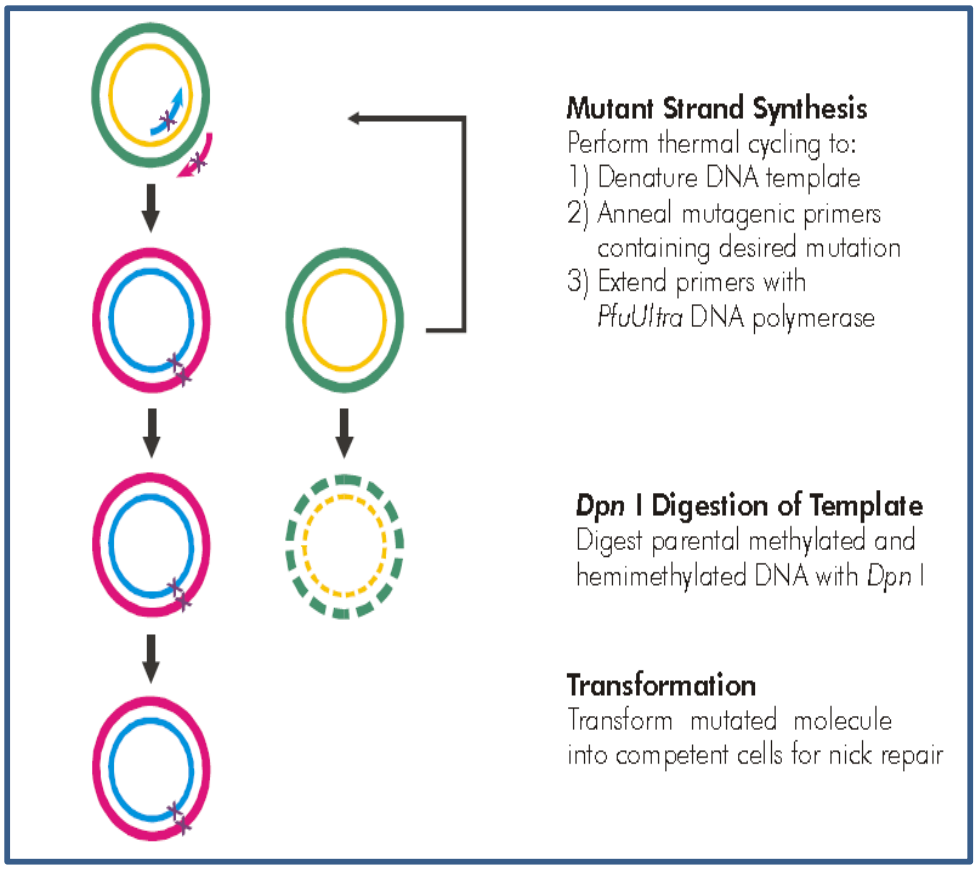

Figure 2.2: Protocol for the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit. This system is similar to the GeneArt® Site-Directed Mutagenesis PLUS Kit mentioned in Figure 2.1. However, in this method, the methylase is not added with the initial PCR reaction, however methylation occurs when the PCR product is mixed with the methylase *Dpn* I. The *Dpn* I digested product is transformed into XL10-Gold® ultracompetent cells which have been exposed to β- mercaptoethanol (β-ME) for increased transformation efficiency. Once the bacteria have been grown in broth, the DNA is extracted (Stratagene QuikChange II XL Site-Directed Mutagenesis Kit Manual).

2.4.3 PCR optimisation

The annealing temperature used during the site-directed mutagenesis PCR reaction was usually 2 ˚C lower than the melting temperature (T_m) of the primers. In cases where the PCR reaction did not yield a product on the first attempt, a PCR optimisation reaction to determine the correct annealing temperatures for the primers was performed. This was performed by using one mutagenic primer and another independent primer (Integrated DNA Technologies, Iowa, USA) with a similar T_m, running them under standard PCR conditions (Saiki et al., 1988). Five PCR reactions were set up with identical proportions of reagents, only differing in the annealing temperature of the reaction. All 5 samples were run on a 1% agarose gel and the annealing temperature of the sample with the most visible band was used for mutagenesis.

2.4.4 Confirmation of successful mutagenesis

To confirm that the mutagenesis process did not result in the addition or deletion of bases, restriction digestions were performed. Mutagenesis products were incubated with either *Nde* I or *Eco* RI restriction enzymes (ThermoScientific, Massachusetts, USA) for 1 hour and the digestion product run on 1% agarose gel at 100 V. The bands produced were compared to that of the wild-type (WT) viral DNA, which was also incubated with the respective restriction enzyme. Confirmation that the desired amino acids in the DNA were mutagenised successfully was performed by sequencing the DNA at the Central Analytical Facilities (University of Stellenbosch, Stellenbosch, South Africa) using a single primer. All mutants generated were subsequently sent for full length sequencing with 12 primers (University of Cape Town, South Africa from Professor Carolyn Williamson) to ensure no PCR errors had been introduced (Figure 2.3).

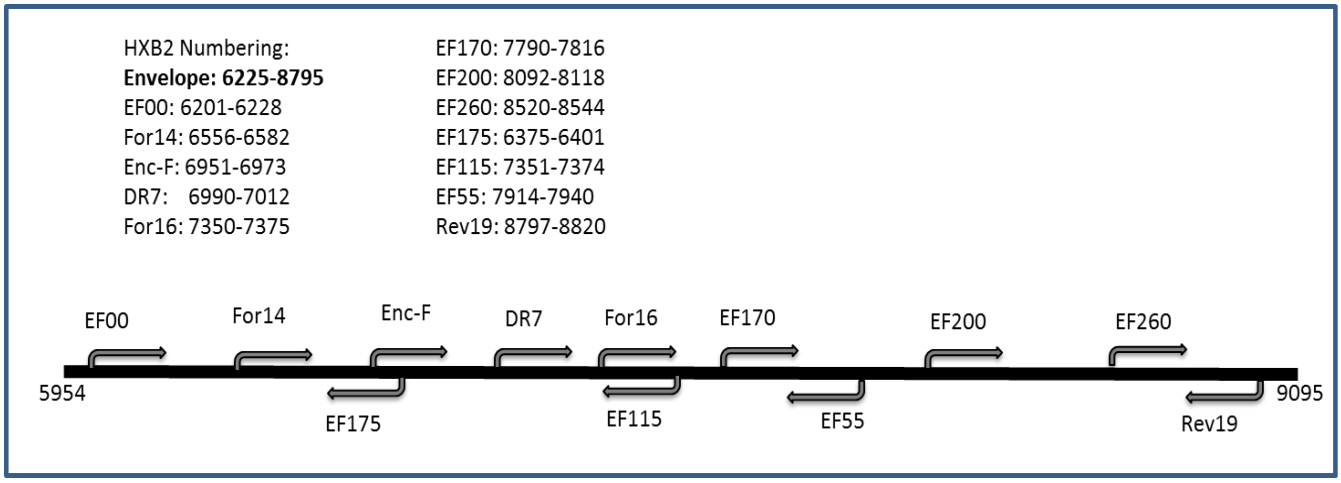

Figure 2.3: Full length sequencing primers. To ensure there were no PCR errors induced in the site-directed mutagenesis process, all mutants were sent for full length sequencing of the *env* gene. 12 primers were selected to span the length of the gene.

2.5 TZM-bl and 293T Cell lines

TZM-bl cells (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. John C. Kappes and Xiaoyun Wu) were used in neutralisation assays. They are susceptible to HIV-1 infection because they are $CXCR4^+$, $CD4^+$, $CCR5^+$ HeLa-derived cells (Montefiori, 2005). They contain a reporter gene for luciferase which is under the control an HIV-1 LTR promoter as well as a β-galactosidase reporter gene under the same (Ozaki et al., 2012, Montefiori, 2005). Human embryonic kidney (HEK) 293T cells (ARRRP, Division of

AIDS, NIAID, NIH, Bethesda, USA from Dr. Andrew Rice) are semi-adherent cells which are highly transfectable and therefore used for pseudovirus production (Ozaki et al., 2012).

2.5.1 Thawing TZM-bl and 293T cell lines

Cells stored in liquid nitrogen were thawed at room temperature. Cells were added to Dulbecco's Modified Eagle Medium (DMEM) (Sigma Chemical Company, Schnelldorf, Germany) constituted with 5% FBS and 1 ug/ml Penicillin-Streptomycin, 1ug/ml Gentamicin and 1% non-essential amino acids (Sigma Chemical Company, Schnelldorf, Germany) and centrifuged at 4000 rpm for 5 minutes. The supernatant was removed to ensure all the DMSO (Sigma Chemical Company, Schnelldorf, Germany) from the freezing solution was removed and the cell pellet was resuspended with 5% DMEM and added to culture plates.

2.5.2 Cell sub-culturing and spitting

Cells were cultured in 5% DMEM and split ever 2-3 days or when they were approximately 80-90% confluent. Adherent TZM-bl cells were removed from culture plates using 0.25% Trypsin-EDTA solution (Invitrogen, Darmstadt, Germany). HEK 293T cells were removed from the culture plates with DMEM. Cells were resuspended in 5% DMEM and centrifuged at 1200 rpm for 5 minutes. The supernatant containing DMEM (and 0.25% Trypsin-EDTA in the case of TZM-bl cells) was removed. Cells were resuspened with 3 ml media. Cells were counted under a light microscope using a haemocytometer following staining with the viability dye Trypan Blue (Sigma Chemical Company, Schnelldorf, Germany). Trypan Blue is taken up by dead cells which have permeable cell membranes but is excluded from live cells because these cells have intact cell membranes and therefore the number of live cells can be differentiated from dead cells (Horan and Kappler, 1977). Cells were returned to culture plates and incubated at 37 ˚C in a 5% CO_2 incubator until required.

2.5.3 Freezing cells

The cell lines were periodically frozen using a freezing solution comprising of 10% DMSO and 90% FBS. Cells were pelleted and resuspended in freezing solution. 1 ml aliquots were added cryovials and frozen overnight at -80 ˚C in styrofoam racks. The cells were then transferred to liquid nitrogen for long term storage. The overnight storage at -80˚C before addition to liquid nitrogen is required as the freezing process is meant to be slow to avoid cell damage.

2.6 Pseudovirus production

Pseudovirus was prepared by co-transfecting molecular clones of gp160 with SG3Δ*env* backbone (AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH Bethesda, USA from Drs. John C. Kappes and Xiaoyun Wu) using XtremeGENE 9 DNA transfection reagent (Roche, Basel, Switzerland). SG3Δ*env* is viral DNA from a Subtype B virus that has been engineered to have functional viral genes but contains a defective *env* gene with a 4 base pair insertion, inducing a frameshift mutation. Therefore, the *env* of interest (for instance, QH343.A10) is combined with SG3Δ*env* to make a pseudovirus capable of infecting a cell. The transfection mixture is then added to 293T cells. This was done according to the standard pseudovirus production methods (Montefiori, 2005). These viruses are known as pseudoviruses because they do not produce infectious progeny virions. This is because the pseudovirus lacks a complete genome (Ozaki et al., 2012, Montefiori, 2005).

Pseudovirus-containing supernatant was harvested 48 hours post-transfection and filtered through a 0.45 μm filer. 10% Fetal Bovine Serum (FBS) was added for preservation. Single-use aliquots were stored at -80˚C until further use. After each pseudovirus production, the virus was titrated in TZM-bl cells to determine the dilution of the virus whereby the relative light units (RLUs) were approximately 50 000.

2.7 Neutralisation Assay

TZM-bl-based neutralisation assays were performed as previously described (Montefiori, 2005). A neutralisation assay is used to determine how well a serum sample or mAbs is able to prevent virions from entering a host cell (Figure 2.4).

Diluted serum samples or mAbs were pre-incubated with pseudovirus in flat bottom 96 well plates for 1 hour at 37 ˚C and then TZM-bl cells were added with 7.5ug/ml Diethylaminoethyl –Dextran (DEAE-Dextran) (Sigma Chemical Company, Schnelldorf, Germany). The plasma-virus-cell mixture was incubated at 37˚C with 5% CO_2 for 48 hours. Cells were then lysed following the addition of the Bright-Glo™ luciferase substrate (Promega, Madison, USA) and the lysate transferred to black 96 well plates. The TZM-bl cells used in this assay have a luciferase gene controlled by an HIV-1 LTR promoter and therefore HIV-1 infection triggers luciferase expression. The luminescence produced by virus-infected cells was measured using a VERITAS MicroPlate Luminometer (Promega, Madison, USA). Luminescence is directly proportional to the number of virions that infect the Tzm-bl cells (Ozaki, 2012).

Percentage neutralisation was calculated as follows:

((Virus control – Sample)/(Virus control– Cell control)) X 100%

During initial screening, the respective viruses were tested against HIV-negative sera as a negative control. No neutralisation was expected to occur in this context.

The percentage neutralisation of a virus by serum was calculated by screening at a single serum dilution using a 1:100 dilution in triplicate. Serum ID_{50} values were generated by titrations of sera starting at a dilution of 1:50, 1:100, 1:150 or 1:300 depending on percentage neutralisation obtained. Titrations were run in duplicate. The ID_{50} is the dilution at which 50% of the virus is prevented from entering target cells. This is opposed to IC_{50} which is the concentration at which 50% of the virus is prevented from entering target cells. ID_{50} is used for serum where the antibody concentrations are unknown and therefore a diluted volume is used. IC_{50} is used for calculating mAb potency as the concentration is known. Serum ID_{50} values were calculated using curve fit functions in Graphpad Prisms 5 (Graphpad, La Jolla, USA).

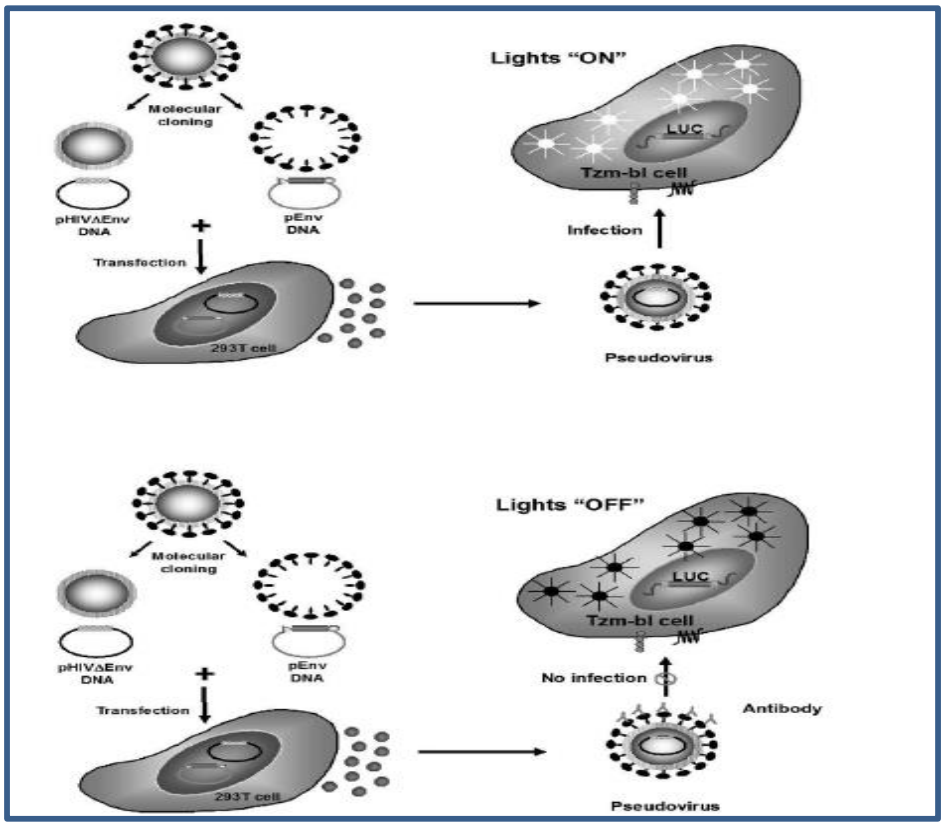

Figure 2.4: Diagrammatic representation of the TZM-bl-based neutralisation assays. Pseudoviruses are able to infect TZM-bl cells stimulating the expression of the luciferase reporter gene which emits luminescence. If the virus is neutralised by antibody before it can infect the TZM-bl cells, no reporter gene expression will occur and there will be no luminescence. (Ozaki et al., 2012).

2.8 Overview of sera identification for epitope mapping

To find broad and potent sera to map out a novel target for BnAbs on the HIV-1 envelope, multiple steps were taken to exclude sera containing antibodies which bound to previously defined targets. The overview of the system used to achieve this is shown below (Figure 2.5).

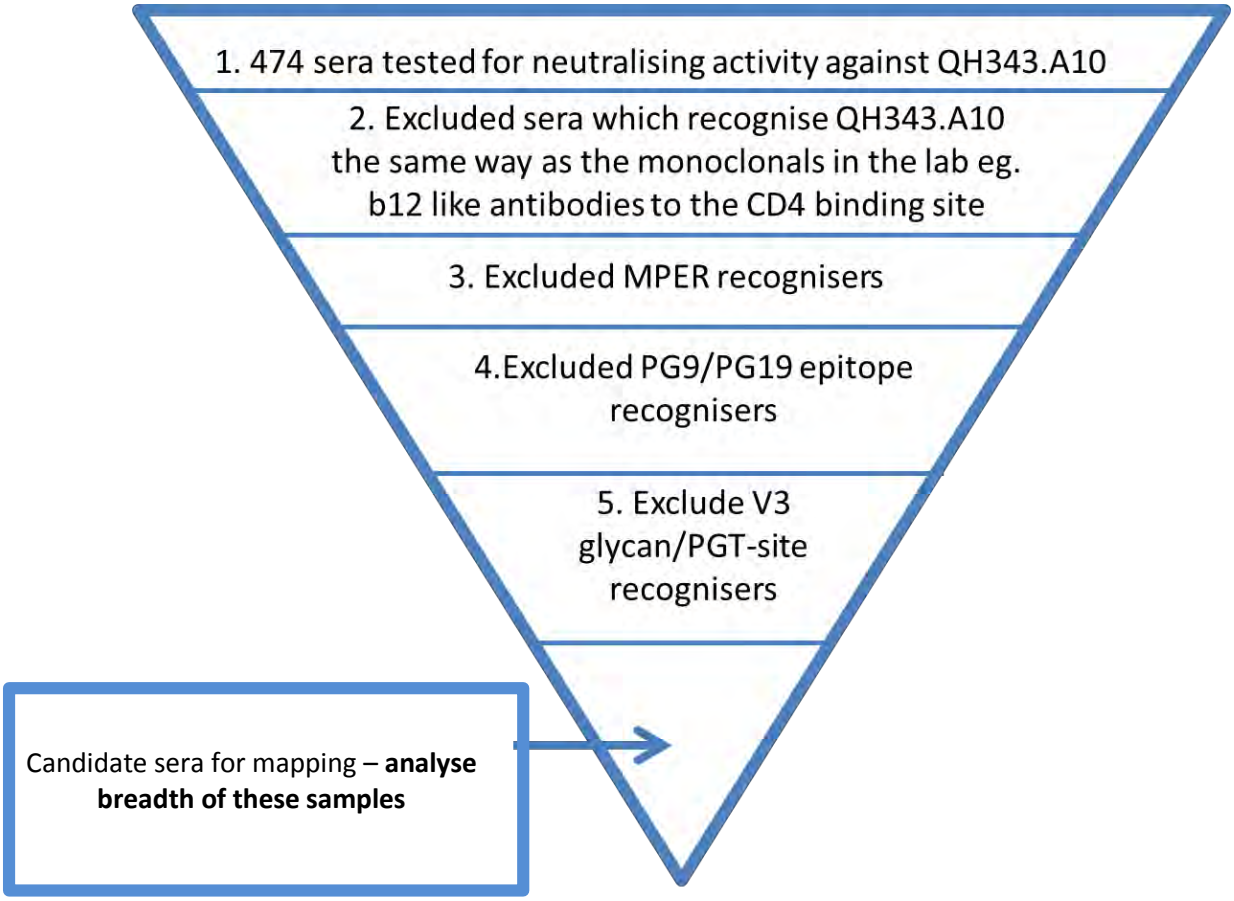

Figure 2.5: Overview of the system used to identify candidate sera for epitope mapping. We developed a step-wise process to obtain candidate sera for the recognition of a novel epitope. Initially, a screening of all the samples was performed against QH343.A10 and this was followed by testing the sera for neutralising activity against MPER. PG9/PG16 resistant mutants of the virus were made and tested against the remaining sera. Once this step was complete, the narrowed down list was tested against the other common target, the V3/glycan site. Breadth and potency were subsequently analysed on the samples remaining and the broad samples were the candidates used for mapping of a novel epitope.

The QH343.A10 virus was tested against 474 serum samples, from chronically infected, ART-naïve participants in Cape Town, to determine their ability to neutralise the virus. This approach was intended to select against sera that recognise QH343.A10 similarly to b12, 2G12, 4E10 and 2F5. Antibodies that bind to the already known epitopes in the same way as these mAbs which do not neutralise the virus were, thus, excluded. QH343.A10-recognising sera that targeted the PG9/16 epitope were excluded by mapping the sera using QH343.A10 mutants which lacked sensitivity to V2/glycan -targetting sera. This further narrowed down the list of candidate sera. The next step was to exclude sera that recognise the other defined epitopes – sera which were not screened out in the initial screening. MPER-recognising sera were excluded by testing the sera on the C1C virus. Exclusion of the V3/glycan site was done by

testing against N332A mutants. From the narrowed down list of sera, the remaining samples were tested for breadth using a 24-virus panel. The broad samples are the samples to focus on in finding a novel epitope for protective antibodies.

2.8.1 Testing QH343.A10 against PG6 and PG16 mAbs

QH343.A10 was tested against the PG9 and PG16 antibodies (Polymun Scientific, Wein, Austria) which target the V2-loop of the HIV-1 envelope. Using the standard TZM-bl based neutralisation assay, a titration was conducted as described in *Section 2.7* of this chapter, with a starting concentration of 20 ug/ml for each mAb.

2.8.2 Detection of anti-MPER Antibodies

A chimeric HIV-2 virus (7312A) displaying an HIV-1 MPER sequence in place of its own (Figure 2.6) was used to detect antibodies to the MPER region. This chimeric virus, known as C1C, displays an exposed subtype C consensus sequence and therefore has frequently been used to detect anti-MPER antibodies in sera (Gray et al., 2011, Binley et al., 2008, Gray et al., 2007). Sera that neutralise this construct at ID_{50}>1000 are considered to have a dominant antibody that targets MPER (Gray et al., 2011). Sera with an ID_{50}<300 have been shown to have no anti-MPER activity (Gray et al., 2011). As sera falling between an ID_{50} of 300-1000 have not yet been well categorised for anti-MPER activity, the threshold used for MPER-recognising sera in this study was an ID_{50}>300 to ensure all MPER-recognising sera were identified and excluded. A comparison was made between the neutralisation of this C1C construct by the sera to the neutralisation of the original 7312A virus to rule out cross-reactivity to other parts of the HIV-2 envelope. The 7312A parent HIV-2 construct and C1C virus (Gray et al., 2011) were both provided by Dr. George Shaw (University of Pennsylvania, Philadelphia, USA).

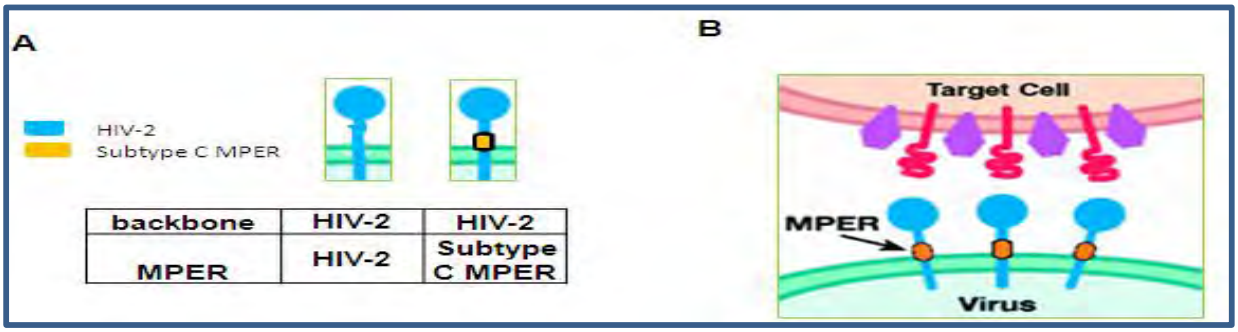

Figure 2.6: Anti-MPER antibody analysis using C1C mutant virus. A. Diagrammatic representation of the C1C virus as compared to the HIV-2 7312A WT virus. **B**. Diagrammatic representation showing C1C envelope and its target cell (not to scale).

The C1C virus was made by transfecting the C1C DNA into HEK 293T cells. No SG3Δ*env* was needed in the virus transfection as the HIV-2 component of the construct provided the backbone needed to form a viable pseudovirus.

2.8.3 Detection of anti- V2/glycan -site Antibodies

Anti- V2/glycan -site antibodies were detected using QH343.A10 viral mutants (N160A, K169E, F159A, R166A, N156A, Q171A) critical for PG9/16 recognition (Gray *et al.*, 2011; Moore *et al.*, 2011; Tomaras *et al.*, 2011; Walker *et al.*, 2009). The reason for choosing six mutants at different amino acids of the V2/glycan epitope was to ensure full coverage of the epitope when testing against the sera in our cohort as not all antibodies bind to the epitope in the same way. V2/glycan -site specific neutralisation activity was identified by a ≥2 fold drop in ID_{50} for at least one of the 6 V2/glycan -site mutants compared to the WT virus.

2.8.4 Detection of V3 glycan Antibodies

Sera containing antibodies against V3 glycan V3/glycan-site were screened for by the use of the QH343.A10 N332A mutant we constructed by site directed mutagenesis described in Section 2.4.2. The glycan at position 332 has been shown to be important for antibody binding to this target (Pejchal et al., 2011, Walker et al., 2011). V3/glycan site neutralisation activity was characterised as a ≥2 fold drop in ID_{50} compared to the WT virus.

2.8.5 Determination of Neutralisation Breadth and Potency

A 24-virus panel was used to rank the candidate sera for neutralisation breadth. The virus panel was chosen to reflect a diversity of viruses in circulation. All the viruses chosen were at least tier 2 ranked viruses; with tier 3 ranked viruses being over represented. Several easy to neutralise viruses were excluded from the panel as it was intended to represent relatively difficult-to-neutralise viruses to obtain more precise breadth measurements. The sera were ranked for neutralisation breadth and potency based on their ability to neutralise the 24 viruses in the panel. The tier 3 viruses (very hard-to-neutralise): PVO.4 (B), 278-50 (CRF02_AG), 253-11 (CRF02_AG), 251-18 (CRF02_AG) and 33-7 (CRF02_AG). Tier 2/3 viruses (hard-to-neutralise): Q461.e2 (A), Du422.1 (C), 001428-2.42 (C), and 928-28 (CRF02_AG). Tier 2 viruses (neutralised moderately): Q168.a2 (A), TRO11 (B), RHPA4259.7 (B), REJO 4541.67

(B), SC422661.8 (B), ZM249M.PL1, CAP45.2.00.G3 (C), Du151.2 (C), 26191-2.48 (C), 16936-2.21 (C), 252-7 (G), 269-12 (CRF02_AG) and 255-34 (CRF02_AG); and viruses not ranked by tier but analysed because of their moderate resistance to sera: QG984.21M.ENV.A3 (A) and QH343.A10 (A) (Sources of viruses: Dr Julie Overbaugh (Fred Hutchinson Cancer Research Centre, Seattle, USA) Professor Lynn Morris (National Institute for Communicable Diseases, Johannesburg, South Africa), Drs D. Ellenberger, B.Li, M. Callahan, and S. Butera (Centers for Disease Control and Prevention, Altanta, USA). The subtypes of the viruses in the panel are shown in brackets.

Sera were also screened against murine leukemia virus (MLV). This is a pseudovirus which contains the envelope of MLV and the SG3Δ*env* backbone. This makes the virus able to infect TZM-bl cells but should not be neutralised by sera from HIV-1 infected individuals. MLV is therefore used as a negative control. If a serum sample is able to neutralise MLV, it means that the individual the serum was taken from was most likely on ART.

Serum samples were screened at a 1:100 dilution, in triplicate, against all 25 viruses. Sera potency was based on the geometric mean of the predicted ID_{50} values of the sera against all the 24 viruses. The threshold used to define potent sera was a geometric mean ID_{50}>250. Breadth was defined as the number of viruses neutralised by a particular serum sample. Broad sera were categorised as those which neutralised 18 or more viruses with a predicted ID_{50}>100.

The predicted ID_{50} value was calculated from the % neutralisation value at a 1:100 serum dilution that was based on a linear regression model (Jacob RA, unpublished data). The equation to obtain the predicted ID_{50} is as follows:

Predicted ID_{50}=exp (1.303 + 0.060962 x % neutralisation)

This prediction model compared known ID_{50} values of serum samples (n=240) to their respective percentage neutralisation at 1:100 dilution. The model was validated using a test set (n=234) which did not overlap the training set (Jacob RA, unpublished data).

Chapter 3: Results

3.1 Criteria for Selection of QH343.A10 for Candidate Sera Identification

The aim of this project was to design a novel system to identify sera likely to target novel broadly neutralising epitopes. This was achieved by taking advantage of the existing characteristics of the QH343.21M.ENV.A10 virus (Blish et al., 2009).

QH343.A10 is a subtype A virus that was isolated from a patient in Kenya in 2005 (Blish et al., 2009). QH343.A10 was chosen for the development of this mapping system because of its key characteristics:

1. It was found to be resistant to all mAbs tested against it (Jacob et al., 2012, Blish et al., 2009). The virus was previously tested in the laboratory against the BnAbs available in the laboratory that target MPER (4E10, 2F5), V3/glycan region (2G12) and the CD4-bs (b12, VRC01) and was found to be resistant to all these mAbs (Table 3.1). QH343.A10 was also resistant to neutralisation by soluble CD4 (sCD4) (Jacob, unpublished data). This makes it a good virus to find a new BnAb target as it presumably cannot be neutralised by antibodies in sera which recognise the virus in the same way as these mAbs.
2. It has been found to be a relatively resistant virus to plasma (Jacob et al., 2012). The fact that this virus is relatively hard to neutralise virus makes it a good candidate to find a novel epitope because hard-to-neutralise viruses tend to be neutralised primarily by broadly neutralising sera. We also wanted a virus that left us neither with too many sera to analyse that had not been well enriched for good candidate sera, nor one that was so hard-to-neutralise that we had too few sera to analyse, even out of our 474 sera that we tested.

The first experiment to initiate this project was to determine whether V2-loop mAbs, which we had newly acquired in the laboratory, PG9 and PG16 could neutralise QH343.A10. We did this by determining the ID_{50} values of PG9 and PG16 against the virus (Table 3.1). We found that these two antibodies, which were not on the original list of IC_{50} values used to choose QH343.A10, were able to neutralise the virus at relatively low IC_{50} values.

Table 3.1: IC_{50} values for mAbs against QH343.A10 targeting various regions of the viral envelope.

mAbs	QH343.A10 IC_{50} (µg/ml)	Targeted Region
sCD4*	**>20**	CD4 binding site
b12*	**>20**	CD4 binding site
VRCO1*	**>20**	CD4 binding site
2G12*	**>20**	V3/glycan site
2F5*	**>20**	MPER
4E10*	**>20**	MPER
PG9	**8.7**	V2/glycan site
PG16	**11.3**	V2/glycan site

*IC_{50} values (in µg/ml) for all the resistant mAbs were reported previously (Jacob et al., 2012, Jacob unpublished data).

3.2 Identification of QH343.A10-recognising Sera

The first step in the novel process we developed was to test our cohort sera for neutralisation against the QH343.A10 virus. We obtained 474 serum samples from our cohort of chronically HIV-infected ART-naïve adults from two HIV wellness clinics in Cape Town. We tested these 474 samples for the ability to neutralise QH343.A10 (Table 1A; appendix). To map sera effectively and be able to detect drops in ID_{50} of at least 2-fold, we wanted sera ID_{50} >150. A 60% neutralisation at 1:100 corresponded approximately with this threshold. ID_{50} values were subsequently measured for those sera neutralising over 60% at 1:100 (Table 3.2).

Table 3.2: Percent neutralisation and ID_{50} values to determine sera which recognise QH343.A10 with sufficient potency for mapping.

BNAB ID	% neut	ID50	BNAB ID	% neut	ID50	BNAB ID	% neut	ID50	BNAB ID	% neut	ID50
BNAB0026	80.0	ND	BNAB0231	94.5	615	BNAB0340	97.6	1386	BNAB0416	83.7	238
BNAB0037	63.0	170	BNAB0238	90.4	106	BNAB0342	79.8	1305	BNAB0420	64.2	137
BNAB0038	78.1	471	BNAB0245	93.4	181	BNAB0344	69.1	419	BNAB0422	91.7	933
BNAB0039	82.5	530	BNAB0252	68.5	316	BNAB0347	80.8	569	BNAB0424	95.0	669
BNAB0071	64.0	132	BNAB0254	70.1	201	BNAB0351	75.6	407	BNAB0426	88.5	606
BNAB0084	82.3	276	BNAB0255	83.4	1440	BNAB0352	86.6	3112	BNAB0431	72.6	243
BNAB0105	75.6	417	BNAB0266	97.6	384	BNAB0355	93.1	372	BNAB0440	66.0	968
BNAB0123	76.0	171	BNAB0268	68.0	507	BNAB0358	67.0	R	BNAB0452	61.6	812
BNAB0136	85.0	883	BNAB0270	91.9	652	BNAB0361	89.8	1376	BNAB0455	66.1	209
BNAB0146	91.9	341	BNAB0279	97.7	365	BNAB0365	94.5	496	BNAB0456	76.0	389
BNAB0149	72.6	138	BNAB0282	63.5	R	BNAB0371	78.3	137	BNAB0463	79.1	654
BNAB0167	68.3	83	BNAB0284	89.7	228	BNAB0373	88.0	696	BNAB0469	82.1	1023
BNAB0171	67.3	1238	BNAB0294	78.8	255	BNAB0376	79.5	434	BNAB0475	70.6	311
BNAB0176	98.2	1066	BNAB0300	78.5	R	BNAB0381	98.9	996	BNAB0478	98.5	872
BNAB0177	98.8	1936	BNAB0308	61.0	186	BNAB0392	84.1	451	BNAB0481	76.2	558
BNAB0179	93.8	75	BNAB0314	70.2	940	BNAB0394	91.2	227	BNAB0488	82.0	196
BNAB0182	96.4	554	BNAB0316	91.7	263	BNAB0400	96.2	460	BNAB0491	75.6	R
BNAB0195	67.3	599	BNAB0328	66.5	R	BNAB0401	89.3	618			
BNAB0197	93.8	606	BNAB0334	95.4	250	BNAB0402	85.9	246			
BNAB0199	79.4	457	BNAB0335	68.5	762	BNAB0410	86.0	437			
BNAB0220	98.9	352	BNAB0337	78.0	R	BNAB0413	77.0	92			

% neut
>90%
70-89%
60-69%

***ND**: not determined; sera was depleted before ID_{50} values could be calculated R: resistant

BNAB IDs highlighted in red indicate samples whose ID_{50} values were less than 150 and therefore were excluded from our list of sera.

If QH343.A10 was resistant (R) to a serum sample, a value of 10 was assigned as the ID_{50}.

Shown in Table 3.2 are the sera which neutralised QH343.A10 by 60% or more in the initial screening and their subsequent ID_{50} values which were determined by titrations. Only 80 samples in our cohort (17%) were able to neutralise the virus by 60% or above. Of the 80 samples that were able to neutralise the virus by 60% and above, 67 samples had an ID_{50}>150 and therefore we continued with these 67 samples. The use of a 60% threshold picked up most of the sera with an ID_{50}>150. 60% neutralisation was a reasonable threshold to use as some sera were excluded which recognised the virus by >60% but did not have an ID_{50}>150.

There are examples of % neutralisation >50% corresponding to an ID_{50} value shown as R (such as BNAB0282). % neutralisation of 50% or above would imply that the virus would be sensitive to the particular serum sample however, % neutralisation determinations are not completely accurate measures. That is the reason why we subsequently revisited these samples and measured the ID_{50} value of each to be certain that they do in fact recognise the QH343.A10 virus. We chose a cut-off ID_{50} value of 150 to consider a serum sample a map-able "QH343.A10 recognisers". Even with our 60% threshold, we had samples with ID_{50} lower than 150 which we excluded from the analysis (eg. BNAB0071). This shows that we thoroughly screened for not only neutralisers of the virus but good neutralisers of the virus which could be mapped more accurately. It was not entirely critical in this process to capture all QH343.A10 recognisers. If we missed a few marginal neutralisers we would still be able to accomplish our aim. Our method was not aimed at catching all of the neutralisers but rather at catching the good neutralisers who we could map easily and effectively.

The relative resistance of QH343.A10 to sera in our cohort made it an excellent candidate for our system as it meant that by screening out non-QH343.A10 recognisers we removed samples which recognise the virus in the same manner as the mAbs shown in Table 3.1, to which the virus was resistant. Sera which do not neutralise the virus may also be recognising it through sites that are not targets for BnAbs. The first step in the selection process had been completed as sera which did not recognise the parent virus were excluded. We then continued with the subsequent steps, which were to exclude as many sera as possible that neutralise QH343.A10 by recognising already known targets of BnAbs.

3.3 Detection of anti-MPER Antibodies in Sera

We then proceeded to remove sera containing anti-MPER antibodies from the 67 QH343.A10 recognisers. Although antibodies in our cohort which recognise QH343.A10 in a similar way to the anti-MPER antibodies 2F5 and 4E10 ought to already be excluded, we would expect that there were still antibodies in the remaining sera that recognised the virus through MPER, just not sera that recognise MPER similarly to 2F5 and 4E10. Therefore, exclusion of all sera containing detectable anti-MPER antibodies was conducted.

The detection of anti-MPER antibodies in sera was performed using an HIV-2/HIV-1 MPER chimera named C1C (Gray et al., 2007). C1C is a virus which consists of an HIV-2 envelope which contains an HIV-1 consensus subtype C MPER in place of the original HIV-2 MPER (Gray et al., 2007). This construct is optimised for MPER detection as the HIV-1 MPER is well exposed in this construct (Gray et al., 2007, Li et al., 2006) and the consensus C sequence for the MPER is expected to maximize the detection of anti-MPER antibodies in individuals infected with subtype C viruses.

Screening of the 67QH343.A10-recognising sera against C1C was performed and % neutralisation determined at 1:100 (Table 3.3). This initial screening was performed to see which sera were able to neutralise the virus and therefore had potential anti-MPER antibodies. The cut-off used was 60% neutralisation. ID_{50} values were determined by titrations on sera with % neutralisation>60. As a negative control, sera were also screened against the 7312A HIV-2 virus which provided the backbone for C1C. % neutralisation of 7312A was subtracted from the C1C neutralisation value obtained. The maximum 7312A % neutralisation was 38% among the 67 QH343.A10 neutralising sera.

Table 3.3: Screening of the 67 QH343.A10 recognising sera against C1C virus to find candidate MPER-neutralising sera.

BNAB ID	% neut	BNAB ID	% neut	BNAB ID	% neut
BNAB0463	100.2	BNAB0478	49.4	BNAB0400	10.4
BNAB0177	99.0	BNAB0455	49.2	BNAB0334	9.3
BNAB0254	97.7	BNAB0270	49.0	BNAB0373	7.1
BNAB0335	97.6	BNAB0231	48.0	BNAB0340	6.5
BNAB0376	97.4	BNAB0314	45.8	BNAB0355	2.5
BNAB0456	97.3	BNAB0426	39.1	BNAB0171	2.0
BNAB0026	97.0	BNAB0105	39.0	BNAB0401	≤0
BNAB0392	96.0	BNAB0123	38.0	BNAB0371	≤0
BNAB0197	95.0	BNAB0182	36.0	BNAB0424	≤0
BNAB0481	92.9	BNAB0352	31.3	BNAB0410	≤0
BNAB0245	88.8	BNAB0342	29.6	BNAB0488	≤0
BNAB0037	84.0	BNAB0347	25.3	BNAB0394	≤0
BNAB0381	83.0	BNAB0440	23.5	BNAB0361	≤0
BNAB0452	80.6	BNAB0252	21.8	BNAB0422	≤0
BNAB0351	79.4	BNAB0469	20.1	BNAB0279	≤0
BNAB0146	78.0	BNAB0039	20.0	BNAB0038	≤0
BNAB0294	76.0	BNAB0344	19.5	BNAB0308	≤0
BNAB0316	70.4	BNAB0416	19.0	BNAB0266	≤0
BNAB0136	70.0	BNAB0220	19.0	BNAB0255	≤0
BNAB0284	66.3	BNAB0195	16.0		
BNAB0176	65.6	BNAB0431	15.6		
BNAB0475	64.3	BNAB0402	12.9		
BNAB0199	64.0	BNAB0268	11.8		
BNAB0084	63.0	BNAB0365	10.9		

Sera with 60% and higher % neutralisation are highlighted in light pink and ID_{50} values were subsequently determined (Table 3.4).

*% neut: determined at 1:100 dilution and is the % neutralisation after subtraction of the HIV-2 7312A virus % neutralisation. 7312A virus forms the backbone of C1C.

The samples which were able to neutralise C1C by 60% and above were titrated against the C1C virus to determine their ID_{50} values (Table 3.4). The cut-off was 60% as an ID_{50}>300 was the threshold used to correspond with sera containing anti-MPER activity and we expect that sera that neutralise at 1:100 dilution below 60% were unlikely to neutralize at ID50>300. ID50<300 against C1C has been shown to be associated with the lack of anti-MPER antibodies in sera (Gray et al., 2011, Gray et al., 2009). Samples with % neutralisation ≤0 represent samples which were unable

to neutralise the C1C virus at all. These samples neutralised the HIV-2 7312A virus more than C1C and therefore gave a negative value. We are therefore certain these samples do not neutralise HIV-1 viruses through the MPER region.

Table 3.4: ID_{50} values of QH343.A10-recognising sera that neutralised C1C by 60% and above.

BNAB ID	ID50	BNAB ID	ID50
BNAB0026	1523	BNAB0294	307
BNAB0037	774	BNAB0316	879
BNAB0084	134	BNAB0335	1145
BNAB0136	188	BNAB0351	527
BNAB0146	139	BNAB0376	54
BNAB0176	R	BNAB0381	104
BNAB0177	12385	BNAB0392	1612
BNAB0197	1974	BNAB0452	46
BNAB0199	75	BNAB0456	2617
BNAB0245	868	BNAB0463	28966
BNAB0254	4387	BNAB0475	349
BNAB0284	303	BNAB0481	R

ID50 value
>1000
300-1000
<300

R: resistant

Titrations consisting of 6 serial dilutions starting at 1:100 (in duplicate) were performed on samples that neutralised C1C by 60% and above. Altogether, 15 serum samples were removed from our list of candidate sera as they contain detectable anti-MPER antibodies that could be responsible for some or all of the neutralisation activity against QH343.A10.

3.4 Detection of Dominant V2/glycan -site Antibodies in Sera

3.4.1 QH343.A10 V2/glycan -site mutant design

We engineered mutants of QH343.A10 to identify sera that recognise QH343.A10 through the recognition of the V2/glycan site. This was achieved by generating 6 single-site mutants by site-directed mutagenesis of the viral DNA at key amino acids. It seemed appropriate to do this as thoroughly as possible because we had no evidence that QH343.A10 was resistant to any PG9/16-like antibody. We chose these 6 V2/glycan -site mutants on their capacity to inhibit

V2/glycan -site-specific CAP256 serum (Moore et al., 2011, Tomaras et al., 2011) and/or the PG9 or PG16 mAbs (Moore et al., 2011, Tomaras et al., 2011. Doores and Burton, 2010) (Figure 3.1). The mutants were: N156A, F159A, N160A, R166A, I169E and Q171A. Each mutant was made by single-site directed mutagenesis.

A

```
HXB2(155-173): I   K   N   C   S   F   N   I   S   T   S   I   R   G   K   V   Q   K   E   Y   A

               ATA AAA AAC TGC TCT TTC AAT ATC AGC ACA AGC ATA AGA GGT AAG GTG CAG AAA GAA TAT GCA
QH343.A10:      M   K   N   C   S   F   N   I   T   T   E   V   R   D   K   I   R   Q   V   Y   S
               ATG AAA AAC TGC TCT TTC AAT ATA ACC ACA GAA GTT AGG GAT AAG ATA CGG CAG GTA TAT TCC
N156A:          M   K   A   C   S   F   N   I   T   T   E   V   R   D   K   I   R   Q   V   Y   S
               ATG AAA GCC TGC TCT TTC AAT ATA ACC ACA GAA GTT AGG GAT AAG ATA CGG CAG GTA TAT TCC
F159A:          M   K   N   C   S   A   N   I   T   T   E   V   R   D   K   I   R   Q   V   Y   S
               ATG AAA AAC TGC TCT GCC AAT ATA ACC ACA GAA GTT AGG GAT AAG ATA CGG CAG GTA TAT TCC
N160A:          M   K   N   C   S   F   A   I   T   T   E   V   R   D   K   I   R   Q   V   Y   S
               ATG AAA AAC TGC TCT TTC GCT ATA ACC ACA GAA GTT AGG GAT AAG ATA CGG CAG GTA TAT TCC
R166A:          M   K   N   C   S   F   N   I   T   T   E   V   A   D   K   I   R   Q   V   Y   S
               ATG AAA AAC TGC TCT TTC AAT ATA ACC ACA GAA GTT GCG GAT AAG ATA CGG CAG GTA TAT TCC
I169E:          M   K   N   C   S   F   N   I   T   T   E   V   R   D   K   I   R   Q   V   Y   S
               ATG AAA AAC TGC TCT TTC AAT ATA ACC ACA GAA GTT AGG GAT AAG GAA CGG CAG GTA TAT TCC
Q171A:          M   K   N   C   S   F   N   I   T   T   E   V   R   D   K   I   R   A   V   Y   S
               ATG AAA AAC TGC TCT TTC AAT ATA ACC ACA GAA GTT AGG GAT AAG ATA CGG GCG GTA TAT TCC
```

B

	Fold difference in ID_{50} value from WT to mutant			
Mutant	**PG9**	**PG16**	**CAP256 PG9/16-like plasma**	**Reference**
N156A	**280**	**1500**	**ND**	Doores and Burton,2010
F159A	**11**	**266.1**	**15**	Moore et al., 2011
N160A	**>1000**	**>1000**	**7.9**	Moore et al., 2011, Tomaras et al., 2011
R166A*	**0.5**	**0.7**	**102.6**	Moore et al., 2011
K169E*	**>1000**	**>1000**	**78.5**	Moore et al., 2011, Tomaras et al., 2011
Q171A	**5.9**	**23.9**	**10.5**	Moore et al., 2011

*R166A was not chosen based on mAbs but rather on the high fold decrease with Cap256 plasma that contains V2/glycan -specific antibodies, to further ensure all antibodies against the PG9/16-site were excluded in our analysis.
*K169E is I169E for QH343.A10; ND= no data in literature

Figure 3.1: QH343.A10 V2/glycan **mutants design. A.** QH343.A10 PG9/PG16-site mutants were created by single-site directed mutagenesis, mutating one amino acid per mutant in the V2 loop region of the viral envelope. Amino acids were converted to either an alanine or a glutamic acid **B.** Mutants were chosen for their ability to inhibit either PG9/16 mAb neutralisation or inhibit binding of Cap256 plasma neutralisation which has previously been shown to have a dominant V2/glycan -site-specific antibody. These data show the fold decrease in neutralisation capacity of the mAbs and sera when tested against pseudoviruses with the mutations. Mutations were introduced into constructs of either a consensus C envelope (ConC) (Moore et al., 2011, Tomaras et al., 2011) or JRCSF (Doores and Burton, 2010).

3.4.2 Site-directed mutagenesis PCR optimisation

For two of the QH343.A10 mutant viruses (F159A and Q171A) a PCR optimisation experiment was necessary to determine the annealing temperature suitable for each primer in the PCR reaction (F159A shown in Figure 3.2). Usually, the annealing temperature for a primer is approximately 2 ˚C lower than its T_m. However, when the PCR reaction did not yield a product, the PCR optimisation reaction was conducted.

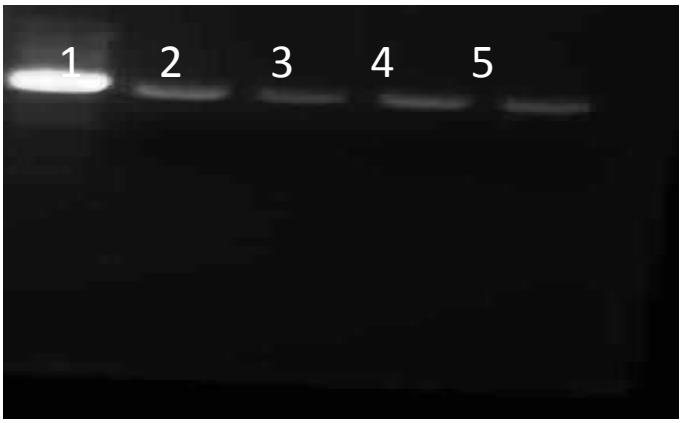

Figure 3.2: Gel electrophoresis of PCR optimisation for QH343.A10 F159A mutant primers. To determine the correct annealing temperature of the site-directed mutagenesis primer, a PCR reaction was performed using one of the mutagenic primers (T_m: 60 ˚C) and another independent primer Rev19 (T_m : 59.6 ˚C) . Five PCR reactions were set up with identical proportions of reagents, only differing in the annealing temperature of the reaction. Lane 1: 52 ˚C; Lane 2: 54 ˚C; Lane 3: 56 ˚C; Lane 4: 58 ˚C and Lane 5: 60 ˚C. All five samples were run on 1% agarose gel and the annealing temperature of the sample with the most visible band was used to set the mutagenesis. No molecular weight marker was used as the size of the PCR product was not expected to change with changing annealing temperature. The QH343.A10 F159A optimisation is shown as a representative for the two PCR optimisation reactions as both had identical optimal annealing temperatures of 52 ˚C. Non-specific bands which appear at the annealing temperature of 52 ˚C were most likely due to the two primers binding with each other or unbound primer from the PCR reaction.

3.4.3 Verification of QH343.A10 V2/glycan -site mutant DNA

After the site-directed mutagenesis PCR, the PCR products were transformed into bacteria and the DNA extracted. The mutant DNA samples were compared to the QH343.A10 WT DNA by restriction digestion. All strands were digested *with Dde I* restriction enzyme and the mutants strands compared to those of the WT DNA (Figure 3.3).

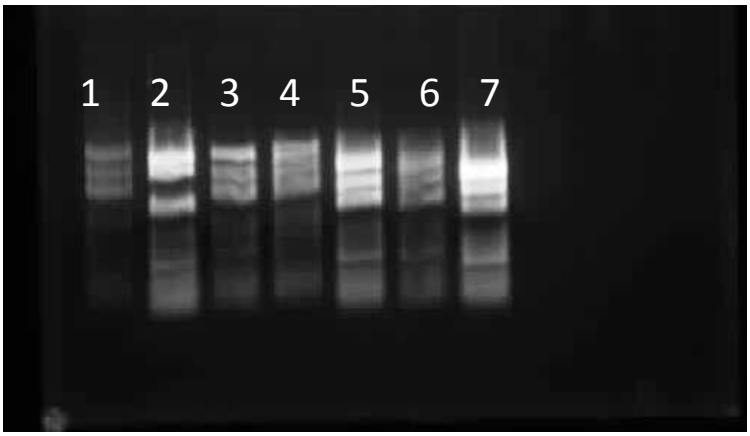

Figure 3.3: QH343.A10 V2/glycan **mutant restriction digestion.** After the DNA extraction of the amplified PCR product, both WT and mutant QH343.A10 DNA were digested by *Dde* I restriction endonuclease. Fragments of mutant DNA were compared with those from wild-type QH343.A10 DNA to ensure that no addition or subtraction of bases had occurred during the PCR product. Lane 1: QH343.A10 WT, Lane 2: N160A mutant, Lane 3: I169E mutant, Lane 4: F159A mutant, Lane 5: N156A mutant, Lane 6: R166A mutant, Lane 7: Q171A mutant. No DNA ladder was used as the comparison was between the WT DNA and mutant DNA solely.

Looking at the gel, it is clear that the bands are not completely identical to the QH343.A10 WT DNA. This could be because the DNA in different wells did not run homogenously or could be a result of differences in the length of the restriction digest bands. Lane 2 which had N160A DNA had a particularly different band from the WT DNA. To verify that no amino acids were added or deleted during PCR, all mutant DNA was sent for full length sequencing with 12 primers that spanned the *env* gene. All sequences came out identical to the parental DNA except in the position of the desired mutation (data not shown). Since the sequencing of the Env region showed no addition or deletion of bases for the N160A mutant, the differences in the band size between this mutant and the WT could be due to changes in the vector backbone. However, this would not affect our experiments as our only focus is on the Env region and it is this region that determines the neutralisation profile of the viruses. *In silico* analysis revealed that no restriction sites were added or removed from any of the sequences.

Using mutant QH343.A10 mutant DNA engineered by site-directed mutagenesis verified by both restriction digestion and full length sequencing, all 6 V2/glycan -site viral mutants were generated. Viruses were harvested and titrations run to determine the dilution of virus to use in subsequent neutralisation assays.

3.4.4 Exclusion of Anti-V2/glycan site Antibodies in Sera

After exclusion of anti-MPER recognising sera from the 66 QH343.A10 recognising sera, we were left with 51 sera. We screened these sera against the 6 QH343.A10 V2/glycan -site-specific mutants (Table 3.5). These mutants were engineered to exhibit a lack of sensitivity to anti-V2/glycan -site antibodies. Therefore, if a drop in neutralisation was observed in these sera as compared to the parent virus, we classified them as V2/glycan -site-recognising sera and excluded them from further analysis (Figure 3.5).

We screened sera for recognition of the V2/glycan -site using a 1:100 dilution of each serum against QH343.A10 WT and each of the 6 mutants. For screening purposes, we used a cut-off of an 8% or higher drop in % neutralisation from QH343.A10 WT compared to any one of the mutants. We performed titrations on samples with an 8% or higher drop in % neutralisation to determine whether they contained dominant V2/glycan -antibodies and calculated their ID_{50} values. We used such a stringent cut off of 8% to ensure that we performed titrations on all samples which could potentially recognise the V2/glycan region. 8% is very low cut-off in respect to % neutralisations and therefore the chances of missing a V2/glycan site recognisers were extremely low. The low specificity and high sensitivity of this cut-off in picking V2/glycan-recognising sera suggests that we have set the cut-off low enough to catch all the positive values. In addition, for samples with a percentage neutralisation of 90% and above for both WT and mutant viruses, we considered that we would be unable to detect ID_{50} difference by just comparing % neutralisation and for these samples, and therefore determined the ID_{50} values at a starting dilution of 1:400 to get more reliable data. If the difference in % neutralisation from QH343.A10 WT and each of the mutants was less than 8% and the % neutralisation of QH343.A10 was less than 90%, these sera were considered to be non- V2/glycan -site-recognisers without further analysis.

Table 3.5: Screening of the remaining 51 sera against V2/glycan -specific QH343.A10 mutants

BNAB ID	QH343 (%neut)	N160A (%neut)	F159A (%neut)	I169E (%neut)	Q171A (%neut)	N156A (%neut)	R166A (%neut)
BNAB0038	83.8	79.2	69.3	94.3	95.5	72.6	83.6
BNAB0039	93.5	84.7	92.7	92.5	90.2	99.5	100.0
BNAB0084	90.7	90.1	101.9	93.4	92.1	92.3	95.5
BNAB0105	82.7	85.6	19.4	87.0	79.7	35.4	27.1
BNAB0123	81.9	88.3	90.6	91.8	89.5	85.0	88.9
BNAB0136	86.7	74.0	99.8	85.4	84.6	93.1	96.8
BNAB0146	94.7	91.2	99.6	87.7	89.1	95.4	98.2
BNAB0171	79.1	61.5	93.5	43.5	59.2	92.6	95.9
BNAB0176	93.4	89.6	98.2	94.8	96.8	90.9	105.0
BNAB0182	47.0	15.2	80.5	74.1	75.0	ND	ND
BNAB0195	75.0	65.8	92.1	73.3	57.1	83.0	91.6
BNAB0199	78.5	70.3	91.2	75.1	84.6	92.5	95.0
BNAB0220	85.0	1.9	-11.5	ND	ND	ND	ND
BNAB0231	88.7	85.0	99.9	95.9	97.5	91.2	101.3
BNAB0252	83.0	74.8	81.6	75.0	84.7	78.1	85.9
BNAB0255	84.5	72.1	49.0	60.9	63.4	74.4	47.0
BNAB0266	87.0	90.4	88.3	85.6	85.5	88.8	81.4
BNAB0268	78.4	72.6	96.0	85.3	68.3	95.6	99.3
BNAB0270	85.7	81.4	97.5	67.1	63.9	89.3	95.9
BNAB0279	76.7	66.4	86.1	73.9	82.3	80.9	90.9
BNAB0308	73.0	77.5	96.7	80.6	73.0	72.4	40.4
BNAB0314	97.7	97.2	51.0	75.0	71.1	81.6	40.0
BNAB0334	64.4	62.8	81.1	83.2	87.3	90.4	89.2
BNAB0340	95.3	93.0	97.2	69.3	61.8	94.1	93.6
BNAB0342	69.1	43.8	98.9	ND	ND	ND	ND
BNAB0344	56.7	33.9	16.9	ND	ND	ND	ND
BNAB0347	71.7	50.9	39.6	ND	ND	ND	ND
BNAB0352	80.4	65.4	96.3	90.4	90.5	82.7	92.5
BNAB0355	88.0	87.1	50.5	ND	ND	ND	ND
BNAB0361	77.4	82.4	57.4	93.0	91.4	51.5	57.5
BNAB0365	90.1	80.3	99.2	83.5	86.8	90.8	99.2
BNAB0373	82.3	79.5	69.8	82.6	78.7	75.4	66.4
BNAB0376	84.1	-2.5	37.3	ND	ND	ND	ND
BNAB0381	90.6	84.2	98.5	52.8	54.4	98.1	97.3
BNAB0394	90.2	81.6	99.5	99.7	99.8	88.4	94.6
BNAB0400	68.4	54.6	96.4	64.3	60.6	96.9	100.2
BNAB0401	69.4	75.4	80.1	80.3	74.1	91.9	78.1
BNAB0402	68.2	59.7	94.2	90.6	93.3	77.1	87.7
BNAB0410	90.4	89.5	99.7	90.1	97.2	87.3	96.8
BNAB0416	75.3	69.9	90.8	91.9	95.9	89.9	97.5
BNAB0422	90.9	94.5	43.8	ND	ND	ND	ND
BNAB0424	70.6	59.6	93.5	71.3	63.9	79.7	98.4
BNAB0426	86.2	83.6	42.2	73.9	82.0	87.7	90.0
BNAB0431	67.6	40.4	91.1	ND	ND	69.8	70.3
BNAB0440	66.7	36.0	90.2	ND	ND	ND	ND
BNAB0452	81.7	84.0	59.1	72.8	71.6	69.8	70.3
BNAB0455	74.3	64.5	87.4	66.7	22.5	97.3	97.8
BNAB0469	86.2	75.8	91.1	51.9	52.0	96.1	99.6
BNAB0478	92.7	82.0	87.5	84.7	81.7	94.0	80.8
BNAB0481	84.9	64.8	78.6	66.7	61.1	ND	ND
BNAB0488	75.6	65.8	78.2	79.12	70.51	82.9	83.8

The remaining samples were screened against the WT virus and the 6 V2/glycan mutants at 1:100 to determine whether they were recognised by the QH343.A10 mutants.

Green shading: samples with % neutralisation higher than 90% and therefore ID50 values were subsequently measured for better resolving power.

Pink shading: samples with more than 8% drop in % neutralisations whose ID50 values were subsequently measured.

ID_{50} values were subsequently measured for all potential V2/glycan -site recognising sera (Table 3.6), i.e. those that had a percentage neutralisation drop of 8% or greater from QH343.A10 WT to at least one mutant: light pink or a QH343.A10 WT % neutralisation greater than 90%: green.

A sample was considered to be a V2/glycan -recognising serum and excluded from our list of candidate sera for epitope mapping if any of the 6 mutants had a 2-fold or greater drop in ID_{50} value compared to QH343.A10 WT. Samples with 90% and higher % neutralisation were run but not shown in the Table 3.6 as none of them had a reduction in % neutralisation and the only reason they were run was because their initial % neutralisation was so high. There was no evidence that they were PG9/16 recognisers from the % neut values and also in their ID_{50} values.

Table 3.6: ID_{50} values of sera to determine presence of anti- V2/glycan **antibodies.**

Sample ID	Qh343.A10 WT ID_{50}	N160A ID_{50}	Ratio	F159A ID_{50}	Ratio	I169E ID_{50}	Ratio	R166A ID_{50}	Ratio	Q171A ID_{50}	Ratio	N156A ID_{50}	Ratio
BNAB0038	471	161	2.9	63	7.5	10	47.1	76	6.2	605	0.8	10	47.1
BNAB0039*	530	306	1.7	ND		ND		ND		ND		ND	
BNAB0105	417	10	41.7	10	41.7	10	41.7	10	41.7	10	41.7	10	41.7
BNAB0171	1238	412	3.0	789	1.6	117	10.6	920	1.3	125	9.9	791	1.6
BNAB0182	155	447	0.3	639	0.2	10	15.5	1154	0.1	10	15.5	393	0.4
BNAB0195	599	531	1.1	ND		93	6.4	ND		10	59.9	ND	
BNAB0199*	457	397	1.2	ND		ND		ND		ND		ND	
BNAB0220	352	10	35.2	ND		58	6.1	ND		ND		10	35.2
BNAB0252	316	88	3.6	ND		ND		ND		ND		ND	
BNAB0255	1440	92	15.7	47	30.7	182	7.9	334	4.3	124	11.6	10	144.0
BNAB0268	507	ND		ND		ND		ND		195	2.6	ND	
BNAB0270	652	1187	0.5	ND		ND				204	3.2	ND	
BNAB0279*	361	1093	0.3	ND				ND		ND		ND	
BNAB0308*	186	ND		ND		ND		1990	0.09	ND		ND	
BNAB0314	940	102	9.2	206	4.6	1046	0.9	49	19.1	647	1.5	169	5.6
BNAB0340*	1386									597	2.3		
BNAB0342	1305	402	3.2	1215	1.1	10	130.5	2175	0.6	10	130.5	1133	1.2
BNAB0344	419	10	41.9	10	41.9	10	41.9	10	41.9	10	41.9	10	41.9
BNAB0347	569	72	7.9	10	56.9	50	11.3	10	56.9	10	56.9	10	56.9
BNAB0352	3112	1050	3.0	811	3.8	385	8.1	1065	2.9	1188	2.6	613	5.1
BNAB0355	372	10	37.2	10	37.2	10	37.2	10	37.2	10	37.2	10	37.2
BNAB0361	1376	198	6.9	10	137.6	247	5.6	366	3.8	241	5.7	10	137.6
BNAB0365*	496	2740	0.2	ND		ND		ND		ND		ND	
BNAB0373	696	305	2.3	342	2.0	ND		219	3.2	ND		ND	
BNAB0376	434	168	2.6	348	1.2	294	1.5	555	0.8	160	2.7	88	4.9
BNAB0381*	1215	ND		ND		934	1.30	ND		ND		ND	
BNAB0394*	227	1166	0.2	ND		ND		ND		ND		ND	
BNAB0400*	648	1326	0.5	ND		ND		ND		ND		ND	
BNAB0402*	246	226	1.1	ND		ND		ND		ND		ND	
BNAB0422	933	10	93.3	10	93.3	855	1.1	95	9.8	671	1.4	10	93.3
BNAB0424*	669	817	0.8	ND		ND		ND		ND		ND	
BNAB0426	606	10	60.6	78	7.8	176	3.4	10	60.6	364	1.7	10	60.6
BNAB0431	243	2223	0.1	7467	0.0	231	1.1	3557	0.1	113	2.2	1412	0.2
BNAB0440	968	101	9.6	518	1.9	133	7.3	854	1.1	46	20.9	10	96.8
BNAB0452	812	10	81.2	10	81.2	151	5.4	10	81.2	281	2.9	10	81.2
BNAB0455	209	84	2.5	ND		10	20.9	ND		242	0.9	ND	
BNAB0469	1023	1193	0.9	ND		290	3.5	ND		153	6.7	ND	
BNAB0478*	872	669	1.3	ND		ND		ND		ND		ND	
BNAB0481	558	141	4.0	162	3.5	159	3.5	374	1.5	196	2.9	56	10.0
BNAB0488*	196	138	1.4	ND		ND		ND		ND		ND	

*ID_{50} values for these samples were measured for the mutant which gave rise to a 8% or higher decrease in % neutralisation in Table 3.5

ND = not determined. Either because the sera already had a 2-fold or higher reduction in neutralisation from mutant(s) already or because the mutant which initially produced an 8% or higher decrease in % neutralisation had no influence on neutralisation by a serum sample.

From the V2/glycan -site-specific sera exclusion procedure, we excluded 28 serum samples, narrowing down our list of candidate sera to map a novel HIV-1 target to 23 samples.

3.5 Detection of anti-V3/glycan Antibodies (N332-dependent PGT121-like antibodies)

3.5.1 QH343.A10 N332A mutant design

To detect anti-V3/glycan antibodies which are dependent on N332 including PGT121-like and 2G12-like antibodies (Walker et al., 2011, Sanders et al., 2002), a QH343.A10 N332A mutant was designed and subsequently engineered by site-directed mutagenesis (Figure 3.4)

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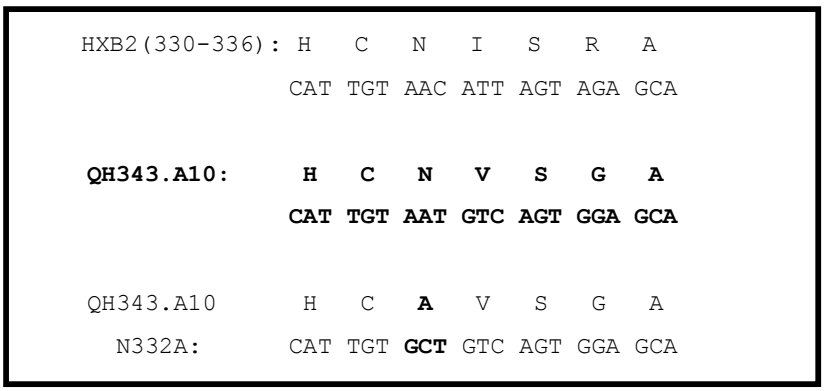

Figure 3.4: QH343.A10 N332A mutant design. A single-point mutation was introduced at position 332, converting the glutamine residue to an alanine.

The point mutation at N332A has previously been reported to diminish binding of the glycan-specific antibodies that target the V3 loop of the HIV-1 envelope (Pejchal et al., 2011, Walker et al., 2011, Sanders et al., 2002).

3.5.2 Verification of QH343.A10 N332A Mutation

Verification that the QH343.A10 N332A DNA had been mutated correctly and no PCR errors were introduced was achieved, first, by restriction digestion by *Dde* I endonuclease (Figure 3.5).

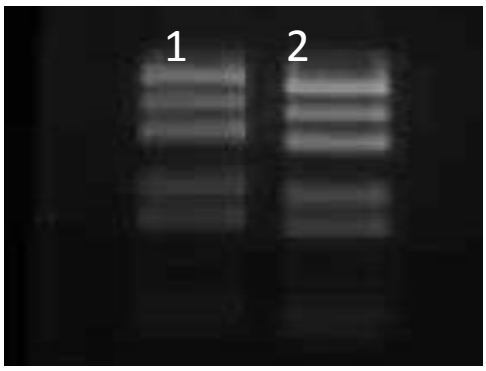

Figure 3.5: QH343.A10 N332A mutant restriction digestion. QH343.A10 N332A DNA was digested with *Dde* I restriction enzyme. QH343.A10 WT DNA was also digested with the same enzyme. The restriction digestion products were run on a 1% agarose gel for 45 minutes at 100V. Lane 1: QH343A10 WT, Lane 2: N332A mutant.

The mutant was identical to the QH343.A10 WT DNA except at the desired mutation site as determined by full-length sequencing of both DNA strands (data not shown).

3.5.3 Detection of anti-V3glycan Antibodies (N332-dependent PGT121-like antibodies)

23 serum samples remained after the exclusion of both anti-MPER and anti- V2/glycan -site-recognising sera. These 23 serum samples were tested for antibodies which target the V3glycan region by comparing the ID_{50} values of the QH343.A10 WT against the QH343.A10 N332A mutant (Table 3.7). V3/glycan site neutralisation activity was characterised as a 2 fold or higher drop in ID_{50} compared to the WT virus.

Table 3.7: ID_{50} values for serum samples against QH343.A10 WT and QH343.A10 N332A mutant to determine presence of V3-glycan-specific antibodies.

BNAB ID	Qh343.A10 WT	QH343.A10 N332A	Fold drop
BNAB0039	370	400	0.9
BNAB0084	276	315	0.9
BNAB0123	171	142	1.2
BNAB0136	105	104	1.0
BNAB0146	341	371	0.9
BNAB0176	1066	1376	0.8
BNAB0199	257	689	0.4
BNAB0231	615	224	2.7
BNAB0266	384	129	3.0
BNAB0279	365	804	0.5
BNAB0308	295	535	0.6
BNAB0334	250	92	2.7
BNAB0365	758	525	1.4
BNAB0381	996	853	1.2
BNAB0394	177	280	0.6
BNAB0400	460	779	0.6
BNAB0401	139	158	0.9
BNAB0402	107	73	1.5
BNAB0410	437	531	0.8
BNAB0416	238	588	0.4
BNAB0424	498	303	1.6
BNAB0478	1860	512	3.6
BNAB0488	181	766	0.2

ID_{50} values were calculated for each of the remaining serum samples for both the QH343.A10 WT and the N332A mutant by titrations. Only 4 serum samples had dominant antibodies that targeted the V3/ glycan region of the QH343.A10 envelope. 19 samples were left in our list of sera for mapping a novel epitope.

3.6 Determination of Sera Neutralisation Breadth and Potency

After exclusion of the V3/glycan region-specific sera, we were left with 19 samples. Because of limitations on materials and expertise in the laboratory, we were unable to conduct CD4-bs and

CD4i-site analysis on the serum samples. We did, nonetheless, assess the breadth and potency of the 19 candidate sera.

% neutralisation values were determined for each of the 19 serum samples against 24 diverse and neutralisation-resistant HIV-1 viruses (Figure 3.8) and MLV which was used as a negative control. After obtaining a % neutralisation value, an ID_{50} prediction model (Jacob et al., unpublished data) was used to calculate a predicted ID_{50} value for each sera against each virus as explained in the Materials and Methods section.

The geometric mean of all the predicted ID_{50} values for a particular serum sample was calculated and used to determine the potency of each serum. Potency is a measure of how efficiently a serum sample can neutralise viruses. Potent neutralisers were characterised as serum samples with geometric mean predicted ID_{50} >250.

The number of viruses that a serum sample could neutralise out of the 24 viruses in the panel was used to determine the breadth of the candidate serum samples. Broad sera were characterised as sera which were able to neutralise 18 viruses or more (Figure 3.8). None of the sera samples were able to neutralise MLV about 45%. 4 samples had MLV values above 25%.

Table 3.8: Breadth and potency analysis on 19 remaining serum samples.

South African Sera Samples																													
			Kenya				Various Locations					Southern Africa				India			Cameroon										
Subtype:			A				B					C							G	CRF02_AG									
Tier:			2	unk		2/3	2				3	2			2/3		2		2			3				2/3	-ve control			
BNAB ID	Geomean of predicted ID50	Number of virused neut	Q168.a2	QG984.21M.ENV.A3	QH343.A10*	Q461.e2	TRO11	RHPA4259.7	REJO4541.67	SC422661.8	PVO.4	ZM249M.PL1	CAP45.2.00.G3	Du151.2	Du422.1	001428-2.42	26191-2.48	16936-2.21	252-7	269-12	255-34	278-50	253-11	33-7	251-18	928-28	MLV (% neut at 1:100)			
BNAB0039	528	23	1003	1247	287	246	143	1487	713	1340	132	795	1392	1359	373	5268	689	1337	125	242	753	129	233	1452	70	504	10			
BNAB0084	621	23	369	614	255	59	396	623	1081	270	426	1212	1243	1634	922	1621	604	1000	1479	1409	1172	411	228	372	785	714	-10			
BNAB0123	113	10	18	123	621	R	R	53	116	60	59	204	98	625	125	442	180	408	89	59	712	66	89	43	39	27	8			
BNAB0136	442	19	947	1122	883	23	80	1435	174	89	1313	442	1473	1536	1390	1647	1296	1171	389	174	405	65	143	1358	927	55	41			
BNAB0146	574	21	1137	1462	155	27	216	1452	879	533	1164	1305	1477	1634	514	1633	467	1655	871	527	1634	411	241	1455	74	86	-6			
BNAB0176	430	21	1420	101	1005	642	1547	1385	142	474	718	R	365	1634	149	870	11	574	445	1562	242	768	2135	390	32	236	-3			
BNAB0199	585	23	88	221	299	62	1240	496	959	412	209	746	1299	1634	190	1661	1251	1529	1264	1322	1464	1463	137	1158	264	145	-7			
BNAB0279	263	16	32	28	652	241	733	451	73	21	1045	661	1286	1524	1246	388	R	171	1570	1602	598	357	54	19	626	17	-33			
BNAB0308	248	19	267	144	144	44	15	1365	160	85	490	132	700	1509	198	887	181	760	124	475	1056	487	72	879	485	28	8			
BNAB0365	522	21	1048	161	496	R	392	37	495	966	1743	45	1061	834	1159	1078	875	1105	1003	387	605	274	1485	666	901	116	10			
BNAB0381	1037	24	1607	1451	1215	1071	1452	1513	1585	1502	1821	239	1661	935	1570	1570	1481	1416	162	652	962	268	1059	1590	1339	706	-7			
BNAB0394	444	23	497	115	1829	20	138	778	312	556	686	193	1530	1066	1054	746	695	793	574	459	162	436	1531	146	1244	184	3			
BNAB0400	619	23	1561	736	648	10	125	1601	542	290	1497	903	1702	1501	1563	1565	1562	1578	545	194	867	588	267	1543	925	102	4			
BNAB0401	161	16	105	408	618	R	17	14	470	138	260	25	1154	128	544	332	125	203	72	33	251	659	80	286	621	54	23			
BNAB0402	105	13	99	126	386	11	124	119	46	249	681	97	362	130	10	617	33	1221	55	14	209	R	30	208	187	27	35			
BNAB0410	391	21	197	363	2026	R	10	155	210	74	429	218	1283	1144	869	1261	869	1365	370	651	785	317	163	495	794	178	23			
BNAB0416	233	19	549	919	105	64	745	808	408	369	1023	24	417	151	126	1052	752	1130	111	51	167	25	39	496	350	76	31			
BNAB0424	425	20	771	838	669	66	125	801	36	356	668	1028	673	1326	919	872	19	1223	1424	862	1385	101	640	1046	630	40	-30			
BNAB0488	238	19	65	108	307	R	368	189	174	81	217	168	399	608	413	243	316	96	83	208	658	707	176	601	296	333	-1			

*ID_{50} values for QH343.A10 were experimentally determined. R=resistant
Red shading= samples with either geometric mean predicted ID_{50} >250 (potency) or number of viruses neutralised above predicted ID_{50} 100 > 18 (breadth).

Breadth and potency were calculated for the final list of 19 serum samples after exclusion of the other known BnAb targets except the CD4-bs and CD4i-site. For each serum/virus, a % neutralisation at 1:100 dilution (triplicate) was determined and predicted ID_{50} values for each were calculated. The geometric mean of the predicted ID_{50} provided information on the potency of the serum sample while the number of viruses neutralised revealed the breadth of the serum. For calculation purposes, if a virus was resistant (R) to a serum sample, an arbitrary ID_{50} of 10 was assigned. If the predicted ID_{50} was less than 10, an ID_{50} value of 10 was also assigned. This assured a high penalty in the calculation for not neutralising a panel virus in the breadth/potency calculation, higher than that the assigned value of 33 used by Walker et al. (2010). MLV was the negative control.

Only sera with neutralisation breadth and potency should be used for attempts to map a novel target for BnAbs. From the 19 samples, 12 of these serum samples were both broad and potent. The next step would be mapping of these 12 sera for dominant anti-CD4-bs and anti-CD4i antibodies and excluding the sera with such antibodies. The remaining handful of samples are to be carried forward to map out a novel epitope targeted by BnAbs.

An overview of the results section is shown in Figure 3.6.

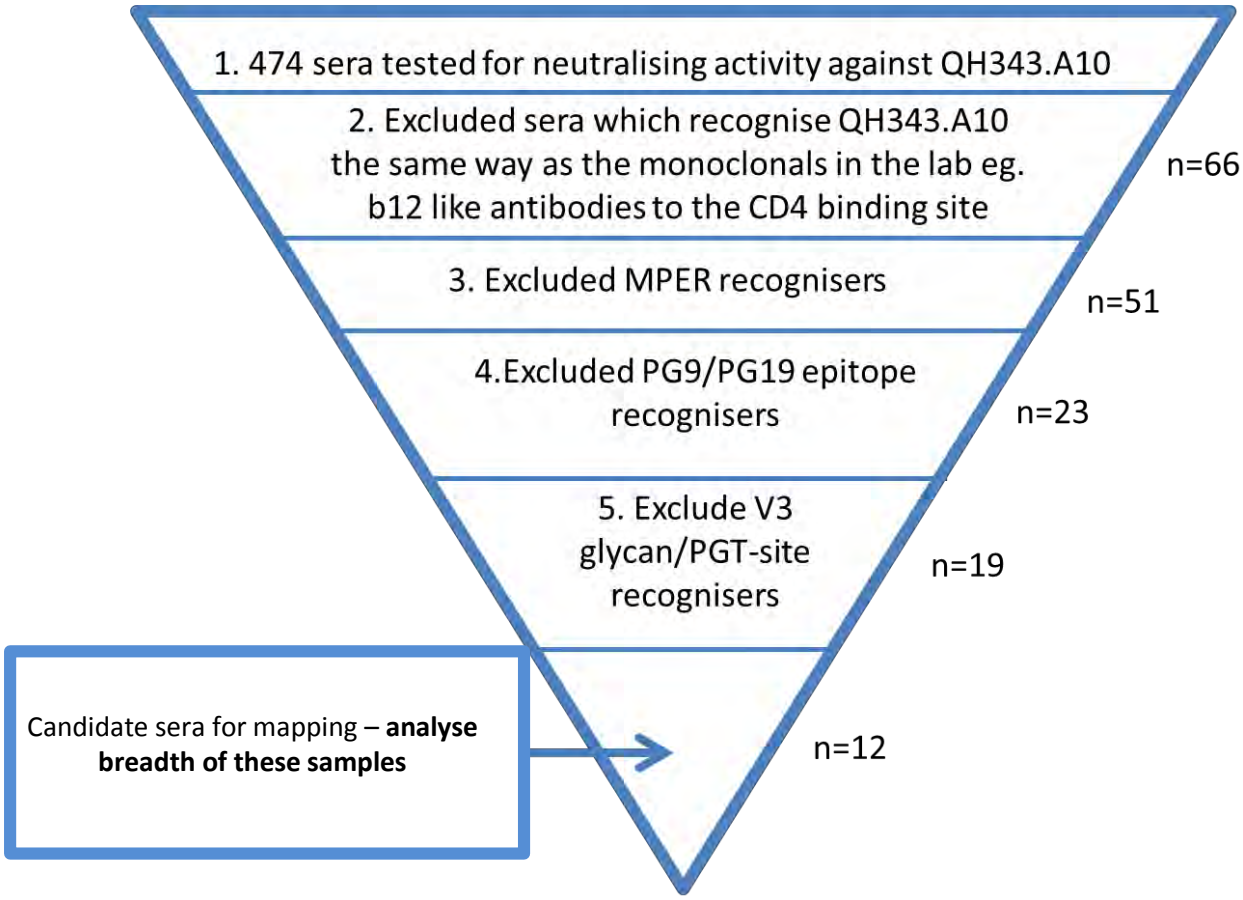

Figure 3.6: Overview of the sera selection process. Sera were tested for activity against the previously defined targets for BnAbs and at each stage sera that recognised these sites were excluded from our analysis. We were finally left with 12 candidate sera which could be mapped to find a novel epitope after CD4-bs-reactive antibodies are excluded.

Chapter 4: Discussion and Conclusions

Eliciting a BnAb response in an HIV-1 vaccine is an attractive prospect for HIV-1 researchers (Palesch and Kirchhoff, 2013, Burton et al., 2012a). The knowledge that nAbs are able to bind to the HIV-1 envelope led to optimism that an efficacious antibody-based vaccine could be developed in a few years (Stamatatos et al., 2009). However, numerous challenges have slowed down the progress in making an effective vaccine (Reardon et al., 2014, Palesch and Kirchhoff, 2013). For example, most BnAbs have undergone extensive somatic hypermutation and as a result usually develop naturally after at least a year of HIV-1 infection (Haynes et al., 2012). Therefore, eliciting BnAb responses in HIV-1-naive individuals has proven to be difficult and it may require a series of immunogens in the correct order to elicit BnAbs (Haynes et al., 2012). Although there is work being done to deliver gp41-proteins in liposomes to elicit anti-MPER antibodies similar to 4E10 and 2F5, these efforts have generally been unsuccessful at eliciting BnAbs in laboratory animals (Reardon et al., 2014). In addition, some of the broadly neutralising anti-MPER antibodies have been found to be autoreactive (Haynes et al., 2005) and therefore eliciting them in a vaccine may be difficult and perhaps even detrimental. As another example, b12 is a broad mAb against the CD4-bs while b13 is narrowly neutralising CD4-bs mAb, however, researchers have been unable to entirely distinguish how the epitope recognised by a broadly neutralising antiCD4-bs antibody is different from the epitope recognised by a narrowly neutralising anti-CD4-bs antibody (Chen et al., 2009).

Another challenge faced in immunogen design is the lack of knowledge of all the BnAb targets present on the HIV-1 envelope (Chuang et al., 2013, Stamatatos et al., 2009). Discovery of novel epitopes may aid in the design of an effective immunogen, capable of activating the humoral immune system to produce BnAbs (Chuang et al., 2013, Stamatatos et al., 2009). In addition, for a broad coverage vaccine, it may be necessary to target more than one or two epitopes. It has been difficult to target the known epitopes of BnAbs and generate neutralising antibodies with a vaccine; in addition, there is some evidence suggesting it may very difficult to target the CD4 binding site (Chen et al., 2009).

The aim of this study was to develop a novel mapping technique for identifying serum samples which can be used to identify a novel epitope of BnAbs. It is a possibility that there are no further targets for BnAbs on the HIV-1 envelope however, if there are still undefined targets, this system

was developed to identify sera able to map these novel targets. As HIV-1 Envelope proteins have several functional constraints, the likelihood appears high that there are still novel epitopes to be found. Our system should aid in finding even rarely occurring BnAbs because of the number of samples that can be efficiently screened.

We chose the virus QH343.A10 to set up our novel system. We chose this virus because of several characteristics including its moderate resistance to neutralisation, particularly to a set of broadly neutralising mAbs. QH343.A10 was previously shown to be completely resistant to mAbs b12 and VRCO1 (both anti-CD4-bs mAb), 2G12 (anti-V3/glycan mAb), 2F5 and 4E10 (both anti-MPER mAbs) and moderately resistant to CRF02_AG plasma samples tested against it (10/12 samples) (Jacob et al., 2012). QH343.A10 was also resistant to neutralisation by sCD4 (Jacob et al., unpublished data). sCD4 neutralisation is thought to correspond to the exposure of the CD4-bs (Daar et al., 1990). This resistance provided the rationale behind using QH343.A10 as the virus in our system to identify sera for mapping a novel epitope. This is because we could easily exclude some of the sera in our cohort which recognised the virus through the known BnAb epitopes due to resistance characteristics of the virus.

Step 1 (Figure 3.6) of our system was to screen the QH343.A10 virus against the 474 sera in our cohort. 66 samples were able to recognise the virus by an ID_{50} value of 150 and above. The fact that the virus was relatively resistant to neutralisation by our subtype C plasma (Jacob et al., 2012) meant that when screening against our serum cohort, we expected that there would not be a large number of serum samples which could recognise the virus. This was beneficial as a large number of QH343.A10-recognising sera would have potentially overwhelmed the screening process. Only 14% of our cohort was able to neutralise the virus efficiently. By choosing a virus of relatively low sensitivity, we would exclude a large proportion of the cohort, leaving us with a better selected group of samples which we could work with. In addition, ability to neutralise QH343.A10 was associated with high neutralisation breadth and potency (Jacob et al., unpublished data). If we used a very sensitive virus, we would have numerous samples capable of neutralising the virus to screen further and most of these samples would be recognising the sensitive virus through the previously defined epitopes.

The screening for QH343.A10-recognising sera was the first selection step in our novel technique. Simply by excluding sera which do not recognise QH343.A10, we had screened out sera which

contain antibodies which target the previously identified epitopes through the same conformations as the mAbs that QH343.A10 was found to be resistant to. After screening out these serum samples we were left with 66 samples.

Sera which recognised QH343.A10 in a similar way to the mAbs 2F5, 4E10, b12, 2G12, VRC01, will likely have already been screened out simply because we expect such antibodies would be unable to neutralise QH343.A10; Step 2 of our process (Figure 3.6). In some of the QH343.A10-recognising sera, there were still, however, antibodies which recognised these previously identified epitopes but through different conformations than the monoclonal antibodies. The next step in our novel system was to exclude sera which recognised QH343.A10 through the MPER, V2/glycan -site and the V3/glycan-site (Figure 3.6)

MPER is a linear epitope found on the gp41 stalk of the HIV-1 envelope (Zwick et al., 2005). In the initial screening for QH343.A10-recognising sera we had screened out sera which target the MPER in a similar way to 2F5 and 4E10 mAbs. 2F5 and 4E10-like antibodies are rare in the sera or plasma of HIV-1 infected individuals (Gray et al., 2007, Li et al., 2006). Therefore, antibodies still present in the QH343.A10-recognising sera would presumably recognise QH343.A10 by recognising MPER differently than 2F5 and 4E10 recognise it.

Step 3 (Figure 3.6), the exclusion of sera containing anti-MPER antibodies was achieved by using the HIV-2/HIV-1 MPER chimeric virus, C1C, which contains an exposed consensus C MPER. We used a threshold of an ID_{50}>300 to classify sera as containing anti-MPER antibodies. We chose this threshold because it has been previously shown that titres of C1C ID_{50}<300 tend not to neutralise HIV-1 pseudoviruses by recognising MPER (Gray et al., 2009). After exclusion of sera with anti-MPER antibodies, we remained with 51 serum samples.

In Step 4 to identify candidate sera to map a novel epitope (Figure 3.6), we excluded the V2/glycan -site-recognisers from our 51 serum samples. This was achieved by mutating the QH343.A10 virus in the V2/glycan -epitope region. We made 6 point mutants which were chosen from literature to inhibit the binding of PG9/16 mAbs and PG9/16-like antibodies in plasma (Moore et al., 2011, Tomaras et al., 2011, Doores and Burton, 2010). We made 6 QH343.A10 mutants to ensure full coverage of the epitope as not all antibodies bind to the epitope in the same way.

We tested the 51 remaining serum samples against all 6 QH343.A10 V2/glycan -site-specific mutants in a two-step process. First, we screened sera for evidence of a change in their

neutralisation capacity from the WT to the mutants. If the sera had an 8% or higher reduction in % neutralisation of any mutant compared to QH343.A10 WT, ID_{50} values were determined which was the second step in the process. We used 8% to make it likely that we captured all of the V2/glycan recognising sera. The 8% cut-off was intended to catch all the potential positives and also many of the negatives. It is the catching of the negatives that argues that we have likely caught most or all of the positives so that they could be properly tested by ID_{50}. We classified a serum sample as having an anti-V2/glycan -site antibody if there was a 2-fold or higher decrease in ID_{50} value from the QH343.A10 WT to any of the 6 mutants. We used this threshold as a 2-fold drop in ID_{50} value implies that 50% of the neutralisation of QH343.A10 comes from a V2/glycan -site recognising antibody.

Our 8% screening cut-off appeared efficient as we had a number of sera which we performed titrations to determine their ID_{50} values and discovered they were not V2/glycan -recognisers. This implied that we had caught most or all of the V2/glycan -recognisers as we had dropped the cut-off so low that we had included non-recognisers in our ID_{50} analysis as well. In addition, for samples with a percentage neutralisation of 90% and above for the QH343.A10 virus, we considered that we would be unable to detect ID_{50} difference by comparing % neutralisation and therefore determined the ID_{50} values at a starting dilution of 1:400 to get more reliable data for these samples. This is because at high % neutralisation, we do not expect that large changes in ID_{50} will result in noticeable changes in % neutralisation at 1:100 dilutions. After exclusion of anti-V2/glycan -site antibodies, we were left with 23 serum samples which were potential candidates for discovering a novel HIV-1 epitope.

The remaining 23 samples were further tested for dominant 2G12/PGT-121-like antibodies which target the V3/glycan region which was Step 5 of the process (Figure 3.6). This was achieved by comparing ID_{50} values obtained from each serum for both the QH343.A10 WT virus and QH343.A10 N332A mutant, using a similar logic as we used with the V2/glycan mutants. A 2-fold or higher drop in ID_{50} value from the WT to the mutant virus classified a serum sample as having anti-V3/ glycan antibodies. A 2-fold drop implies that 50% of neutralisation is caused be an antibody neutralising through the V3/ dependent glycan region and therefore these samples were excluded. Because fewer samples remained at this step, we dispensed with the process of screening using 1:100 dilutions of the test sera and measured ID_{50} values for all 23 remaining sera.

After the exclusion of anti-V3/-glycan site antibodies in sera, we were left with 19 samples. Although there are other glycans such as N301 which are bound by broadly neutralising V3/glycan-specific antibodies such as PGT127 and PGT130 (Walker et al., 2011), we used the N332A mutant as it has been widely shown to diminish binding of antibodies to this target (Julien et al., 2013, Mouquet et al., 2012, Pejchal et al., 2011, Walker et al., 2011). We are currently making the QH343.A10 N301A N332A double mutant for further V3/glycan testing.

The next step in selecting sera to be used to map a novel epitope would be identifying the sera which had antibodies against the CD4-bs and CD4i-site. However, our laboratory lacks the protein production and handling capacity that the assay requires. Gray et al (2011) described the process of mapping antibodies to the CD4-bs using a gp120 polyprotein with a D368R mutation. The D368 residue has been found to be essential for antibody binding to the CD4-bs (Gach et al., 2013, Gray et al., 2011, Li et al., 2007). The I420R mutation could also be engineered into the gp120 monomer (Gray et al., 2011) or into a transfected *env* gene to inhibit co-receptor binding (Klein et al., 2012). We aim to contact collaborators who would be willing to screen our sera for anti-CD4-bs and anti-CD4i-site antibodies and thus narrow down our list of candidate sera by excluding all the CD4-bs- and CD4i-site-specific sera.

Although, we did not perform the CD-bs analysis, QH43.A10 is resistant to VRC01 and b12 antibodies – both anti-CD4-bs antibodies. VRC01 is an extremely potent and broad next-generation antibody, which was capable of neutralising 91% of isolates tested against it *in vitro* (Wu et al., 2010). VRC01 has been used in preclinical trials in nonhuman primates (Pegu et al., 2011) and has been found to be effective in a topical gel formulation tested in humanized mice (Veselinovic et al., 2012). It was therefore, particularly striking that QH343.A10 is resistant to mAb VRC01, which is unusually broad and potent.

Step 6 (Figure 3.6) was performed to ensure that the novel epitope we potentially map out with these candidate sera is targeted by potent BnAbs. We had to exclude both narrowly neutralising antibodies as well as antibodies with low potency. This was achieved by screening the sera against a 24-virus panel and calculating both the potency of the sera as well as their breadth. Only the broad and potent sera were selected as candidate sera for the mapping of a novel epitope targeted by BnAbs.

The % neutralisation of each sera to each virus tested was converted to a predicted ID_{50} by a prediction model developed in the laboratory (Jacob et al., unpublished data

Out of the 19 samples tested for breath and potency, 12/19 samples had a geometric mean predicted ID_{50} values of above 250 and were able to neutralise 18 or more viruses with an ID_{50}>100. We used the high threshold of ID_{50}>250 to ensure the sera we chose had high potency. It must be noted that all the sera, except BNAB0279, that were able to neutralise 18 or more viruses all had a geometric mean ID_{50}>200. Therefore, even with a threshold of geometric mean predicted ID_{50}>200, we would add 3 more samples to our list (15/19).

We also used a severe threshold of 18 viruses neutralised to classify a serum sample as broad. Note that our panel viruses are, on average, unusually resistant, see below. Using this stringent threshold ensured we remained with only the very broad sera. We assigned virus/sera pairs an ID_{50} value of 10 if the virus was resistant to a particular serum sample for the purposes of calculation of the geometric mean ID_{50}. We used 10 instead of 33 which had been previously reported (Simek et al., 2009) as it puts a higher mathematical cost on the sera for not being able to neutralise a virus. In comparison of our 24-virus panel method to that of Simek (2009), we used a harder to neutralise panel, used a similar threshold (250 here versus 300 for them). Also, we used a value of 10 for resistance, while they used 33. Lastly, we also imposed a rule for the number of panel viruses a serum should neutralise, 18/24, in order to include a criterion based mostly on breadth without a large component of neutralisation potency.

The 24-virus panel included relatively resistant viruses from 5 different subtypes. These viruses were hard-to-neutralise viruses containing 5 tier 3 viruses, 4 viruses falling in either tier 2 or 3, 13 tier 2 viruses and 2 viruses of unknown tier, including QH343.A10 (Seaman et al., 2010). Using harder-to-neutralise viruses increases the threshold of the potency data as it shows that the potent sera are able to neutralise viruses which are not easily neutralised by other sera. Using viruses from different subtypes shows that the broad sera are not only broad in terms of neutralising a wide variety of subtype-matched viruses but can also neutralise across the different HIV-1 subtypes.

Of the QH343.A10-recognising sera tested against the 24-virus panel, most (12/19) of the sera were both broad and potent (15/19 if one considers the ID_{50}>200 potency threshold). This could be because QH343.A10 is very hard-to-neutralise and therefore much more likely to be neutralised by broadly neutralising sera. In addition, the fact that we used sera from subtype C infected

individuals and tested it against QH343.A10, a subtype A virus, could also have selected for more broad and potent sera.

These 12 samples are the candidate sera to map out a novel epitope. As QH343.A10 is a relatively hard-to-neutralise virus, this is not a surprising result as the sera which recognise the virus are already selected towards being both broad and/or potent based on the characteristics of the virus. CD4-bs and CD4i-site analysis must still be performed on these 12 sera but it is a large enough number of sera that a select few samples are likely to remain which can be mapped for a novel epitope.

Overall, by choosing a relatively resistant HIV-1 virus, QH343.A10, and screening it against our 474 serum samples we were able to identify at least 12-15 serum samples which contain both broad and potent anti-HIV-1 antibodies that do not detectably bind to any of the previously identified targets of BnAbs; CD4-bs and CD4i-site analysis must still take place. To reach our final list of candidate sera we eliminated QH343.A10-recognising sera which recognised the virus through the MPER region, the V2/glycan site and the V3/-dependent glycan site; known targets of BnAbs. Therefore, we successful developed a system which identified 12-15 candidate serum samples for mapping a novel epitope that is targeted by BnAbs.

No other study to date has screened for BnAbs in this way and therefore our system is completely novel and unique. Other viruses which have similar characteristics to QH343.A10 could also be used in a similar approach and therefore our method is also reproducible. As no effective HIV-1 immunogen has been developed to date, finding a novel BnAb target may be beneficial in the development of an effective, global HIV-1 vaccine that could potentially control the HIV/AIDS pandemic.

Future Work

Although we have successfully found candidate sera for epitope mapping, several experiments still need to be done to map out the novel epitope targeted by the BnAbs in these sera. Based on the research from this thesis, the following are recommended:

Short-term experiments to be conducted:

- Exclude sera which contain antibodies against the CD4-bs and CD4i-site because these are the last remaining known targets for BnAbs that we did not assess.

- Exclude sera which recognise the V3/glycan region through the N301 glycan. The N301 glycan is also targeted by a range of BnAbs such as PGT126, 127 and 128 and the N301A mutant has been shown to diminish neutralisation of V3/glycan-specific antibodies (Walker et al., 2011). All the PGT antibodies analysed by Walker et al. (2011) were dependent either on N332 or N301. Work is currently underway to produce the QH343.A10 N301A mutant to identify sera with dominant antibodies dependent upon N301.

- Consider performing MPER-depletion confirmatory experiments on samples who we classified as containing anti-MPER antibodies. In depletion experiments, MPER-peptides are bound onto magnetic beads and the sera is run through the beads. The neutralisation against C1C by MPER-depleted sera is then compared to control-depleted sera - which was exposed to peptides with a scrambled sequence. Drops in neutralisation >2 fold with the MPER-depleted sera coincide with the presence of anti-MPER antibodies. This experiment will be helpful to verify that we effectively excluded samples with anti-MPER antibodies and did not exclude samples in error.

- To map out the epitope(s) targeted by the candidate sera, we propose the use of the domain swapping technique which is described in Section 1.7.6.

- Once the region of the novel epitope(s) has been located, alanine-scan mutants can be made to determine the exact amino acids that the epitope(s) consists of (Gray et al., 2009, Walker et al., 2009, Zwick, 2005).

Long-term recommendations:

- Neutralising mAbs from key candidate sera can be isolated. It is important to isolate mAb to allow for the study of single specificities of the antibodies found in sera. It is possible for sera breadth to be coming from multiple antibodies although this appears to be rare for the sera with the very highest neutralisation breadth (Walker et al., 2010).

- Characteristics of the isolated mAbs can be studied. For example, autoreactivity, extent of somatic hypermutation and frequency of recognition of these mAbs in HIV-1-infected individuals. The protective properties of the mAbs may then be assessed in non-human primate models. If the mAbs have desirable characteristics they may be ideal candidates to be elicited in an HIV-1 vaccine.

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