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Identification and characterization of HIV-1 specific neutralizing antibodies from HIV-1 seropositive patients and autoimmune (HIV-1 seropositive or seronegative) participants.

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Identification and characterization of HIV-1 specific neutralizing antibodies from HIV-1 seropositive patients and autoimmune (HIV-1 seropositive or seronegative) participants.

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

JOHANNESBURG 2011

DECLARATION

I, Thenusha Naidoo declare that the work presented in this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine in the University of Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination at this or any other University.

……

……day of ……, 2011

DEDICATION

This work is dedicated to my parents, Neethea and Kogie Naidoo, the rest of my family and to my fiancé Enver Naidoo. Thank you for your continued love and support.

ABSTRACT

Since the discovery of HIV-1, the production of an effective prophylactic or therapeutic vaccine remains elusive. An effective vaccine must be able to elicit a potent humoral and cellular immune response. Neutralizing antibodies target the envelope glycoproteins on the surface of HIV-1 virions thereby preventing viral entry. Unfortunately, to date only a handful of neutralizing antibodies have been identified that are capable of neutralizing different viral strains within diverse subtypes, and none have been isolated from HIV-1 subtype C infected patients. In this study, we screened four different HIV-1 subtype C infected patient cohorts for the presence of neutralizing antibodies against a panel of 5 subtype C and 1 subtype B pseudovirus/es in a pseudovirion based neutralizing antibody assay. The CT cohort comprised 9 slow progressor plasma samples, the FV cohort consisted of 11 antiretroviral drug naïve HIV-1 subtype C infected plasma samples. Plasma samples from 10 antiretroviral treatment experienced HIV-1 subtype C infected patients failing first line therapy made up the DR cohort and the JM cohort consisted of 10 serum samples from HIV-1 seropositive or seronegative individuals with an autoimmune disorder. A pseudovirion neutralizing antibody assay was successfully established, and all plasma and serum samples were heat inactivated and screened using this assay. Analysis of the percentage neutralization and IC_{50} data showed no correlation between the presence of neutralizing antibodies and delayed disease progression in the SP cohort. High levels of neutralizing antibodies were observed in the DR cohort, however future studies are required to confirm if the measured neutralization is due to residual antiretroviral drugs in the plasma or neutralizing antibodies. No samples within the

FV cohort showed promising neutralizing antibody activity however the JM cohort harboured 3 serum samples (TN5, TN6 and TN8) that exhibited a greater than average breadth of neutralization and are worth investigating further in future studies. Patients TN5, TN6 and TN8 were all HIV-1 positive with an additional autoimmune disease. The availability of stored bone marrow samples for TN5, TN6 and TN8 will allow for the generation of antibody phage display libraries and isolation of monoclonal antibodies, with potentially broadly cross reactive activity.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
aCL	Anti cardiolipin
ADCC	Antibody dependant cellular cytotoxicity
ADCVI	Antibody dependent cell-mediated viral inhibition
APOBEC3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
APC	Antigen presenting cell
APS	Anti phospholipid Syndrome
ART	Antiretroviral Therapy
ARV	Antiretroviral
CA	Capsid protein
CCR5	Chemokine (C-C Motif) Receptor 5
CCL3	C-C motif chemokine ligand-3
CCL5	C-C motif chemokine ligand-5
cDNA	Complimentary DNA
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CDR	Complimentarity determining region

CRFs	Circulating Recombinant Forms
CXCR4	CXC Chemokine Receptor 4
C1-C5	Conserved regions 1 to 5
DEAE	Diethylaminoethyl
dH2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribose nucleic acid
dsDNA	Double stranded deoxyribose nucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra acetic Acid
ELISA	Enzyme linked immunosorbant Assay
Env	Envelope
EFV	Efavirenz
FCS	Fetal calf serum
FP	Fusion peptide
gp120	Surface glycoprotein 120
gp160	Envelope glycoprotein 160
gp41	Transmembrane glycoprotein 41
HAART	Highly Active Antiretroviral Therapy

HEK 293T Cells	Human Embryonic Kidney 293T Cells
HIV	Human Immunodeficiency Virus
HIVIG	Human Immunodeficiency Virus Immune Globulin
HR	Heptad Repeat
HTLV	Human T-Cell Leukaemia Virus
IAVI	International AIDS Vaccine Initiative
IN	Integrase
ITP	Immune thrombocytopenic purpura
kDa	KiloDaltons
LTNP	Long term non progressors
LB	Luria Bertani
MA	Matrix Protein
MHC	Major Histocompatibility Complex
MPER	Membrane-Proximal External Region
Mw	Molecular Weight Marker
NC	Nucleocapsid Protein
Nef	Negative factor
NIH	National Institute of Health
NVP	Nevirapine

PBS	Phosphate Buffered Saline
PIC	Preintegration Complex
Pol	Polymerase
PR	Protease
R5	CCR5
Rev	Regulator of virion
RLU	Relative light units
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
SANBS	South African National Blood Services
SDF-1	Stromal cell derived Factor 1
SIV	Simian Immunodeficiency Virus
SLE	Systemic Lupus Erythematosis
SST	Serum separating tubes
TAE Buffer	Tris-Acetate-EDTA Buffer
Tat	Trans-activator of transcription
$TCID_{50}$	50% Tissue Culture Infectious Dose
TCR	T Cell Receptor

V1/2/3/4/5	Variable Loop 1/2/3/4/5
Vpu	Viral protein U
Vpr	Viral protein R
Vif	Viral infectivity factor
X4	CXCR4

CHAPTER 1: INTRODUCTION

1.1. Human immunodeficiency virus (HIV) and Acquired immunodeficiency syndrome (AIDS)

1.1.1 HIV and the global AIDS pandemic

AIDS was first identified in humans in 1981 and is defined as a condition characterized by severe immune suppression followed by a decline in $CD4^+$ T-lymphocytes as well as the emergence of opportunistic infections such as tuberculosis or illnesses such as Kaposi's sarcoma (UNAIDS 2010). Since the reports of the first AIDS cases, the number of deaths among individuals with AIDS has rapidly escalated. In the 1980's AIDS was most prevalent among homosexual men and injection drug users and heterosexual contact accounted for the minority of infected individuals (CDC 1981). By contrast, the incidence of AIDS among individuals exposed through heterosexual contact has increased substantially (UNAIDS 2010).

The causal agent of AIDS was initially believed to belong to the family of human T-lymphotropic retroviruses (HTLV) but differed biologically and morphologically from previous isolates, HTLV-I and HTLV-II (Barre-Sinoussi, Chermann et al. 1983; Gallo, Salahuddin et al. 1984). Eventually in 1986, the retrovirus was named HIV (Coffin, Haase et al. 1986).

HIV and AIDS-related illnesses remain one of the leading causes of death worldwide with Southern Africa being the most heavily affected by this epidemic, where more than 10% of the population is infected with HIV (UNAIDS 2010)

(Figure 1.1). In 2009, there were 2.6 million new infections and 1.8 million AIDS-related deaths reported globally (UNAIDS 2010). In Sub Saharan Africa alone, 1.3 million AIDS-related deaths were reported in 2009 with 1.8 million new infections reported among adults and children (UNAIDS 2010). Although the rate of newly acquired HIV infections in Sub Saharan Africa has decreased from 2008 (2.7 million), the total number of individuals living with HIV continues to rise where poverty, limited access to good health care systems and poor education fuels the spread of the virus (UNAIDS 2010). The situation continues to deteriorate by the lack of an effective HIV vaccine. Therefore there is an urgent need for the development of a prophylactic or therapeutic vaccine.

1.1.2 HIV Classification

HIV can be divided into 2 types, HIV-1 and HIV-2 (Clavel, Guetard et al. 1986; Coffin, Haase et al. 1986). HIV-1 can be categorized into four phylogenetic groups. Group M, group O, group N and the recently identified group P (Simon, Mauclere et al. 1998; Plantier, Leoz et al. 2009). Group M is responsible for the worldwide HIV-1 epidemic and can be further divided into 9 subtypes (A, B, C, D, F, G, H, J and K) as well as combinations of one or more subtypes known as circulating recombinant forms (CRF) (Robertson, Anderson et al. 2000).

HIV-2 became evident in a Sengalese woman in 1985 (Barin, M'Boup et al. 1985). Phylogenetic analysis shows that HIV-2 is closely related to Simian Immunodeficiency virus (SIV) from the sooty mangabey monkey native to West Africa, whereas HIV-1 appears to be closely related to viruses found in

chimpanzees (Chen, Luckay et al. 1997). HIV-2 can be divided into 7 different groups (A, B, C, D, E, F and G) (Chen, Luckay et al. 1997; Yamaguchi, Devare et al. 2000; Damond, Worobey et al. 2004).

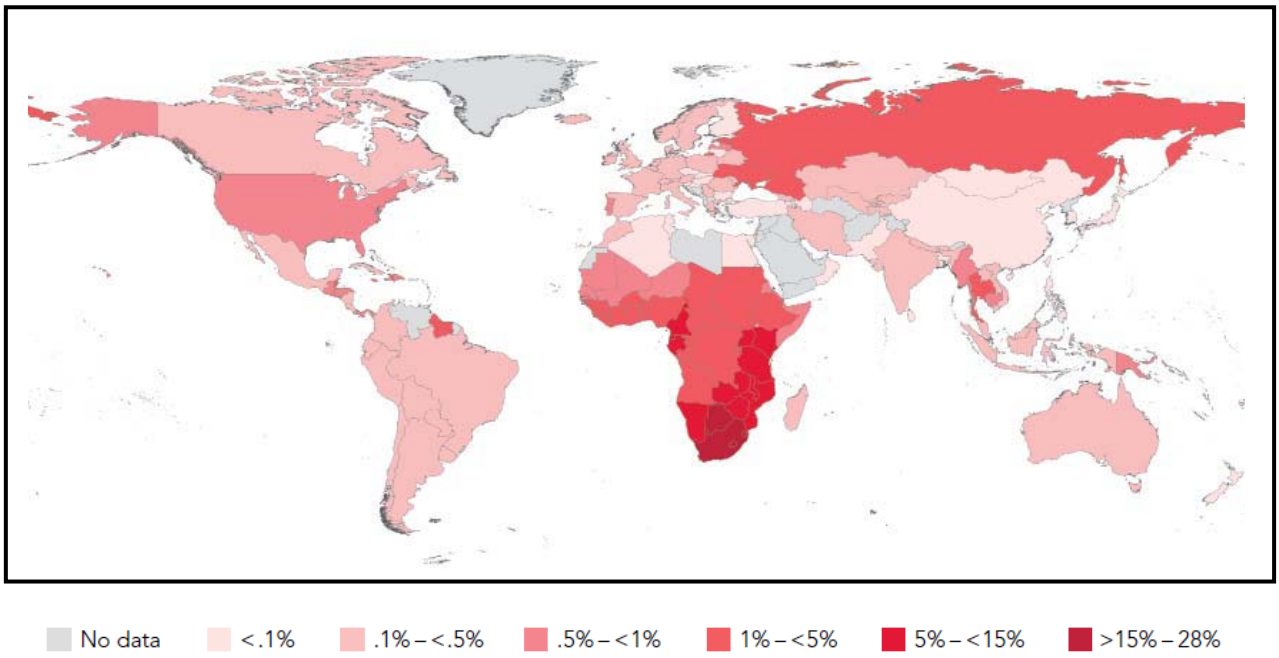


Figure 1.1: World map showing the prevalence of HIV-1 infection in adults across the globe. The total population of individuals living with HIV-1 within different parts of the world is indicated by the colour coded key. Figure copied from the 2010 report on the global AIDS epidemic (UNAIDS 2010).

1.1.3 HIV-1 structure and genome organization

HIV-1 is a lentivirus belonging to the retroviridae family. A common feature of all retroviruses is that they synthesize DNA from their RNA genome via the error prone reverse transcriptase enzyme (Leis, Baltimore et al. 1988). The mature HIV-1 virion has a spherical shape and is approximately 100nm in diameter (Gelderblom, Özel et al. 1988). The virion is composed of two identical copies of plus-strand RNA together with the error-prone reverse transcriptase (RT), integrase (IN), RNase H and protease (PR) enzymes encapsulated within the viral core (CA; p24), which is enclosed within the matrix (MA; p17) (Leis, Baltimore et al. 1988) (Figure 1.2). The envelope precursor (gp160) is cleaved by host proteases to generate the surface glycoprotein, gp120 and the transmembrane glycoprotein, gp41 (Pinter, Honnen et al. 1989). Three identical copies of gp120 are non-covalently linked to three identical gp41 molecules, forming the functional Env trimer, arranged as spikes on the HIV-1 virion (Leis, Baltimore et al. 1988) (Figure1.2).

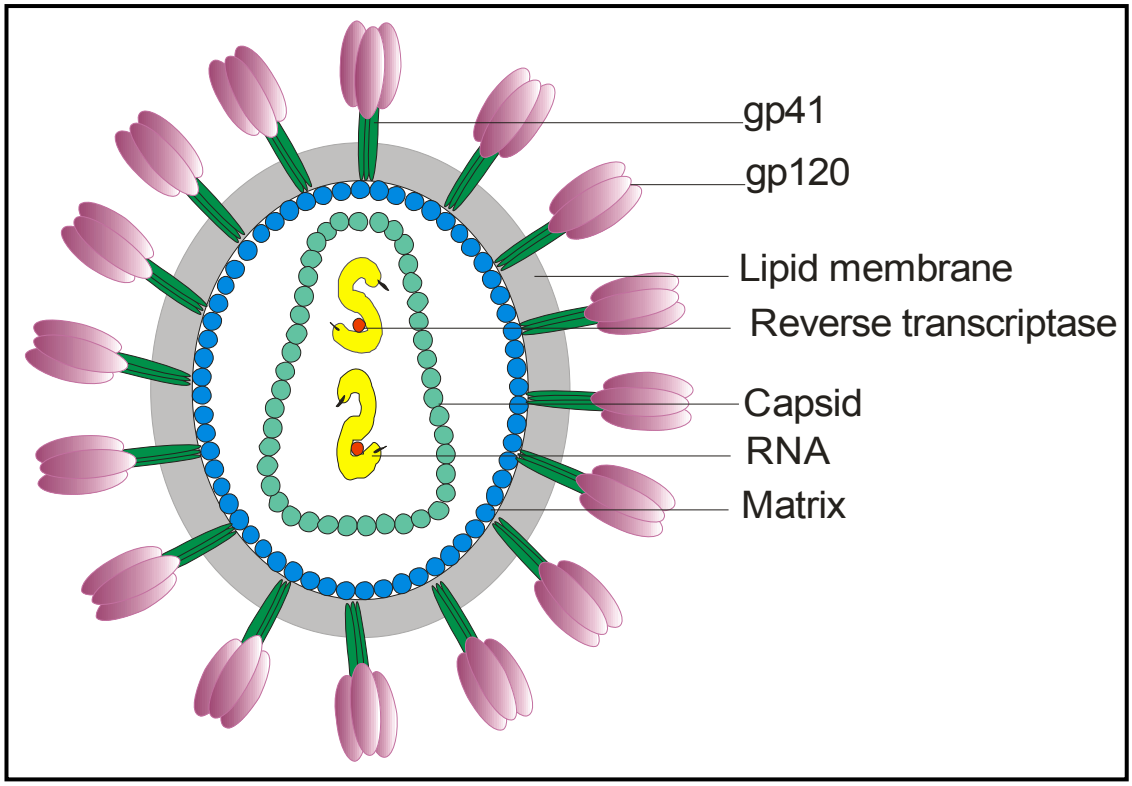


Figure 1.2: Schematic diagram of a mature HIV-1 virion. The envelope glycoprotein spikes, composed of gp120 (purple) and gp41 (green) are embedded in a host derived lipid membrane (grey). Two identical copies of plus strand RNA and reverse transcriptase is shown in yellow and red respectively, enclosed within the viral capsid (green). The viral capsid is enclosed within the matrix (blue).

The complex HIV-1 genome (9.8kb) contains only nine genes which can be categorized into 4 accessory genes, 2 regulatory genes and 3 structural genes, flanked by two identical long terminal repeat (LTR) regions (Leis, Baltimore et al. 1988). *Vpu, vpr, vif* and *nef* comprise the accessory genes of HIV-1 (Subbramanian and Cohen 1994). Vpu functions to downregulate CD4 receptor expression via ubiquitin-mediated degradation to promote the extracellular release of viral particles (Terwilliger, Cohen et al. 1989; Chen, Maldarelli et al. 1993) and Vpr is involved in the translocation of the pre-integration complex (PIC) from the

cytoplasm into the nucleus (Heinzinger, Bukinsky et al. 1994). Vif is essential for viral maturation as well as infectivity and interacts with the host-specific factor, apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) and inhibits the antiviral properties of APOBEC3G (Stopak, de Noronha et al. 2003). Nef functions to downregulate CD4 expression by interacting with cellular proteins involved in signal transduction to alter T-cell receptor signalling (Aiken, Konner et al. 1994; Miller, Warmerdam et al. 1994).

Regulatory genes include *tat* and *rev.* Tat functions to increase the transcription of HIV-1 genes (Jeang 1994; Leitner, Foley et al. 2005) and Rev is involved in the export of unspliced and single spliced mRNA from the nucleus to the cytoplasm (Feinberg, Jarrett et al. 1986; Nekhai and Jeang 2006). *Gag, pol* and *env* comprise the structural genes of HIV-1. The Gag precursor (assemblin) is processed by HIV-1 protease (PR) into the viral capsid (CA), matrix (MA) and nucleocapsid (NC) proteins (Freed 1998) (Figure 1.2). The *pol* gene encodes the viral enzymes; protease (PR), reverse transcriptase (RT), RNAse H and integrase (IN), which are essential for viral integration into the host genome, (Jacks, Power et al. 1988; Leis, Baltimore et al. 1988) (Figure 1.3). The *env* gene encodes the viral envelope glycoprotein, synthesized as a single polyprotein, gp160 in the endoplasmic reticulum. Post-gp160 synthesis, the polyprotein is heavily glycosylated in the Golgi complex before host proteases cleave it into the surface glycoprotein, gp120 and the transmembrane glycoprotein, gp41 (Leis, Baltimore et al. 1988). The gp120 functions to recognise the host receptor and chemokine co-receptor, CD4 and CCR5/CXCR4 respectively (Moore, Trkola et al. 1997). On the other hand,

gp41 functions in viral-host membrane fusion interactions (Wyatt and Sodroski 1998).

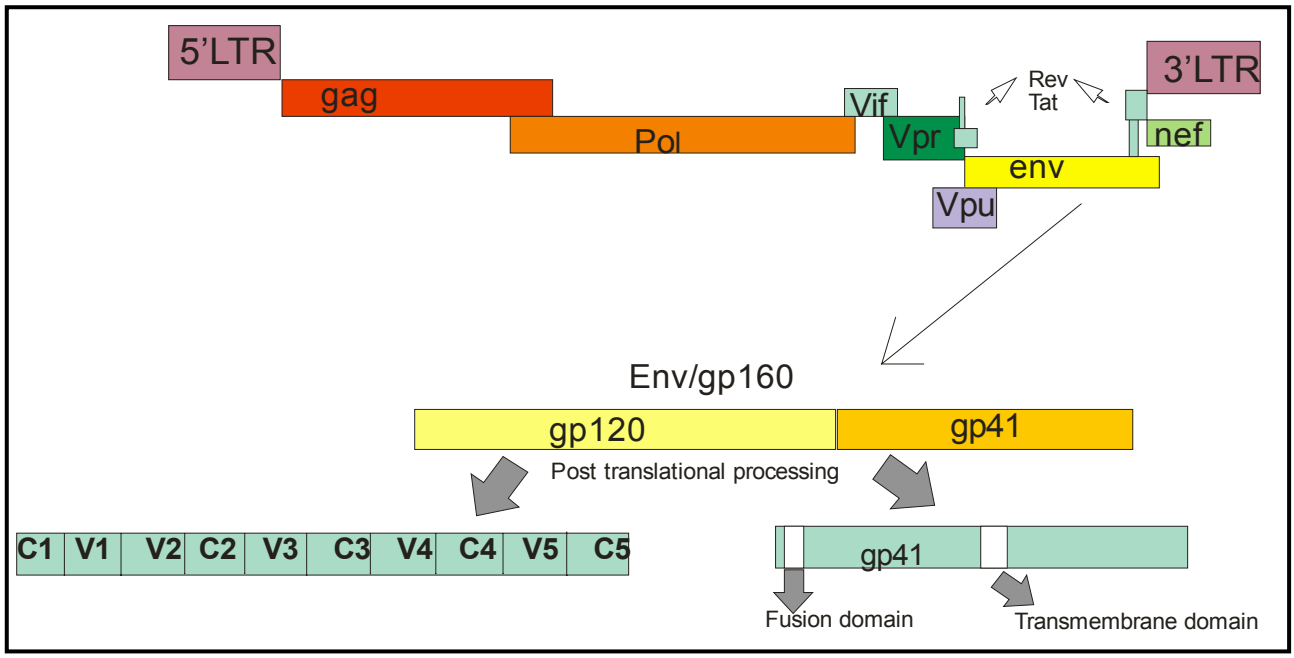


Figure 1.3: Schematic diagram of HIV-1 genome organization**.** The gp120 conserved and variable genes, C1-C5 and V1-V5, respectively are illustrated as well as the fusion and transmembrane domains of gp41.

1.2. HIV-1 Life cycle

1.2.1 Overview of viral entry

HIV entry into host target cells is initiated by the interaction between the gp120 and the host receptor CD4 (Dalgleish, Beverley et al. 1984; Bour, Geleziunas et al. 1995) (Figure 1.4, points 1). Post gp120-CD4 interaction, conformational changes in the gp120 expose the co-receptor binding site, allowing for the interaction between gp120 and its chemokine co-receptor, CCR5 or CXCR4 (Sattentau, Moore et al. 1993; Moore, Trkola et al. 1997). HIV strains which utilise CCR5 as the co-receptor are known as R5- or M-tropic viruses and strains utilizing CXCR4 are referred to as X4- or T-tropic viruses. Strains of HIV exist with the ability to utilise both co-receptors and are known as dual tropic or R5X4 viruses (Moore, Trkola et al. 1997).

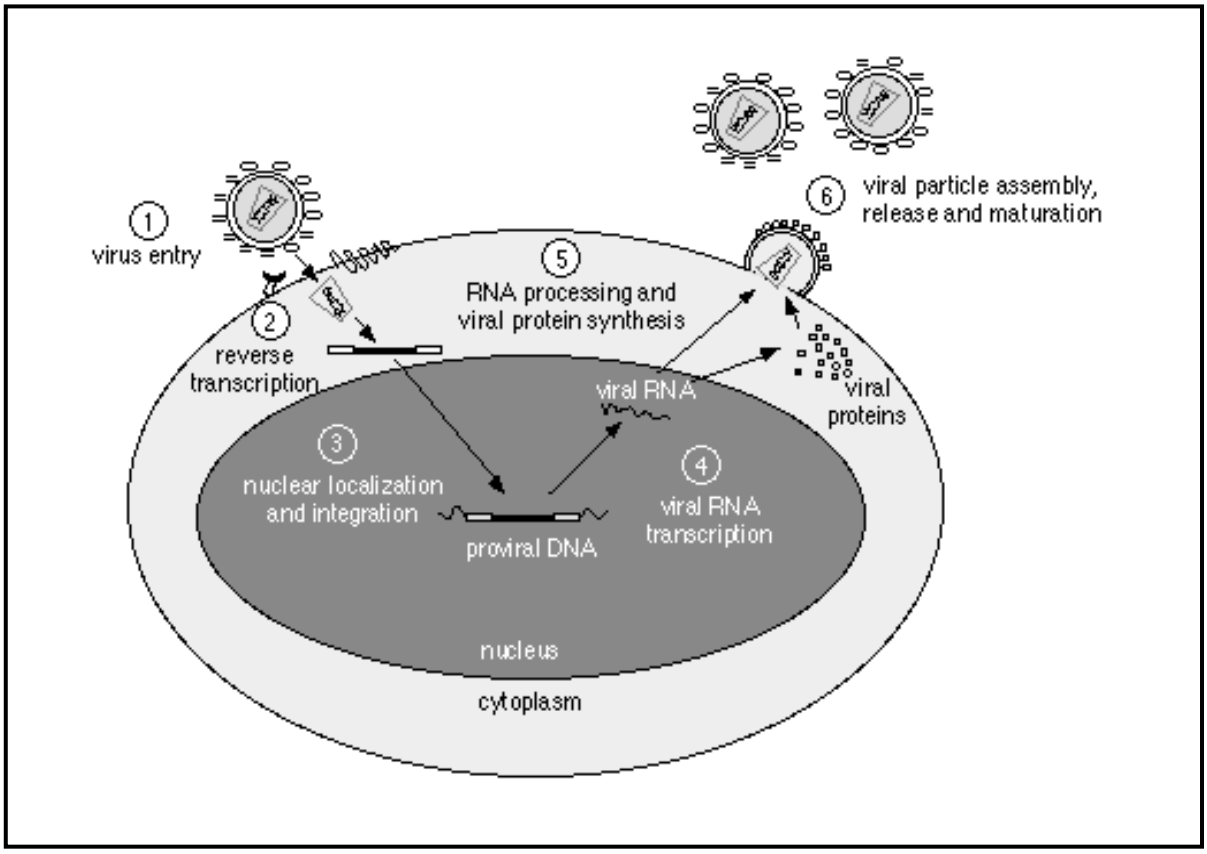


Figure 1.4: Schematic diagram of the HIV-1 life cycle. Viral life cycle steps are indicated by points 1 to 6. Figure copied from (Ramezani and Hawley 2002).

Once gp120 interacts with its primary receptor and co-receptor, further conformational changes result in the shedding of gp120 from the virion (Hart, Kirsh et al. 1991), ultimately exposing the fusogenic domain of the gp41 as well as heptad repeat regions one and two (HR1 and HR2) of gp41 (Wyatt and Sodroski 1998) (Figure 1.5A). Conformational changes in the transmembrane region of gp41 culminate in the insertion of HR1 into the host membrane. This subsequently results in the formation of a trimeric coiled coil structure known as the six helix bundle, where HR2 folds back on to HR1 in an anti-parallel manner bringing the

viral and host cell membranes closer together (Chan, Fass et al. 1997) (Figure 1.5B), thereby facilitating fusion and internalization.

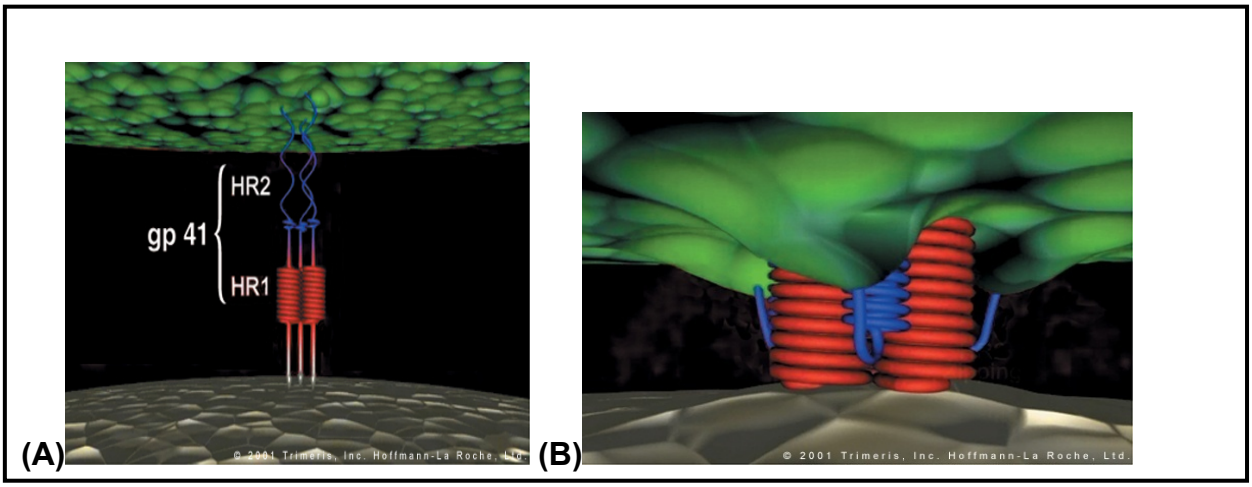


Figure 1.5: (A) Schematic representation of the gp41 molecule exposing the HR1 and HR2 regions shown in red and blue, respectively. Insertion of the HR1 region into the host cell membrane (grey) is illustrated. (B) Formation of the six helix bundle. The viral membrane is depicted in green and the host cell membrane is illustrated in grey. Figure copied from (Roche 2001).

Once the virus has entered the target cell, its capsid is shed, releasing two copies of single plus-strand viral RNA, which is subsequently reverse transcribed into double stranded complementary DNA (cDNA) by the error-prone reverse transcriptase (Gelderblom 1991) (Figure 1.4, point 2). An HIV-1 pre integration complex (PIC) is then formed and contains viral integrase, reverse transcriptase, Vpr and matrix proteins (Farnet and Haseltine 1991). The PIC is responsible for the transport of the newly synthesized cDNA into the nucleus and integration of the cDNA into the host genome (Figure 1.4, point 3) where the transcription of viral

genes takes place together with host genes (Bukrinsky, Sharova et al. 1992) (Figure 1.4, point 4). The mRNA is then transported from the nucleus to the cytoplasm where translation of viral and host proteins occurs (Goto, Nakai et al. 1998). RNA processing occurs, where double spliced RNA species translate into the viral proteins Tat and Rev, (Figure 1.4, point 5) and single spliced long length RNA is translated into Gag, Pol and Env (Goto, Nakai et al. 1998) (Figure 1.4, point 5). Host proteases cleave gp160 into the surface gp120 and transmembrane gp41 subunits (Wyatt and Sodroski 1998). The immature virions travel to the host membrane where they assemble and bud out of the cell taking part of the host lipid membrane (Figure 1.4, point 6). Viral protease continues to cleave long strands of protein (*gag* and *pol*) into smaller strands which are used to form mature viral particles that can initiate a new round of infection (Goto, Nakai et al. 1998) (Figure 1.4, point 6).

1.2.2. Host cellular proteins involved in the entry process

1.2.2.1 The CD4 receptor

The cluster of differentiation 4 (CD4) receptor molecule is a 58kDa T-cell transmembrane protein consisting of extracellular and transmembrane regions with a cytoplasmic tail at the C terminus (McDougal, Kennedy et al. 1986). The CD4 receptor is expressed on a subset of T-lymphocytes known as $CD4^+$ T-cells and presents antigens to the major histocompatibility complex II molecule (MHC class II) found on the surface of antigen presenting cells (APC) to initiate an immune response (Germain 1997).

CD4 was first recognized as the primary cellular receptor used for HIV-1 entry in the 1980's (Klatzmann, Barre-Sinoussi et al. 1984). Structural analysis of the CD4 molecule has allowed for the identification of residues on CD4 that are involved in gp120 interactions (Ryu, Kwong et al. 1990) (Figure 1.6). It has been shown that the gp120 binding site on CD4 is situated in the N-terminus of the first extracellular domain of CD4 and involves a Phe43 residue which reaches into a hydrophobic cavity of gp120 (Sattentau, Arthos et al. 1989; Ryu, Kwong et al. 1990) (Figure 1.6).

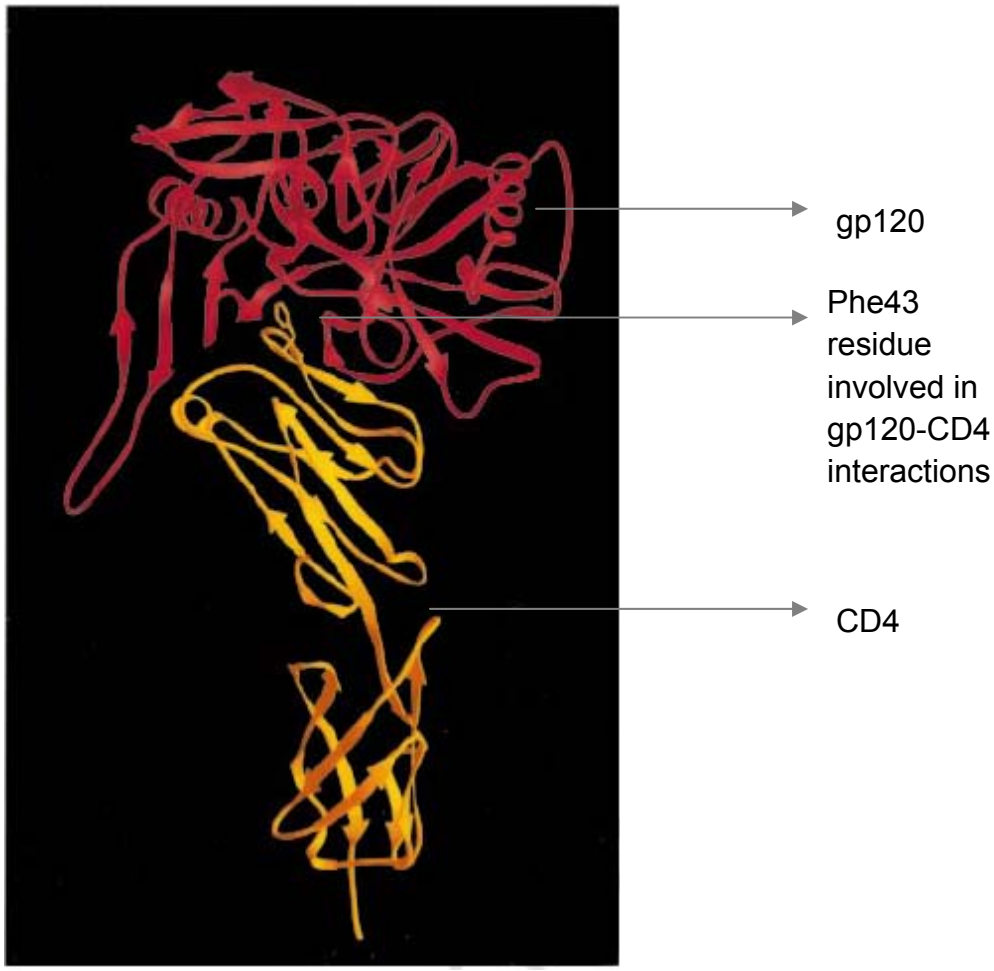


Figure 1.6: Ribbon diagram of CD4 molecule in complex with HIV-1 gp120.

The Ph43 residue is also depicted reaching into a recessed cavity of gp120. Figure adapted from (Kwong, Wyatt et al. 1998).

1.2.2.2 Chemokine co-receptors CCR5 and CXCR4

Chemokine receptors are defined as small molecules which mediate the chemotaxis of leukocytes (Yoshie, Imai et al. 1997). The α chemokine (CXC) receptor 4 (CXCR4) is normally expressed on the surface of haematopoietic cell types and is the receptor for the α-chemokine, stromal derived factor 1 (SDF-1) (Oberlin, Amara et al. 1996). The β chemokine (CC) receptor 5, CCR5 is commonly expressed on the surface of dendritic cells, T-cells and macrophages and binds to β-chemokines such as C-C motif chemokine ligand-5 (CCL5), C-C motif chemokine ligand-3 (CCL3) and C-C motif chemokine ligand-4 (CCL4), previously known as RANTES, MIP-1α and MIP-1β, respectively (Alkhatib, Combadiere et al. 1996). CCR5 and CXCR4 belong to the seven transmembrane-spanning G protein-coupled receptors (Feng, Broder et al. 1996). HIV-1 strains which utilize CCR5 as their co-receptor are referred to as M-tropic viruses and strains which utilize CXCR4 for entry are known as T-tropic viruses (Moore, Trkola et al. 1997).

1.2.3 Viral proteins involved in the entry process

1.2.3.1 gp120

The gp120 is a conformationally flexible and heavily glycosylated protein consisting of five conserved regions (C1-C5), interspersed between five variable regions (V1-V5) (Modrow, Hahn et al. 1987) (Figure 1.3). The variable regions of gp120 play an essential role in viral infectivity, viral tropism and the shielding of neutralization epitopes. In particular the V3 loop (Figure 1.7) is involved in viral tropism but is well occluded from neutralizing antibody recognition and possesses diverse sequence variability (Ait-Khaled and Emery 1993). The conserved regions, C1 to C5 are vital for the interactions between gp120 and gp41 as well between gp120 and the primary CD4 receptor and co-receptor CCR5/CXCR4. On the native trimeric Env, the conserved regions are masked by the variable regions (Starcich, Hahn et al. 1986). Unfortunately, the variable regions of gp120 have a high degree of sequence diversity and subsequently present different Env epitopes to the immune system which allows for the continued viral immune escape from antibody recognition (Ait-Khaled and Emery 1993). The Env is also heavily glycosylated with poorly immunogenic glycans creating a glycan shield which can be rearranged without altering the functionality of the Env (Wyatt, Kwong et al. 1998). In addition, gp120 adopts a different conformation once bound to CD4, allowing for the exposure of the co-receptor binding site (Sullivan, Sun et al. 1998). Post gp120 co-receptor interactions, further conformational changes occur which requires large bonding energy to allow for the reordering of the gp120 structure (Myszka, Sweet et al. 2000). The gp120 is subsequently shed from the virion, allowing the gp41 to facilitate viral fusion. The shed gp120 is extremely

immunogenic and acts as an immune decoy, exposing predominantly non neutralizing epitopes (Moore, Cao et al. 1994).

Despite the many immune escape mechanisms employed by gp120, neutralizing antibody responses have been detected targeting conserved regions such as the CD4 binding site, the poorly immunogenic glycan shield on the surface of gp120, as well as co-receptor binding sites and variable regions of gp120. X-ray crystallography of gp120 has allowed for the identification of regions involved in CD4 and antibody binding, for example the X5 neutralizing antibody which targets the co-receptor binding site (Huang, Tang et al. 2005) (Figure 1.7).

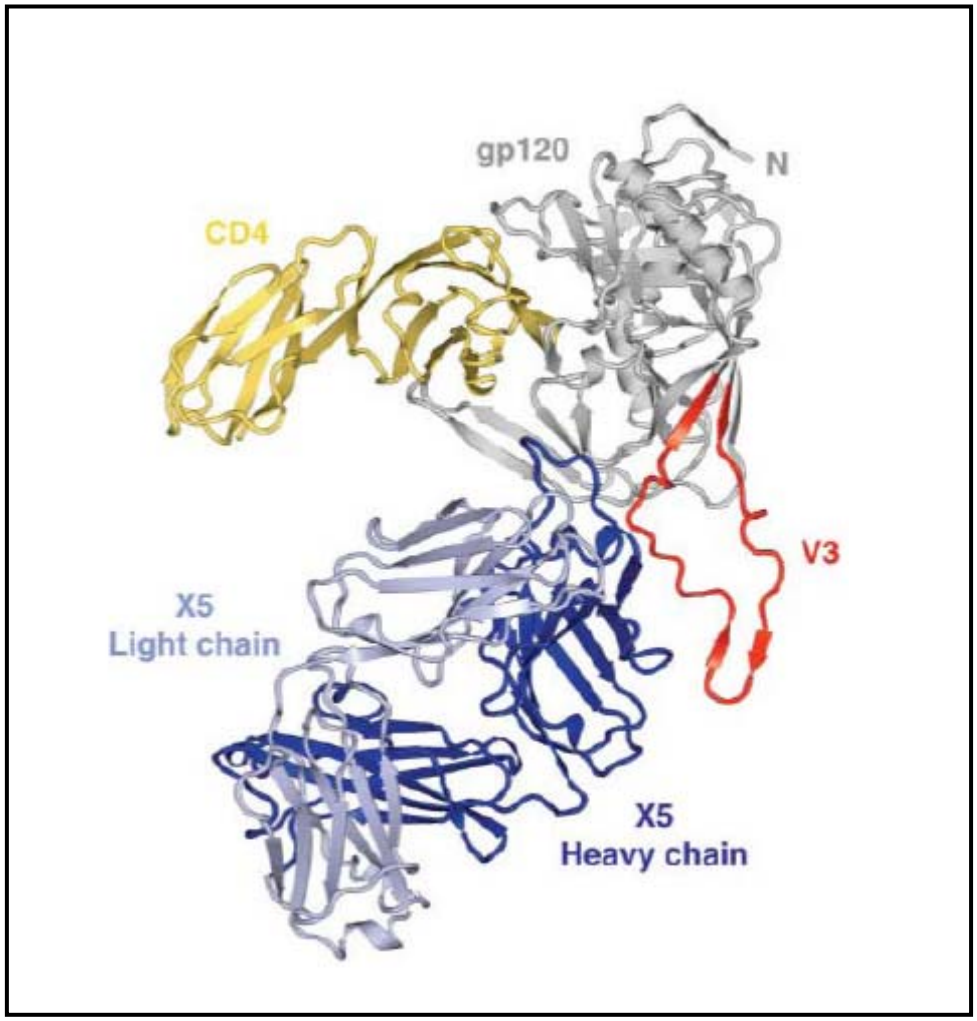


Figure 1.7: Structure of the HIV-1 V3-containing gp120 core shown in red and grey bound to the CD4 receptor illustrated in yellow. The Fab portion of an anti-gp120 antibody (X5) is indicated in light and dark blue. Figure copied from (Huang, Tang et al. 2005).

1.2.3.2 gp41

The gp41 transmembrane protein plays an essential role in viral fusion and consists of three domains; the ectodomain, transmembrane domain and the cytoplasmic tail (Chan, Fass et al. 1997). The ectodomain contains the membrane proximal external region (MPER), the fusion peptide as well as 2 hydrophobic regions, N-terminal heptad repeat (HR1) and C-terminal heptad repeat (HR2) which can self assemble in an anti parallel manner to form the six helix bundle, essential for viral fusion (Eckert, Malashkevich et al. 1998) (Figure 1.5). The gp41 is anchored into the viral membrane via a tryptophan-rich transmembrane domain and the C terminal cytoplasmic tail (Gallaher, Ball et al. 1989). Antibody responses have been detected targeting the gp41 region; however most do not succeed at inhibiting viral entry and subsequent infection, due to viral escape mechanisms employed by HIV-1 to evade immune responses such as steric hindrance and the transient exposure of neutralizing epitopes (Purtscher, Trkola et al. 1994; Jiang, Lin et al. 1998; Zwick, Labrijn et al. 2001).

1.3. HIV-1 disease progression to AIDS

HIV-1 undergoes a multiphasic disease progression which can be divided into primary infection, acute infection, chronic infection and AIDS (Figure 1.8). After transmission and successful infection, primary infection is characterized by a rapid decline in $CD4^+$ T-cells together with a rapid increase in HIV-1 viral loads (Schnittman, Greenhouse et al. 1990). During acute infection, the virus is allowed to replicate uncontrollably to very high levels and a peak in viremia is observed (Little, McLean et al. 1999) (Figure 1.8). Cellular immune responses such as anti HIV-1 CD8+ cytotoxic T-lymphocytes are subsequently elicited , ultimately bringing viral replication under control, and reducing viral loads to a steady viral set point (Koup, Safrit et al. 1994; Moore, Cao et al. 1994). At the same time, humoral immune responses are elicited, and the presence of binding antibodies can be detected (seroconversion). Binding antibodies do not control viral replication. Control of viral replication by anti HIV-1 CD8+ cytotoxic T-lymphocytes allows for the partial recovery of $CD4^+$ T cells (Figure 1.8), and the infected individual enters the chronic or asymptomatic phase of infection. This phase of HIV-1 infection can last from years to decades, during which time the infected individual remains clinically asymptomatic. During this time, the infected individual can mount vigorous cellular and humoral (including neutralizing antibody) immune responses against HIV-1. Eventually, viral loads escalate again and CD4+ T-cell counts decline to the extent where they can no longer support the immune system (Mellors, Munoz et al. 1997). This results in severe immune suppression, allowing for opportunistic infections to occur such as tuberculosis or Kaposi's Sarcoma, culminating in the progression to AIDS (Pantaleo, Graziosi et al. 1993) (Figure

1.8). An AIDS diagnosis is made when the HIV-1 infected individual has a CD4 T-cell count less than 200 cells/μl. Death usually occurs within 2 years of AIDS in the absence of antiretroviral therapy and the chance of death tends to increase with decreasing CD4 T-cell counts (WHO 2006). The introduction of highly active antiretroviral therapy (HAART) has substantially reduced the mortality and morbidity among HIV-1 infected individuals (Porter, Babiker et al. 2003).

AIDS patients are placed on HAART, and with successful therapy viral loads can decrease to levels below detection (<50 RNA copies/ml) within 12 weeks (Markowitz, Saag et al. 1995). Unfortunately, the emergence of drug resistance in patients on HAART is inevitable. Patients placed on a first line treatment regimen usually have to adjust their treatment combinations to a new regimen once HIV-1 drug resistant variants emerge (Little, Daar et al. 1999).

The rate at which HIV-1 infected individuals progress to AIDS is variable and can be categorised into rapid progression, intermediate progression and slow progression (Buchbinder, Katz et al. 1994; Cao, Qin et al. 1995; Pantaleo and Fauci 1996). Ten to fifteen percent of HIV-1 infected individuals are rapid progressors, characterised by rapid $CD4^+$ T-cell decline and hence progress to AIDS from as little as 6 months to a few years after initial infection (Anzala, Nagelkerke et al. 1995). The majority of infected individuals are intermediate progressors, characterised by disease progression to AIDS approximately 10 years after initial infection. Only about 5% of infected individuals are characterized as long term non-progressors (LTNP) (Cao, Qin et al. 1995), elite controllers (Rosenberg, Billingsley et al. 1997) or slow progressors. Long term non

progressors are a group of individuals who maintain normal CD4 counts and remain asymptomatic for more than 7 years (Hogervorst, Jurriaans et al. 1995). Slow progressors are a subgroup of LTNP's and are immunologically and clinically comparable to LTNP for about 4 years before showing a decline in CD4 cell counts and eventually clinical symptoms of AIDS within about 7 years (Hogervorst, Jurriaans et al. 1995). Elite controllers are another subgroup of LTNP's and are characterized by their ability to maintain low viral loads (<50 RNA copies/ml) in the absence of antiretroviral therapy (Rosenberg, Billingsley et al. 1997). These groups of rare individuals, and are important to study further since they may harbour useful information about unexplored mechanisms of HIV-1 control.

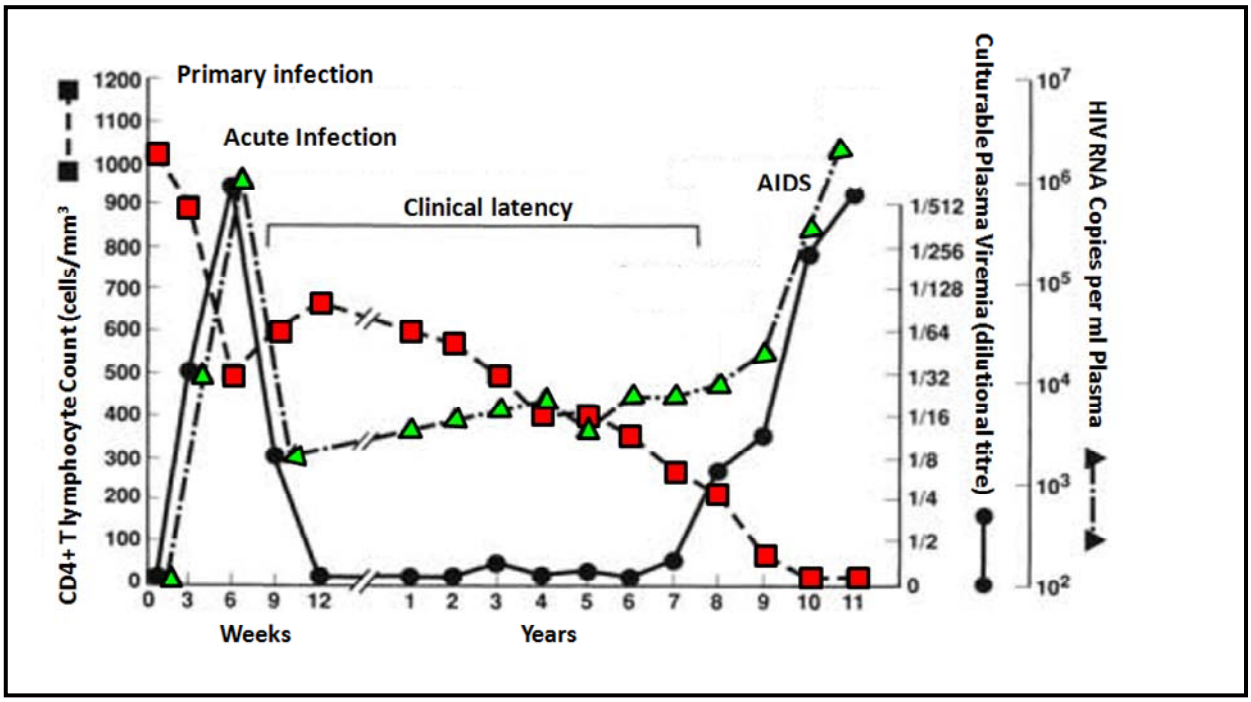


Figure 1.8: Graphical representation of HIV-1 disease progression. The dashed line with squares represents CD4 T-lymphocyte count (cells/mm^3). The solid line with circles represents culturable plasma viremia and the dashed line with triangles represent viral load measured in viral RNA copies per ml. Figure copied from (Pantaleo, Graziosi et al. 1993).

1.4. Antibody responses to HIV-1

Throughout HIV-1 infection an abundance of antibodies are elicited. These include binding antibodies, neutralizing antibodies and antibodies that work together with innate immune cells to kill off virus infected cells. The latter process is referred to as antibody dependent cell-mediated cytotoxicity (ADCC) and antibody dependent cell-mediated viral inhibition (ADCVI) (Sarmay, Lund et al. 1992). The majority of antibodies elicited early during HIV-1 infection are known as binding antibodies but are ineffective at preventing infection. By contrast, neutralizing antibodies aim to prevent viral entry and subsequent infection by binding to exposed regions on the Env, however once HIV-1 has entered into its target cells there is little or no role for these antibodies.

1.4.1. ADCC and ADCVI

ADCC antibodies bind to viral epitopes exposed on the surface of HIV-1 infected cells. The biological activity of ADCC antibodies is mediated through the F_C region of the antibody (Sarmay, Lund et al. 1992). F_C receptors are expressed on natural killer cells, monocytes, macrophages, dendritic cells and neutrophils (Sarmay, Lund et al. 1992). ADCC occurs by a 3-way interaction between an infected target cell which displays antigens on its surface, ADCC antibodies and an effector cell expressing the F_C receptor. These interactions ultimately result in the killing of the infected target cell (Baum, Cassutt et al. 1996). ADCVI also involves the interaction between a target cell, ADCVI antibody and an effector cell. However, rather than causing cell death, ADCVI antibodies aim to reduce the viral output from infected target cells. It has been confirmed that HIV-specific ADCC and

ADCVI antibodies are present in most HIV-1 infected individuals during early infection (Ljunggren, Bottiger et al. 1987), however with disease progression, natural killer cells lose their function and are unable to mediate ADCC activity (Baum, Cassutt et al. 1996). ADCC antibodies differ from neutralizing antibodies; ADCC antibodies target infected cells and result in cell killing whereas neutralizing antibodies target cell free virus and results in viral inhibition.

1.4.2. Binding antibodies

Binding antibodies are characterized by their inability to prevent viral infection and are produced at high levels throughout the lifetime of an infected individual. These antibodies recognize highly immunogenic and variable regions of the HIV-1 virion. Even though they do not prevent viral entry, binding antibodies are useful as a diagnostic indication of whether the virus is present or not. For example, the detection of anti-p24 (core) antibodies by enzyme-linked immunosorbant assay (ELISA) is used as a diagnostic test to detect seroconversion in the acute phase of infection (Daar, Little et al. 2001).

1.4.3. Neutralizing antibodies

1.4.3.1. Autologous neutralizing antibodies

Most HIV-1 infected individuals produce antibodies capable of inhibiting their own virus (autologous virus) but incapable of neutralizing heterologous viruses and are

known as autologous neutralizing antibodies (Arendrup, Nielsen et al. 1992). These antibodies target immunogenic, exposed regions of the HIV-1 virion, however their neutralization capacity is transient due to viral mechanisms to escape antibody recognition (Arendrup, Nielsen et al. 1992). Once the humoral immune system recognizes a particular viral variant and generates an appropriate antibody response, a new escape variant will subsequently emerge (Arendrup, Nielsen et al. 1992), possibly due to a combination of the immune pressure and the error prone reverse transcriptase. Therefore autologous neutralizing antibody responses tend to lag behind viral escape variants.

A study reported on the detection of autologous Nab as early as 52 days after detection of HIV-specific antibodies in acutely infected patients (Wei, Decker et al. 2003). Another study by evaluated autologous and heterologous neutralizing antibody responses in 14 HIV-1 subtype C acutely infected individuals (Gray, Moore et al. 2007). Env-clones were used which were obtained within the first 2 months of infection. Their results revealed that potent autologous neutralizing antibodies are produced within 3 to 12 months post-infection with an increase in autologous antibody production observed within the first 6 months (Gray, Moore et al. 2007). Interestingly, it was noted that potent autologous neutralization correlated with shorter Env variable region lengths as well as the presence of fewer glycoslation sites in the V1 to V2 region of Env (Gray, Moore et al. 2007).

1.4.3.2. Broadly neutralizing antibodies

Neutralizing antibodies with cross reactivity have been identified in a minority of HIV-1 infected individuals, and have the unique ability to effectively neutralize multiple viral isolates across diverse strains and subtypes. Target epitopes of neutralizing antibodies include the Env glycoprotein subunits, gp120 and gp41.

Numerous studies have evaluated and characterized the neutralizing antibody responses elicited during natural infection among serum from HIV-1 infected individuals worldwide. For example, a study by van Gils et al. (2009) evaluated neutralizing antibody responses in 35 participants with rapid or slow HIV-1 infection. The findings from this study suggest that neutralizing antibody titers increase with time during infection. A study by Binley et al. (2008) characterized the epitope specificities recognized by 24 subtype B and C infected participants with broadly neutralizing serum. They found that the majority of neutralizing antibodies present in these individuals recognized unidentified epitopes in addition to the more conserved CD4 and co-receptor binding sites.

Broadly neutralizing antibodies are rarely elicited during natural infection. However a few broadly neutralizing antibodies have been characterized and isolated from HIV-1 positive individuals. These include IgG1b12 (Roben, Moore et al. 1994), 2G12 (Trkola, Purtscher et al. 1996), PG9, PG16 (Walker, Phogat et al. 2009), VRC01 and VRC02 (Zhou, Georgiev et al. 2010) that target the gp120, as well as 2F5 (Muster, Steindl et al. 1993), 4E10 (Buchacher, Predl et al. 1994) and Z13 (Zwick, Labrijn et al. 2001) which target the gp41.

The broadly neutralizing monoclonal antibody IgG1b12 recognizes the highly conserved CD4 binding site on gp120 and acts as a competitive inhibitor of CD4 (Roben, Moore et al. 1994). IgG1b12 interacts with its epitope via its unique extended long complementarity-determining region, CDR3 loop from the variable domain of its heavy chain. (Roben, Moore et al. 1994) (Figure 1.9). The CD4 binding site on gp120 is a recessed pocket which forms a contact site for a Phe-43 amino acid residue that protrudes from the loop on the CD4 molecule (Wyatt and Sodroski 1998). Without this interaction gp120 binds too quickly to CD4 and will not allow the fusion process to occur (Wyatt and Sodroski 1998). Upon binding to its epitope, IgG1b12 induces little entropic and conformational changes suggesting a favourable fit into the CD4 binding site of gp120 (Kwong, Doyle et al. 2002). In a study by Binley et al. (2004), IgG1b12 was evaluated for its cross reactivity against a diverse panel of primary and pseudoviruses representing all major subtypes (A, B, C, D, F, and AC and AE recombinants) as well as 25 primary isolates. IgG1b12 was able to neutralize 50% of all tested viruses including viruses from all subtypes, confirming that IgG1b12 is a potent broadly neutralizing antibody (Binley, Wrin et al. 2004).

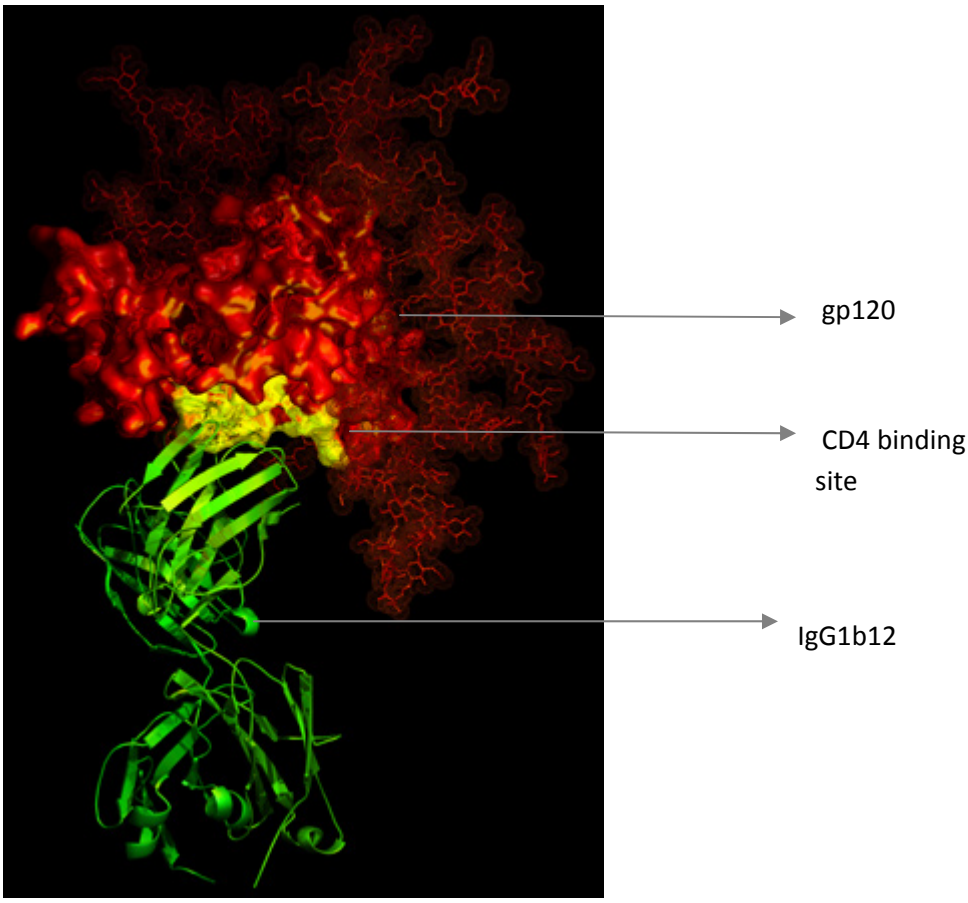


Figure 1.9: X-ray crystallography capturing IgG1b12 bound to the CD4 binding site on gp120. Figure adapted from (Chen, Kwon et al. 2009).

Two recently identified somatically related monoclonal antibodies, PG9 and PG16 (Figure 1.10) were isolated from cloned B cells of a subtype A infected patient (Walker, Phogat et al. 2009). These 2 antibodies have the ability to neutralize 70 – 80% of circulating HIV-1 strains/subtypes by recognizing conserved epitopes in the V2 and V3 loops of trimeric gp120 (Walker, Phogat et al. 2009). Structural analysis of PG9/PG16 has revealed 3 distinct features; N-linked glycosylation, affinity maturation and the longest observed CDR H3 region in human antibodies (Pancera, McLellan et al. 2010). The extensive glycosylation was found not to be required for neutralization. The long CDR H3 region forms an axe-shaped

subdomain (Figure 1.10) which comprises about 42% of the CDR surface. Affinity maturation was shown to play a major role in the neutralization breadth of these 2 antibodies (Pancera, McLellan et al. 2010).

Wu et al. (2010) have recently developed antigen resurfaced glycoproteins that are specific for the CD4 binding site of gp120. These were used as probes to identify sera with neutralizing antibodies to the CD4 binding site and subsequently isolate B cells from identified individuals to produce monoclonal antibodies. As a result, three potent neutralizing antibodies were identified, two somatic variants VRC01 (Figure 1.11) and VRC02 as well as VRC03. VRC01 and VRC02 showed a striking neutralization breadth of 91% against viral isolates across diverse subtypes (A, B, C, D, G) as well as circulating recombinant forms (CRF01_AE and CRF07_BC) (Wu, Yang et al. 2010; Zhou, Georgiev et al. 2010). VRC03 showed less neutralization potency than VRC01 and VRC02, with the ability to neutralize 57% of HIV-1 diverse primary isolates, including subtypes A, B, C, D, G and CRF01_AE and CRF07_BC (Wu, Yang et al. 2010). VRC01 binds to a highly conserved portion of the CD4 binding site. Interestingly there is a significant correlation to the residues on gp120 involved in CD4 binding as well as VRC01 binding (Zhou, Georgiev et al. 2010). This unique antibody partially mimics the CD4 molecule with 73% homology with the CD4 N-terminal domain (Zhou, Georgiev et al. 2010). An interesting feature of the VRC01 antibody is that it targets the outer domain of the CD4 binding site which is occluded by glycan shields (Zhou, Georgiev et al. 2010). The VRC01 light chain makes contact with the N-linked glycan at residue 276 on gp120; therefore VRC01 uses the glycan for binding rather than being occluded by it (Zhou, Georgiev et al. 2010). Only 13% of

contact is made with the bridging sheet of gp120 and is found not to be essential for binding. By contrast, the CD4 molecule makes 33% of its contact with the gp120 bridging sheet and is essential for binding (Kwong, Wyatt et al. 1998). The $Phe43_{CD4}$ interaction with gp120 is not observed in VRC01-gp120 binding however the $Arg59_{CD4}$ interaction with gp120 is mimicked by VRC01 (Zhou, Georgiev et al. 2010).

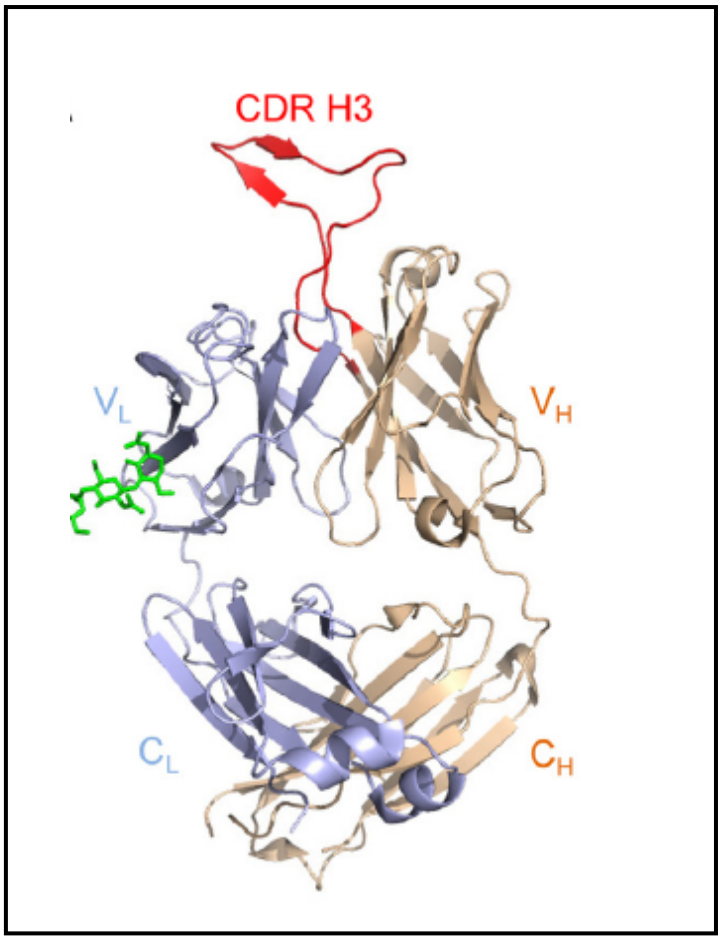


Figure 1.10: Crystal structure of the antigen binding fragment (Fab) of neutralizing antibody PG16. Heavy and light chains are represented in tan and blue respectively and the CDR H3 region is shown in red. The variable regions comprise the top half of the image and the constant regions comprise the bottom.

A single N-linked glycan is shown by the green stick representation. Figure copied from (Pancera, McLellan et al. 2010).

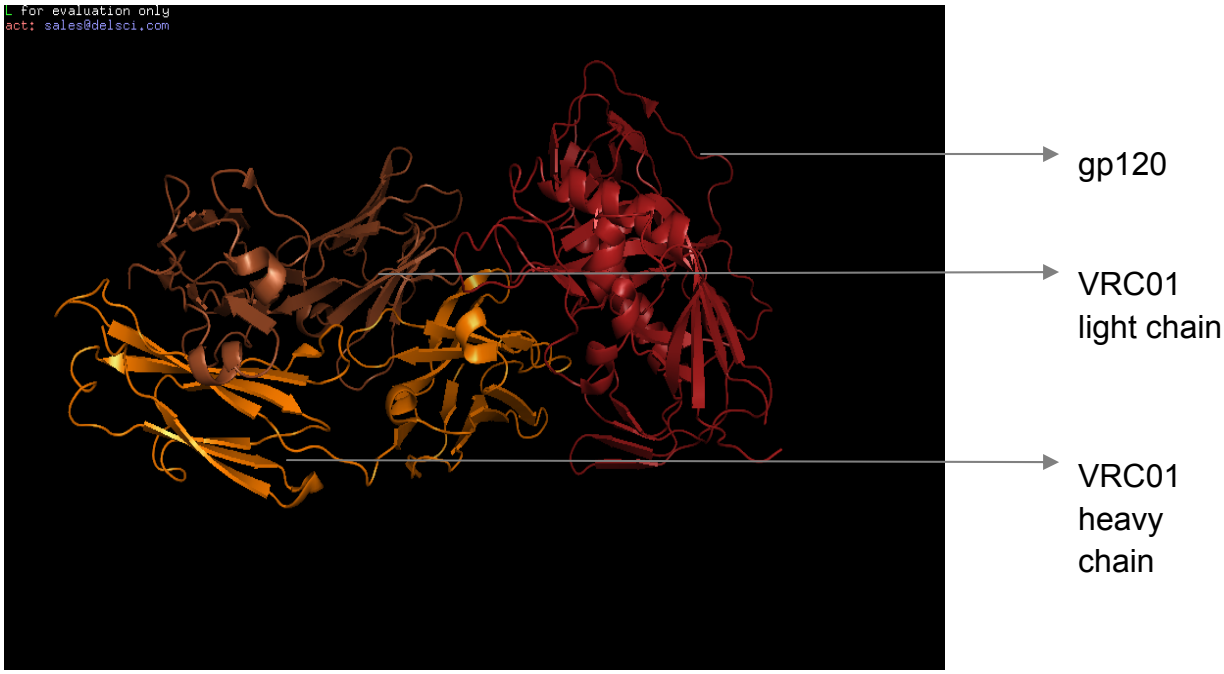


Figure 1.11: Crystal structure of gp120 complexed with the neutralizing antibody, VRC01. Figure adapted from (Zhou, Georgiev et al. 2010).

The monoclonal antibody 2G12 has a unique structure in which the arms of the variable heavy chain domains are swapped (Figure 1.12), and is unique in its ability to target gp120's non immunogenic glycan shield (Trkola, Purtscher et al. 1996) (Figure 1.12). 2G12 recognizes glycans at residues 332 and 392 and is dependant on glycans at positions 295, 339 and 386 (Scanlan, Pantophlet et al. 2002). 2G12 has been shown to bind to Manα1-2Man at the D1 and D3 terminals of an oligomannose sugar on the gp120 silent face (Calarese, Lee et al. 2005).

Similar to IgG1b12, 2G12 induces little conformational changes when bound to its epitope (Kwong, Doyle et al. 2002).

2G12 was tested in a neutralization assay against pseudoviruses representing subtypes A, B, C, D, F, AC and AE as well as 25 primary viral isolates in order to determine the neutralization breadth of the antibody (Binley, Wrin et al. 2004). Results revealed that 2G12 could effectively neutralize 41% of the tested viruses none of which were from subtype C or E (Binley, Wrin et al. 2004). The fact that 2G12 does not exhibit cross neutralization against subtype C isolates may be due to the lack of a glycan at position 295 at the N-terminal base of the V3 loop in subtype C viruses (Binley, Wrin et al. 2004).

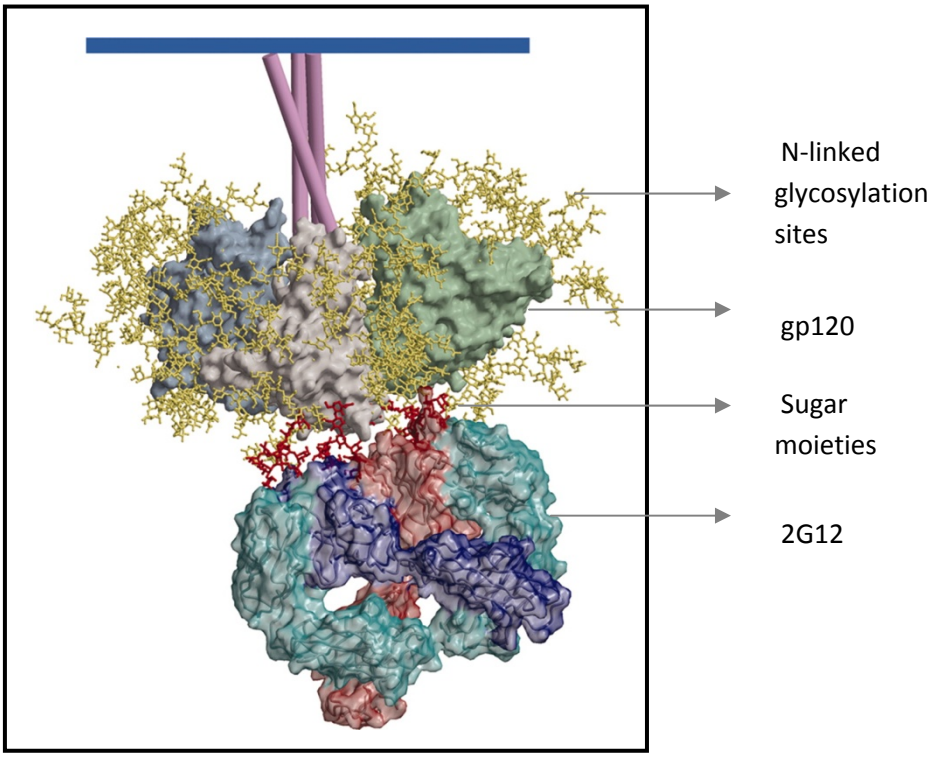


Figure 1.12: Schematic diagram of 2G12 bound to the Env spike. The heavy chains of 2G12 heavy chains are shown in dark blue and light red and the light chains are shown in light blue. Sugar moieties are indicated in dark red. The

gp120 trimer is shown in dark grey, light grey and green with potential N-linked glycosylation sites depicted in yellow. The gp41 is shown in purple with the viral membrane depicted in blue. Figure adapted from (Burton, Stanfield et al. 2005).

2F5, 4E10 and Z13 are potent cross reactive neutralizing antibodies which recognize overlapping epitopes in the conserved membrane proximal external region (MPER) at the base of gp41 (Muster, Steindl et al. 1993; Buchacher, Predl et al. 1994; Zwick, Labrijn et al. 2001) (Figure 1.13). 2F5 recognizes the epitope ELDKWA and the DKW motif is the minimum core requirement for 2F5 neutralization (positions 664-666 on gp41) (Stiegler, Kunert et al. 2001). 2F5 effectively neutralizes isolates from subtypes A, B, D and E however shows poor neutralization capacity against subtype C isolates, possibly due to the fact that most subtype C viruses have a DSW motif in place of DKW (Purtscher, Trkola et al. 1996; Stiegler, Kunert et al. 2001). 4E10 was previously described to recognize the NWFDIT epitope; however Binley et al. (2004) showed that the minimum requirement for 4E10 neutralization is the WFXI motif. The authors also confirmed that 4E10 has potent cross-reactive neutralization activity against 90 viruses from subtypes A, B, C, D, F, G, J and AC, AE, AG, BF and BG recombinants. The extensive cross reactive neutralization capacity of 2F5 was confirmed by its ability to recognize and bind to 31 variations of the MPER (Bryson, Julien et al. 2009). 2F5 also exhibits faster binding to the MPER and is more thermodynamically favourable compared to 4E10 binding (Dennison, Stewart et al. 2009); the slow binding of 4E10 could be attributed to the membrane immersion depth of its epitope compared to the 2F5 epitope (Dennison, Stewart et al. 2009). Dennison et al. (2009) showed that the neutralization breadth of antibodies targeting the MPER

is dependant of the membrane immersion depth of their epitopes. Z13 was isolated in 2001 from an antibody phage display library and was shown to recognize an epitope similar to that of 4E10 but with the ability to neutralize a limited set of primary viruses (Zwick, Labrijn et al. 2001). Recently, a high affinity variant of Z13 designated Z13e1 was isolated recognising an epitope overlapping that of 2F5 and 4E10 (WASLWNWFDITN) and exhibited about 100-fold better affinity for the MPER as well as enhanced neutralization capacity against sensitive strains of HIV-1 (Nelson, Brunel et al. 2007).

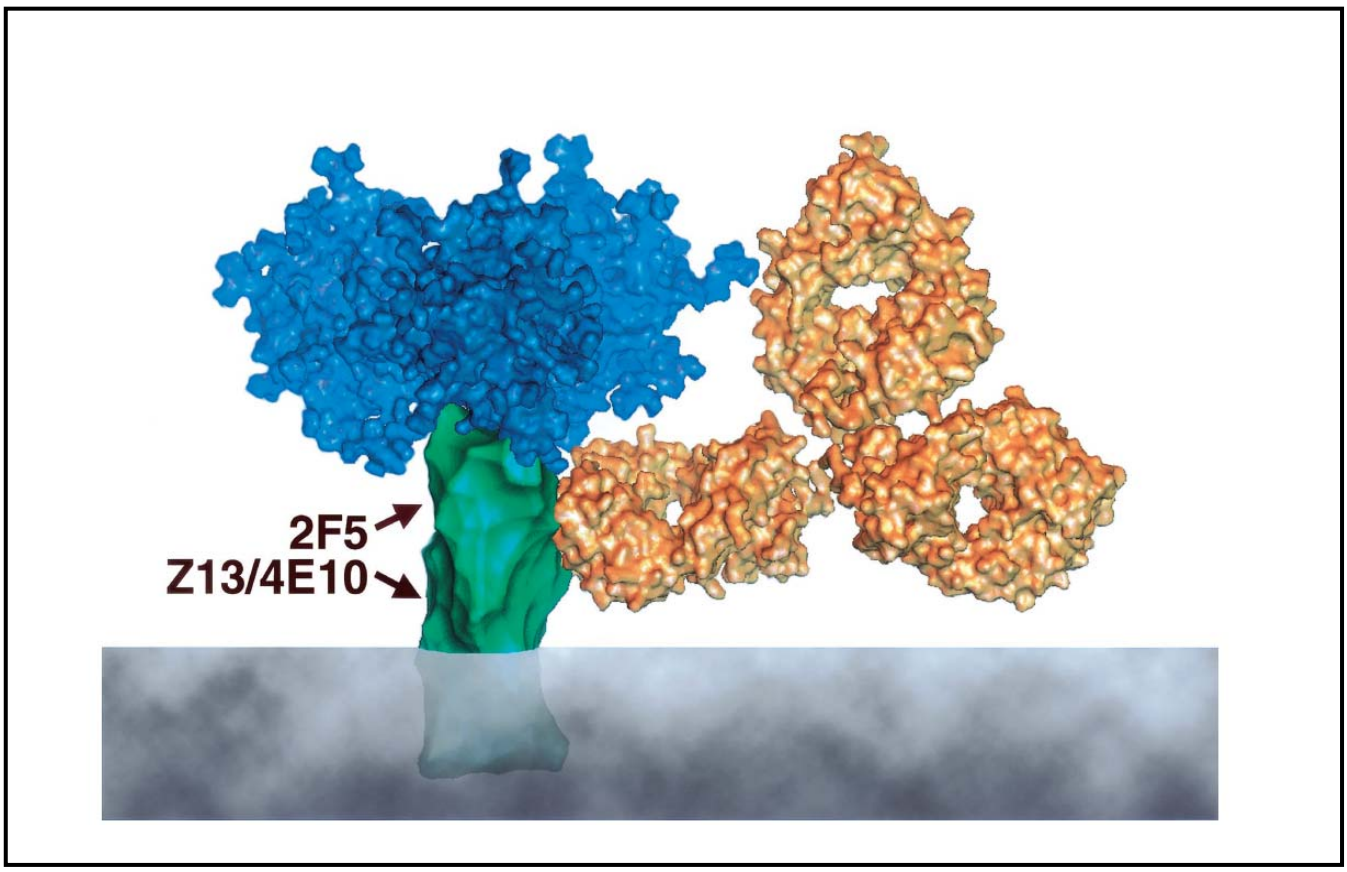


Figure 1.13: Animated model of the HIV-1 trimeric envelope spike. The HIV-1 gp120 is illustrated in blue and the membrane proximal external region (MPER) of gp41 is depicted in green. The 2F5, 4E10 and Z13 recognition sites are also

indicated. An IgG molecule, represented in orange is shown to scale in proximity to the indicated antibody binding regions. Figure copied from (Zwick, M.B et al. 2001).

There are several antibodies that have been well described that exhibit some degree of neutralization against HIV-1. Previously, the V3 loop of gp120 was referred to as the principle neutralizing domain/epitope (Hwang, Boyle et al. 1991). However this was only true for laboratory adapted viruses (Hwang, Boyle et al. 1991). Primary virus isolates have been shown to sequester the V3 region making it inaccessible to neutralizing antibodies, therefore antibodies directed against the V3 loop are generally type specific with limited neutralization capacity (Bou-Habib, Roderiquez et al. 1994).

The anti-V3 neutralizing antibody 447-52D shows moderate neutralization breadth by interacting with a conserved GPGR motif in subtype B isolates via its long CDR H3 loop in a sequence independent manner (Conley, Gorny et al. 1994). However, for CCR5-utilizing viruses, the V3 loop is poorly immunogenic before CD4 binding therefore 447-52D has limited neutralization breadth against these isolates (Lusso, Earl et al. 2005). Interestingly, 447-52D has been shown to neutralize 45% of subtype B viruses but only 7% of other subtypes (A, B, C, D, F, AC and AE as well as 25 primary viral isolates) (Binley, Wrin et al. 2004). Neutralization of non-B subtypes largely depends on the presence of the GPGR motif (Binley, Wrin et al. 2004).

Another monoclonal antibody 2219 (Gorny, Williams et al. 2002) recognizes the V3 loop but with less efficiency than 447-52D. Unfortunately, HIV-1 is capable of escaping recognition by both these antibodies by reducing its accessibility to the extent where 447-52D and 2219 cannot recognise their epitopes anymore (Gorny, Williams et al. 2002). The monoclonal antibody, 17b recognizes CD4 induced (CD4i) epitopes, in particular the gp120 bridging sheet essential for gp120-co-receptor binding (Zhang, Godillot et al. 2001) (Figure 1.14). Unfortunately 17b is not able to effectively neutralize a diverse range of isolates. This could be due to the fact that the co-receptor binding site is only transiently exposed on gp120 during viral fusion and therefore access to the 17b epitope is limited (Labrijn, Poignard et al. 2003). Although CD4i antibodies are elicited in most HIV-1 infected individuals, they are unable to access their epitopes before CD4 binding and are therefore poorly neutralizing (Labrijn, Poignard et al. 2003; Decker, Bibollet-Ruche et al. 2005).

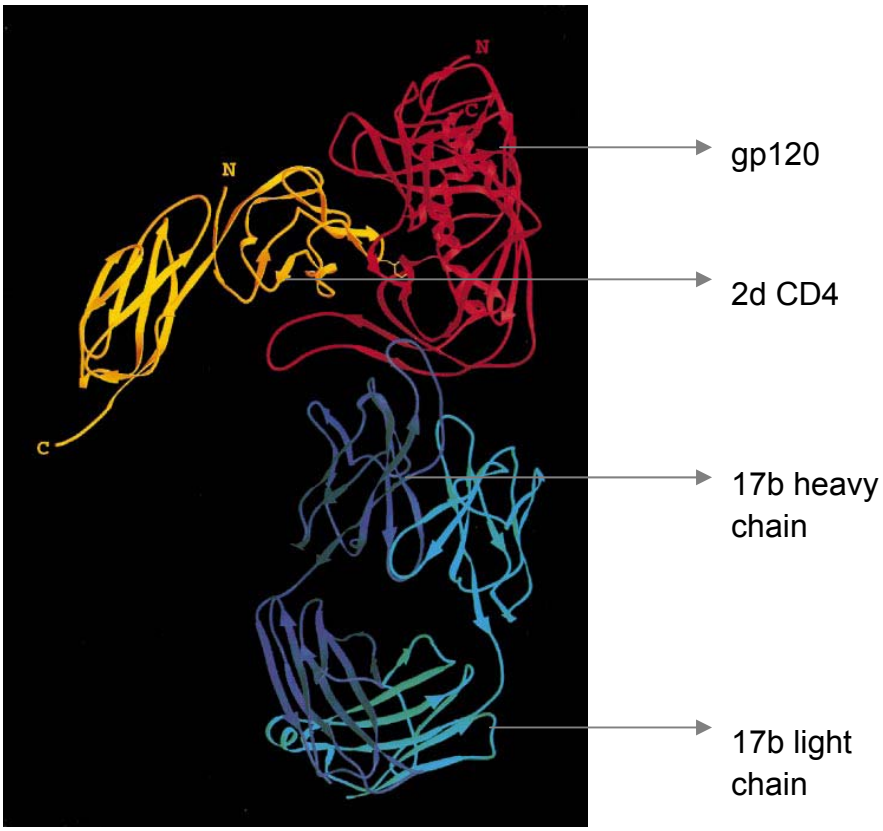


Figure 1.14: Ribbon diagram of gp120 complexed with neutralizing antibody 17b. Figure adapted from (Kwong, Wyatt et al. 1998).

1.4.4 Antibodies as therapeutics

Neutralizing antibodies are highly effective in controlling HIV-1 replication *in vitro.* To have a better understanding of the role of neutralizing antibodies in controlling HIV-1 infection *in vivo*, passive immunization studies have been conducted using animal models and more recently in human clinical trials. Conley et al. (1996) demonstrated the ability of the monoclonal antibody 2F5 to partially protect chimpanzees against intravenous challenge with a primary virus. In this study, 2 chimpanzees were intravenously infused with the 2F5 monoclonal antibody and subsequently challenged with a primary HIV-1 isolate. Among the control

chimpanzees which received no antibody, infection was established immediately and seroconversion occurred within 4 weeks. By contrast, seroconversion occurred by week 14 in the 2F5 infused chimpanzees (Conley, Kessler et al. 1996). Using non human primate models to study the role of neutralizing antibodies in HIV-1 infection is difficult because most HIV-1 primary isolates do not infect non human primates. Mascola et al. (1999) demonstrated the use of a simian/human immunodeficiency virus (SHIV) using the Env of a primary isolate (HIV-89.6). HIV-1 immune globulin (HIVIG) as well as 2 monoclonal antibodies 2F5 and 2G12 were passively infused into rhesus macaques in a double dose (2F5,2G12) or triple dose (HIVIG, 2F5, 2G12) 24 hours before viral challenge (Mascola, Lewis et al. 1999). Out of 6 macaques that received the triple dose, 3 were completely protected from viral challenge and 2 out 3 macaques that received the double dose showed reduced viral loads (Mascola, Lewis et al. 1999).

The passive immunization of monoclonal antibodies in animals has provided useful information of the control of these antibodies *in vivo*. However human studies are still needed to evaluate the safety and tolerability of these antibodies in humans. The broadly neutralizing monoclonal antibodies, 2F5, 2G12 and 4E10 were evaluated for safety and tolerability in humans during a phase I clinical trial (Armbruster, Stiegler et al. 2002). These 3 monoclonal antibodies were subsequently used in a proof-of-concept study using human subjects (Trkola, Kuster et al. 2005). This study demonstrated that high doses of a cocktail of 3 monoclonal antibodies (2F5, 2G12 and 4E10) resulted in delayed viral rebound in patients who already had suppressed viral loads by ART before the passive administration of the antibodies (Trkola, Kuster et al. 2005). Eight chronically

infected and 6 acutely infected patients were included. Interestingly, most of the inhibitory effects of the cocktail could be attributed to 2G12. 2G12 was found to have a significantly slower systemic clearance than 4E10 or 2F5 (Trkola, Kuster et al. 2005). The plasma samples from this study were later assessed to determine estimates for effective neutralization titers (Trkola, Kuster et al. 2008). The contribution of each monoclonal antibody to the total neutralization activity in patients was assessed. Their findings suggested that the stage of HIV-1 infection influenced the quantities of neutralizing antibody needed. For example, lower neutralizing antibody levels showed an effect in acute infection compared to chronic infection (Trkola, Kuster et al. 2008). These findings suggest that a therapeutic antibody-based vaccine may be more efficient if administered early in HIV-1 infection.

In another proof-of-concept study 3 monoclonal antibodies (2G12, 2F5 and 4E10) were passive administered to 10 HIV-1 infected individuals who received ART during acute or chronic infection (Mehandru, Vcelar et al. 2007). 2G12, 2F5 and 4E10 were intravenously administered at equal concentrations. Results showed that 8 out of 10 patients experienced viral rebound and 2 patients remained aviremic throughout the study. Seven out of 8 patients with viral rebound showed resistance to 2G12 and the depletion of CD4 T-cells (Mehandru, Vcelar et al. 2007).

1.4.5 Autoreactivity of broadly Neutralizing antibodies and other anti-HIV-1 antibodies

The fact that antibodies to the MPER can be elicited in some individuals is noteworthy, since the 2F5 and 4E10 epitopes are in close proximity to the host plasma membrane. Therefore, such antibodies could show auto reactivity and should be negatively selected for during B-cell differentiation (Haynes, Fleming et al. 2005). 2F5, 4E10 and Z13 do show some degree of auto reactivity to a host phospholipid, cardiolipin (Golding, Robey et al. 1988; Haynes, Fleming et al. 2005). Anti-cardiolipin (aCL) antibodies are observed in patients with the auto immune disorder antiphospholipid syndrome (APS) (Mackworth-Young, Loizou et al. 1989). They are also found in HIV-1 infected patients with systemic lupus erythematosus (SLE) (Petrovas, Vlachoyiannopoulos et al. 1999). 4E10 has also been shown to react with SLE auto antigen SS-A/Ro.B12 as well as double-stranded DNA (dsDNA), centromere B, and histones (Haynes, Fleming et al. 2005; Haynes, Moody et al. 2005). This has been hypothesized to be a result of the cross reactivity between self and non-self antigens (Haynes, Fleming et al. 2005), or molecular mimicry (Deas, Liu et al. 1998).

B-cell tolerance is controlled in $CD4^+$ T-lymphocytes. T-dependent antigens require the recognition of both T- and B-cells in order for an effective humoral immune response to occur and the appropriate antibody to be released. During the maturation process of T-cells in the thymus and B-cells in the bone marrow, they are exposed to host antigens for positive and negative selection. Immune cells that are able to recognize host antigens can induce tolerance (Starr, Jameson et al.

2003) however during HIV-1 infection, thymic function is severely impaired (Douek, McFarland et al. 1998). This may provide a possible explanation for the production of anti-self antibodies.

A study by Bermas et al. (1994) showed that sera from HIV uninfected individuals with SLE had antibodies directed against conserved regions of gp120. In addition, another study showed that a single chain antibody fragment isolated from a lupus antibody phage library had the ability to bind to the conserved CD4 binding site on gp120. The antibody fragment also showed good neutralizing activity against X4 and R5 HIV-1 strains from subtypes B, C and D (Karle, Planque et al. 2004). Interestingly, It has also been documented that the light chain portion of antibodies isolated from lupus patients can neutralize gp120 (Nishiyama, Karle et al. 2007).

Detection of 2G12-like antibodies was not associated with any auto antibodies however detection of the broadly neutralizing antibody IgG1b12 was associated with the presence of aCL and anti cytoplasmic antibodies (Martinez, Diemert et al. 2009). During the budding process by which HIV-1 exits the cell, HIV-1 takes certain host proteins with it from the lipid membrane, therefore antibodies against self antigens found in patients with autoimmune diseases may cross react with HIV-1 Env proteins found close to the host derived viral membrane such as the 2F5, 4E10 and Z13 epitopes (Scherl, Posch et al. 2006).

The auto reactivity of the above-mentioned broadly neutralizing antibodies raises concern regarding the elicitation of such antibodies through a vaccine incorporating the Env glycoprotein and provides a possible explanation for the rare

occurrence of these antibodies during natural infection (Gray, Taylor et al. 2009; Martinez, Diemert et al. 2009). One broadly neutralizing antibody in particular, m44 targets an epitope within the gp41 region and is of special interest because it can neutralize diverse strains of HIV-1 isolates without any reactivity to self antigens such as cardiolipin (Zhang, Vu et al. 2008). From these findings, it is evident that patients with autoimmune diseases are worthwhile investigating further for the presence of broadly neutralizing antibodies against HIV-1.

1.4.6 Features of Env that make it difficult to elicit broadly neutralizing antibodies

HIV-1 employs various different escape mechanisms which allow the virus to avoid and escape antibody recognition. Firstly, the conformational flexibility of the gp120 creates thermodynamic and kinetic barriers to neutralization and complex tertiary and quaternary structures renders a large portion of the functional Env trimer inaccessible to these antibodies (Wyatt and Sodroski 1998).

The extensive glycosylation of gp120 which masks conserved epitopes renders a large portion of the protein poorly immunogenic, and the re-arrangement of the glycan shield without altering the functionality of Env, allows for antibody escape (Wei, Decker et al. 2003). The CD4 binding site and co-receptor binding sites are shielded by variable regions of the Env. While these immunogenic regions are targeted by neutralizing antibodies during the course of infection the virus is however able to continuously escape antibody responses by sequence diversity (Burton, Stanfield et al. 2005). In addition, there is extensive sequence diversity as

a result of the error prone reverse transcriptase enzyme. This results in non synonymous changes in important epitopes leading to amino acid substitutions, insertions and/or deletions to avoid antibody recognition. In addition there is sequence diversity among different subtypes of HIV-1, with up to 35% of Env sequence diversity between the different HIV-1 subtypes (Gaschen, Taylor et al. 2002).

Furthermore, due to the labile gp120-gp41 interaction, monomeric gp120 is shed from the virion and acts as a major immunogen *in vivo*, eliciting high titers of non-neutralizing or binding antibodies by exposing epitopes that are buried or not formed on the functional trimeric Env spike (Rong, Li et al. 2009). In addition, functional epitopes that could inhibit gp120-CD4 interactions or gp120-co-receptor interactions and epitopes exposed during viral fusion are only transiently exposed, thus not allowing sufficient time for the production of the appropriate antibody.

1.5. Vaccine development

Numerous attempts have been made in the development of an HIV-1 vaccine but have been met with little success. Previous traditional vaccine strategies have focused on live attenuated viruses, whole killed viruses and protein subunits (Baba, Liska et al. 1999; Learmont, Geczy et al. 1999). These approaches have proven successful against other viruses such as the influenza virus but raise great safety concerns with regard to HIV-1. More recent vaccine strategies have made use of gene delivery technologies such as plasmid DNA vaccines or recombinant viral vectors expressing HIV-1 antigens (Casimiro, Chen et al. 2003). Unfortunately, plasmid DNA vaccines require high doses in humans in order to

elicit effective immune responses (Graham, Koup et al. 2006). Therefore recent HIV-1 vaccine candidates comprise recombinant viral vectors (attenuated or replication-incompetent viruses) such as adenoviruses (Priddy, Brown et al. 2008) or poxviruses (Harari, Bart et al. 2008).

The international AIDS vaccine initiative (IAVI) is an organization that aims to develop an effective HIV-1 vaccine and ensure its delivery to countries where it is most needed such as Sub Saharan Africa and Asia (Chataway and Smith 2006). IAVI funds potential vaccine candidates at the developmental stage as well as clinical trials. Currently, IAVI operates in 22 countries and works closely with it's scientific partners who perform research on the development of an HIV-1 vaccine (Chataway and Smith 2006). Recently, in October 2010 IAVI announced the start of a phase I prime/boost clinical trial known as B003/IPCAVD-004 (IAVI 2010). This trial aims to evaluate the safety and immunogenicity of the 2 vaccine candidates (B003) and (IPCAVD-004) in a regimen where vaccines will be given sequentially in an attempt to boost immune responses to HIV-1. The vaccine candidates comprise adenoviral vectors (serotype 26 and 35) containing the Env gene. The study aims to include 212 healthy participants between the ages of 18 and 50 (IAVI 2010). An overview and update of all HIV-1 vaccine human clinical trials to date can be found on http://www.iavi.org.

A vaccine trial by Merck made use of a candidate which comprised a recombinant adenovirus vector that expressed HIV-1 subtype B *gag, pol* and *nef* genes (Priddy, Brown et al. 2008). However participants with pre-existing antibodies against the adenovirus vector showed suppressed immune responses to the vaccine

(Kostense, Koudstaal et al. 2004). Phase 2b proof of concept studies were subsequently initiated to evaluate 3000 subjects for HIV-1 specific cellular immune responses elicited by this vaccine regimen. This was known as the STEP study and was conducted in America, Caribbean and Australia (McElrath, De Rosa et al. 2008). Unexpectedly, this study was brought to an early halt due to safety concerns. It was hypothesized that the recombinant adenovirus vector may have increased the acquisition of HIV-1 infection in some individuals (Sekaly 2008; Watkins, Burton et al. 2008).

The first efficacy trial (Vax004) began in 1998. The Vax004 trial was aimed at determining the efficacy of a recombinant bivalent subtype B gp120 vaccine (AIDSVAX) in preventing sexual transmission of HIV-1 in North America and the Netherlands, where subtype B infections are prevalent (Berman 1998). The trial included 5108 men who have sex with men (MSM) and 309 high risk women. Although the AIDSVAX vaccine elicited strong antibody responses, it failed to protect against HIV-1 infection and had no effect on the viral loads of participants who acquired HIV-1 infection after vaccination (Gilbert, Ackers et al. 2005). Interestingly, a renewed interest in the Vax004 trial began last year (Gilbert, Wang et al. 2010). Gilbert et al (2010) analysed the neutralizing antibody responses in the participants of the Vax004 trial against a panel of very sensitive (tier 1) and moderately sensitive (tier 2) HIV-1 strains. Results revealed that the participants produced high titers of neutralizing antibodies against the tier 1 viruses but little or weak antibody responses against the panel of tier 2 viruses. The authors reported that the overall lack of tier 2 virus neutralization was consistent with the lack of antibody protection in the Vax004 trial.

The latest results from the RV144 trial (using a canary pox viral vector expressing gp120, *gag* and p*ol* to prime immune responses followed by the AIDSVAXgp120 vaccine to boost the immune response) showed, for the first time, a statistically significant 30% protective efficacy (Rerks□Ngarm, *et al*., 2009). This suggests that an effective HIV-1 vaccine should include components that elicit responses in both arms of the immune system.

Although it has been a great challenge to elicit effective broadly neutralizing antibody responses in humans through vaccination, progress has been made in understanding the Env structure, viral escape mechanisms and the mechanisms by which identified rare neutralizing antibodies exhibit cross reactivity (Hoxie 2010). The RV144 Thai trial provided scientists with some hope that a preventative HIV-1 vaccine is possible (Rerks-Ngarm, Pitisuttithum et al. 2009). The exact mechanism of protection in this trial is not clearly defined however it is believed by some that the low level of protection may be a result of a combination of antibodies including neutralizing antibodies, antibodies that mediate ADCC, antibodies that mediate the induction of β chemokines or binding antibodies and CD4i antibodies (Rerks-Ngarm, Pitisuttithum et al. 2009). The results from previous vaccine studies highlights the urgent need to better understand the humoral immune response towards HIV-1 for more effective vaccine design in the future. While broadly neutralizing antibodies are infrequently elicited among infected individuals for reasons not clearly defined, B-cell tolerance mechanisms and viral escape mechanisms are however believed to play a major role. A better

understanding of why this occurs would assist in eliciting such responses through vaccination (Srivastava, Ulmer et al. 2005).

Another promising approach for identifying a vaccine candidate is to dissect the natural immune response in individuals who are able to control HIV-1 infection such as slow progressors. Although the mechanism of control among slow progressors remains elusive, several studies provide possible explanations. A study showed that slow progressors have decreased Env function resulting in reduced replication capacity, possibly contributing to viral suppression (Lassen, Lobritz et al. 2009). Another study (Chien, Chen et al. 2004) demonstrated that antibodies directed against the CD4 binding site on gp120 interferes with the presentation of gp120 to T-helper cells and hence further weakens the immune system of HIV-1 infected individuals. Interestingly they showed that slow progressors have very little or no anti-CD4 antibodies compared to rapid progressors who have higher anti-CD4 antibody responses (Chien, Chen et al. 2004). Host genetic factors may also contribute to delayed disease progression such as HLA polymorphisms or co-receptor polymorphisms (Roger 1998).

1.6. Relevance of this study

The identification of few broadly neutralizing antibodies to date have provided valuable information of conserved epitopes as potential vaccine targets. However, HIV-1 specific broadly neutralizing antibody responses are rarely elicited during natural infection, illustrating the poor immunogenicity of their epitopes. Some individuals however, produce neutralizing antibodies capable of inhibiting diverse

viral isolates across different subtypes. A better understanding of these individuals may be useful in providing information for vaccine development aimed at eliciting similar immune responses (Binley 2009). The auto-reactivity of 2F5, 4E10 and IgGb12 may explain why these antibodies are rarely produced in HIV infected individuals with B-cell tolerance mechanisms preventing their production. If this is the case, then patients with auto immune diseases display some protection against HIV-1 (Haynes, Fleming et al. 2005).

None of the above mentioned neutralizing antibodies were derived from HIV-1 subtype C infected individuals and efforts to generate monoclonal antibodies from these individuals have lagged. Three identified broadly neutralizing antibodies show less potency against subtype C viral isolates. For example, IgG1b12 effectively neutralizes 75% of subtype B isolates and less than 50% of non-subtype B strains (Binley, Wrin et al. 2004), and 2G12 and 2F5 have limited neutralization capacity against subtypes A and C (Gray, Meyers et al. 2006; Blish, Nedellec et al. 2007). It is therefore important to systematically screen for the presence of HIV-1 subtype C specific neutralizing antibody responses among infected patients, and to generate HIV-1 specific broadly neutralizing monoclonal antibodies.

The overall objective of this study was to identify individuals with broad cross-reactive neutralizing antibodies against HIV-1 subtype C from a range of four different cohorts. These included HIV-1 negative and positive patients with autoimmune diseases, antiretroviral (ARV) drug-naïve AIDS patients, HIV-1

positive patients failing first line therapy and HIV-1 positive patients classified as slow progressors.

This was achieved by the following aims**:**

1.6.1. To collect, isolate and store serum, plasma, peripheral blood mononuclear cells and matched bone marrow from participants with autoimmune disease (HIV-1 seronegative or seropositive).

1.6.2. To collect plasma samples from HIV-1 infected slow progressors, ARV drug naïve patients and HIV-1 positive patients failing first line therapy.

1.6.3. To establish a pseudovirion-based neutralizing antibody assay.

1.6.4. To screen the serum and plasma samples from all four cohorts for the presence of greater than average breadth of antibody neutralization using the pseudovirion-based neutralizing antibody assay.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study population

A total of 40 patient samples were collected or available for the purposes of this study. These included participants from a CT cohort (9), FV cohort (11), DR cohort (10) and JM cohort (10). The available demographic and clinical data of the participants is shown in Table 2.1.

The CT cohort included plasma samples from nine HIV-1 seropositive slow progressor patients obtained from Professor Caroline Tiemessen (National Institute for Communicable Diseases). Eleven HIV-1 subtype C seropositive plasma samples from antiretroviral (ARV) drug naive patients comprised the FV cohort and were available for use in the laboratory (Connell, Michler et al. 2008). Ten plasma samples from HIV-1 subtype C seropositive ARV treatment experienced patients who were failing first line therapy were available for use in the laboratory and made up the DR cohort.

The JM cohort consisted of peripheral blood with matching bone marrow samples obtained from patients referred to the Haematology Department at the Charlotte Maxeke Johannesburg Academic Hospital for bone marrow aspiration as part of their routine diagnostic workup. Ten participants were selected over a period of 12 months (2009) according to the following inclusion criteria; *Participants must have a clinical indication for performing a bone marrow aspirate procedure. Participants must have their HIV serostatus already established, Participants must have a result of autoimmune screen or have had clinical assessment of autoimmune disease status, and participants must be willing to sign an informed consent form.*

Bone marrow aspirations were performed as per standard procedure by Professor Johnny Mahlangu and an additional 5 ml of bone marrow was collected into a PAXgene blood tube (Qiagen, Maryland, USA) or Ethylene Diamine Tetra acetic Acid (EDTA) blood tube for the study. Five millilitres of matching peripheral blood was collected into EDTA and serum separating tubes (SST). The blood and matching bone marrow samples were transported to the laboratory at room temperature.

2.1.1 Ethics

Informed Consent was obtained from each study participant prior to sample collection. Ethics clearance was obtained from the University of the Witwatersrand committee for research on human subjects (protocol number M090547, (Appendix A.1).

Table 2.1: Epidemiological and clinical data from the participants used in this study

Cohort name	Participant sample name	Age in years/Sex	CD4 count (cells/µl)	Viral load (RNA copies/ml)	Clinical
CT	SP1	M	384	49000	HIV positive. Asymptomatic
	SP2	F	327	2772	HIV positive. Asymptomatic
	SP3	M	904	240	HIV positive. Asymptomatic
	SP4	M	1130	<40	HIV positive. Asymptomatic
	SP5	F	511	25500	HIV positive. Asymptomatic
	SP6	M	267	<400	HIV positive. Asymptomatic; ART treatment (Truvada and Efavirenz)
	SP7	M	426	71700	HIV positive. Asymptomatic
	SP8	M	728	<400	HIV positive. Asymptomatic
	SP9	F	1059	52	HIV positive. Asymptomatic
FV	FV2	46/M	159	22900	HIV positive. Asymptomatic. ART naive
	FV3	28/F	101	N/A	HIV positive. Oral candidiasis; HSV; retinal necrosis. ART naïve
	FV5	43/M	133	750 000	HIV positive. Previous T.B. ART naïve
	FV6	28/M	187	179 000	HIV positive. Asymptomatic. ART naive
	FV10	32/M	6	750 000	HIV positive. T.B, on T.B treatment. ART naive
	FV11	38/M	124	750 000	HIV positive.Previous TB. ART naïve
	FV14	24/F	16	N/A	HIV positive. ART naive
	FV15	33/F	122	343 000	HIV positive. previous TB. ART naive
	FV23	34/F	108	37 000	HIV positive. Asymptomatic. ART naïve
	FV26	49/F	N/A	N/A	HIV positive. Asymptomatic. ART naïve
	FV27	29/F	11	158 000	HIV positive. ART naive
DR	FVDR1	59/F	280	64100	Failing first line treatment regimen: Stavudine (d4T), lamivudine (3TC) and nevirapine (NVP) or Efavirenz (EFV)
	FVDR4	43/F	54	6700	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
	RES5	27/F	118	353 000	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
	RES6	40/M	53	30800	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
	RES7	41/F	43	74300	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
	RES8	37/F	112	9320	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
	RES10	46/F	57	>750 000	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
	RES12	60/F	301	6680	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
	RES13	N/A	N/A	N/A	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
	RES14	31/F	105	8880	Failing first line treatment regimen; d4T, 3TC, NVP or EFV
JM	TN1	39	660	Below detection	Thrombocytopenia
	TN2	20	204	6000	HIV positive. Thrombocytopenia
	TN3	48	155	12565	HIV positive. Thrombocytopenia
	TN4	50	45	94500	HIV positive. Thrombocytopenia,
	TN5	28	N/A	N/A	HIV positive. Pancytopenia
	TN6	36	101	N/A	HIV positive. Rheumatoid arthritis,bicytopenia
	TN7	N/A	N/A	N/A	N/A
	TN8	44	255	Below detection	HIV positive
	TN9	39	587	Below detection	HIV positive. Bicytopenia
	TN10	25	55	35000	HIV positive. APS, cytopenias

2.2 Reagents used throughout the course of this study

2.2.1 Reagents obtained from the NIH AIDS Research and Reference Reagents Program

Twelve subtype C and two subtype B envelope glycoprotein (Env*)* plasmids together with a backbone plasmid were obtained from the NIH AIDS Research and Reference Reagents Program (Table 2.2). All recombinant plasmids carried resistant genes for ampicillin and were expanded by transformation into competent *Escherichia coli* (*E. coli)* DH5α cells (see section 2.4.1.2).

Human HIV-1 neutralization serum (catalogue number 1984) and Human HIV-1 negative serum (catalogue number 2411) samples were obtained from the NIH AIDS Research and Reference Reagents Program, and reconstituted in 0.25ml distilled water for use as controls in the pseudovirion neutralizing antibody assay.

Table 2.2: Env plasmids and the backbone vector used for the production of pseudoviruses.

VIRUS NAME	CLONING VECTOR	SUBTYPE	CAT NO	DISSOLVED IN
CAP45	pcDNA3.1/V5-His©TOPO	C	11316	TE Buffer
CAP210	pcDNA3.1D/V5-His TOPO©	C	11317	TE Buffer
ZM53	pCR3.1	C	11313	TE Buffer
ZM109	pCR3.1	C	11314	TE Buffer
ZM135	pCR3.1	C	11315	TE Buffer
ZM197	pCDNA3.1D/V5-His TOPO	C	11309	TE Buffer
ZM214	pCDNA3.1D/V5-His TOPO	C	11310	TE Buffer
ZM233	pCDNA3.1D/V5-His TOPO	C	11311	TE Buffer
ZM249	pcDNA3.1/V5-His©TOPO	C	11312	TE Buffer
Du156	pcDNA3.1/V5-His©TOPO	C	11306	TE Buffer
DU172	pcDNA3.1D/V5-His TOPO	C	11307	TE Buffer
DU422	pcDNA3.1D/V5-His TOPO©	C	11308	TE Buffer
B15	pcDNA3.1(+)	B	11037	TE Buffer
B11	pcDNA3.1D/V5-His TOPO©	B	11022	TE Buffer
pSG3$^{\Delta env}$	pTZ19U	Backbone plasmid	11051	TE Buffer

2.2.2 Cell lines

HEK 293T cells and TZM-bl cells were available for use in the laboratory. HEK 293T cells are derived from human embryonic kidney cells and were used for the production of Env-pseudoviruses. The TZM-bl cell line is a HeLa cell clone which is engineered to stably express CD4, CCR5 and CXCR4, and was previously obtained from the NIH AIDS Research and Reference Reagents Program (catalogue number 8129). This cell line also contains a luciferase reporter gene that is linked to the HIV-1 LTR region and is under the control of HIV-1 *tat*. Therefore the expression of luciferase is driven by a production HIV-1 infection. The TZM-bl cell line was used to measure the neutralizing antibody activity of patient plasma/serum in the pseudovirion neutralizing antibody assay.

2.2.3 South African National Blood Services (SANBS) plasma samples

Eighteen HIV-1 seropositive plasma samples were purchased from the SANBS. Plasma samples were heat inactivated in a 56°C water bath for an hour and the eighteen samples were screened in the pseudovirion neutralizing antibody assay. The sample with the best neutralizing antibody activity was subsequently used as the positive control throughout the study.

Blanket ethics consent was obtained from the SANBS for the use of the plasma samples and therefore no other ethics was required.

2.3 Participant sample processing

2.3.1 CT cohort

In order to determine whether optimal results would be obtained from heat inactivation of plasma samples, 200µl from each plasma sample was removed and stored until used at -80°C. The remaining volumes of plasma were heat inactivated (described in section 2.2.3) and stored at -80°C for use in the pseudovirion neutralizing antibody assay.

2.3.2 FV cohort and DR cohort

Plasma samples were heat inactivated as previously described in section 2.2.3 and stored at -80°C until used.

2.3.3 JM cohort

Prior to processing the blood and bone marrow samples, 3 ml of Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) was added to 15 ml UNI-SEP tubes (Novamed, Indiana, USA) and centrifuged at 400xg for one minute at 4°C. All patient blood vials were centrifuged at 290xg for 10 minutes at 4°C. During this time, sterile cryovials (Nunc, Roskilde, Denmark) were prepared and labelled as: serum, $EDTA_{PLASMA}$, $EDTA_{PBMC}$ and $bone\ marrow_{PLASMA}$ or $bone\ marrow_{PBMC.}$ After centrifugation, the serum (SST tubes) and plasma (EDTA tubes) portion was carefully removed and aliquoted into the relevant cryovials (Nunc). The buffy coat

layer from the EDTA tubes was then transferred into a separate 15 ml UNI-SEP tube (Novamed) and diluted 1:1 with complete RPMI-1640 media (Appendix B.1.1) (Sigma-Aldrich, Missouri, USA) and centrifuged at 1000xg for 20 minutes at 18°C. The contents were then transferred into a 15 ml tube (Nunc) and 10 ml of RPMI-1640 media was added to the contents of the tube (Appendix B.1.1) (Sigma-Aldrich) and centrifuged at 230xg for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed by resuspending in RPMI-1640 media (Appendix B.1.1) (Sigma-Aldrich) and recentrifuged. This washing step was repeated until the supernatant appeared clear. After the final wash, the supernatant was discarded and the pellet was resuspended in 1 ml of freezing mix (Appendix B.1.2) and transferred immediately to -80°C until used.

EDTA tubes containing bone marrow aspirates were processed by adding 10 volumes of *RNAlater* (Qiagen, Maryland, USA) directly to the blood tube containing the bone marrow sample, in order to stabilise the RNA. The samples together with the PAXgene blood tubes were then stored at 4°C overnight before transferring to -80°C for long term storage.

All serum samples were heat inactivated as described previously in section 2.2.3 before use in the pseudovirion neutralizing antibody assay.

2.4 Pseudovirion neutralizing antibody assay set-up

2.4.1 Preparation of recombinant plasmids

2.4.1.1 Generation of competent E. coli DH5α cells

Competent *E. coli* DH5α cells were generated under sterile conditions and were used in transformation experiments with the 12 subtype C and 2 subtype B Env plasmids as well as the backbone plasmid (Table 2.2). Briefly, ten microlitres of *E. coli* DH5α cell stocks (available in the laboratory) were inoculated into 10 ml Luria Bertani (LB) broth (Appendix C.1.1) containing no antibiotics and incubated overnight in a 37°C shaking incubator. The following day the culture was diluted 1:40 with fresh LB broth before incubation for 1 hour in a 37°C shaking incubator to allow optimal growth to an optical density of 0.4 at 600nm. The culture was then centrifuged for 10 minutes at 300xg at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml of ice cold transformation buffer (Appendix C.1.2) and transferred immediately on ice for 30 minutes. The reaction was then centrifuged for 10 minutes at 300xg at 4°C. Finally, the supernatant was discarded and the pellet was resuspended in 1 ml of transformation buffer (Appendix C.1.2). The competent *E. coli* DH5α cells were stored in 100µl aliquots at -80°C until used.

2.4.1.2 Bacterial transformation of Env and backbone plasmid/s

Twelve subtype C Env plasmids; CAP45, CAP210, ZM53, ZM109, ZM135, ZM197, ZM214, ZM233, ZM249, DU156, DU172, DU422, and 2 subtype B Env plasmids, SVPB11 and SVPB15 as well as the backbone plasmid $pSG^{\Delta env}$ (Table 2.2) were transformed into competent *E. coli* DH5α cells. Briefly, 25 µl of competent *E. coli* DH5α cells were added to 5 µl of each Env or backbone plasmid and incubated on ice for 30 minutes. The cells were then heat shocked in a 42°C water bath for 90 seconds and immediately transferred onto ice for 5 minutes. Two hundred microlitres of LB broth (Appendix C.1.1) was added to each reaction and incubated at 37°C in a shaking incubator for one hour. The backbone plasmid was incubated at 34°C to ensure optimal growth. The cells were then spread onto ampicillin (Appendix C.1.4) (Roche, Mannheim, Germany) containing agar plates and incubated overnight at 37°C in a non shaking incubator for 34°C for the backbone plasmid. Successfully transformed *E. coli* DH5α cells containing the relevant plasmid are ampicillin resistant and therefore form colonies on the ampicillin contained agar plates. Under sterile conditions, a single colony was picked from the ampicillin (Appendix C.1.4) (Roche) containing agar plates and inoculated into 100 ml LB broth (Appendix C.1.1) containing sterile ampicillin (Appendix C.1.4) (Roche). This was incubated overnight at 37°C in a shaking incubator or 34°C for the backbone plasmid.

Glycerol stocks were produced for the 14 Env transformed and backbone transformed *E. coli* DH5α cells. Briefly, 200 µl of 100% glycerol (Merck, Hohenbrunn, Germany) was gently added to 800 µl of each bacterial culture and

stored in sterile cryovials (Nunc) at -80°C until used. The glycerol protects the *E. coli* DH5α cells from damage caused by ice crystals which form in the LB broth during freezing.

2.4.1.3 Large scale recombinant plasmid preparations

Plasmid midi preparations were performed on all 14 Env plasmids as well as the backbone plasmid as per manufacturer's instructions (Qiagen). Briefly, each recombinant *E. coli* DH5α culture was inoculated into 100 ml LB broth (Appendix C.1.1) and incubated overnight in a dry 37ºC shaking incubator. The bacterial cells were then harvested by centrifugation at 6000xg for 15 minutes at 4ºC. The pellet was resuspended in 4 ml resuspension buffer P1 containing RNase H (Appendix C.1.5.1). Four millilitres of lysis buffer P2 (Appendix C.1.5.2) was then added, mixed and incubated at room temperature for 5 minutes to allow for the shearing of genomic DNA. During the incubation, the filter cartridge was prepared, by screwing the cap onto the nozzle of the QIAfilter midi cartridge and placing the cartridge in a convenient tube. Four millilitres of chilled neutralization buffer P3 (Appendix C.1.5.3) was added and mixed thoroughly. The lysate was then poured into the QIAfilter midi cartridge and incubated at room temperature for 10 minutes. Four millilitres of equilibration buffer QBT (Appendix C.1.5.4) was added to a QIAGEN-TIP 100 and the column was allowed to empty by gravity flow. The cap was then removed from the QIAfilter midi cartridge and the plunger was gently inserted into the cartridge filtering the cell lysate into the previously equilibrated QIAGEN-TIP 100. The cleared lysate was allowed to enter the resin by gravity flow. The column was then washed twice with 10 ml of wash buffer QC (Appendix

C.1.5.5) and the DNA was eluted with 5 ml of elution buffer QF (Appendix C.1.5.6) into a 15 ml tube. The eluted DNA was precipitated by the addition of 3.5 ml room temperature isopropanol (Merck) which was mixed and centrifuged at 15000xg for 30 minutes at 4ºC. The supernatant was discarded and the remaining pellet was washed with 2 ml room temperature 70% ethanol (Merck) and centrifuged at 15000xg for 10 minutes. After discarding the supernatant, the pellet was air dried at room temperature for 5 – 10 minutes and redissolved in 150µl distilled water. All plasmids were run on a 1% agarose gel (Appendix C.2.3) to verify the presence of DNA.

2.4.2 Generation of pseudovirion stocks

2.4.2.1 Mammalian cell lines

HEK 293T and TZM-bl cells were maintained in complete Dulbecco's Modified Eagle Medium (DMEM), containing DMEM (Sigma-Aldrich, Steinheim, Germany), 10% foetal calf serum (Gibco, Grand Island, USA), L-Glutamine (Gibco), penicillin/streptomycin (Sigma-Aldrich) (Appendix B.1.3). All cell lines were cultured in 75cm^3 flasks and incubated at 37°C in a humidified 5% CO_2 incubator.

Cells were trypsinized when confluent, or as needed. The media was discarded from the flask, and the cells were rinsed with 5 ml 1X PBS (Sigma-Aldrich). After discarding the PBS, 1 ml of 0.25% trypsin/EDTA (Gibco) was added to the cells to enable the detachment of the cells from the flask, the cells were incubated at room temperature for 30 seconds. The cells were then incubated at 37°C in a humidified, 5% CO_2 incubator for 2 – 5 minutes before the addition of 10 ml of

complete DMEM to inactivate the trypsin and dilute the cells (Appendix B.1.3). The cells were detached from the flask by vigorously pipetting the cells and media, and diluted as required.

2.4.2.2 HEK 293T cell counting

Cells were counted on a haemocytometer as required. Briefly, freshly trypsinized cells were diluted 5 times in 0.4% Trypan blue (Sigma-Aldrich) and loaded onto the haemocytometer with a pipette before counting the cells. The average number of cells was multiplied by the dilution factor (5) and by the volume of the haemocytometer (10^4) to give the number of cells per ml.

2.4.2.3 HEK 293T cell stocks

Stocks of all cells lines were made by centrifuging freshly trypsinized cells at 200x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 3 ml freezing mix (Appendix B.1.2) and stored in 1 ml aliquots. Cells were slowly frozen to -80°C by transferring them into the Nalgene™ Cryo 1°C freezing container (Cat# 5100-0001) to achieve a -1°C/min rate of cooling and transferred to -80°C for short term storage or liquid nitrogen for long term storage. Each time new cell stocks were transferred to the freezing container, the isopropanol (Merck) within the container was replaced.

2.4.2.4 Co-transfection experiments

HEK 293T cells were co-transfected in 25cm^3 flasks (Nunc) with the appropriate Env plasmid and the backbone plasmid (pSG3$^{\Delta env}$) containing all the HIV encoded genes, but with a premature stop codon in gp160. HEK 293T cells were cultured as described previously. Once the cells reached 70-80% confluency, 1,5x10^6 HEK 293T cells were seeded in 25cm^3 flasks (Nunc) the day before transfection and incubated overnight at 37°C in a humidified, 5% CO_2 incubator. After 24 hours, 2µg of the appropriate Env plasmid and 2µg of the backbone plasmid (pSG3$^{\Delta env}$) was added to 100µl of DMEM (no additives). Forty microlitres of transfection reagent polyfect® (Qiagen) was added to each reaction, gently mixed and incubated at room temperature for 10 minutes. The reaction tube contents were then gently added to the 25cm^3 flask of HEK 293T cells and incubated for 5 hours at 37°C in a humidified, 5% CO_2 incubator. After 5 hours, the media was replaced with complete DMEM (Appendix B.1.3) and incubated for 48 hours at 37°C in a humidified, 5% CO_2 incubator.

After 48 hours, the generated pseudoviruses were harvested. Briefly, the content of the flasks were transferred into labelled 15 ml tubes (Nunc) and centrifuged at 400xg at 4°C for 10 minutes. The supernatant containing the pseudovirus was filter sterilised (0.22µM Acrodisc® 25mm syringe filters) (Pall Corporation, Ann Arbor, USA) with a 10 ml syringe into 15 ml tubes (Nunc) and the pellet was discarded. The supernatant was then supplemented with FCS (Gibco) up to a concentration of 20% and aliquoted into cryovials (Nunc) and stored at -80°C until used.

2.4.2.5 $TCID_{50}$ determination of pseudovirus stocks

The tissue culture infectious dose ($TCID_{50}$) is defined as the dilution of virus that is required to infect 50% of inoculated cell cultures. The $TCID_{50}$ was determined for each pseudovirus stock produced. Briefly, TZM-bl cells were cultured as described previously and once 70-80% confluency was reached, $1x10^5$ cells/ml were counted. One hundred microlitres of complete DMEM (Appendix B.1.3) was added to each well of a 96 well microplate. Twenty five microlitres of pseudovirus was added to column 1 (row A-D) and 25µl of a second pseudovirus was added to rows E-H. A 5-fold dilution of the pseudoviruses were performed by transferring 25µl from column 1 to 2, etc and mixing thoroughly each time. Twenty five microlitres was discarded from column 11. Column 12 served as the cell control (no pseudovirus added). One hundred microlitres of $1x10^5$ TZM-bl cells containing a final concentration of 15µg diethylaminoethyl (DEAE) dextran (Sigma-Aldrich) was added to each well. The reaction was incubated for 48 hours at 37°C in a humidified, 5% CO_2 incubator.

After 48 hours, a luciferase assay was performed to detect the reduction in luciferase activity, as measured in relative light units, from the TZM-bl cells. The reduction in luciferase activity is indicative of viral inhibition by neutralizing antibody activity. Briefly, all the media was discarded from the 96 well plates. Ninety microlitres of Glo lysis Buffer (Promega, Madison, USA) was added to each well and incubated at room temperature for 5 minutes. Fifty microlitres was then transferred into the corresponding wells of a white luminometer plate (Promega).

Fifty microlitres of BriteGlo reagent (Promega) was then added to each well and luciferase activity was measured immediately in a luminometer (Veritas™ Microplate Luminometer, Turner Biosystems).

The $TCID_{50}$ was calculated by the use of a macro obtained from the NIH AIDS Research and reference reagent program (http://www.aidsreagent.org). The program's calculation is based on the Reed and Muench equation and is explained below (Reed and Muench 1938).

i. **P**roportionate **D**istance = $\frac{\text{(\% mortality at dilution above 50\%) – (50\%)}}{\text{(\% mortality at dilution above 50\%) – (\% mortality at dilution below 50\%)}}$

ii. -Log = dilution above 50% mortality (i.e. 10^{-3} would be -3)

iii. ((PD)+(-log(dilution factor))

iv. $TCID_{50} = 10^{(ii + iii)}$

2.5 Pseudovirion neutralizing antibody assay

Neutralizing antibody assays were performed in 96 well microplates (Nunc). The first two columns were used as the cell control (containing no sample and no virus) and virus control (containing no sample), respectively. One hundred and fifty microlitres of complete DMEM (Appendix B.1.3) was added to the first column (cell

control) and 100µl was added to the second column (virus control). For the 4 point dilution format (Figure 2.1A), 140 µl of medium was added to rows D and H and 100 µl was added to the rest of the 96 well microplate. For the eight point dilution format 140 µl was added only to row H only (Figure 2.1B). Eleven microlitres of heat inactivated sera/plasma was added to the relevant wells in rows D and H (or only row H for eight point dilutions) and serially diluted 3-fold by transferring 50 µl from row D to C and so on until row A, and from row H to G until row E for the four point dilutions and 50 µl was discarded from rows A and E, respectively (whereas 50 µl was discarded from row A only for the 8 point dilution format). Fifty microlitres of the relevant pseudovirus at 4000$TCID_{50}$ was then added to each well on the entire 96 well microplate except the cell control. The reaction was then incubated for 1 hour at 37ºC in a humidified 5% CO_2 incubator. One hundred microlitres of TZM-bl cells (1X10^5cells/ml) was then added to the entire plate and incubated for 48 hours at 37ºC in a humidified 5% CO_2 incubator.

After 48 hours the luciferase assay was performed as described previously (Section 2.4.2.5). The 4-point dilution format was used to set up and optimize the pseudovirion based neutralizing antibody assay using the NIH AIDS Research and Reference Reagents Program sera, and SANBS plasma samples. The subsequent screening of cohorts was performed using the 8 point dilution format (Figure 2.1A and B).

Percentage inhibition was calculated as per the following calculation

PERCENTAGE INHIBITION = (Test Well-Cell Control) / (Virus Control-Cell Control) X 100

The cell control was calculated as the average of the cell control column in RLU and the virus control was calculated as the average of the virus control column in RLU.s

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
B	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
C	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
D	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
			Samples 1 & 2		*Samples 3 & 4*		*Samples 5 & 6*		*Samples 7 & 8*		*Samples 9 & 10*	

(A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8
B	CC	VC	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7
C	CC	VC	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6
D	CC	VC	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
			Sample 1		*Sample 2*		*Sample 3*		*Sample 4*		*Sample 5*	

(B)

Figure 2.1: Dilution formats used for pseudovirion neutralizing antibody assay (A) Four point dilution (1/20 to 1/540) format used for the set up for the pseudovirion based neutralizing antibody assay (B). Eight point dilution (1/20 to 1/43740) format

used for the screening of participant samples. The cell control, virus control and dilution wells are abbreviated as CC, VC and Dil1 to 4 or 1 to 8, respectively.

2.5.1 Screening of NIH serum and SANBS plasma samples

Plasma is a portion of blood with clotting factors such as fibrinogen which is responsible for the coagulation of blood. Serum contains the same components of plasma except fibrinogen and other clotting factors.

The positive and negative serum samples (NIH) were screened in the pseudovirion neutralizing antibody assay against the subtype C pseudovirus CAP210 and the subtype B pseudovirus B15, using the four point dilution format. In addition, the 18 seropositive plasma samples (SANBS) and an HIV-1 seronegative plasma sample (available in our laboratory) were screened in the pseudovirion neutralizing antibody assay using the 4 point dilution format against CAP210 for the identification of a plasma sample with a greater than average breadth of neutralization for use as a positive and negative plasma control.

2.5.2 Testing of participant samples for the presence of neutralizing antibodies

2.5.2.1 CT cohort

A pseudovirion neutralizing antibody assay was performed with plasma samples SP2 to SP7 with and without heat inactivation to determine which would produce

optimal neutralizing antibody results. All plasma samples (SP1 to SP9) were subsequently heat inactivated, and screened in the pseudovirus neutralizing antibody assay against 5 subtype C and 1 subtype B generated pseudoviruses using eight dilutions.

2.5.2.2 FV and DR cohort

Plasma samples from both, the FV and DR cohort, were tested for the presence of neutralizing antibodies against the 6 generated subtype C and B pseudoviruses. All pseudovirion neutralizing antibody assays were performed using the eight point dilution format.

2.5.2.3 JM cohort

The required amount of heat inactivated serum sample aliquots were thawed at room temperature and tested for the presence of neutralizing antibodies against the 6 generated subtype C and B pseudoviruses. All pseudovirion neutralizing antibody assays were performed using the eight point dilution format.

CHAPTER 3: RESULTS

3.1 Study participants' demographics and clinical data

Epidemiological data of all the participants used in this study are shown in Table 2.1. From the available clinical data amongst the four cohorts, 12 participants were male and 17 were female, ranging from 20 to 60 years of age. The CD4+ T-cell counts ranged from 6 to 1130 cells/μl and viral loads ranged from below detection to greater than 750 000 RNA copies/ml.

Plasma, serum and peripheral blood mononuclear cells (PBMC's) were successfully obtained from all 10 participants in the JM cohort and bone marrow samples were obtained from 8 of the 10 individuals in this cohort. Plasma samples from 30 participants in the remaining three cohorts were previously obtained during routine diagnosis and were already available for use in the laboratory.

3.2. Preparation of recombinant plasmids for generation of pseudoviruses

The pSGΔenv backbone plasmid and 14 different Env plasmids were all successfully transformed into and isolated from *E. coli* DH5α competent cells (Figure 3.1). The DNA concentrations of all 15 purified recombinant plasmids are shown in Table 3.1. Isolated plasmids were used to generate pseudovirion stocks.



Figure 3.1: Agarose gel electrophoresis (1%) of large scale plasmid preparations of the pSGΔenv backbone plasmid and 10 different Env plasmids. The 10kb molecular weight marker (Fermentas, Ontario, Canada) is shown in Lane 1 and the backbone plasmid $pSG^{\Delta env}$ is illustrated in lane 2. Lanes 3 to 12 include the subtype C plasmids CAP210, CAP45, DU156, DU422, ZM53, DU172, ZM197, ZM233, ZM109 and the subtype B, B11 plasmid, respectively.

Table 3.1: DNA concentrations of the 14 purified recombinant Env and backbone plasmid/s used during the study.

Plasmid name	**DNA concentration (ng/µl)**
CAP210	818.1
CAP45	362.0
DU156	750.0
DU422	1119.0
DU172	1340.1
ZM214	850.0
ZM53	251.8
ZM249	113.3
ZM109	846.5
ZM135	371.6
ZM197	1289.8
ZM233	230.0
B15	1356.6
B11	229.4
Backbone plasmid ($pSG^{\Delta env}$)	938.0

3.3. Generation of pseudoviruses

Co-transfection experiments of each of the Env plasmids together with the $pSG^{\Delta env}$ backbone plasmid in HEK 293T mammalian cell lines, followed by pseudovirion infection of TZM-bl cells resulted in the identification of only 6 functional pseudoviruses, despite repeated attempts. The tissue culture infectious dose

($TCID_{50}$) values of all generated pseudoviruses for the TZM-bl cell line are listed in Table 3.2. The 6 functional pseudoviruses with a $TCID_{50}$ greater than 10 000 included 5 subtype C and 1 subtype B pseudovirus/es (highlighted in yellow in Table 3.2) which were subsequently used throughout the course of this study. The pseudovirion stocks were depleted or lost infectivity with storage over time, therefore the co-transfection experiments and $TCID_{50}$ determinations were repeated as required (see duplicates, and dates prepared in Table 3.2).

Table 3.2: $TCID_{50}$ values of all pseudoviruses in the TZM-bl cell line.

Pseudovirion name	**$TCID_{50}$**	**Date**
CAP210	91376	17-08-2009
	31250	30-04-2010
CAP45	10687	09-09-2009
	69877	15-09-2010
DU156	31250	09-09-2009
	10687	02-08-2010
DU422	13975	02-09-2009
	69877	02-08-2010
DU172	2795	18-09-2009
	<50	20-10-2009
ZM214	<50	18-09-2009
	2795	20-10-2009

ZM53	91376	02-09-2009
	349386	28-07-2010
ZM249	<50	15-07-2009
	<50	02-08-2010
ZM109	3489	15-07-2009
	699	18-09-2009
ZM135	<50	15-07-2009
	<50	18-09-2009
ZM197	559	18-09-2009
	<50	25-09-2009
ZM233	2795	17-08-2009
	559	24-08-2009
B15	<50	11-02-2010
	<50	02-08-2010
B11	53437	11-02-2010

3.4 Establishment of the HIV-1 pseudovirion based neutralizing antibody assay

Initial attempts to establish the pseudovirion neutralizing antibody assay made use of positive and negative serum (NIH) which were tested against the subtype C CAP210 and subtype B B15 pseudoviruses (Figure 3.2). The positive serum (NIH) did not show promising neutralizing antibody activity against both CAP210 and B15, with less than 100% inhibition at the first dilution which rapidly decreased with increasing dilutions of the serum (Figure 3.2).

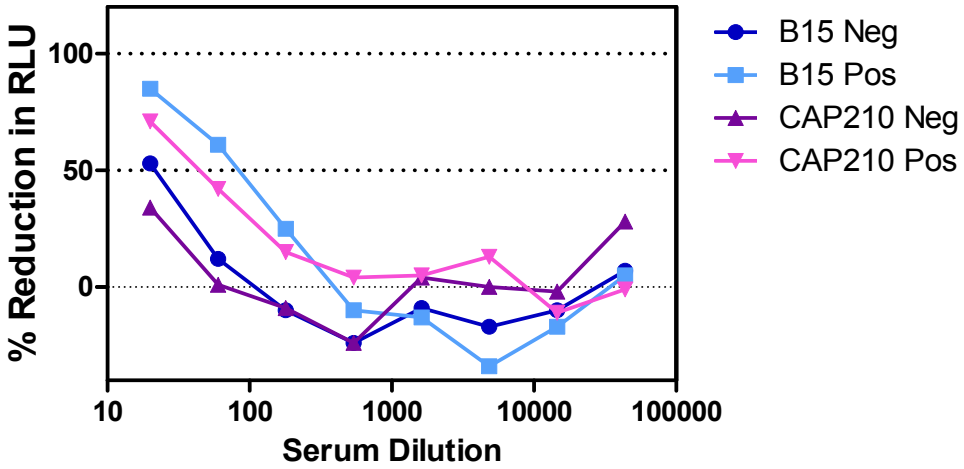
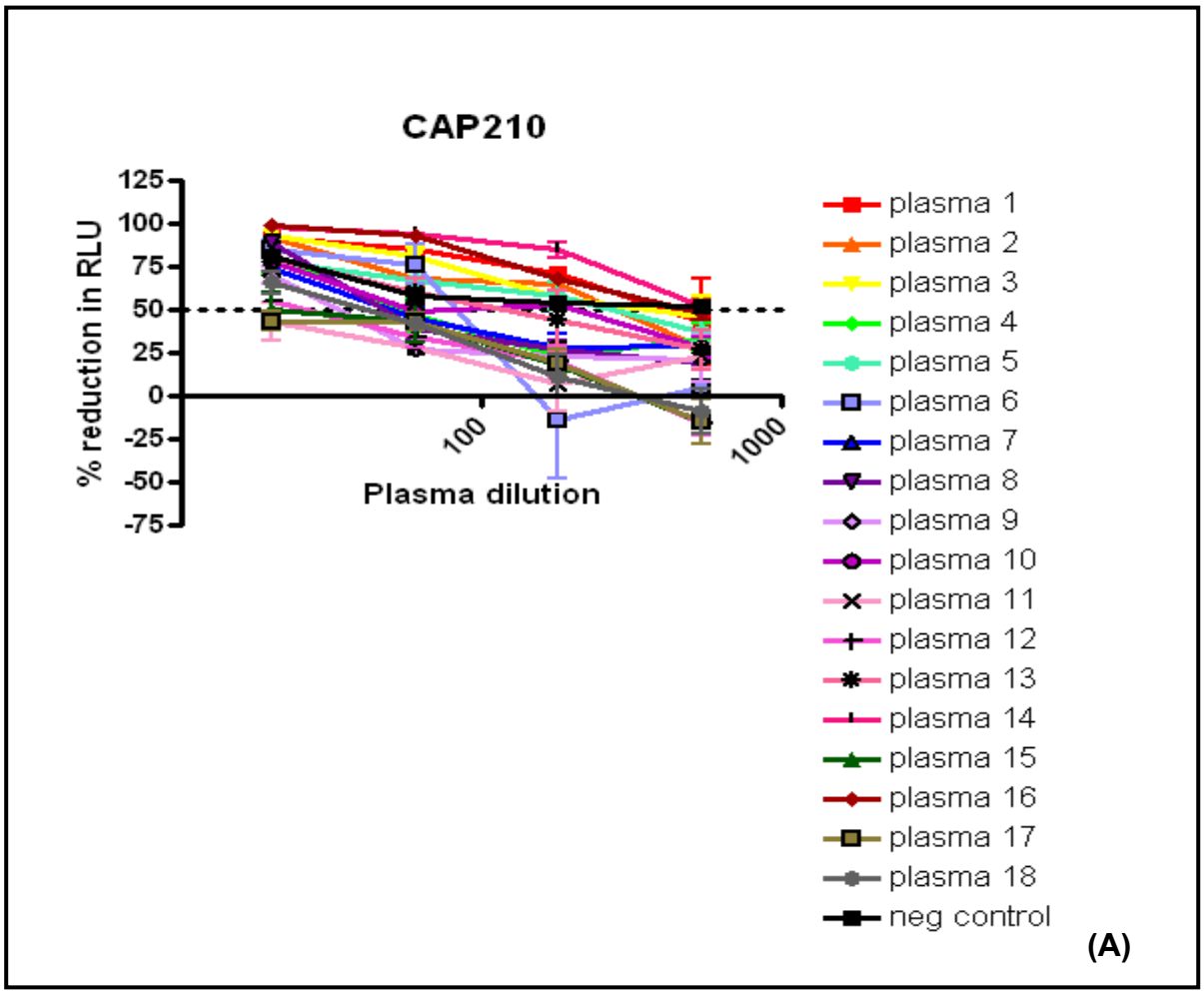


Figure 3.2: Percentage inhibition curves for the NIH positive and negative serum samples against the subtype C CAP210 and the subtype B B15 pseudoviruses.

Subsequently, 18 plasma samples were purchased from the South African National Blood Services (SANBS) and screened against the subtype C CAP210 pseudovirus for the presence of greater than average neutralizing antibody activity in an attempt to identify a positive control. These experiments were set up using the 4 point dilution format therefore too few points were available for the

determination of IC_{50} (the concentration of an antibody required for 50% virus inhibition) values (Figure 3.3A).

During the initial screening, the negative control plasma showed some degree of activity at the lowest dilution ranging from about 50% to 75% inhibition but rapidly decreased after the second dilution (Figure 3.3A). Screening of the 18 plasma samples led to the identification of three plasma samples (plasma 6, 14 and 16) which showed greater than average breadths of neutralization and were subsequently retested against CAP210 using 8 dilutions, ranging from 1/20 to 1/43740 (Figure 3.3B).



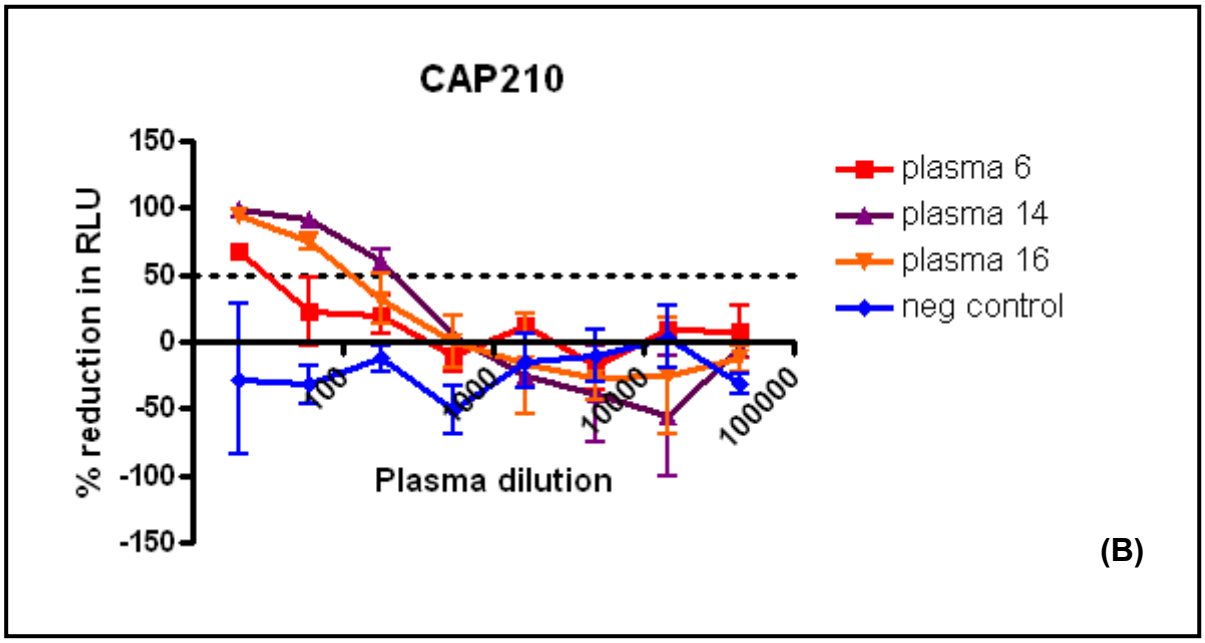


Figure 3.3: (A) Percentage inhibition curves of 18 plasma samples (SANBS) against the subtype C CAP210 pseudovirus**.** Dilutions ranged from 1/20 to 1/540. (B) Percentage inhibition curves for the three plasma samples from A with greater than average neutralizing antibody activity were selected for further screening against CAP210 using 8 dilutions (1/20 to 1/43740).

Table 3.3: IC_{50} values for SANBS plasma tested against the CAP210 pseudovirus

Plasma sample number	**IC_{50} value**
Plasma 6	33
Plasma 14	208
Plasma 16	116

Plasma sample 14 showed 100% inhibition at the lowest plasma dilution which gradually decreased with increasing dilutions and was subsequently selected as the positive control for use in the pseudovirion neutralizing antibody assays (Figure 3.3B) and showed the highest IC_{50} value of 208 (Table 3.3). A 200 ml pack of plasma sample 14 was subsequently purchased from the SANBS, heat inactivated (described in section 2.2.3) and stored in 100µl aliquots at -80°C until required for use. For each experiment a 100µl vial of plasma sample 14 was thawed together with a 100µl vial of HIV negative plasma. Any leftover sample was subsequently discarded and a fresh vial was thawed for each new experiment.

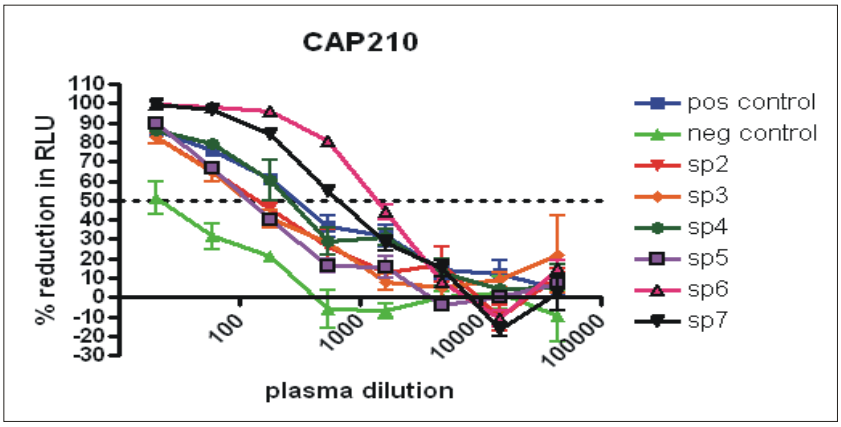
3.5 Screening of cohorts for the presence of neutralizing antibodies

The 40 patient samples available from the 4 different cohorts (Table 2.1) were evaluated against the 5 subtype C and 1 subtype B pseudovirus/es for the presence of neutralizing antibodies (highlighted yellow in Table 3.2). Results of all percentage inhibition curves are shown in Figures 3.5 to 3.8.

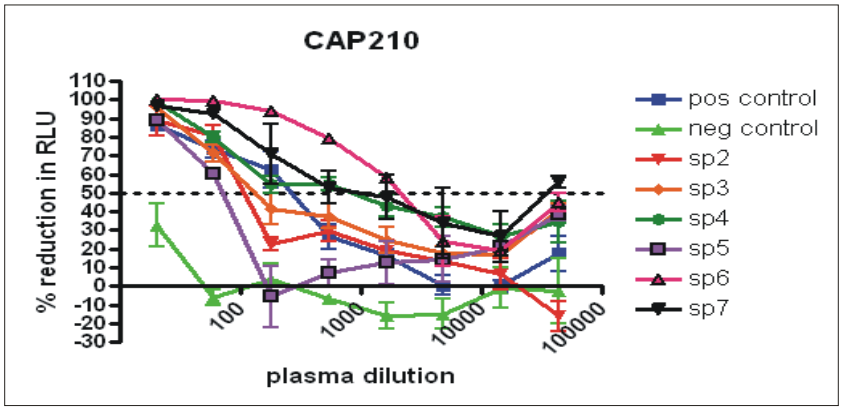
3.5.1 Neutralizing antibody activity within the CT cohort

In order to determine whether optimal results would be obtained from heat inactivation of plasma samples or not, a pseudovirion neutralizing antibody assay was performed using the SP2 to SP7 samples, with or without heat inactivation against the subtype C CAP210 pseudovirion (Figure 3.4A and B). Similar results were obtained for both samples, however since heat inactivated samples are more commonly used, in the literature, it was decided that all plasma samples

would be heat inactivated before use in the pseudovirion neutralizing antibody assay.



(A)

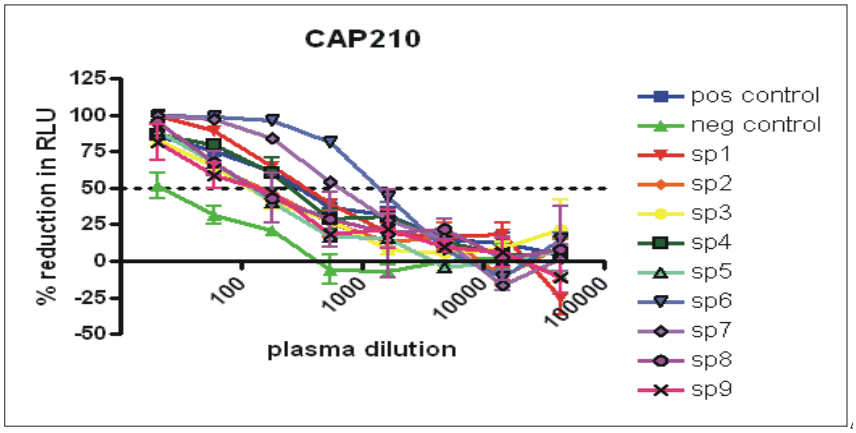


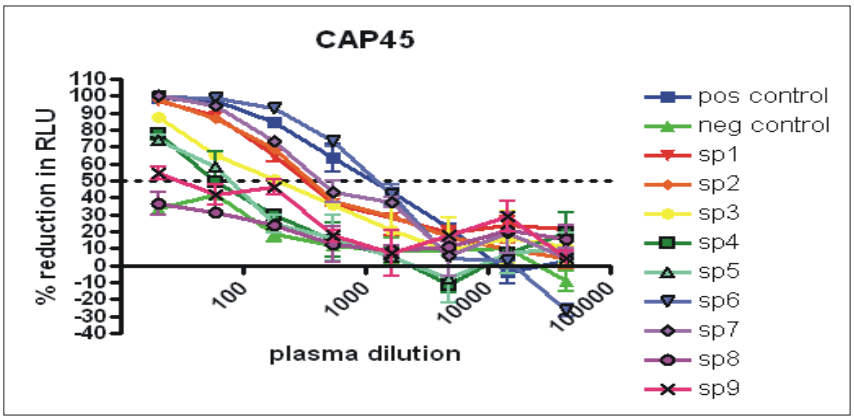
(B)

Figure 3.4: (A) Heat inactivated plasma samples SP2 to SP7 tested in the pseudovirion neutralizing antibody assay against the subtype C CAP210 pseudovirus. (B) Plasma samples SP2 to SP7 without heat inactivation, tested against the subtype C CAP210 pseudovirus. Dilutions ranged from 1/20 to 1/43740.

Percentage inhibition curves of the 9 slow progressor participants (SP1 to SP9) against all 6 pseudoviruses are represented in Figure 3.5A and B. Plasma sample SP6 contained the greatest neutralizing antibody activity within the CT cohort against CAP210 (Figures 3.4 and 3.5A), maintaining 100% inhibition after the third dilution, with the highest recorded IC_{50} value of 1445 against CAP210 (Table 3.4). SP7 was able to maintain 100% inhibition after the second dilution against CAP210 (Figure 3.5A) with an IC_{50} value of 630 (Table 3.4). The CAP45 pseudovirus was effectively neutralized by SP6 showing a similar percentage inhibition curve to that of the positive control (Figure 3.5A) with an IC_{50} value of 1202 (Table 3.4). Plasma sample SP7 showed a greater than average neutralization breadth against DU156 and showed similar percentage inhibition curves to that of SP6, maintaining 100% inhibition at the second dilution (Figure 3.5A) with high IC_{50} values of 1737 and 1202, respectively (Table 3.4).

SP6 and SP7 exhibited promising neutralizing antibody activity against DU422 with IC_{50} values of 1258 and 724, respectively and SP6 showed the greatest neutralizing antibody activity against ZM53 and B11, maintaining 100% inhibition at the third dilution with high IC_{50} values of 1063 and 1096, respectively as shown in Figure 3.5B.





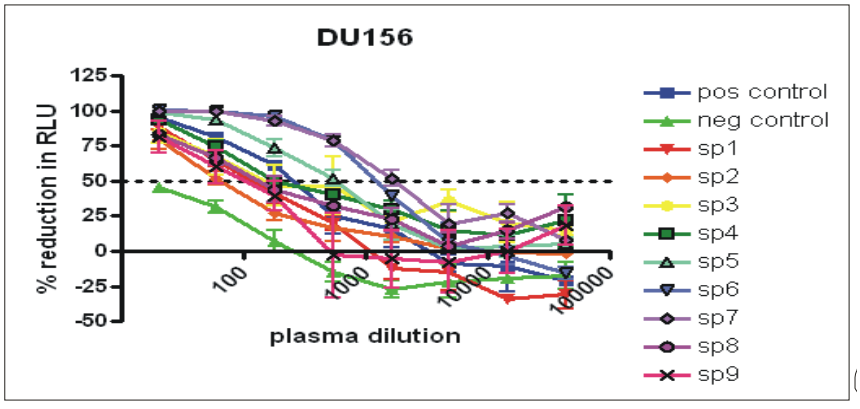
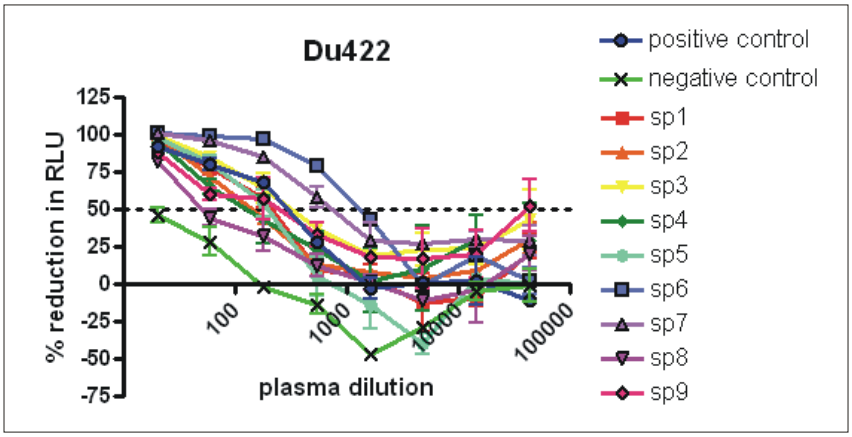
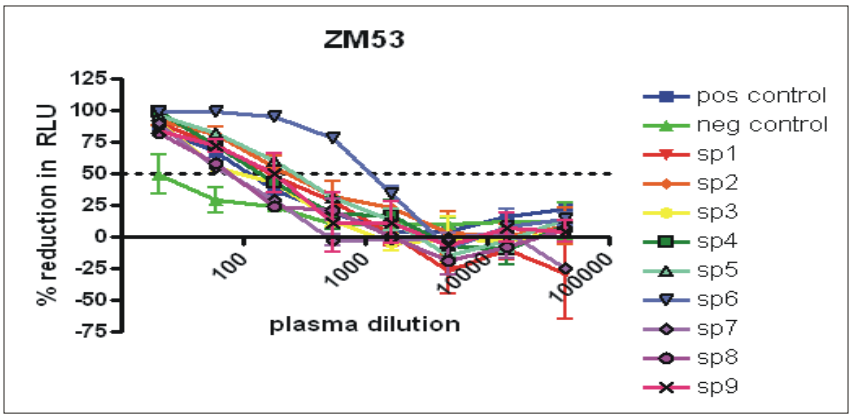


Figure 3.5: (A) Percentage inhibition curves of plasma samples SP1 to SP9 from the CT cohort, with dilutions ranging from 1/20 to 1/43740, against the subtype C CAP210, CAP45, DU156 pseudoviruses.





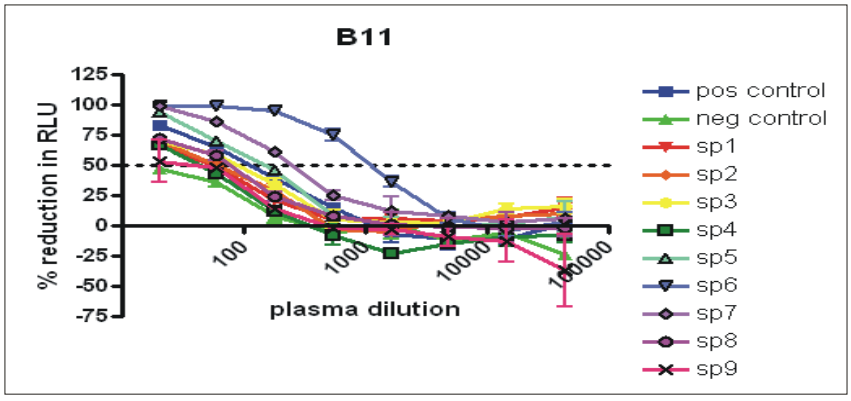
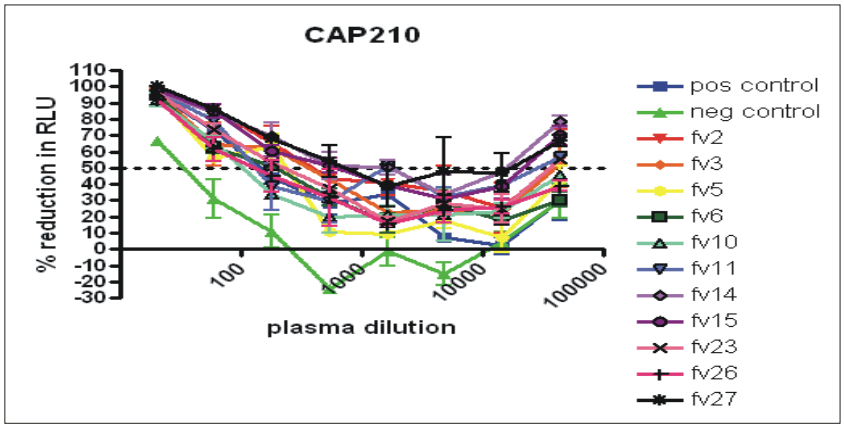


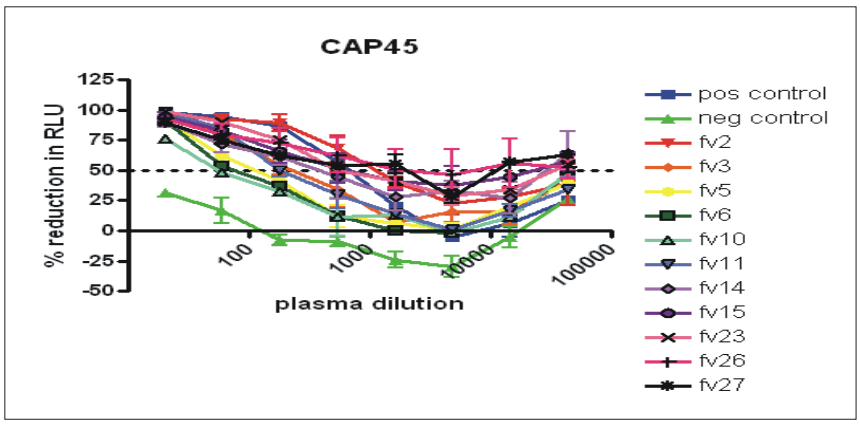
Figure 3.5: (B) Percentage inhibition curves for plasma samples SP1 to SP9 from the CT cohort, with dilutions ranging from 1/20 to 1/43740, against the subtype C DU422, ZM53 and the subtype B B11 pseudoviruses.

3.5.2 Neutralizing antibody activity within the FV cohort

Neutralizing antibody activity from the 11 HIV-1 subtype C infected and antiretroviral treatment naïve participants within the FV cohort are shown in Figures 3.6A and B against all 6 pseudoviruses. Samples from the FV cohort generally did not neutralize CAP210 effectively and the % inhibition (as measured in relative light units) rapidly decreased after the first plasma dilution (Figure 3.6A). However, FV2 and FV26 showed some degree of neutralization against CAP45 (Figure 3.6A) with IC_{50} values of 1000 and 1792, respectively (Table 3.4). DU156 was effectively neutralized by plasma samples FV5, FV26 and FV27 (Figure 3.6A). FV5 and FV26 were able to maintain 100% inhibition after the second plasma dilution and FV27 showed a similar percentage inhibition curve to that of the positive control. IC_{50} values obtained for these 3 samples included 1063, 2154 and 1584, respectively (Table 3.4).

Samples FV14, FV26 and FV27 showed high IC_{50} values of 1015, 1047 and 4713, respectively against DU422 (Figure 3.6B, Table 3.4). Interestingly, plasma samples FV2 and FV15 showed neutralization curves that could still inhibit ZM53 at the second dilution (Figure 3.6B), however these samples revealed low IC_{50} values of 541 and 292, respectively (Table 3.4). FV27 was able to neutralize the subtype B pseudovirus B11 effectively (Figures 3.6B) with a high IC_{50} value of 1634 (Table 3.4).





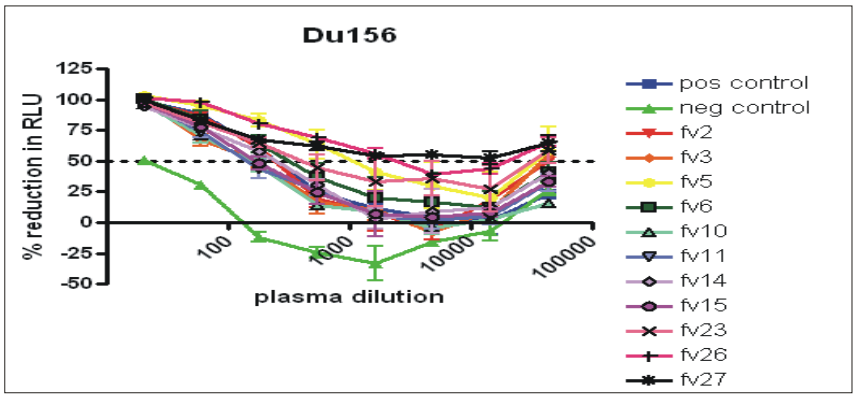
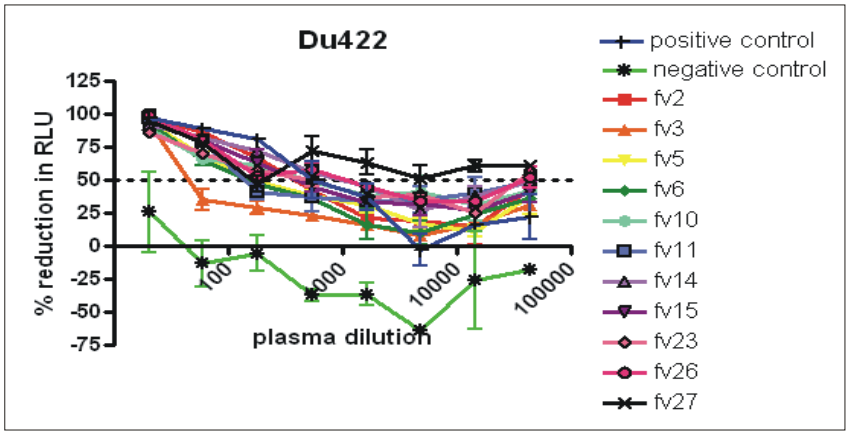
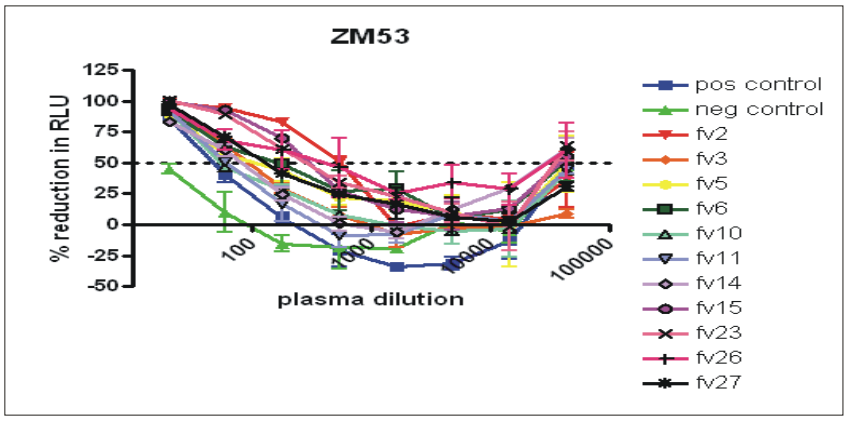


Figure 3.6: (A) Percentage inhibition curves of plasma samples FV2-FV27 from the FV cohort, with dilutions ranging from 1/20 to 1/43740, against the subtype C CAP210, CAP45, DU156 pseudoviruses.





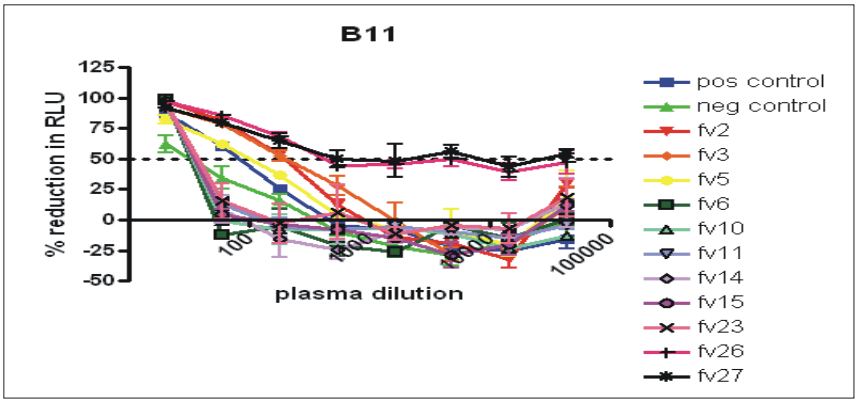
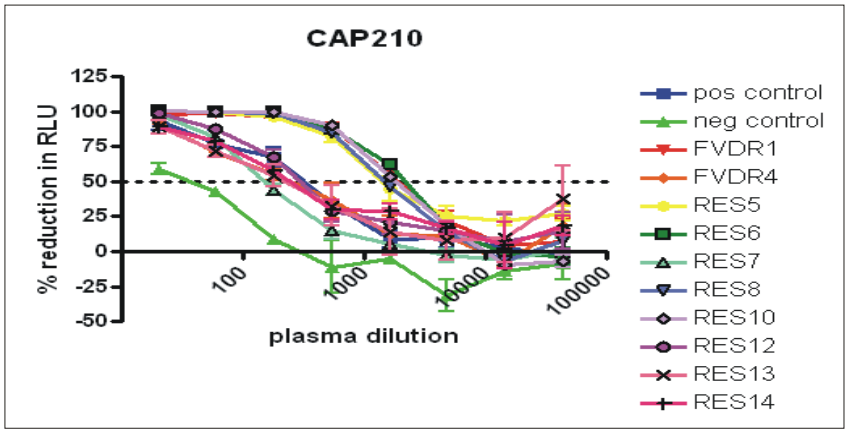
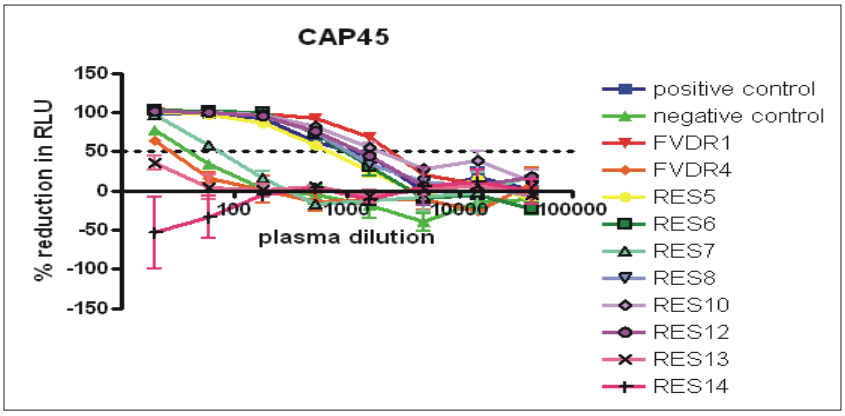


Figure 3.6: (B) Percentage inhibition curves of plasma samples FV2-FV27 from the FV cohort, with dilutions ranging from 1/20 to 1/43740, against the subtype C DU422, ZM53 and the subtype B B11 pseudoviruses.

3.5.3 Neutralizing antibody activity within the DR cohort

Percentage inhibition curves of the 10 HIV-1 subtype C infected participants failing first line ARV therapy from the DR cohort against all 6 pseudovirions are illustrated in Figure 3.7A and B. FVDR1, RES5, RES6, RES8 and RES10 effectively neutralized CAP210 and showed similar percentage inhibition curves and patterns of neutralization to one another with high IC_{50} values of 1792, 1359, 2154, 1318 and 1737, respectively as shown in (Figure 3.7A and Table 3.4) while plasma samples FVDR1, RES8, RES10 and RES12 showed potent neutralizing antibody activity against CAP45 showing similar percentage inhibition curves to each other (Figure 3.7A) with IC_{50} values of 2256, 1047, 1934 and 1338, respectively (Table 3.4). In contrast, RES7, RES13 and RES14 showed little or no neutralizing antibody activity against CAP45 (Figure 3.7A). DU156 was effectively inhibited by FVDR1, RES5, RES6, RES8 and RES10 (Figure 3.7A) with the plasma samples maintaining 100% inhibition after the third dilution with high IC_{50} values of 3066, 1015, 2326, 2796 and 1995, respectively (Table 3.4). FVDR1, RES5, RES6 and RES10 exhibited potent neutralization against DU422 (Figure 3.7B) maintaining 100% inhibition by the fourth plasma dilution and high IC_{50} values were obtained for these samples of 2796, 1423 and 1609, respectively. FVDR1, RES5, RES6, RES10 and RES8 was effective at inhibiting ZM53 with FVDR1 maintaining 100% inhibition by the fourth dilution and the others maintaining 100% inhibition at the third dilution (Figure 3.7B). B11 was successfully inhibited by FVDR1, RES6, RES8 and RES10, and all maintained 100% inhibition at the third plasma dilution (Figure 3.7B) with IC_{50} values of 2590, 1063, 2221, 2026 and 2363 respectively (Table 3.4).





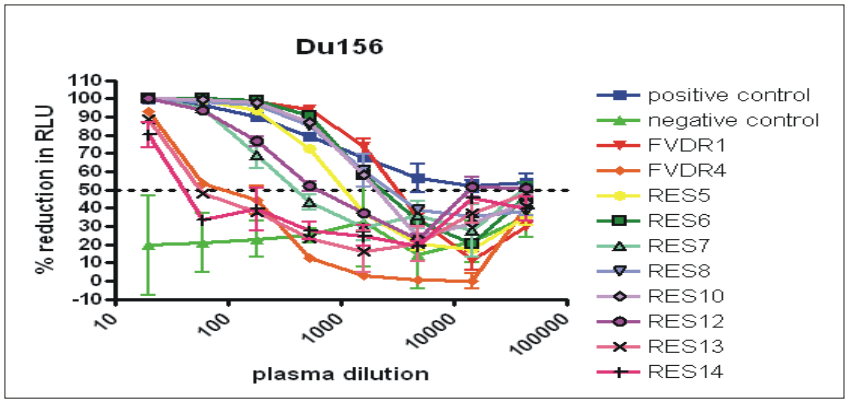
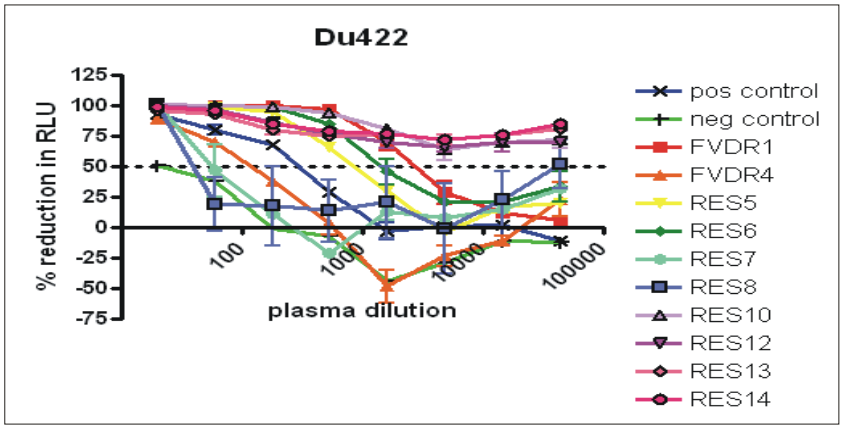
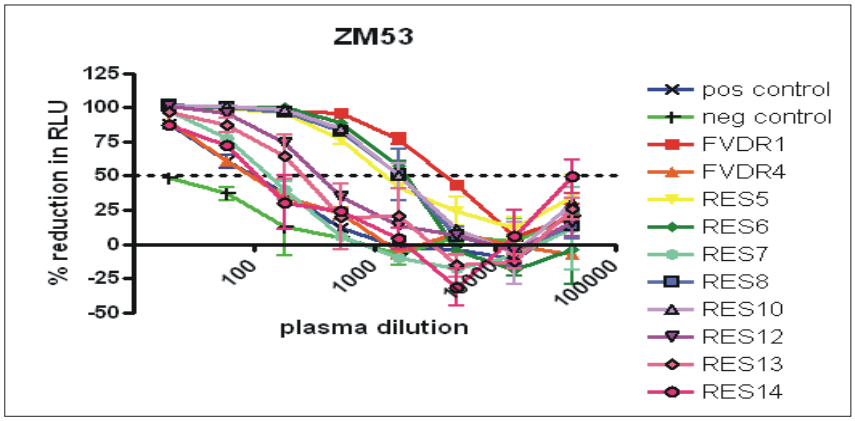


Figure 3.7: (A) Percentage inhibition curves of plasma samples FVDR1 to RES14 from the DR cohort, with dilutions ranging from 1/20 to 1/43740, against the subtype C CAP210, CAP45, DU156 pseudoviruses.





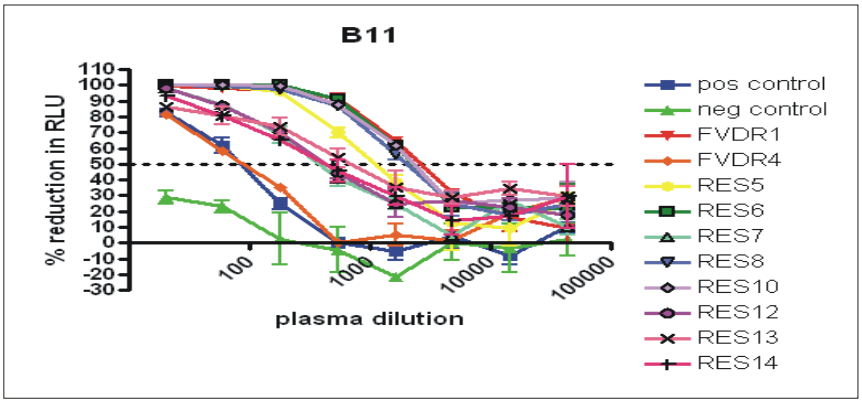


Figure 3.7: (B) Percentage inhibition curves of plasma samples FVDR1 to RES14 from the DR cohort, with dilutions ranging from 1/20 to 1/43740, against the subtype C DU422, ZM53 and the subtype B B11 pseudoviruses.

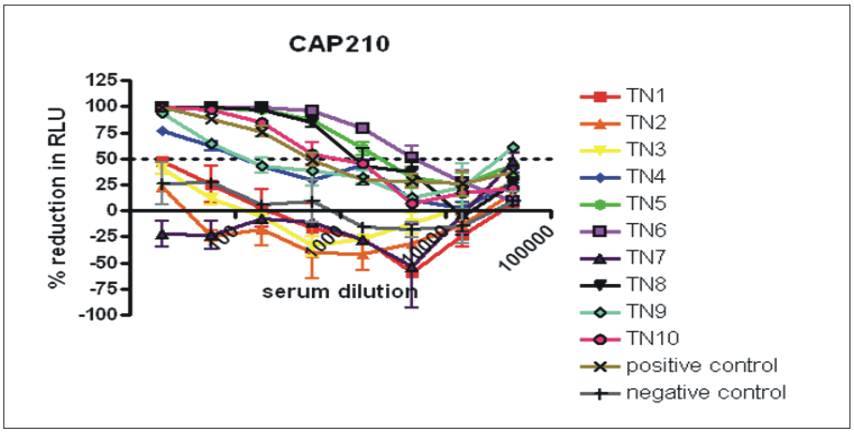
3.5.4 Neutralizing antibody activity within the JM cohort

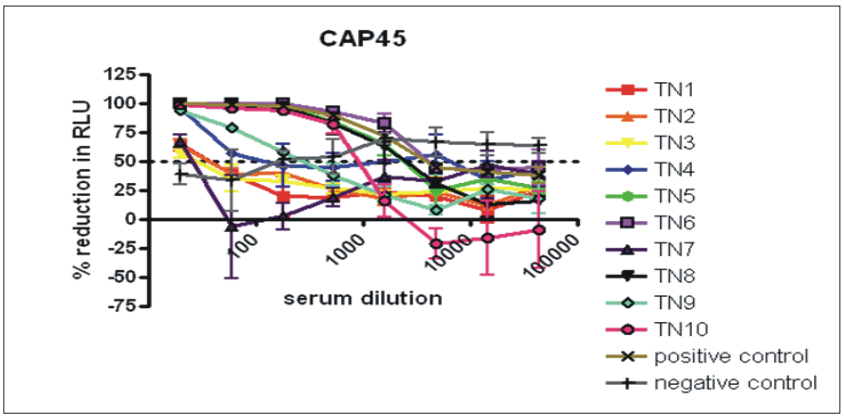
Neutralization activities of the 10 seropositive or seronegative participants with autoimmune diseases from the JM cohort were evaluated against all 6 pseudoviruses and are represented in Figure 3.8A and B. The calculated IC_{50} values are listed in Table 3.4.

The relative light units (RLU) measured for sera TN1, TN2, TN3 and TN7 showed less than 50% inhibition against CAP210, CAP45, Du156, Du422 and ZM53 at the first dilution indicative of little or no neutralizing antibody activity (Figure 3.8A to Figure 3.8B) therefore IC_{50} values could not be obtained for these samples. TN8 and TN10 exhibited potent neutralizing antibody responses against the CAP210 (Figure 3.8A) with IC_{50} values of 1584 and 831, respectively (Table 3.4). Similarly, TN6 showed promising neutralizing antibody activity against CAP210 with 100% inhibition observed at the fourth dilution and the highest IC_{50} value of 3414 (Table 3.4). In addition, TN6 showed a similar percentage inhibition curve to the positive control against CAP45 (Figure 3.8A) with an IC_{50} of 4105 (Table 3.4). Serum sample TN5 showed a greater than average breadth of neutralization against DU156 maintaining almost 100% inhibition at the third dilution with an IC_{50} of 4105 (Table 3.4). In addition, TN6 also showed potent neutralizing antibody activity against DU156 with almost 100% inhibition at the fourth dilution (Figure 3.8A). DU422 was most potently neutralized by TN6 (Figure 3.8B) with the highest IC_{50} value of 13594, TN5 and TN8 also showed greater than average neutralizing antibody activity against DU422 (Figure 3.8B) with IC_{50} values of 1239 and 1634 respectively (Table 3.4).

TN5, TN6, TN8 and TN10 showed neutralizing antibody activity greater than the positive control against ZM53 (Figure 3.8B). TN6 had the highest IC_{50} value of 2590 against ZM53, together with TN5 and TN8 with IC_{50} values of 2026 and 1685 respectively whereas TN10 showed an IC_{50} value of 363 (Table 3.4).

B11 was effectively neutralized by, maintaining 100% inhibition at the third dilution with an IC_{50} value of 2590 (Table 3.4). TN2, TN5, TN8, TN9 and TN10 showed greater antibody neutralization activity compared to the positive control. TN1, TN3 and TN7 showed little or no neutralizing antibody activity against B11.





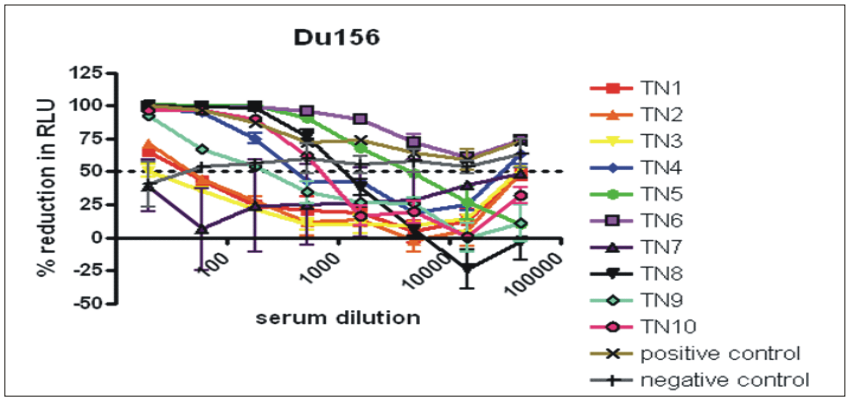
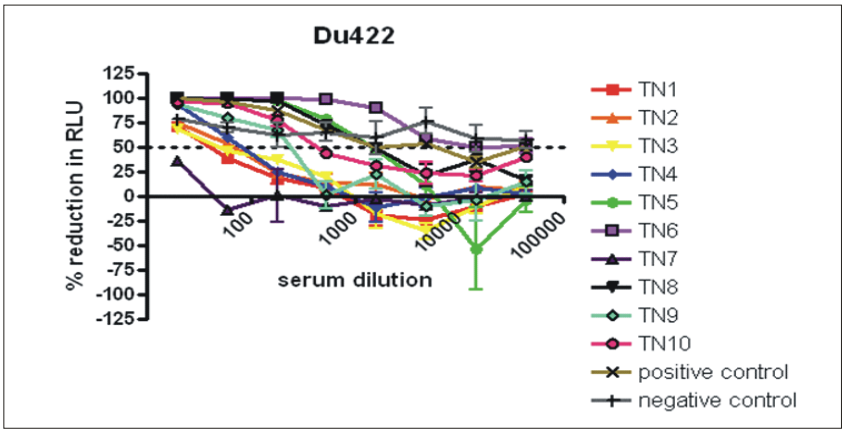
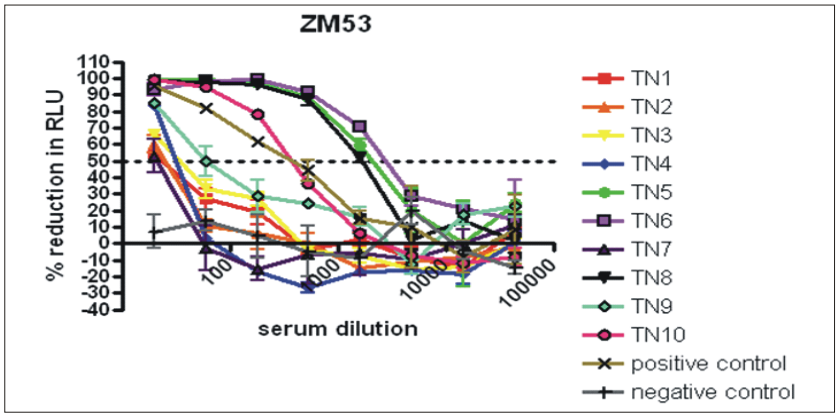


Figure 3.8: (A) Percentage inhibition curves of sera TN1 to TN10 from the JM cohort, with dilutions ranging from 1/20 to 1/43740, against the subtype C CAP210, CAP45, DU156 pseudoviruses.





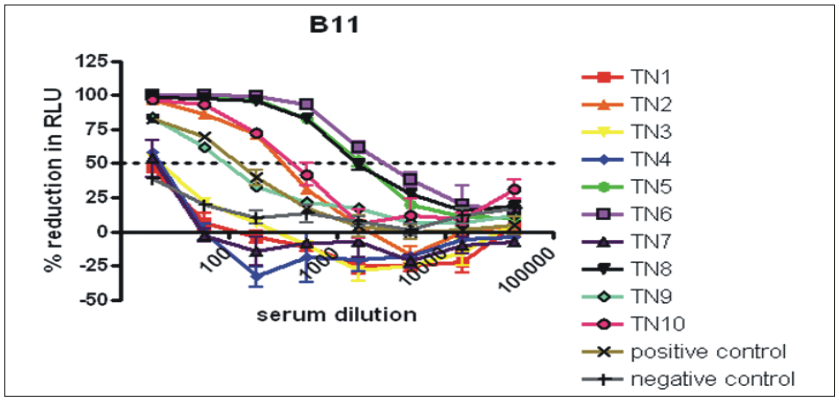


Figure 3.8: (B) Percentage inhibition curves of sera TN1 to TN10 from the JM cohort, with dilutions ranging from 1/20 to 1/43740, against the subtype C DU422, ZM53 and the subtype B B11 pseudoviruses.

Table 3.4: IC_{50} values of 40 patient samples from four different cohorts against 6 pseudoviruses in a neutralizing antibody assay.

PATIENT COHORT	CAP210	CAP45	DU156	DU422	ZM53	B11
CT						
Positive control	292	**1278**	259	**2786**	106	116
SP1	331	302	127	212	163	57
SP2	131	341	65	138	215	59
SP3	113	184	179	297	85	85
SP4	259	50	179	122	140	45
SP5	116	78	558	187	267	153
SP6	**1445**	**1202**	**1202**	**1258**	**1063**	**1096**
SP7	630	423	**1737**	724	67	243
SP8	131	n/a	163	50	78	71
SP9	140	27	109	255	179	47
FV						
Positive control	153	650	243	558	47	78
FV2	363	**1000**	251	392	541	196
FV3	363	222	168	45	91	190
FV5	229	120	**1063**	176	50	100
FV6	179	65	321	142	179	31
FV10	106	59	158	501	52	33
FV11	131	190	149	146	65	36
FV14	650	363	229	**1015**	88	35
FV15	575	611	158	404	292	35
FV23	222	558	410	486	267	37
FV26	113	**1792**	**2154**	**1047**	478	423
FV27	630	508	**1584**	**4713**	131	**1634**
DR						
Positive control	311	**1015**	**14678**	306	95	83
FVDR1	**1792**	**2256**	**3066**	**2796**	**3920**	**2590**
FVDR4	208	27	87	122	92.6	88
RES5	**1359**	621	**1015**	844	**1187**	**1063**
RES6	**2154**	984	**2326**	**1423**	**1934**	**2221**
RES7	153	66	416	56	133.86	386
RES8	**1318**	**1047**	**2796**	39	**1659**	**2026**
RES10	**1737**	**1934**	**1995**	**1609**	**1560**	**2363**
RES12	292	**1338**	660	471	346.74	450
RES13	208	n/a	51	35	247.36	691
RES14	222	n/a	38	n/a	107.98	423
JM						
Positive control	311	**3981**	n/a	**1537**	386	127
TN1	20.2	36	41	40	23	18
TN2	n/a	35	45	62	22	316
TN3	n/a	27	19	51	33	23
TN4	109	104	416	81	28	21
TN5	**2026**	**2435**	**4105**	**1239**	**2026**	**1685**
TN6	**3414**	**4105**	n/a	**13594**	**2590**	**2590**
TN7	n/a	23	n/a	n/a	20	21
TN8	**1584**	**2290**	**1239**	**1634**	**1685**	**1584**
TN9	123	275	229	236	57	85.7
TN10	831	857	691	423	363	386

Overall, the negative control showed some non-specific neutralization activity from approximately 50% at the first/lowest dilution which rapidly decreased by the second and higher dilutions (Figures 3.4 to 3.8). Neutralizing antibody activity of the positive control was comparable amongst the JM and CT cohorts, showing high IC_{50} values and good activity against the subtype C CAP45 and DU422 pseudoviruses (Table 3.4). However the activity of the positive control was not comparable amongst the FV and DR cohort. No promising neutralization was observed against the FV cohort however CAP45 and DU156 were effectively neutralized by the positive control in the DR cohort (Table 3.4).

In summary, plasma sample SP6 contained promising neutralizing antibody activity with the ability to neutralize all 5 subtype C and 1 subtype B pseudovirus/es with the highest IC_{50} values among the slow progressor participants in the CT cohort. Although the FV cohort did not include any plasma samples that could neutralize all 6 pseudoviruses, FV26 and FV27 effectively neutralized 3 of the 6 pseudoviruses. The DR cohort included 5 plasma samples with a greater than average breadth of neutralization. FVDR1 and RES10 effectively neutralized all 6 pseudoviruses, RES6 and RES8 neutralized 5 of the 6 pseudoviruses and RES5 neutralized 4 of the 6 pseudoviruses. The serum samples with the best neutralizing antibody activity in the JM cohort included TN5, TN6 and TN8 (Table 3.4) which were effectively able to neutralize all 6 pseudoviruses.

CHAPTER 4: DISCUSSION

Humoral immunity involves antibody protection against pathogens and subsequent neutralization of pathogens in the body. Most HIV-1 infected individuals produce an autologous neutralizing antibody response during the first year of infection however such antibodies lag behind viral escape mechanisms. One of the major goals for an effective HIV-1 vaccine is to elicit an appropriate cross reactive or broadly neutralizing antibody response capable of controlling infection. To date, only a handful of broadly neutralizing antibodies have been identified which are produced in the minority of infected individuals, illustrating the poor immunogenicity of neutralizing antibody epitopes (Binley 2009). Interestingly some HIV-1 infected individuals develop cross reactive neutralizing antibody responses. Further analysis of such patients provides insight into conditions that favour the production of broadly neutralizing antibodies that could be useful for vaccine development. In this study we screened plasma or serum samples from four different cohorts for the presence of greater than average neutralization breadths against a panel of 5 subtype C and 1 subtype B Env-pseudoviruses.

4.1 Generation of Env-pseudoviruses

Twelve subtype C and 2 subtype B Env-pseudoviruses were generated for use in the pseudovirion neutralizing antibody assay which was used to screen plasma or serum from four different patient cohorts for the presence of HIV-1 specific neutralizing antibodies. Six pseudoviruses were functional and showed a $TCID_{50}$ greater than 10 000 which were subsequently used throughout the study. These

included 5 subtype C pseudoviruses, CAP210, CAP45, DU156, DU422, ZM53 and 1 subtype B pseudovirus, B11 (Table 3.2).

The subtype C Env-pseudoviruses mentioned above are included in a South African subtype C Env-pseudovirus panel (Li, Salazar-Gonzalez et al. 2006). A similar Env panel was developed with subtype B pseudoviruses, of which B11 was included (Li, Gao et al. 2005). Clinical and geographic information is available for all Env isolates used. Env clones with the CAP prefix were isolated from participants in Durban enrolled in a study of acute or early HIV transmission organized by the Center for the AIDS Program of Research in South Africa (CAPRISA) in 2005 (Li, Salazar-Gonzalez et al. 2006). The CAP210 Env was isolated 5 weeks after the estimated infection date from an individual with a viral load of 127 000 RNA copies/ml and a CD4 count of 461 cells/µl and CAP45 was also isolated 5 weeks after the estimated infection date from a participant with a viral load of 236 000 RNA copies/ml and a CD4 count of 974 cells/µl. Env clones with a DU prefix were obtained from commercial sex workers at truck stops between Durban and Johannesburg (Li, Salazar-Gonzalez et al. 2006). The participant from which DU156 Env was obtained showed a viral load of 22 122 RNA copies/ml and a CD4 count of 404 cells/µl. The DU156 Env was isolated in 1999 less than 4 weeks after the patient's estimated infection date. DU422 was isolated in 1998, 8 weeks after the estimated infection date from a patient with a viral load of 17 118 RNA copies/ml and a CD4 count of 409 cells/µl. The ZM53 Env clone was obtained from a participant involved in a study of HIV-1 discordant couples in Lusaka, Zambia. The Env was isolated in 2000, less than 14 weeks after the estimated date of infection. This participant showed a viral load of 26 643 RNA copies/ml, the CD4 count was not available (Li, Salazar-Gonzalez et al.

2006). The subtype B pseudovirus, B11 (also known as PVO4) was isolated in 1996 from a patient in Italy who had been infected for 4 weeks prior to the collection of Env. The patient had a viral load of 2 127 976 RNA copies/ml and a CD4 count of 311 cells/μl (Li, Gao et al. 2005). All Env isolates were R5-tropic.

Recently, Seaman et al. (2010) assembled a panel of 109 Env-pseudoviruses representing a diverse range of isolates to allow for the systemic characterization of neutralizing antibody responses elicited by vaccine immunogens. All major subtypes were included; Envs from post-transmission, early and chronic phases of infection as well as geographically diverse Envs were also included in the panel. HIV-1 positive plasma samples were pooled together to assess the sensitivity of the generated pseudoviruses. All pseudoviruses were classified as either Tier 1A (very high sensitivity to neutralization), Tier 1B (above average neutralization sensitivity), Tier 2 (moderate neutralization sensitivity) and Tier 3 (low neutralization sensitivity). According to this classification, all the subtype C generated pseudoviruses used in our study were classified as Tier 2 (Seaman, Janes et al. 2010).

Another study screened 24 plasma samples from patients with broadly neutralizing antibody activity (Binley, Lybarger et al. 2008). The plasma samples were screened in a TZM-bl pseudovirion based neutralizing antibody assay against a panel of 17 subtype C and 20 subtype B pseudoviruses to confirm cross reactivity. Pseudoviruses were categorised according to neutralization susceptibility. Among other pseudoviruses generated, CAP45, CAP210, DU156, DU422 and ZM53 were included in their subtype C panel. According to the results of their study, CAP45

was most sensitive to neutralization followed by DU156. CAP210 was moderately neutralized by the plasma samples whereas ZM53 and DU422 were quite resistant to neutralization by plasma samples. They also noted that the subtype B infected plasma samples were not able to neutralize the Durban subtype C pseudoviruses, including DU156 and DU422 as effectively as the other subtype C pseudoviruses. By contrast, subtype C infected plasmas were able to neutralize the Durban as well as other subtype C pseudoviruses with no difference in neutralization capacity (Binley, Lybarger et al. 2008). Another study evaluated the neutralization profiles of 70 plasma samples from HIV-1 chronically infected individuals (Gray, Taylor et al. 2009). According to their results, CAP210 was the least sensitive to neutralization and CAP45 showed moderate neutralization sensitivity with DU156 being among the pseudoviruses most sensitive to neutralization (Gray, Taylor et al. 2009). The results of our study are similar to that Gray et al; our data reveal that DU156 was most sensitive to neutralization. In total, 12 out of 40 participant samples were able to effectively neutralize DU156. While CAP45, DU422 and B11 showed moderate neutralization sensitivity, with 10 samples neutralizing these pseudoviruses with high titers. CAP210 and ZM53 were the least sensitive to neutralization in this study, neutralized by only 9 samples. However, the neutralization sensitivity differed within cohorts. For example, within the CT cohort, only 2 out of 9 plasma samples were able to neutralize DU156 whereas only 1 sample neutralized the remaining pseudoviruses. DU156 and DU422 were most easily neutralized within the FV cohort, effectively neutralizing 3 out of 11 plasma samples. Within the DR cohort, 5 out of 10 plasma samples neutralized CAP210, DU156, ZM53 and B11 and within the JM cohort, 3 samples were effectively able to neutralize all 6 pseudoviruses.

4.2 Establishment of the HIV-1 pseudovirion based neutralizing antibody assay

A standardized and reproducible neutralizing antibody assay for the measurement of neutralizing antibodies is needed for the accurate comparison of antibody responses against a panel of diverse viral strains among different laboratories (Polonis, Brown et al. 2008). Two types of neutralizing antibody assays are currently used which make use of virus propagated in peripheral blood mononuclear cells (PBMC) or viruses produced by pseudotyping (Polonis, Brown et al. 2008). Viruses derived from PBMC's represent a heterogeneous viral population since the viruses use the error prone reverse transcriptase to replicate. By contrast, pseudoviruses are derived from co-infection of HEK 293T cells with a DNA plasmid encoding the HIV genome lacking the Env component together with an Env-encoding plasmid from a strain of choice. Pseudoviruses allow for the production of a homogeneous viral population with only one Env strain incorporated. Pseudotyped viruses are used to infect TZM-bl cells whereas PBMC derived viruses are used to infect PBMC's (Polonis, Brown et al. 2008). The pseudovirion neutralizing antibody assay is advantageous because it reduces variables such as viral growth kinetics or other non-antibody related factors which may influence the results, however this assay format has concerns regarding the reproducibility of results. Although the PBMC based neutralizing antibody assay is a better representation of natural infection, this assay format shows variation in results possibly due the genetic variability from different PBMC donors (Mascola, D'Souza et al. 2005; Polonis, Brown et al. 2008).

Eighteen plasma samples (SANBS) were purchased for the set up of the pseudovirion neutralizing antibody assay. Plasma sample 14 which was selected as the positive control for this study did not show reproducible results throughout all pseudovirion neutralizing antibody experiments due to variations in the assay, as noted by other researchers (Mascola, D'Souza et al. 2005; Polonis, Brown et al. 2008). However, the negative control plasma was fairly consistent and did not exceed 50% inhibition at the first dilution and rapidly decreased by the second dilution (Figures 3.4 to 3.8).

4.3 CT Cohort

Slow progressors are of great interest because they may provide information about HIV control mechanisms which could be used for vaccine design and development. However, the role of neutralizing antibodies in delayed disease progression among these individuals is controversial. Several studies have implicated the cellular immune system, in particular CD8+ cytotoxic T lymphocytes as a major contributor in the control of HIV-1 infection among slow progressors (Ogg, Kostense et al. 1999; Gillespie, Kaul et al. 2002). In addition, the major histocompatibility complex (MHC) class I allele HLAB*57 is found to be over represented in these individuals (Costello, Tang et al. 1999; Gillespie, Kaul et al. 2002; Altfeld, Addo et al. 2003). MHC class I molecules are involved in interactions with CD8+ cytotoxic T-lymphocytes to initiate killing of infected cells. By contrast, little or no role for the humoral immune response has been reported in patients with slow disease progression (Zwart, van der Hoek et al. 1994; Cecilia,

Kleeberger et al. 1999). Cecilia et al. (1999) evaluated neutralizing antibody responses in rapid progressors and long term non progressors (LTNP). Their results showed no significant differences in neutralizing antibody titers against primary HIV-1 isolates during early infection (when both groups were asymptomatic). Alternatively, at a later time point (when rapid progressors showed signs of clinical AIDS), long term non progressors exhibited increased neutralizing antibody titers whereas rapid progressors showed decreased neutralizing antibody levels. Another study by Zwart et al. (1994) studied the rate of disease progression after seroconversion and the level of IgG antibody responses in a group of rapid progressors and slow progressors. No significant difference was observed in neutralizing antibody responses against Env epitopes between the two groups. However more vigorous neutralizing antibody responses against the V3 domain and gp41 epitopes were observed in rapid progressors a few months after seroconversion. A study by Doria-Rose et al. (2009) also showed that there was no significant different between the neutralizing antibody responses of progressors and slow progressors. In their study, serum samples were screened from three cohorts, long term non progressors, slow progressors and progressors, for the presence of broadly neutralizing antibodies. Their results revealed that progressors had the highest titer (42%) of broadly neutralizing antibody responses against a panel of 5 Env-pseudotyped viruses and among the slow progressor cohort, 41% of the samples showed broadly neutralizing antibodies. Long term non progressors showed the lowest titre (25%) of broadly neutralizing antibodies. The progressor and slow progressor cohorts did not differ significantly in the proportion of patients with good neutralization breadth (Doria-Rose, Klein et al. 2009). This suggests that neutralizing antibodies play a little role in the delayed

disease progression evident among slow progressors (Altfeld, Addo et al. 2003; Bailey, Lassen et al. 2006; Euler, van Gils et al. 2010). By contrast, other studies claim that neutralizing antibodies may play a significant role in viral control. Montefiori et al. (1996) evaluated the serum from LTNP and progressors for the presence of neutralizing antibodies and infection enhancement. Their results revealed that LTNP had significantly higher neutralizing antibody titers when compared to progressors. Similarly another study showed that LTNP exhibited strong neutralizing antibody response against a diverse panel of primary isolates (Cao, Qin et al. 1995).

The above studies highlight the controversy around the role of neutralizing antibodies in individuals with slow disease progression. In this study, we evaluated the presence of neutralizing antibodies in participants with slow disease progression. Our results revealed 2 plasma samples (SP6 and SP7) with exceptionally good neutralizing antibody activity against a panel of 6 pseudoviruses (5 subtype C and 1 subtype B). The remaining 7 samples did not harbour a greater than average breadth of neutralizing antibodies. SP6 was able to neutralizing all 6 generated pseudoviruses with high neutralizing antibody titers. From the available clinical data, SP6 showed a CD4 T-cell count of 267 cells/µl and a viral load of below 400 RNA copies/ml. At the time of sample collection, no other clinical data was available, but it was determined that patient SP6 was on antiretroviral treatment at the time of sample collection (Truvada and Efavirenz), which could account for the higher neutralization detected (see below). No clinical symptoms of AIDS were reported.

SP7 showed promising percentage inhibition curves against 3 subtype C pseudoviruses (CAP210, DU156 and DU422) with a high IC_{50} value obtained for DU156 (1737) only. Clinical data revealed that SP7 was asymptomatic at the time of sample collection with a CD4 count of 426 cells/µl and a viral load of 71700 RNA copies/ml. Thus SP7 is a promising candidate for further evaluation and characterization of their neutralizing antibody responses.

The potent neutralization activity of SP6 may be attributed to the ARV treatment. There is evidence of immune reconstitution in patients undergoing treatment with HAART (Gulick, Mellors et al. 1997). Among patients who initiate HAART therapy during chronic infection, it has been documented that there is an increase or reconstitution of neutralizing antibody responses (Gulick, Mellors et al. 1997). This is supported by findings which demonstrated that spontaneous reconstitution of neutralizing antibody responses were observed in 4 out of 19 chronically HIV-1 infected patients undergoing HAART therapy (Kimura, Yoshimura et al. 2002).

Possible mechanisms for the delayed disease progression in the remaining 7 subjects may include host genetic factors such as polymorphisms in their HLA or chemokine receptors or infection with attenuated viruses such as *nef*- defective particles associated with slow progression as well as strong CD8+ cytotoxic T lymphocyte responses (Dyer, Zaunders et al. 2008). In addition, structural observations have been made about the length of the variable loops among slow progressors. The length of the variable loops on the Env appears to correlate with antibody escape; longer loop lengths are associated with escape from antibody recognition (Gray, Moore et al. 2007). Interestingly, the V1V2 loop lengths are

shorter in HIV controllers than in non controllers during early and chronic infection (Sagar, Wu et al. 2006). The number of N-linked glycosylation sites on the surface of gp120 correlates with viral escape from immune recognition and HIV controllers were found to have a significantly lower number of potential N-linked glycosylation sites compared to non controllers, suggesting that viral adaptive escape mechanisms are limited in these individuals (Sagar, Wu et al. 2006; Gray, Moore et al. 2007). In addition, Bello et al. (2005) found that the rate of viral evolution tends to be much lower in slow progressors compared to progressors, similarly the diversity of the virus emerged at a much lower rate in patients who could control HIV infection compared to non controllers.

Van Gils et al. (2009) studied 35 subjects in the Amsterdam cohort, comprising 20 long term non progressors and 15 progressors for the presence of cross reactive neutralizing antibodies against subtype A, B, C and D pseudoviruses at 2 and 4 years post-seroconversion. This study concluded that 2 years after seroconversion 20% of the subjects had developed cross reactive neutralizing antibody responses and this figure increased to 31% 4 years after seroconversion. They also revealed that high viral loads and low CD4 counts were associated with early development of cross reactive neutralizing antibodies and neutralizing antibody titers were shown to increase during the course of infection in 91% of the participants (van Gils, Euler et al. 2009). Importantly, this study concluded that no significant differences were observed in neutralizing antibody serum activity between long term non progressors and progressors.

According to the results of this study, no direct correlation can be made between the presence of neutralizing antibodies and slow disease progression. Although one sample (SP6) was identified with exceptionally broad neutralizing antibodies, further studies are required to confirm that the ARV treatment is not contributing to the overall measured neutralization effect.

4.4 FV cohort

Eleven HIV-1 subtype C infected patients who were naive to ARV treatment were included in the FV cohort. Screening of the 11 plasma samples for the presence of neutralizing antibodies revealed several patients with moderate neutralization capacity against a panel of 6 pseudoviruses. FV26 exhibited greater than average breadths of neutralization against 3 subtype C pseudoviruses (CAP45, DU156 and DU422). Unfortunately no clinical data was available for this patient. FV27 in particular, is of special interest with its ability to effectively neutralize the subtype B pseudovirus, B11 as well as the subtype C pseudoviruses, DU156 and DU422, suggesting that FV27 contains cross reactive neutralizing antibodies against subtypes B and C isolates. Interestingly, this patient has a very low CD4 T-cell count of 11 cells/µl and a viral load of 158 000 RNA copies/ml. Plasma sample FV14 also had a very low CD4 T-cell count of 16 cells/µl but the viral load was not available. This patient showed neutralizing antibodies capable of inhibiting the subtype C pseudovirus DU422.

Patient plasma sample FV2 had a CD4 T-cell count of 159 cells/µl and a viral load of 22900 RNA copies/ml. This patient was able to neutralize 2 subtype C

pseudoviruses (CAP45 and ZM53) with moderate neutralization breadth. FV5 showed a very high viral load of 750 000 RNA copies/ml and a CD4 T-cell count of 133 cells/µl, FV5 was able to neutralize one subtype C pseudovirus (DU156) with a greater than average breadth of neutralization.

Consistent with the data obtained by van Gils et al. (2009), the results of our study show that patients with exceptionally low CD4 T-cell counts (FV14 and FV27) can exhibit potent neutralizing antibodies capable of neutralizing subtype C as well as subtype B (plasma samples FV27) isolates.

Kelly et al. (2005) evaluated the neutralizing antibody patterns and viral escape in 10 HIV-1 chronically infected patients with non-B subtypes. All patients included in the study were ARV treatment naive. Their results revealed that all 10 subjects had autologous neutralizing antibodies that could neutralize virus obtained 6 months earlier but not later time point viruses. However this study used primary virus rather than pseudoviruses and it has been documented that primary viruses are generally more difficult to neutralize than pseudovirus (Moore, Cao et al. 1995).

Devito et al. (2006) evaluated the autologous and heterologous neutralizing antibody responses before and during ARV treatment of 6 subjects. Before the initiation of ARV treatment, the subjects had viral loads ranging from 70 000 to 800 000 RNA copies/ml and CD4 counts ranging from 20 to 360 cells/µl. Before ARV treatment, results revealed that all subjects showed very little and weak autologous neutralizing antibody responses ranging from 0 to 38% as well as

weak heterologous neutralizing antibody responses. Patients designated A, B and C neutralized 3, 2 and 1 out of 8 heterologous viruses respectively before antiretroviral treatment was initiated. These patients had all had low CD4 counts and high viral loads. Patient A had a very low CD4 count of 20 cells/μl and a viral load of 70 000 RNA copies/ml. Patient B had a CD4 T-cell count of 110 cells/μl with a very high viral load of 800 000 RNA copies/ml and patient C and a CD4 count of 80 cells/μl with a viral load of 70 000 RNA copies/ml (Devito, Hejdeman et al. 2006). ARV treatment was initiated for a 12 to 19 month period and serum samples were subsequently re-analysed for neutralization activity. Results post-treatment revealed no significant improvement in autologous or heterologous neutralizing antibody responses despite successful ARV treatment and increased CD4 counts (Devito, Hejdeman et al. 2006). The data obtained from this study before treatment are consistent with our data, in that generally weak neutralization responses were observed within the FV cohort and the subjects with very low CD4 counts and very high viral loads exhibited greater than average neutralizing antibody activity.

Consistent with some of the literature (Zwart, van der Hoek et al. 1994; Cecilia, Kleeberger et al. 1999; Doria-Rose, Klein et al. 2009), the neutralizing antibody responses between patients with slow disease progression and patients with AIDS were similar. The CT cohort revealed 2 samples (SP6 and SP7) with 1 (SP6) sample that exhibited potent neutralization breadth against 6 pseudoviruses and the FV cohort revealed 2 (FV26 and FV27) with greater than average breadth of neutralization against 3 pseudoviruses. This suggests that neutralizing antibodies may be a poor predictor of disease progression.

4.5 DR cohort

The use of antiretroviral therapy (ART) has transformed the management of HIV-1. However, one of the major challenges with ART is that prolonged use or non-adherence can lead to virus escape mutants who are no longer susceptible to the drug and so drug resistance develops (Menendez-Arias 2002). Studies have found that certain mutations conferring drug resistance in viruses may be considered favourable in some cases, providing new ways to target the virus by increasing susceptibility to neutralizing antibodies (Reeves, Lee et al. 2005; Hu, Mahmood et al. 2007). Certain mutations may occur in the N-linked glycosylation sites of gp120 rendering the virus more susceptible to neutralization by the broadly neutralizing antibody 2G12 (Hu, Mahmood et al. 2007). A recent study demonstrated that a virus strain resistant to an anti-HIV agent cyclotriazadisulfonamide (CADA), targeting the CD4 region of gp120 showed increased sensitivity to the 2G12 neutralizing antibody as well as soluble CD4 (Vermeire, Van Laethem et al. 2009). This study found that anti-CD4 monoclonal antibodies bound more efficiently to the CADA escape mutant compared to the wild type isolates. In the case of the CADA resistant gp120, the escape mutations involved the reduction of N-linked glycosylation sites on gp120 which rendered the virus more susceptible to neutralization by neutralizing antibodies (Vermeire, Van Laethem et al. 2009).

In order to evaluate the neutralizing antibody profiles of treatment experienced patients failing therapy, we screened 10 plasma samples from HIV-1 subtype C infected individuals who were treatment experienced and failing their first line treatment regimen for the presence of neutralizing antibodies.

Our results revealed 5 plasma samples with exceptionally good neutralizing antibody responses, these included plasma samples FVDR1, RES5, RES6, RES8 and RES10. Interestingly, 4 out 5 samples (FVDR1, RES6, RES8 and RES10) showed promising neutralizing antibody activity against the subtype B, B11 as well as subtype C pseudoviruses. This implies that these participants may contain cross reactive neutralizing antibodies in their plasma or that the ARV treatment played a role in the potent neutralization breadths of these individuals. The above plasma samples all showed common clinical data, with low CD4 counts and high viral loads. However this was not unique among samples which exhibited good neutralization activity since low CD4 counts and high viral loads are usually expected among individuals who are failing ARV therapy. Therefore no conclusion can be drawn from the clinical data of these individuals. The presence of ARV drugs in the plasma of these individuals may have contributed to some extent to the measured neutralization. This may have occurred through the carry over of any ARV drugs present in the plasma to the pseudovirion neutralizing antibody assay (Dreyer, Kallas et al. 1999).

The effect of HAART on the humoral immune response to HIV-1 was evaluated by Binley et al. (2000). They concluded that when HAART is initiated during acute infection, the humoral response to HIV-1 is suppressed. They also argued that when HAART is initiated after several years of infection, it has little effect on neutralizing antibody responses. Morris et al. (2001) performed a longitudinal study, evaluating the humoral immune responses in 19 chronically infected patients. The patients were categorised into 2 groups, HAART successes (viral load of <500 RNA copies/ml within 12 weeks of therapy and continued

suppression for 1 year) and HAART failures (less than 10 fold decrease in viral load within 1 year on HAART). They collected plasma samples before and 1 year after initiation of HAART. Their results before therapy revealed that neutralizing antibody titers against the HIV-1 T-cell line adapted strain MN was significantly higher in patients with successful HAART therapy compared to patients who were failing therapy, however MN neutralizing antibody titers decreased in treatment successes over time whereas the titers remained stable during the same time period in treatment failures. Interestingly, when neutralizing antibody titers were evaluated against clinical subtype B isolates, a different neutralization pattern was observed. Before HAART was initiated, neutralizing antibody titers were higher in treatment successes than treatment failures. One year after HAART initiation, the treatment successes showed a decrease in cross neutralizing antibody titers. By contrast HAART failures exhibited more cross reactive neutralizing antibodies after 1 year of HAART (Morris, Katzenstein et al. 2001). Overall, neutralizing antibody titers and breadth was greater in patients failing therapy compared to patients with successful therapy. This study suggests that the suppression of viremia as a result of successful HAART therapy correlates with a decreased humoral response; this may be due to decreased antigen exposure and stimulation (Morris, Katzenstein et al. 2001). This is consistent with the data from our study where 50% of the samples within the DR cohort show potent neutralization breadth and 40% of the samples exhibited cross reactive neutralizing antibodies.

Overall, treatment experienced patients failing their first line therapy regimens harbour increased neutralization levels compared to treatment naive patients. This is consistent with the findings of Morris et al (Morris, Katzenstein et al. 2001). The

observed increase in neutralization capacity of treatment experienced patients may however be due to the suppression of viral loads during therapy, allowing time for the partial recovery of the humoral immune system, therefore once viral loads increase again in treatment failures, the immune system is able to mount a potent humoral response against the virus (Bailey, Lassen et al. 2006). However, longitudinal studies would need to be performed to make a direct comparison of the neutralizing antibody responses in patients before and during or after HAART.

4.6 JM Cohort

The 2 broadly neutralizing antibodies, 2F5 and 4E10 which show reactivity to the host phospholipid, cardiolipin also share similar structural features to antibodies produced in autoimmune disease patients (Haynes, Moody et al. 2005). This could provide a possible reason for why they are so rarely produced in infected individuals due to B-cell tolerance mechanisms preventing their production (Verkoczy, Diaz et al.). Molecular mimicry may play a vital role in the auto reactive nature of these antibodies. For example, the HIV-1 V3 loop shares homology with a conserved part of the T-cell receptor (Lake, Schluter et al. 1994). Similarly the gp41 region of the HIV-1 envelope shares homology with a conserved domain of the MHC class II molecule (Golding, Robey et al. 1988; Fraziano, Montesano et al. 1996). Interestingly, the C5 region of gp120 also shows homology with host MHC class I and class II molecules (Cadogan, Austen et al. 2008).

To evaluate the presence of HIV-1 specific neutralizing antibodies among individuals with autoimmune diseases, we screened the serum of 10 HIV-1

seropositive or seronegative participants with autoimmune disorders. Our results revealed 3 serum samples TN5, TN6 and TN8 that showed greater than average neutralization breadths against a panel of 5 subtype C and 1 subtype B Env-pseudoviruses. Further analysis and characterization studies are required to map the neutralizing epitopes of these antibodies and determine if 2F5 or 4E10-like antibodies are present in the serum of these individuals. All 3 serum samples were isolated from HIV-1 seropositive individuals.

Participant TN6 had a CD4 count of 101 cells/μl; however, the viral load was not available. This patient also presented with the autoimmune disorders, rheumatoid arthritis and bicytopenia. Rheumatoid arthritis is a systemic autoimmune disorder disease affecting the synovial tissues. Interestingly it has been found that the chemokine receptor, CCR5 plays a major role in disease progression of rheumatoid arthritis (Gómez-Reino, Pablos et al. 1999). Participant TN5 had a CD4 count of 250 cells/μl and presented with pancytopenia and TN8 was isolated from an HIV-1 infected individual with a CD4 counts of 255 cells/μl and an undetectable viral load while TN10 was isolated from a patient who presented with antiphospholipid syndrome (APS) as well as cytopenias. This participant had a very low CD4 count of 55 cells/μl and a viral load of 35 000 RNA copies/ml. APS is characterized by the production of auto antibodies against phospholipids such as cardiolipin and β_2-glycoprotein I, also known as apolipoprotein H (Rand 2007). APS is often seen in conjunction with other autoimmune disorders such as Immune Thrombocytopenia Purpura (ITP) which is characterized by platelet destruction and impaired platelet production.

Serum samples TN2 and TN9 are of great interest since they were only able to neutralize the subtype B pseudovirus, B11 but none of the subtype C pseudoviruses. This suggests that these subjects harbour neutralizing antibodies which recognize subtype B viruses better than subtype C viruses. These may include 447-52D-like antibodies or 2G12-like antibodies which recognize epitopes that are better exposed on subtype B viruses than subtype C viruses. As mentioned previously, 447-52D recognizes the GPGR motif present on the V3 loop of subtype B viral isolates and 2G12 neutralization is dependent on glycans at positions 295, 339 and 386 however the glycan at position 295 is not present in the majority of subtype C isolates (Binley, Wrin et al. 2004). 2F5 also exhibits better neutralization against subtype B isolates compared to subtype B because the minimum requirement epitope (DKW) is substituted with DSW in most subtype C viruses (Purtscher, Trkola et al. 1996; Stiegler, Kunert et al. 2001).

Clinical data showed that TN2 was HIV-1 seropositive and presented with thrombocytopenia and TN9 was HIV-1 seropositive with bicytopenia. Interestingly, all the serum samples with greater than average neutralizing antibody activity showed presented with cytopenias, (pancytopenia, bicytopenia or thrombocytopenia). Cytopenia refers to the reduction in the number of blood cells (Pizzo PA. 1999). Thrombocytopenia refers to the destruction of platelets whereas bicytopenia is characterized by the depressed bone marrow production of 2 cell lines and pancytopenia is characterized by the reduction in erythrocytes, leukocytes and platelets (Pizzo PA. 1999).

A study by Rawson et al (2007) showed that chronic infection with HIV-1 also results in the production of autoreactive CD8+ T-cells that present a set of self-peptides on MHC class 1 that have escaped thymic tolerance. This is suggestive that HIV-1 exposure results in the breaking of tolerance mechanisms in the host making it susceptible to cellular and humoral autoreactivity. It has also been documented that HIV-1 infection causes severe damage to memory B-cells responsible for antibody production (Cagigi, Nilsson et al. 2008). These findings provide possible explanations for the elicitation of cross reactive neutralizing antibodies with auto reactivity in HIV-1 infected patients.

Bone marrow samples were collected from 8 out of the 10 participants for future studies of participant samples with exceptionally broad neutralizing antibody activity. RNA would be isolated from the bone marrow of these samples and used to generate an antibody phage display library in an attempt to generate monoclonal antibodies with broadly neutralizing activity against diverse viral isolates.

4.7. Concluding remarks

In this study we evaluated the presence of neutralizing antibodies in patients with HIV-1 infection with slow disease progression, HIV-1 subtype C chronically infected treatment naive patients, HIV-1 subtype C infected treatment experienced patients failing first line therapy and HIV-1 seropositive or seronegative participants with autoimmune diseases. All samples were screened against a

panel of 6 generated pseudoviruses comprising 5 subtype C and 1 subtype B Env-pseudoviruses.

No correlation was observed between the presence of neutralizing antibodies and slow disease progression within the CT cohort. In addition, subjects who had progressed to AIDS in the absence of antiretroviral therapy showed similar neutralizing antibody levels to the slow progressors. These results suggest that the delayed progression observed in the 9 slow progressor participants is not due to the presence of neutralizing antibodies. Further analysis into the participants' genetics and cellular immune responses are required for the characterization of slow progression in these individuals (these studies are currently underway in the Tiemessen laboratory). Although the DR cohort showed high neutralizing antibody titers, our assay could not confirm that it was due to neutralizing antibodies and not antiretroviral drugs. To confirm that the measured neutralization of these samples are due to neutralizing antibodies, future studies are required and should include HIV-1 negative controls with the addition of the predicted ARV drug levels or the removal of drugs before the assay via dialysis, or the purification of antibody from the plasma samples prior to the neutralization assays. The latter would be the most accurate measurement of the presence of neutralizing antibodies in these plasma samples (Dreyer, Kallas et al. 1999). Cross sectional analysis of neutralizing antibody responses before and during HAART reveal that treatment experienced patients failing first line therapy harbor increased neutralizing antibody titers compared to treatment naïve patients.

Within the JM cohort, 3 participants (TN5, TN6 and TN8) are worth investigating further against a larger panel of pseudoviruses representing diverse subtypes and the isolation of RNA may be required from bone marrow samples that were obtained from these patients during this study for the generation of monoclonal antibodies.

In conclusion, this study has identified 6 individuals out of 40 with exceptionally broad neutralizing antibodies capable of neutralizing all 6 subtype C and B generated pseudoviruses used in this study. In addition, 5 individuals were identified that were able to neutralize at least 3 out of 6 generated pseudoviruses. Further characterization of the antibody responses in these individuals is necessary to provide useful insight into the specificities of broadly neutralizing antibody epitopes which may be useful for the generation of vaccine immunogens.

APPENDICES

Appendix A

A.1 Ethics

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Maria Papathanasopoulos

CLEARANCE CERTIFICATE	**M090547**
PROJECT	Identificationand Characterization of HIV-1 Specific Neutralizing antibodies in Patients with with HIV-1 Infection or autoimmune Disorders with or without HIV-1 Infection
INVESTIGATORS	Dr Maria Papathanasopoulos.
DEPARTMENT	Molecular Medicine & Haematology
DATE CONSIDERED	09.05.29
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 09.05.29 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr J Mahlangu

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

A.2 Patient information leaflet

Introduction

Good day, we are from the Department of Molecular Medicine and Haematology at the University of the Witwatersrand Medical School. We are doing research on HIV to help us understand how to make a vaccine against this disease. We would like to invite you to consider participating in a research study, entitled 'Identification and characterization of HIV-1 specific neutralizing antibodies from HIV-1 seropositive patients and autoimmune (HIV-1 seropositive or seronegative) participants.'

1. Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks, discomforts as well as the alternative procedures that are available to you as a study participant.

2. This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to take part in this study.

3. If you have any questions, do not hesitate to ask me.

4. If you choose to participate in this study, you will be free to withdraw from the study any time without giving reasons for your withdrawal. This will not affect your right to future care at the hospital.

5. If you choose to participate in this study, you will be asked to sign this document to confirm that you understand the study.

The following information describes the study and your role as a possible participant. We will answer any questions you may have about this information sheet and about the study. Please read this information carefully and do not hesitate to ask any questions about the study information provided below.

Why are we doing this study?

The aim of this study is to investigate if you have certain special proteins called neutralizing antibodies against HIV. The reason we are interested in the neutralizing antibodies is that they will help us understand how the HI virus infects the body and how the body responds to the virus. These antibodies will also help us to develop vaccine against the HI virus. Currently there is no vaccine against the HI virus.

Why are we interested in you as participant in this study?

Your doctor has requested us to perform a procedure called bone marrow aspiration. This procedure is necessary for your doctor to be able to find out what is wrong with you. Bone marrow aspiration involves putting a needle into your bone space and sampling the content of the bone space called bone marrow. Bone marrow is then sent to the laboratory for analysis and for reporting.

Whilst doing this procedure we would like to take an additional sample of bone marrow to be used for this research. The sample taken will be approximately a teaspoon (5ml) of bone marrow.

In addition to the bone marrow, we would like to request that you donate a tablespoon (10ml) of blood which will be taken from your arm vein. This blood and bone marrow samples will then be taken to laboratory for further analysis.

What happens to your blood and bone marrow samples in the laboratory?

The samples taken will be handled differently depending the nature of analysis to be done. Blood samples will be spun, separated, frozen and stored. They will then be analysed for neutralizing antibodies. Bone marrow samples will be spun, separated and also frozen. The tests done on these samples will be only those outlined in this study. Consent will need to be obtained from the Human Research Ethics committee if there is a need to do other testing on the remaining samples.

What are the risks and discomforts in this study?

Blood sampling will be exactly the same as it done during your normal hospital visit. Drawing blood may result in fainting, dizziness, inflammation of the vein, pain, bruising, bleeding, or infection at the site of puncture. The amount of blood taken is small and will therefore have negligible effects to your health. Bone marrow sampling is associated with pain. This pain is minimized by injecting local anaesthesia at the site of aspiration. Drawing bone marrow may result in fainting, dizziness, inflammation, pain, bruising, bleeding, or infection at the site of puncture. The pain is further minimized by giving you a pain tablet before and after the procedure.

What are the benefits of participating?

You will not personally benefit from participating in this study. You will not be paid or compensated in any way for participating in the study.

What if you do not want to participate in the study?

You may choose not to participate in this study. If you decide not to participate in this study, you will continue to receive your standard care in this hospital.

How is confidentiality of the study information protected?

All the information collected from you, including samples, will be identified with a number to ensure your identity will be kept confidential. Only the study doctors hold the information that allows the number to be linked to your name.

The information collected will be processed, analyzed and reported by study doctors. The result will also be published in scientific articles. You will not be identified in person in any reports/publications.

You have the right to ask to be shown what data about you has been collected and if you think anything is incorrect you may ask to have it corrected.

Representatives of the Ethics Committee staff may require access to your medical records to ensure the study is properly conducted and that the data collected is correct. Your privacy will be respected.

Study-related Injury

If you suffer an injury or illness because of your direct participation in this research study, you will be given any medical treatment that is necessary to help you recover from the injury or illness.

Whom to contact if needing further information on this study?

Your study doctor or his/her delegate will answer any questions you may have. The 24-hour telephone number, through which you can reach your study doctor or another authorised person, is 083 644 5659.

If you want any information regarding your rights as a research participant, or complaints regarding this research study, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301.

Voluntary Participation

Your participation in this study is voluntary. No study procedures will be performed until you have read, understood, and signed this informed consent form. You may refuse to participate or may discontinue participation at any time during the study without penalty or loss of benefits to which you are otherwise entitled.

If you decide to participate in this study, you will be given a signed copy of this information sheet.

Ethical Approval

This study protocol has been submitted to the University of the Witwatersrand, Human Research Ethics Committee (HREC) and written approval has been granted by that committee.

The study has been structured in accordance with the Declaration of Helsinki (last updated: September 2004), which deals with the recommendations guiding doctors in biomedical research involving human participants. A copy may be obtained from your study doctor should you wish to review it.

A.3 Patient informed consent

INFORMED CONSENT (FOR PARTICIPANTS 18 YEARS OR OLDER)

- I have read this information sheet and my questions have been answered to my satisfaction.
- I voluntarily consent to participate.
- I understand that if I choose to not participate or to withdraw, my current medical care will not be affected by this decision.
- I authorize the release of my medical records to the Authorized Representatives, as specified in this consent form.
- By signing and dating this consent form, I have not waived any of the legal rights that I would have if I were not a participant in a research study.

1. PARTICIPANT:

Printed Name Signature / Mark or Thumbprint Date and Time

2. STUDY DOCTOR/NURSE:

Printed Name Signature Date and Time

3. TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT…….. **(DESIGNATION):**

Printed Name Signature Date and Time

Appendix B

B.1. Tissue culture reagents

B.1.1 Complete RPMI-1640

50 ml 10% heat inactivated foetal calf serum (FCS) (Gibco,USA), 5 ml L-glutamine (Gibco,USA) and 2.5 ml antibiotics (0.01mg/ml streptomycin and 100 units/ml penicillin) (Sigma-Aldrich) were added to 500 ml RPMI-1640 (Sigma-Aldrich) and mixed thoroughly before storage at 4°C until use.

B.1.2 Freezing mix

9 ml FCS (Gibco, USA) was added to 1 ml DMSO (Fluka, USA) and mixed thoroughly before use.

B.1.3 Complete Dulbecco's Modified Eagle Medium (DMEM)

50 ml 10% heat inactivated FCS (Gibco, USA), 5 ml of 2mM L-glutamine and 2.5 ml antibiotics (Sigma-Aldrich) (0.01mg/ml strep and 100 units/ml penicillin) were added to 500 ml DMEM (Sigma-Aldrich) and mixed thoroughly before storage at 4°C until use.

Appendix C

C.1. Solutions for bacterial cell culture

C.1.1 LB Broth

10g tryptone (Oxoid Inc; Hampshire, England), 5g yeast extract (Biolab; Gauteng, South Africa) and 10g NaCl (Sigma-Aldrich) were dissolved in 1 litre deionised water (Adcock Ingram). The broth was then autoclaved for 20 minutes on the liquid cycle (121ºC, 1kg/cm^2, 20 minutes) and stored at room temperature until use.

C.1.2. Transformation buffer

1.4702 g $CaCl_2.2H_2O$ (Merck), 0.3024g PIPES (Boehringer Mannheim; Germany) and 15 ml of autoclaved glycerol were made up to a final volume of 100 ml with distilled water (Adcock Ingram). The pH of the solution was adjusted to 7.0 with 10M NaOH (Merck). The buffer was autoclaved on the liquid cycle (121ºC, 1kg/cm^2, 20 minutes) and stored at -20 ºC until use.

C.1.3. Nutrient Agar Plates

2g tryptone (Oxoid Inc; Hampshire, England), 1g yeast extract (Biolab; Gauteng, South Africa), 2g NaCl (Sigma-Aldrich) and 3g agar powder (Sigma-Aldrich) were dissolved in 200 ml deionised water. The Agar was then autoclaved for 20 minutes

on the liquid cycle (121ºC, 1kg/cm^2, 20 minutes) and stored at room temperature until use. The solution was allowed to cool before the addition of ampicillin (100mg/ml) (Roche) and poured into 90 mm Petri dishes and allowed to set.

C.1.4 Ampicillin (100mg/ml)

1g Ampicillin (Roche) was dissolved in 10 ml of 70% ethanol and stored in 1 ml aliquots at -20°C until used.

C.1.5 Composition of buffers for large scale plasmid preparations, taken from Qiagen Plasmid Purification Handbook (Qiagen)

C.1.5.1 Buffer P1 (Qiagen formulation)

6.06g Tris base and 3.72g $Na_2EDTA.2H_2O$ were dissolved in 800 ml distilled water and the pH was adjusted to 8.0 with HCl. The total volume was adjusted to 1litre with distilled water and 100mg RNase A was added per litre of P1. The solution was stored at 4°C after the addition of RNase A.

C.1.5.2 Buffer P2 (Qiagen formulation)

8.0g of NaOH pellets was dissolved in 950 ml distilled water and 50 ml 20% SDS (w/v) was added to a final volume of 1 litre.

C.1.5.3 Buffer P3 (Qiagen formulation)

294.5g potassium acetate was dissolved in 500 ml distilled water. The pH was adjusted to 5.5 with glacial acetic acid (~110 ml). The volume was adjusted to 1litre with distilled water.

C.1.5.4 Buffer QBT (Qiagen formulation)

43.83g NaCl and 10.46g MOPS (free acid) were dissolved in 800 ml distilled water. The pH was adjusted to 7.0 with NaOH. 150 ml pure isopropanol and 15 ml 10%Tirton X-100 solution was added to the mixture and the volume was adjusted to 1 litre with distilled water.

C.1.5.5 Buffer QC (Qiagen formulation)

58.44g NaCl together with 10.45g MOPS (free acid) were dissolved in 800 ml distilled water. The pH was adjusted to 7.0 with NaOH. 150 ml pure isopropanol was added and the volume was adjusted to 1litre with distilled water.

C.1.5.6 Buffer QF (Qiagen formulation)

73.05g NaCl 6.06g Tris base were dissolved in 800 ml distilled water and the pH was adjusted to 8.5 with HCl. 150 ml pure isopropanol was added and the total volume was adjusted to 1 litre with distilled water.

C.2. Solutions for agarose gel electrophoresis

C.2.1 50 X Tris acetate EDTA (TAE) Buffer

242g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5M EDTA were mixed with distilled water to a final volume of 1L and stored at room temperature until use. The solution was diluted to 1X TAE by mixing 20 ml of 50X TAE with 980 ml of distilled water.

C.2.2 6X DNA Loading Buffer

0.25% Bromophenol Blue (Saarchem; Merck Chemicals, Gauteng, South Africa) and 30% glycerol were made up in 10 ml of dH_2O. The buffer was stored in 1 ml aliquots at -20°C until required.

C.2.3 1% Agarose gel

1g agarose powder (Sigma-Aldrich) was added to 100 ml of 1XTris acetate EDTA (TAE) buffer and heated in a microwave until the agarose completely dissolves. The solution was then allowed to cool at room temperature before the addition of 7.5µl Ethidium Bromide (0.5µg/ml). (Promega, USA). The agarose was then poured and allowed to set in a Bio-Rad Gel Chamber System (Bio-Rad).

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