Characterisation of subtype C HIV-I envelope glycoproteins and their recognition by llama antibody fragments

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Signed Declaration:

I, Willie Wee-Lee Koh, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Subtype C HIV-1 is currently responsible for the majority of new infections in the world, particularly in parts of Africa where the adult prevalence rate is as high as 15%. In the absence of a viable vaccine in the near future, the study of new neutralising antibodies that can inhibit virus entry is urgently needed. To understand the subtype C HIV-1 envelopes, the *env* gene was cloned directly from 15 patient plasma samples obtained from a few countries in Africa and in the UK, and 18 replication-competent chimeric viruses were created. These envelopes were then characterised and compared with other envelopes in standard reference panels. We then exploited the unique properties of llama heavy-chain antibodies to create antibody fragments (VHH) that can recognise HIV-1 envelopes and prevent infection. Four VHH that recognise a conformation dependent epitope on gp41 were isolated from a llama that was immunised with recombinant gp140 derived from a subtype B'/C isolate after panning of the phage libraries on recombinant gp41. These VHH were more potent in neutralising subtype C isolates than subtype B isolates. Based on the success of an earlier study on VHH that recognise an epitope overlapping the CD4 binding site on gp120, a novel strategy was used to isolate variants of the VHH to create a family-specific VHH library. Thirty-one new VHH were characterised and grouped according to their neutralisation breadth against 3 subtype C viruses. The neutralisation breadth of the VHH correlated with its dissociation rate with gp120, and was found to be dependent on 3 amino acid residues in the third complementarity determining region of the VHH. These VHH may have further use in applications such as HIV-1 microbicides development and immunogen design through reverse immunology.

List of Abbreviations

Ab	antibody
ABSF	acetylbenzenesulfonyl fluoride
AIDS	acquired immunodeficiency syndrome
bcnAb	broadly cross neutralising antibody
CD4bs	CD4 binding site
CDR	complementarity determining region
CRF	circulating recombinant form
DMEM	Dulbecco's Modified Eagle Medium
Env	Envelope
FCS	foetal calf serum
FFU	focus forming unit
FR	framework region
HIV	human immunodeficiency virus
IC	inhibitory concentration
LANL	Los Alamos National Laboratory
LTNP	long term non progressor
mAb	monoclonal antibody
MPER	membrane proximal external region
NHS	normal human serum
PNLG	potential N-linked glycosylation
RLU	relative light unit
RPMI	Roswell Park Memorial Institute
sCD4	soluble CD4
SHIV	simian/human immunodeficiency virus
SIV	simian immunodeficiency virus
TCID	tissue culture infectious doses
TCLA	T cell line adapted
VLP	virus like particles

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1 Introduction

This thesis will describe the characterisation of subtype C HIV-1 envelope glycoproteins obtained from primary isolates, and the subsequent isolation of llama antibody fragments that can recognise these envelopes. This chapter will serve to introduce the various topics addressed in this thesis. The first section will give a scientific overview of the human immunodeficiency virus type 1 (HIV-1) and acquired immunodeficiency syndrome (AIDS), with particular emphasis on the molecular virology of HIV-1. The second section will concentrate on the immune response to HIV-1, with particular attention to neutralising antibodies (Abs) and research work involving vaccines and microbicide development. The third and final section will introduce the humoral immune system of camelids and how single-chain antibody fragments from llamas can be utilised to inhibit HIV-1 infection.

1.1 HIV-1 and AIDS

1.1.1 The start of the AIDS pandemic and the discovery of HIV

The origins of the AIDS pandemic can be traced back to June 1981 when the Centers for Disease Control and Prevention (CDC) in the USA published a report in the Morbidity and Mortality Weekly Report on five cases of rare *Pneumocystis carinii* pneumonia in previously healthy young homosexual men in Los Angeles (Centers for Disease Control, 1981b). As more cases emerged, reports of this disease being associated with a depletion of T-helper cells were described (Gottlieb et al., 1981; Masur et al., 1981). In that same year, reports of a rare and aggressive form of Kaposi's sarcoma in young homosexual men in both New York and California

appeared (Centers for Disease Control, 1981a; Hymes et al., 1981), which were also associated with a loss of T-helper cells (Stahl et al., 1982). Similar cases were also reported in Europe (du Bois et al., 1981; Rozenbaum et al., 1982; Vilaseca et al., 1982). This condition was later officially named acquired immunodeficiency syndrome, or AIDS, by the end of 1982 (Centers for Disease Control, 1982).

In a retrospective article jointly written by R. Gallo and L. Montagnier (Gallo and Montagnier, 2003), they described their search for the causative agent of AIDS during the early years of the epidemic. As the cases of AIDS were observed to transmit through blood and sexual activity, from mother to child, as well as through filtered blood products containing clotting factors for haemophilia, a virus was thought to be the transmissible agent. Coupled with the observations that the depletion of CD4+ T-helper cells was the biological marker in AIDS patients, and that animal models with AIDS-like wasting syndrome that was caused by lymphotropic retroviruses were known, a search for a retrovirus or a variant of the human leukaemia virus (HTLV) as the causative agent of AIDS was made.

The first direct association between AIDS and HIV was made in May 1983, when the group of Luc Montagnier at the Pasteur Institute in France described the isolation of a novel retrovirus from a lymph node of a patient with AIDS-like illness (Barré-Sinoussi et al., 1983). Reverse transcriptase activity was detected in the culture supernatant after 15 days of culturing the lymph node lymphocytes, indicating the presence of a retrovirus that was then observable under electron microscopy. Antisera to HTLV-1 did not react with cells infected with the novel virus, indicating that the new virus was distinct from HTLV. A protein of similar size to the p24 core protein of HTLV-1 was found, but was not recognised by antibodies to the HTLV-1 p24 protein. Based on these observations, Barré-Sinoussi *et al*. concluded that the

novel virus belonged to a general family of T lymphotropic retroviruses, which was related to but distinct from HTLV. The virus was subsequently called lymphadenopathy virus (LAV). This was then again independently isolated from other patients by another group (Vilmer et al., 1984).

A year later in May 1984, the group of Robert Gallo reported the isolation of a novel retrovirus from individuals with AIDS and evidence of it being the aetiological agent for AIDS in a series of articles (Gallo et al., 1984; Popovic et al., 1984; Sarngadharan et al., 1984; Schupbach et al., 1984). The virus was called human T lymphotropic virus type 3, or HTLV-3. In August 1984, Jay Levy *et al*. (Levy et al., 1984) reported an additional isolation of retroviruses from patients with AIDS. This virus was called AIDS-associated retrovirus, or ARV.

By 1985, it was clear through molecular cloning and sequencing techniques that LAV, HTLV-3 and ARV belonged to the same species (Ratner et al., 1985a; Wain-Hobson et al., 1985a). The reported nucleotide sequences, together with additional genetic and morphological studies (Gonda et al., 1985; Rabson and Martin, 1985; Sonigo et al., 1985; Wain-Hobson et al., 1985a), suggested that the AIDS virus is more related to the *Lentivirus* genus of the *Retroviridae* family than to the *Deltavirus* genus to which HTLV belongs. In 1986, the AIDS virus was named the human immunodeficiency virus, or HIV (Coffin et al., 1986).

Another novel lentivirus, related to but distinct from the HIV strains already described, was isolated from West African individuals with AIDS in 1986 by Clavel *et al.* (Clavel et al., 1986). Serological evidence for a novel lentivirus distinct from previous HIV isolates had, however, already been presented the year before (Barin et al., 1985). This virus was eventually termed HIV-2, while the HIV isolated by Barré-

Sinoussi and colleagues was called HIV-1. Françoise Barré-Sinoussi and Luc Montagnier subsequently shared the 2008 Nobel Prize in Medicine for their discovery of HIV.

1.1.2 Epidemiology of HIV today

With the first identification of AIDS in North America and Europe in the mid-1981, it soon became apparent by the mid-1980s that the AIDS epidemic was a global phenomenon. At a World Health Organisation (WHO) meeting that was held in November 1983 to assess the global AIDS situation, which was also the start of the global surveillance by the WHO, it was reported that AIDS was present in many parts of North and Central America, Europe, Africa, Australia and Japan (World Health Organisation, 1983). In August 1985, there were 15, 948 cases of AIDS in the USA alone (Curran et al., 1985). Twenty-six years after the identification of HIV as the cause of AIDS, there are an estimated 33.2 million people living with HIV worldwide in 2007, according to the joint report by the United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organisation (UNAIDS/WHO, 2007). Figures for 2008 are not yet available at the time of writing.

Although the global HIV incidence had peaked at the end of the 1990s, there were an estimated 2.5 million new infections in 2007 alone, with 2.1 million deaths (UNAIDS/WHO, 2007). The AIDS pandemic is caused by HIV-1, whereas HIV-2 is the cause of a small epidemic limited to relatively few countries in western Africa, with some presence in Portugal and also in India (Reeves and Doms, 2002; UNAIDS/WHO, 2007; van der Loeff et al., 2006). Although the high-risk groups include sex workers, men who have sex with men, and intravenous drug users and their partners, the main mode of transmission is heterosexual contact. As the global

AIDS pandemic is mainly caused by HIV-1, the rest of this thesis will concentrate solely on HIV-1.

Figure 1-1 shows the distribution of regions most affected by the AIDS pandemic, and Table 1-1 gives the breakdown of HIV statistics according to geographical regions. The worst affected region is sub-Saharan Africa, with an adult prevalence rate that is more than 6 times higher than the global average and 68% of the total number of infections. In this region, AIDS remains the major cause of death, with 76% of the global annual AIDS deaths (UNAIDS/WHO, 2007). In eight sub-Saharan countries (Botswana, Lesotho, Mozambique, Namibia, South Africa, Swaziland, Zambia and Zimbabwe) the adult prevalence is more than 15% (UNAIDS/WHO, 2007). The HIV pandemic continues to grow in many countries and regions, such as South-East Asia, China, Russia and Ukraine in Eastern Europe, North America, Brazil and Western Europe. South Africa has the highest number of infections in the world, and is closely followed by India (UNAIDS/WHO, 2007).

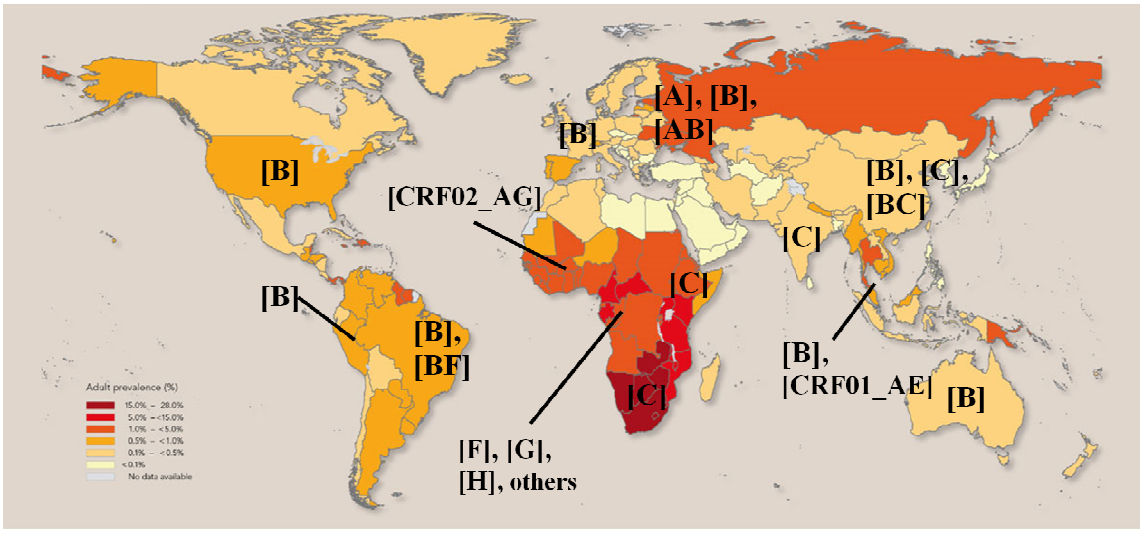


Figure 1-1. A global view of HIV infection in 2007 and associated subtypes

With about 33 million people currently living with HIV, the above figure shows the global adult prevalence rates. Countries shaded in darker colours have higher adult prevalence rates. The most affected region is sub-Saharan Africa, with the highest adult prevalence in Botswana, Lesotho and Swaziland. The prevalent subtypes and recombinant forms are shown in square brackets. This figure was adapted from the WHO website (www.who.int/hiv/facts).

Table 1-1. Regional HIV statistics for 2007[a]

	People living with HIV	People newly infected with HIV	Adult prevalence (%)
Sub-Saharan Africa	22.5 million	1.7 million	5.0
Middle East and North Africa	380 000	35 000	0.3
South and South-East Asia	4 million	340 000	0.3
East Asia	800 000	92 000	0.1
Oceania	75 000	14 000	0.4
Latin America	1.6 million	100 000	0.5
Carribean	230 000	17 000	1.0
Eastern Europe and Central Asia	1.6 million	150 000	0.9
Western and Central Europe	760 000	31 000	0.3
North America	1.3 million	46 000	0.6
Total	**33.2 million**	**2.5 million**	**0.8**

[a] Figures obtained from the report: AIDS epidemic update 2007 (UNAIDS/WHO, 2007)

1.1.3 Diversity and origins of HIV

Phylogenetically, HIV-1 can be classified into three divergent lineages, with each lineage apparently arising from a separate transmission events from chimpanzees (McCutchan, 2006). These three lineages are group M (major), N (non-major, non-outlier) and O (outlier). Very recently, a new group P was proposed after an isolate was found to group very closely to SIV_{gor} (Plantier et al., 2009). Groups N and O of HIV-1 are rare and are limited to a few individuals in Central Africa, whereas Group M accounts for over 99% of the world's infections with extraordinary diversity and can be classified into various genetic subtypes (McCutchan, 2006).

Group M can be divided into 9 subtypes: A, B, C, D, F, G, H, J and K, plus a number of circulating recombinant forms (CRFs). Subtypes are defined by significant clustering across the genome in phylogenetic analyses whereas CRFs are defined by significant clustering plus an identical recombinant structure, both of which require three epidemiologically unlinked complete sequences (Robertson et al., 2000). Out of these, subtypes A, B, C, D, CRF01_AE and CRF02_AG dominate the global pandemic, with subtype C accounting for around 50% of infections worldwide and currently infecting more people than any other subtype especially in eastern and southern Africa and India (Hemelaar et al., 2006; McCutchan, 2006; Taylor et al., 2008). The geographical distribution of common HIV-1 group M subtypes and CRFs is shown in Figure 1-1.

HIV was thought to have been brought into the human population by several cross-species transmissions of simian immunodeficiency viruses (SIVs) from chimpanzees (SIV_{cpz}) and sooty mangabeys (SIV_{sm}) to cause HIV-1 and HIV-2 respectively (Hahn et al., 2000). Although it was commonly thought that SIVs are not pathogenic in

their hosts, a recent study has shown that SIV_{cpz} can cause AIDS-like immunopathology in chimpanzees (Keele et al., 2009). HIV-1 was the confirmed to be derived from SIV_{cpz} from the *Pan troglodytes troglodytes* subspecies of chimpanzees (Keele et al., 2006). They showed that HIV-1 of group M (major or pandemic group) and N (non-major, non-outlier) arose from two distinct SIV_{cpz} strains, circulating in two geographically separated chimpanzee populations present in west central Africa. HIV-1 of group O (outlier), on the other hand, may have been brought into the human population as the result of a cross-species transmission of SIV_{gor} in gorillas, as related SIVs have been found in both chimpanzees and gorillas (Van Heuverswyn et al., 2006).

HIV is perhaps the most variable of human pathogens, and it exists as a swarm of highly related but non-identical viral genomes known as "quasispecies". The sources of this variation are due to these three factors: The rapid replication rate of the virus, which is estimated to produce 10^{10} virions per day in an infected individual (Ho et al., 1995), the error-prone reverse transcriptase (RT), and the high recombination rate due to the alternate copying from the two RNA molecules found in each virion (Robertson et al., 1995). These will be discussed in greater detail in Section 1.1.7.2.

1.1.4 HIV-1 genome

HIV-1 belongs to the *Retroviridae* family of viruses, whose members are enveloped viruses with diploid, linear, single-stranded RNA genomes of positive polarity, which use a virus-encoded reverse transcriptase to convert their genomic RNA into DNA. The DNA is subsequently incorporated into the host genomic DNA where it resides as a provirus, and consists of three main open reading frames, the *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) genes (Coffin et al.,

1997). Sequencing of the HIV-1 genome in 1985 classified the virus as part of the *Lentivirus* genus (Sonigo et al., 1985), whose other members include the sheep visna/maedi lentivirus that also cause slow disease syndromes in mammals (Narayan and Clements, 1989).

HIV-1 has a diploid genome composed of two identical positive-sense RNA molecules of approximately 9 kb (Ratner et al., 1985b; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985b), and a genomic map is shown in Figure 1-2. The *gag* gene encodes a 55 kDa Gag precursor polyprotein ($Pr55^{Gag}$), which is cleaved by the viral protease into matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7) and p6 proteins, as well as the two smaller spacer peptides SP1 and SP2 (Henderson et al., 1992). The *pol* gene encodes the viral enzymes protease (PR or p15), reverse transcriptase (RT or p66 and p51) and integrase (IN or p31). These enzymes are initially synthesised as part of a Gag-Pol precursor polyprotein ($Pr160^{Gag\text{-}Pol}$), which is produced by ribosome frameshifting near the 3' end of *gag*, but cleaved into individual enzymes by the viral protease (Frankel and Young, 1998; Swanstrom, 1997; Vogt, 1997). The *env* gene encodes an Env precursor glycoprotein (gp160), which is cleaved and processed by cellular enzymes to produce a non-covalent complex of a surface glycoprotein (SU or gp120) and a transmembrane glycoprotein (TM or gp41) (Allan et al., 1985; Robey et al., 1985; Swanstrom, 1997; Veronese et al., 1985).

The HIV-1 genome also contains genes encoding regulatory and accessory proteins, located downstream of the *pol* gene (Frankel and Young, 1998; Vogt, 1997). The *tat* and *rev* genes contain two exons each and encode the gene regulatory proteins Tat (Transactivator of transcription) and Rev (regulator of virion), whereas the *vif*, *vpr*,

vpu and *nef* genes encode the accessory proteins Vif (viral infectivity factor), Vpr (viral protein R), Vpu (viral protein U) and Nef (negative factor) (Vogt, 1997).

The HIV-1 genome is flanked by two long terminal repeats (LTRs) at both the 5' and 3' end of the integrated provirus genome, and are 630-640 bp long (Starcich et al., 1985; Wain-Hobson et al., 1985b). The LTRs consist of the U3 region that contains the viral promoter and enhancer sequences, the R region that contains the polyadenylation signal, and the transactivation response element (TAR) that serves as the binding site for the viral Tat protein (Berkhout et al., 1989; Laspia et al., 1989).

The secondary structure of the complete single-stranded RNA genome of HIV-1 was recently determined, which can function to regulate the translation and facilitate the proper folding of proteins with implications for viral fitness (Watts et al., 2009).

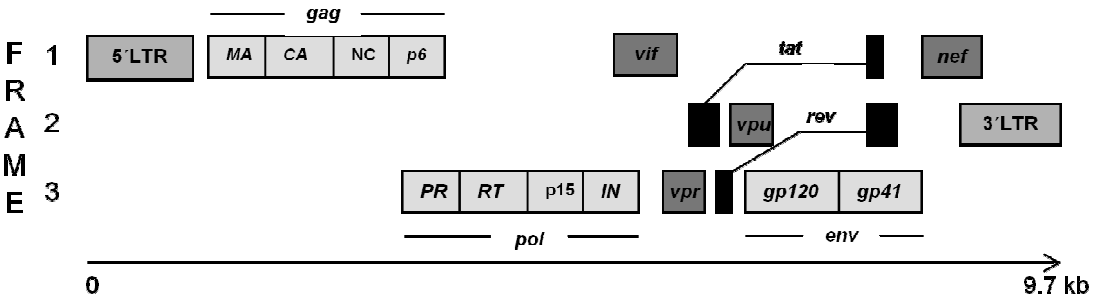


Figure 1-2. Schematic representation of the HIV-1 genomic map

Open reading frames are represented as rectangles showing their relative positions on the genome, and positioned according to the reading frame. The *gag*, *pol* and *env* open reading frames encode structural proteins and enzymes, whereas *rev* and *tat* encode gene regulatory proteins and *vif*, *vpr*, *vpu* and *nef* encode accessory proteins. The HIV-1 genome is flanked by two long terminals repeats (LTRs).

1.1.5 HIV-1 structure

1.1.5.1 HIV-1 virion structure

The average diameter of a mature HIV-1 virion is about 145 nm (Briggs et al., 2003), and is enveloped with host cell-derived lipid bilayer embedded with virus-encoded Env consisting of the surface glycoprotein gp120 non-covalently linked to the transmembrane glycoprotein gp41. The functional viral envelope spike exists as a trimer of gp120/gp41 on the virus surface (Wyatt and Sodroski, 1998; Zanetti et al., 2006; Zhu et al., 2006). Below the virus envelope is a layer of trimeric MA proteins which interacts with the lipid bilayer via amino (N)-terminal myristoyl groups (Hill et al., 1996). The MA shell surrounds a cone-shaped core, consisting of hexameric CA proteins forming a hexagonal lattice (Briggs et al., 2003; Li et al., 2000; Mortuza et al., 2004). The conical core contains the nucleocapsid (NC) protein, which is closely associated with the viral genomic RNA. The NC protein is a small, 55 amino acid residue-protein, which contains zinc-finger motifs common to many proteins that bind nucleic acids (Frankel and Young, 1998). The RNA genome is diploid and the two molecules are associated at the 5′ end. Dimerisation is mediated by so-called kissing-loop interactions between hairpin structures in the dimer initiation site located downstream of the U5 region (Hoglund et al., 1997; Mujeeb et al., 1998; Paillart et al., 2004; Paillart et al., 1996). The viral core also contains PR, RT and IN, as well as Vpr. A schematic representation of a mature HIV virion is shown in Figure 1-3.

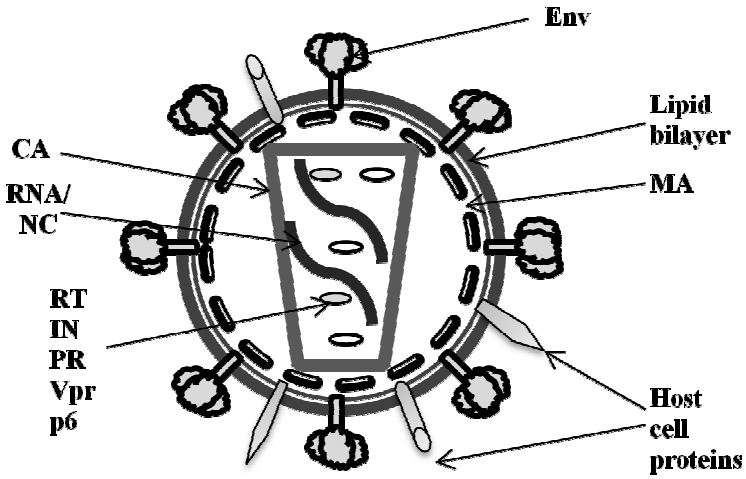


Figure 1-3. Schematic structure of the mature HIV-1 virion.

The lipid bilayer membrane contains the viral Env spikes made up of trimeric gp120/gp41. The matrix protein (MA) forms a layer beneath the membrane. The capsid protein (CA) forms the conical core. Within it contains the diploid viral RNA genome, that is associated with the nucleocapsid protein (NC), and several other viral proteins such as reverse transcriptase (RT), integrase (IN), protease (PR), viral protein R (Vpr) and p6.

1.1.5.2 HIV-1 envelope spike structure

As the major part of this thesis is focused on the envelope glycoprotein of HIV-1 and antibodies that can recognise them, this section will aim to provide an in depth review on what is currently known about the HIV-1 envelope spike.

The HIV-1 envelope spike is made up of the surface glycoprotein gp120 bound non-covalently to the transmembrane glycoprotein gp41 (Allan et al., 1985; Helseth et al., 1991; Robey et al., 1985; Veronese et al., 1985). The functional HIV-1 envelope spike exist as a trimer of the gp120/gp41 heterodimers (Chan et al., 1997; Weissenhorn et al., 1997; Zanetti et al., 2006; Zhu et al., 2006). In addition to functional trimeric spikes, there is evidence for non-functional spikes on the virus surface, such as gp120/gp41 monomers or other oligomeric forms, as well as gp41 stumps formed after shedding of gp120 (Moore et al., 2006; Poignard et al., 2003).

The transmembrane protein gp41 contains a cytoplasmic tail, a membrane-spanning domain and an ectodomain (Wyatt and Sodroski, 1998). The ectodomain consists of a fusion peptide, an N-terminal helical heptad repeat (N-HR), a loop region, a C-terminal heptad repeat (C-HR) and a membrane-proximal external region (MPER). High-resolution structural data for gp41 in the context of the native envelope spike is not yet available, but such structural data is available for the gp41 ectodomain in its fusogenic state, revealing a six-helix bundle consisting of a central parallel trimeric coiled-coil of the three N-HR helices, surrounded by the three C-HR helices in an anti-parallel hairpin fashion (Weissenhorn et al., 1997; Yang et al., 1999). Formation of the six-helix bundle is essential for fusion of the cellular and viral membranes.

Antibody binding studies have indicated that in the context of the functional spike, gp41 is relatively occluded from recognition by antibodies (Nyambi et al., 2000; Sattentau et al., 1995). Immunising rabbits with an engineered recombinant trimeric molecule containing the ectodomain of gp41 and the C1 and C5 regions of gp120, and reported to keep gp41 in a pre-fusion configuration, has supported these observations, as it failed to elicit neutralising antibodies (Qiao et al., 2005). The MPER is, however, accessible to antibodies, at least on a fusion intermediate form of gp41, as demonstrated by the isolation of neutralising antibodies directed against this highly conserved region (Muster et al., 1993; Stiegler et al., 2001; Zwick et al., 2001). There are two atomic structures available of fragments of the MPER in complex with two broadly neutralising monoclonal antibodies, 2F5 and 4E10 (Cardoso et al., 2005; Ofek et al., 2004).

The surface glycoprotein gp120 consists of five conserved (C1-C5) and five variable (V1-V5) regions (Starcich et al., 1986; Willey et al., 1986). Data from antibody competition studies predicted the variable regions to be located on the outside of

gp120, as they were well-exposed to antibodies, whereas the constant regions were less exposed (Moore et al., 1994a; Moore and Sodroski, 1996). The C1 and C5 regions were concluded to be the regions interacting with gp41, as they were found to be accessible to antibodies on recombinant monomeric gp120 but not in the context of gp120/gp41 constructs (Moore et al., 1994a; Moore et al., 1994b) and mutations in these regions could abolish gp120/gp41 interactions (Helseth et al., 1991). Similarly, observations made in mutational studies also indicated that the variable regions mask more conserved sites of gp120, such as the CD4bs (Pollard et al., 1992; Wyatt et al., 1993). For example, antibodies to the receptor-binding sites have been observed to bind with higher affinity to gp120 with deleted V1, V2 and V3 regions (Cao et al., 1997; Moore et al., 1994a; Sullivan et al., 1998; Wyatt et al., 1995; Wyatt et al., 1993). The V3 region was also found to protrude from the gp120 core, which could explain its immunodominant properties (Huang et al., 2005). CD4 binding was also observed to be dependent on sequences within the C3 and C4 regions (Cordonnier et al., 1989; Lasky et al., 1987; Olshevsky et al., 1990).

Of great interest is the CD4 binding site (CD4bs) on gp120 as blocking this site on gp120 effectively prevents the virus from entering a cell. High-resolution structural data of a deglycosylated gp120 core liganded to soluble CD4 and an antibody to a CD4-induced epitope, 17b, was solved using X-ray crystallography on HXB2 and a primary isolate (Kwong et al., 2000; Kwong et al., 1998). A fully glycosylated and unliganded SIV gp120 structure was also solved (Chen et al., 2005a). The core of monomeric, CD4-liganded gp120 is made up of five α-helices, twenty-five β-strands and ten loop segments, all arranged into an outer and an inner domain linked by a bridging sheet. The inner domain is made up predominantly by the C1 and C5 regions and is suggested to be the gp41 interaction site, based on its lack of

glycosylation and its sequence conservation (Kwong et al., 1998; Wyatt et al., 1998). The outer domain is thought to be located on the outside of the envelope spike and contains the V3, V4 and V5 variable regions (Huang et al., 2005; Kwong et al., 1998). It is heavily glycosylated, making it less visible to the host immune system (Kwong et al., 1998; Wyatt et al., 1998).

The CD4bs lies at the interface of the outer domain, the bridging sheet and the inner domain (Kwong et al., 1998). Twenty-six amino acid residues in gp120 (on the V1/V2 region, the bridging sheet, a β-strand in the outer domain, and so called CD4-binding loop on gp120) make contact with twenty-two residues in the D1 domain of CD4 (Kwong et al., 1998). The CD4-gp120 interaction is mainly electrostatic but also results from 219 van der Waals contacts and twelve hydrogen bonds located within two cavities on the surface of gp120 (Kwong et al., 1998). Comparison of the CD4-liganded HIV-1 gp120 and the unliganded SIV gp120 structures showed that binding of CD4 to gp120 resulted in substantial conformational changes in gp120 (Chen et al., 2005a), as was previously observed in antibody binding experiments (Sattentau and Moore, 1991) and changes in entropy in thermodynamic studies (Myszka et al., 2000). Many of the residues involved in CD4 binding are located in a long, narrow and hydrophobic cavity made up by the α-helices of the inner domain, part of the outer domain and the β20-β21 ribbon, explaining the relative inaccessibility of the CD4bs to many antibodies (Chen et al., 2005a).

The coreceptor binding site is less clearly defined, due to the lack of crystal structure data. The V3 region of gp120 has been shown to be critical for co-receptor binding (Bandres et al., 1998; Lapham et al., 1996; Trkola et al., 1996a; Wu et al., 1996) and is thought to interact with the N-terminus and second extracellular loop of the co-

receptor (Brelot et al., 1997; Doranz et al., 1997; Dragic, 2001; Huang et al., 2007a). A comparison of the CD4-liganded and unliganded gp120 core structure has revealed that the coreceptor binding site is only assembled and presented after CD4 binding (Chen et al., 2005a; Lapham et al., 1996; Pantophlet and Burton, 2006; Trkola et al., 1996a; Wu et al., 1996; Wyatt and Sodroski, 1998).

The high-resolution crystal structures of the HIV-1 envelope glycoproteins were based on recombinant monomeric gp120 and gp41 in its fusogenic state, and these recombinant proteins might not be a good representation of the envelope glycoproteins as they appear in the functional envelope spike. This is supported by the observation that antibody ability to bind to recombinant proteins does not necessarily correlate with ability to neutralise the corresponding virus. Instead, neutralisation has been shown to depend on the ability to recognise functional envelope spike (Fouts et al., 1997; Moore et al., 1995; Moore et al., 2006; Parren et al., 1998; Poignard et al., 2003; Sattentau and Moore, 1995). Unfortunately, a high-resolution structure is not available due to the labile and flexible nature of the spike and the high carbohydrate content of a functional envelope spike.

However, electron microscopy tomography studies of envelope spikes on the surface of chemically fixed HIV-1 virions have provided some insight into the three-dimensional nature of native HIV-1 envelope spikes and provided the first conclusive evidence for the presence of trimers on actual virions (Zhu et al., 2003). Heterogeneity was observed among different spikes, possible reflecting the heterogeneity and the existence of non-functional spikes predicted by observations made using biochemical assays (Moore et al., 2006). Another study using cryo-electron microscopy tomography on frozen, unfixed, unstained and hydrated HIV-1 and SIV particles gave a much better resolution and observed to have an average of

14 spikes per virion which clustered together rather than being uniformly dispersed on the virion surface (Zhu et al., 2006). A three-dimensional density map is reproduced in Figure 1-4A, which shows a multi-lobed structure and a tripod-like foot consisting of three well-separated legs.

Another report using cryo-electron tomography on virion-associated SIV envelope spikes revealed a slightly different structure. Instead of tripod-like structures, the envelope spike was found to be made up of three distinct gp120 subunits on top of a narrow gp41 stalk with the presence of a cavity at the interface of gp120 and gp41 (Zanetti et al., 2006), as shown in Figure 1-4B.

A third study on virion-associated HIV-1 envelope spikes revealed yet another slightly different shape, but does share the narrow gp41 stalk feature revealed by Zanetti *et al*. (Zanetti et al., 2006). The density map of the HIV-1 envelope spike as revealed by Liu *et al*. (Liu et al., 2008) is shown in Figure 1-4C. Fitting of gp120 atomic structures into the density map indicates that the variable regions and the glycosylated residues of gp120 are located on the outside of the spike, with the V3 region near the top of the spike, and the CD4 binding site is located about 20Å from the top of the spike in a recessed cavity (Liu et al., 2008). Liu *et al*. also observed that the spike undergoes a drastic quaternary conformational rearrangement upon binding to sCD4 and the monoclonal antibody 17b, resulting in a vertical upward displacement of gp120, a more open spike structure, and exposure of the gp41 stalk (Liu et al., 2008). These observations are likely to further the understanding of HIV-1 interaction with cellular receptors and entry into target cells, which has implications for vaccine design. However, due to the differences in the three observed structures, the true structure of the envelope spike still remains unclear (Roux and Taylor, 2007; Subramaniam, 2006).

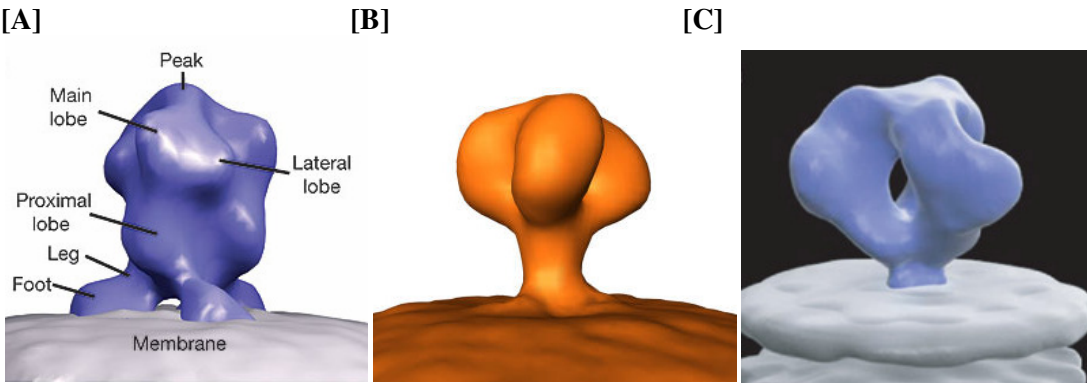


Figure 1-4. Three-dimensional models of the SIV and HIV-1 envelope spikes based on cryo-electron tomography studies

[A Density map of an unliganded SIV envelope spike constructed by Zhu *et al.*. Image obtained from Nature, 441:847-52. **[B]** Density map of an unliganded SIV envelope spike constructed by Zanetti *et al*. Image obtained from PLoS Pathogens, 2:e83. **[C]** Density maps of an unliganded HIV-1 envelope spike constructed by Liu *et al*. Image obtained from Nature 455:109-13.

1.1.6 HIV-1 cellular receptors

Understanding of the cellular receptors for HIV-1 is crucial as the virus needs to interact with these receptors in order to gain entry into the host cell. Blocking of these interactions, for example through the use of antibodies, can potentially block virus infection of the cell. The cellular receptor for HIV-1 was identified to be CD4 in 1984 (Dalgleish et al., 1984; Klatzmann et al., 1984), when they found that monoclonal antibodies (mAbs) to CD4 were able to block HIV-1 infection. The CD4 antigen is a transmembrane glycoprotein belonging to the immunoglobulin superfamily of receptors (Maddon et al., 1985; Terhorst et al., 1980), and is expressed predominantly on T helper cells but to a lesser extent on other cells types such as monocytes, macrophages, dendritic cells, eosinophils and mast cells (Germain, 2002; Lee et al., 1999; Lucey et al., 1989; Wood et al., 1983). CD4 on T helper cells acts as a coreceptor by binding to the non-polymorphic regions of MHC class II molecules on antigen-presenting cells (Wang et al., 2001), and is involved in

the activation of antigen-driven T cell responses (Germain, 2002). It has a molecular mass of 55 kDa and consists of an extracellular part, a transmembrane domain and an intracellular domain. The extracellular portion consists of four immunoglobulin-like domains, D1-D4 (Maddon et al., 1985; Ryu et al., 1990). In the initiation of the HIV-1 entry process, the gp120 portion of the envelope spike was discovered to bind to the D1 domain only (Kwong et al., 1998).

It was soon realised that a coreceptor is required for HIV-1 entry, as non-human cells engineered to express CD4 only were not susceptible to infection (Lores et al., 1992; Maddon et al., 1985) and other human components or cofactors are involved (Weiner et al., 1991). This missing coreceptor for HIV-1 entry was identified to be CXCR4 in 1996, where Feng *et al.* showed that co-expression of both CD4 and CXCR4 are required to render previously non-permissive cells susceptible to infection (Feng et al., 1996). However, this was found to support the infection of T cell line-tropic isolates of HIV-1 and not with macrophage-tropic strains and primary T-cell tropic strains, indicating that yet another co-factor was responsible for mediating entry for the latter isolates. The coreceptor for macrophage-tropic HIV-1 was identified by several groups to be CCR5, which is also the receptor for various chemokines such as RANTES, MIP-1α and MIP-1β (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). Other coreceptors, such as CCR2 and CCR3, can also mediate entry of some HIV-1 strains (Choe et al., 1996; Doranz et al., 1996).

Chemokine receptors belong to the superfamily of seven-transmembrane G-protein-coupled receptors (GPCRs). They are integral membrane proteins that consist of an extracellular N-terminal domain followed by seven helical membrane-spanning regions connected by three intracellular and three extracellular loops and a

cytoplasmic tail (Allen et al., 2007). The gp120 portion of the envelope spike was found to bind to the first and third extracellular domains of CCR5 and CXCR4 (Brelot et al., 1997; Doranz et al., 1997; Huang et al., 2007a).

All primary HIV-1 isolates use one or both of CCR5 and CXCR4 as co-receptor (Lusso, 2006). In addition, some virus isolates have been shown to use alternative chemokine receptors *in vitro*. At least a dozen other seven-transmembrane GPCRs, such as CCR3, CCR2b, CCR8, Strl33 and D6, have been reported to function as co-receptors *in vitro* (Clapham and McKnight, 2002; Neil et al., 2005). CCR3 is commonly used as a minor coreceptor for HIV-1 in the central nervous system, especially in microglial cells (He et al., 1997).

Before the coreceptors for HIV-1 were discovered, two distinct phenotypes of HIV-1 were observed with different tropism for CD4+ cells and appear to coreleate with clinical severity (Asjo et al., 1986; Cheng-Mayer et al., 1988; Connor and Ho, 1994). Viruses with a high turnover phenotype were able to induce syncytia in immortalised T cell lines, whereas viruses with a low turnover phenotype and also non-syncytia inducing were found to replicate in macrophages but not T cell lines (Collman et al., 1989; Schuitemaker et al., 1992; Tersmette et al., 1988). With the identification of CXCR4 and CCR5 as coreceptors for HIV-1, the confusion between replication kinetics, syncytia formation and tropism began to clear up (Dittmar et al., 1997). Virus isolates are now classified based on coreceptor usage, and are referred to as CCR5- or CXCR4-using viruses, or R5 and X4 viruses, or R5X4 for dualtropic viruses (Berger et al., 1998). Also, isolates that have been adapted for growth in T cell lines are referred to as T cell line-adapted (TCLA), whereas viruses only cultured in primary PBMC or macrophages are referred to as primary isolates. CCR5 using viruses were observed to be preferentially transmitted over CXCR4 using

viruses (Margolis and Shattock, 2006; van't Wout et al., 1994), although CXCR4 using viruses have been associated with lower CD4+ T cell counts and faster progression to AIDS (Connor et al., 1997a; Daar et al., 2007; Scarlatti et al., 1997; Zhu et al., 1993)

Although coreceptor usage does not differ significantly between the different HIV-1 subtypes (Moyle et al., 2005; Zhang et al., 1996), the majority of studies on HIV-1 coreceptor usage and tropism have been carried out on subtype B viruses. However, CXCR4 usage has been less frequently observed among subtype C isolates, compared to among HIV-1 of subtype B, even in isolates from individuals with AIDS (Cecilia et al., 2000; Peeters et al., 1999; Ping et al., 1999; Tscherning et al., 1998). In addition, CXCR4 usage has been reported to be more prevalent among subtype D viruses compared to among subtype A viruses (Huang et al., 2007b).

As most of the studies on coreceptor usage were focused on CCR5 and CXCR4, I have investigated the use of CCR3 as a coreceptor by the different HIV-1 subtypes, and this is described in Chapter 3 of this thesis.

1.1.7 HIV-1 life cycle and replication

1.1.7.1 Virus entry

The HIV-1 entry process is initiated by attachment of the gp120 subunit of the viral envelope spike to the primary cellular receptor CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984). Binding of gp120 to CD4 triggers a series of conformational rearrangements in gp120 and in the envelope spike as a whole, resulting in the exposure of the coreceptor binding site (Liu et al., 2008; Myszka et al., 2000; Sattentau and Moore, 1991; Sattentau et al., 1993), thus allowing either of the main cellular co-receptors CCR5 or CXCR4 to bind to gp120 (Lapham et al., 1996; Trkola

et al., 1996a; Wu et al., 1996). The V3 loop, in particular the amount of net positive charges on the V3 loop, is the main determinant for co-receptor usage and thereby cell tropism (Hartley et al., 2005; Hwang et al., 1991), although sequence changes in the V1, V2, C3 and C4 regions of gp120 and in gp41 have been implicated (Aasa-Chapman et al., 2006b; Boyd et al., 1993; Hartley et al., 2005; Huang et al., 2008).

Binding of gp120 to CD4 and to the coreceptor results in conformational changes in gp41, leading to the formation of a pre-hairpin intermediate which later springs out to insert the gp41 fusion peptide into the host cell membrane, resulting in fusion of viral and cellular membranes (Doms and Moore, 2000; Gallo et al., 2003; Roux and Taylor, 2007). Although the exact mechanism for membrane fusion is unclear, the formation of the pre-hairpin intermediate and the insertion of the fusion peptide is thought to be followed by the energetically favoured formation of a six-helix bundle (Chan and Kim, 1998; Yang et al., 1999). Formation of the six-helix bundle is thought to coincide with fusion of the viral and cellular membranes, which is aided by the energy release during bundle formation (Melikyan et al., 2000).

The number and exact stochiometry of envelope spikes required for HIV-1 entry has not been determined precisely, and was found to require between one to seven functional spikes per virion (Herrera et al., 2006; Sougrat et al., 2007; Yang et al., 2005). Virus entry can also take place through cell-cell transfer via a virological synapse (Jolly et al., 2004; McDonald et al., 2003) or via filopodial bridges (Sherer et al., 2007) or long nanotubes (Sowinski et al., 2008), and are all dependent on HIV-1 envelope glycoprotein and CD4. Such cell-cell spread of virus and its ability to evade antibody responses remain unclear. Although the virus is generally thought to enter a cell through fusion with the plasma membrane, the virus has also been observed to enter via endocytosis in a pH-independent step (Miyauchi et al., 2009).

1.1.7.2 Reverse transcription

After virus entry, the little understood process of viral core uncoating and formation of a reverse transcription complex (RTC) occurs (Aiken, 2006; Nisole and Saib, 2004). Uncoating involves the disassembly of CA and release of the viral ribonucleoprotein complex. This process remains to be elucidated but has been suggested to involve both viral and cellular factors (Aiken, 2006), in particular the cellular protein cyclophilin A (Franke et al., 1994; Luban et al., 1993; Thali et al., 1994). The cellular tripartite motif protein TRIM5α can associate with the CA and promote its degradation, leading to accelerated core disassembly and uncoating, and thus restrict retroviral infection in a species-specific manner (Stremlau et al., 2004; Stremlau et al., 2006). The RTCs are large nucleoprotein structures consisting of packed filaments containing IN and Vpr (Fassati and Goff, 2001; Nermut and Fassati, 2003) and interacts with the cytoskeleton (Bukrinskaya et al., 1998).

Reverse transcription of the viral genomic RNA into a double-stranded DNA genome is thought to take place essentially within the RTC by the viral RT, which possess both DNA polymerase and RNase H activities to degrade the genomic RNA template (di Marzo Veronese et al., 1986; Freed, 2001). The RT polymerase lacks any proof-reading ability and is therefore highly error-prone, making approximately 3.4×10^{-5} errors per base pair per cycle to cause the extreme sequence variation observed among HIV-1 isolates (Mansky and Temin, 1995). In addition, RT binds with low affinity to its template and can hence make frequent jumps between the two RNA genomic molecules. If the two genomic RNA molecules are different, this ability results in the generation of genetically recombinant DNA genomes, thus contributing to genetic variability (Temin, 1993).

The reverse transcription process can be inhibited by a cellular cytidine deaminase protein called APOBEC3G, and thus restrict retroviral replication by deaminating cytosine residues in the synthesis of the first retroviral DNA strand and leading to hypermutation of the viral DNA (Sheehy et al., 2002). In HIV-1, this restriction is overcome by the viral Vif protein, which mediates polyubiquitylation and proteasomal degradation of APOBEC3G, thus preventing APOBEC3G incorporation into virions and its modulation of the reverse transcription process (Sheehy et al., 2003; Yu et al., 2003).

1.1.7.3 Nuclear import, integration and transcription

HIV-1 replication requires that the virus enters the nucleus. Once the reverse transcription is complete, the RTCs are gradually transformed into pre-integration complexes (PICs) in a process that is little understood (Nisole and Saib, 2004). The PICs contain the viral genomic DNA and viral protein IN (Farnet and Haseltine, 1991), and are actively imported into the nucleus (Bukrinsky et al., 1992) with the help of importin 7 (Zaitseva et al., 2009), and the viral protein Vpr which contains nuclear localisation signals (Lu et al., 1993).

Once the pre-integration complex has been imported into the nucleus, the viral DNA is integrated into the cellular genomic DNA and resides as a provirus in the host genome (Brown, 1997). This is solely mediated by viral IN (Bushman et al., 1990), and shown to specifically integrate into transcriptionally active regions of the host genome (Schroder et al., 2002).

Once integrated into the host genome, the provirus can stay latent or be transcribed by the cellular machinery (Cullen, 1991). Prior to the production of the viral transactivator Tat, the HIV-1 promotor on the 5'LTR is activated by cellular

transcription factors alone, resulting in low transcriptional levels and short transcripts. Once Tat is produced, it strongly enhances transcriptional activation and elongation in a positive feedback loop (Sodroski et al., 1985).

After transcription, the viral RNAs are polyadenylated, spliced and exported from the nucleus. The obtained transcripts are either unspliced (9 kb), incompletely spliced (around 4 kb), or fully spliced RNAs (around 2 kb). The primary HIV-1 RNA transcript or unspliced RNAs are used for expression of Gag and Gag-Pol precursors or packaged into virions as genomic RNA. Incompletely spliced mRNAs have the *gag-pol* region spliced out and can be used for expression of Env, Vif, Vpr and Vpu, whereas fully spliced mRNAs have the *gag*, *pol* and most of *env* removed, contain no intron sequences, and are used to express Tat, Rev and Nef (Robert-Guroff et al., 1990; Schwartz et al., 1990). The viral Rev protein promotes the nuclear export of unspliced and incompletely spliced viral RNAs by binding to a highly structured RNA region called the Rev responsive element (RRE) (Malim et al., 1989; Zapp and Green, 1989).

1.1.7.4 Synthesis, assembly, and processing of viral proteins

The Gag ($Pr55^{Gag}$) and Gag-Pol ($Pr160^{Gag\text{-}Pol}$) polyproteins are synthesised on polyribosomes in the cytoplasm, and are important for membrane targeting and binding, multimerisation of Gag, encapsidation of viral genomic RNA and association with viral envelope glycoproteins anchored to the plasma membrane for particle budding and release (Freed, 1998; Swanstrom, 1997). The MA protein is important for membrane targeting and binding (Hill et al., 1996). Gag multimerisation is mediated by the viral NC protein at the plasma membrane and a layer of Gag-particles associated with the inner layer of the plasma membrane that

will eventually form a spherical immature virus particle, with the encapsidation of viral genomic RNA by NC (Freed, 1998; Ganser-Pornillos et al., 2008; Swanstrom, 1997).

The HIV-1 envelope glycoprotein, gp160, is first synthesised as a precursor polyprotein on the rough endoplasmatic reticulum (ER) where it undergoes glycosylation, folding and oligomerisation (Freed and Martin, 1995; Maggioni and Braakman, 2005; Wyatt and Sodroski, 1998). The gp160 polypeptide is co-translationally glycosylated in the ER (Swanstrom, 1997), and it contains around 30 potential N-linked glycosylation sites (Muesing et al., 1985; Ratner et al., 1985b; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985b), which are made up of asparagine-X-serine/threonine motifs where X is any amino acid except proline. Proper folding of gp160 is mediated by the isomerisation of disulphide bonds and the signal peptide then cleaved (Berman et al., 1988; Land et al., 2003). The gp160 also undergoes oligomerisation before exiting the ER and into the Golgi (Earl et al., 1990; Lu et al., 1995; Pinter et al., 1989; Weiss et al., 1990). Although dimers and tetramers have been observed, the functional spike exists as a trimer (Chan et al., 1997; Pantophlet and Burton, 2006; Weissenhorn et al., 1997; Wyatt and Sodroski, 1998; Zanetti et al., 2006; Zhu et al., 2006). To prevent the premature interaction of gp160 with CD4 in the ER, the viral protein Vpu has been shown to mediate CD4 degradation through ubiquitin-mediated proteolysis (Margottin et al., 1998). CD4 cell-surface expression is also down-regulated by the viral protein Nef, which mediates internalisation and degradation of CD4 via clathrin-coated pits and lysosomes (Malim and Emerman, 2008).

The oligomeric gp160 is then transferred from the ER to the Golgi, where the newly added N-linked high-mannose glycans are modified by mannosidase enzymes,

initiating the formation of complex N-linked carbohydrate glycans (Kozarsky et al., 1989; Stein and Engleman, 1990). The variability in glycosylation and processing of glycans contributes to the heterogeneity of the HIV-1 envelope. The trimeric gp160 then undergoes endoproteolytic cleavage in the Golgi to form the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 by cellular serine proteinases (McCune et al., 1988; Stein and Engleman, 1990; Willey et al., 1988). Cleavage occurs at a highly conserved lysine/arginine-X-lysine/arginine-arginine motif (where X is any amino acid) and is essential for the fusogenic ability of the viral envelope spike. After cleavage, gp120 and gp41 associate non-covalently with each other.

The envelope spikes are then directed to the plasma membrane through the secretory pathway and incorporated into virions (Swanstrom, 1997). Several studies have indicated that an interaction between the cytoplasmic tail of gp41 and the MA domain of Gag is responsible for the recruitment of envelope glycoproteins to Gag (Cosson, 1996).

1.1.7.5 Assembly and budding of virions

Virus assembly and budding is generally believed to occur at the plasma membrane of infected cells (Swanstrom, 1997). However, conflicting reports suggests that the location is dependent on cell types. For example, HIV-1 assembles and buds at the plasma membrane for T cells, but the same occurs in late endosomes or intracellular multivesicular bodies for macrophages (Ono and Freed, 2004). Real-time imaging of Gag assembly in HeLa cells has further supported the notion that HIV-1 assembles at the plasma membrane (Jouvenet et al., 2008). The viral protein p6 is crucial for the release of virus particles, as the deletion of p6 has been shown to

cause accumulation of assembled virus particles tethered to the plasma membrane (Gottlinger et al., 1991). A recently described protein, tetherin, was found to tether virus particles to the cell surface (Neil et al., 2008), but was antagonised by the presence of the viral Vpu protein which was previously known to enhance virus release (Terwilliger et al., 1989).

HIV-1 buds from the membrane of infected cells in an immature, non-infectious form (Swanstrom, 1997). Upon budding, the viral PR is activated and cleaves the Gag precursor $Pr55^{Gag}$ in a stepwise fashion into the MA, CA, NC and p6 proteins. PR also cleaves the Gag-Pol polyprotein $Pr160^{Gag\text{-}Pol}$, generating PR, RT and IN. Cleavage of the Gag and Gag-Pol polyproteins leads to structural rearrangement of the individual Gag proteins and the formation of a mature, infectious HIV-1 virion containing the characteristic conical core.

1.1.8 HIV-1 transmission

HIV-1 can be transmitted along three major routes. The first route is through direct exposure to HIV-1 positive blood or blood products, either in donated blood or contaminated needles, and represents about 5-10% of HIV-1 infections worldwide (Hladik and McElrath, 2008). The second route is via mother-to-child transfer, and represents about 15% of new infections in 2007 (UNAIDS/WHO, 2007). The final route is through sexual contact primarily at the genital and rectal mucosa (Royce et al., 1997), and represents the vast majority of all new infections (UNAIDS/WHO, 2007). Sexual transmission is the focus of discussion in this section.

The probability of infection through sexual contact can vary greatly, and is dependent on the viral dose and also on whether the virus is transmitted directly into

the blood via breaks in the epithelium or across intact mucous membranes (Haase, 2005; Royce et al., 1997).

In men, HIV-1 transmission across the genital mucosa occurs most frequently through the inner foreskin and perhaps also through the penile urethra. The inner foreskin is lined by a multilayer stratified squamous epithelium whereas the urethra is lined by layers of columnar epithelial cells, both of which contain target cells for HIV-1 infection and foreskin explants were susceptible to HIV-1 infection (McCoombe and Short, 2006; Patterson et al., 2002). Circumcision confers a reduced risk of HIV-1 infection for men, highlighting the important role of the foreskin in HIV-1 transmission (Gray et al., 2007b).

Transmission to women during vaginal intercourse can occur through the vaginal, ectocervical, or endocervical mucosa (Hladik and McElrath, 2008). The intact vaginal and ectocervical mucosa is made up of several layers of stratified squamous epithelium, whereas the endocervix is lined by a single-layer columnar epithelium. Studies on macaques have shown that the vaginal mucosa is sufficient for SIV transmission (Miller et al., 1992b). Various factors can lead to an increased risk of HIV-1 transmission, such as physical abrasion (Baleta, 1998), abnormalities in the vaginal flora (Sewankambo et al., 1997) or genital ulcers caused by sexually transmitted diseases (Weiler et al., 2008).

Although the events on how HIV-1 can lead to an established infection when it reaches the epithelium is not clearly understood, studies on explants models have shown that HIV-1 can directly infect Langerhans cells, subepithelial DCs, macrophages and CD4+ T cells (de Witte et al., 2008; Hladik and McElrath, 2008; Hladik et al., 2007; Maher et al., 2005; Miller and Shattock, 2003).

1.2 The immunology of HIV-1

1.2.1 Progression of HIV-1 infection

After mucosal transmission of HIV-1, infection is established locally at the point of entry which then spreads to the draining lymph nodes and subsequently to other lymphoid tissues via the circulation (Haase, 2005). Once established, natural HIV-1 infection is characterised by three phases, which are also seen in infection of macaques by pathogenic strains of SIV.

The first phase is the primary acute infection phase, which is associated with intense viral replication, a rapid increase in viral load and a decline in CD4+ T cells, especially the mucosal gut-associated lymphoid tissue (GALT) which comprises most of total body CD4+ T cells (Brenchley et al., 2004; Veazey et al., 1998). Over the following weeks, as antiviral immune responses develop in the host, the number of circulating CD4+ T cells start to recover and plasma viral load decreases to a set-point.

The second phase is the chronic, and in general clinically asymptomatic, infection that is characterised by a gradual increase in plasma viral load and a progressive decrease in circulating CD4+ T cells that can span for ten years or more in untreated patients. Despite the lack of clinical symptoms, persistent viral replication occurs throughout this phase (Ho et al., 1995; Wei et al., 1995).

The third phase is characterised by the terminal failure of the immune system, symptomatic infection and onset of AIDS. AIDS is defined, by the CDC, as the onset of immune deficiency leading to the occurrence of any of more than twenty opportunistic infections or HIV-related cancers with a circulating CD4+ T cell count below 200 cells/μl of blood (Centers for Disease Control, 1992).

1.2.2 Host immune response to HIV-1

The course of HIV-1 infection and the rate of disease progression (as described above) vary between individuals, but none of any infected individuals has been able to contain the persistent viraemia and to clear the HIV-1 infection. Host genetic factors, such as the CCR5Δ32 allele and some human leukocyte antigen (HLA) genotypes (Lama and Planelles, 2007), as well as cellular factors that can restrict retroviral replication such as TRIM5α, APOBEG3G and Fv1 (Bieniasz, 2004), can affect the rate of disease progression in an infected individual. Innate and adaptive immune responses play a big role in determining disease progression. In this section, only the background of the adaptive host immune response, in particular the humoral response, will be discussed.

Within weeks of the initial primary infection, HIV-1 specific CD8+ T cells emerge to help in controlling the viraemia, coinciding with the decline in viral load and restoration of circulating CD4+ T cells in humans and macaques (Pantaleo et al., 1994; Schmitz et al., 1999). However, cellular-mediated immunity soon comes under pressure from the selection of cytotoxic T lymphocyte (CTL) escape mutants in primary infection (Borrow et al., 1997). Research work in cellular-mediated immunity has received renewed focus recently after the failure of the STEP vaccine clinical trials (Fauci et al., 2008), which will be discussed in Section 1.2.3.1.

The humoral or antibody response to HIV-1 infection is primarily directed against the gp120, gp41, CA and MA viral proteins, but only antibodies that recognise gp120 and gp41 epitopes are able neutralise HIV-1 infection, and this antibody response varies widely between individuals (Burton et al., 2005; Frost et al., 2008; Li et al., 2009; Montefiori et al., 2007). Neutralising antibodies can be observed within

weeks of initial infection and can help in the control of primary viraemia, and are also detectable after acute infection when viral load declines. The initial neutralising antibody response is, in general, highly specific for the autologous early virus variants and shows limited cross-neutralisation properties (Aasa-Chapman et al., 2004; Albert et al., 1990; Ariyoshi et al., 1992; Frost et al., 2005; Gray et al., 2007a; Lathey et al., 1997; Li et al., 2006a; Moog et al., 1997; Pellegrin et al., 1996; Richman et al., 2003; Wei et al., 2003). Neutralising anti-envelope antibodies are thought to constitute only a fraction of the total antibody response, as the majority of anti-envelope antibodies are unable to bind functional envelope glycoprotein spikes on the virus surface (Moore et al., 2006; Parren et al., 1997; Poignard et al., 2003).

Neutralisation escape mutants of the virus can be isolated with the emergence of neutralising antibodies, which then again prompts the generation of new antibodies that are able to neutralise the escape mutants in an evolutionary cycle to control the virus infection (Burton et al., 2005; Richman et al., 2003; Wei et al., 2003). Although the neutralising antibody response may be very vigorous, it is probably not able to keep up with the high replicative and mutational rates of the virus (Mansky and Temin, 1995; Perelson et al., 1996; Richman et al., 2003). This selective pressure thus helps to drive virus evolution, and contributes to the extreme genetic diversity observed in the *env* gene and the variation in neutralisation sensitivity amongst the HIV-1 variants. This also leads to the gradual broadening of serum neutralising activity, sometimes producing broadly neutralising antibodies that are often found in long term non-progressors (Braibant et al., 2006; Frost et al., 2005; Pilgrim et al., 1997; Richman et al., 2003). However, there may be limits to the ability of HIV-1 to continuously evolve to escape neutralising antibody responses in the chronic stage (Deeks et al., 2006; Draenert et al., 2006).

In addition to being able to directly neutralise virus, anti-envelope antibodies can induce additional antiviral immune functions, mediated by interaction of the Fc portion of the antibody with Fc receptors (FcRs) or with complement that can lead to the clearance of infected cells (Huber and Trkola, 2007; Perez et al., 2009). Such antiviral immune functions can be mediated by both neutralising as well as non-neutralising antibodies which recognise non-functional spikes present on the virus surface (Moore et al., 2006). The relevance of FcR-mediated effector functions *in vivo* has been highlighted by the recent observation that the ability of the broadly neutralising mAb b12 to protect macaques against SHIV challenge was reduced if mutations that impair FcR binding were introduced into the Fc portion of b12 (Hessell et al., 2007).

The role of neutralising antibodies in the protection against HIV-1 infection is further demonstrated in passive immunisation studies. Studies have found that macaques can be protected from a SHIV challenge through the transfer of neutralising antibodies (Gauduin et al., 1997; Veazey et al., 2003), and can also help to control the plasma virus load post-infection (Trkola et al., 2005; Yamamoto et al., 2007).

1.2.3 HIV-1 vaccines and prevention strategies

Improvements in anti-retroviral therapy (ART) have advanced tremendously in recent years, especially with the introduction of highly-active anti-retroviral therapy (HAART) in 1996 which helped to reduce mortality and sustain the quality of life in infected individuals (Hogg et al., 1998). Although such therapy can control viral load and prevent progression to AIDS, it does not clear the infection completely and issues remain concerning costs and toxicity, especially in Africa where it is needed

most. In order to control the HIV-1 pandemic in the world's most vulnerable regions, there is an urgent need for preventive measures to lower transmission rates and to protect from infection. Such strategies would obviously include barriers to block transmission, such as condoms, but the Holy Grail to prevent transmission would be an effective HIV-1 vaccine.

1.2.3.1 HIV-1 vaccine development

Despite many years of intensive research in HIV-1 vaccine development, an effective vaccine against HIV-1 is still unavailable. Several features of HIV-1 make it a challenging target for vaccine design, in particular its high mutation rate and its remarkable genetic diversity. The immune evasive properties of the HIV-1 envelope glycoproteins are other major obstacles in vaccine development, as is the ability of HIV-1 to remain latent in cells.

Nevertheless, the observation that passively transferred neutralising antibodies can protect macaques against SHIV and SIV challenge (Baba et al., 2000; Mascola et al., 2000; Shibata et al., 1999; Van Rompay et al., 1998) indicates that antibodies could potentially prevent infection once elicited. As T cell responses also play a role in controlling viraemia in HIV-1 and SIV infection, it is generally believed that a successful vaccine against HIV-1 will need to elicit both humoral and cellular immune responses in order to prevent infection, disease, or both (Fauci et al., 2008; Letvin, 2006; Walker and Burton, 2008).

The vaccine candidates that have so far provided the best protection in the macaque model are live attenuated SIVs (Berkley and Koff, 2007; Friedrich and Watkins, 2008; Koff et al., 2006). Immunising macaques with live attenuated SIV has been shown to provide protection against challenge with homologous pathogenic SIV for

up to more than two years after immunisation, but was less effectvive against heterologous SIV isolates (Daniel et al., 1992; Gauduin et al., 2006; Mansfield et al., 2008; Nilsson et al., 1998; Whatmore et al., 1995; Wyand et al., 1999; Wyand et al., 1996). There are safety issues with this approach however, as there is a possibility that the virus may revert back to a pathogenic form, or prove to be pathogenic after a prolonged period of time or in immunocompromised individuals, as have been observed in macaque studies (Baba et al., 1999; Whatmore et al., 1995).

Other vaccine candidates include inactivated or killed virus preparations that may be safer, but were found to offer only short-lived protection in the macaque model and only against an identical viral challenge (Lifson et al., 2004). Non-infectious virus-like particles (VLPs) were found to offer a slightly broader immune response (McBurney et al., 2007). DNA vaccines encoding HIV-1 proteins are also under investigation, but they were found to be of low immunogenicity in non-human primates compared to in mice (Letvin, 2006; McMichael, 2006), and might be better used as priming vaccines in various prime-boost strategies (Amara et al., 2001; Hanke et al., 1999; Shiver et al., 2002; Wang et al., 2006). Mucosal administration of several vaccine candidates have been shown to reduce viral loads in macaques after mucosal challenge with SHIV (Belyakov et al., 2001; Bertley et al., 2004; Fuller et al., 2002).

Subunit vaccines containing crude virus extracts or recombinant proteins can be easily produced and are generally thought to be safest. These were also the first HIV-1 vaccine candidates to enter phase II/III clinical trials in humans. VaxGen (San Francisco, CA, USA) produced two vaccine candidates containing recombinant gp120 derived from HIV-1 of subtype B and A/E, which were shown to protect chimpanzees against challenge with homologous virus (Berman et al., 1990). Both

candidates were brought forward to phase III clinical trials in the USA and/or in Thailand, but they did not prevent infection, had no effect on subsequent viral load, and did not elicit antibodies that could neutralise primary isolates of HIV-1 *in vitro* (Cohen, 2003; Flynn et al., 2005; Pitisuttithum et al., 2006).

The failures of efficacy in the VaxGen trials were perhaps not too surprising, given what we now know about the immune evasive mechanisms of the HIV-1 envelope glycoproteins and the extreme HIV-1 genetic diversity. Great efforts are currently focused on designing immunogens that can elicit a broader and more potent neutralising antibody response required of a vaccine. This includes the generation of stable recombinant trimeric envelope proteins that mimic the functional HIV-1 envelope spike, as well as peptide and protein constructs that better present conserved epitopes recognised by a handful of broadly neutralising antibodies to the HIV-1 envelope glycoproteins (Walker and Burton, 2008; Zwick and Burton, 2007). Unfortunately, such an immunogen has yet to be developed.

In light of the difficulties in eliciting a neutralising antibody response to HIV-1, along with the evidence for a role of T cells in the control of SIV and HIV-1 infection, much focus has been on developing vaccines that elicit T cell responses, which even though they may not prevent infection may contain viral replication, leading to lower viral load set-points and delayed onset of disease (Letvin, 2006; Walker and Burton, 2008).

Merck developed a T-cell based vaccine candidate that went into clinical trials. This is based on the incorporation of HIV-1 genes into viral vectors to mimic the efficacy of a live attenuated vaccine. Their vaccine candidate consists of a replication-defective adenovirus type 5 vector engineered to express the HIV-1 Gag, Pol and

Nef proteins. Results from a macaque model showed some protection against subsequent viral challenge with pathogenic SHIV89.6P, with immunised animals showing reduced viral load and rarely developed disease (Shiver et al., 2002). This vaccine candidate was taken forward for clinical trials in 2004 by Merck and the HIV Vaccine Trials Network, and was called the STEP trial. This vaccine candidate had been shown to induce CD8+ T cell responses in clinical phase I and II trials of safety and immunogenicity in humans. However, the Merck vaccine candidate not only failed to prevent infection and to reduce the viral load in subsequently infected individuals in phase III trials, it has enhanced the rate of infection in vaccinated individuals with pre-existing high-titre antibodies to adenovirus of serotype 5 (Cohen, 2007a; Cohen, 2007b; Ledford, 2007; Steinbrook, 2007).

A vaccine candidate developed by Sanofi-Pasteur is currently in clinical efficacy trials in Thailand, and is based on a canarypox vector in combination with the recombinant gp120 vaccine candidate developed by VaxGen in a prime-boost regime. This vector expresses HIV-1 Gag, Pol and envelope glycoproteins. Unfortunately, the immunogenicity of this vaccine regimen has been shown to be fairly low in clinical phase I and II trials and preliminary data has suggested no effect on viral load in individuals who have become infected (Lee et al., 2004; Nitayaphan et al., 2004).

So far, none of the vaccine strategies have been able to protect macaques against SIV challenge (Friedrich and Watkins, 2008; Watkins et al., 2008). The two candidates that have completed large-scale efficacy trials in humans have failed to prevent or control infection. Moreover, no vaccine candidate has been able to elicit neutralising antibodies, in any species, of the breath and potency likely required of a vaccine. The lack of known correlates of immune protection makes it difficult to estimate the

efficacy of vaccine candidates prior to testing in costly human trials, and results obtained in animal models do not necessarily correlate with or predict outcome in humans (Friedrich and Watkins, 2008; Sattentau, 2008; Walker and Burton, 2008; Watkins et al., 2008). Virus transmission in the best available animal model, the SHIV/SIV macaque model, is likely to differ substantially from HIV-1 transmission in humans (Friedrich and Watkins, 2008; Watkins et al., 2008). For example, the artificial virus preparations used in animal models may not be representative of the virus swarms involved in sexual transmission of HIV-1. Moreover, the limited number of SIV or SHIV isolates available may not accurately represent the vast majority of the circulating HIV-1 strains. A vaccine against HIV-1 therefore remains elusive.

1.2.3.2 Topical strategies against HIV-1

As a viable vaccine candidate for HIV-1 will not be realised any time soon, an alternative would be the use of a topical microbicide to inhibit sexual transmission of HIV-1. Microbicides are agents that can be applied topically in the vagina or rectum, and are advantageous over the use of condoms, which is largely male-controlled and may not be compatible with marriage, nor can it be combined with reproduction. As microbicides could potentially be inexpensive, readily available and socially acceptable, they could offer a promising preventive intervention for sexual transmission of HIV-1 (Balzarini and Van Damme, 2007; Klasse et al., 2008; Klasse et al., 2006; Lederman et al., 2006).

Similar to HIV-1 vaccine development, there are many challenges to overcome in HIV-1 microbicide development. Firstly, a microbicide must be effective against the relevant local variants of HIV-1, which will be difficult given the extraordinary

genetic heterogeneity observed among HIV-1 isolates (Taylor et al., 2008). Secondly, a microbicide must be safe to use. A potential microbicide should not cause inflammation or have adverse effects on the resident microflora or the structural integrity of the mucosal epithelia that might increase the risk of transmission. Thirdly, an effective microbicide needs to be stable at a range of temperatures, easy to use, inexpensive, readily accessible, compatible with condom use, acceptable to users and sexual partners, and yet retain its potency in the presence of semen (Balzarini and Van Damme, 2007; Klasse et al., 2008; Klasse et al., 2006; Lederman et al., 2006; Neurath et al., 2006; Shattock and Moore, 2003). Finally, there are the same issues as for vaccines with efficacy evaluation and result correlation between *in vitro* experiments, animal models and actual human trials (Grant et al., 2008; Klasse et al., 2006; van de Wijgert and Shattock, 2007). Current evaluation models include the human explants models (Cummins et al., 2007), humanised mice models (Denton et al., 2008), and the macaque vaginal transmission model (Ambrose et al., 2008; Harouse et al., 2001; Lederman et al., 2004; Veazey et al., 2003).

Compounds being evaluated as microbicides include detergents and surfactants that inactivate free virus by disrupting the viral envelope, and may also be active against other sexually transmitted pathogens, including enveloped viruses such as herpes simplex virus (Lederman et al., 2006). Nonoxynol-9 was the first such compound to be tested in clinical trials, which was originally developed in the 1960s as a spermicide used in lubricants and to coat condoms, and was shown to demonstrate anti-HIV-1 activity in vitro and in animal models (Hicks et al., 1985; Miller et al., 1992a). Although signs of inflammation could be detected when using nonoxynol-9 (Stafford et al., 1998), it went ahead in clinical trials. The results revealed that

nonoxynol-9 not only had no effect on HIV-1 incidence, but had increased the risk of transmission in some cases by disrupting the membranes of the epithelium and the recruitment of target cells to the site due to inflammation (Fichorova et al., 2001; Hillier et al., 2005; Roddy et al., 1998; Van Damme et al., 2002). Another surfactant called C31G (Savvy) went into phase III clinical trials but it did not reduce the incidence of HIV-1 and might have increased the hazards of receiving HIV-1 (Feldblum et al., 2008).

Other microbicides being tested in clinical trials involve other modes of activity. One big category is the use of anionic polymers or polyanions which can bind to positively charged regions of gp120, such as the V3 region and the co-receptor binding site (Moulard et al., 2000). The first of these is cellulose sulphate, or known as Ushercell, which had shown good activity against HIV-1, HSV, HPV, *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Gardnerella vaginalis* in preclinical studies (Anderson et al., 2002; Christensen et al., 2001; Malonza et al., 2005; Simoes et al., 2002). Unfortunately, Ushercell failed to protect against HIV-1 infection in phase III trials, and might even possibly enhance the risk of infection (Van Damme et al., 2008). The second polyanion to enter clinical trials was Carraguard, which is a sulphated polysaccharide extracted from a red seaweed that has been shown to have anti-HIV-1 activity in vitro (Schaeffer and Krylov, 2000), but again did not demonstrate any efficacy in phase III trials (The Population Council, 2008). The failure of these two candidates was thought not to be surprising, as they had been shown to have limited potency in vitro, especially against CCR5-using viruses and in the presence of semen (Grant et al., 2008; Hartley et al., 2005; Neurath et al., 2006). A third polyanion, PRO 2000/5, which is a synthetic naphthalene sulphonate

polymer in clinical efficacy trials, has been shown to be active against HIV-1 in vitro (Keller et al., 2006) and against SHIV in the macaque model (Weber et al., 2001).

Another class of molecules being considered for use as candidate microbicides are HIV-1 specific entry inhibitors that specifically bind to gp120 or gp41. These include plant lectins which recognise glycans on the viral envelope. One of these is cyanovirin-N, which has been shown to prevent infection of macaques after vaginal SHIV challenge (Tsai et al., 2004). MAbs to gp120 and gp41 are also being considered (Lederman et al., 2006) and have already been determined to be effective in macaques (Veazey et al., 2003). These include the mAbs b12, 2G12 and 2F5, which will be discussed in Section 1.2.4.2. Other entry inhibitors under consideration include small molecules such as BMS-378806 which binds to gp120 (Wang et al., 2003), gp41 peptides which can inhibit virus entry such as T-20 (Klasse et al., 2008), RNA aptamers that bind to gp120 (Dey et al., 2005), CD4 mimics (Martin et al., 2003), CCR5 ligands such as RANTES (Lederman et al., 2004), the CCR5 antagonist Maraviroc (Fatkenheuer et al., 2005), as well as the CXCR4-binding small-molecule AMD3465 (Klasse et al., 2008). An innovative but controversial microbicide agent involves the genetic engineering of live commensal bacteria, such as the human vaginal isolate of *Lactobacillus jensenii*, to secrete a two-domain version of CD4 or produce the lectin cyanovirin-N that can inhibit HIV-1 in vitro (Chang et al., 2003; Pusch et al., 2006).

With disappointing results in all clinical efficacy trials completed to date, increasing efforts are being made to develop microbicides containing existing antiretroviral drugs such as the NRTIs and NNRTIs (Grant et al., 2008; Klasse et al., 2008). Rectal application of Tenofovir has been shown to protect macaques from SIV infection following rectal challenge (Cranage et al., 2008). However, the outcome of the use

of therapeutic drugs in microbicides and its contribution to the spread of drug resistance remains uncertain (Wilson et al., 2008).

Just like the field of HIV-1 vaccine development, research work to produce an effective anti-HIV microbicide is still in a very nascent stage.

1.2.4 Role of neutralising antibodies in tackling HIV-1

As mentioned in Section 1.2.2, neutralising antibodies are produced by the host immune response to an HIV-1 infection in an effort to clear the disease. Although HIV-1 quickly evolves its envelope glycoprotein to escape neutralisation, some individuals known as long term non-progressors (LTNP) are able to produce broadly cross neutralising antibodies (bcnAbs) that are able to keep viraemia levels in check (Braibant et al., 2006).

1.2.4.1 Antigenic profile of the HIV-1 envelope glycoprotein

The structure of the HIV-1 envelope spike has been introduced earlier in Section 1.1.5.2. This section will introduce the antigenic profile of the HIV-1 envelope glycoprotein and means to evade recognition by antibodies.

Since the envelope spike exists as a trimer of gp120/gp41 heterodimer, the antigenic profile of gp120 possess a non-neutralising face that is located on the inner domain of gp120 and a neutralising face that is exposed in the context of the envelope spike (Kwong et al., 1998; Moore and Sodroski, 1996; Wyatt et al., 1998). The non-neutralising face is normally buried within the trimer and interacts with gp41 or other gp120 protomers, but can become exposed on shed gp120 or on non-functional spikes on the virus surface (Moore et al., 2006; Poignard et al., 2003). The non-neutralising face can be highly immunogenic (Palker et al., 1987) and only elicit

non-neutralising anti-gp120 antibodies (Moore and Sodroski, 1996; Wyatt et al., 1998). On the other hand, the neutralising face of gp120 which includes parts of the bridging sheet and the inner and outer domains, including the V2 and V3 regions (Wyatt et al., 1998), contains the receptor-binding sites and epitopes for a number of neutralising antibodies (Moore and Sodroski, 1996; Pantophlet and Burton, 2006). However, conserved and functionally important regions of gp120, such as the CD4bs, are often occluded from antibody recognition (Pantophlet and Burton, 2006). The V3 region is particularly immunogenic but typically elicit only isolate-specific antibodies (Hartley et al., 2005; Pantophlet and Burton, 2006).

Much of gp41 is almost completely occluded from antibody recognition in the context of the HIV-1 envelope spike, as it is masked by glycans, gp120, and interactions with other gp41 molecules within the trimer (Nyambi et al., 2000; Qiao et al., 2005; Sattentau et al., 1995). However, several regions of gp41 have been found to be highly immunogenic and a large number of anti-gp41 antibodies have been isolated from infected individuals (Binley et al., 1996; Gnann et al., 1987; Xu et al., 1991). As these immunodominant regions are thought to be exposed only on non-functional or disassembled spikes, or on gp41 in the post-fusion state, the majority of anti-gp41 antibodies are non-neutralising. The membrane-proximal external region (MPER) of gp41, which is located adjacent to the transmembrane domain of gp41 and is the recognition site for the two well characterised broadly neutralising mAbs 2F5 and 4E10, is usually not well exposed but can be accessible to antibodies on a fusion intermediate form of gp41 (Muster et al., 1993; Stiegler et al., 2001; Zwick et al., 2001). However, immune response against the MPER is relatively small (Braibant et al., 2006; Dhillon et al., 2007; Li et al., 2007).

As can be seen, the HIV-1 envelope spike is highly complex and possesses a number of strategies to evade neutralising antibodies. As previously mentioned, the *env* gene is highly diverse due to the high mutation rate, and neutralising antibodies are often strain specific (Pantophlet and Burton, 2006). The glycan shield renders the outside of the envelope spike less visible to the immune system and its position is constantly evolving to escape neutralising antibodies (Cheng-Mayer et al., 1999; Wei et al., 2003). The variable regions of gp120 are thought to mask both the receptor and coreceptor binding sites (Sullivan et al., 1998; Wyatt et al., 1995; Wyatt et al., 1993). Unliganded gp120 is also thought to posses a high degree of conformational flexibility and potent neutralising antibodies were found to recognise most of the different conformations (Myszka et al., 2000; Yuan et al., 2006). The presence of non-functional spikes on the virus surface, such as gp41 stumps, gp120/gp41 monomers and uncleaved gp160, and shed gp120 may all serve to divert the antibody response (Moore et al., 2006; Poignard et al., 2003; Zwick and Burton, 2007).

Therefore, targeting the least conformationally variable regions of the functional envelope spike may produce a potent neutralising antibody and be valuable in vaccine design. Such regions could be identified by studying broadly and potently neutralising antibodies to the HIV-1 envelope glycoproteins.

1.2.4.2 Neutralising antibodies to HIV-1

As previously discussed, most of the anti-envelope antibodies developed during HIV-1 infection are non-neutralising but studies on the humoral response in LTNP can give insights into the types of neutralising antibodies that are needed to control viraemia. Most of the neutralising antibodies from infected individuals with broadly neutralising sera were found to bind to the CD4bs on gp120, to gp120 epitopes not

present on monomeric gp120, or to carbohydrate epitopes on gp120, but these neutralising antibodies did not bind to the MPER of gp41, or to the V1/V2/V3 regions of gp120 (Dhillon et al., 2007; Li et al., 2007; Yuste et al., 2006). It was also found that broadly neutralising sera from most LTNP contain antibodies that were able to compete with the monoclonal antibody b12 for binding to the CD4bs, and with the monoclonal antibody 2F5 for binding to the MPER (Braibant et al., 2006)

Only a handful of mAbs have been found to be broadly neutralising across various HIV-1 subtypes (Zwick and Burton, 2007). Two of these broadly neutralising mAbs are directed against gp120. The mAb b12, which binds to an epitope that in part overlaps a subset of the CD4bs (Barbas et al., 1992; Burton et al., 1991; Burton et al., 1994; Zhou et al., 2007), and 2G12, which recognises a carbohydrate motif on gp120 (Buchacher et al., 1994; Sanders et al., 2002a; Scanlan et al., 2002; Trkola et al., 1996b). Another two broadly neutralising mAbs, 2F5 and 4E10, are directed against the MPER of gp41 (Buchacher et al., 1994; Muster et al., 1993; Stiegler et al., 2001; Zwick et al., 2001). The Fab fragment X5 (Moulard et al., 2002), which recognises an epitope on gp120 that is better exposed after CD4 binding also display some neutralising activity across HIV-1 subtypes.

The mAb b12 (also referred as IgG1b12) is one of the most extensively studied mAbs to HIV-1 envelope. It was initially isolated in 1991 as an Fab fragment, through panning of a combinatorial phage library displaying monoclonal Fab fragments from an asymptomatic HIV-1-infected individual on recombinant monomeric gp120 (Burton et al., 1991) and has since been expressed as a full immunoglobulin. The mAb b12 has been shown to be able to neutralise a large proportion of HIV-1 isolates from a wide range of subtypes (Barbas et al., 1992; Binley et al., 2004; Burton et al., 1994; Pantophlet and Burton, 2006) and to protect

macaques against SHIV challenge (Parren et al., 2001; Veazey et al., 2003). The crystal structure of b12 in complex with gp120 has confirmed that b12, which possess an unusually long and protruding heavy-chain CDR3, binds to an epitope that overlaps part of the CD4 binding site and revealed that b12 binds to gp120 only with its heavy-chain (Zhou et al., 2007). Amino acid residues in all three complementarity determining regions (CDRs) of b12 make contact with the functionally conserved CD4 binding loop and the outer domain of gp120.

The mAb 2G12 is also broadly neutralising, and recognises a cluster of oligomannose residues on gp120. It has an unusual structure in that it has undergone a domain-swap, where the heavy-chain variable regions of each Fab have swapped positions so that they interact with the light-chain variable region of the neighbouring arm (Buchacher et al., 1994; Sanders et al., 2002a; Scanlan et al., 2002; Trkola et al., 1996b). This antibody has shown efficacy in monkey models when used in combination with other mAbs (Baba et al., 2000; Hofmann-Lehmann et al., 2001), and also in humans through intravenous administration (Trkola et al., 2005). Although 2G12 is able to neutralise many of the viruses tested, it does not neutralise subtype C viruses (Binley et al., 2004).

The mAbs 2F5 (Buchacher et al., 1994; Muster et al., 1993) and 4E10 (Stiegler et al., 2001; Zwick et al., 2001) target adjacent epitopes on the conserved MPER gp41. The mAb 2F5 binds to an epitope overlapping the conserved sequence ELDKWA (Muster et al., 1993; Ofek et al., 2004), whereas 4E10 recognises the epitope NWF(D/N)IT (Cardoso et al., 2005; Zwick et al., 2001) which is just C-terminal to the 2F5 epitope. The epitopes for both 4E10 and 2F5 have been shown be slightly hydrophobic and possibly membrane-bound (Alam et al., 2007; Haynes et al., 2005; Sanchez-Martinez et al., 2006). In neutralisation studies, 2F5 was not able to

neutralise most subtype C viruses as they lack the DKW motif required for 2F5 recognition (Binley et al., 2004), whereas 4E10 was able to neutralise most viruses tested (Binley et al., 2004; Li et al., 2005; Li et al., 2006b) and is possibly the broadest neutralising mAb to HIV-1 described to date.

Other similar mAbs that target the CD4 binding site of gp120 and the MPER of gp41 were isolated. These are the mAbs m14 (Zhang et al., 2004b), F105 (Clayton et al., 2007), and Z13 (Zwick et al., 2001), but they were found to display less broad and potent neutralisation activity compared to b12, 2F5 and 4E10.

All of the broadly neutralising mAbs reported in the literature to date are from individuals infected with HIV-1 of subtype B (Zwick and Burton, 2007), which may explain why the mAbs 2G12 and 2F5 do not recognise viruses from subtype C. As most circulating strains are currently of subtype C, new broadly neutralising antibodies with better coverage of the circulating strains are needed.

1.2.4.3 HIV-1 immunogens and vaccine design

Efforts are being made to design an immunogen that is able to elicit broadly neutralising antibodies like those described in the previous section. The envelope constructs should mimic the functional envelope spike (Pantophlet and Burton, 2006; Phogat and Wyatt, 2007), as previous attempts at using HIV-1 envelope glycoprotein constructs as immunogens (such as gp120 monomers or gp140 monomers or gp140 trimers) has typically elicited only antibodies with weak neutralisation activity against heterologous isolates. Recombinant oligomeric gp140 molecules are gp160 molecules that have been truncated just before the transmembrane region of gp41 (Beddows et al., 2005; Chakrabarti et al., 2002; Earl et al., 2001; Grundner et al., 2005; Jeffs et al., 2004; Kim et al., 2005; Li et al., 2006c; Sanders et al., 2002b;

Selvarajah et al., 2005; Srivastava et al., 2002; Yang et al., 2000; Yang et al., 2001; Zhang et al., 2001). However, it is thought that none of these recombinant constructs fully mimic the functional viral spike (Pancera and Wyatt, 2005; Pantophlet and Burton, 2006; Schulke et al., 2002).

Other envelope constructs have also been engineered, such as deglycosylated gp120 and gp120 with deleted immunodominant variable regions, gp120 locked in a CD4-bound conformation, and masking of non-neutralising regions (Barnett et al., 2001; Dey et al., 2007; Kim et al., 2003; Pantophlet and Burton, 2003). Various scaffolds that hold the MPER neutralising epitopes are also under evaluation (Law et al., 2007; Zwick, 2005), as is presenting envelope glycoproteins in the context of VLPs (Crooks et al., 2007). A trimeric construct of the MPER of gp41 was also constructed and used to immunise rabbits, but was not able to produce neutralising antibodies (Hinz et al., 2009).

To deal with the heterogeneity of the HIV-1 sequence, polyvalent immunogens consisting of envelopes from different subtypes are also being considered and was shown to produce antibodies with better cross reactivity (Chakrabarti et al., 2005; Cho et al., 2001; Zwick and Burton, 2007). Perhaps some envelopes are also conformationally presented in a better way. Rabbits immunised with a recombinant gp140 derived from an HIV-1-infected individual, whose serum was found to be broadly neutralising, were also found to produce antibodies with cross-subtype reactive neutralising activity (Zhang et al., 2007).

In light of the recent failures in vaccine clinical trials, more attempts are needed to design an appropriate immunogen that is able to elicit broadly neutralising antibodies. Reverse immunology, in which antigens can be engineered from epitopes

recognised by neutralising antibodies, is another strategy that can be utilised to design such an immunogen. For that to happen, more broadly neutralising antibodies are needed to characterise the relevant epitopes on the envelope glycoprotein for use in immunogens.

1.3 Single-chain camelid antibodies

As discussed earlier, more broadly neutralising antibodies are needed to understand and to interrogate epitopes on the HIV-1 envelope glycoprotein that can then be used to design immunogens which can elicit such broadly neutralising antibodies, in particular against subtype C viruses. We decided to exploit the unique characteristics of the llama immune system to isolate antibodies that can neutralise diverse strains of HIV-1, and these are described in Chapters 4 and 5 of this thesis. This section will give an introduction on camelid antibodies and single-chain antibody fragments in particular, and methods to isolate antibodies of interest.

1.3.1 Basic features and properties of camelid single-chain antibodies

Conventional IgG antibodies found in higher vertebrates are composed of a pair each of heavy chains and light chains. The heavy chains consist of a constant domain (CH), which is organised into three constant domains (CH1-CH3), and one variable domain (VH). A hinge region connects the CH1 and CH2 domains. The light chains are composed of one constant (CL) and one variable domain (VL). The antigen binding region or paratope is formed by the paired VH and VL domains.

Hamers-Casterman *et al*. reported in 1993 that members of the *Camelidae* family were able to produce fully functional antibodies that are devoid of light chains, called heavy-chain antibodies, in addition to conventional antibodies (Hamers-Casterman et al., 1993). The *Camelidae* family includes the camel (*Camelus bactrianus*), dromedary (*Camelus dromedarius*), llama (*Lama glama*), alpaca (*Lama pacos*), guanaco (*Lama guanicoe*) and vicuna (*Lama vicugna*). These heavy-chain antibodies also lack the first constant domain (CH1) of the constant region of the heavy chain (Figure 1-5). The antigen-binding properties of the heavy-chain antibodies are provided by one single fragment, the variable region of the heavy-chain (Hamers-Casterman et al., 1993), and has been termed the VHH. A schematic representation of the camelid heavy-chain antibody is shown in Figure 1-5.

The heavy-chain IgG fraction has been estimated to constitute up to 50% of the total serum IgG content in camelids, and IgA, IgD and IgM fractions do not appear to lack the light chains (Maass et al., 2007; Nguyen et al., 2001). The residues involved in antibody effector functions, mediated by binding of the Fc to complement and Fcγ receptors are conserved in the heavy-chain antibodies, indicating that they retain their ability to mediate effector functions (Vu et al., 1997).

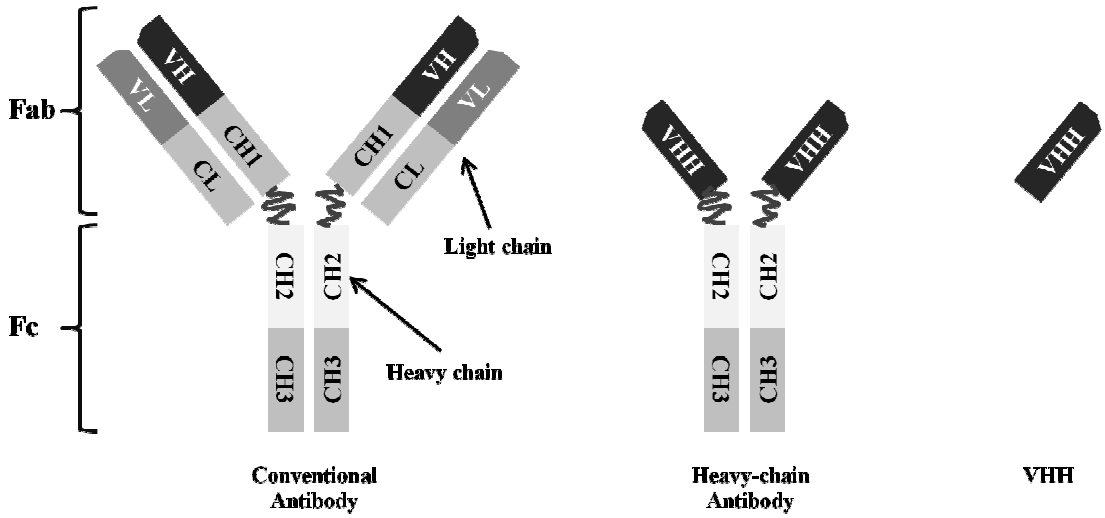


Figure 1-5. Schematic representations of camelid heavy-chain antibody

Conventional mammalian IgG consist of two heavy chains made up of three constant domains (CH1-CH3) and one variable domain (VH), and two light chains made up of a constant (CL) and a variable (VL) domain. Camelid heavy-chains lack the light chains and the first constant domain of the heavy chains. Their variable domain has been termed the VHH.

Figure 1-6. Schematic representation of VHH domains

Like conventional VH domains, the VHH domain of heavy-chain antibodies contains four framework regions (FR1-FR4) and three complementarity-determining regions (CDR1-CDR3).

Sequence and structural analysis of the VHH reveal that they share many features with VH domains on conventional antibodies (Harmsen and De Haard, 2007; Harmsen et al., 2000; Muyldermans et al., 2001; Vu et al., 1997). The VHH is organised into four relatively conserved framework regions, FR1-FR4, and three hypervariable complementarity-determining regions, CDR1-CDR3, which determines antigen specificity (Figure 1-6). On average, camelid VHH display longer CDRs compared to the conventional VH counterparts (Harmsen et al., 2000; Muyldermans et al., 1994; Vu et al., 1997). The CDR3 loops of llama VHH are on average 15 amino acids long, compared to an average of 12 amino acids in humans

and 9 amino acids in mouse (Vu et al., 1997). The extent of somatic hypermutation is found to be higher in VHH compared to in VH, resulting in an overall increased sequence diversity compared to VH, thus allowing them to exhibit diverse antigen-binding properties (Harmsen and De Haard, 2007; Muyldermans et al., 2001; Nguyen et al., 2000).

Despite their single-domain nature and small size of approximately 14 kDa, VHH have characteristics in terms of affinity and specificity that are similar to those of conventional antibodies (van der Linden et al., 1999), and the size of the interface surface areas are also similar (De Genst et al., 2006). The VHH of heavy-chain antibodies have been shown to have a preference for binding into active site clefts using its protruding CDR3 (De Genst et al., 2006; Lauwereys et al., 1998) and to be able to recognise cryptic epitopes typically occluded to conventional antibodies (Stijlemans et al., 2004), compared to conventional antibodies which tend to recognise planar or convex surfaces.

1.3.2 Exploitation of VHH in medical research

The single domain and the diverse antigen-binding properties of VHH make them a very useful tool in medical research, and VHH have many biotechnological and therapeutic applications. Individual VHH can be easily isolated from phage display libraries, without the need for random recombination of the VH and VL domains in conventional antibody Fab fragments (Harmsen and De Haard, 2007). Selected VHH can then be readily cloned and expressed to high levels in bacteria or yeast (Frenken et al., 2000). Furthermore, VHH have been shown to be thermally and conformationally stable, exhibiting thermodynamic stabilities that have not been observed for functional conventional antibody fragments, such as the retention of

binding properties at temperatures up to 90°C and fully reversible refolding of structure after denaturation through chemically induced unfolding (Dumoulin et al., 2002; Perez et al., 2001; van der Linden et al., 1999). The stability and solubility of VHH also allows for engineering of multivalent and bispecific molecules, and these have been shown to exhibit better binding and neutralising properties than their monovalent counterparts (Coppieters et al., 2006; Els Conrath et al., 2001; Roovers et al., 2007).

These unique properties of VHH allow it to have diverse applications against a wide range of biological targets in biomedical research. VHH have been used to target the fungus *Malassezia furfur* implicated in the formation of dandruff (Dolk et al., 2005), and to target human lysozyme to inhibit amyloid fibril formation (Dumoulin et al., 2003). Other applications include cancer therapy (Cortez-Retamozo et al., 2004; Roovers et al., 2007) and infectious diseases (Baral et al., 2006; Garaicoechea et al., 2008; Harmsen et al., 2007; Kruger et al., 2006; van der Vaart et al., 2006). Biotechnological applications include immune-affinity chromatography (Verheesen et al., 2003) and intracellular targeting and tracing of antigenic structures within living cells (Rothbauer et al., 2006).

1.3.3 VHH to target HIV-1

Given the unique properties of VHH, such as its small size and diverse antigen-binding properties and wide applications, VHH can also be used to target the HIV-1 envelope spike to inhibit virus entry. Coupled with the VHH preference for binding to active site clefts with their protruding CDR3 loops, VHH may be ideal candidates to bind into the CD4 and coreceptor binding sites. The broadly neutralising mAbs b12, 2F5 and 4E10 were all found to possess long CDR3 loops. The stability,

solubility, ease of cloning and high production yield of VHH makes it an ideal candidate for microbicide development in the prevention of HIV-1.

A previous PhD student in the lab, Anna Forsman, has isolated three VHH which show potency against cross-subtype HIV-1 (Forsman et al., 2008). These were isolated from a llama immunised with recombinant monomeric gp120 derived from a subtype B'/C primary isolate. A functional selection approach was used to pan for VHH with competitive elution with soluble CD4, in a bid to increase the likelihood of isolating VHH that can target the CD4 binding site. The three isolated VHH (C8, D7 and A12 VHH) were found to recognise an area around the CD4 binding site of gp120 and were able to bind with high affinities, thus inhibiting virus entry into host cells. These VHH were found to target various primary isolates of subtypes B and C, some of which were not recognised by the mAb b12. Broadly neutralising antibodies are usually isolated from infected individuals and this is the first report of obtaining such antibodies from an immunised animal.

1.4 Approach to this thesis

Based on the initial success in isolating VHH that can inhibit HIV-1 entry, more neutralising VHH would be useful. These can have further applications in therapy as entry inhibitors or prevention reagents in microbicides, and can also be useful in the design of an HIV-1 vaccine by defining neutralising and non-neutralising epitopes in reverse immunology. MAbs to the HIV-1 envelope glycoproteins may be useful in the design of an HIV-1 vaccine as they can help define neutralising and non-neutralising epitopes. MAbs able to neutralise a wide range of HIV-1 isolates can provide information about vulnerable, conserved and functionally important sites on the HIV-1 functional envelope spike, such as the receptor-binding sites.

Therefore, the aim of this thesis is to utilise the non-conventional immune system of llamas to generate more VHH against the HIV-1 envelope spike, in particular to primary isolates of subtype C.

Chapter 3 will describe the isolation and cloning of gp120 and gp160 envelopes from individuals in Africa and in the United Kingdom who are infected with subtype C HIV-1, and the insertion of these envelope fragments into HIV-1 backbone vectors to produce chimeric viruses that are replication competent. These envelopes are then characterised with various antibodies, and are used as neutralisation targets in subsequent chapters. Chapter 4 will describe the isolation of four different anti-gp41 VHH from a llama that was immunised with trimeric gp140 derived from the same subtype B'/C recombinant that is mostly of subtype C in its envelope. Chapter 5 will describe the creation of C8- and D7-family specific VHH libraries, from the original immune library of the llama used to isolate the C8, D7 and A12 VHH, to create more VHH that are able to recognise the CD4 binding site of gp120. The CDRs of these VHH are then analysed and amino acid residues that are important for binding to HIV-1 are postulated.

But first, the materials and methods will be described in Chapter 2, and the final chapter, Chapter 6, will discuss and summarise the findings of this thesis.

2 Materials and Methods

2.1 Materials

This section provides details of the buffers and solutions, recombinant proteins and antibodies used in this study.

2.1.1 Buffers and solutions

The buffers and solutions used in this study are listed in Table 2-1.

2.1.2 Recombinant HIV-1 envelope glycoproteins

Recombinant gp120 derived from the HIV-1 subtype B'/C (CRF07_BC) primary isolate CN54 (Su et al., 2000), produced in a baculovirus expression system, was kindly provided by I. Jones, Reading University, UK, through the European Microbicide Project (EMPRO). Recombinant gp140 CN54 and gp140 derived from the HIV-1 subtype A primary isolate 92UG037 (Jeffs et al., 2004), produced in a Chinese hamster ovary (CHO) cell system, were kindly provided by S. Jeffs, Imperial College London, UK. Recombinant gp120 derived from the HIV-1 subtype B TCLA isolate IIIB (Popovic et al., 1984), produced in a baculovirus expression system, was obtained from the Centralised Facility for AIDS Reagents (CFAR, catalogue number EVA607) at the National Institute for Biological Standards and Control (NIBSC), Herts, UK. Recombinant gp120 derived from the HIV-1 subtype C primary isolate 92BR025 (Gao et al., 1996), were cloned, expressed and purified as detailed in Section 2.7.1.1. Recombinant gp41Δ from subtype B HIV-1 MN, consisting of only the ectodomain region (aa 546-682, 16-28kDa) of gp41, was obtained from CFAR (catalogue number ARP680). Full length recombinant IIIB gp41 that was expressed in an yeast system (catalogue number R65890) was procured from Biodesign International (Saco, Maine, USA). Recombinant trimeric

gp140 96ZM651.02, 92UG037.A9 and Bx08 (Moog et al., 1997) were obtained from Polymun Scientific GmbH, Vienna, Austria, as part of the Bill & Melinda Gates Foundation Collaboration for AIDS Vaccine Discovery (CAVD).

2.1.3 Monoclonal antibodies to HIV-1 gp120 and gp41

Monoclonal antibodies b12 (Burton et al., 1991) were kindly provided by D. Burton, Scripps Institute, La Jolla, USA. MAbs 2G12 (Buchacher et al., 1994), 2F5 (Muster et al., 1993) and 4E10 (Stiegler et al., 2001) were obtained from Polymun Scientific GmbH, Vienna, Austria, as part of the Bill & Melinda Gates Foundation Collaboration for AIDS Vaccine Discovery (CAVD). Mouse mAb 1577 which targets gp41 peptide 735-752 (CFAR catalogue number ARP317, original source M. Ferguson), and human mAbs 50-69D and 98-6D (CFAR catalogue numbers ARP3222 and ARP3223 respectively, original source S. Zolla-Pazner) were all obtained from the Centralised Facility for AIDS Reagents (CFAR), NIBSC. MAb 50-69D reacts to a conformational dependent epitope on gp41, whereas mAb 98-6D reacts with aa 644-663 of gp41.

2.1.4 Sera and plasma from HIV-1-seropositive individuals

Quality control (QC) sera 1, 2, and 6 from subtype B HIV-1-seropositive individuals have been described previously (McKeating et al., 1989). Plasma from individuals infected with subtype C HIV-1 was kindly provided by D. Yirrel, Glasgow, UK for envelope cloning. Serum and plasma samples were heat-inactivated to destroy complement by incubation at 56°C for 1 h prior to use in neutralisation assays.

Table 2-1. Buffers and solutions

Buffer/solution	Composition
2 × tryptone/yeast (TY) medium	1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl
Luria-Bertani (LB) medium	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl
LB agar	LB medium plus 1.5% (w/v) agar
Phosphate buffered saline (PBS)	137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM K_2HPO_4, pH 7.4
PBS-T	0.05% (v/v) Tween 20 in PBS
Super optimal broth with catabolite repression (SOC)	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
Tris acetate EDTA (TAE)	40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA, pH 7.8
Tris buffered saline (TBS)	20 mM Tris-HCl, 120 mM NaCl, pH 7.6
TBS-T	0.05% (v/v) Tween 20 in TBS
TMT/BS	4% (w/v) milk powder and 10% (v/v) bovine serum (BS) in TBS-T
TMT/GS	4% (w/v) milk powder and 10% (v/v) goat serum (GS) in TBS-T
Transfer Buffer	25mM Tris, 192mM Glycine, 0.1% SDS
His elution buffer	50 mM Na_2HPO_4, 300 mM NaCl, 150 mM imidazole, pH 7.0
His equilibrium buffer	50 mM Na_2HPO_4, 300 mM NaCl, pH 8.0
His pre-elution buffer	50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 7.0
FACS buffer	0.1% azide, 1%FCS in PBS

w/v, weight/volume; v/v, volume/volume; Tris, tris-(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Imidazole, 1,3-diazole; Tween 20, polyoxyethylene sorbitan monolaurate.

2.1.5 Recombinant sCD4

Recombinant soluble CD4 (sCD4) consisting of the extracellular domains D1-D4 of human CD4 expressed in Sf21 cells in a baculovirus expression system was obtained from R&D Systems (Minneapolis, USA, catalogue number 514-CD-050/CF).

2.2 Cell culture techniques

2.2.1 Cell lines and culture media

The TZM-bl cell line (Derdeyn et al., 2000; Platt et al., 1998; Wei et al., 2002), previously designated JC53-bl (clone 13), is a HeLa cell line which stably expresses human CD4, CXCR4 and CCR5 and contains Tat-responsive firefly luciferase and β-galactosidase genes under control of the HIV-1 promoter. TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Programme (catalogue number 8129) from J. C. Kappes, X. Wu and Tranzyme Inc, and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) containing 10% (v/v) foetal calf serum (FCS).

The NP2 cell lines (Soda et al., 1999) are human glioma cell lines expressing human CD4 and either of the HIV-1 chemokine co-receptors, CCR3 (NP2/CD4/CCR3) or CXCR4 (NP2/CD4/CXCR4) or CCR5 (NP2/CD4/CCR5). NP2 cells were cultured in DMEM (Invitrogen) containing 5% (v/v) FCS.

The 293T cell line, of human embryonic kidney origin (Graham et al., 1977), was cultured in DMEM GlutaMAX (Invitrogen), which is DMEM containing L-analyl-L-glutamine instead of L-glutamine, supplemented with 10% (v/v) FCS. The use of DMEM GlutaMAX minimises ammonia build-up in cell cultures, as L-analyl-L-glutamine does not degrade into ammonia like L-glutamine.

The H9 (HUT78) cell line, which is of human cutaneous T cell lymphoma origin (Mann et al., 1989), was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% (v/v) FCS.

2.2.2 Mammalian cell line maintenance

All cells were grown in cell culture flasks at 37°C in a humidified atmosphere containing 5% CO_2, except for 293T cells, which were kept in a humidified atmosphere containing 10% CO_2. Cell lines were passaged every 2-5 days and split at a ratio of 1:3-1:10 depending of the cell line and cell density. For adherent cell lines, cell monolayers were disrupted and removed by treatment with trypsin-EDTA (0.25% (w/v) trypsin, 1 mM EDTA; Invitrogen) at confluency, prior to passaging. Trypsinisation was performed by washing cells once in sterile PBS, followed by incubation with approximately 4 ml of trypsin-EDTA (Invitrogen) at 37°C. Once cells had detached from the flask, fresh culture medium was added and cells were diluted in the appropriate growth medium to the desired density and split into an appropriate number of flasks. NP2 cells engineered to express CD4 were occasionally passaged in selective medium containing 1 mg/ml Geneticin G418 sulphate (Invitrogen) to maintain stable CD4 expression, and 1ug/ml puromycin for CXCR4 and CCR5 selection.

2.2.3 Freezing and thawing of cells

Adherent cells were trypsinised and then pelleted by centrifugation at 325 *g* for 10 min, and approximately 10^7 cells/ml were resuspended in the appropriate cell culture medium containing 20% (v/v) FCS and 10% (v/v) dimethyl sulphoxide (DMSO). Resuspended cells were aliquoted into cryovials and subsequently transferred to -80°C. To allow for slow freezing, vials were put into a polystyrene box. The next

day, the vials were transferred to the vapour phase of liquid nitrogen, for long-term storage. When needed, cryovials containing frozen cells were transferred from the vapour phase of liquid nitrogen to a 37°C water bath. Once thawed, cells were added to 10 ml of growth medium and pelleted at 325 *g* for 10 min. Pelleted cells were resuspended in growth medium, transferred to cell culture flasks and kept at 37°C.

2.2.4 Transfection of 293T cells

A day before transfection, 293T cells were plated in a 6-well dish at a density of $4x10^5$ cells/well. The following day, the medium was replenished before transfection. Six µl of Fugene-6 (Roche, Germany) was premixed with 4 µg of DNA in 200 µl of serum-free Opti-MEM (Invitrogen, UK) for 15 min at room temperature, and then added dropwise to each well. After overnight incubation at 37°C, the medium was replenished and harvested 42 h later.

2.2.5 Preparation of peripheral blood mononuclear cells (PBMC)

Buffy coats from the blood bank (Brentwood blood transfusion centre, London) were diluted 1:1 (v/v) in sterile PBS. Forty-five ml of the diluted Buffy coat were then layered onto 15ml of Lymphoprep (Nycomed, Norway) in 50 ml falcon tubes, and then centrifuged at 1500 *g* for 30 min without brakes. The lymphocytes layer was harvested, washed twice with sterile PBS by centrifugation at 800 *g* for 10 min. The cells were then incubated with 10 ml of red blood cell lysis buffer (Sigma, UK) for 10 min at RT. Lymphocytes were then harvested by centrifugation, and diluted into RPMI. The cells were resuspended at a concentration of 10^6 cells/ml in RPMI-10% FCS containing phyto-haemagglutinin (PHA, Murex, UK) at a final concentration of 20U/ml and grown for 3 days at 37°C. The cells were then centrifuged and 10^6

cells/ml was resuspended in RPMI supplemented with 20U/ml of IL-2 (Roche, Germany).

2.3 Virus stocks and manipulations

2.3.1 HIV-1 strains

HIV-1 IIIB (Popovic et al., 1984) and HIV-MN (Gallo et al., 1984), which are T-cell line adapted (TCLA) CXCR4-using subtype B lab strains, were originally isolated from the PBMC of HIV-1 seropositive individuals in the 1980s. These were obtained from the Centralised Facility for AIDS Reagents (CFAR) as cell-free cell-culture supernatant from infected H9 cells.

HIV-1 BaL (Gartner et al., 1986) and HIV-1 SF162 (Cheng-Mayer and Levy, 1988), originally isolated from human infant lung tissue and adult brain tissue respectively, are both subtype B CCR5-using strains propagated in PBMC. These were also obtained from the Centralised Facility for AIDS Reagents (CFAR). PBMC-propagated HIV-1 primary isolates 92UG037 and 92BR025 were obtained from the WHO-UNAIDS collection of primary isolates (Gao et al., 1994).

HIV-1 subtype B and subtype C reference panels of envelope clones (Li et al., 2005; Li et al., 2006b) from acute and early HIV-1 infections were obtained through the NIH AIDS Research and Reference Reagent Programme (ARRRP; catalogue numbers 11227 and 11326). The reference panels were designed for use as Env-pseudotyped viruses to facilitate standardized Tier 2/3 assessments of HIV-1-specific neutralizing antibodies (Mascola et al., 2005). The Env-pseudotyped viruses exhibit a neutralization phenotype that is typical of most primary HIV-1 isolates, and they are all CCR5-using. No clone is unusually sensitive or resistant to neutralization. The gp160 genes were cloned from sexually acquired, acute/early infections and

comprise a wide spectrum of genetic, antigenic and geographic diversity within subtype B and C.

The subtype B panel consists of gp160 clones 6535.3, QH0692.42, SC422661.8, PVO.4, TRO.11, AC10.0.29, RHPA4259.7, THRO4156.18, REJO4541.67, TRJO4551.58, WITO4160.33 and CAAN5342.A2, whereas the subtype C panels consists of clones Du156.12, Du172.17, Du422.1, ZM197M.PB7, ZM214M.PL15, ZM233M.PB6, ZM249M.PL1, ZM53M.PB12, ZM109F.PB4, ZM135M.PL10a, CAP45.2.00.G3 and CAP210.2.00.E8. The subtype C gp160 clones 93MW965.26 and 96ZM651.02 were kindly provided by D. Montefiori (Duke University Medical Centre, Durham, USA) through the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA-VIMC) as part of the CAVD. These HIV-1 envelope pseudotyped viruses were produced in 293T cells by co-transfection with the pSG3Δ*env* plasmid, and are then capable of a single round of infection in TZM-bl cells.

Replication-competent chimeric molecular clones CA6 and CB7, which contain subtype C gp120 held in the pHXB2Δ*env* vector (McKeating et al., 1993), were kindly provided by M. Aasa-Chapman (UCL, London, UK).

A collection of 20 HIV-1 subtype C infected plasma samples were obtained from David Yirell (Specialist Virology Centre in Glasgow, Scotland), with randomised patient ID numbers given from C20 to C39. Replication competent chimeric molecular clones C202, C222, C250, C261, C271, C281, C291, C344, C351, C361, C381 and C391 contain HIV-1 Env gp120 from the patients and held in the pHXB2Δ*env* vector. Similar molecular clones C27b, C27d, C33.52, C37.42, C38d, C38g, C38.22, and C38.32 containing HIV-1 Env gp160 from the patients were also

produced and held in the pNL43Δ*env* vector (Zheng and Daniels, 2001). The cloning and characterisation of these viruses are described in Chapter 3.

Table 2-2. HIV-1 isolates and molecular clones used in this study

Virus	Subtype	Type[a]	Source[b]	Geographical origin
IIIB	B	TCLA	PBMC	USA
MN	B	TCLA	PBMC	USA
BaL	B	PBMC	Lung	USA
SF162	B	PBMC	Brain	USA
QH0692.42	B	PSV	ccPBMC	Trinidad
PVO.4	B	PSV	ccPBMC	Italy
92BR025.C1	C	MC	ccPBMC	Brazil
93MW965.26	C	PSV	No info.	Malawi
96ZM651.02	C	PSV	No info.	Zambia
ZM214M.PL15	C	PSV	Plasma	Zambia

[a]TCLA, T cell line-adapted isolate; PBMC, Primary PBMC-propagated isolate; PSV, pseudotyped viruses containing full-length envelopes; MC, molecular gp120 clone slotted into pHXB2Δ*env*.

[b]Source of original isolate. ccPBMC, co-cultured PBMC; for molecular clone, the envelope was cloned directly from ccPBMC.

2.3.2 Growth of viral stocks

HIV-1 BaL and SF162 were propagated in PHA-stimulated PBMC. Approximately 1-2 x10^7 PBMC (pooled from two donors) were incubated with virus at 37°C in a total volume of 1-2 ml. After 2 h of incubation, growth medium (RPMI 1640 supplemented with 10% (v/v) FCS and 20 U/ml of interleukin-2) was added to the cells to a total volume of 10 ml. Virus production was monitored by evaluating the p24 content in the cell culture supernatant using the Vironostika HIV Uni-Form II Ag/Ab p24 assay (bioMérieux, Marcy l'Etoile, France) as per manufacturer's protocol. After 6-7 days, the cells were pelleted by centrifugation at 325 *g* for 10 min and co-cultured (in fresh growth medium) with fresh, uninfected PHA-stimulated PBMC from two donors at a 1:1 ratio. Cell density was adjusted to approximately 1-2 x 10^6 cell/ml. This procedure was repeated every 6-7 days for 4 weeks or until a sufficient volume and titre of virus had been obtained. The p24 content of the cell culture supernatant was monitored throughout. At the peak of p24 production, the cell culture supernatant was harvested by centrifugation of the cultures at 325 *g* for 10 min. The supernatant was subsequently filtered (0.45 μm) and stored in liquid nitrogen in 0.5 ml aliquots.

Laboratory-adapted CXCR4-tropic strains of HIV-1 (IIIB and MN) were grown in H9 cells. A virus stock was thawed and incubated with 10^6 H9 cells for 2 h at 37 °C, after which it was resuspended in 5 ml of RPMI supplemented with 10% (v/v) FCS, and allowed to incubate for 3 days at 37 °C. The infected cells were then centrifuged and allowed to incubate with a further 5x10^6 cells for 2 h. The cells were resuspended in 12 ml of media for another 3 days, until syncytia were visible under the microscope. The cells were then pelleted and the supernatant, containing the virus particles, was aliquoted and stored in liquid nitrogen.

2.3.3 Production of HIV-1 envelope pseudotyped viruses and virus from replication-competent molecular clones in 293T cells

HIV-1 envelope pseudotyped viruses were produced in 293T cells by co-transfection with the pSG3Δ*env* plasmid, which contains the entire HIV-1 genome except for the *env* gene. For each 10 cm cell culture dish of 293T cells, 4 μg of envelope clone and 8 μg of pSG3Δ*env* was used. Virus stocks from replication-competent HIV-1 molecular clones were also prepared by transfection of 293T cells. Transfection of 293T cells was carried out as described in Section 2.2.4. The virus-containing cell culture supernatant was harvested at 48-72 h after transfection, and filtered through a 0.45 μm filter. It was then aliquoted into cryovials and stored in liquid nitrogen.

2.3.4 HIV-1 p24 intracellular immunostaining

HIV-1 infection of NP2 cells was detected using *in situ* intracellular p24 immunostaining as previously described (Sonza et al., 1991). The cell culture medium was removed and the cells were fixed for 10 min at room temperature using 200 μl per well of an ice-cold mixture of methanol and acetone (at a 1:1 ratio). The cells were then subsequently washed twice in PBS+1% FCS. The fixed cells were then incubated with 200 μl per well of an equal mixture of mouse anti-p24 mAbs 38:96K and EF7 (corresponding to reagents ADP365 and ADP366 at the Centralised Facility for AIDS Reagents, NIBSC) at 1:40 dilution in PBS+1% FCS, for 1 h at room temperature. After another two washes in PBS+1% FCS, the cells were incubated with 200 μl per well of a goat anti-mouse immunoglobulin antibody conjugated to β-galactosidase (Southern Biotechnology Associates, Birmingham, USA; catalogue number 1010-06) at 2.5 μg/ml in PBS+1% FCS, for 1 h at room temperature. The stained cells were then washed twice in PBS+1% FCS and once in PBS, and subsequently incubated with 200 μl per well of β-galactosidase substrate

solution (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactopyranoside in PBS containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide and 1 mM $MgCl_2$) at 37°C overnight. Cells infected with HIV-1 will develop a deep-blue colour, and individual or grouped stained cells were counted under the microscope as focus-forming unit (FFU).

2.3.5 Titration of virus stocks on NP2 cells

HIV-1 titres were assessed on NP2/CD4/CXCR4 cells or NP2/CD4/CCR5 cells, depending on the co-receptor usage of the HIV-1 strain. Cells were split and $2x10^4$ cells were seeded per well in a 48-well plate, and allowed to incubate overnight at 37°C. A vial containing the viral stock was thawed and 10-fold serially diluted in DMEM+10% FCS. One hundred µl of the serial dilutions were allowed to incubate with cells in duplicate wells for 2 h at 37°C. The cells were then washed once with medium and incubated with 500 µl of DMEM+10% FCS per well for 48-72 h at 37°C. The medium from each well were then removed and fixed with ice cold methanol/acetone. Virus infection was detected with intracellular HIV-1 p24 immunostaining as described in Section 2.3.4. Focus forming units (FFU) were counted microscopically and the virus titre determined as FFU/ml.

2.3.6 Neutralization assays on NP2 cells

NP2 cells were plated overnight in 48-well cell culture plates at a density of 2×10^4 cells per well. Virus (100 FFU) was then incubated in triplicates with 2-fold serial dilutions of test antibody (from 25 µg/ml) or serum (from 1:10 dilution) in 100µl DMEM+10% FCS for 2 h at 37°C. The cells were then gently washed once with DMEM+10% FCS and incubated with 500 µl of DMEM+10% FCS per well, and incubated for 3 days at 37°C. The cells were then fixed and virus infection was

detected with HIV-1 p24 intracellular immunostaining as described in Section 2.3.4. FFU were counted microscopically and neutralisation titres were determined as the antibody concentration (or dilution) required to give 50% reduction of infection (IC_{50}) compared to a negative control antibody (or normal human serum).

2.3.7 Detection of infection of TZM-bl cells

TZM-bl cells contain the integrated reporter gene for firefly luciferase, which is induced in trans by viral Tat protein soon after infection. Luminescence production was detected using the Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Briefly, 100 µl of cell culture medium was removed from each well, and 100 µl per well of Bright-Glo reagent was subsequently added. Lysis was allowed to occur for 2 min and luminescence (in relative light units; RLU) was then detected using a GloMax 96 Luminometer (Promega).

2.3.8 Titration of virus stocks on TZM-bl cells

Viral stocks were five-fold serially diluted in cell culture medium in quadruplicate wells, in a total of 100 µl per well. This was performed in opaque 96-well cell culture plates (Nuclon, Nunc). Eight of the wells contain 100 µl of cell culture medium only for virus-controls. Approximately $1x10^4$ newly trypsinised TZM-bl cells in 100 µl of DMEM+10% FCS and 30 µg/ml diethylaminoethyl (DEAE)-dextran (Sigma-Aldrich) were then added to each well, giving a final concentration of 15 µg/ml of DEAE-dextran. The plates, containing serially diluted virus and equal amounts of TZM-bl cells, were allowed to incubate at 37°C for 48 h. Luminescence was then detected as described in Section 2.3.7, and background luminescence (i.e. luminescence in wells containing cells but no virus) was subtracted. The dilution where 50% of wells were positive, i.e. the TCID50, was calculated using an Excel

macro set up by D. Montefiori at the Duke University Medical Centre, Durham, NC, USA.

2.3.9 HIV-1 neutralisation assay on TZM-bl cells

This assay measures the reduction in luciferase reporter gene expression in TZM-bl cells following a single round of virus infection. Serial three-fold dilutions of monoclonal antibody (starting at 25 µg/ml) or QC serum (starting at 1:10 dilution) were prepared in growth medium in duplicate wells of opaque 96-well cell culture plates, in a total volume of 50 µl per well. Approximately 200 TCID50 of virus, in 50 µl of growth medium, was then added to each well, and the plates were subsequently incubated at 37°C. After 1 h of incubation, $1x10^4$ newly trypsinised TZM-bl cells in 100 µl of growth medium containing 30 µg/ml of DEAE-dextran (Sigma-Aldrich) were added to each well. The plates were then incubated at 37°C for 48 h, after which the cells were lysed and luminescence was detected as described in Section 2.3.7. For each plate, 8 wells containing cells only, and another 8 wells containing cells and virus only, were included. Neutralisation was measured as the reduction in RLU in test wells compared to virus control wells after subtraction of background luminescence (from cells only wells). The lowest antibody concentration (or serum dilution) required to give 50% reduction in RLU (IC_{50}) were determined using the XLFit 4 software (IDBS). A negative control VHH (VHH #3; kindly provided by A. Gorlani, Utrecht University, the Netherlands) or normal human serum was tested in parallel.

2.4 Molecular biology techniques

2.4.1 Isolation of RNA

RNA was isolated from plasma of HIV-1 infected individuals using the QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Briefly, virus was lysed by the addition of QIAGEN AVL lysis buffer and incubated at room temperature for 10 min, in the presence of polyA carrier RNA. RNA was then purified on the QIAamp Mini Spin columns and eluted in QIAGEN AVE elution buffer.

2.4.2 Isolation of plasmid DNA

Five ml of LB medium containing the appropriate antibiotic for selection (typically 100 μg/ml of ampicillin or carbenicillin) was inoculated with a single bacterial colony from an agar plate. Cultures were grown overnight at 37°C (or 30°C for bacteria with large vectors such as full-length HIV-1 molecular clones or gp160 clones) with orbital shaking at 250 revolutions per minute (rpm). The bacteria were then pelleted by centrifugation at 3000 *g* for 10 min at 4°C. Plasmid DNA was isolated from pelleted bacteria using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Briefly, the pelleted bacteria were resuspended in resuspension buffer P1, containing RNase A, and subsequently lysed using buffer P2, with gentle inversion to avoid shearing of bacterial chromosomal DNA. The lysate was then neutralised and adjusted to high-salt binding conditions by the addition of the neutralising buffer N3. The samples were mixed by gentle inversion and precipitated SDS, chromosomal DNA, cellular debris, and denatured proteins were pelleted by centrifugation at maximum *g*. The supernatant was added to QIAprep Spin columns containing a silica membrane, and adsorbed plasmid DNA

was purified in a series of washing steps and eluted in 50 µl of QIAGEN EB elution buffer (10 mM Tris-HCl, pH 8.5).

2.4.3 Restriction enzyme (RE) digestion of plasmid DNA

Plasmid DNA was digested using restriction enzymes obtained from Promega (Southampton, UK) for 1 h under conditions specified by the manufacturer, usually at 37°C with an appropriate buffer. Digested DNA was separated using agarose gel electrophoresis.

2.4.4 Agarose gel electrophoresis of DNA

DNA fragments were separated according to size by agarose gel electrophoresis. The gels were made with 1% (DNA fragments >500 bp) or 1.5-2% (DNA fragments <500 bp) agarose (w/v; Roche Diagnostics, Lewes, UK) in 1x TAE buffer containing 0.5 µg/ml of ethidium bromide (Sigma-Aldrich, Poole, UK). A molecular weight marker of appropriate size (GeneRuler 1 kb DNA ladder or GeneRuler 100 bp DNA ladder; Fermentas Life Sciences, York, UK) was run in parallel. Electrophoresis was carried out in 1x TAE buffer at 100 V for an appropriate amount of time. DNA was visualised by illumination with UV light.

2.4.5 Extraction of DNA fragments from agarose gels

DNA bands of the expected size were excised from the agarose gel while minimising exposure to UV light, and the DNA fragments were extracted using the QIAquick Gel Extraction kit (QIAGEN) according to the manufacturer's instructions.

2.4.6 DNA ligation

DNA ligation of the DNA inserts into a vector backbone was carried out using a 2x Rapid Ligation Buffer together with T4 DNA ligase (both from Promega), with an

appropriate amount of vector and insert DNA. The mixture was allowed to incubate for 30 min at room temperature.

2.4.7 Transformation of competent bacteria

Ligation reactions were transformed into chemically competent *E. coli* TOP10 cells (Invitrogen, Paisley, UK) by adding 1 µl of ligation reaction to 25-50 µl of competent bacteria, followed by incubation on ice for 30 min. The bacteria were subsequently subjected to heat-shock at 42°C for 30 s, followed by incubation on ice for 1 min and addition of 250 µl of SOC medium (Invitrogen). The bacteria were then incubated for 1 h at 37°C (or 30°C for bacteria transformed with vectors containing full-length HIV-1 molecular clones or gp160 clones) with orbital shaking at 250 rpm, and subsequently plated onto LB agar plates containing 100 µg/ml ampicillin or carbenicillin. Plates were then incubated overnight at 37°C (or 30°C for bacteria transformed with vectors containing full-length HIV-1 molecular clones or gp160 clones).

Electrocompetent *E. coli* TG1 cells (Stratagene, La Jolla, USA) were transformed by adding 1 µl of ligation reaction or plasmid DNA to 40 µl of bacteria. Samples were then transferred to 0.1 cm electroporation cuvettes (Bio-Rad Laboratories, Hemel Hempstead, UK), which had been pre-chilled on ice, and electroporated using a MicroPulser electroporator (Bio-Rad Laboratories) according to the manufacturer's instructions (1.7 kV, 200 Ω, 25 µF). After addition of 1 ml of pre-warmed SOC medium, the bacteria were incubated for 1 h at 37°C with orbital shaking at 250 rpm and subsequently plated onto LB agar plates containing 100 µg/ml of ampicillin (or carbenicillin) and supplemented with 2% glucose. Plates were then incubated overnight at 37°C.

2.4.8 DNA sequencing

Plasmid DNA was sequenced using the CEQ DTCS Quick Start Kit (Beckman Coulter, High Wycombe, UK) according to the manufacturer's instructions. Briefly, sequencing reactions were carried out in a total volume of 10 µl containing 100 ng of DNA, 1.6 *p*mol of sequencing primer and 1x CEQ DTCS Quick Start master mix. Thermal cycling conditions consisted of 30 cycles of 96°C for 20 s, followed by 50°C for 20 s and 60°C for 4 min. For each sample, the sequencing reaction was stopped by the addition of 1 µl of 3 M NaOAc (pH 5.2), 1 µl of 100 mM Na_2-EDTA (pH 8.0), and 0.5 µl of 20 mg/ml of glycogen. The reactions were then cleaned through ethanol precipitation and re-suspended in 20 µl of sample loading solution (provided with the CEQ DTCS Quick Start Kit). Resuspended sequencing reactions were covered by one drop of mineral oil and plates were loaded onto a CEQ 2000 DNA Analysis System sequencer (Beckman Coulter).

Most of the DNA sequencing was however carried out by the DNA Sequencing Service at the Wolfson Institute, University College London, UK, using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). DNA sequencing of VHH clones was carried out by Ablynx NV, Porto, Portugal. Predicted amino acid sequences were aligned using the Clustal W2 multiple sequence alignment software (Larkin et al., 2007).

2.4.9 Determination of nucleic acid concentration

Nucleic acid concentration and purity was determined spectrophotometrically using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) by measuring absorbance at 260 nm with prior zeroing of the machine with EB solution.

2.4.10 Determination of protein concentration

Protein concentration was determined using a combination of two methods. The first was method was spectrophotometry using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) by measuring absorbance at 280nm with prior zeroing with PBS. Since protein measurements with spectrophotometry were often prone to errors, a second method was employed by running the samples with dilutions of a standard amount of BSA in SDS-PAGE and Coomassie staining.

2.4.11 SDS-PAGE and Coomassie blue staining

Proteins were resolved using SDS-polyacrylamide gel electrophoresis (PAGE). Separating polyacrylamide gels were made with 15% Bis-polyacrylamide (Bio-Rad Laboratories) and 0.1% SDS in 375 mM Tris-HCl (pH 8.8), with 100 µl of 10% ammonium persulphate (APS) and 10 µl of N'-tetramethylethylenediamine (TEMED) per 10 ml of gel, to polymerise. Stacking gels were made with 4% Bis-polyacrylamide and 0.1% SDS in 125 mM Tris-HCl (pH 6.8), and APS and TEMED as above.

Samples were mixed with sample buffer to a final concentration of 2% (w/v) SDS, 2 mM dithiothreitol (DTT), 4% (v/v) glycerol, 40 mM Tris-HCl (pH 6.8) and approximately 0.01% (w/v) bromophenol blue, boiled for 5 min, loaded onto gels and run at 200 V in running buffer containing 25 mM Tris-HCl, 200 mM glycine and 0.1% (w/v) SDS.

Alternatively, proteins were separated on pre-cast NuPAGE Novex 10% Bis-Tris gels (Invitrogen) in 3-(N-morpholino) propane sulphonic acid (MOPS) SDS running buffer. Samples were mixed with 1x NuPAGE LDS Sample Buffer and 1x NuPAGE Reducing Agent and heated to 70°C for 10 min prior to being loaded onto gels and

electrophoresed at 200 V. The PageRuler (Fermentas Life Sciences) or BenchMark (Invitrogen) pre-stained protein ladders were run in parallel, as molecular weight markers.

Gels were stained using 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) in 10% acetic acid and 40% methanol for 1 h and subsequently destained in 10% acetic acid and 40% ethanol until an appropriate resolution is obtained.

2.4.12 Western Blots

Proteins were first separated in polyacrylamide gels as described in Section 2.4.11, and then transferred to a Hybond-P PVDF membrane (Amersham/GE, UK) using an Amersham SemiPhor unit (Amersham/GE, UK) for semi-dry transfer. The membrane was first pre-soaked for 30 s in methanol before placing it under the gel and in between 4 pieces of blotting paper already soaked in transfer buffer added with 10% methanol. The transfer conditions were 60 mA for 1 h. After the transfer, the membrane was soaked in TBS-T with 5% (w/v) skim milk for 1h at room temperature with shaking. The membrane was then washed with 2 changes of TBS-T for 10 min each. To probe the antigens on the membrane, VHH or antibody (at 1ug/ml unless otherwise stated) in TBS-T was introduced for 1 h with shaking, and then washed off with 3 changes of TBS-T for 10 min each. These were repeated again with secondary mAb conjugated with HRP (at 4000-fold dilution). To detect the probed samples, 8 ml of ECL detection reagent (RPN2106, Amersham/GE, UK) was used on the membrane for 1 min, and the membrane was exposed on an x-ray film (Hyperfilm ECL, Amersham, UK) in a dark room for 15-45 s.

2.4.13 Enyzme-linked immunosorbent assay (ELISA)

Opaque 96-well Maxisorp plates (Nunc, Denmark) were coated overnight at 4 °C with 1 µg/ml of antigen (unless otherwise stated) in 0.1 M $NaHCO_3$, pH 8.5. After washing 3 times with TBS-T, non-specific protein binding was blocked by incubating wells with 200 µl per well of 4% (w/v) milk powder in TBS for 1 h at room temperature. Wells were then washed 3 times with TBS-T and subsequently incubated with 50 µl per well of serial dilutions of antibody in 1% (w/v) milk powder in TBS, for 1 h at room temperature. Each dilution of antibody were assayed in duplicate. Blank wells were included, or negative control antibodies were used, together with positive controls. This was washed again 3 times with TBS-T, and then incubated for 1 h with 100 µl per well of a detection antibody conjugated with alkaline phosphatase (AP) in 1% (w/v) milk powder in TBS. After 3 washes with TBS-T, 100 µl per well of Lumi-Phos Plus substrate (Aureon Biosystems) was added and chemiluminescence was detected after 0.5 h of incubation at 37°C. Blank-corrected RLU were plotted against the antibody dilution. For detection with horse-radish peroxidase (HRP) conjugated antibodies, the SureBlue TMB Microwell Substrate (Kirkegaard and Perry Laboratories, Maryland, USA) was added and allowed to develop for 30 min, and the reaction was stopped with the addition of 25 µl per well of 1 M H_2SO_4. The A_{490} was then measured and background subtracted values were plotted on a graph against protein concentration.

2.5 Cloning of subtype C *env* genes and virus characterisations

This section will describe most of the procedures used in Chapter 3.

2.5.1 Sources of RNA

HIV-1 gp120 and gp160 were amplified from viral RNA isolated from infected patient plasma samples as described in Section 2.4.1. These plasma samples were infected with subtype C HIV-1 and were obtained from David Yirell (Specialist Virology Centre in Glasgow, Scotland), with randomised patient ID numbers given from C20 to C39.

2.5.2 Primers and primer design

The primers used to amplify HIV-1 gp120 and gp160 are listed in Table 2-3. Primers o_envf and o_envr were designed by Anna Forsman (UCL, London, UK) to target nucleotide stretches conserved among most HIV-1 group M subtype A, B, C, and D as well as from HIV-1 of group O. Primers fenv3 and renv3, which amplify gp160 and contain restriction sites for *Xho*I and *Eco*RI (underlined in Table 2-3) to facilitate subsequent sub-cloning, were modified from primers described by Zheng and Daniels (Zheng and Daniels, 2001). Primers 125Y and 626L contain restriction sites for *Bst*EII and *Mlu*I (underlined in Table 2-3) were used to amplify gp120, whereas primers 944S and E400010_His were used for gp120 recombinant protein expression. The primer 944S contains a natural Kozak sequence and a start codon, while the primer E400010_His contains a 6-histidine tag, a stop codon, as well as the recognition motif (VVQREKR) for sheep polyclonal antibody D7324 (Aalto Bio Reagents, Dublin, Ireland), which recognises a conserved motif at the very C-terminus of gp120 (APTKAKRRVVQREKR). These primers were provided by M. Aasa-Chapman (Aasa-Chapman et al., 2006a). The nucleotide position refers to numbering on the HIV-1 HXB2 genome (GenBank accession number K03455) which corresponds to the 5'-end of the primer.

Table 2-3. Primers used for amplification of HIV-1 gp160 and gp120

Primer	Sequence (5´to 3´)[a]	Position[b]
o_envf	TYTCCTATGGCAGGAAGAAGC	5964
o_envr	TAACCCWTCCAGTCCCCCCTTTT	9096
fenv3	AGCTCGAGCAGAAGACAGTGGCAATGA	6202
renv3	CTATGGAATTCTTCGACCACTTGCCCCCCATTT	8827
626L	GTGGGTCACCGTCTATTATGGG	6326
125Y	CACCACGCGTCTCTTTGCCTTGGTGGG	7742
944S	AGAAAGAGCGGCCGCCAGTGGCAATG	6202
E400010_His	TTAATGATGATGATGATGATGTCTTTTTTCTCTCTGCACCACT	7778

[a.] Underlined portions denote the restriction enzyme digestion site. The primers fenv3, renv3, 626L and 125Y contain *Xho*I, *Eco*RI, *Bst*EII, and *Mlu*I digestion sites respectively.

[b.] Nucleotide positions according to HXB2 numbering

Table 2-4. Primers used for sequencing of HIV-1 gp160 and gp120

Primer	Sequence (5´to 3´)	Position[a]
gp160 +1	ATGGGGACACAGAGGAATTATC	6234
gp160 +298	GAGGATGTAATCAGCTTATG	6540
gp160 -317	CATAAGCTGATTACATCCTC	6559
gp160 +580	CAAGCCTGTCCAAAGATCTC	6831
gp160 -600	GAGATCTTTGGACAGGCTTG	6850
gp160 +901	ATAGGACCAGGACAAACATTC	7155
gp160 +1198	TGCAGATCAAATATCACAGG	7557
gp160 +1581	GTCTGGTATAGTGCAACAGC	7859
gp160 +1881	GCTTGAAGAATCACAAAGCCAG	8159
gp160 +2191	GTGAGCGGATTCTTAGCACT	8469
gp160 +2431	GTAGCTGAGGGAACAGATAG	8688
gp160-C21R	AAGTTCTTGTGGGTTGGGGT	6476
gp160-C3	CGCAAAACCAGCGGAGCACA	6896
gp160-C5	TGGCTTAATTCCATGTGTAC	6983
gp160-C6	AGACTTGATATAGTACCACT	6755

[a.] Nucleotide positions according to HXB2 numbering

Primers used for DNA sequencing of gp120 and gp160 clones are shown in Table 2-4. Primers gp160 +1, gp160 +298, gp160 -317, gp160 +580, gp160 -600, gp160 +901, gp160 +1198, gp160 +1581, gp160 +1881, gp160 +2191, and gp160 +2431 were designed by K. Aubin (UCL, London, UK). Primers gp160-C21R, gp160-C3, gp160-C5, and gp160-C6 were designed by me to cover gaps in the sequences and ensure complete sequence coverage for certain isolates when the other sequencing primers failed to work.

2.5.3 RT-PCR

Specific first-strand cDNA was synthesised from viral RNA and subsequently amplified in a one-step RT-PCR using the Titan One Tube RT-PCR Kit (Roche Diagnostics) and primers o_envf and o_envr (Table 2-3) according to the manufacturer's instructions. The Titan One Tube RT-PCR Kit uses avian myeloblastosis virus (AMV) reverse transcriptase and the DNA polymerase mix from the Expand Long Template PCR System (Roche Diagnostics). Reverse transcription is initiated at the reverse primer. The reaction was carried out in a total of 50 µl containing 1x Titan RT-PCR reaction buffer, 1 µl of Titan enzyme mix, 500 µM of each of the four dNTPs, 300 nM each of forward (o_envf) and reverse (o_envr) primers, 5 mM of DTT, 5 U of RNase inhibitor, and up to 1 µg of RNA. For the reverse transcription step, the reaction mix was incubated at 48°C for 30 min. The reverse transcription step was immediately followed by the PCR step, which consisted of initial denaturation at 96°C for 5 min, followed by 35 cycles of denaturation at 96°C for 1.5 min, annealing at 60°C for 1 min and elongation at 68°C for 10 min, followed by a final elongation step of 20 min at 68°C. Negative control reactions containing no template RNA were included in each RT-PCR run to

monitor possible contamination. Control reactions where reverse transcription was not carried out, containing the enzyme mix from the Expand Long Template PCR System instead of the Titan enzyme mix were also included to exclude amplification from genomic DNA. A 1-5 µl aliquot from the RT-PCR was taken forward to a second round of PCR using nested primers, to amplify gp120 or gp160.

2.5.4 PCR amplification of gp120 and gp160

Full-length HIV-1 gp120 and gp160 was amplified from proviral DNA or plasmid DNA in two rounds of PCR using the Expand Long Template PCR System (Roche Diagnostics). In addition to Taq DNA polymerase, the Expand Long Template PCR System enzyme mix contains the Tgo DNA polymerase, which has proofreading activity. The first round of PCR was carried out in a total of 50 µl containing 1x Expand Long Template Buffer 3, 500 µM of each of the four dNTPs, 300 nM each of the forward (o_envf) and reverse primer (o_envr), 3.75 U of the Expand Long Template enzyme mix and 300-500 ng of template genomic DNA. It consisted of an initial denaturation step of 5 min at 96°C, followed by 35 cycles of denaturation at 96°C for 1.5 min, annealing at 60°C for 1 min and elongation at 68°C for 10 min, followed by a final elongation step of 20 min at 68°C.

A 1-5 µl aliquot from the first round of PCR amplification (or from RT-PCR amplification) was taken forward to a second round of PCR using nested primers that amplified gp120 or gp160. If the gp120 was going to be cloned into the pcDNA3.1-TOPO expression vector (Section 2.7.1.1) for recombinant protein expression in a T7 RNA polymerase-recombinant vaccinia virus system, gp120 was amplified using primers 944S and E400010_His. If the gp120 was going to be slotted into cloning vectors (Section 2.5.5), gp120 was amplified from the outer PCR product, or re-

amplified from selected gp120 clones held in the pcDNA3.1-TOPO expression vector, using primers 125Y and 626L. Full-length gp160 was amplified using primers fenv3 and renv3. The nested PCRs consisted of an initial denaturation step of 5 min at 96°C, followed by 35 cycles of denaturation at 96°C for 1.5 min, annealing at 52°C (primers fenv3/env3), 55°C (primers 944S/E400010_His), or 57°C (primers 125Y/626L) for 1 min, and elongation at 68°C for 5 min, followed by a final elongation step of 10 min at 68°C. For primers that had not been previously described, the PCR conditions were optimised by varying annealing temperatures and elongation times. Negative control reactions containing no template DNA were included in each PCR to monitor possible contamination. PCR products were separated using agarose gel electrophoresis and DNA fragments of the expected size were purified from the gel as described in Sections 2.4.4 – 2.4.5 .

2.5.5 Cloning of amplified gp120 into expression vectors

Gel-purified gp120 92BR025 amplified using primers 944S/E400010_His was ligated into the expression vector pcDNA3.1-TOPO (Invitrogen) according to the manufacturer's protocol. The ligation reactions were subsequently transformed into chemically competent TOP10 bacteria. Screening of pcDNA3.1-TOPO plasmids carrying an insert of the expected size was carried out by restriction enzyme digestion followed by agarose gel electrophoresis (refer to molecular techniques described in Section 2.4). Clones producing functional gp120 when transfected into T7 RNA polymerase-recombinant vaccinia virus-infected 293T cells (Section 2.2.4) were sequenced for verification.

2.5.6 Cloning of amplified gp120 and gp160 into cloning vectors

Gel-purified gp120 and gp160 clones were ligated into pCR2.1-TOPO (Invitrogen) cloning vectors according to the manufacturer's protocol. Competent bacteria were transformed with the ligation reactions (Section 2.4.7) and plated onto LB/ampicillin plates added with 40 µl of 40 mg/ml of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) in N,N´-dimethylformamide (Promega). As successful cloning of an insert into the pCR2.1-TOPO vectors interrupts the coding sequence of β-galactosidase, recombinant clones were identified by blue/white colour screening. Clones containing an insert of the correct size were subsequently identified using restriction enzyme digestion (Section 2.4.3) and agarose gel electrophoresis.

2.5.7 Sub-cloning of gp120 into the pHXB2Δ*env* backbone

To generate chimeric infectious HIV-1 molecular clones containing heterologous gp120, full-length gp120 was sub-cloned from the pCR2.1-TOPO vectors into the pHXB2Δ*env* vector (McKeating et al., 1996), by means of the *BstE*II and *Mlu*I recognition sites present in primers 626L and 125Y respectively. The pHXB2Δ*env* backbone is based on HIV-1 HXB2 (subtype B) and allows incorporation of heterologous gp120 sequences from amino acid residue 38 to six amino acids before the gp120/gp41 junction. The gp120 DNA fragment was extracted from the pCR2.1-TOPO cloning vectors by double digestion with *BstE*II and *Mlu*I. Digested plasmid DNA was separated using agarose gel electrophoresis and the gel-purified DNA fragments of the expected size were then ligated into the pHXB2Δ*env* backbone (which had also been digested with the *BstE*II and *Mlu*I enzymes and gel-purified) using T4 DNA ligase (Promega). For some virus isolates, the gp120 contained an internal *BstE*II recognition site. Partial restriction digests were then carried out and bands of the expected size were purified from the gel and ligated into pHXB2Δ*env*.

2.5.8 Sub-cloning of gp160 into the pNL43-based C2 cassette

The C2 cassette (Zheng and Daniels, 2001), which is based on the infectious HIV-1 NL4.3 (subtype B) molecular clone, was used to generate chimeric infectious HIV-1 molecular clones containing heterologous full-length *env* genes. Full-length gp160 was extracted from the pCR2.1-TOPO vectors by double digestion with enzymes *Xho*I and *Eco*RI, whose recognition sites were present in primers fenv3 and renv3 respectively. Digested gp160 fragments were then gel-purified and transferred into the similarly digested and gel-purified pNL43-based C2 cassette using the T4 DNA ligase (Promega).

2.5.9 Production of infectious gp120/HXB2 and gp160/NL43 chimeras

The gp120-pHXB2Δ*env* and gp160-pNL43Δ*env* ligation reactions were transformed into competent bacteria (Section 2.4.7). Clones containing inserts of the correct sizes were identified through restriction enzyme digestion and used for virus production in 293T cells as described in Section 2.3.3. Clones producing infectious virus were identified by titration of the cell culture supernatant from transfected 293T cells onto NP2/CD4/CCR5 or NP2/CD4/CXCR4 target cells, as described in Section 2.3.5. Clones producing infectious virus were then sequenced (Section 2.4.8) using the sequencing primers listed in Table 2-4.

2.5.10 Envelope sequence manipulations and analysis

Chromatograms from the sequencing reactions were analyzed using Sequencher 4 software (Gene Codes Corp., Ann Arbor, MI) and individual sequence fragments for each *env* clone were assembled and edited from a minimum of two different sequencing reactions to ensure full coverage. These curated sequences are available from GenBank, and the accession numbers are listed in Table 3-3. Protein

translations and deduced Env amino acid sequences were aligned using Clustal W2 (Larkin et al., 2007) and comparatively analyzed. The alignments were fed into the SeqPublish tool found on the Los Alamos National Laboratory (LANL) HIV database website (www.hiv.lanl.gov) to produce publication ready alignments. The number of potential glycosylation sites for each clone was checked using the N-GlycoSite tool (Zhang et al., 2004a) from the LANL HIV database. The variable loops on gp120 were determined by the positions of the cysteine residues, and the number of amino acid residues within the variable loops was counted.

2.5.11 Phylogenetic tree construction

The DNA sequence for the gp120 portions of the viruses were first aligned based on the Hidden Markov model (HMM) to the curated DNA alignments on the LANL HIV database, and the subtype assignment was confirmed by clustering with the subtype reference alignments (Leitner et al., 2005). This consists of 4 different strains for each HIV-1 subtype and recombinant forms. A phylogenetic tree using the maximum likelihood approach was then constructed with the PhyML tool (Guindon and Gascuel, 2003). Pairwise distance matrices were calculated using the General Time Reversible (GTR) algorithm with a transition/transversion ratio of 2.0. The consistency of the phylogenetic clustering was tested using bootstrap analysis with 100 replicates. DNA sequences from Li's African subtype C standard reference panel, consisting of Du123.6, Du151.2, Du156.12, Du172.17, Du422.1, ZM197M.PB7, ZM214M.PL15, ZM215F.PB8, ZM233M.PB6, ZM249M.PL1, ZM53M.PB12, ZM55F.PB28a, ZM106F.PB9, ZM109F.PB4, ZM135M.PL10a, CAP45.2.00.G3, CAP210.2.00.E8 and CAP244.2.00.D3 (Li et al., 2006b) were also included in the phylogenetic tree analysis for comparison. The tree is rooted with three subtype D strains (NDK, ELI and 94UG114).

2.6 Anti-gp41 VHH from gp140-immunized library

2.6.1 Immunization of animal and library construction

Immunisation of llamas and construction of phage libraries expressing the VHH repertoires of the immunised llamas were carried out by staff of Ablynx, NV, Ghent, Belgium as part of the EMPRO Consortium. One adult *Lama glama* (L48) was immunized subcutaneously with recombinant CN54 gp140 (kindly provided by Ian Jones, Reading University). The llama received six intramuscular injections at weekly intervals. Each injection consisted of a freshly prepared 4.5 ml water-in-oil emulsion, prepared by vigorous mixing of 2 volume units of antigen (50 or 100 µg) with 2.5 volume units of the adjuvant Stimune (CEDI Diagnostics, Lelystad, the Netherlands), and was administered intramuscularly in the neck divided over two spots. One hundred µg of the immunogen was administered on days 0 and 7, and 50 µg on days 14, 21, 28 and 35. Preimmune sera was obtained on day 0. Four days after the last antigen injection (on day 39 post-immunisation), a 150 ml blood sample and a lymph node biopsy were collected as the source of B cell containing tissues. After another four days (on day 43 post-immunisation) a second 150 ml blood sample was collected. ELISAs were carried out on the titrated sera to determine the presence of an appropriate immune response against the immunogen (gp140 CN54).

Total RNA was isolated from peripheral blood lymphocytes and lymph node biopsies collected post-immunisation by acid guanidinium thiocyanate-phenol-chloroform extraction. The total RNA isolated from the two different tissues was pooled and reverse transcribed into cDNA using oligo-dT primers with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The VHH repertoires were amplified in two rounds of PCR. In the first PCR, the repertoire of both

conventional (1.6 kb) and heavy-chain (1.3 kb) antibody gene segments were amplified using a forward primer specific for the leader sequence and a reverse oligo-dT primer. To allow for efficient separation of cDNA fragments encoding conventional and heavy-chain antibodies, the resulting PCR product was then treated with the *Bgl*I restriction enzyme, since the gene segment coding for the CH1 domain, present only in conventional antibodies, contains a unique *Bgl*I recognition site. Following restriction enzyme digestion and agarose gel electrophoresis and purification, the resulting 1.3 kb fragment was used as a template in a nested PCR using a mixture of forward primers specific for the FR1 region of VHH, which also introduced a *Sfi*I recognition site, and a reverse oligo-dT primer. The resulting PCR products were subsequently treated with *Sfi*I and *BstE*II (naturally occurring in FR4) restriction enzymes and separated using agarose gel electrophoresis. The VHH-encoding DNA fragment (400 bp) was extracted from the gel and 330 ng was ligated into 1 µg of the pUC-derived phagemid vector pAX050, followed by transformation into electrocompetent *E. coli* TG1 cells.

To obtain recombinant bacteriophages expressing the VHH as fusion proteins with the bacteriophage gene III product, the transformed TG1 cells were grown to logarithmic phase and then infected with helper phage M13KO7. The phage particles were precipitated with polyethylene glycol 6000 (PEG; Sigma-Aldrich) to remove free VHH as described in Section 2.6.2.4. From each library, a colony PCR using an M13 reverse primer and a gene III primer was carried out on 24 randomly picked colonies, and the proportion of clones containing an insert of the correct size was estimated.

2.6.2 Bio-panning and selection for anti-gp41 VHH

The panning of the phage display library on immobilised antigens will be described in this section. An overview of this method is shown in the figure below.

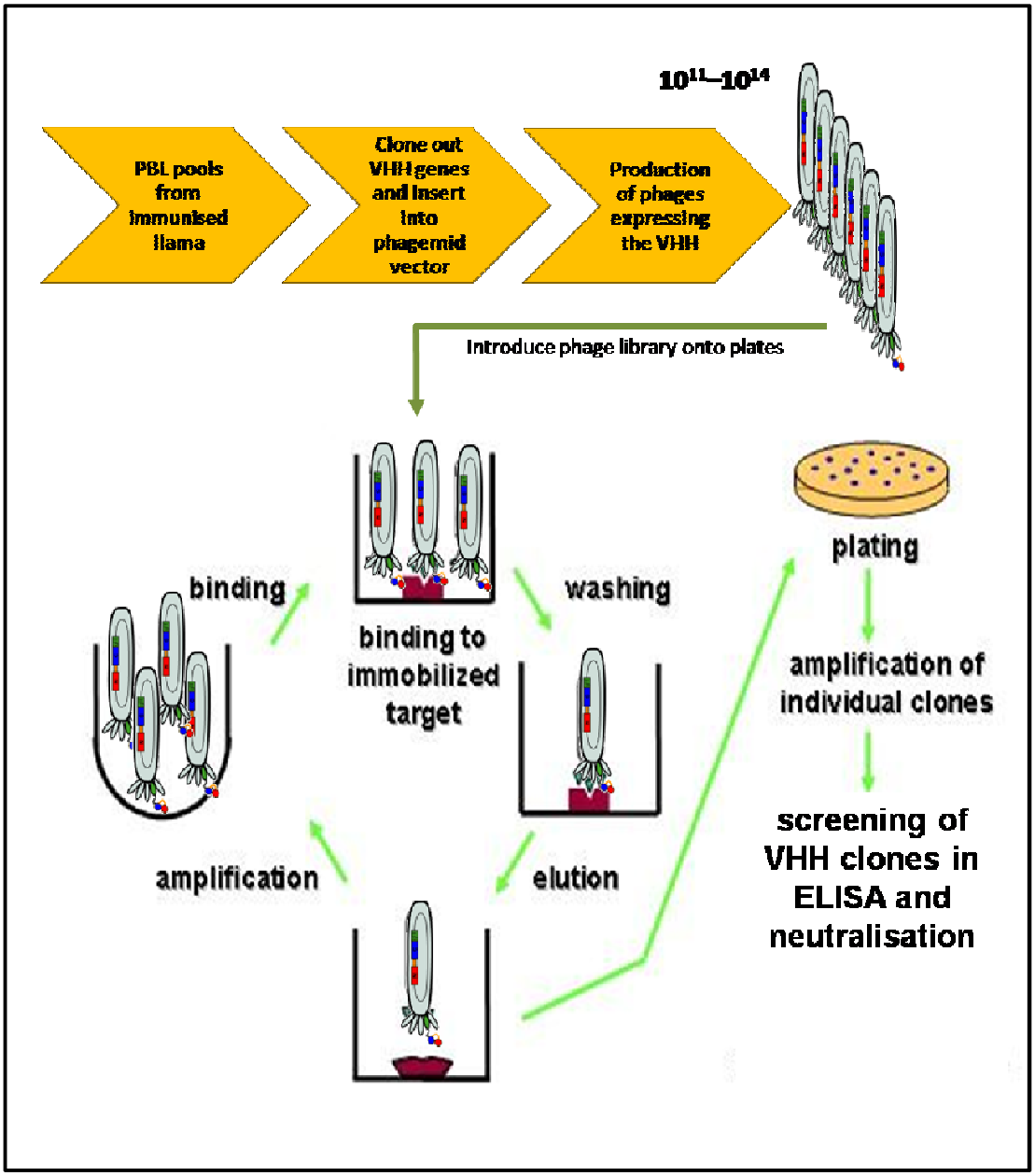


Figure 2-1. Schematic overview of the phage panning process

2.6.2.1 Plate preparation and phage binding

Maxisorp plates (Nalgene) were separately coated with gp41 and gp41Δ at 5, 1 and 0.2 µg/ml concentration in PBS in a total volume of 100 µl/well. Different concentrations of antigens were used to determine the optimum concentration to elute out phage binders. Negative control wells containing only PBS were also included. After an overnight incubation at 4°C, the plates were washed four times with PBS-T, and non-specific protein binding was subsequently blocked by filling the wells with 1% (w/v) casein in PBS and incubating for 1 h at room temperature and orbital shaking at 200 rpm. After the blocking step, the plates were washed four times with PBS-T.

Phages, expressing the cloned VHH repertoires as prepared earlier, were then diluted 1:10 in 0.2% (w/v) casein in PBS and subsequently pre-incubated at room temperature for 10 min to allow for unspecific binding. One hundred µl of diluted phages were then incubated with wells containing immobilised gp41 or gp41Δ for 1 h at room temperature with orbital shaking at 200 rpm, and thereafter the wells were washed 20 times in PBS-T to remove unbound or unspecifically bound phages. To increase the stringency of the washing step, every five washes the plates were incubated with PBS-T for 15 min at room temperature and orbital shaking at 200 rpm. Plates were then finally washed twice in PBS.

Bound phages were eluted using a variety of methods; namely trypsin, glycine and antibody competition. Trypsin elutes out every bound phage through the digestion of the gene III product at the base of the VHH on the phage, and this was done by incubating 100 µl per well of trypsin (1mg/ml) for 30 min at room temperature and orbital shaing at 200 rpm, and then neutralised with 5-fold molar excess (210 uM) of

trypsin blocker, acetylbenzenesulfonyl fluoride (ABSF; Sigma). Glycine elutes phages through low pH shock, and was performed by incubating wells with 100 µl per well of 1 mg/ml glycine. After 30 min at room temperature and orbital shaking at 200 rpm, the glycine-eluted phage (100 µl) was transferred to 50 µl of 1 M Tris-HCl pH 7.5 for neutralisation. In antibody competition elution, bound phages were eluted with a high concentration (100 µg/ml) of mAbs 2F5 or 4E10 to target phages carrying VHH that target these epitopes.

2.6.2.2 Titration of eluted phage

Eluted phages from each well were titrated onto *E. coli* TG1 cells to determine the success of the selection process. Serial dilutions of the output phage were prepared (up to 10^{-4}) by sequentially transferring 10 µl to 90 µl of PBS. A 5 µl aliquot from each dilution (dilution factor 20) was used to infect 95 µl of exponentially growing TG1 cells (at an optical density at 600 nm of approximately 0.65) in V-bottomed 96-well microtitre plates. After incubation at 37°C without shaking for 30 min, 5 µl of bacteria were spotted in duplicates onto LB agar plates containing 100 µg/ml of ampicillin and 2% (w/v) glucose, and grown overnight at 37 °C.

2.6.2.3 Amplification and rescue of eluted phage

Eluted phages from the biopanning were multiplied in TG1 cells and rescued by superinfection with helper phage. A 50 µl aliquot of eluted phage was used to infect 0.5 ml of exponentially growing TG1 cells for 30 min at 37 °C without shaking. The cells were then spun down at 4500 x *g* for 5 min, and then resuspended in 10 ml of 2xTY medium containing 100 µg/ml of ampicillin and 2% (w/v) glucose, and subsequently incubated overnight at 37°C and 250 rpm. The overnight culture was used to prepare glycerol stocks. To rescue the phages, 1 µl of helper phage M13KO7

(corresponding to approximately 10^{11} plaque-forming units; PFU) were added to 2.5 ml of the overnight culture, and allowed to incubate for 30 min at 37 °C without shaking. Cells were then pelleted and resuspended in 50 ml of 2xTY medium containing 100 μg/ml of ampicillin and 25 μg/ml kanamycin and incubated at 37°C overnight with shaking at 250 rpm.

2.6.2.4 Phage precipitation

After the rescue of the phages, the cells were pelleted and 40 ml of the supernatant (containing free phages) were added to 10 ml of 20% (w/v) polyethylene glycol 6000 (PEG; Sigma-Aldrich) and 2.5 M NaCl, and then incubated on ice for 30 min. The precipitated phages were then spun for 15 min at 4500 x *g* at 4 °C, and the supernatant removed. The pellet was then resuspended into 1 ml of PBS for washing, and pelleted again at maximum speed on a tabletop centrifuge for 5 min to remove cell debris. The supernatant was collected, transferred to a new 1.5 ml tube containing 250 μl of 20% (w/v) PEG and 2.5 M NaCl, and incubated on ice for 10 min. It was then centrifuged at maximum speed for 5min, and the pellet was resuspended again in 1 ml PBS. To remove the last bits of cell debris, the suspension was centrifuged for 5 min and the supernatant was transferred into a new eppendorf tube. These new phage stocks were titrated onto TG1 cells and taken forward to subsequent rounds of panning.

2.6.2.5 Further rounds of selection

A repeat of the above procedure was carried out in a second round of selection to further enrich the phage pool for binders to gp41.

2.6.3 Isolation and small scale expression of VHH

The eluted phages were cloned into expression vectors and individual clones were picked and the VHH subsequently expressed in *E. coli* TG1 cells.

2.6.3.1 Cloning of VHH repertoire into expression vectors

Five ml of the overnight cultures were mini-preped to harvest the plasmids containing the VHH gene fragments. The plasmids were first digested with *Sfi*I at 50°C for 2 h, followed by *BstE*II at 50 °C for another 2 h. The digested products were gel purified on a 1.5% agarose gel and fragments of about 300 bp were cut out and the fragments were ligated into pAX051 for 15 min at room temperature using T4 DNA Ligase (Promega). The pAX051 expression vector is a derivative of pUC19 (Yanisch-Perron et al., 1985) and contains the LacZ promoter, which enables a controlled induction of expression using isopropyl-ß-D-thiogalactopyranoside (IPTG). Expression from the pAX051 vector incorporates a 6-histidine and a c-myc-tag to the C-terminus of the VHH. The ligated mixture (2 µl) was electrotransformed into 40 µl of electrocompetent TG1 cells (Stratagene, UK), and 1 ml of 2xTY medium with 2% glucose was added for recovery. Various dilutions of the transformed cells were plated onto LB agar plates containing 100 µg/ml of ampicillin and 2% glucose and left to incubate overnight at 37 °C. Ninety-six clones from each output were picked and used to inoculate 1 ml of LB medium containing 100 µg/ml of ampicillin (or carbenicillin) and 2% glucose in 96-well deep-well plates. Plates were incubated overnight at 37°C with 250 rpm. Glycerol stocks were prepared from the overnight cultures and stored at -80°C in 96-well plates as "masterplates".

2.6.3.2 DNA Fingerprinting

Colony PCR was performed on the clones selected by using sterile toothpicks to dip the cells into a 10 µl PCR mastermix (M13F/M13R standard primers) on a 96-well format. The PCR products were digested with *Hin*fI for DNA fingerprinting by incubating the restriction enzyme for 2-3h at 37 °C and then analyzed on a 2.5% agarose (TBE) gel.

2.6.3.3 Small scale expression of VHH in 96-well format

The same toothpick used for colony picking was used to inoculate a 96-well plate containing 100 µl of 2xTY containing 100 µg/ml of ampicillin and 2% glucose, in the exact same well position, and allowed to incubate overnight at 37 °C. The next day, 10 µl of the overnight culture was used to inoculate 1 ml of 2xTY containing 100 µg/ml of ampicillin and 0.1% glucose in 96-deep well plates, whilst the residual culture were frozen in glycerol. The deep well plates were incubated at 37 °C with shaking at 250 rpm. When the OD_{600} reached approximately 0.8 (approximately 3 h later), 10 µl of 0.1M IPTG was added into each well to induce the production of VHH. After 4 h of further incubation at 37 °C with shaking, the cells were pelleted by spinning at 4500 x *g* for 10 min at 4 °C, and the pellet was allowed to freeze overnight after the removal of the supernatant. The frozen pellet was then resuspended in 100 µl of PBS. The freeze-thaw action allows the escape of the VHH trapped in the periplasmic layer of the cells. The periplasmic extracts (containing the expressed VHH) was harvested from the supernatant after spinning down to remove the spheroplasts, and transferred to new 96-well plates and stored at -80 °C.

2.6.4 Large scale expression of VHH

VHH found to be neutralising or binding were expressed and purified as described in this section. Some of the *E. coli* TG1 produced stocks of VHH were also obtained from Ablynx NV (Ghent, Belgium).

2.6.4.1 Expression of selected VHH

Glycerol stocks of *E. coli* TG1 cells containing selected VHH clones (held in the pAX051 vector) were used to inoculate 50 ml of LB medium containing 100 µg/ml of ampicillin and 2% (w/v) glucose. The cultures were incubated overnight at 37 °C and 250 rpm, and a 5 ml aliquot was subsequently used to inoculate 1 litre of 2xTY medium containing 100 µg/ml of ampicillin and 0.1% (w/v) glucose. This culture was incubated at 37 °C and 250 rpm until an OD_{600} of 0.7 was reached. VHH expression was then induced with 1 mM of IPTG and the cultures incubated overnight at 30 °C with 250 rpm. Aliquots of pre-induced and induced samples were kept and analysed by SDS-PAGE (Section 2.4.11). After the overnight incubations, bacteria were harvested by centrifugation of 4 x 250 ml aliquots at 4000 *g* for 30 min at 4 °C. The pelleted bacteria were then frozen at -80 °C overnight. The day after, the bacteria were thawed, resuspended in 4 x 25 ml of PBS, incubated at 8 °C for 1 h at 250 rpm, and subsequently subjected to centrifugation at 4000*g* for 30 min at 4 °C. The resulting supernatants, corresponding to the VHH-containing *E. coli* periplasmic extracts, were collected.

2.6.4.2 Purification of expressed VHH

Expressed VHH were purified from *E. coli* periplasmic extracts by means of the 6-histidine-tag using TALON Metal Affinity Resin (Clontech, Mountain View, CA, USA), according to the manufacturer's instructions. Briefly, the TALON Metal

Affinity Resin suspension was washed twice in 50 ml of VHH equilibrium buffer. Approximately 1 ml of TALON Metal Affinity Resin was subsequently resuspended in 50 ml of VHH-containing *E. coli* periplasmic extract in 50 ml Falcon tubes (BD Biosciences, Oxford, UK) and incubated at room temperature for 1 h with head-over-head rotation. The resin was then pelleted by centrifugation at 700 *g* for 2 min at 4°C. The supernatant was discarded and an aliquot was saved for analysis by SDS-PAGE (unbound sample). The resin was then washed twice in 50 ml of VHH equilibrium buffer, by incubating the resin with buffer at room temperature for 10 min with head-over-head rotation followed by centrifugation at 700 *g* for 2 min at 4 °C. The resin (approximately 1.5-2 ml per VHH) was then resuspended in a total of 10 ml of VHH equilibrium buffer and packed into Poly-Prep gravity/flow columns (Bio-Rad Laboratories). Packed columns were then washed twice with one bed volume of VHH equilibrium buffer. One bed volume of VHH pre-elution buffer was then added and the eluate collected. Bound protein was then eluted with approximately 4 x 0.5 bed volumes of VHH elution buffer and the eluate collected in 4 fractions.

The eluted fractions were analysed by SDS-PAGE and Coomassie blue staining and fractions containing the bulk of the eluted VHH were pooled and dialysed for 8 h at 4°C against 1 litre of PBS using Slide-A-Lyzer Dialysis Cassettes (Pierce Protein Research Products, Thermo Fisher Scientific, Rockford, USA) with a molecular cut-off of 3.5 kDa. After dialysis, the purified VHH were analysed by SDS-PAGE and Coomassie blue staining and the protein concentration was determined (Sections 2.4.10 – 2.4.11).

2.6.5 Screening of selected anti-gp41 VHH with ELISA

The periplasmic extracts and purified VHH were tested for their ability to bind to the selecting antigen (either recombinant gp41 or gp41Δ) in ELISAs as described in Section 2.4.13. Opaque 96-well Maxisorp plates (Nunc) were coated overnight with 50 µl of either antigen at 1 µg/ml. After blocking and washing of the plates, 50 µl of 50-fold diluted periplasmic extracts were allowed to incubate for 1 h with the antigen. The positions of the wells on the ELISA plates were matched with the selected VHH clones on the VHH masterplate. After washing, 100 µl of 0.5 µg/ml mouse anti-c-myc mAb (catalogue number 11667149001, Roche Diagnostics) was introduced for 1 h to detect the c-myc tagged VHH. After washing as before, 100 µl per well of 0.5 µg/ml of an AP-conjugated goat anti-mouse IgG antibody (catalogue number 1030004, Oxford Biotechnology) was introduced for another 1 h. After a final washing step, 100 µl of Lumi-Phos (Lumigen, Southfield, MI, USA) was allowed to incubate at 37 °C for 30 min. The luminescence (RLU) was then determined using a plate reader.

2.6.6 Screening of the selected anti-gp41 VHH in neutralisation

All the selected VHH were tested for their ability to neutralise HIV-1 using the NP2 assay described in Section 2.3.6. To enable a fast screening of all the selected VHH, they were tested at a single dilution (1:10) of periplasmic extracts with 100 FFU of virus per well. Viruses included in the preliminary neutralisation screening include IIIB, MN, 92Br025 and 92UG037. In the TZM-bl cell-based neutralisation assay (Section 2.3.9), 50 µl of VHH-containing periplasmic extract was pre-incubated 200 TCID50 of virus, per well. Irrelevant (negative control) *E. coli* periplasmic extracts were included on each plate.

2.6.7 Western Blot of anti-gp41 VHH

The recombinant gp41 antigen was first resolved in polyacrylamide gels and then transferred to a PVDF membrane as described in Sections 2.4.11 and 2.4.12. After washing of the membranes, 1 µg/ml of purified VHH in TBS-T supplemented with 4% (w/v) skim milk was introduced for 1 h with shaking to probe the antigens on the membrane. These were then washed off and 0.5 µg/ml of mouse anti-myc mAb (Roche) in TBS-T supplemented with 4% (w/v) skim milk was introduced for another hour. After washing, the antibody was probed with a 4000-fold diluted anti-mouse IgG conjugated with HRP (NXA931, Amersham/GE, UK). ECL detection reagent (Amersham/GE, UK) was then used and the membrane exposed on an x-ray film.

A modified Western Blot was carried out whereby the purified VHH was first resolved in the polyacrylamide gels and then transferred to a PVDF membrane. Its ability to bind antigen in solution form was determined by incubating the membrane with 1 µg/ml of recombinant gp41 in PBS. Captured gp41 by the VHH on the membrane was probed with an anti-gp41 mouse mAb (ARP 317, NIBSC) and detected with a 4000-fold diluted anti-mouse IgG conjugated with HRP. This was then visualised on an x-ray film after addition of ECL detection reagent.

2.6.8 Flow cytometry of HIV-1 infected cells

H9 T-cells chronically infected with HIV-1 MN were used in this assay. Cells ($5x10^5$ cells/tube) were washed once with 4 ml of PBS (by spinning at 250 x *g* for 5 min, then re-suspending), and then washed again with 4 ml of FACS buffer. Blocking of the cells was carried out using 10% goat serum in FACS buffer for 10 min at 4 °C. The cells were then spun down and resuspended in 20 µl of VHH (at 1 µg/ml), and allowed to incubate for 1 h at 4 °C. The cells were then washed twice in FACS

buffer, and resuspended in 50 µl of 10-fold diluted mouse anti-c-myc mAb (Roche), and allowed to incubate for another hour at 4 °C. After 2 more washes in FACS buffer, a sheep anti-mouse Ab conjugated with FITC (at 10 µg/ml) was introduced for 1 h at 4 °C. The cells were again washed twice with FACS buffer, and then once with PBS. The cells were fixed with 3.8% formal saline and stored at 4 °C prior to analysis. Cells were acquired using a Becton Dickinson FACS LSRII (BD Biosciences, San Jose, CA) and the data analyzed using FlowJo (Tree Star, Inc, Ashland, OR). The anti-gp41 mAb (ARP317) was used as a positive control, and a negative control was performed without any primary Ab. H9 cells without HIV-1 MN infection were run concurrently as negative controls.

2.6.9 VHH competition with each other for binding to gp41 in ELISA

VHH competition with each other was also assayed by ELISA. A set of the anti-gp41 VHH were first biotinylated, as described in Section 2.7.1.2, and allowed to compete with unbiotinylated anti-gp41 VHH in ELISA. Opaque 96-well Maxisorp plates (Nunc) were coated overnight with 1 µg/ml of gp41 in 0.1 M $NaHCO_3$, pH 8.5. After washing four times with TBS-T, non-specific protein binding was blocked by incubating wells with 200 µl per well of 4% milk powder in TBS for 1 h at room temperature. Wells were then washed four times with TBS-T. Serial dilutions of the different unbiotinylated VHH in 1% milk powder in TBS were introduced at 50 µl/well and allowed to bind to gp41 in duplicates. Biotinylated VHH at an optimum concentration (0.005 µg/ml) were then introduced at 50 µl/well to compete with the unbiotinylated VHH for binding to gp41. Bound biotinylated anti-gp41 VHH was then detected with 100 µl of 0.5 µg/ml of an AP-conjugated streptavidin (RPN1234, Amersham/GE, UK). Luminescence was detected as above and blank-corrected RLU were plotted against VHH concentration. The A12 VHH, which recognise the CD4

binding site of gp120, was added as a negative control; and the mAb 2F5 was used to determine if any of the VHH binds to the MPER of gp41.

2.6.10 Epitope mapping of anti-gp41 VHH

To determine the possible binding sites on gp41, the anti-gp41 VHH were tested on a set of 15-mer linear peptides, with 11 amino acid overlaps between sequential peptides, from the MN strain (catalogue ARP7113 from NIBSC). The peptides were coated onto Maxisorp plates at 10 µg/ml in 0.1 M $NaHCO_3$, pH 8.5, and an ELISA was carried out as described in Section 2.6.5.

In a separate attempt to determine the epitopes of the anti-gp41 VHH, these VHH were sent to Hans Langedijk (Pepscan Presto BV, The Netherlands) for testing on their proprietary combinatorial peptide arrays. In a variation of the ELISA, this involved the testing of the VHH for its ability to bind to a proprietary collection of short 20-mer linear and cyclic peptide fragments overlapping by 19 amino acids of gp41 of subtype A and C (MW965 and UG037).

2.6.11 Denaturing gp41 ELISA

To determine if the anti-gp41 VHH recognise linear or conformational dependent epitopes, a denaturing ELISA was carried out. Gp41 was denatured by diluting 1 µg/ml of the antigen in 1% SDS/50 mM dithiothreitol (DTT, Sigma Aldrich) with 0.1 M $NaHCO_3$, pH 8.5 and incubating for 10 min at 100°C. Two parallel Maxisorp plates were set up, one coated with 1 µg/ml of gp41 in 0.1 M $NaHCO_3$, pH 8.5 (untreated) and the other coated with the denatured gp41. Coating of the plates was allowed to progress overnight at 4°C, as before. The VHH were then added on, as described in Section 2.6.5.

2.7 Creation and characterisation of C8- and D7-family specific VHH library

2.7.1 Preparation of recombinant gp120 antigen

This part describes the steps involved in preparing the recombinant gp120 for use as an antigen in the panning for C8- and D7-family specific VHH library.

2.7.1.1 Expression of recombinant gp120

Recombinant gp120 derived from HIV-1 92BR025 was expressed as described by Aasa-Chapman *et al*. (Aasa-Chapman et al., 2004) using a bacteriophage T7 RNA polymerase-recombinant vaccinia virus system (Fuerst et al., 1986). Primers E400010_His (Table 2-3) specific for subtype C gp120 envelopes were used to amplify the gp120 fragments from HIV-1 92BR025, which is a Brazilian subtype C virus. The primer E400010_His incorporates a 6x histidine tag at the C-terminus of the product for downstream purification of the expressed protein. Following the procedures as described in Sections 2.4.6 and 2.4.7, the gp120 DNA fragments were first subcloned into TOPO vectors before cloning into a mammalian expression vector pcDNA3.1-TOPO (Invitrogen, UK). Sequencing was carried out to verify that the clone contained the correct sequence in the correct orientation.

Exponentially dividing 293T cells, seeded the day before at a density of 4×10^5 cells per well of a 6-well plate, were washed once in serum-free DMEM and subsequently overlaid with 2 ml per well of serum-free DMEM. Cells were then incubated with approximately 4×10^4 50% tissue culture infectious doses (TCID50) per well of T7 RNA polymerase recombinant vaccinia virus (vTF7-3, American Tissue Culture Collection number VR-2153) at 37°C. After 2 h of incubation, cells were washed once in DMEM, overlaid with 2 ml per well of DMEM/GlutaMAX supplemented

with 10% (v/v) of FCS, and subsequently transfected with the gp120-pcDNA3.1-TOPO plasmids. Transfections were carried out as described in Section 2.2.4 using 4 µg of plasmid DNA and 12 µl of FuGENE 6 (Roche Diagnostics). Cell culture supernatants were harvested 72 h after transfection, treated with NP40 detergent (Sigma-Aldrich) to inactivate vaccinia virus, filtered and stored at -20°C. To monitor residual infectious vaccinia virus, cell culture supernatants were titrated onto Vero cells (African green monkey kidney) and cytopathic effects were observed microscopically. The gp120-containing cell culture supernatants were then used directly in an enzyme-linked immunosorbent assays (ELISA) as described in Section 2.4.13. If a high expression titre was achieved, the gp120 was purified using Ni Sepharose 6 Fast Flow (GE Healthcare, Little Chalfont, UK) by means of the 6-histidine-tag encoded by primer, according to the manufacturer's instructions.

2.7.1.2 Biotinylation of recombinant gp120

One hundred µg of both 92BR025 and IIIB gp120 recombinant proteins were dialyzed using Microcon YM-50 Centrifugal Filter Units (Milipore, UK) with a 50kDa cut-off to remove contaminating proteins and buffers in the solution. The concentrated protein residue was washed 3 times with 300 µl of 50 mM carbonate buffer at pH 8.0, and harvested by spinning the inverted columns into new eppendorf tubes. Biotin was added in a 6x molar excess ratio using the EZ-Link Biotinylation kit (Pierce, UK) and allowed to incubate for 2 h at 4°C. The NHS groups of biotin will interact with any free NH2 groups on a protein. The reaction mixture was then put back into new YM-50 filter units, and washed 3 times with 200 µl of PBS. The biotinylated recombinant proteins were eluted out by spinning the inverted columns into new eppendorf tubes.

The 5 different anti-gp41 VHH described in Chapter 4 were also biotinylated in a similar way for testing in competitive binding to gp41 in ELISA (Section 2.6.9).

2.7.1.3 Gp120 functionality check

The recombinant gp120 proteins were then checked for the successful integration of biotin and also for the non-disruption of integral epitopes that are essential for binding to VHH. ELISAs were performed as described in Section 2.4.13. Maxisorp plates (Nunc) were first coated overnight with 4 different concentrations of biotinylated and unbiotinylated gp120 samples, and then blocked with 4% skim milk in TBS. After washing with TBS-T, biotinylation of the samples were detected with HRP-conjugated streptavidin. The substrate was added and allowed to develop for 30 min, and the reaction was stopped with the addition of 25 µl of 1 M H_2SO_4. The OD490 was then measured and background subtracted values were plotted on a graph against protein concentration. Another set of ELISA was also set up similarly as above, but the bound gp120 were probed directly with the C8 and D7 VHH, followed by mouse anti-c-myc mAb, and the detected with HRP-conjugated donkey anti-mouse antibody.

Western blots were also carried out (as described in Section 2.4.12) by first resolving the biotinylated gp120 in polyacrylamide gels, and transferring them onto PVDF membranes. The PVDF membranes were then reversibly visualised with the protein stain, Ponceau S (Sigma Aldrich), to ensure the successful transfer of proteins. The membrane was then washed in water to remove the stain and blocked with 1% skim milk. After washing in TBS-T, 0.5 µg/ml of HRP-conjugated streptavidin was then introduced to probe for the gp120. This was then visualised on an x-ray film after treatment with ECL detecting reagent.

2.7.2 Creation of C8- and D7-family specific VHH libraries

Total RNA (60 µg) that was previously isolated from the PBL pools from llama 44 (L44), was reverse transcribed into cDNA using random hexamers (SuperScript III, Invitrogen, Carlsberg, USA) and cleaned up with QIAquick PCR purification kit (Qiagen, Germany). The llama (L44) was immunised with recombinant CN54 gp120 and from which the C8, D7 and A12 VHH were isolated from (Forsman et al., 2008). (Refer to Section 2.6.1)

Based on the sequences of the C8 and D7 VHH, unique degenerate reverse primers were designed to pull out VHH gene fragments with similar sequences to C8 and D7 through nested PCR. These reverse primers, C8p (5'-TGAGGAGACGGTGACCTGGGTCCCCTGGCCCCAGTCTGTATAGTCGC) and D7p (5'-TGAGGAGACGGTGACCTGGGTCCCCTGGCCCCAGTMGTWRTAATCYGAATTCG), extends into the last 4-6 codons of the CDR3 loop region (underlined). In conjunction with a framework 1 (FR1) specific primer, the PCR was carried with Expand High Fidelity (Roche, UK) using the following programme: 94 ºC for 2 min, followed by 28 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 90 s, and a final extension at 72 ºC for 7 min. Twenty-eight cycles were carried out to prevent over saturation of the amplified products, and a 300 bp band was cut out after separation on an agarose gel. After restriction enzyme digestion with *BstE*II and *Sfi*I and gel-purification, the digested DNA fragments were ligated into a phagemid vector (pAX050) for display on filamentous bacteriophage and electro-transformed into *E. coli* TG1 electro-competent cells. The transformed cells were titrated on agar plates to determine the library size, and a colony PCR was performed on a selection of colonies to determine the presence of DNA inserts in the vector. (Refer to Section 2.6.1) The creation of these libraries was carried out at Ablynx NV.

2.7.3 Bio-panning and Selection for C8 and D7-like VHH

For the selection of C8- and D7-like VHH, a variation from the previously described bio-panning method was used. Instead of antigens coated directly onto a solid substrate, the biotinylated recombinant gp120 was allowed to bind to the phages in solution form, followed by the subsequent capture of the phages-biotinylated gp120 complexes through the use of neutravidin coated plates.

2.7.3.1 Plate preparation and phage binding

Maxisorp plates (Nunc) were coated with 100 µl of neutravidin (5 µg/ml; Pierce Biotech) to capture biotinylated-gp120 and allowed to incubate overnight at 4 °C. Neutravidin is a neutrally charged derivative of the streptavidin molecule that binds to biotin with minimal nonspecific binding through the removal of carbohydrate groups which can bind to lectins. The next day, the plates were washed 3 times with PBS-T, and blocked with 200 µl of 1% casein in PBS at room temperature for 1 h with shaking, and washed 5 times with PBS-T thereafter.

Another set of uncoated U-bottom 96 well plates were blocked with 200 µl of 1% casein in PBS at room temperature for 1 h with shaking, and then washed 5 times with PBS-T. Biotinylated-gp120 (IIIB and 92BR025) were serially diluted to various concentrations from 10nM to 0.01nM in PBS supplemented with 0.2% casein and 50 µl of each of these dilutions were allowed to incubate with 50 µl of 5-fold diluted C8- and D7-specific phage library stock (created in Section 2.7.2) in the pre-blocked plates. This was to allow the binding of the phages to gp120 in solution form. Negative control blank wells were included where no antigen was added. After 2 h of incubation with shaking at room temperature, 75 µl of the gp120/phage mixture was applied to the neutravidin coated plates, and allowed to incubate for not more than 30 min with shaking at room temperature. The plates were then washed 20

times with PBS-T to remove uncaptured phages. Captured phages were eluted with 100 µl of 1 mg/ml trypsin for 30 min with shaking at room temperature, and then neutralized with 5-fold molar excess (210 µM) of trypsin blocker ABSF (Sigma).

Alternatively, plates were coated with 10 µg/ml of the antibody D7324 (Aalto Bio Reagents), a sheep polyclonal antibody raised against a conserved motif at the C-terminus of gp120. After an overnight incubation at 4°C, D7324-coated plates were washed and blocked as described above, and subsequently incubated with serially diluted 92BR025 gp120 in cell culture supernatant (from Section 2.7.1.1) in 0.2% casein in PBS. Negative control wells containing D7324 only, or D7324 plus cell culture supernatant from a negative control transfection experiment (i.e. transfections carried out as described in Section 2.7, but without addition of gp120 plasmids) were included. After 1 h at room temperature, the plates were washed five times with PBS-T.

2.7.3.2 Titration, rescue and precipitation of phages

The eluted products from the first round of selection were titrated to determine the effectiveness of the selections. The phages were then rescued and precipitated to prepare for the second round of selections. These procedures are detailed earlier in Sections 2.6.2.2 to 2.6.2.4.

2.7.3.3 Further rounds of selection

The second round of selection was similar to the first, but lower concentrations of biotinylated gp120 were used, with 10-fold serial dilution steps from 1 nM to 0.1 *p*M. The phage stocks used were diluted 50-fold in the second round, instead of 5-fold dilution in the first round. As in the first round of selection, 50 µl of biotinylated gp120 and 50 µl of the diluted phage stocks were allowed to incubate separately on a

pre-blocked plate for 2 h with shaking. Seventy-five µl of the mixture was then transferred to the pre-blocked neutravidin-coated plates or D7324-coated plates for 30 min and with shaking. The phages were then harvested with trypsin, and the phages were rescued according to the procedures described above.

2.7.3.4 Clone isolations and expression of periplasmic extracts

After 2 rounds of selections, 47 clones were picked from each selected output after subcloning into the expression vector pAX051 and their DNA was fingerprinted to check for clonal diversity. Periplasmic extracts containing the VHH were grown and then harvested for further testing and analysis. These procedures are described in Sections 2.6.3.1 to 2.6.3.3.

2.7.4 Surface plasma resonance

VHH affinity for recombinant IIIB gp120 was determined by Bart Hoorelbeke at Ablynx NV (Ghent, Belgium), using surface plasmon resonance techniques. All experiments were carried out using a BIAcore 3000 (BIAcore, GE Healthcare, Uppsala, Sweden). An estimate of the dissociation rates were first measured for each of the VHH in the periplasmic extracts. A selected few were then subsequently purified and their affinity constants determined. Briefly, approximately 2100 response units (RU) of biotinylated gp120 IIIB was non-covalently captured by streptavidin that was pre-immobilised onto a SA sensor chip (BIAcore).

The VHH, serially diluted to 80, 60, 40, 20 and 10 nM in 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% (w/v) surfactant P20 (HBS-EP buffer; BIAcore), were injected onto the chip at a flow rate of 45 µl/min for 3 min and were allowed to dissociate for 15 min. Regeneration was achieved by washing with 10 mM HCl for 3

min. The association rate constants (k_a) and dissociation rate constants (k_d) were computed from the binding curves using the BIAevaluation software (1:1 interaction). The equilibrium dissociation constants K_D were calculated as $K_D = k_d/k_a$.

2.7.5 Screening of selected VHH in neutralisation

Individual VHH were screened for their ability to neutralise HIV-1 using the TZM-bl cell-based neutralisation assay as described in Section 2.3.9. Briefly, 50 µl of VHH-containing periplasmic extract was pre-incubated 200 TCID50 of virus, per well. Irrelevant (negative control) *E. coli* periplasmic extracts were included on each plate. Purified VHH samples were tested at a maximum concentration of 25 µg/ml.

2.7.6 VHH competition with sCD4 for binding to envelope proteins in ELISA

The ability of the VHH to inhibit sCD4 binding to gp120 was evaluated in ELISA. Opaque 96-well Maxisorp plates (Nunc) were coated with sCD4, blocked and washed, as described in Section 2.4.13. Serial dilutions of VHH in 1% milk powder in TBS were pre-incubated with purified gp120 IIIB at 0.5 µg/ml in 1% milk powder in TBS in a separate plate, for 1 h at room temperature. The pre-incubated VHH-gp120 mixtures were then added to duplicate wells of the sCD4-coated plates (50 µl per well). Wells lacking sCD4 were included, as was an irrelevant VHH, and negative controls. After 1 h at room temperature, the plates were washed 3 times with TBS-T and bound gp120 was then detected using 100 µl of 1 µg/ml antibody D7324 (Aalto Bio Reagents) and thereafter, by 100 µl of 0.5 µg/ml of AP-conjugated rabbit anti-sheep IgG antibody (catalogue number. ab6748-1, Abcam, Cambridge, UK). Luminescence was detected as before and blank-corrected RLU were plotted against VHH concentration.

3 Virus cloning and characterisation

3.1 Introduction

This chapter describes the cloning of HIV-1 envelopes directly from primary samples obtained from patients. When this project was started in 2005, most of the published research and virological studies were performed on subtype B viruses that are prevalent in North America and Europe. However, subtype C viruses account for more than half of all new infections worldwide, and are prevalent in sub-Saharan Africa, South Asia and Brazil. There is a need to study and characterise the envelopes of these viruses to determine the neutralisation sensitivity of circulating strains to monoclonal antibodies and for vaccine design studies.

To achieve this, plasma samples were obtained from patients infected with subtype C HIV-1. The *env* gene was cloned directly from the viral RNA in plasma samples, and then subcloned into HIV-1 vectors to produce replication competent viruses. The vectors used were pHXB2*Δenv* and pNL43*Δenv*, which allow the insertion of heterogeneous gp120 or gp160 gene respectively (McKeating et al., 1996; Zheng and Daniels, 2001). By amplifying the envelopes directly from patient plasma samples, we have isolated viral envelopes that have not been through isolation procedures that can potentially select for virus strains able to replicate faster in PMBC cultures and may omit viruses adapted for replication in *in vivo* cell types. This facilitated the comparison of the proteins encoded by *env* in each virus at the time of sampling, and provided a realistic picture of the circulating envelopes.

Other research groups in the world have also recognised the scarcity of subtype C envelopes for research in neutralisation and vaccine studies, and had set out to characterise the subtype C circulating strains. In the interim period of this research, Brown *et al.* had created a panel of 60 viruses; 10 each from subtypes A, B, C, D, CRF01_AE, and CRF02_AG (Brown et al., 2005). The 10 subtype C viruses were obtained from 6 different countries; five African and one Indian. However, all the isolates were obtained from primary PBMC coculture. Propagation of HIV-1 in cell culture can restrict the diversity of the quasispecies present in the original blood sample and can favour the replication of some members of the quasispecies. Li *et al.* then created a reference panel of 12 African subtype C pseudoviruses and deposited them in the NIH AIDS reagent program (Li et al., 2006b). The envelopes were cloned from a mixture of cocultured PBMC, uncultured PBMC, and plasma samples. These were sourced from only two countries in Africa. The WHO panel of primary isolates (Gao et al., 1994) contain one subtype C isolate, 92BR025, which is used in the later part of this thesis.

The viruses described in this chapter are all replication-competent with the envelopes cloned directly from plasma samples. The 18 different envelope clones were derived from seropositive patients obtained from 8 different countries; 7 in Africa and also from within the United Kingdom. These chimeric viruses were then analysed for their coreceptor usage, and sensitivity to established broadly cross-neutralising monoclonal antibodies (bcnAbs), and compared with the subtype C reference strain panel described by Li *et al.* (Li et al., 2006b).

3.2 Patient samples

Plasma samples from HIV-1 infected individuals were obtained from David Yirrell of the National Surveillance System in Scotland. Some of the sources of infection were originally acquired from Africa (Yirrell et al., 2004). The samples were obtained from 20 different patients (numbered 20 to 39), all of which were typed as subtype C based on *gag* sequences. Samples from patient numbers 3 and 6 were previously cloned and described by Aasa-Chapman et al. (Aasa-Chapman et al., 2004). The sample origin and the virus load at the time of sampling are listed in Table 3-1.

Table 3-1. Sample origin and virus load at time of sampling

Patient	Infection origin	Plasma viral load (copies/ml)	Source	Subtype
3	Scotland, UK	293,000	plasma	C
6	Mozambique	>100,000	plasma	C
20	Zambia	36,300	plasma	C
21	South Africa	79,700	plasma	C
22	Malawi	76,900	plasma	C
23	Gambia	22,600	plasma	C
24	Congo	249,000	plasma	C
25	Scotland, UK	130,000	plasma	C
26	Zimbabwe	2,680,000	plasma	C
27	Malawi	200,000	plasma	C
28	Scotland, UK	329,000	plasma	C
29	Zimbabwe	27,600	plasma	C
30	Malawi	98,900	plasma	C
31	South Africa	440,000	plasma	C
32	Zimbabwe	71,000	plasma	C
33	Zimbabwe	35,300	plasma	C
34	Zimbabwe	21,800	plasma	C
35	Zambia	194,000	plasma	C
36	Scotland, UK	>750,000	plasma	C
37	South Africa	541,000	plasma	C
38	Nigeria	257,000	plasma	C
39	Burundi	38,800	plasma	C

3.3 HIV-1 envelope cloning

Viral RNA was first extracted from the plasma samples using the QIAamp Viral RNA Mini Kit (QIAGEN). First-strand cDNA was synthesised from the viral RNA and subsequently amplified in a one-step RT-PCR using the Titan One Tube RT-PCR Kit (Roche Diagnostics) and primers o_envf and o_envr according to the methods described in Section 2.5.3. Nested PCR were then carried out to clone out either gp120 or gp160 using primers 626L/125Y (for gp120) or fenv3/renv3 (for gp160). The amplified fragments were separated on an agarose gel, and bands of the expected sizes were excised and purified using the Qiagen Gel Extraction kit.

The purified DNA fragments were first inserted into pcDNA3.1-TOPO vectors and transformed into chemically competent TOP10 *E. coli*. The plasmids were then isolated and digested with either *Bst*EII/*Mlu*I (for gp120) or *Eco*RI/*Xho*I (for gp160), and subcloned into pHXB2Δenv (for gp120) or pNL43Δenv (for gp160) HIV-1 backbones. These restriction sites were encoded within the primers. pHXB2Δenv allows the incorporation of heterologous gp120 sequences from amino acid 38 (seven amino acids after the signal peptide) to six amino acids prior to the gp120/gp41 junction, whilst pNL43Δenv allows the incorporation of heterologous gp160 sequences (McKeating et al., 1996; Zheng and Daniels, 2001).

Plasmid minipreps from multiple colonies of transformed TOP10 cells (Invitrogen) were screened for the correct inserts by restriction enzyme digestion. The resulting HIV-1 molecular clones encode replication-competent chimeric viruses containing envelopes from primary isolates. An average of 8 clones from each of the gp120/HXB2 and gp160/NL4.3 chimeric constructs from each patient sample was screened for the ability to infect NP2 cells. Figure 3-1 shows the agarose gels of the

DNA fragments after digestion of the cloned products. Obtaining productive gp120 chimeric viruses were more efficient than with gp160 chimeric viruses from all the patients.

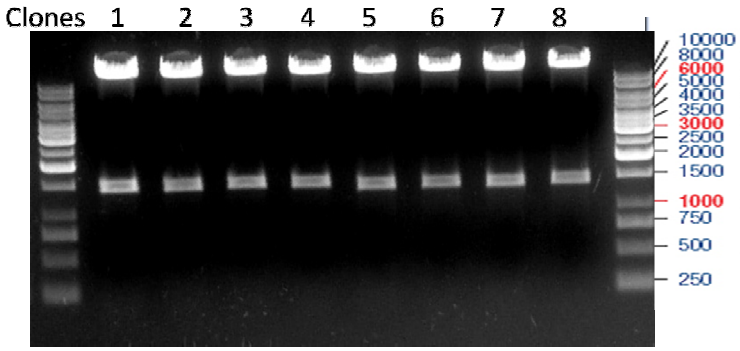
Plasmids containing the correct inserts were transfected into 293T cells and replication competent viruses were harvested from the supernatant. Clones which successfully produced replication competent chimeric viruses are shown in Table 3-2.

Table 3-2. Clones that gave productive chimeric viruses[a]

Patient Number	gp160/ NL4.3 viruses	gp120/ HXB2 viruses
3	-	CA6[b]
6	-	CB7[b]
20	-	C202
21	-	-
22	-	C222
23	-	-
24	-	-
25	-	C250
26	-	C261
27	C27b, C27d	C271
28	-	C281
29	-	C291
30	-	-
31	-	-
32	-	-
33	C33.52[c]	-
34	-	C344
35	-	C351
36	-	C361
37	C37.42[c]	-
38	C38d, C38g, C38.22[c]	C381
39	-	C391

[a.] The envelopes (both gp120 and gp160) were cloned from the plasma samples from each patient and inserted into either NL4.3 or HXB2 backbones to produce replication competent chimeric viruses. Dashes indicate the failure to obtain productive chimeric viruses.
[b.] These virus clones were produced by Craig Seymour.
[c.] These virus clones were produced by Anna Forsman.

[A]



[B]

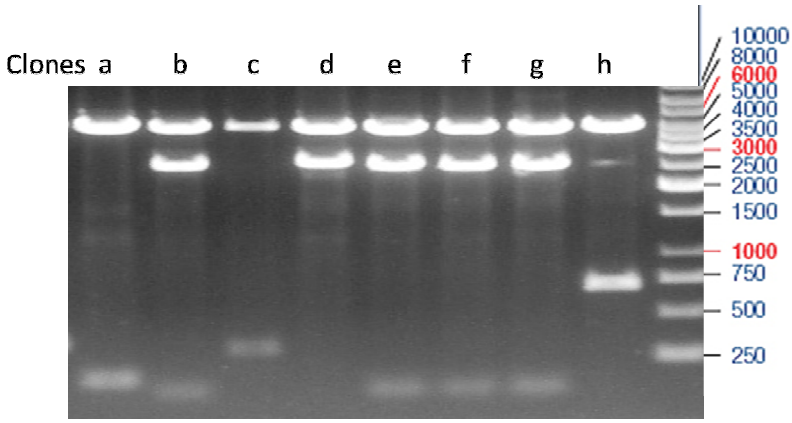


Figure 3-1. Agarose gels showing the DNA fragments after restriction enzyme digestion.

Eight clones from each patient sample were screened for the correct inserts by restriction enzyme digestion. [A] Digestion of cloned gp120 envelopes in the HXB2 vector gave 2 fragments: a 1.5kb fragment for gp120, and an 11kb fragment for the vector backbone. All the clones produced inserts of the correct size. [B] Digestion of cloned gp160 envelopes in the TOPO vector gave 2 fragments: a 2.5kb fragment for gp160, and a 4kb fragment for the vector. Only 5 out of the 8 clones (clones b, d, e, f, and g) produced inserts of the correct size. The above show a representative gel from each set of digested clones.

3.4 Sequencing of the cloned HIV-1 envelopes

The envelopes were sequenced using an assortment of 11 different primers to ensure sufficient coverage of the cloned products (as described in Section 2.5.2), and to exclude any identical envelope clones during the picking of colonies. The sequences were then assembled using the Sequencher4 software and are shown in Figure 3-2. Accession numbers of the sequences that were uploaded onto Genbank are shown in Table 3-3.

Gp120 primary envelope isolates

>CA6
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GCTTGATATAGTACCAATTGAAAGCTCTAATGGGAACTCTACTGAGTATAGATTAATAAACTGTAATACTTCAACCATAGCACAA
GCCTGTCCAAAGATCTCTTTTGATCCAATACCTATACATTATTGTGCTCCTGCTGGTTATGCGATTCTAAAGTGTAATAATAAGA
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>CB7
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>C202
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>C222
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Figure 3-2. (Cont. on next page)

>C250
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>C261
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>C271
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>C281
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Figure 3-2. (Cont. on next page)

>C291
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>C344
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>C351
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>C361
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Figure 3-2. (Cont. on next page)

>C381
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>C391
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Gp160 primary envelope isolates

>C27.b
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Figure 3-2. (Cont. on next page)

>C27.d
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>C33.52
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Figure 3-2. (Cont. on next page)

>C37.42
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>C38.d
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Figure 3-2. (Cont. on next page)

>C38.g
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>C38.22
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Figure 3-2. Sequences of the cloned envelopes in fasta format

3.5 Sequence analysis of the cloned gp120 envelopes

The amino acid sequences were computationally translated from the DNA sequences, and all the clones had an uninterrupted *env* open reading frame. The amino acid sequences of the gp120 regions were then aligned using the programme ClustalW2 (Larkin et al., 2007) and compared with the consensus subtype C sequence on the Los Alamos National Laboratory (LANL) HIV database. The aligned amino acid sequences are shown in Figure 3-3.

The cysteine residues (C) at the bases of the V1, V2, V3, and V4 loops of gp120 were highly conserved in the cloned samples. When compared with the consensus subtype C sequence, considerable amino acid sequence variability was seen in V1, V2, V4, V5 and a region immediately C-terminal of V3. Amino acid sequence variability within V3 itself was relatively low. The 34 amino acids immediately C-terminal to V3 were remarkably variable, as is typical of subtype C viruses (Gnanakaran et al., 2007; Li et al., 2006b).

The positions of potential N-linked glycosylation (PNLG) sites are known to influence neutralisation patterns (Reitter et al., 1998), and these are shown in bold and underlined in Figure 3-3. PNLG occur at sites with an NX[ST]X motif, where X is any amino acid other than proline (P), followed by either serine (S) or threonine (T) within the square brackets. It appears that the V1, V2, V4 and V5 of gp120 were the most heavily glycoyslated regions. Fourteen PNLG sites were highly conserved (>90%) in the subtype C *env* clones studied, and these are denoted with open triangles in Figure 3-3. The number of PNLG sites in each gp120 clone is shown in Table 3-3. The average number of PNLG sites in all the *env* clones is 26.7, with the lowest number being 23 (found in C202, C361 and CA7) and the highest number

being 30 (found in C37.42). This is similar to the numbers found in the subtype C reference clones as described by Li *et al.* (Li et al., 2006b), with the exception that the reference clones contained only nine conserved PNLG sites. One highly conserved PNLG is in the N-terminus of V3, which was shown to mask neutralisation epitopes in the V3 loop of subtype B viruses. (Back et al., 1994; Schonning et al., 1996).

The number of amino acids in the variable loops of gp120 can affect the neutralisation phenotype (Etemad-Moghadam et al., 1998; Moore and Sodroski, 1996; Wei et al., 2003). The numbers were highly varied amongst the clones, with values of up to 50% variability in some of the clones, and are comparable to the average number of amino acids within the variable loops in the subtype C reference panel (Li et al., 2006b). The only exception was the V3 loop, with all *env* clones possessing a constant number of 35 amino acids (Table 3-3)

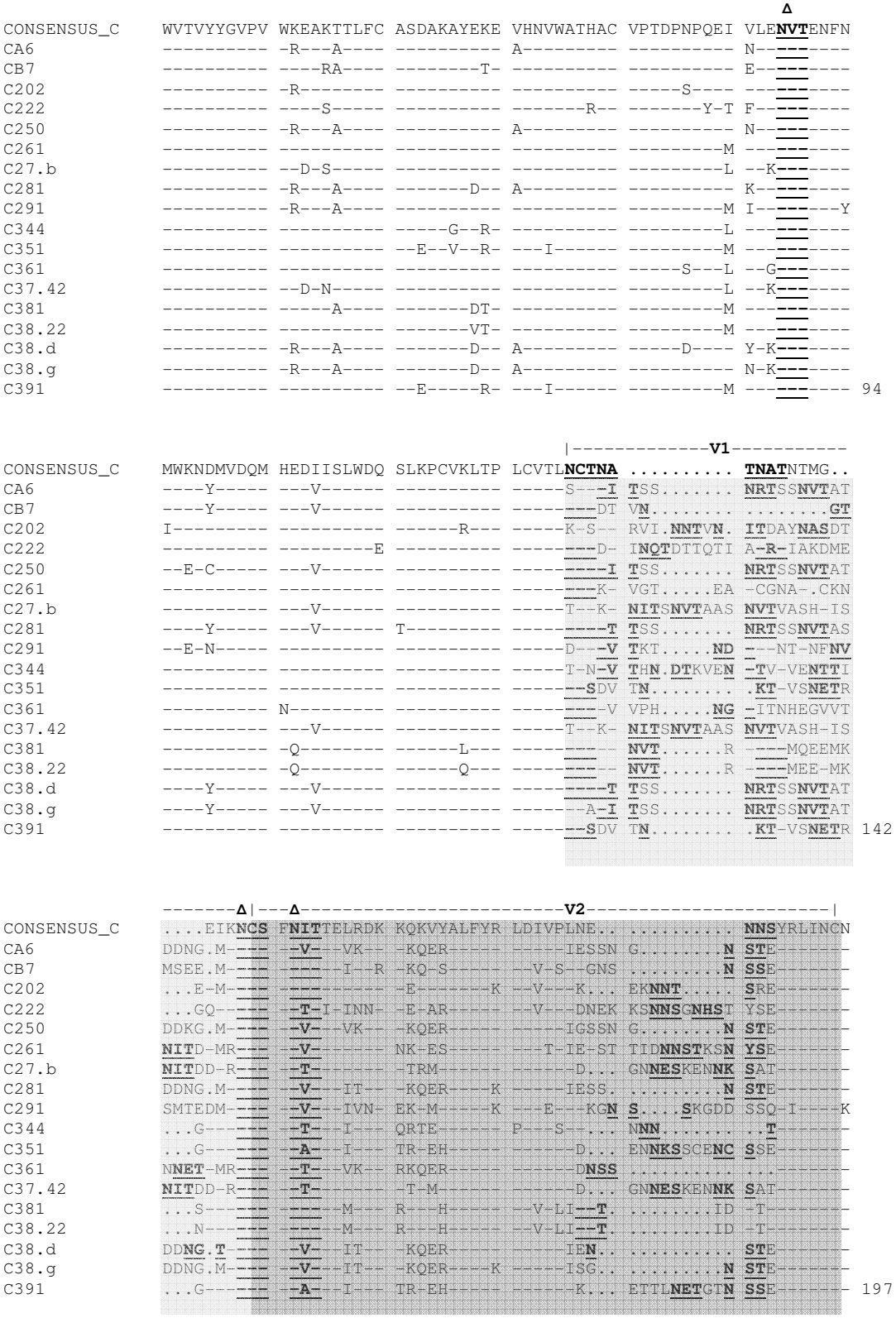

Figure 3-3 (cont. on next page)

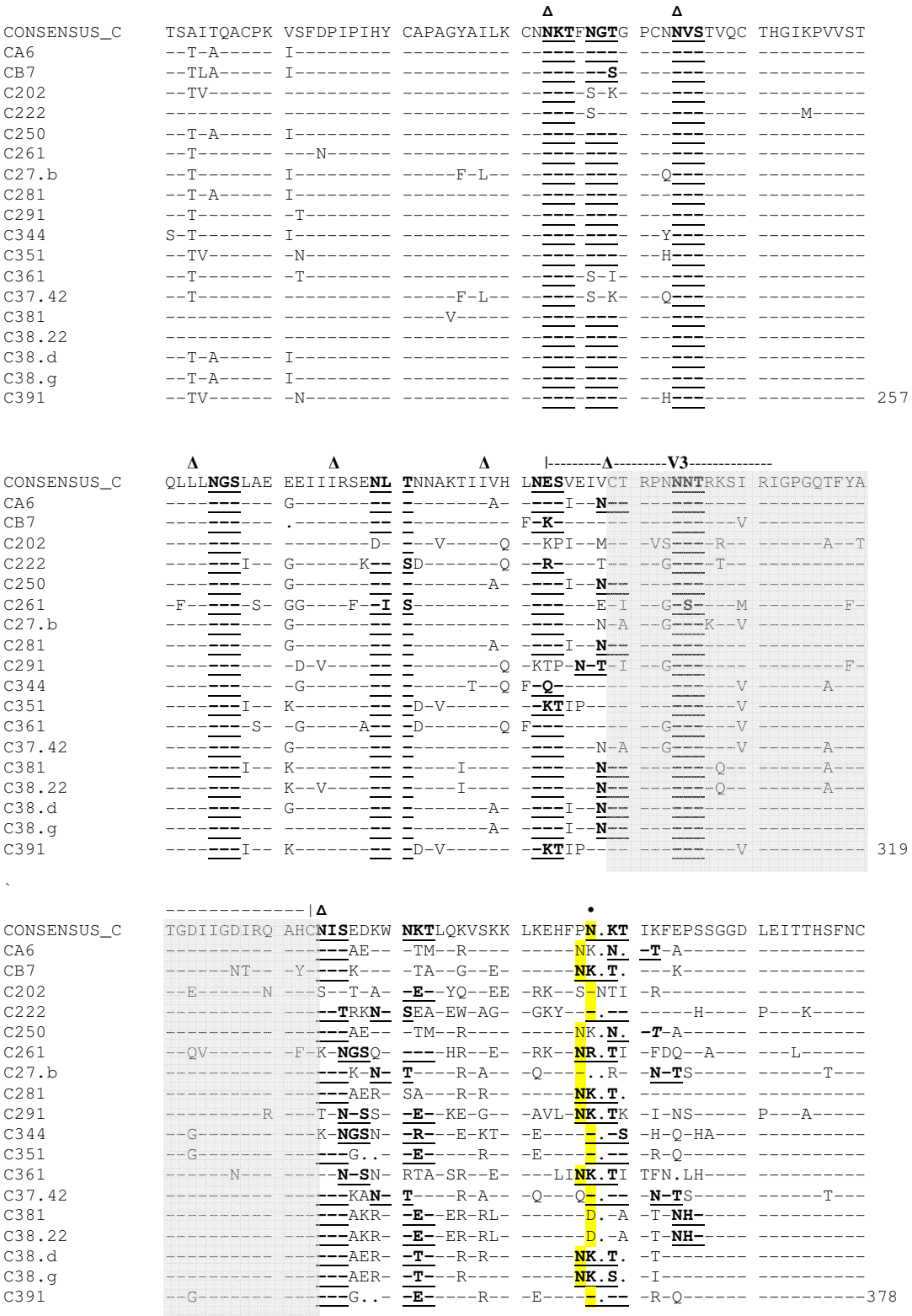

Figure 3-3 (cont. on next page)

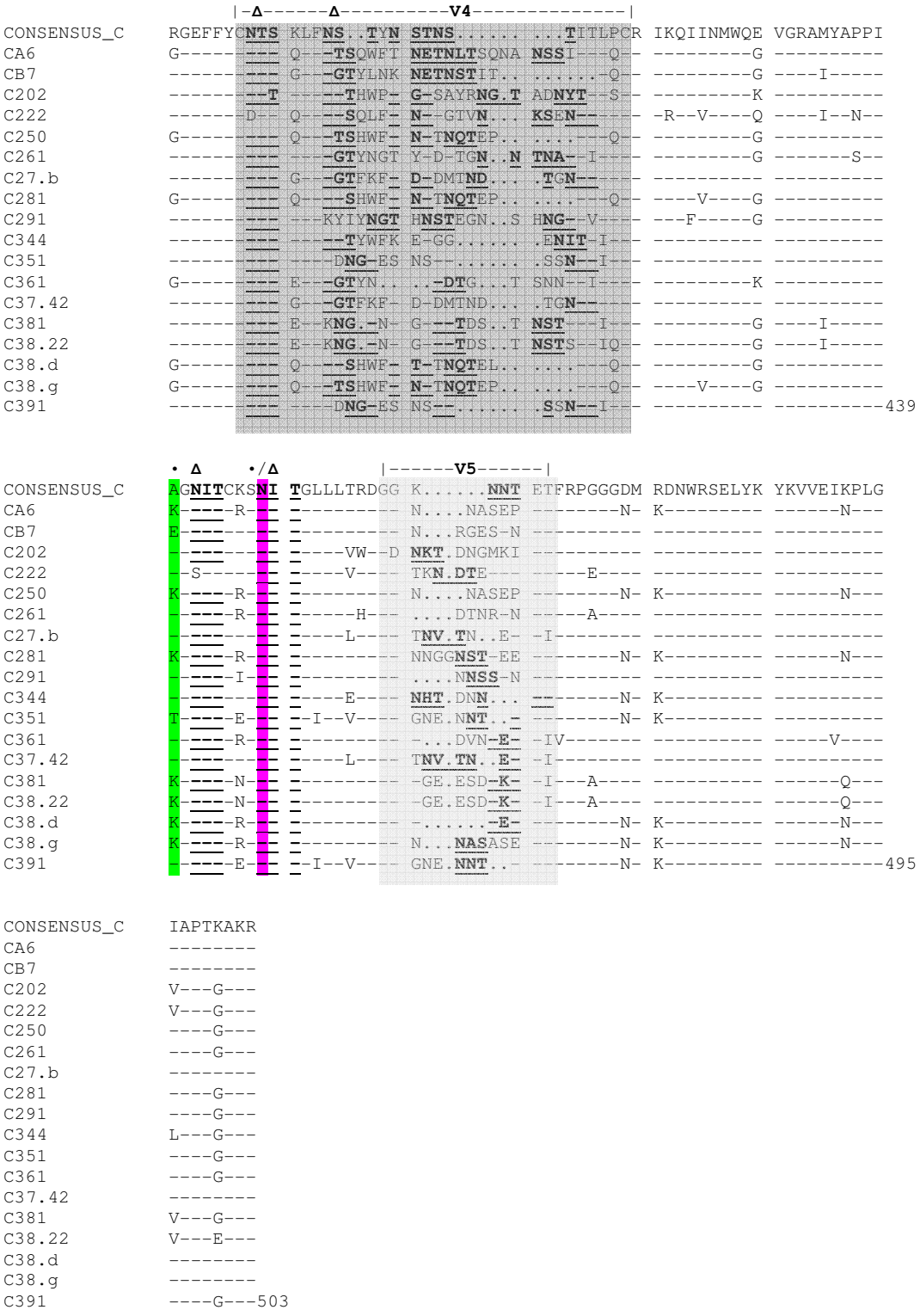

Figure 3-3. Amino acid alignments of cloned gp120 envelopes.

Nucleotide sequences of the gp120 *env* gene from subtype C patient samples were translated, aligned, and compared with the subtype C consensus sequence with ClustalW2. Numbering of amino acid residues is according to the HXB2 numbering sequence. Dashes denote sequence identity, while dots represent gaps introduced to optimize alignments. V1, V2, V3, V4 and V5 regions (shaded boxes) designate hypervariable HIV-1 gp120 domains. Potential N-linked glycosylation sites (NXYX motif,

where X is any amino acid other than proline and Y is either serine or threonine) are in bold and underlined. Highly conserved sites of potential N-linked glycosylation are denoted with open triangles. Shaded residues with a solid bullet are positions 356, 440, and 448, which were previously determined to be important for CCR3 usage.

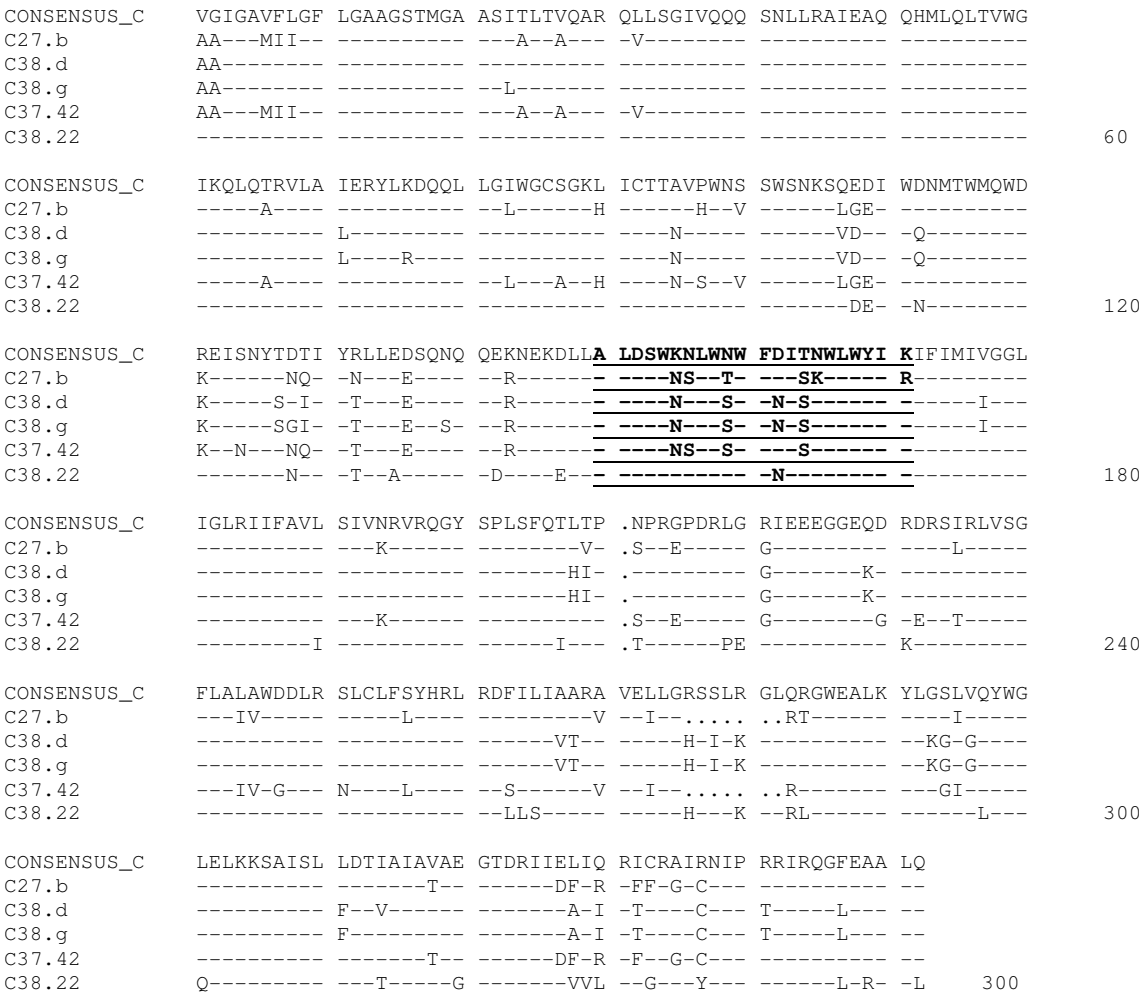

Figure 3-4. Amino acid alignments of the gp41 sequence from the gp160 envelope clones.

The amino acid sequence of the gp41 portions from the subtype C gp160 envelope clones were aligned together with the consensus C sequence obtained from the LANL HIV database using ClustalW2. The underlined sequences denote the MPER of gp41 containing the equivalent of the 2F5 and 4E10 epitope. The 2F5 epitope is missing in the subtype C sequences, whereas the 4E10 epitope is present.

Table 3-3. Genbank accession numbers, number of PNLG sites, and number of amino acids in variable loops

Virus Clone[1]	Genbank accession number	No. of N-linked glycosylation sites	No. of amino acids in variable loops				
			V1	V2	V3	V4	V5
Consensus_C		25	17	38	35	22	7
CA6	FJ977089	26	25	42	35	33	9
CB7	FJ977090	23	16	41	35	25	10
C202	FJ977078	23	28	42	35	32	12
C222	FJ977079	26	30	50	35	30	12
C250	FJ977080	27	25	42	35	27	9
C261	FJ977081	28	28	50	35	31	9
C27.b*	FJ977091	29	33	47	35	29	10
C281	FJ977082	27	25	40	35	27	13
C291	FJ977083	28	28	46	35	31	9
C344	FJ977084	26	29	38	35	26	9
C351	FJ977085	27	21	47	35	26	10
C361	FJ977086	23	28	37	35	26	10
C37.42*	FJ977094	30	33	47	35	29	10
C381	FJ977087	28	24	41	35	30	12
C38.d*	FJ977092	28	25	38	35	27	7
C38.g*	FJ977093	27	25	39	35	27	10
C38.22*	FJ977095	28	24	41	35	30	12
C391	FJ977088	27	21	47	35	26	10
Mean		26.7	25.8	43.4	35.0	28.6	10.2

[1] Replication-competent molecular clone containing gp120, or gp160 (indicted with asterisk, *), cloned directly from patient plasma and inserted into an HIV-1_{HXB2}, or HIV-$1_{NL4.3}$ (*), based vector.

3.6 Phylogenetic studies of cloned gp120 envelopes

The amino acid sequences of the cloned envelopes were compared with the subtype reference alignments on the LANL HIV database, which consist of at least 4 representative strains from each subtype and recombinants in the M-group. Phylogenetic studies of all the envelope nucleotide sequences confirmed that the newly isolated clones grouped within subtype C. Two reference subtype C strains, 92BR025.8 from Brazil and SK164B1from South Africa (Gao et al., 1996; Kiepiela et al., 2004), clustered closely to our cloned sequences (Figure 3-5).

A phylogenetic tree was then constructed using the GTR (General time reversible) PhyML algorithm (Guindon and Gascuel, 2003) with bootstrap replications of 1000. The tree was rooted with three subtype D strains (NDK, ELI and 94UG114) and is shown in Figure 3-6. In addition to the 18 viral *env* sequences used in this study, sequences from the subtype C reference panel were also included for comparison. Some of the new subtype C envelopes clustered closely to the reference panel, indicating close relationships. For example, C361, C222 and C381 were found to be quite similar to ZM109F.PB4, ZM135M.PL10a and ZM233M.PB6 respectively. However, a few strains were well separated from the reference panel. These are the C281 and C344 clusters, as well as isolates C261, C291, C351, and CB7. These isolates can be used as further strains in testing for the neutralisation breadth of a candidate antibody. In terms of geographical clustering, the isolates C281, C250 and CA6 were all from Scotland, UK and formed one cluster. The Nigerian isolate C38.d and C38g were also clustered together. However, 4 isolates from different regions were clustered together, and these were C27.b, C37.42, C351 and C391.

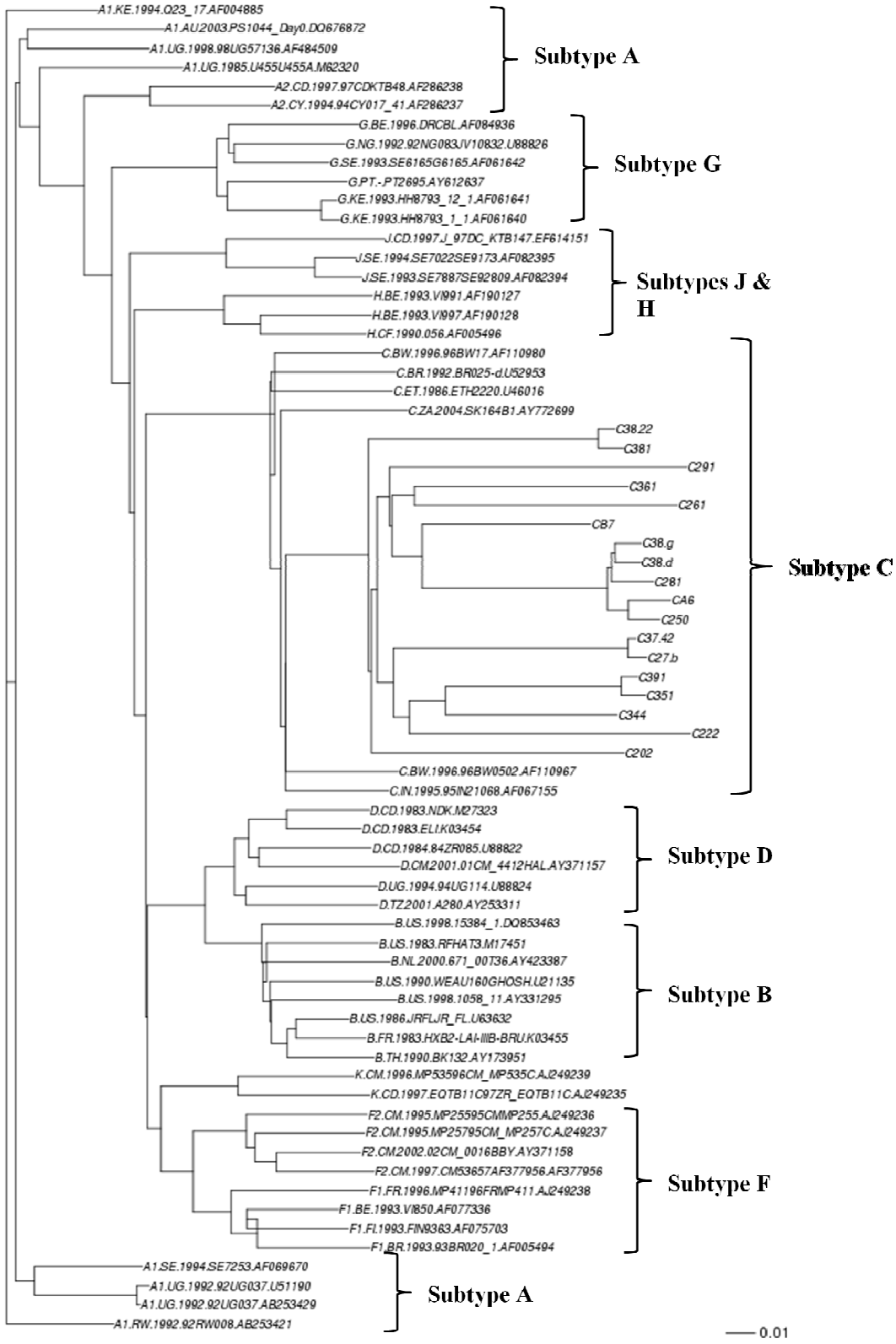

Figure 3-5. Phylogenetic clustering of the cloned gp120 envelopes with the subtype reference alignments.

Phylogenetic clustering was performed together with the subtype reference alignments using the Neighbour Treemaker function on the LANL HIV database website. The cloned envelopes were found to cluster together with other subtype C viruses from the database.

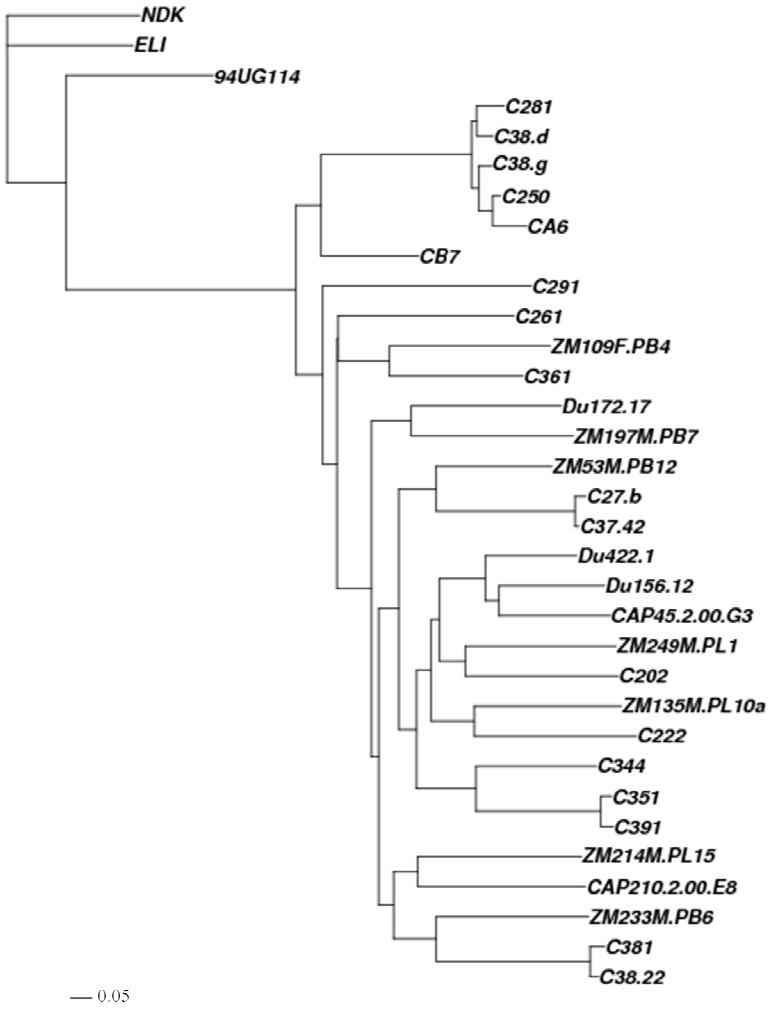

Figure 3-6. Phylogenetic relationships of subtype C env clones.

Newly characterised subtype C sequences (with a C-prefix) are compared with the subtype C reference clones from Li *et al.* 2006. Horizontal branch lengths are drawn to scale (the scale bar represents 0.05 nucleotide substitutions per site), but vertical separation is for clarity only. The phylogenetic tree was rooted with subtype D env sequences (NDK, ELI and 94UG114).

3.7 Titration of chimeric viruses and coreceptor usage

The chimeric viruses obtained after the transfection were first titrated on NP2 indicator cell lines (Soda et al., 1999) that stably express CD4 and the coreceptor CCR5 (NP2/CD4/CCR5) to determine the infectivity of the chimeric viruses, as described in Section 2.3.5. Infected NP2 cells were stained blue in a p24 β-gal assay, and these were counted under the light microscope. The titres of the virus stocks were then determined by calculating the focus-forming units (FFU) per ml of virus used and the results are shown in Table 3-4. The envelope clones C27d, C33.52, and C271 produced chimeric viruses of insufficient titre to be useful in further characterisations, and were not included in the table.

To determine if the primary envelopes were able to use other coreceptors to enter the cells, the same virus stocks were then titrated on other NP2/CD4 cells expressing either CXCR4, CCR3, CCR8, or APJ. To determine if the chimeric viruses were coreceptor independent, they were also titrated on NP2/CD4 cells not expressing any coreceptor. The titre in FFU/ml were determined as above and shown in Table 3-4.

All the 18 chimeric viruses were found to use CCR5 as the main coreceptor and were mostly mono-tropic. None of the cloned envelopes, except for C344, were able to use CXCR4 efficiently. The envelope clone C344 was multi-tropic for R3/X4/R4/R8. Three other virus clones; C261, C281 and C381, were dual-tropic for CCR5 and CCR3 usage. The virus clone C202 was the only virus, other than C344, to use CCR8 as a coreceptor. None of the virus clones was found to use APJ as a coreceptor.

Table 3-4. Coreceptor usage of envelope clones

Virus clone[1]	Infectivity (x 10^3 FFU/ml) for NP2/CD4					Classification[2]	% CCR3 usage compared to CCR5
	CCR5	CXCR4	CCR3	CCR8	APJ		
CA6	31	-[3]	0.2	nt[4]	nt	R5	0.6
CB7	1,800	-	10	nt	nt	R5	0.6
C202	32	0.4	2	5.5	-	R5/R8	6.3
C222	2,100	0.1	1.6	3.2	-	R5	0.08
C250	73	1.5	4.1	4.1	-	R5	5.6
C261	21	0.8	2.5	0.7	-	R5/R3	12
C27.b*	2,800	-	21	1.0	-	R5	0.8
C281	300	0.5	95	1.9	0.3	R5/R3	32
C291	1,100	0.5	15	1.6	0.2	R5	1.4
C344	38	7.6	11	9.2	0.7	R5/X4/R3/R8	29
C351	1,200	0.4	34	1.5	0.4	R5	2.8
C361	43	0.7	2.4	1.5	0.2	R5	5.6
C37.42*	3	-	-	-	-	R5	0
C381	7.7	0.5	1.0	0.6	0.3	R5/R3	13
C38.d*	6.7	-	-	-	-	R5	0
C38.g*	12	-	0.6	-	-	R5	5.0
C38.22*	500	-	9.8	nt	nt	R5	2.0
C391	4,100	0.5	230	2.0	0.2	R5	5.6
Median							3.9

[1] Replication-competent molecular clone containing gp120, or gp160 (indicted with asterisk, *), cloned directly from patient plasma and inserted into an HIV-1_{HXB2}, or HIV-$1_{NL4.3}$ (*), based vector.
[2] Viruses were classified as dual- (or multi-)-tropic if their titre on alternative coreceptors were within 1 $\log_{10}$ of the titre obtained on the dominant coreceptor line.
[3] -, <100 (FFU/ml).
[4] nt, not tested.

The median percentage of CCR3 usage compared to CCR5 was 3.9% in this study of 18 cloned subtype C viruses. It was observed that the proportion of viruses that were able to use both CCR3 and CCR5 were lower in this study of subtype C envelopes, compared to the previous findings on subtype B envelopes where >70% of the viruses tested were able to use CCR3 efficiently (Aasa-Chapman et al., 2006a). Other groups have also reported high CCR3 usage for subtype B viruses (Agrawal et

al., 2009; Peters et al., 2004). This may indicate a different pattern of coreceptor usage between different subtypes of HIV-1.

To investigate this further, the CCR3 usage patterns in subtypes B and C envelope reference panels (Li et al., 2005; Li et al., 2006b) were similarly studied. The median value of CCR3 usage compared to CCR5 in the panel of 11 subtype B envelopes was 9.8%, compared to a much lower median value of 0.05% in the panel of 12 subtype C envelopes (Table 3-5 and Table 3-6). The median values for the CRF07_BC and CRF02_AG reference strains panels were 0.18% and 0.53% respectively (Table 3-7 and Table 3-8). Therefore, CCR3 usage in subtype B viruses appears to be higher than in other subtypes. A student's *t*-test performed on the data confirmed that viruses in the subtype B reference strain panels had a significantly higher usage of CCR3 compared to CCR5 than the viruses in the subtype C reference strain panel, with a p value of 0.015.

Table 3-5. CCR3 use by the Tier 2 subtype B reference strain panel of gp160 clones[1]

Virus	Source[2]	Infectivity (x 10^3 FFU/ml) for NP2/CD4		% CCR3 usage compared to CCR5
		CCR5	CCR3	
6535.3	ccPBMC	645	93	14
QH0692.42	ccPBMC	7,050	290	4.1
SC422661.8	plasma	2,350	230	9.8
PVO.4	ccPBMC	2,165	145	6.7
TRO.11	ccPBMC	149	23	16
AC10.0.29	ccPBMC	135	110	81
RHPA4259.7	plasma	1,550	305	19.7
REJO4541.67	plasma	860	7.8	0.9
TRJO4551.58	plasma	2,150	4.5	0.2
WITO4160.33	plasma	149	23	15
CAAN5342.A2	plasma	1,635	28	1.7
Median[3]				9.8 (0.2-81)

[1]Li et al. (2005) Human immunodeficiency virus type 1 *env* clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol., 79:10108-25.
[2]ccPBMC, co-cultured PBMC.
[3]Median level of CCR3 usage (and range) in percentage (%) for the panel.

Table 3-6. CCR3 use by the Tier 2 African subtype C reference strain panel of gp160 clones[1]

Virus	Source[2]	Infectivity (x 10^3 FFU/ml) for NP2/CD4		% CCR3 usage compared to CCR5
		CCR5	CCR3	
Du156.12	ccPBMC	640	0.14	0.02
Du172.17	ccPBMC	95	0.06	0.06
Du422.1	ccPBMC	1,685	0.16	0.01
ZM197M.PB7	ucPBMC	38	0.02	0.05
ZM214M.PL15	plasma	7,050	43	0.61
ZM233.PB6	ucPBMC	410	0.05	0.01
ZM249M.PL1	plasma	89	0.18	0.20
ZM53M.PB12	ucPBMC	735	0.37	0.05
ZM109F.PB4	ucPBMC	2,745	10.8	0.39
ZM135M.PL10a	plasma	625	0.13	0.02
CAP45.2.00.G3	plasma	15	0.01	0.07
CAP210.2.00.E8	plasma	198	0.02	0.01
Median[3]				0.05 (0.01-0.61)

[1]Li et al. (2006) Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular *env* clones from acute and early heterosexually acquired infections in southern Africa. J. Virol., 80:11776-90.
[2]ccPBMC, co-cultured PBMC; ucPBMC, uncultured PBMC.
[3]Median level of CCR3 usage (and range) in percentage (%) for the panel.

Table 3-7. CCR3 use by the Tier 2 CRF07_BC reference strain panel of gp160 clones[1]

Virus	Source[2]	Infectivity (x 10^3 FFU/ml) for NP2/CD4		% CCR3 usage compared to CCR5
		CCR5	CCR3	
CH038.12	ccPBMC	9,125	116	1.3
CH064.20	ccPBMC	1,150	0.18	0.02
CH070.1	ccPBMC	17,400	61	0.35
CH091.9	ccPBMC	4,550	5.9	0.13
CH110.2	ccPBMC	3,100	3.9	0.13
CH111.8	ccPBMC	0.40	0.03	7.5
CH181.12	ccPBMC	1,505	0.21	0.01
CH120.6	ccPBMC	1,380	7.7	0.56
CH119.10	ccPBMC	3,100	23	0.74
CH117.4	ccPBMC	1,420	1.5	0.10
CH115.12	ccPBMC	2,485	0.04	0.002
CH114.8	ccPBMC	215	0.50	0.23
Median[3]				0.18 (0.002-7.5)

[1]Registry of Molecularly Cloned HIV-1, SIV and SHIV gp160 Genes for Use as Env-Pseudotyped Viruses in Neutralizing Antibody assays (http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm

[2]ccPBMC, co-cultured PBMC.

[3]Median level of CCR3 usage (and range) in percentage (%) for the panel.

Table 3-8. CCR3 use by the Tier 2 CRF02_AG reference strain panel of gp160 clones[1]

Virus	Source[2]	Infectivity (x 10^3 FFU/ml) for NP2/CD4		% CCR3 usage compared to CCR5
		CCR5	CCR3	
T257-31	ccPBMC	1,505	6	0.40
928-28	ccPBMC	55	0	0
271-11	ccPBMC	131	5.9	4.5
T33-7	ccPBMC	383	14	3.7
263-8	ccPBMC	110	26	26
T250-4	ccPBMC	240	0.02	0.008
T251-18	ccPBMC	1,025	30	2.9
T278-50	ccPBMC	748	0.56	0.07
T255-34	ccPBMC	12	0.01	0.08
235-47	ccPBMC	7.5	0.04	0.53
266-60	ccPBMC	490	4.2	0.86
Median[3]				0.53 (0-26)

[1]Registry of Molecularly Cloned HIV-1, SIV and SHIV gp160 Genes for Use as Env-Pseudotyped Viruses in Neutralizing Antibody assays (http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm
[2]ccPBMC, co-cultured PBMC.
[3]Median level of CCR3 usage (and range) in percentage (%) for the panel.

Three amino acid residues were found to be linked to the use of CCR3 as a co-receptor by subtype B envelopes (Aasa-Chapman et al., 2006b). These were asparagine N356, arginine/serine R/S440 and asparagine N448 (numbering according to HXB2 sequence, and these positions are shaded in Figure 3-3). The two asparagine positions are PNLG sites, and if removed, the viruses were found to be mono-tropic for CCR5. The four CCR3-using subtype C envelopes (C261, C281,

C344, and C381) contain both PNLG sites, except for C381 which possess only the latter PNLG site (N448). Interestingly, none of the subtype C envelopes tested in this study possess either an arginine or serine in position 440. The subtype C virus clones tested here do not follow the rules that were previously determined for subtype B viruses (Aasa-Chapman et al., 2006b). Other determinants must therefore be involved in CCR3 coreceptor usage in subtype C viruses.

3.8 Neutralisation phenotype of cloned envelopes

The 18 cloned replication-competent subtype C viruses were tested for their neutralization sensitivity to four well-established bcnAbs, and to soluble CD4 (sCD4). The bcnAbs tested were the mAbs b12 and 2G12 which target the CD4 binding site and the glycan shield of gp120 respectively, and mAbs 2F5 and 4E10 which recognize adjacent epitopes in the membrane-proximal external region of gp41 (Muster et al., 1993; Purtscher et al., 1994; Sanders et al., 2002a; Scanlan et al., 2002; Stiegler et al., 2001; Trkola et al., 1996b; Zhou et al., 2007; Zwick et al., 2001). Only gp160 subtype C cloned viruses were tested for sensitivity to 2F5 and 4E10. The cloned viruses were also tested for their sensitivity to two different patient serum samples and an uninfected normal human serum (NHS) as negative control. These neutralisation assays were tested on TZM-bl cells using the luciferase assay as described in Section 2.3.9. The maximum concentration of antibody tested was 50 µg/ml and a 1:10 dilution for serum samples. Two subtype B lab-adapted strains (NL4.3 and MN) and a sensitive Tier-1 subtype C strain (93MW965.26) were included for comparison. The relative light units (RLU) from the luciferase assay were measured in a luminometer. The percentage of RLU in test wells compared to in virus only control wells (after subtraction of background luminescence) was used to determine the inhibitory effects of the antibody or serum, and this was then plotted

against the concentration of the antibody or dilution of the serum used. These graphs are shown in Figure 3-7. The inhibitory concentration of antibody required to cause 50% neutralisation of the virus (IC_{50}) are listed Table 3-9.

3.8.1 Neutralisation by soluble CD4

The sensitivity of the chimeric viruses to neutralisation by sCD4 was tested to understand the conformational traits of the CD4 binding site of the cloned envelopes. This is important for Chapter 5 where some of these viruses were tested for sensitivity to VHH that targets the CD4bs.

The results showed that many of the cloned viruses were sensitive to inhibition by sCD4 alone, with only 3 of the cloned viruses (CA6, C281, and C38.g) not inhibited at 50 µg/ml of sCD4, suggesting some form of epitope exposure in and around the CD4 binding site of gp120 in the sensitive isolates. Resistance to neutralisation by sCD4 have been observed among primary isolates (Daar et al., 1990; Pugach et al., 2004). Out of these 3 viruses, only CA6 was sensitive to b12 neutralisation, which suggest conformational differences around the CD4 binding site. The mAb b12 was able to neutralise only 6 of the viruses at 50 µg/ml. The virus clone C351 was particularly sensitive to b12, with IC_{50} value of <0.02 µg/ml. This is significantly more sensitive to the other viruses in the subtype B and C reference panels. Interestingly, llama antibody fragments that target the CD4 binding site were able to neutralise 3 isolates (C38.22, C222, and C261) which were not neutralised by b12 (Forsman et al., 2008).

3.8.2 Neutralisation by monoclonal antibodies

The cloned viruses were resistant to neutralisation by the glycan-specific 2G12, except for C38.g. Similar broad resistance to 2G12 has been reported for other

subtype C viruses (Binley et al., 2004; Gray et al., 2006). The cloned viruses were insensitive to the mAb 2F5, due to the absence of the DKW motif on subtype C viruses. The mAb 4E10 was able to neutralise all the subtype C cloned envelope viruses tested. None of the viruses contain the NWFNIT epitope previously thought to be required for 4E10 recognition (Zwick et al., 2001), although they all contain the WFXI motif that has been reported to be essential for recognition (Binley et al., 2004; Li et al., 2005).

When compared to the neutralisation results obtained with the subtype C reference panels (Li et al., 2006b), the sensitivity of the subtype C reference envelopes with 2G12 and 2F5 were similar to the cloned subtype C envelopes in this study. In contrast, all the subtype C viruses in the reference panel were neutralised by sCD4, and a higher proportion were neutralised by b12, compared to the lower sensitivity to both sCD4 and b12 by the viruses created in this study. In addition, the geometric mean titre (GMT) of 7.6 µg/ml for the mAb 4E10 was 7 times higher than that obtained in the subtype C reference panels. The viruses obtained in this study were therefore slightly more resistant to neutralisation than those in the Li *et al.* subtype C standard reference panel (Li et al., 2006b).

3.8.3 Neutralisation by human serum samples

The subtype C viruses in this study were also tested on two serum samples (QC1 and QC2) obtained from chronic subtype B HIV-1 patients, together with a serum pool from uninfected individuals. These results were compared with the two subtype B lab adapted strains (NL4.3 and MN), and a Tier-1 subtype C isolate (93MW965.26). All the cloned viruses in this study were broadly sensitive to QC1, and 11 of the 18 viruses were sensitive to QC2. Most of the cloned viruses were less sensitive than

93MW965.26 to neutralisation by the sera. From the results, some of the viruses tested were clearly more sensitive than others, and they can be grouped according to their sensitivity to the QC sera. The viruses C261, C291, C344, C351, C361, C381 and C391 were highly sensitive and easily neutralised by high dilutions of QC sera. These have similar sensitivity to 93MW965.26, and more sensitive than MN and NL4.3 and can be classified together with the easily neutralised "Tier-1" viruses (Mascola et al., 2005). In contrast, the less sensitive virus clones CA6, CB7, C27.b, C37.42, C38.d, C38.g, and C38.22 require high concentrations of QC1 to be neutralised, and were not neutralised by QC2. These are a 100-fold less sensitive than 93MW965.26 and 10-fold less sensitive than MN and NL4.3, and they can therefore be classified as "Tier-2" viruses (Mascola et al., 2005).

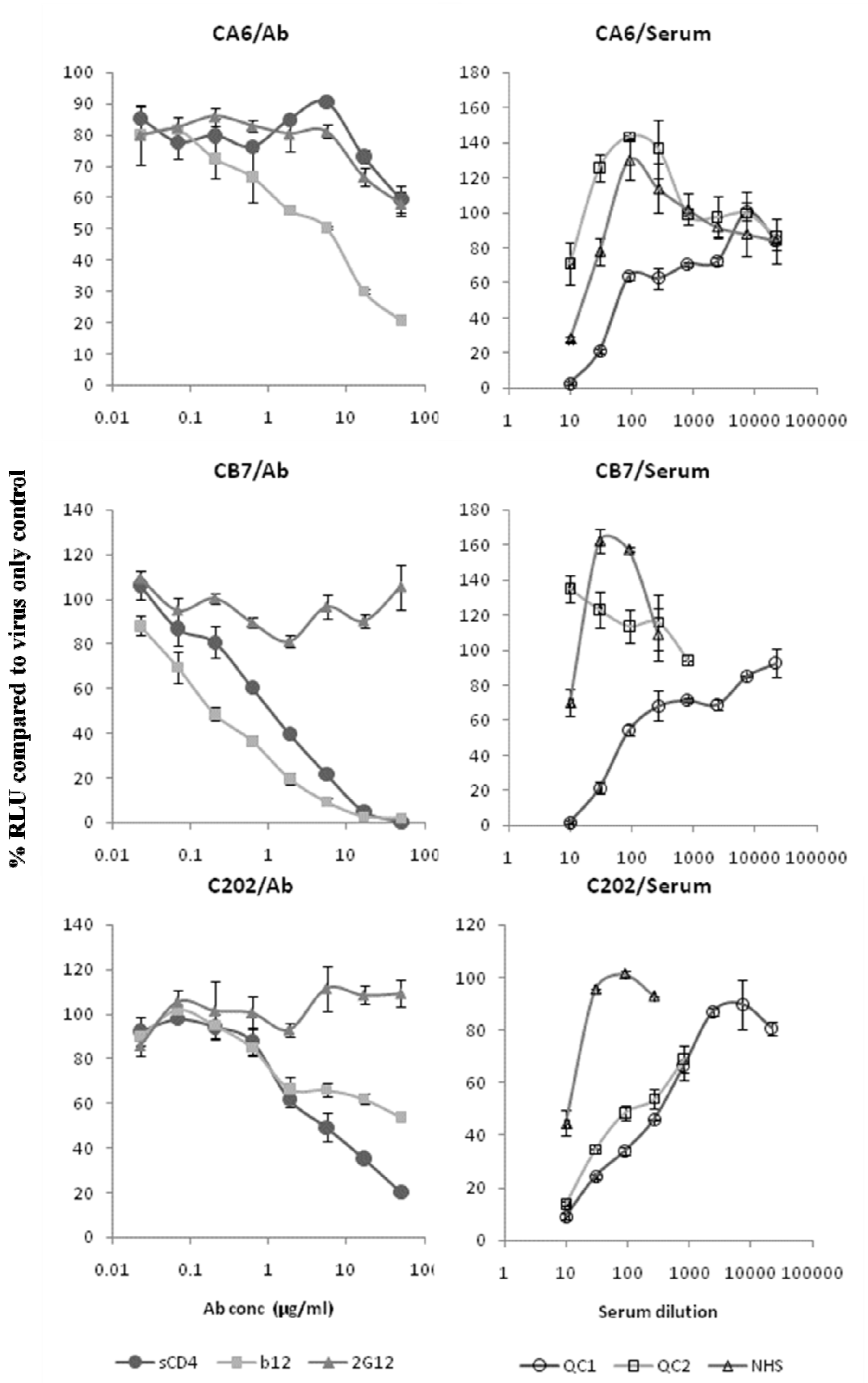

Figure 3-7. (cont. on next page)

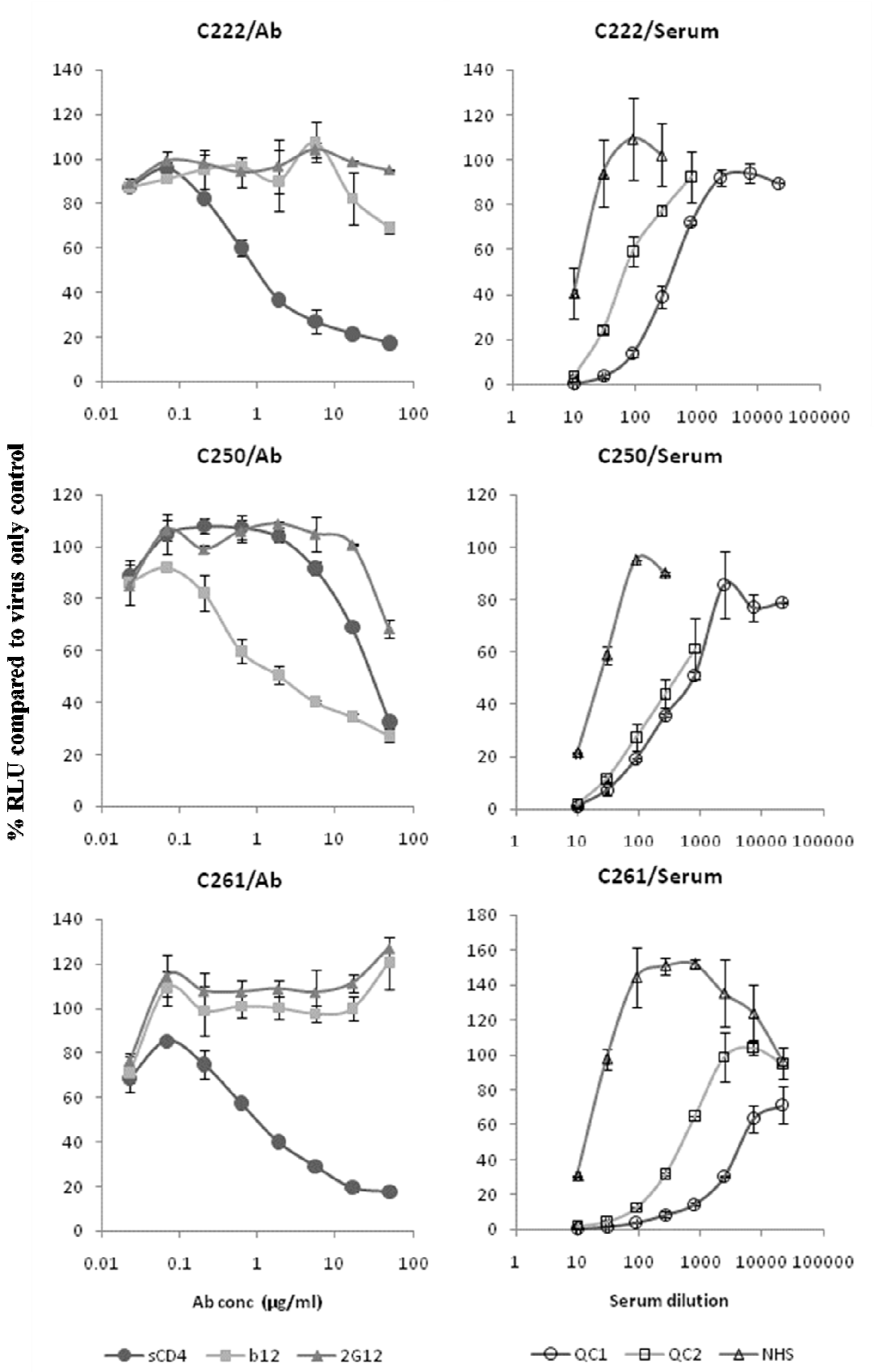

Figure 3-7. (cont. on next page)

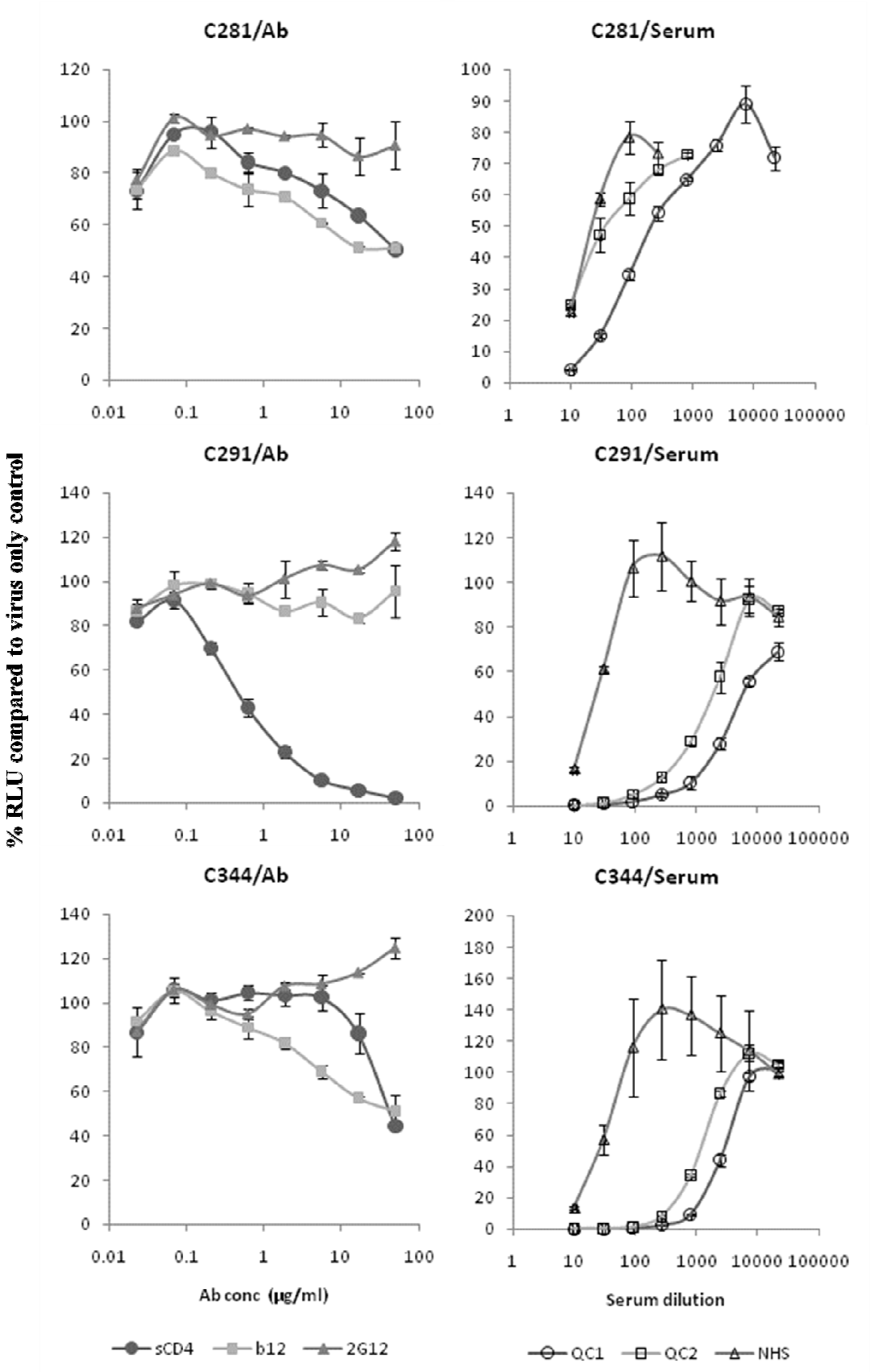

Figure 3-7. (cont. on next page)

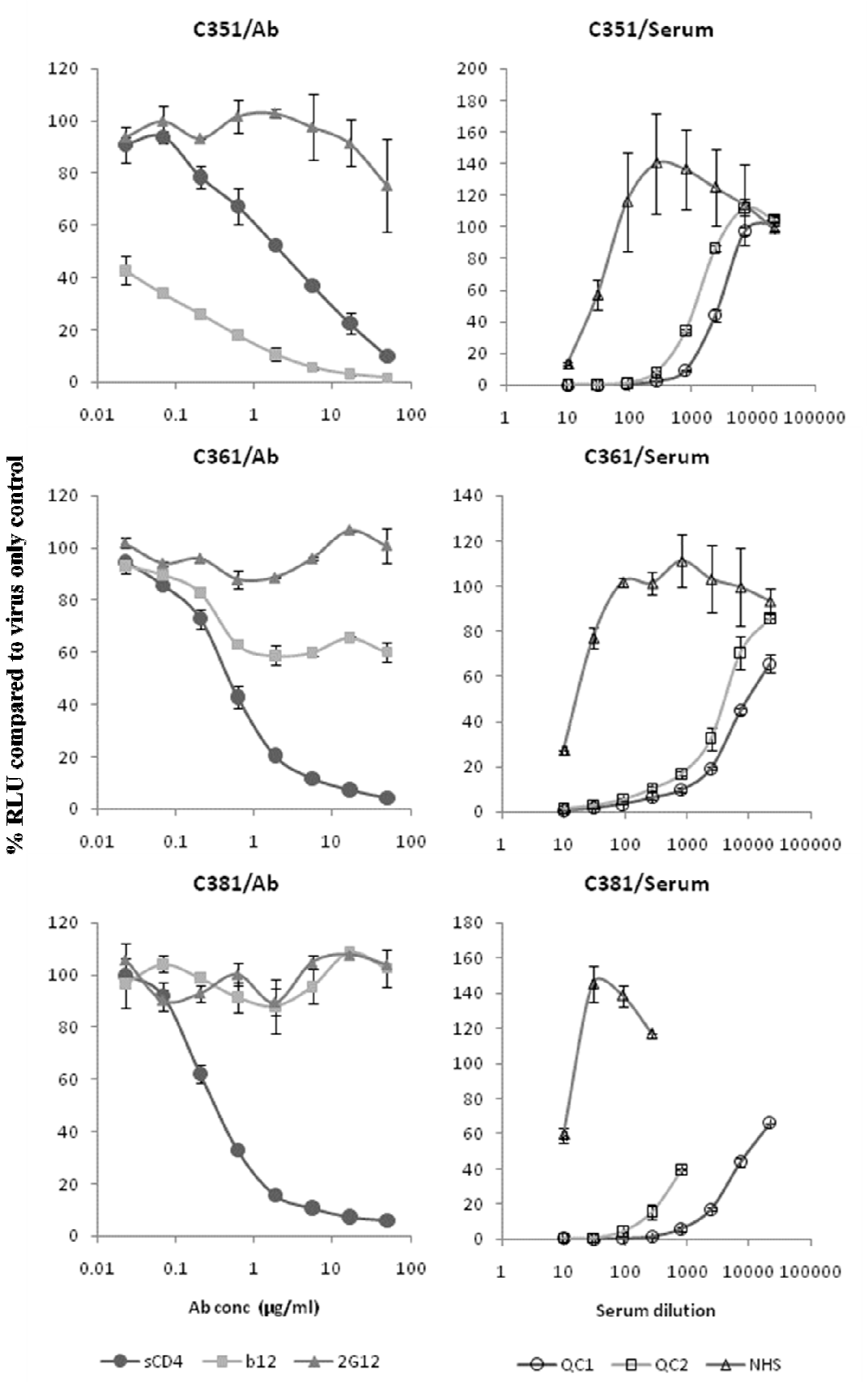

Figure 3-7. (cont. on next page)

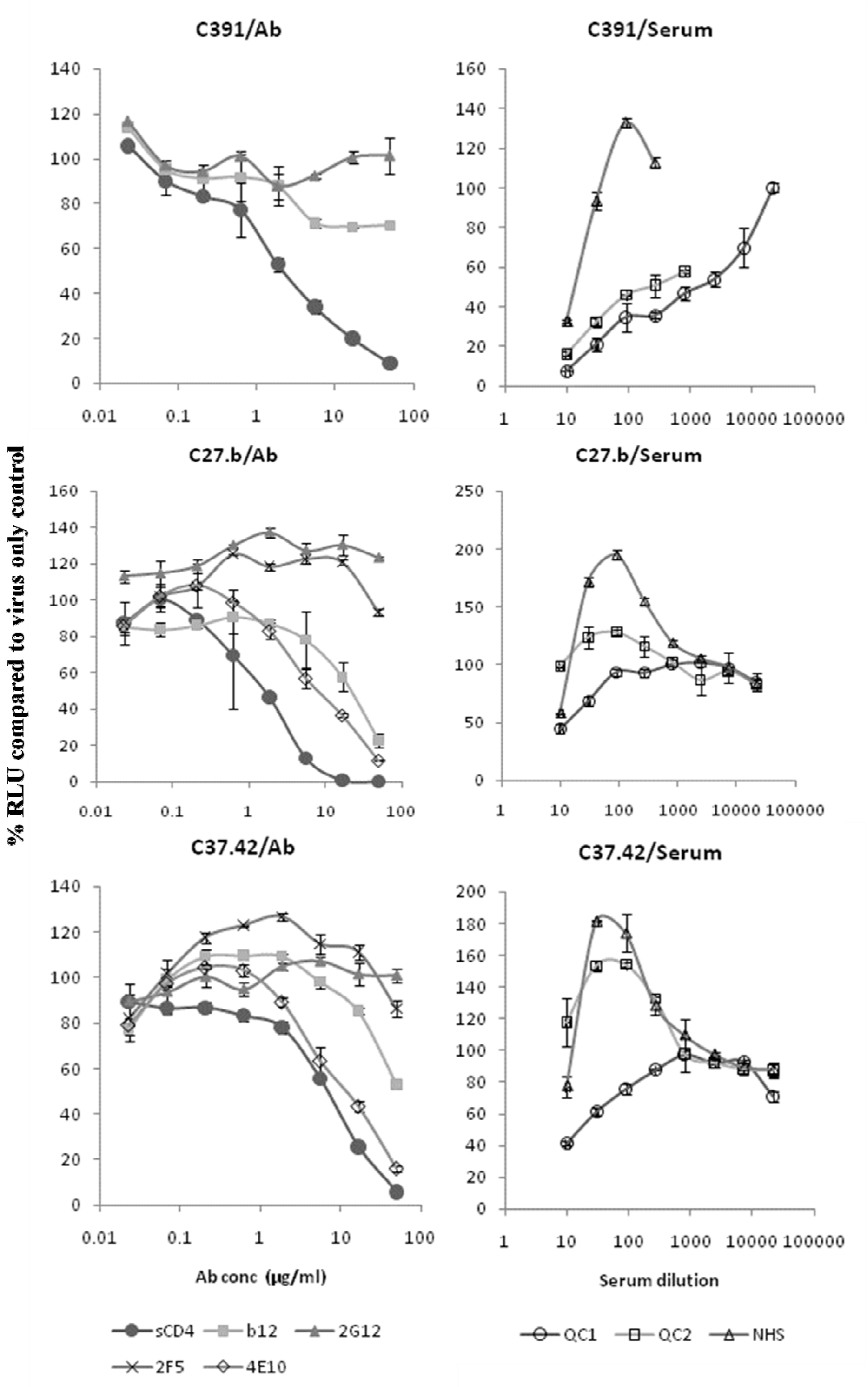

Figure 3-7. (cont. on next page)

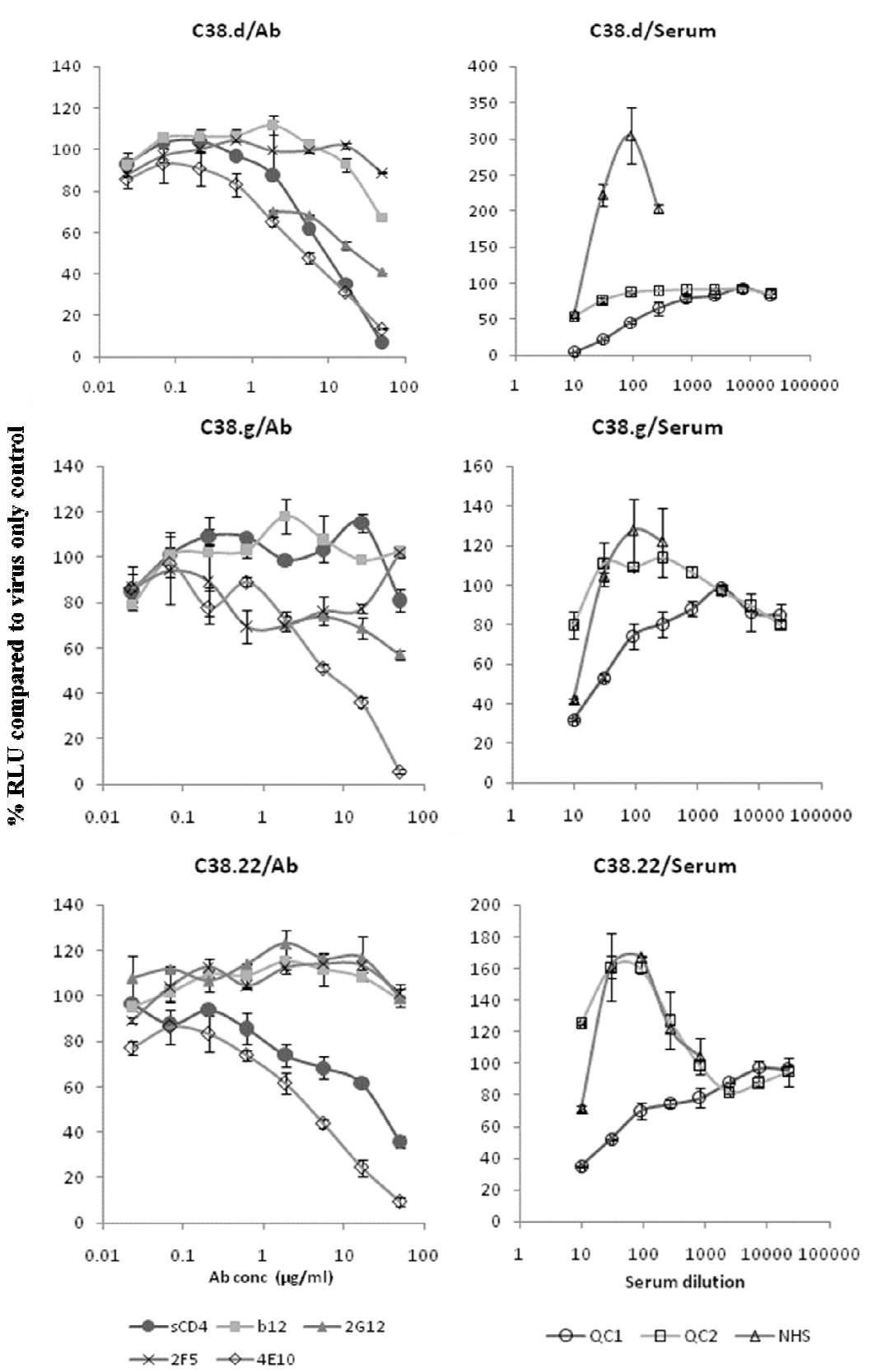

Figure 3-7. (cont. on next page)

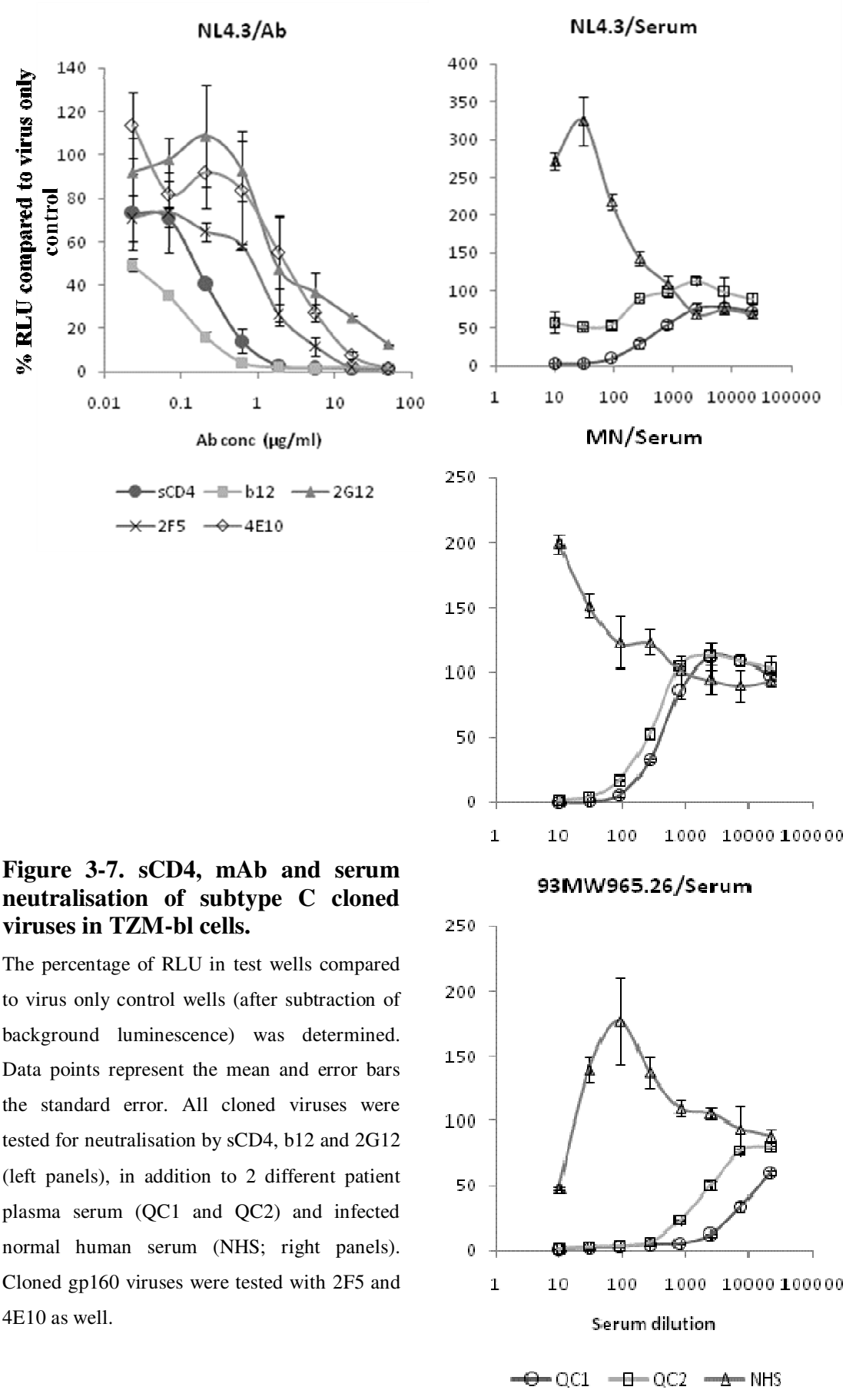

Figure 3-7. sCD4, mAb and serum neutralisation of subtype C cloned viruses in TZM-bl cells.

The percentage of RLU in test wells compared to virus only control wells (after subtraction of background luminescence) was determined. Data points represent the mean and error bars the standard error. All cloned viruses were tested for neutralisation by sCD4, b12 and 2G12 (left panels), in addition to 2 different patient plasma serum (QC1 and QC2) and infected normal human serum (NHS; right panels). Cloned gp160 viruses were tested with 2F5 and 4E10 as well.

Table 3-9. Neutralisation profiles

	IC_{50} in TZM-bl cells[b]							
	Conc. (µg/ml)					Reciprocal serum dilution		
Virus clone[a]	**sCD4**	**IgG1b12**	**2G12**	**2F5**	**4E10**	**QC1**	**QC2**	**Normal pool**
NL4.3	0.15	0.02	1.9	0.8	2.3	517	84	<10
MN	nd	nd	nd	nd	nd	345	169	<10
93MW965.26	nd	nd	nd	nd	nd	13,723	1,590	10
CA6	>50	6.2	>50	nd	nd	76	<10	14
CB7	1.0	0.19	>50	nd	nd	86	<10	<10
C202	5.3	>50	>50	nd	nd	301	115	11
C222	1.1	>50	>50	nd	nd	370	65	<10
C250	32	1.8	>50	nd	nd	528	382	24
C261	1.1	>50	>50	nd	nd	5,667	665	12
C27.b*	1.5	19	>50	>50	8.2	12	<10	<10
C281	>50	>50	>50	nd	nd	216	38	21
C291	0.5	>50	>50	nd	nd	6,166	1,650	19
C344	25	>50	>50	nd	nd	3,798	1,103	22
C351	2.3	<0.02	>50	nd	nd	2,147	358	16
C361	0.49	>50	>50	nd	nd	12,204	2,251	18
C37.42*	6.9	43	>50	>50	11	13	<10	<10
C381	0.32	>50	>50	nd	nd	9,410	1,187	<10
C38.d*	9.3	>50	25	>50	4.0	99	19	11
C38.g*	>50	>50	>50	>50	7.7	44	<10	11
C38.22*	29	>50	>50	>50	7.5	32	<10	<10
C391	2.3	>50	>50	nd	nd	1,146	180	14
GMT[c]	5.3			>50	7.6	301	65	11

[a] Clones marked with an asterisk are gp160 *env* clones

[b] Neutralisation values are the concentration or dilution at which RLU were reduced by 50% compared to virus control wells. QC1 and QC2 are serum samples from individuals infected with subtype B HIV-1.

[c] Geometric mean titre against the 18 replication competent subtype C viruses containing cloned primary isolate Env.

3.9 Discussion

3.9.1 Genotype of cloned subtype C envelopes

The sequences of the cloned subtype C envelopes showed variations in the lengths of the variable loop regions, as well as variations in the positions and number of PNLG sites, thus demonstrating the heterogeneity of these cloned subtype C envelopes. Variations in these regions have been shown to mask epitopes and influence neutralisation patterns (Cao et al., 1997; Reitter et al., 1998; Wei et al., 2003; Wyatt et al., 1995). In this study sample, the V3 loop was relatively conserved, followed by a region of 34 amino acids downstream that was highly variable. This is similar to other observations on newly transmitted subtype C and R5 subtype B viruses (Engelbrecht et al., 2001; Milich et al., 1997; Ping et al., 1999). As with Li's subtype C envelope reference panel, all the viruses in this study contain a highly conserved PNLG near the start of the V3 loop (position 301, HXB2 numbering) that is known to mask V3 epitopes on subtype B viruses (Back et al., 1994; Schonning et al., 1996). Based on the phylogenetic studies, many of the cloned viruses in this study clustered into separate groups from those in the subtype C reference strain panel.

3.9.2 Coreceptor usage of cloned subtype C envelopes

All the subtype C envelopes in this study used CCR5 as a coreceptor, as is typical of newly transmitted subtype B and C viruses regardless of the stage of infection (Cecilia et al., 2000; Connor et al., 1997b; Li et al., 2006a; Ping et al., 1999; Williamson et al., 2003; Zhang et al., 1993). Previous findings had shown that CCR3 usage is prevalent in *in vivo* replicating samples; such as brain tissue samples (Agrawal et al., 2009; He et al., 1997; Peters et al., 2004), but was diminished after

PBMC coculture (Aasa-Chapman et al., 2006a). The panel of subtype C envelopes which were cloned directly from plasma samples in this study had a median CCR3 usage of 3.9%, compared to a median of 0.05% in the subtype C reference panel that were cloned from a mixture of plasma, uncultured and cocultured PBMC. This observation has therefore validated our previous findings with regards to diminished CCR3 usage in PBMC cocultures.

All previous studies on CCR3 usage were performed predominantly on subtype B isolates. Coreceptor use was found to be dependent on the third variable (V3) region of gp120 (Choe et al., 1996), and the determinants were mapped to three residues on gp120 (Aasa-Chapman et al., 2006b). However, the R3/R5 subtype C viruses in this study did not possess all the requisite amino acid residues needed for CCR3 use. R3/R5 subtype C viruses possibly possess a different conformational pattern for coreceptor binding compared to subtype B viruses.

In these subtype C patient samples, we observed that CCR3 usage is much lower than previous studies performed on subtype B samples. To corroborate this finding, the CCR3 usage of the envelopes in the subtype B, C, CRF07_BC and CRF02_AG standard reference panels was investigated. The use of CCR3 as a coreceptor to enter cells was 100-fold more in subtype B envelopes than in the other subtypes and recombinant forms. The reasons as to why subtype B envelopes are more prone to use CCR3 as a coreceptor than other subtypes should be further investigated, and the amino acid residues required for CCR3 usage can be determined in future studies. These results may have implications in the development of CCR5 entry inhibitors and provide insight into alternative entry pathways for each HIV- subtype.

While a manuscript was being prepared with the results of this chapter, a paper describing very similar results was very recently published. Nedellec *et al.* reported identical results where they found increased CCR3 usage in subtype B viruses, and lower incidence of CCR3 usage in subtypes A, C and D viruses (Nedellec et al., 2009). They have also used the NP2 cell assay to determine coreceptor usage. However, instead of cloning HIV-1 envelopes into replication competent chimeric viruses, they have used pseudotyped viruses.

3.9.3 Neutralisation profile of cloned subtype C envelopes

The neutralisation profile of the subtype C viruses in this study is slightly more resistant to neutralisation by the sCD4 and the common bcnAbs than Li's subtype C reference panel. For example, the IC_{50} GMT titre with the mAb 4E10 was nearly a 10-fold higher for the viruses in this study. Li *et al.* (Li et al., 2006b) noted a trend in which HIV-1 envelopes cloned from cocultured PBMC viruses were more sensitive than those cloned from plasma viruses. None of the cloned viruses in this study was obtained from PBMC cultures. The envelopes in this study were all cloned directly from patient plasma samples and can be classified as Tier-2 viruses as they were more resistant to neutralisation by antibodies (Mascola et al., 2005).

Although some of the isolates in this study were phylogenetically similar to the subtype C reference panels, a few of them (in particular C261, C291, C351, and CB7) clustered into distinct groups. These isolates can be used as further strains for use in testing the neutralisation breadth of a candidate antibody or for vaccine studies, and were used in our studies on llama antibody fragments (termed VHH) which target the CD4 binding site of gp120 (Forsman et al., 2008) and also in the investigation of the neutralisation breadth of serum samples in East London patients.

3.9.4 Summary

In summary, the *env* genes were amplified from 15 patients with subtype C HIV-1 from a few countries in Africa and in the UK. These were inserted into HIV-1 vectors to create replication-competent viruses, and 18 of these were characterised here. Some of the chimeric viruses in this study were more resistant to neutralisation and were phylogenetically distinct from Li's subtype C reference panel. These cloned subtype C viruses are therefore useful to supplement the standard reference strains for use in characterising the neutralisation potency of antibodies. These viruses were used to study the neutralisation breadth of VHH in Chapter 5. This is also the first study on CCR3 usage by subtype C viruses. The use of CCR3 as a coreceptor was found to be less prevalent in subtype C viruses, and indeed in the other subtype reference panels, than in the commonly studied subtype B strains. Even in cases where CCR3 use was determined, the required amino acid residues required for CCR3 interaction differed from those for subtype B envelopes.

4 Anti-gp41 VHH

4.1 Introduction

Of the four broadly cross-neutralising monoclonal antibodies (bcnAbs) described to date, two of them target the gp41 portion of the HIV-1 envelope glycoprotein (refer to Introduction in Section 1.2.4.2). These are the human mAbs 2F5 and 4E10, which were generated from individuals naturally infected with a subtype B HIV-1 strain (Buchacher et al., 1994; Muster et al., 1993; Stiegler et al., 2001; Zwick et al., 2001). While 4E10 is able to neutralise most subtype C strains of HIV-1, 2F5 is often ineffective against subtype C HIV-1 as it lacked an epitope on the MPER of subtype C gp41 (Li et al., 2006b). This finding is confirmed by the results as detailed in Chapter 3. The gp41 portion is also more conserved than gp120, possibly making gp41 a better target than gp120 when it comes to generating broad neutralising antibodies, although such instances have been extremely rare (refer to Section 1.2.4). As subtype C HIV-1 accounts for more than half of the infections in the world and is currently infecting more people than any other subtype (Hemelaar et al., 2006), there is a need to focus on generating and characterising neutralising antibodies that are effective against subtype C HIV-1.

Although the bcnAbs described to date were all obtained from HIV-1 infection in humans, rather than from immunisation (Pantophlet and Burton, 2006), we decided to exploit the non-conventional immune system of the camelids to generate novel antibodies against a subtype C envelope. Camelid VHH are small in size (about 15 kDa), have a preference for cleft recognition due to their long protruding CDR3 loops (De Genst et al., 2006; Vu et al., 1997), and are easily expressed in large quantities using yeast cultures (van de Laar et al., 2007). These qualities make VHH

a good candidate for use as a microbicide (refer to Chapter 1 for detailed introduction).

This chapter will describe the selection of anti-gp41 VHH isolated from a llama immunised with recombinant trimeric CN54 gp140 derived from a subtype C HIV-1. The selection of anti-gp120 VHH from the same llama was performed by Anna Forsman and described elsewhere (Forsman et al, 2008). The anti-gp41 VHH isolated in this study were then characterised for their ability to neutralise HIV-1 and an attempt at epitope mapping was made.

4.2 Llama 48 immunisation and phage library construction

The work described in this section was undertaken by staff at Ablynx NV, Ghent, Belgium. Llama immunisations were carried out as part of the European Microbicide Project (EMPRO). One llama (L48) was immunised with recombinant gp140 from the HIV-1 CN54 strain, which is a subtype B'/C recombinant (CRF07_BC) that was originally isolated in China, which is entirely subtype C in envelope apart from the very N-terminus (Rodenburg et al., 2001; Su et al., 2000). This strain was chosen as the immunogen as it was a non-subtype B virus and the recombinant glycoprotein (Chen et al., 2005b) was available in large amounts within the EMPRO consortium.

The llama received six intramuscular injections at weekly intervals (as described in Section 2.6.1). The humoral immune response was evaluated in ELISA on gp120 or gp140 coated plates, using serum samples from day 0 and 43 post immunisation, and shown in Figure 4-1. The ELISA showed the presence of anti-HIV-1 envelope antibodies in the llamas, thus indicating successful immunisation. The immune response against gp140 was almost twice as high as gp120, indicating a robust recognition of the gp41 region.

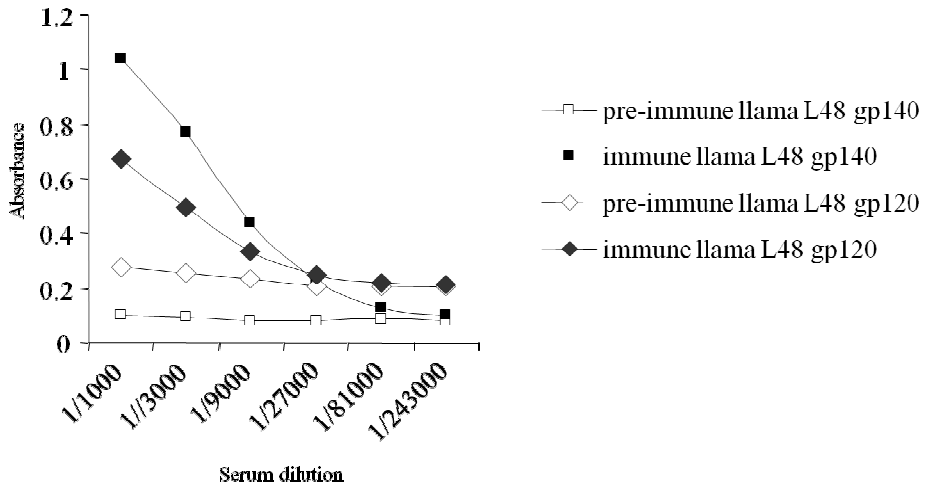

Figure 4-1. Anti-envelope antibody response in llama 48.

Llama 48 (L48) was immunised with recombinant CN54 gp140, which was introduced intramuscularly in 6 weekly doses. Blood samples were taken on days 0 and 43 post-immunisation and the serum anti-gp120 and anti-gp140 response was evaluated in ELISA on solid-phase CN54 gp120 and CN54 gp140. The immune response against gp140 was almost twice as much than in gp120, indicating a robust recognition of the gp41 region.

To enable the recovery of VHH repertoires of extensive diversity, B cells were isolated from both blood and lymph nodes from the immunised llamas. Blood samples were collected at day 39 and 43 post immunisation. In addition, a lymph node biopsy was taken at day 39. For each llama, total RNA was isolated from peripheral blood lymphocytes as well as lymph node lymphocytes, and subsequently pooled. Oligo dT-primed cDNA was synthesised and the VHH repertoire was amplified and cloned into the pAX050 phagemid vector, followed by transformation into *E. coli* TG1 cells. The library derived from llama 48 was found to contain 1.4×10^7 clones, and 62% were found to contain an insert of the correct size. To obtain recombinant bacteriophages expressing the VHH as fusion proteins with the phage gene III product, the transformed TG1 cells were grown to logarithmic phase and then infected with helper phage M13KO7. The phage particles were precipitated with polyethylene glycol to remove free VHH.

4.3 Characterisation of recombinant gp41 and gp41Δ with monoclonal antibodies

The recombinant gp41 and gp41Δ used in this study were obtained from BioDesign, Inc and the Centralised Facility for AIDS Reagents (CFAR) respectively. The recombinant gp41 was made from constructs based on IIIB HIV-1 (subtype B) and contains the full length gp41 sequence, whereas recombinant gp41Δ was made from MN HIV-1 (subtype B) and contains only the ectodomain region of gp41. Both recombinant proteins are monomeric.

To determine the integrity and presence of essential epitopes, these antigens were probed with various mAbs in ELISAs. Most important of these were the human mAbs 2F5 and 4E10, which were used during the panning process for the selection of VHH from the phage library (described in the next section). The other two human mAbs were 50-69D, which recognises a conformational dependent epitope on gp41, and 98-6D, which recognises a linear epitope (aa 644-663) on gp41. The recombinant proteins were coated overnight at a concentration of 1 µg/ml, and probed with serial dilutions of the mAbs. ELISAs were carried out as described in Section 2.4.13. The ELISA results are shown in Figure 4-2, which confirms the presence of both the 2F5 and 4E10 epitopes on gp41 and gp41Δ. Additionally, the mAbs 50-69D and 98-6D were able to bind to gp41, thus demonstrating that the recombinant protein was properly folded.

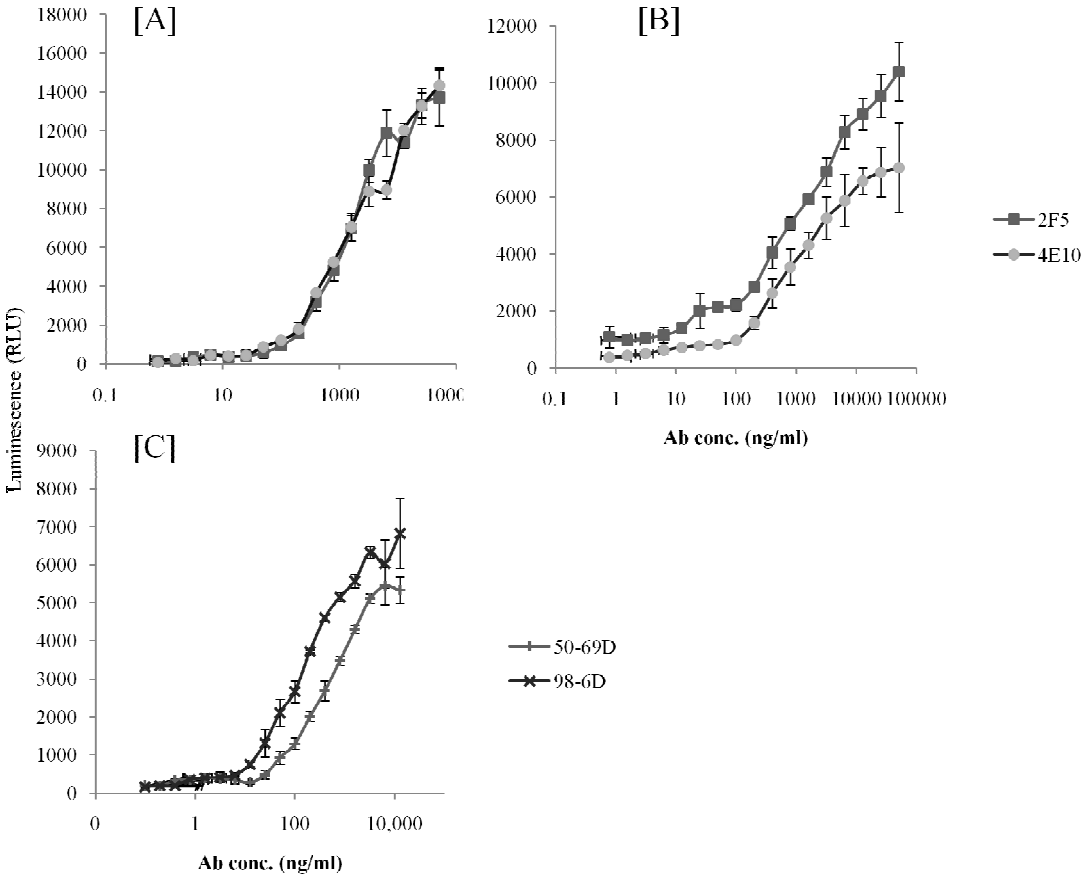

Figure 4-2. Binding of mAbs 2F5, 4E10, 50-69D and 98-6D to gp41 and gp41Δ in ELISA

Maxisorp plates were coated overnight with gp41 [A, C] and gp41Δ [B] at a concentration of 1 µg/ml, and probed with serially diluted mAbs 2F5, 4E10 [A, B] and 50-69D, 98-6D [C]. These were then detected with an AP-conjugated goat anti-human IgG antibody. Chemiluminescence was detected with the addition of substrate and luminescence readings (in RLU) were normalised to readings obtained from blank control wells. The results showed that all 4 mAbs bind to gp41 and, 2F5 and 4E10 bound to gp41Δ in a dose-dependent manner. The 2F5 and 4E10 epitopes were therefore present on the recombinant gp41, and the proteins were correctly folded.

4.4 Panning for anti-gp41 VHH from phage libraries

This section describes the panning for anti-gp41 VHH using phage display. This process was divided into three sections, representing three different strategies to select for VHH that target gp41. The first method employed trypsin and glycine to

elute out all phages that bind to gp41. The second method employed the mAbs 2F5 and 4E10 in competitive elution to dislodge phages that recognised the MPER of gp41. The final method involved the panning of phages that recognised the gp41 portion of trimeric gp140, with the prior removal of phages that recognised gp120 by preincubating the phage library with gp120.

4.4.1 Panning of phage libraries on gp41 with trypsin and glycine elutions

VHH targeting gp41 of the HIV-1 envelope were selected by panning the phage library for 1 h on recombinant gp41, which were coated onto plates at concentrations of 5, 1, and 0.2 µg/ml. The full-length and truncated recombinant gp41s were used for selections. Blank control wells containing PBS only were also included. Bound phages were washed extensively with PBS to remove unspecific binding of phages, and the phages were eluted with either trypsin or glycine in parallel. Treatment with trypsin will elute out all phages bound to gp41 through the digestion of the gene III product, whilst treatment with glycine will elute out phages due to low pH changes. The eluted phages were harvested and titrated on *E. coli* TG1 cells. Selections where a larger number of clones were eluted out compared to blank control wells were taken forward to a second round of selections. In the second round of selections, only 1 and 0.2 µg/ml concentrations of gp41 and gp41Δ were used. This was based on the results after the first round of selections where 5 µg/ml of antigen was found to produce an excessive number of eluted phages. After two rounds of panning, selections where a dose-dependent enrichment of clones was observed were chosen and individual VHH were expressed. (Full methods described in Section 2.6.2)

Figure 4-3 shows the outcome of the titration after the selections on gp41 and gp41Δ. In general, no correlation was observed between the number of clones eluted by trypsin or glycine, nor with the use of either gp41 or gp41Δ. Selections where a larger number of clones were eluted compared to blank controls were taken forward to a second round of panning, as marked by the block arrows in Figure 4-3 [A]. These were all selected on 0.2 µg/ml of gp41 and gp41Δ. Phages eluted in the blank controls were able to bind unspecifically to the wells and represent the background noise levels. The outputs from the trypsin and glycine elutions that were selected on gp41 and gp41Δ were combined (denoted with 'Φ41a' and 'Φ41b' respectively in Figure 4-3) and used in the first round of panning in the next section.

The titration of the eluted phages after the second round of panning was similarly carried out. All the selections again gave a greater number of eluted phages compared to blank controls, as shown in Figure 4-3 [B]. Outputs from the 1 µg/ml gp41 and 0.2 µg/ml gp41Δ produced a good number of eluted phages and were brought forward to the next stage of picking and expression of individual clones.

From each of the outputs, the eluted phage repertoires were allowed to infect *E. coli* TG1 cells and plasmids containing the VHH gene fragments were harvested through miniprep. The plasmids were digested with *BstE*II and *Sfi*I and inserted into the pAX051 expression vector before transforming back into electrocompetent TG1 cells (refer to methods in Section 2.6.3). Ninety-six clones were picked from each output and the VHH harvested from the periplasmic fractions. The clones were also DNA fingerprinted with *HinF*I to determine the heterogeneity of the selected clones. This is shown in Figure 4-4.

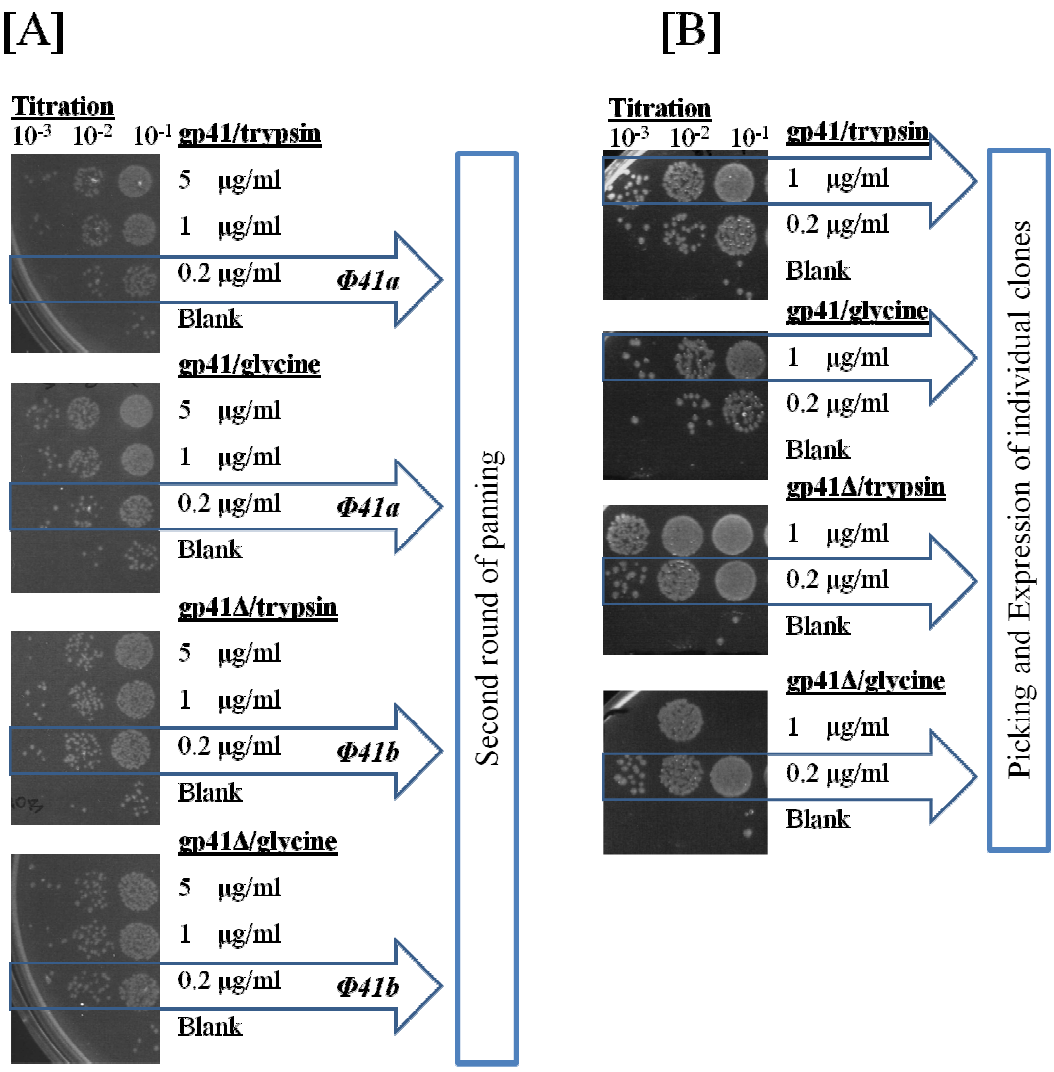

Figure 4-3. Titrations of eluted phage after trypsin and glycine elutions

The phage library was panned on immobilised gp41 and gp41Δ of different concentrations, and bound phages were eluted with either trypsin or glycine. In all cases, the number of phages eluted was higher than in the blank control wells. There was no visible difference between selections carried out on gp41 or gp41Δ, nor elutions performed using either trypsin or glycine. [A] After the titration of eluted phages from the first round of selection, the outputs from the 0.2 µg/ml concentration was brought forward to a second round of panning, as shown by the block arrows. The phage outputs from both trypsin and glycine elutions on gp41 and gp41Δ (denoted with 'Φ41a' and 'Φ41b' respectively) were used in the first round of panning for the next section. [B] After the titration of eluted phages from the second round of selection, the outputs from the 1 µg/ml gp41 and 0.2 µg/ml gp41Δ were brought forward to the next stage for the picking and expression of individual clones. There was an error in the serial dilution of the outputs of 1 µg/ml gp41Δ/glycine (bottom panel of [B]) which gave zero titres but the outputs of 0.2 µg/ml gp41Δ/glycine were fine.

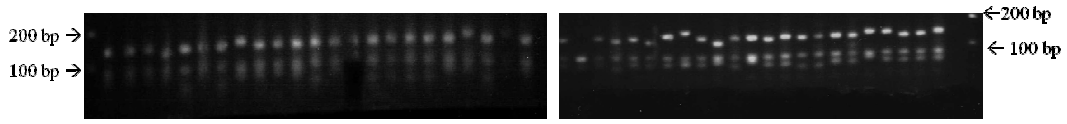

Figure 4-4. DNA fingerprint of selections from trypsin and glycine elutions

The final outputs from the selections were subcloned into expression vectors for VHH production. Ninety-six different clones were picked for VHH expression, and their DNA was fingerprinted by digestion with *HinF*I. These were then separated on agarose gel electrophoresis and visualised. The heterogeneity of the band sizes show that the clones selected were fairly diverse. A representative sample of the digested clones is shown here.

4.4.2 Panning of phage libraries on gp41 with competitive elution by the monoclonal antibodies 2F5 and 4E10

In the previous section, all phages that bound to gp41 were eluted out with trypsin or glycine. In this section, selections using a similar setup were carried out, except that phages were eluted out with competitive elution by the human mAbs 2F5 and 4E10. A high concentration (100 µg/ml) of the mAbs was used to saturate the binding site on the gp41 antigen and potentially dislodge phages that recognise the MPER of gp41. The negative (or irrelevant) control used was the human mAb Remicade, which is an anti-TNFα IgG_1, also at a concentration of 100 µg/ml. As before, both gp41 and gp41Δ were used as the selecting antigens, but at concentrations of 1 and 0.2 µg/ml for the first round of panning. In addition to the phage library from llama 48, the outputs from the first round of selections on gp41 and gp41Δ that were obtained in the previous section (denoted with 'Φ41a' and 'Φ41b' in Figure 4-3) were also used as starting libraries. These libraries were enriched with phages that can bind to gp41 after one round of panning.

As before, the eluted phages were harvested and titrated on *E. coli* TG1 cells. The titrations after the first round of selections are shown in Figure 4-5 [A]. The elutions with the original phage library from llama 48 gave better titrations than those phage

libraries obtained from the outputs of the previous selections (Φ41a and Φ41b), as a dose-dependent output could be seen. The indistinct titration results could be due to the 5-fold dilutions that were carried out, instead of the 10-fold dilutions used previously. The outputs of the selections on both gp41 and gp41Δ were similar and could not be easily differentiated, although there was a slight dose dependent reduction in eluted phages from those selected from Φ41a and Φ41b. All the outputs from 0.2 µg/ml of gp41Δ were brought forward to the second round of panning, and the outputs from gp41 were discarded.

Figure 4-5 [B] shows the titrations of the eluted phages from the second round of selections. As in the first round of panning, the eluted phages were indistinct from that obtained with Remicade as control. A dose dependent reduction in eluted phages corresponding to a reduction in antigen concentration was also not present. Nevertheless, the outputs from 0.2 µg/ml of gp41Δ were carried forward to the next stage of picking and expression of individual clones.

As before, the eluted phage repertoires from each of the final outputs were allowed to infect *E. coli* TG1 cells and the plasmids containing the VHH gene fragments were harvested through miniprep. The plasmids were digested with *BstE*II and *Sfi*I and inserted into the pAX051 expression vector before transforming back into electrocompetent TG1 cells (refer to methods in Section 2.6.3). Ninety-six clones were picked from each output and the VHH was harvested from the periplasmic fractions. The clones were also DNA fingerprinted with *HinF*I to determine the heterogeneity of the selected clones, and shown in Figure 4-6. Compared to the previous selections, VHH obtained from 2F5 and 4E10 elutions were less heterogeneous.

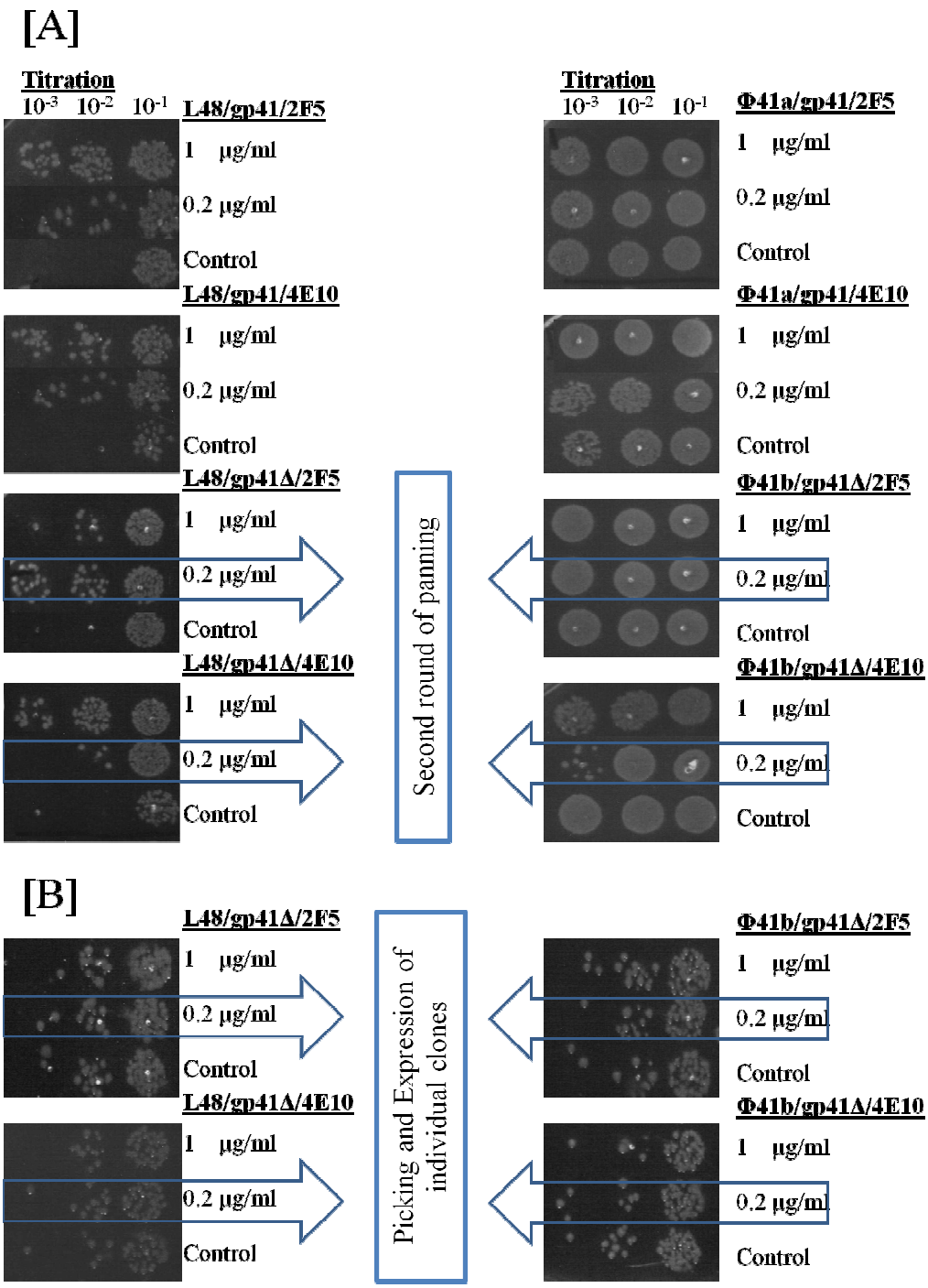

Figure 4-5.Titrations of eluted phage after 2F5 and 4E10 elutions

The phage libraries from llama 48 and outputs, Φ41a and Φ41b from the previous selections, were used as inputs in the first round of panning on immobilised gp41 and gp41Δ. Bound phages were eluted with 2F5, 4E10 and Remicade as control. In contrast to the previous selections, these selections using 2F5 and 4E10 gave high background levels with Remicade. A dose dependent dilution was also not distinct. [A] After the first round of selections, only elutions from 0.2 µg/ml of gp41Δ were carried forward to the second round of panning. [B] After the second round of selections, the elutions from 0.2 µg/ml of gp41Δ were brought forward to the next stage for the picking and expression of individual clones.

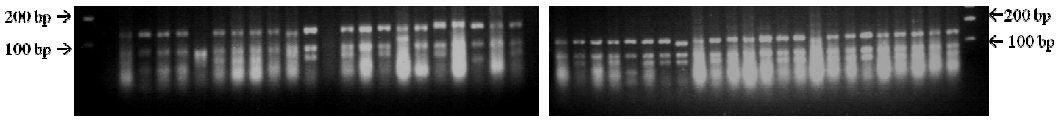

Figure 4-6. DNA fingerprint of selections from 2F5 and 4E10 elutions

The final outputs from the selections were subcloned into expression vectors for VHH production. Ninety-six different clones were picked for VHH expression, and their DNA was fingerprinted by digestion with *HinF*I. These were then separated on agarose gel electrophoresis and visualised. The digested fragments show only slight variations in the band sizes, thus suggesting that the clones obtained in these selections were less diverse than those obtained in the previous selections. A representative sample of the digested clones is shown here.

4.4.3 Panning of phage libraries on CN54 gp140 with gp120 subtraction

In this last method to select for anti-gp41 VHH, the phage library from llama 48 was allowed to incubate with immobilised CN54 gp140 (at a concentration of 0.5 µg/ml) on a plate. The phage library was first diluted 100- and 1000-fold to produce an optimal concentration of phages, and then allowed to preincubate with 10 µg/ml of CN54 gp120 for 1 h to mop up phages that recognise gp120 before putting onto the gp140 coated plates. Another plate without gp120 preincubation was also run in parallel. Blank control wells without gp140 were also included. The bound phages were eluted with trypsin, as previous experience showed that this gave a more complete eluate.

The eluted phages were then titrated on TG1 cells as before. Figure 4-7 [A] shows the titration after the first round of selection. The number of eluted phages in the 1/100 dilutions were more than those in the 1/1000 dilutions. However, they were not significantly more than the blank control wells and the presence of gp120 did not seem to affect the number of phages eluted. Nevertheless, the eluted phages from

both the 1/100 dilution with and without gp120 preincubation were moved to the second round of panning. For consistency in the second round of panning, the outputs from the panning without gp120 preincubation were also brought forward. Figure 4-7 [B] shows the titration after the second round of selection. There was a dose dependent reduction in the number of eluted phages corresponding to the dilution factor of the input phage, but the background levels were still very high. The number of eluted phages between those with gp120 preincubation appear to be similar to those without gp120 preincubation. Both the outputs from the plate with gp120 preincubation were brought forward for picking and expression of individual clones.

As before, 96 clones were picked from each output and the VHH were harvested from the periplasmic fractions. The clones were also DNA fingerprinted with *HinF*I to determine the heterogeneity of the selected clones, and shown in Figure 4-8. The selected clones appear to be fairly heterogeneous, according to the variation in sizes of the DNA bands.

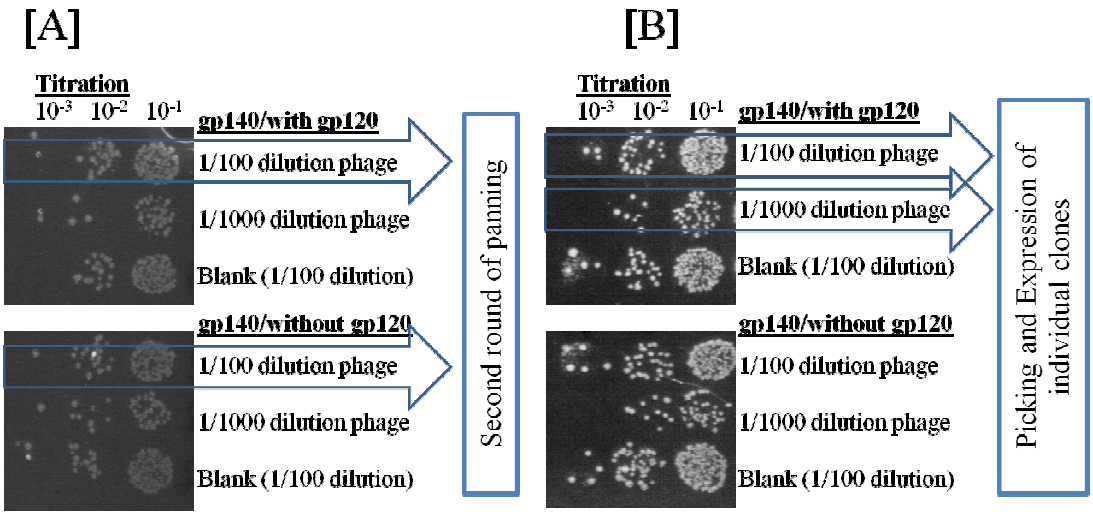

Figure 4-7. Titrations of eluted phage after panning on gp140

The phage library (of two different dilutions) was panned on immobilised CN54 gp140 after preincubation with CN54 gp120. Phages bound to gp120 will not bind to gp140, thus leaving phages that recognise gp41 to bind to the immobilised gp140. [A] After the first round of panning, the number of eluted phages was less with an increase in dilution factor. However, background levels were high as seen in the blank wells. Phages eluted in the 100-fold dilution were brought forward to the next round. Phages eluted in the wells without gp120 preincubation were also brought forward in the second round of panning for consistency. [B] The results of the titration in the second round of panning were similar to the first round. There was a dose-dependent reduction in eluted phages, but background levels were also high. Elutions from the wells with gp120 preincubation were brought forward to the next stage of picking and expression of individual clones.

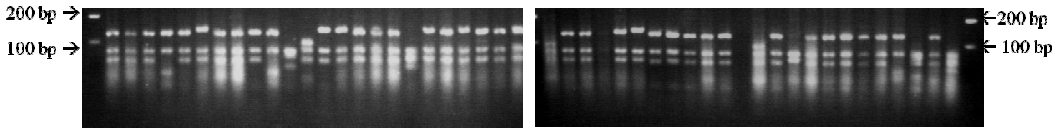

Figure 4-8. DNA fingerprint of selections from gp140 panning with gp120 subtraction

The final outputs from the selections were subcloned into expression vectors for VHH production. Ninety-six different clones were picked for VHH expression, and their DNA was fingerprinted by digestion with *HinF*I. These were then separated on agarose gel electrophoresis and visualised. The digested fragments show slight variations in the band sizes, thus suggesting that the clones obtained in these selections were fairly heterogeneous. A representative sample of the digested clones is shown here.

4.4.4 Summary of anti-gp41 VHH selections

From each selection output, 96 different clones were picked and grown in 96-well microtitre plates. A total of 10 different outputs were obtained after the selection process. The phage library expressing the VHH repertoire from llama 48 (ΦL48 in Figure 4-9) was the starting material for 8 of the selections, and 2 of the selections started off with the outputs from the first round of panning on gp41 (Φ41b in Figure 4-9). Two of the selections were panned on gp140, and another 2 were panned on gp41. The rest of the 6 selections were panned on gp41Δ. Six of the selections were eluted with either trypsin or glycine, whilst 4 of the selections were eluted by using 2F5 and 4E10. A schematic overview of the whole selection process is shown in Figure 4-9.

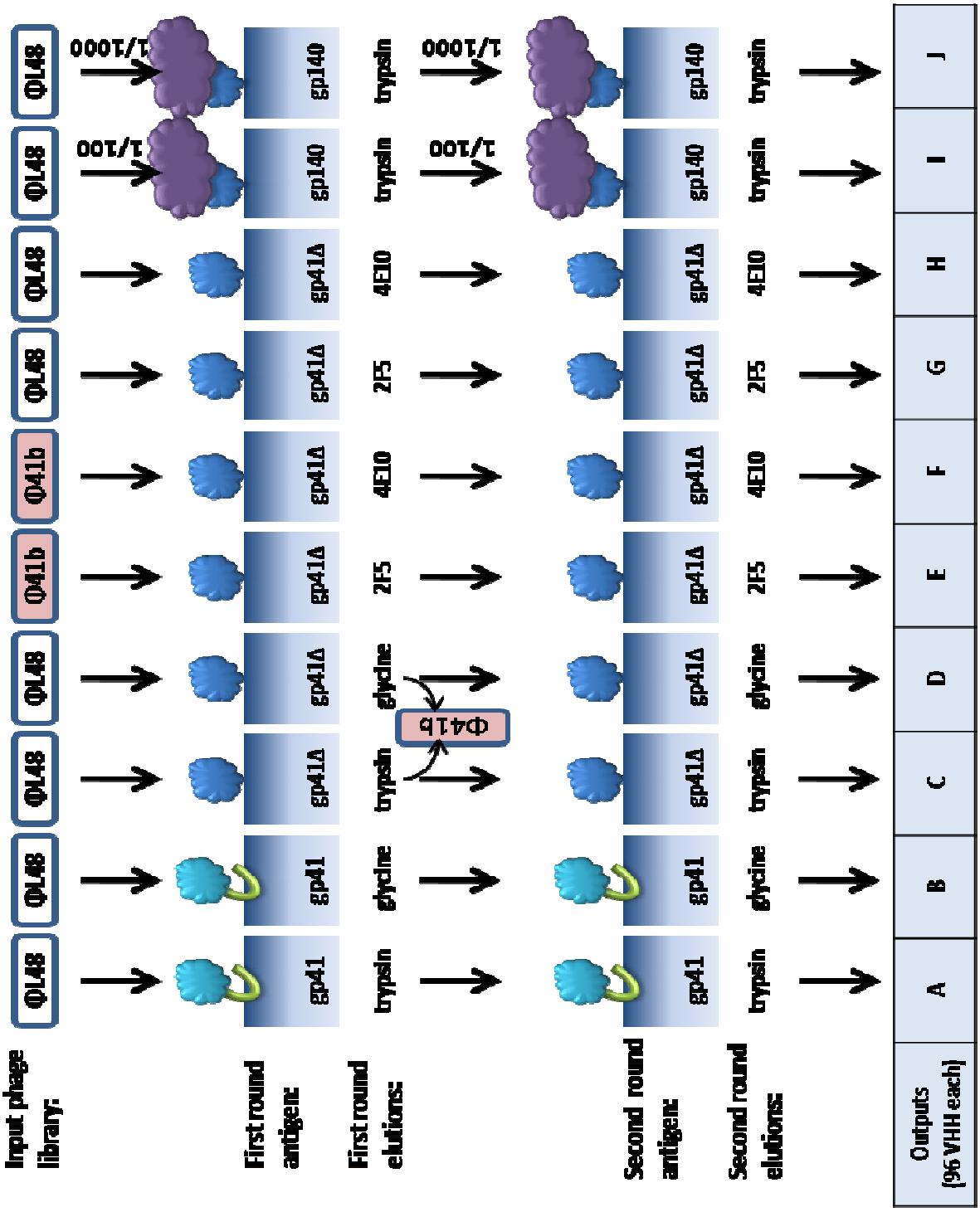

Figure 4-9. Schematic showing the overview of the selection process

4.5 Screenings of the selected anti-gp41 VHH

The 960 individual clones selected in the previous sections were grown overnight in *E. coli* TG1 cells in 1 ml of medium in 96 deep-well plates. For each clone, approximately 90 µl of *E. coli* periplasmic extract containing the expressed VHH was prepared and screened for its ability to bind to gp41 and to neutralise HIV-1. The methods are described in Section 2.6.3.

4.5.1 Screening of anti-gp41 VHH in ELISA

The first step in the screening of the selected anti-gp41 VHH was to test their ability to bind to gp41 in ELISA (full methods described in Section 2.4.13). These were assayed on plates precoated with gp41Δ. As only a limited amount of periplasmic extract containing VHH was produced, there was enough material to carry out the ELISA only once, but in duplicate. Two µl of periplasmic extract from each clone was diluted into 48 µl of PBS, and these were introduced into each well of the maxisorp plate in duplicate. Each VHH clone was also tested in a blank control well which was precoated with only PBS. Bound VHH were detected with a mouse anti-c-myc mAb, and then with an AP-conjugated goat anti-mouse IgG antibody. Chemi-luminescence was detected with the addition of substrate, and the relative light units (RLU) for each clone were averaged and the blank control values subtracted. These were then plotted against the position numbers of the clones on the 96-well plates (refer to Figure 4-10).

The graphs [A] to [J] correspond to the outputs shown in the schematic diagram (Figure 4-9). Many of the VHH clones were found to bind to gp41 very strongly. To establish the number of positive clones from each output, a cut-off RLU value was determined. This value was defined as 2 standard deviations above the average blank

control values on each plate, and worked out to have an average RLU value of 1500. In cases where the blank control value was very low, a minimum RLU value of 1500 was imposed.

Table 4-1 gives the summary of the results of the ELISA screening of the selected anti-gp41 VHH. From the outputs, an average of 84 out of 96 clones on each plate was found to be positive for binding to gp41, or an 88% success rate.

Table 4-1. Summary of results from ELISA screening

Output[a]	Number of positive clones	Percentage value[b]
A	80/96	83%
B	88/96	92%
C	86/96	90%
D	91/96	95%
E	87/96	91%
F	93/96	97%
G	93/96	97%
H	87/96	91%
I	61/96	64%
J	74/96	77%
Average[b]	**84/96**	**88%**

[a] The alphabet outputs corresponds to the outputs from the selections as shown in Figure 4-9.

[b] The percentage values were rounded to the nearest whole number.

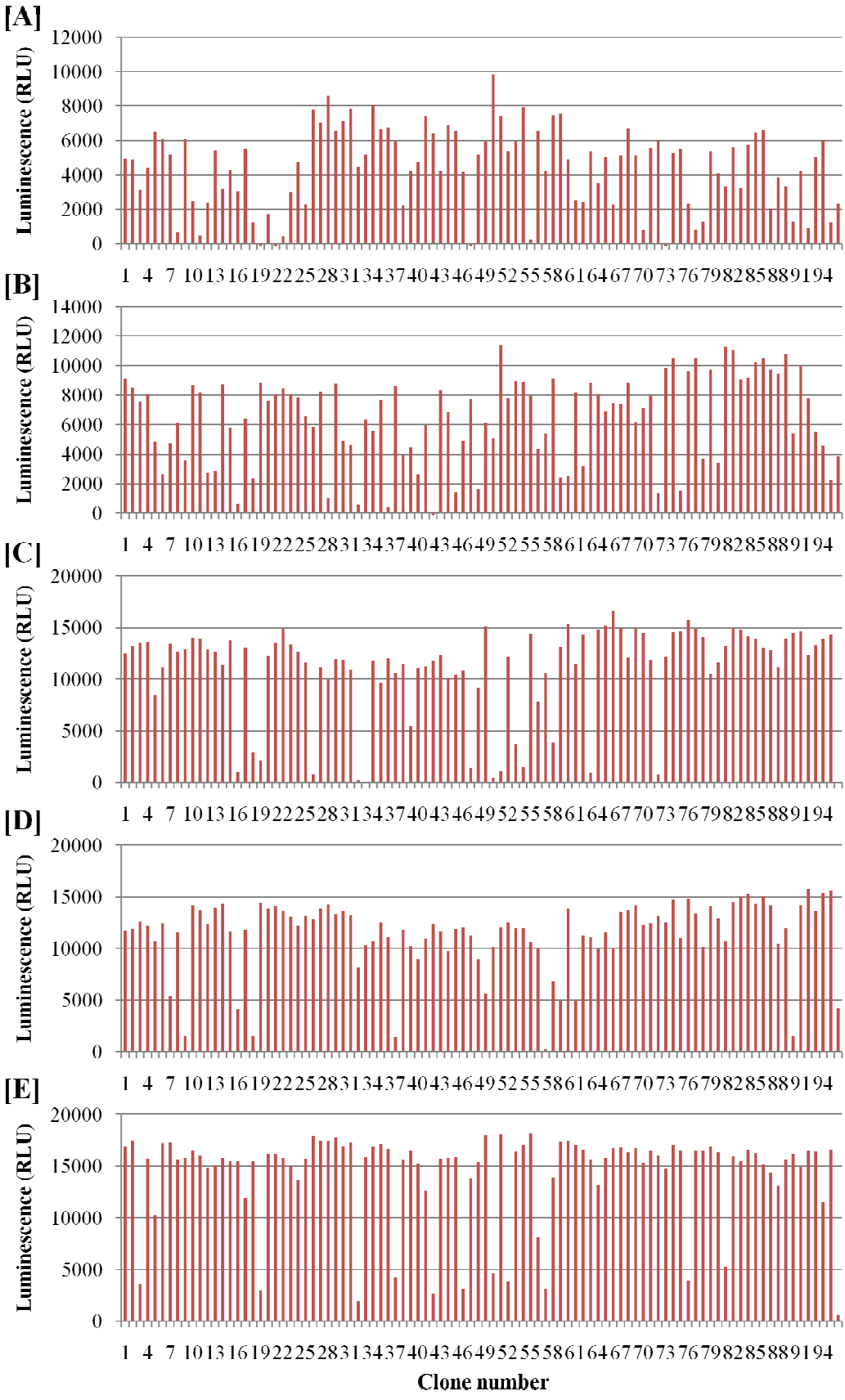

Figure 4-10 (cont. next page)

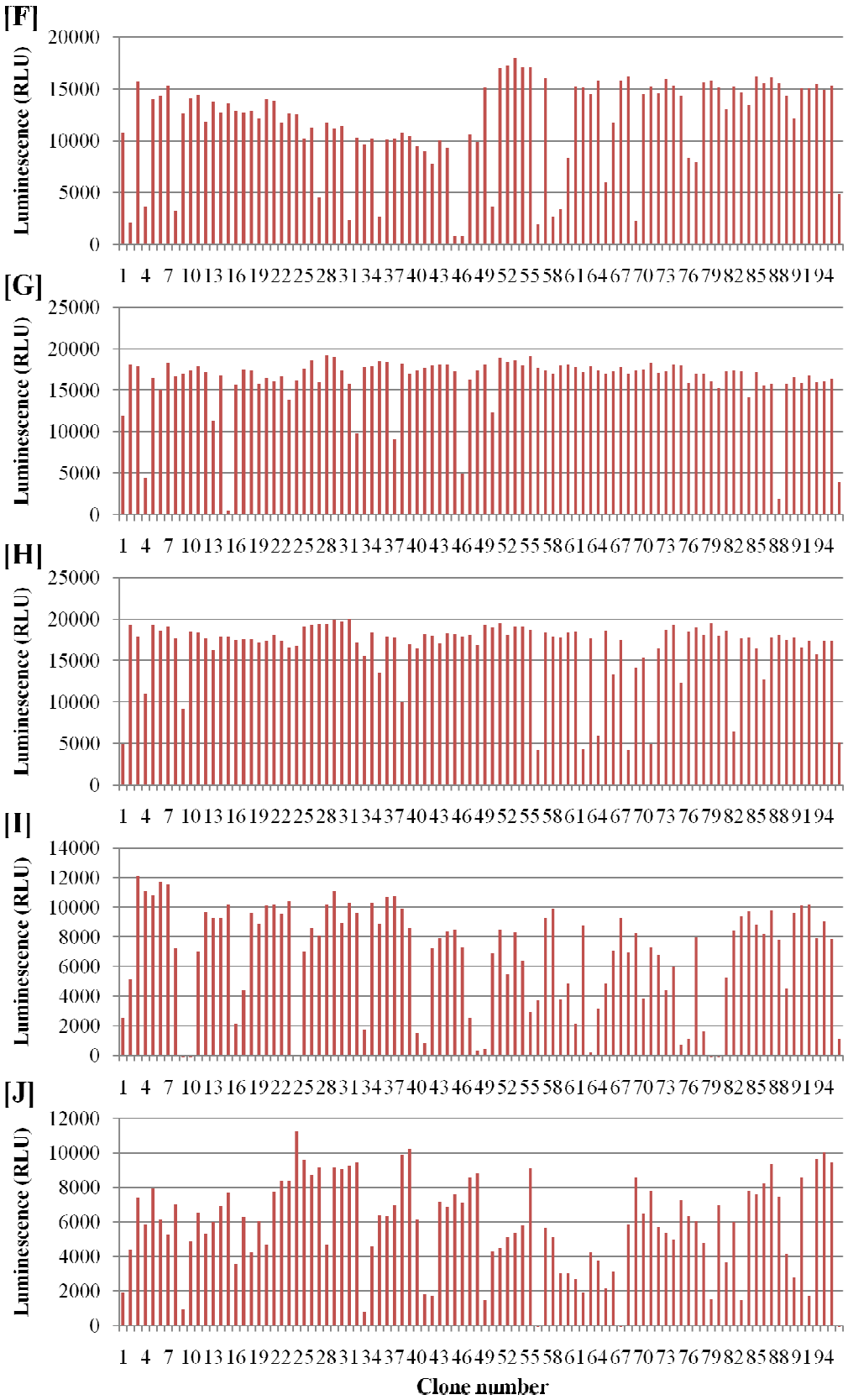

Figure 4-10. Screening of anti-gp41 VHH from periplasmic extracts in ELISA

VHH from the various outputs were tested for their ability to bind to gp41 in ELISA. The blank control adjusted RLU values were plotted against the clone numbers from each output.

4.5.2 Screening of anti-gp41 VHH in neutralisation

Although it was encouraging to find 840 (out of 960) VHH that were positive for binding to gp41, the ELISA screenings did not eliminate enough VHH to enable further characterisations of just a handful of VHH. A functional screen, through the use of neutralisation assays, was carried out to assess if any of the VHH were good neutralisers. All the 960 individual VHH were screened for their ability to neutralise HIV-1 IIIB, based on the rationale that IIIB is generally sensitive to neutralisation. HIV-1 CN54 was not included in the neutralisation screening, due to difficulties in propagating the isolate in PBMC to a high enough titre, and a molecular clone bearing the full envelope of CN54 was also found to be unviable.

The HIV-1 neutralisation screen of VHH was evaluated in NP2/CD4/CXCR4 target cells. The periplasmic extracts containing the VHH were tested along with negative control periplasmic extracts without any VHH. Due to the limited amounts of periplasmic extracts obtained, 50 µl of each clone was tested in one well only. Since the periplasmic extracts were estimated to contain approximately 10 µg/ml of VHH, the approximate amount of VHH assayed per well is 0.5 µg (or a final concentration of 2.5 µg/ml of VHH). At 72 h post infection, cells were fixed and stained for HIV-1 p24 protein in a β-galactosidase assay. FFU were counted microscopically and the reduction in FFU in test wells compared to virus control wells was determined for each clone. (Methods described in Section 2.6.6)

The results from the NP2 neutralisation assay showed that all the 960 VHH obtained were either not neutralising or just weakly neutralising. The percentage neutralisation averaged 30-40%, reaching a maximum neutralising value of 65% for only a handful of clones. None of the VHH was able to neutralise IIIB to more than

90%. From previous experience in the laboratory, periplasmic extracts containing VHH that targeted gp120 can achieve neutralisation values of 90%. However, as periplasmic extracts can also contain cell debris and other proteins, this might have contributed to the high background levels, and making the interpretation of results difficult. Negative control periplasmic extract was, on its own, able to cause an average of 15% neutralisation on HIV-1 IIIB. Nevertheless, the 12 VHH clones to give the highest neutralisation values were chosen for futher characterisation, after verifying that they also bound to gp41 in ELISA. These VHH then had their DNA sequenced to ensure that they were not identical clones.

4.5.3 Summary of anti-gp41 VHH screening

The results from the ELISA binding screening showed that 840 of the selected VHH clones were able to bind to gp41. The panning of the phage library from llama 48 had therefore worked very well indeed. However, the results from the neutralisation screen on HIV-1 IIIB were very disappointing, as none of them were found to be strongly neutralising. Nevertheless, the 12 best neutralisers were chosen and verified that they were also positive for binding in the ELISA screen. These 12 VHH were sequenced and 4 were chosen for further characterisation. Their translated sequences are shown below in Figure 4-11.

The VHH 4F1 and 4G6 were obtained from output H, which were obtained by panning the phage library from llama 48 on gp41Δ with 4E10 elution; whilst the VHH 5A1 and 5G1 were obtained from output G, which were similarly panned but with 2F5 elution. Despite the difference in elution methods, the VHH 4F1, 4G6 and 5G1 share similar sequences, especially in the CDR3. The VHH 5A1, on the other hand, was vastly different in all the CDRs when compared to the other three.

```
4F1             EVQLVESGGGLVQPGGSLRLSCAVSGTPFSFTGVAWFRQAPGKQREPVAVMTSTG-TTNY 59
4G6             QVKLEESGGGLVQPGGSLRLSCAVSGITFRFTGVAWFRQAPGKQREPVAVMTSTG-TTNY 59
5A1             QVQLVESGGGLVQAGDSLRLSCAASGRSFNSYTMGWFRQNPGKEREFVAAIHFNGYTTWY 60
5G1             EVQLVESGGGLVQPGGSLRLSCAVSGISFSFTGVAWFRQAPGKQREKIAVMTSGG-TTAY 59
                :*:* ********.*.*******.** .*    :.**** ***:** :*.:   * ** *

4F1             EDSVKGRFTISRDNAKRTVYLQMNSLKPEDTAVYYCFVEDS---EAGGLEITGYWGQGIQ 116
4G6             EDSVKGRFTISRDNAKRTVYLQMNSLKPEDTAVYYCFVEDS---EAGGLEITGYWGQGIQ 116
5A1             ADSVKGRFTSSRDDAKSTVYMQMNSLKPEDTAVYYCAAKQFGSWRSTGVSEYNYWGRGTP 120
5G1             EDSVNGRFSISRDNAKRTVYLQMNSLKPEDTAVYYCFVEDS---EAGGLEITGYWGQGIQ 116
                 ***:***: ***:** ***:*************** .::    .: *:.  .***:*

4F1             VTVSS 121
4G6             VTVSS 121
5A1             VTVSS 125
5G1             VTVSS 121
                *****
```

Figure 4-11. Amino acid sequences of the four anti-gp41 VHH

The 4 VHH chosen were obtained by panning the phage library from llama 48 on gp41Δ. 4F1 and 4G6 were obtained with 4E10 elution, and 5A1 and 5G1 were obtained with 2F5 elution. These VHH bound to gp41 in ELISA screenings and were able to neutralise HIV-1 IIIB to 50%. After sequencing these VHH, they were translated and aligned using ClustalW2. Even though they were obtained from different selection procedures, the VHH 4F1, 4G6 and 5G1 share similar sequences whereas 5A1 is distinct from the rest. The CDRs are shown shaded.

4.6 Large scale expression of VHH

To be able to further characterise the individual VHH, they have to be expressed in greater quantities and purified. After the expression of VHH in *E. coli* TG1 cells and subsequent purification by means of the C-terminal 6-Histidine-tag in nickel column beads, the result of the purification was monitored by SDS-PAGE. The full methods are described in Section 2.6.4. From the gel image, the eluates from the purification process contain a clean and prominent band near the 19kDa mark, which corresponds to the approximate size of the VHH.

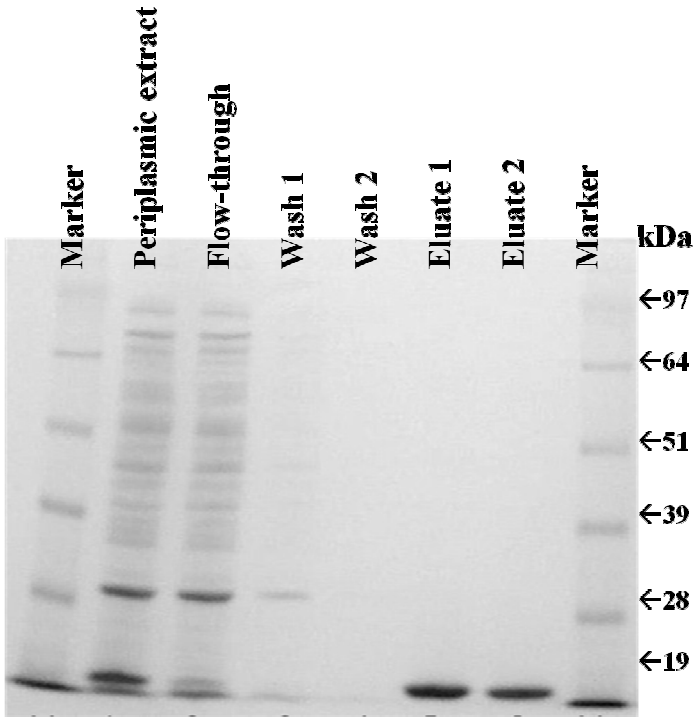

Figure 4-12. Expression and purification of VHH

Recombinant VHH were expressed in *E. coli* TG1 cells and purified by means of the C-terminal 6-Histidine-tag. The purification was monitored by SDS-PAGE followed by Coomassie blue staining. Shown is a representative analysis of the purification of a VHH. The first and last lanes contain the molecular weight marker (SeeBlue Plus 2, Invitrogen), followed by unpurified *E. coli* periplasmic extracts, the unbound flow-through fraction, followed by 2 washes, and finally 2 eluates. The flow-through seems to contain some uncaptured VHH, as with Wash 1. By Wash 2, all unbound proteins were washed out. The two eluates gave a clear distinct band near the 19 kDa mark.

4.7 Neutralisation of HIV-1 by anti-gp41 VHH

The purified VHH were tested again in neutralisation assays, because previous tests were carried out using periplasmic extracts which contain cell debris and other proteins. As the TZM-bl cell assay was later developed and set up in our laboratory, this assay was used instead of the NP2 cell assay, which involves the time-consuming and laborious counting of FFUs microscopically. This assay is described in Section 2.3.9. The TZM-bl neutralisation assay employs a luciferase read-out using a luminometer and is a relatively high-throughput process. This assay utilises

TZM-bl cells, which are HeLa cells expressing endogenous CXCR4 and engineered to express CD4 and CCR5 and to contain a Tat-induced, LTR-driven firefly luciferase reporter gene. The assay measures neutralisation as a reduction of Tat-induced luciferase expression (Derdeyn et al., 2000; Montefiori, 2004; Platt et al., 1998; Wei et al., 2002).

Virus was incubated with serial dilutions of VHH (up to a maximum of 50 µg/ml) for 1 h and subsequently incubated with TZM-bl cells. At 48 h post infection, cells were lysed with the addition of Promega luciferase assay reagent. Luminescence was measured and background luminescence subtracted. The percentage of relative light units (RLU) in test wells, compared to in virus only control wells, was then determined. The lowest VHH concentration required to achieve 50% reduction of infectivity (IC_{50}) compared to a virus control was calculated using the XLfit 4 software with sigmoidal regression analysis. To ensure reproducible results, the neutralisation activity of each VHH was assayed in duplicate and on two separate occasions. Against each virus included in the study, a negative control VHH (VHH #3 that is directed against an irrelevant epitope; at 50 µg/ml) was included in at least two wells.

The virus isolates tested in this assay included IIIB, MN, 93MW965.26 and 96ZM651.02. The TCLA isolates IIIB and MN are commonly used subtype B viruses, and are known to be generally sensitive to antibody-mediated neutralisation (Moore et al., 1993; Wrin et al., 1995). The subtype C isolates are 93MW965.26 and 96ZM651.02, which are Tier 1 and 2 viruses respectively. The Tier 1, 2 and 3 classification is based on the sensitivity of the envelope pseudotyped viruses to neutralisation, with Tier 1 viruses being the most sensitive to neutralisation (Mascola

et al., 2005). It was not possible to test the VHH for neutralisation to the autologous virus HIV-1 CN54 as virus yields in PBMC cultures were of low titres and cloning of the *env* gene did not produce any infectious clones.

The graphs in Figure 4-13 show the results of the neutralisation assays with the respective viruses, and Table 4-2 shows the IC_{50} values of the anti-gp41 VHH. Surprisingly, only the 5G1 VHH was able to neutralise both IIIB and MN to 50%. On closer inspection of the graphs, all the VHH showed some neutralising activity only when quantities were higher than 17 µg/ml. The 5G1 VHH gave IC_{50} values of 49.2 µg/ml and 37.8 µg/ml with IIIB and MN respectively.

The anti-gp41 VHH fared better against the subtype C isolates, particularly against the Tier 2 isolate 96ZM651.02. All the four VHH were able to neutralise 96ZM651.02 with IC_{50} values between 6.2 – 45.1 µg/ml, but only 4G6 and 5G1 were able to neutralise 93MW965.26.

In summary, the anti-gp41 VHH were not able to potently neutralise the Tier 1 subtype B viruses tested at concentrations up to 50 µg/ml. This contradicts the results of the NP2 assay which was carried out in the screening of the selected VHH. On the other hand, these VHH were found to neutralise the two subtype C viruses tested, especially against 96ZM651.02 which is a Tier 2 virus. These results are discussed in Section 4.12.

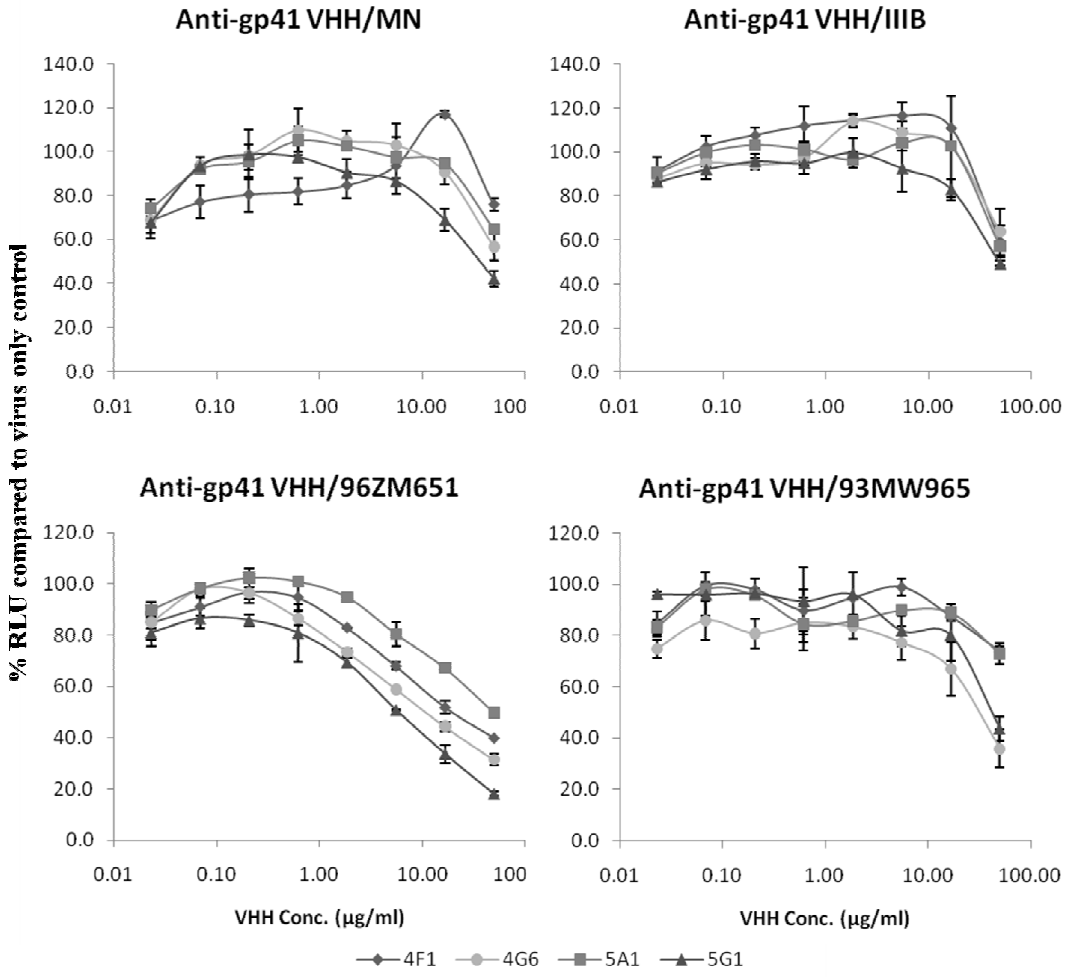

Figure 4-13. Anti-gp41 VHH neutralisation of HIV-1 in TZM-bl cells

The percentage of RLU in test wells compared to in virus only control wells (after subtraction of background luminescence) was determined for VHH concentrations up to a maximum of 50 µg/ml. None of the VHH were able to neutralise either IIIB or MN to 50%. All of the VHH were able to neutralise 96ZM651.02, but only 4G6 and 5G1 were able to neutralise 93MW965.26.

Table 4-2. Anti-gp41 VHH IC_{50} (µg/ml) titres against HIV-1

VHH	IC_{50} against virus isolates (µg/ml)			
	IIIB	MN	93MW	96ZM
4F1	>50	>50	>50	22.4
4G6	>50	>50	35.4	12.0
5A1	>50	>50	>50	45.1
5G1	49.2	37.8	43.9	6.2

4.8 Enzyme linked immunosorbent assays (ELISA)

4.8.1 VHH titrations

The purified VHH were also checked to determine if they bind to gp41 in ELISA. These were first titrated on Maxisorp plates coated with 1 µg/ml of gp41 to determine the best range of VHH concentration to use in ELISA, starting from 1 µg/ml of VHH and 5-fold serially diluted thereafter.

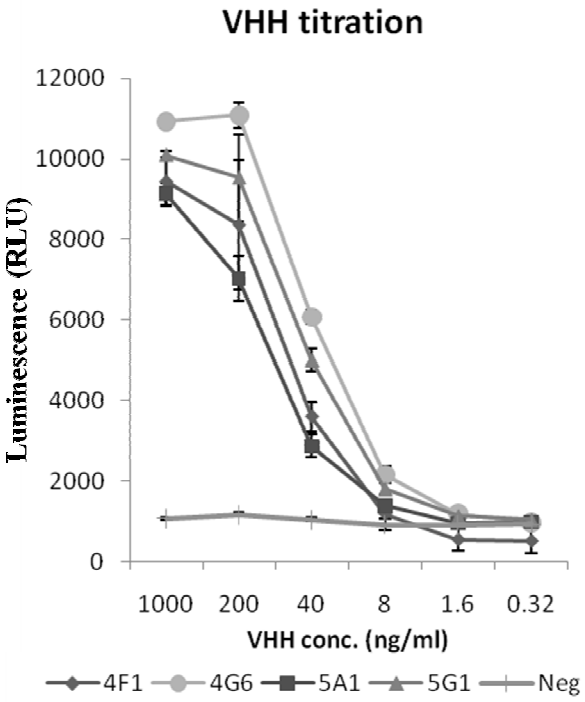

Figure 4-14. Titration of anti-gp41 VHH in ELISA

The purified VHH were titrated on gp41 coated plates. The results showed a dose-dependent signal, with maximum peak values at around 1 µg/ml of VHH and dropping to a low at around 8 ng/ml. The negative control VHH produced background level signals.

Figure 4-14 shows the graph of the purified VHH titration on gp41. The various VHH gave a peak luminescence signal at 1000 ng/ml, which drops down to a minimum at around 8 ng/ml. The negative control VHH produced only a background signal in the ELISA.

4.8.2 VHH binding to recombinant gp41 and gp140

The four different purified VHH were then tested for binding in ELISA against four different antigens, this time in 2-fold dilution starting from 1 µg/ml. The four antigens were recombinant gp41 IIIB, and recombinant trimeric gp140 obtained from isolates 92UG037 (subtype A), Bx08 (subtype B), and 96ZM651.02 (subtype C). The CN54 immunogen was not included as supplies were exhausted at this stage

of the project. The plates were first coated overnight with 1 µg/ml of the antigens, and the ELISA was carried out as described in Section 2.6.5. The mAb 2F5, which recognise the MPER of gp41, was included as a positive control.

Figure 4-15 shows the graphs from the ELISAs. As expected, all the VHH and 2F5 bound to gp41 in a dose-dependent manner. When comparing the curves with recombinant gp140, a striking difference emerged. The mAb 2F5 was able to recognise 92UG037 and Bx08, which are isolates from subtypes A and B respectively, but none of the VHH were able to bind to these two antigens. Interestingly, all the VHH were able to bind to gp140 96ZM651.02. The mAb 2F5 was not able to bind to 96ZM651.02 as the 2F5 epitope are often not present on subtype C isolates (Li et al., 2006b).

The results of the ELISA correspond well with the results from the neutralisation tests in the previous section. VHH binding to gp140 96ZM651.02 results in the neutralisation ability of the corresponding virus isolate. Absence of binding to gp140 Bx08 corresponds to low neutralisation ability of subtype B isolates IIIB and MN. Although the VHH were unable to bind to trimeric forms of gp41 presented in the context of gp140 Bx08, the VHH were able to bind to monomeric forms of gp41 IIIB. This could be due to the more 'exposed' structure of gp41 compared to gp140. It should also be noted that these VHH were selected through panning on gp41 MN, and not on gp140. In summary, the results from the neutralisation and ELISA tests show that the VHH seem to have a preference for subtype C isolates. This is perhaps not too surprising as the llama was immunised with subtype C gp140 CN54.

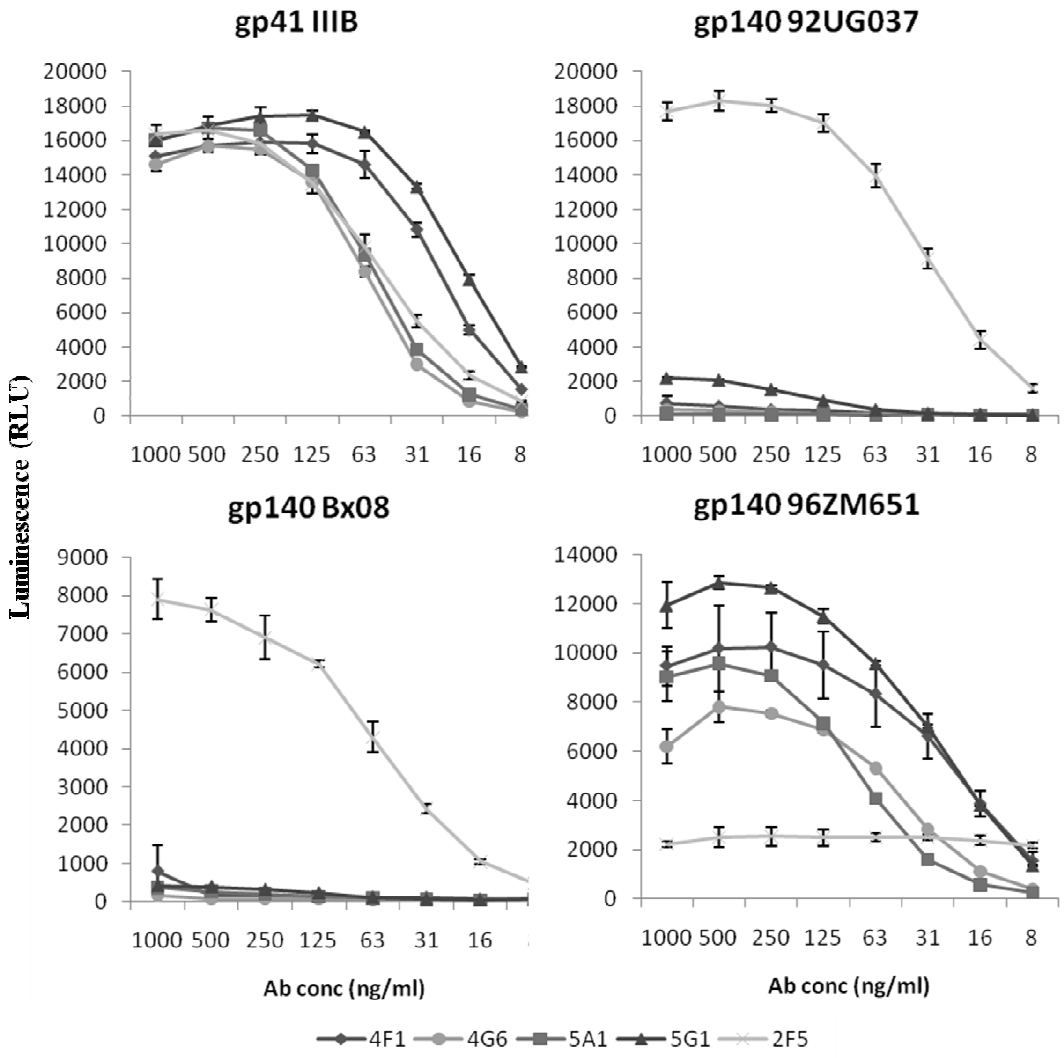

Figure 4-15. Binding of anti-gp41 VHH to recombinant gp41 and gp140 in ELISA

Plates were first coated overnight with the respective antigens at 1 µg/ml. The VHH were introduced as described in the text. All the VHH were able to bind to gp41 IIIB and gp140 96ZM651.02 in a dose dependent manner, but they were unable to bind to gp140 92UG037.A9 and Bx08. The mAb 2F5 was used as a positive control, and it bound to all except gp140 96ZM651.02 where the epitope was absent.

4.8.3 VHH competition in ELISA

In an initial attempt to map the epitopes of the anti-gp41 VHH and to determine if they bind to similar epitopes, the VHH were allowed to compete for each other in binding in an ELISA. In order to distinguish and detect the test sample from the other VHH, each of the VHH was biotinylated using a procedure described in Section 2.7.1.2. To check for successful biotinylation, the biotinylated VHH were titrated on gp41 coated Maxisorp plates and detected with an AP-conjugated streptavidin. Unbiotinylated VHH at the highest concentration was also included in the titration. From the titration graph in Figure 4-16, a concentration-dependent signal was observed, and they were all higher than their respective unbiotinylated samples. The biotinylation of the VHH samples was therefore successful.

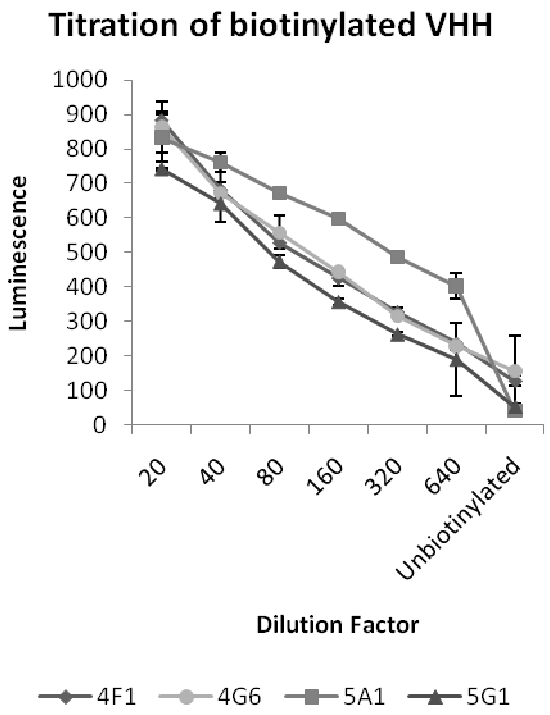

Figure 4-16. Titration of biotinylated VHH
To check that each VHH was successfully biotinylated, a titration was carried out in ELISA and the VHH was detected with an AP-conjugated streptavidin. Unbiotinylated VHH at the highest concentration was included in the titration. From the graph, all the biotinylated VHH were successfully biotinylated and produced a signal which was above the unbiotinylated samples.

The VHH were then checked to see if they competed with each other for binding to similar epitopes. The procedure is described in Section 2.6.9. Briefly, titrated amounts of unbiotinylated VHH and mAb 2F5 were allowed to bind to gp41 coated Maxisorp plates in separate wells and in duplicate. The anti-gp120 VHH, A12, was

added as an irrelevant negative control. A fixed amount of biotinylated VHH (optimised at 5 µg/ml) was introduced to all the wells and allowed to compete for binding to epitopes on gp41. The amount of gp41-bound biotinylated VHH was detected with an AP-conjugated streptavidin, and the luminescence was detected after the addition of substrate.

The graphs in Figure 4-17 show the results of the competition ELISA. The negative control A12 VHH does not bind to gp41, and hence it did not impede the binding of the biotinylated test samples to give a flat curve in each graph. This observation was also true for 2F5, and hence none of the VHH competes for binding to the 2F5 epitope. Each of the biotinylated test sample will compete with its corresponding unbiotinylated VHH. Hence lower levels of biotinylated VHH was detected when a greater concentration of its own unbiotinylated VHH was present in each well, thus producing the dose-dependent curves seen.

Of the four different VHH tested, 5A1 was the only VHH which did not compete with the other VHH for binding to the same epitope. The presence of high concentrations of the other three VHH did not inhibit the binding of 5A1 to gp41. The 4F1, 4G6 and 5G1 VHH all compete with each for binding to a similar epitope on gp41.

In summary, the 4F1, 4G6 and 5G1 VHH all recognised a similar epitope on gp41, whereas the 5A1 VHH recognised a different epitope on gp41. This was hardly surprising as the sequences of the CDRs for 4F1, 4G6 and 5G1 were quite similar, compared to 5A1 which was quite different from the rest (Figure 4-11).

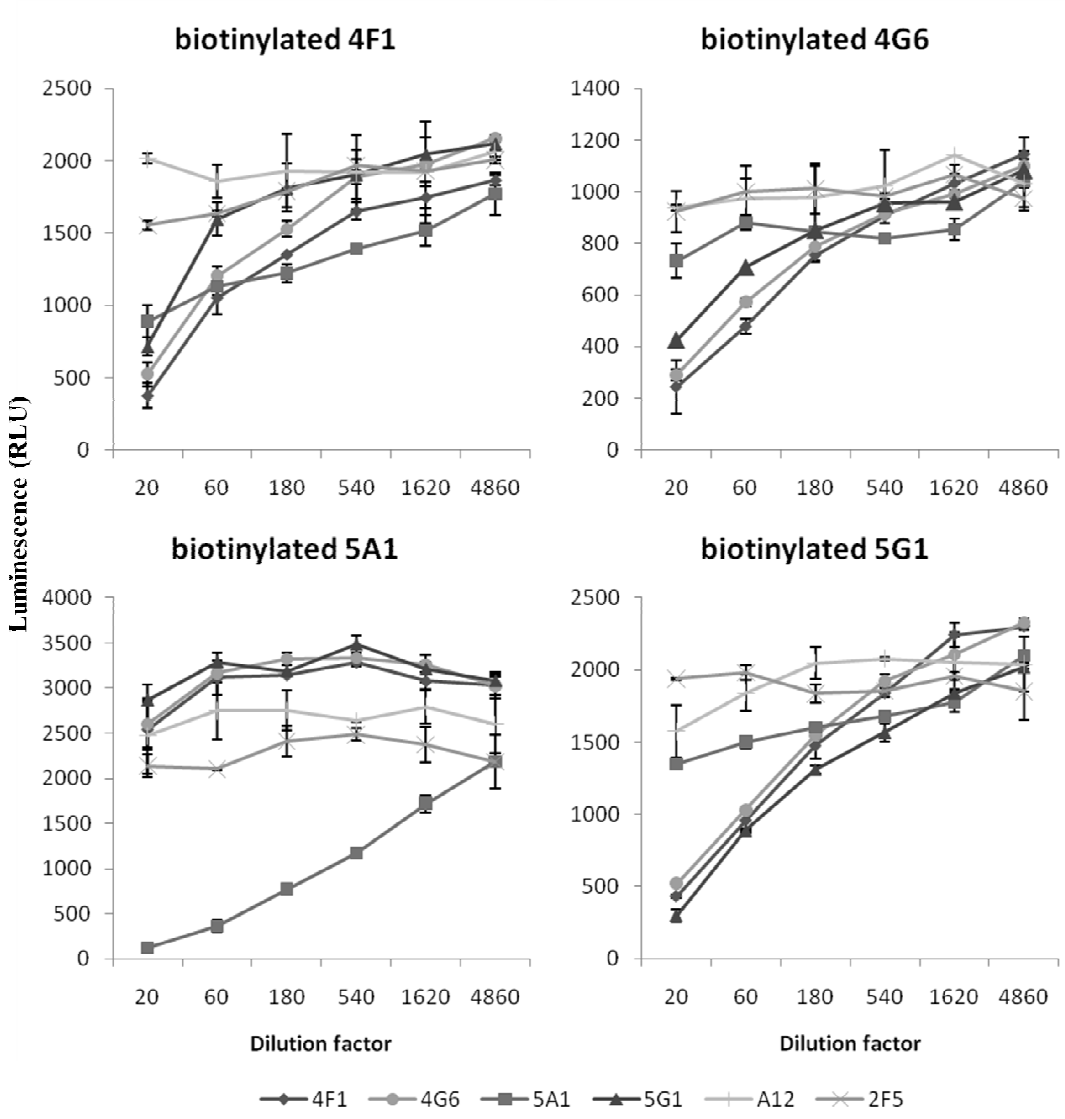

Figure 4-17. Competitive binding to gp41 in ELISA

Titrated amounts of unbiotinylated VHH and 2F5 were allowed to bind to gp41 coated plates. The anti-gp120 VHH, A12, was included as an irrelevant negative control VHH. A fixed amount of biotinylated VHH was added to all the wells for competitive binding to gp41 and then detected with AP-conjugated streptavidin. The biotinylated VHH will compete for binding to its own corresponding unbiotinylated sample. None of the VHH tested was able to compete with 2F5 for binding. The 4F1, 4G6 and 5G1 VHH competed with each other for binding to gp41, whereas 5A1 recognised a different epitope compared to the rest.

4.9 Western blots using anti-gp41 VHH

To verify that the VHH were able to recognise gp41 in a specific manner, western blots were carried out according to procedures described in Section 2.6.7. Briefly, about 10 ng of gp41 was resolved, without the boiling of samples, in multiple lanes in SDS-PAGE and then blotted onto a PVDF membrane. The individual lanes were cut out and probed with the different VHH in separate receptacles. The mAb 50-69D, which reacts to a conformational dependent epitope on gp41, was used as a positive control; and an irrelevant VHH, E7, was used as a negative control. The western blots were visualised using ECL detection reagent and x-ray film.

Figure 4-18[A] shows the individual lanes blotted with gp41. The four different anti-gp41 VHH gave distinct prominent bands near the 39 kDa marker, indicating a specific recognition of recombinant gp41. The positive control mAb produced multiple bands, which might be an indication of impurities in the sample. The negative control E7 VHH did not give a signal in the western blot.

In a variation of the above method, the VHH were then resolved in SDS-PAGE and blotted onto a PVDF membrane. Even though the VHH were subjected to high temperatures and chemically denatured, the VHH have a remarkable ability to renature itself (Dumoulin et al., 2002; Perez et al., 2001; van der Linden et al., 1999). The VHH were tested for their ability to capture recombinant gp41 in solution form. Captured gp41 were detected with the mAb 50-69D and visualised on an x-ray film and shown in Figure 4-18[B]. All the four different VHH were able to capture gp41, as indicated by a prominent band at the 14 kDa mark, which is the approximate size for a VHH. The negative control VHH (E7) was not able to capture gp41, as shown by the absence of a band.

Both western blots thus showed that the isolated anti-gp41 were able to bind to and capture gp41 in a specific manner.

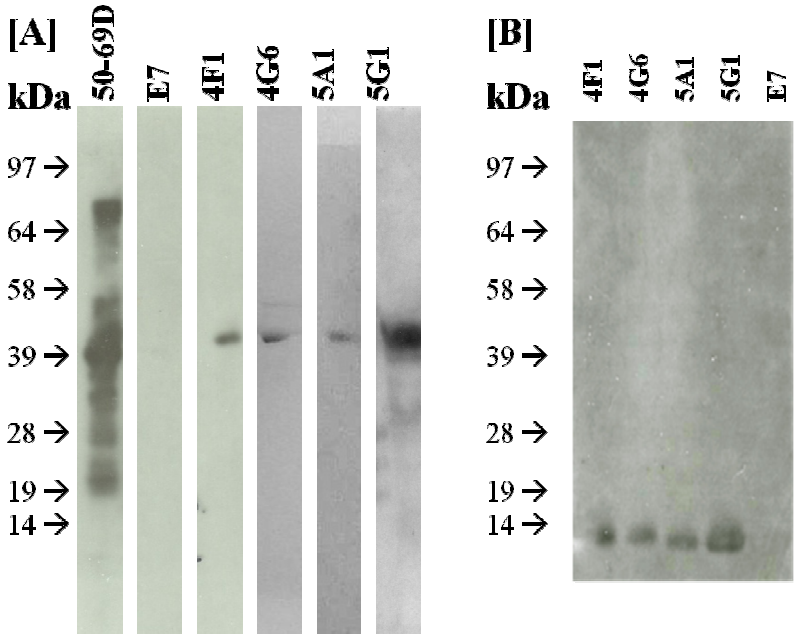

Figure 4-18. Western blots of anti-gp41 VHH

[A] Recombinant gp41 were blotted onto a PVDF membrane in six separate lanes, and the lanes were then cut out. These were then individually probed with various antibodies and VHH and detected with ECL reagent. All the VHH produced a single prominent band near the 39 kDa mark, except for the negative control E7 VHH. The positive control mAb 50-69D produced a prominent band near the 39 kDa mark, but also detected other impurities in the sample. [B] The VHH were blotted onto a PVDF membrane in the lanes as shown. These were then allowed to capture gp41 in solution form, which was in turn probed with the anti-gp41 mAb 50-69D. The results show that the anti-gp41 VHH were able to capture gp41 in solution form, but not the negative control E7 VHH.

4.10 Epitope mapping

In an attempt to map the binding epitopes of the four different anti-gp41 VHH, they were tested for their ability to bind to a set of 15-mer peptides, with 11 amino acid overlaps between sequential peptides. The sequences of these peptides were based on the MN strain. To ensure complete coverage of the gp41 sequence, a total of 42 different peptides were tested. These peptides were coated overnight onto Maxisorp

plates at a concentration of 10 µg/ml, and an ELISA was carried out as described in Section 2.6.10.

None of the VHH was able to bind to any of the peptides in the set. A few positive controls using known mAbs with established binding epitopes showed that the experiment was working properly. This was repeated three times and the results were consistently negative.

Through the Collaboration for AIDS Vaccine Discovery (CAVD), we have access to reagents and technologies developed by other groups. One of these groups, Pepscan Presto BV (The Netherlands), has developed a proprietary set of combinatorial peptide arrays covering the sequences of gp160 from the subtype A and C isolates 92UG037 and 93MW965 respectively. Their peptide arrays contain 15 to 18-mer linear and cyclic peptide fragments that overlaps by 19 amino acids. The data of their results are presented in Figure 4-19.

The positions of the gp160 peptides are laid out on the horizontal axis, and the vertical axis indicates the presence of binding. The VHH produced a high background signal to peptides from 92UG037 and these are not shown. All the VHH produced a binding signal to peptides covering the gp41 portion of 93MW965. However, multiple peaks were observed for all the VHH samples, indicating that they do not bind to a singular linear epitope. In all instances of 4F1, 4G6 and 5G1, three to four prominent peaks were detected on linear peptides from 93MW965. These were highlighted in circles in Figure 4-19. The 5A1 VHH produced high background signals and with peaks that were different to the rest.

The consensus binding sequences of the three VHH were found to be SITLTVQARQLLS and LTVWGIKQL. These amino acids correspond to positions

534-546 and 568-576 on the HXB2 Env numbering system. To a lesser extent, these VHH were also found to recognise WEREIDNYTH, which corresponds to position numbers 631-640.

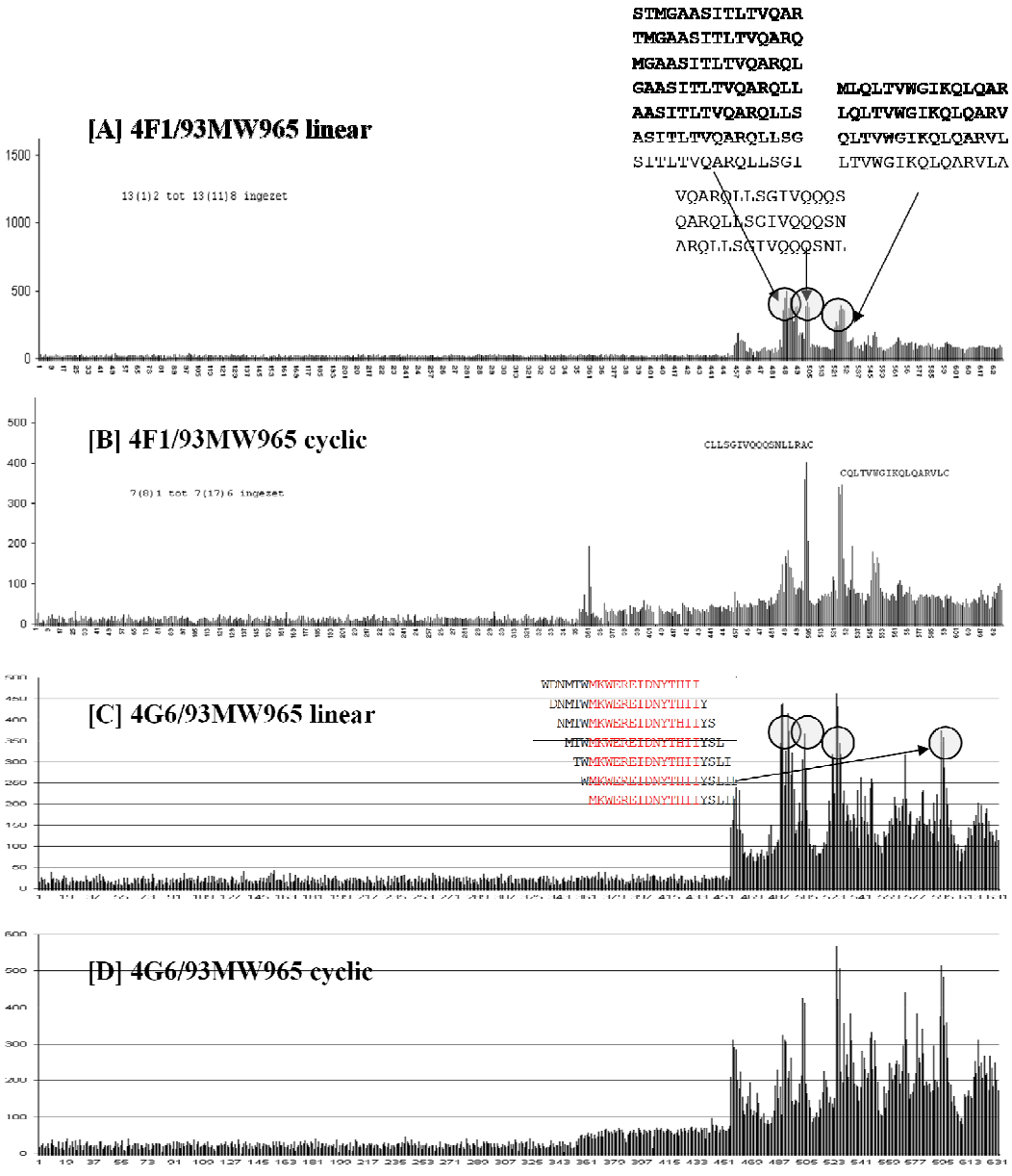

Figure 4-9 (cont. next page).

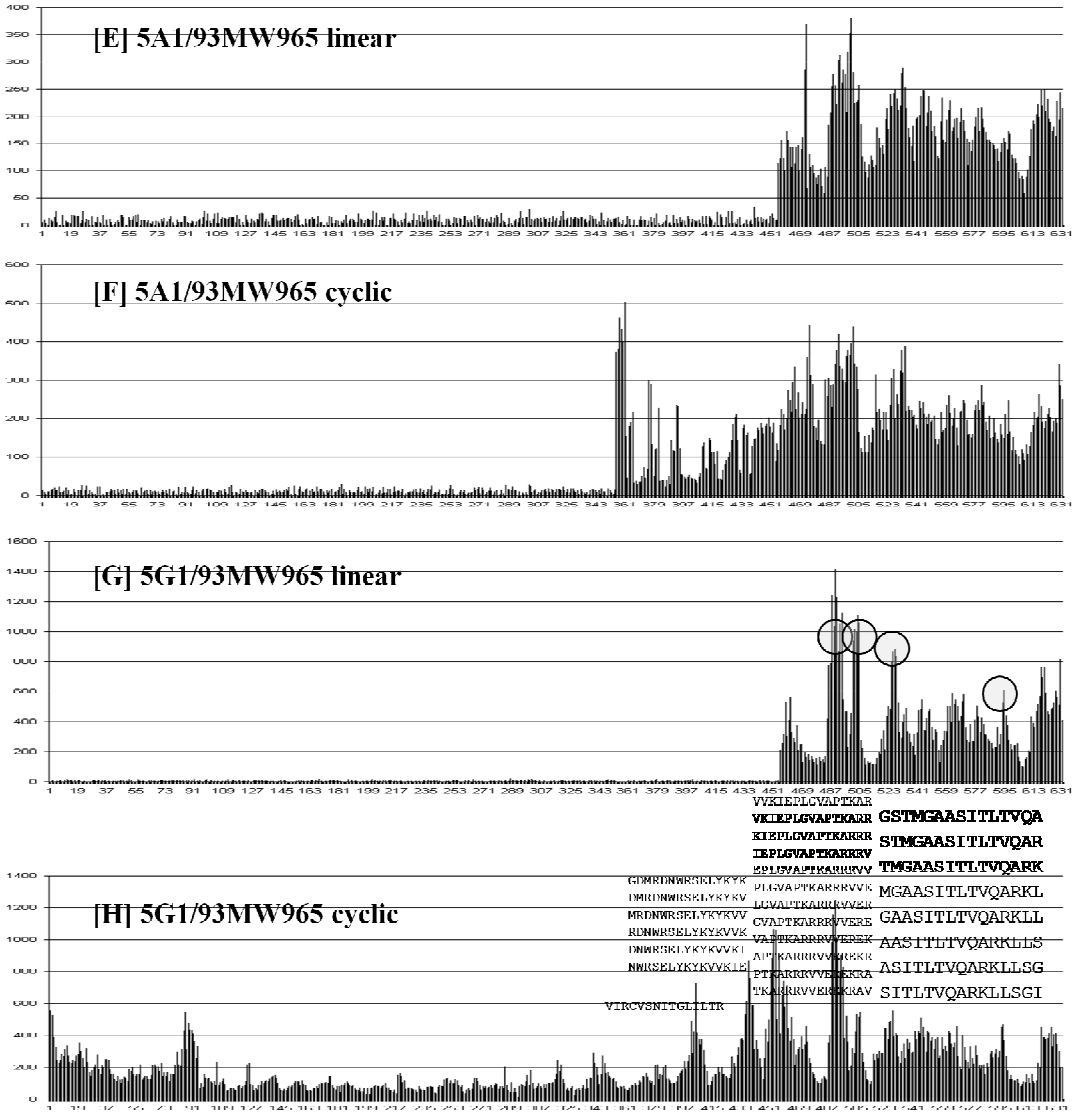

Figure 4-19. Results of epitope mapping from Pepscan

Linear and cyclic peptides from gp160 93MW965 are shown lined up on the horizontal axis, and binding of the VHH to the peptides would be shown as peaks on the vertical axis. All the VHH tested were found to bind to the gp41 peptides. Similar peaks (circled above) were identified for 4F1, 4G6 and 5G1, whereas those from 5A1 produced inconclusive results.

An attempt was made to understand the relationship of these epitopes on the structure of g41. Unfortunately, the crystal structure of a native gp41 in the context of an envelope spike is as yet unsolved. The predicted structure of gp41 was obtained from Zolla-Pazner's review (Zolla-Pazner, 2004) and adapted to show the relative positions of the identified epitopes. These are marked with red boxes in Figure 4-20. Interestingly, the identified epitopes for the three VHH (4F1, 4G6, and 5G1) occupy adjacent positions on gp41, thus adding strength to the results obtained from epitope mapping. The binding epitopes for 5A1 were not similarly identified.

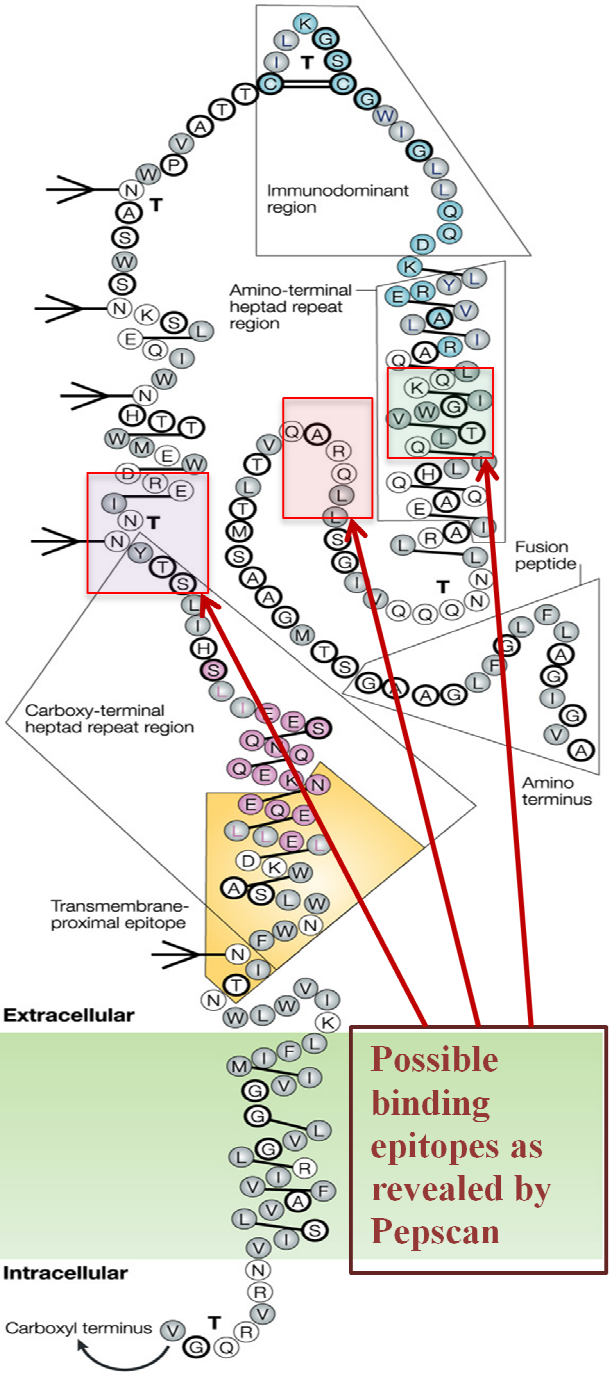

Figure 4-20. Illustration of epitope positions on gp41

The epitopes identified by Pepscan were mapped onto the above illustrated structure of gp41. It was found that the possible epitopes occupy adjacent positions on gp41. Figure adapted from *Nat Rev Immunol* 4(3):199-210. (Zolla-Pazner, 2004)

4.11 Conformational dependent epitope

To confirm that the VHH bind to a conformational dependent epitope on gp41, a denaturing ELISA was carried out as described in Section 2.6.11. Briefly, two aliquots of gp41 at 1 µg/ml were coated onto Maxisorp plates. However, one of the gp41 aliquot was treated with 1% SDS, 50 mM DTT and then boiled for 5 minutes to denature the protein. The four different VHH were then allowed to bind to both samples of gp41 and run in parallel. Two mAbs were included as controls; 2F5 recognises a linear epitope whilst 50-69D recognises a conformational dependent epitope on gp41.

The results of the ELISA are shown in Figure 4-21. On the plate with untreated gp41, all the VHH and mAbs were able to bind to gp41 as per usual. However, none of the VHH and 50-69D were able to bind to the denatured gp41. Only 2F5, which does not require a conformational dependent epitope, was able to bind to denatured gp41. Therefore, all the VHH were found to recognise a conformational dependent epitope.

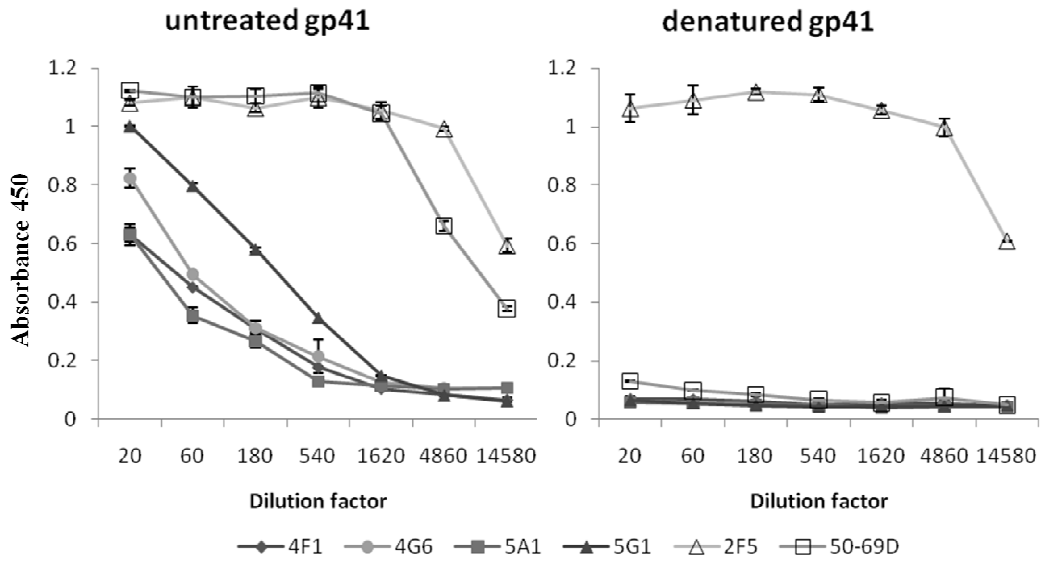

Figure 4-21. VHH binding to denatured gp41 in ELISA

Two ELISA plates were set up in parallel, one coated with gp41 and the other coated with denatured gp41. The VHH were allowed to bind to both forms of gp41. The mAbs 2F5 and 50-69D, which recognise a linear and conformational dependent epitope respectively, were included as controls. On the plate with untreated gp41, all the VHH and mAbs were able to bind. However, none of the VHH and 50-69D except 2F5 was able to bind to the denatured gp41. Therefore, all the four VHH recognised a conformational dependent epitope.

4.12 Discussion

In this chapter, a phage library containing the antibody repertoire from a llama immunised with a subtype C trimeric gp140 CN54 was panned on recombinant gp41. A total of 10 different selection methods were used. This consisted of using recombinant gp41, gp41Δ or gp140 as the selecting antigen, and different elution methods using trypsin, glycine or antibody competition were used. Although 88% of all the selected VHH were found to bind to gp41, all of them displayed very weak neutralising ability in the initial neutralisation screen using NP2 cells.

Four anti-gp41 VHH, 4F1, 4G6, 5A1 and 5G1, were brought forward for further characterisation. These 4 were panned on gp41Δ, but 4F1 and 4G6 were eluted with

4E10, whereas 5A1 and 5G1 were eluted with 2F5. However, the 4F1, 4G6 and 5G1 VHH were found to be quite similar based on their amino acid sequences, whereas 5A1 possessed CDRs which were comparatively different.

The VHH were tested for their ability to neutralise a few strains of HIV-1 in the newer TZM-bl cell assay. Differences between assays have been previously observed in our laboratory, and also in the global collaboration working on the standardisation of HIV-1 neutralisation assays (Polonis et al., 2008). Discrepancies between results obtained in different neutralisation assays may be expected for non-potent mAbs. This is because the different assays employ different target cells, virus inputs, incubation times, as well as the use of the polycation DEAE-dextran to enhance the level of infection in TZM-bl cells.

In the TZM-bl assay, all the 4 VHH were unable to neutralise the two neutralisation-sensitive TCLA isolates IIIB and MN to high potency (<10 µg/ml). Instead, these VHH were found to be more potent against subtype C isolates (i.e. 93MW965.26 and 96ZM651.02). The mAb 4E10 was also found to neutralise at similar levels (10-50 µg/ml) against subtype C viruses (Binley et al., 2004), indicating similar neutralising potencies. Binding tests in ELISA showed that these VHH do not bind well to trimeric gp140 from subtype A and B, but they bound to trimeric gp140 subtype C and monomeric subtype B gp41. This observation was perhaps not surprising as these VHH were isolated from a llama that was immunised with a subtype C envelope. A positive correlation between the subtype-specific antigen binding with subtype-specific neutralisation was therefore observed, as also reported elsewhere (Crooks et al., 2008; Fouts et al., 1997; Moore et al., 2006). In future work, more

isolates from subtypes B and C can be tested for sensitivity to neutralisation by these VHH.

In an attempt to identify their binding epitopes, all the VHH were found to bind to a conformational dependent epitope. Nevertheless, epitope mapping using peptides from the 93MW965 strain has revealed the possible binding site on gp41 for 4F1, 4G6 and 5G1, which also cross-compete with each other for binding on gp41. The epitope for 5A1 was unable to be determined, but competition studies have shown that 5A1 does not compete for binding with the other three VHH. The exact binding epitopes for these VHH could perhaps be determined in the future by alanine scanning of gp41.

In summary, four anti-gp41 VHH were isolated from a llama immunised with gp140 CN54 which showed a preference for neutralising subtype C HIV-1 isolates. These VHH do not recognise the MPER of gp41, but bind to a conformational dependent epitope on gp41.

5 C8- and D7- Family Specific VHH Libraries

5.1 Introduction

In addition to llama (L48) that was immunised with recombinant gp140 CN54 (described in Chapter 4), another llama (L44) was separately immunised with recombinant gp120 CN54 and a phage display VHH library was created. These were all carried out as part of the European Microbicide Project (EMPRO), in collaboration with Ablynx NV, Ghent, Belgium.

Anna Forsman, a former PhD student in the lab, has described the isolation of three VHH (C8, D7 and A12 VHH) from llama 44 that were able to inhibit different subtypes of HIV-1 (Forsman et al., 2008). These three VHH were able to block CD4 binding to HIV-1 gp120 and were able to neutralise various subtype B and C isolates of HIV-1 to high potency. These VHH were selected after two rounds of panning on gp120 IIIB with competitive elution with soluble CD4 (sCD4).

In the continuing quest for more broadly neutralising antibodies against the HIV-1 envelope, it will be useful to select for variants of an antibody that has already been shown to be broadly neutralising. A "family-specific" approach was therefore devised to allow the amplification of a "family" of affinity variants that had been generated *in vivo* in the llama, and followed by the subsequent selection for such variants. In this chapter, two family-specific VHH libraries containing members that are similar to the parental C8 and D7/A12 VHH were created. These members were amplified from the immune library that was originally created from llama 44. The C8-family specific library only produced identical members to the C8 VHH, whereas the creation of the D7/A12-family (thereafter named as D7- family) specific library

was a success. This method of creating a family-specific library is a novel approach to VHH or antibody selection. Members of this family were then characterised and an attempt at identifying the crucial amino acid residues involved in binding were made.

5.2 Library creation

5.2.1 Primer design

To create a family-specific phage display library where the members have similar antigen recognition ability as the parental VHH, the sequences of the members should share some homology to the parental VHH. Because the H chain CDR3 possesses the greatest flexibility and conformational variability of the CDRs, it could have the greatest influence on antigen binding (Nuttall et al., 2000). In order to extract out VHH with similar properties as the parental VHH, C8- and D7/A12-specific primers that recognised the last stretch of amino acids (4-6 codons) in the CDR3 were designed.

Figure 5-1 shows the sequences of the C8, A12 and D7 VHH, with the CDR3 sequences highlighted in grey. The A12 and D7 share similar sequences and are part of the same family. Degenerate reverse primers were designed to correspond to the underlined sequences, with one primer to capture both D7- and A12-like sequences. The sequences of the primers were: C8, 5'- TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC CCA GTC TGT ATA GTC GC -3' and D7, 5'- TGA GGA GAC GGT GAC CTG GGT CCC CTG GCC CCA GTM GTW RTA ATC YGA AT TCG -3'. The underlined region of the primers corresponds to the specific CDR3 of the VHH. The forward primer used was specific for the FR1 region of VHH.

Figure 5-1. Nucleotide sequences of the parental VHH

The nucleotide sequences of the parental VHH were aligned and displayed above, with the CDR3 sequences highlighted in grey. Two unique degenerate reverse primers (shown above with arrows for the C8 and D7 primers), which corresponds to the underlined sequences, were designed to extend into the CDR3 region to create the family specific phage library.

5.2.2 C8- and D7- family specific phage library construction

The procedures for the creation of the C8- and D7/A12- like (D7 for short) dedicated libraries were described in Section 2.7.2 and were carried out by Ablynx NV. Briefly, the primers designed above were used to amplify out VHH genes with similar CDR3 sequences from the cDNA library that was originally isolated from the PBL pools of llama 44. The amplified fragments were then digested and gel purified, before insertion into a phagemid vector (pAX050) for display on filamentous bacteriophage. The family-specific phage libraries was then titrated out, and the size of the libraries was determined to be 5.7 x 10^6 and 2.0 x10^6 clones for the C8- and D7-specific library respectively. Through colony PCR on a selection of clones, the

percentage of functional inserts within the library were determined to be 100% and 91.3% for the C8- and D7-specific library respectively.

5.3 Preparation of the recombinant gp120 antigen

5.3.1 Recombinant gp120 92BR025 expression and purification

As the aim of the EMPRO project was to obtain antibodies that can better target subtype C HIV-1, selecting for VHH that can recognise a subtype C envelope will be highly desirable. The only readily available subtype C recombinant gp120 was from the CN54 isolate, but there were doubts about the antigenic profile of this isolate. Anna Forsman (a former PhD student in the lab) has demonstrated that recombinant gp120 CN54 does not bind efficiently to soluble CD4 and did not mediate infection when expressed on a virus particle. As there was a need to create a subtype C gp120 recombinant protein for use as an antigen in the panning process, she has cloned the full-length gp120 from a subtype C primary isolate (92BR025) which was obtained from the WHO-UNAIDS collection of primary isolates (Gao et al., 1994). These primary isolates represent strains that were circulating in areas chosen for vaccine trials. The gp120 92BR025.C1 clone was able to mediate infection when expressed on the HXB2 backbone and was chosen for recombinant protein expression.

The procedure for recombinant gp120 92BR025 expression is described in Section 2.7.1.1. Briefly, amplified gp120 fragments were cloned into a mammalian expression vector, and recombinant gp120 was expressed in 293T cells infected with a T7 RNA polymerase recombinant vaccinia virus to enhance expression. Secreted envelope protein was harvested 48 h post-transfection and purified by means of the 6-Histidine-tag which was incorporated into the reverse primer. Expression of recombinant gp120 in the cell culture supernatant was captured onto ELISA plates

by the sheep antibody D7324. Since the D7324 antibody recognises the C-terminus of gp120, this method captures antigens that are fully translated and secreted. Captured gp120 was detected using human anti-HIV-1 sera. A negative control supernatant (mock transfected cells) and gp120 CN54 of known concentration were also included in the ELISA.

Figure 5-2 shows the results of the ELISA from gp120 92BR025 expression. A dose-dependent curve was observed for both gp120 92BR025 and CN54. Based on the luminescence signals of both curves, the approximate amount of gp120 92BR025 expressed in the supernatant was determined to be approximately 3 µg/ml.

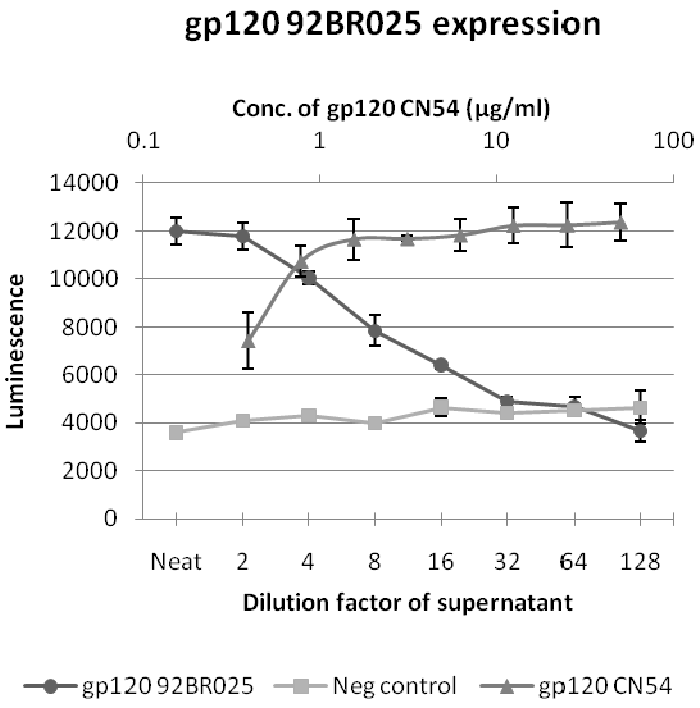

Figure 5-2. Detection of gp120 92BR025 expression in ELISA

Serial dilutions of cell culture supernatant were incubated on D7324 coated plates to capture gp120, which was detected with human anti-HIV serum followed by an AP-conjugated goat anti-human IgG antibody. A serial dilution of gp120 CN54, starting from 50 µg/ml, was also included as a positive control. A mock expression was included as a negative control.

The recombinant gp120 92BR025 was then purified with nickel columns using the method described in Section 2.6.4.2. The purified samples were dialysed overnight in PBS with a 90 kDa cut-off membrane. Figure 5-3 shows the image of the SDS-PAGE of the samples after purification. Most of the supernatant proteins (between 40 – 70 kDa) were removed, but the presence of impurities was still observed after His-tag purification. Dialysis of the purified samples did not remove all the impurities, as shown in the highlighted ellipse.

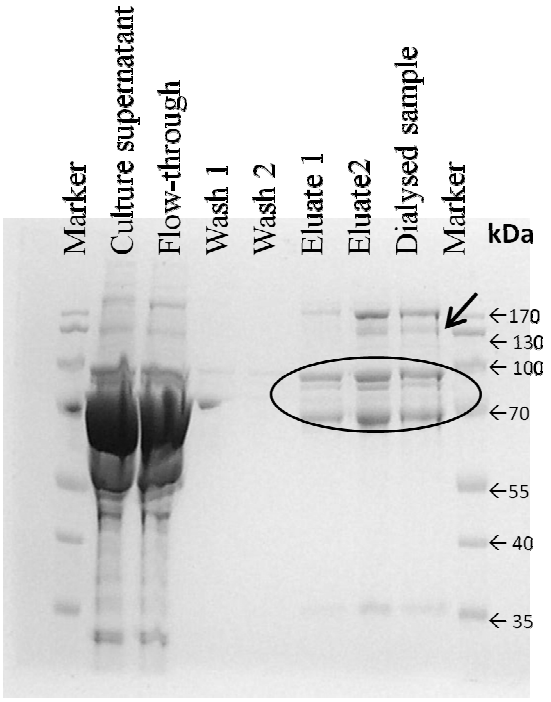

Figure 5-3. SDS-PAGE of gp120 92BR025 purification

The products before and after nickel column purification were analysed in SDS-PAGE. The culture supernatant and the flow-through showed the presence of huge quantities of culture proteins. After elution of the His-tag proteins, some of the impurities were still present in the sample. These are marked in the ellipse above. These impurities were not removed even after dialysis of the sample. The recombinant gp120 92BR025 with the His-tag is marked with the bold arrow.

5.3.2 Biotinylation of recombinant gp120

Antigen that is coated onto a solid substrate can cause antibodies to 'stick' onto the substrate, leading to avidity effects. In a new strategy to isolate VHH of higher affinity without the associated avidity effects, the phages were allowed to bind to the antigen in solution form, instead of coated onto plastic plate. The bound phages were captured by means of a biotin-avidin interaction on avidin coated plates. For that to occur, the antigen of interest needs to be biotinylated.

The gp120 of both IIIB and 92BR025 were biotinylated according to the method described in Section 2.7.1.2, which involves the attachment of biotin to any free NH2 groups on a protein. To check that the biotinylation of the antigens have succeeded, both ELISA and Western blots were carried out.

In the ELISA, Maxisorp plates were coated with both the biotinylated and unbiotinylated forms of gp120. Biotin was detected with a streptavidin-PO conjugate. The substrate was added and A_{490} measured. Figure 5-4 shows the graph from the ELISA. Both biotinylated gp120 IIIB and 92BR025 produced higher signals than the corresponding unbiotinylated samples and the blank control wells, indicating that both recombinant gp120 were successfully biotinylated. The recombinant gp120 were also probed with HRP-conjugated streptavidin in Western blots and visualised with ECL reagent on an X-ray film.

Figure 5-5 shows the image of the Western blot. Biotinylated gp120 IIIB gave a clear prominent band near the 130 kDa marker, whereas biotinylated gp120 92BR025 only gave a faint band at the same position. The presence of other bands in the 92BR025 sample indicated that impurities were present, as was already highlighted in the previous section.

The biotinylated gp120 samples were also probed with the C8 and D7 VHH in ELISA, and these VHH were able to bind. Hence, the epitopes for C8 and D7 were not disrupted during the biotinylation process (Data not shown).

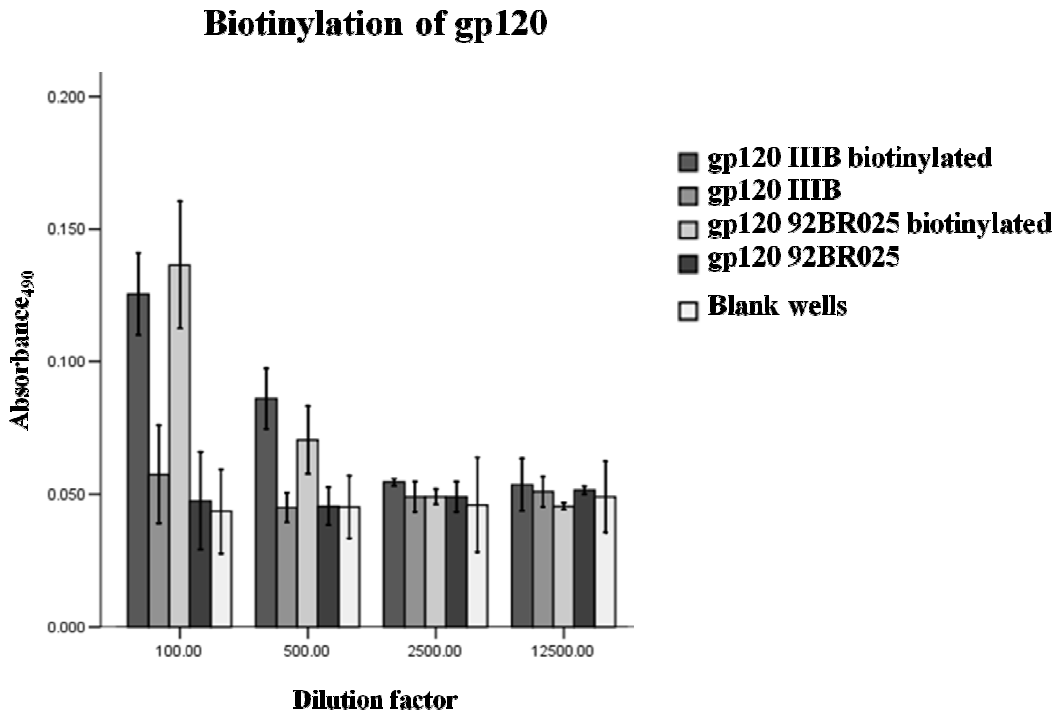

Figure 5-4. Detection of biotinylated gp120 in ELISA

Both biotinylated and unbiotinylated gp120 IIIB and 92BR025 were coated onto Maxisorp plates and detected with streptavidin in ELISA. The results show that the biotinylated samples gave a higher signal than unbiotinylated samples, which were comparable to the readings from blank control wells.

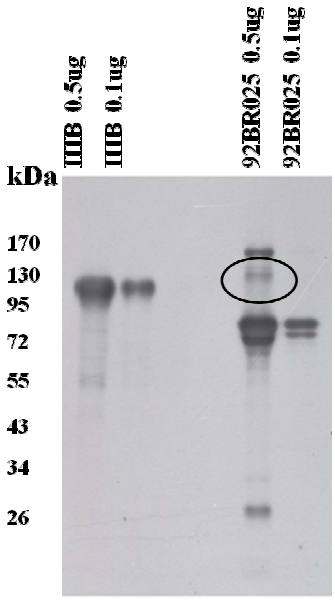

Figure 5-5. Western blot of biotinylated gp120 IIIB and 92BR025

Biotinylated gp120 IIIB and 92BR025 of two different concentrations were blotted onto PVDF membranes and probed with streptavidin. Biotinylated gp120 IIIB produced a clean band near the 130 kDa mark, whereas 92BR025 produced only a faint band (shown circled). Impurities were also present in the biotinylated gp120 92BR025 sample, as shown by the presence of bands near the 72 kDa mark.

5.4 Biopanning and selection of C8- and D7- family specific phage libraries

The C8- and D7-family specific phage libraries that were created above were sequentially panned on either biotinylated gp120 IIIB or 92BR025. Recombinant gp120 IIIB was used in the selection protocol due to its availability, purity and good binding to sCD4 in ELISA. HIV-1 IIIB is, however, a CXCR4-using TCLA isolate and does not represent the majority of the transmitted HIV-1 isolates. In addition, IIIB is a subtype B virus; a subtype which accounts for only about 10% of infections worldwide. Nevertheless, since the parental C8 and D7/A12 VHH were isolated from selections using gp120 IIIB, this antigen was also used in the following selection.

To facilitate selection of VHH that could recognise HIV-1 primary isolates, as opposed to the TCLA virus HIV-1 IIIB, the family specific phage libraries were also panned on recombinant gp120 derived from the subtype C primary isolate 92BR025 described in the previous sections. The phage libraries were panned twice on each subtype to enrich for VHH that can recognise that particular subtype of HIV-1.

In an attempt to remove avidity effects during the binding of phages to the antigen, the phages were allowed to bind to low concentrations of antigen in solution form in pre-blocked plastic plates. This solution was then transferred to neutravidin coated plates to capture the biotinylated gp120 antigen. Neutravidin is a neutrally charged derivative of the avidin molecule that binds to biotin with minimal nonspecific binding. Selections with biotinylated gp120 92BR025 were found to produce high background levels, and this might be due to the presence of impurities in the sample.

We decided that the sheep antibody D7324 (which recognises the C-terminus of gp120) would be coated onto plates to capture the phages bound to gp120 92BR025.

This method of allowing the phages to bind to antigen in solution form is in contrast to the previous selections where the phages were allowed to bind to antigen that was pre-coated on Maxisorp plates. After the transfer of bound phages to the plates, the wells were then washed eight times to dislodge unbound phages and antigen. We assumed that the family specific phage library would have similar properties as the parental C8 and D7 VHH, and should therefore all recognise the CD4 binding site-related epitopes of gp120. Therefore, trypsin was used to elute out all bound phages, instead of a competitive sCD4 elution used in the selections for C8 and D7. After two rounds of selections on each biotinylated antigen, the outputs that gave the best enrichment of binders from the optimum concentration of antigen were harvested. These procedures are detailed in Section 2.7.3.

5.4.1 Bio-panning on biotinylated gp120 IIIB and 92BR025

In the first round of selection, the family specific phage library was allowed to incubate with serially diluted biotinylated gp120 IIIB and 92BR025 ranging from 10 nM to 0.01 nM. Blank controls containing PBS only were also included. These were then transferred to neutravidin and D7324 coated plates respectively, and bound phages were captured through the attachment of the antigen onto the plates. After the removal of unbound phages through washing, the bound phages were eluted with trypsin. Eluted phages were titrated onto *E. coli*, as described in Section 2.6.2.2, and shown in Figure 5-6 [A]. In each selection, there was an enrichment in the number of clones eluted, compared to the blank control wells. However, the selections using gp120 92BR025 appear to give a higher background compared to using gp120 IIIB.

The eluted phages from the first round of selection were amplified in *E. coli* TG1 cells, and the phages were rescued to be used as inputs in the second round of selection following the methods described in Section 2.6.2. The same selection procedure was carried out as before, except that the amount of antigen used was decreased. The eluted phages were again titrated on *E. coli*, then on agar plates and shown in Figure 5-6 [B]. Two outputs from each selection were chosen for VHH expression by subcloning the selected repertoire into the pAX051 expression vector (refer to methods in Section 2.6.3). Ninety-six clones were picked from each output and the VHH harvested from the periplasmic fractions.

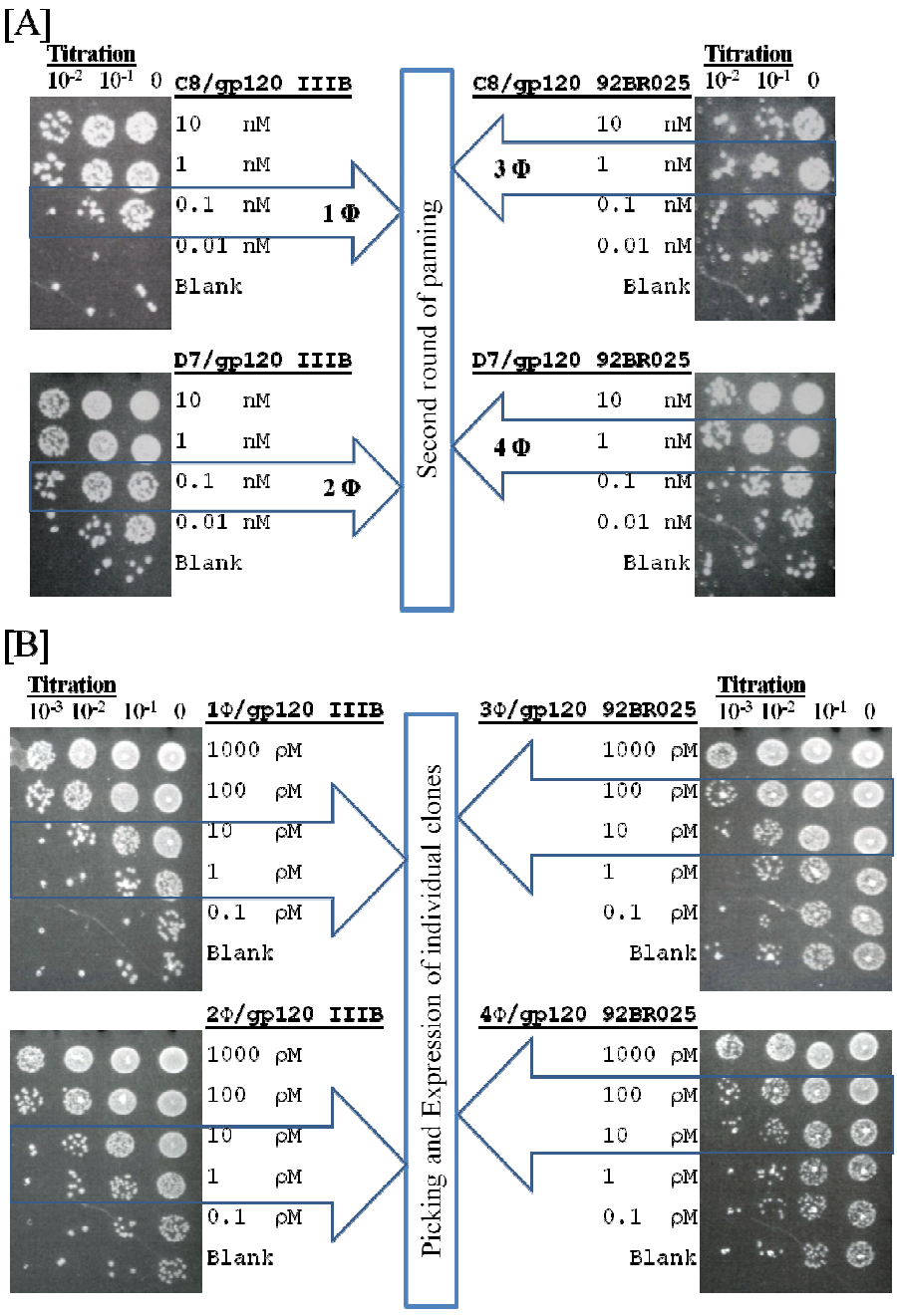

Figure 5-6. Titration on eluted phages from C8- and D7-family specific libraries

The C8 and D7 family specific phage libraries were sequentially panned with biotinylated gp120 IIIB or 92BR025 in 2 rounds of selections. [A] In the first round of selection, the eluted phages (1Φ, 2Φ, 3Φ, and 4Φ) were brought forward to a second round of panning, as shown in the block arrows above. [B] After the titration of eluted phages from the second round of selection, the outputs from two of each selection were brought forward to the next stage of picking and expression of individual clones (as shown in the block arrows). In all cases, the number of phages eluted was more than the blank control wells.

A representative sample of the selected clones was DNA fingerprinted with *HinF*I to determine their heterogeneity. This is shown in Figure 5-7. Compared to the fingerprints generated in Chapter 4, the selected clones here appear to show much less variation from each other. This is to be anticipated since all the selected clones have homologous sequences that belong to the same family.

A total of 8 different plates containing 96 different clones each were selected, containing 4 plates each from the C8 and D7 family specific library. A schematic of the selection process and the outputs are shown in Figure 5-8.

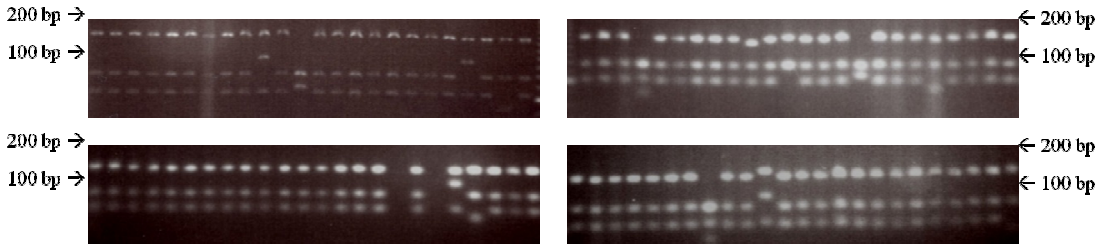

Figure 5-7. DNA fingerprint of selected clones from family specific libraries

The final outputs from the selections were subcloned into expression vectors for VHH production. Ninety-six different clones were picked for VHH expression, and their DNA was fingerprinted by digestion with *HinF*I. These were then separated on agarose gel electrophoresis and visualised. The similarity of the fragment sizes show that the clones selected were not very diverse. A representative sample of the digested clones is shown here.

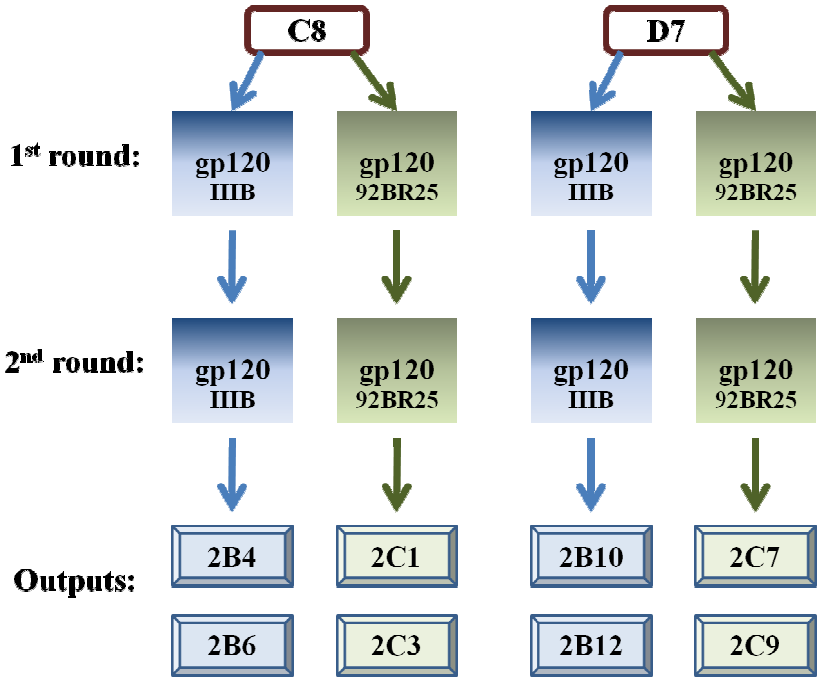

Figure 5-8. Schematic of the C8- and D7- family specific libraries selection

5.4.2 Polyclonal sequencing of the outputs

To determine if the family specific phage library construction and the panning of sister clones were successful, a sequencing reaction was carried out on the polyclonal outputs of each selection. Since the outputs from each selection contain the selected VHH repertoire, sequencing of the polyclonal outputs will produce variations in the nucleotide sequence if the outputs were of a high diversity.

Figure 5-9 shows the sequences of the polyclonal outputs aligned with the sequence of the parental VHH. In the selections from the C8-specific library, all the outputs were identical in sequence to the parental C8 VHH. This meant that only identical clones were produced during the construction of the C8-family specific phage library. This could be due to the C8-primer being too specific and only pulled out

sequences identical to C8 during the construction of the C8-family specific phage library, or no other clones like C8 were present in the llama.

Fortunately, the polyclonal outputs from the D7-family selections showed variations in its translated sequences and are denoted with an 'X' to represent an undeterminable amino acid sequence in Figure 5-9. On closer inspection, many of these variations occur within the CDRs, indicating that the selections had managed to pull out sister clones which were fairly diverse and could represent affinity matured clones.

In summary, selections on the C8-family specific phage library produced only identical clones to the parental C8 VHH. On the other hand, selections on the D7-family specific phage library have succeeded in producing a family of homologous clones with similar sequences as the parental D7/A12 VHH.

C8-family outputs

```
HIV-1B2D        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-1B2C        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-2B4D        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-2B4E        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-2B6D        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-2B6E        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-1C9B        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-1C9C        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-2C1C        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-2C3C        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-2C3D        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
HIV-2C1D        EVQLVESGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
PMP9C8          AVQLVDSGGGLVQAGGSLRLSCVVSGSIFSINAMGWYRQAPGKQRDLVARISGDSSTYYI 60
                :****:**************************************************

HIV-1B2D        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-1B2C        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-2B4D        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-2B4E        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-2B6D        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-2B6E        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-1C9B        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-1C9C        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-2C1C        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-2C3C        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-2C3D        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
HIV-2C1D        DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
PMP9C8          DSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAARRLPIGDYTDWGQGTQVTVSS 118
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D7-family outputs

```
                                          CDR1                    CDR2
HIV-2B10D       EVQLVESGGGLVQAGGSLRLSCTXSGRXSSSXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
HIV-1C2C        EVQLVESGGGLVQAGGSLRLSCTXSGRXSSSXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
HIV-1B5E        EVQLVESGGGLVQAGGSLRLSCTASGRXXSSXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
HIV-1B5D        EVQLVESGGGLVQAGGSLRLSCTXSGRIXSSXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
HIV-2B10E       EVQLVESGGGLVQAGGSLRLSCTXSGRIXSXXDMGWFRQAPGKEREFVAAIXWSGGTTDY 60
HIV-2B12D       EVQLVESGGGLVQAGGSLRLSCTXSGRIXSSXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
HIV-1C2B        EVQLVESGGGLVQAGGSLRLSCTXSGRXSSSHDMGWFRQAPGKEREFVAAISWSGGTTDY 60
PMP9D7          AVQLVESGGGLAQAGGSLRLSCTVSGRTSSSHDMGWFRQAPGKEREFVAAISWSGGTTNY 60
HIV-2C7C        EVQLVESGGGLVQAGGSLRLSCTASGRISSSXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
HIV-2C9C        EVQLVESGGGLVQAGGSLRLSCTASGRISSSXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
HIV-2C7D        EVQLVESGGGLVQAGGSLRLSCTASGRISSXXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
HIV-2C9D        EVQLVESGGGLVQAGGSLRLSCTASGRISSXXDMGWFRQAPGKEREFVAAISWSGGTTDY 60
                 **********.***** ***** ***  *  ****************** ****** *

                                                  CDR3
HIV-2B10D       ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSXYPSNSDYYYWGQGTQVTVSS- 127
HIV-1C2C        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSXXPSNSDYYYWGQGTQVTVSS- 127
HIV-1B5E        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSXXPSNSDYYYWGQGTQVTVSS- 127
HIV-1B5D        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDXPSNSDYYYWGQGTQVTVSS- 127
HIV-2B10E       ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSXYPSNSDYYXWGQGTQVTVSS- 127
HIV-2B12D       ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSXYPSNSDYYXWGQGTQVTVSS- 127
HIV-1C2B        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDXPSNSDYYYWGQGTQVTVSS- 127
PMP9D7          ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDNPSNSDYNYWGQGTQVTVSS- 127
HIV-2C7C        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDYPSNSDYYDWGQGTQVTVSS- 127
HIV-2C9C        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDYPSNSDYYDWGQGTQVTVSS- 127
HIV-2C7D        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYXDYPSNSDYYDWGQGTQVTVSS- 127
HIV-2C9D        ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDYPSNSDYYDWGQGTQVTVSS- 127
                ******************************************   ******  ***********
```

Figure 5-9. Polyclonal sequencing of the selected outputs

The polyclonal outputs from the selections were sequenced and the nucleotide sequence was translated and aligned using ClustalW2. Positions marked with an 'X' refer to an undeterminable amino acid, probably due to variations in that particular position. Outputs from the C8-family were found to be identical to the parental C8 VHH, whereas variations were observed in the CDRs in the outputs of the D7-family.

5.4.3 Sequencing of the individual VHH

Sequencing of all the individual clones selected from the D7-specific family was carried out to determine the diversity of the VHH in this library. Out of the 376 clones picked, there were 49 unique amino acid sequences. Many identical clones were observed in both the selection strategies, using either biotinylated gp120 IIIB or 92BR025, and therefore the antigen subtype did not help in the enrichment of a particular set of clones. One may postulate that there were at least 49 unique members with sequences similar to D7 and A12 in the original phage library repertoire from llama 44. All the VHH showed high sequence homology to the parental D7 VHH, and variations in the sequences were observed in both the framework regions, as well as the CDRs of the VHH.

A total of 31 unique VHH were chosen, based on the differences in their amino acid sequences, for large scale expression and purification of the VHH to enable further characterisation. The DNA encoding the VHH fragments were all subcloned into the pAX051 expression vector providing a C-terminal 6x Histidine tag for purification. The methods are described in Section 2.6.4.

5.5 Variations in Neutralisation potencies

The 31 individual VHH from the D7-family specific library were tested for their neutralisation activity by assaying on T-cell line adapted viruses, recombinant replication-competent chimeric viruses and psuedoviruses expressing envelopes cloned from plasma of infected individuals. The neutralisation activity was evaluated on the TZM-bl cell line that express CD4, the co-receptors CXCR4 and CCR5, as well as a Tat-induced luciferase reporter when infected with HIV-1. The TZM-bl

assay is described in Section 2.3.9, and the highest VHH concentration tested was at 25 µg/ml. The lowest VHH concentration required to achieve 50% reduction of infectivity (IC_{50}) compared to virus control were determined.

The numbers of VHH tested from the 2B10, 2C7 and 2C9 outputs (refer to Figure 5-8) were 11, 9 and 11 respectively. These were chosen based on differences in their amino acid sequences, especially in the CDR regions. A comparison of the neutralisation potency with the b12 mAb was also included, which is one of the most extensively studied bcnAb to HIV-1, which also binds to an epitope that overlaps the CD4bs of gp120 (Zhou et al., 2007). A total of seven different strains of viruses were used, of which 3 were subtype B viruses (IIIB, QH0692.42, PVO.4) and 4 were subtype C viruses (C222, C27d, 92BR025, ZM214M). The QHO692.42, PVO.4 and ZM214M isolates are psuedoviruses which are Tier-2 CCR5 using envelopes, and are part of the subtype standard reference panels (Li et al., 2005; Li et al., 2006b). The C222 and C27d primary isolates were described in Chapter 3, and 92BR025 is a PBMC isolate obtained from the WHO-UNAIDS panel of primary isolates (Gao et al., 1994). The envelopes from these isolates were cloned into HIV-1 backbones producing chimeric replication competent viruses.

From the results in Table 5-1, the VHHs can be broadly categorised into three groups according to their ability to neutralise different strains of HIV-1. Most of the VHH showed similar neutralisation potencies. For instance, all the VHH tested were able to neutralise HIV-1 IIIB and C27d at less than 1 µg/ml, whereas none were able to neutralise PVO.4 and ZM214M. All the VHH were also able to neutralise QHO692.42 to moderate levels with IC_{50} values of between 10-25 µg/ml.

However, two of the subtype C viruses tested revealed significant differences in the neutralising potencies of the VHH. Most of the VHH were able to neutralise both C222 and 92BR025 to high potencies with IC_{50} values of less than 1 µg/ml and 5 µg/ml respectively. This group of VHH is termed the broad neutralisers. The A12 VHH belongs to this group. A group of 7 different VHH was unable to neutralise both C222 and 92BR025, and are termed 'narrow' neutralising VHH. The D7 VHH belongs to this group. Three of the VHH were able to neutralise C222 with IC_{50} values of less than 15 µg/ml, but they were not able to neutralise 92BR025. This group was termed 'intermediate' neutralisers.

Table 5-1. VHH and mAb b12 IC_{50} (ug/ml) titres against HIV-1

	VHH[a]	Subtype B			Subtype C			
		IIIB	QH0692.42	PVO.4	C222	92BR025	C27d	ZM214M
Broad	2B10A2	0.07	19.73	>25	0.13	2.01	0.02	>25
	2B10C2	0.16	18.66	>25	0.12	1.57	0.03	>25
	2B10D5	0.57	19.63	>25	0.96	1.95	nd	>25
	2B10D7	0.12	21.63	>25	0.20	1.42	0.03	>25
	2B10D11	0.18	15.48	>25	0.36	4.47	0.03	>25
	2B10G5	0.40	16.10	>25	0.04	0.28	0.05	>25
	2B10H1	0.29	17.21	>25	0.08	0.74	0.04	>25
	2B10H3	0.29	19.14	>25	0.06	0.39	0.04	>25
	2C7B8	0.15	10.78	>25	0.27	0.78	0.14	>25
	2C7D2	0.17	20.71	>25	0.28	0.60	0.14	>25
	2C7E3	0.07	12.16	>25	0.13	0.48	0.03	>25
	2C7G11	0.16	16.08	>25	0.24	1.76	0.08	>25
	2C9A8	0.30	nd	>25	0.41	2.40	0.07	>25
	2C9B11	0.24	nd	>25	0.30	3.41	0.24	>25
	2C9C5	0.12	nd	>25	0.16	1.23	0.12	>25
	2C9D9	0.37	nd	>25	0.61	3.64	0.30	>25
	2C9E4	0.35	nd	>25	0.50	1.76	0.14	>25
	2C9E7	0.30	20.87	>25	0.34	0.90	0.15	>25
	2C9F1	0.13	19.31	>25	0.48	0.95	0.13	>25
	2C9F10	0.13	18.70	>25	0.16	0.61	0.12	>25
	2C9H5	0.17	17.28	>25	0.19	0.73	0.03	>25
Inter-mediate	2C7F7	0.34	17.31	>25	4.53	>25	0.24	>25
	2C7H5	0.12	16.50	>25	10.20	>25	0.06	>25
	2C9E2	0.20	19.39	>25	4.08	>25	0.16	>25
Narrow	2B10B10	0.54	19.40	>25	>25	>25	nd	>25
	2B10E2	0.46	15.90	>25	>25	>25	nd	>25
	2B10E8	0.49	18.54	>25	>25	>25	nd	>25
	2C7D1	0.24	16.29	>25	>25	>25	0.17	>25
	2C7D5	0.13	20.04	>25	>25	>25	0.08	>25
	2C7E11	0.21	17.27	>25	>25	>25	0.09	>25
	2C9F6	0.18	14.54	>25	>25	>25	0.15	>25
	A12	0.09	13.00	>25	0.07	0.23	0.02	>25
	D7	0.30	17.00	>25	>25	>25	0.03	>25
	b12	0.21	0.70	>25	>25	>25	0.02	12.29

[a.] The VHH are grouped into 3 categories according their neutralising potencies against C222 and 92BR025. The neutralisation potencies are shaded in different shades, with darker shades indicating higher potencies.

5.6 Sequence analysis of the CDRs

All the 31 VHH possessed unique sequences with variations within the framework regions (FR) as well as in the complementarity determining regions (CDRs). To understand the basis of the variations in neutralising potencies amongst the members of the D7-specific VHH family, the amino acid sequences of the CDRs of the VHH were grouped according to their three neutralising categories, and these are shown in Table 5-2. Only the sequences of the CDRs are shown as these are crucial in determining antigen recognition. The positions of the CDRs residues are as follows: 24-33 for CDR1, 52-59 for CDR2 and 99-116 for CDR3. Variations in the amino acid residues are highlighted in grey.

When comparing between the broad and narrow neutraliser categories, the only region that is distinct between them is the presence of an Tyr-Tyr-Asp (YYD)116 motif at the C-terminal end of the CDR3 region for the broad neutralisers. There is an absence of this motif in all the narrow neutralisers. Mutation of this YYD116 motif to YYY, YNY or YND seems to hinder the neutralising abilities of these particular viruses. Within the broad neutraliser category, mutations in the other CDR or framework residues did not hinder neutralising abilities.

To demonstrate that the YYD116 motif is crucial for the broad neutralising ability of the VHH, we compared the sequences of all the VHH obtained from this family. In Figure 5-10(A), the sequences of both 2C7D2 and 2C9E2 are exactly the same, except for a single YYD116 to YYY116 mutation. This single mutation resulted in a change in neutralisation category from broad to intermediate. Similarly for 2B10G5 and 2C7D1, a single YYD116 to YNY116 mutation resulted in a neutralisation category change from broad to narrow, as shown in Figure 5-10(B). The 2C7H5 and

2C7E11 VHH belong to the intermediate and narrow neutralisation category, and they both exhibit a YND116 mutation. Although these two VHH contain a few other mutations in its sequence, these mutations are also found in the other broad neutraliser (or YYD) variants and therefore do not affect its neutralising potential, as shown in Figure 5-10(C). The 2C9E2 VHH possesses a S63F mutation in the framework region, in addition to the presence of a YYY motif that caused a change from broad to poor neutralisation ability. This is shown in Figure 5-10(D). The YYY, YNY and YND motifs are therefore associated with less broad neutralising potential when compared to the YYD motif.

Certain residues within the CDRs were found to show mutations in high frequencies. These are residues 24, 28, 31, and 32 within CDR1, residue 59 in CDR2, and residues 106, 108, 115 and 116 in CDR3. Except for residues 115 and 116 which are part of the YYD motif, mutations in all the other residues did not affect the neutralisation ability of the VHH, and are therefore not as critical to function.

However, the presence of a YYD motif in itself is not sufficient to confer broad neutralising ability to the VHH against these subtype C strains tested. Even though the 2C7F7 VHH contains a YYD motif, it falls under the intermediate category. From Table 5-2, 2C7F7 contain three other point mutations within the CDRs. There is one unique H/Y32F mutation within the CDR1 that is not found in any of the other VHH within the family. A unique phenylalanine residue, that is nonpolar and hydrophobic, has replaced the polar histidine or tyrosine residues that are usually found in that position. This particular mutation might therefore affect the folding or binding of 2C7F7 and thus affect its neutralisation ability.

Table 5-2. Sequence comparison of the CDRs

VHH[a]	CDR1(24-33)[b]	CDR2(52-59)[b]	CDR3(99-116)[b]
Broad Neutralisers			
2B10D5	VSGRTSSSHD	SWSGGTTD	KWRPLRYSDNPSNSD**YYD**
2B10C2	VSGRTSSSHD	SWSGGTTD	KWRPLRYSDNPSNSD**YYD**
2C9A8	VSGRTSSSHD	SWSGGTTD	KWRPLRYNDNPSNSD**YYD**
2C9B11	VSGRTSSSHD	SWSGGTTD	KWRPLRYNDNPSNSD**YYD**
2B10A2	VSGRTSSSHD	SWSGGTTN	KWRPLRYSDNPSNSD**YYD**
2B10D11	VSGRISSSHD	SWSGGTTD	KWRPLRYSDNPSNSD**YYD**
2C7E3	ASGRISSSHD	SWSGGTTN	KWRPLRYSDSPSNSD**YYD**
2C9C5	ASGRISSSHD	SWSGGTTN	KWRPLRYSDSPSNSD**YYD**
2C9F10	APGRISSSHD	SWSGGTTN	KWRPLRYSDSPSNSD**YYD**
2C7D2	ASGRISSSHD	SWSGGTTD	KWRPLRYSDDPSNSD**YYD**
2B10G5	ASGRISSSHD	SWSGGTTD	KWRPLRYSDDPSNSD**YYD**
2C9E7	AFGRISSSHD	SWSGGTTD	KWRPLRYSDDPSNSD**YYD**
2B10H3	ASGRISSSHD	SWSGATTD	KWRPLRYSDDPSNSD**YYD**
2C7B8	ASGRISSSYD	SWSGGATD	KWRPLRYSDYPSNSD**YYD**
2B10D7	ASGRISSSYD	SWSGGTTD	KWRPLRYSDYPSNSD**YYD**
2B10H1	ASGRISSSYD	SWSGGTTD	KWRPLRYSDYPSNSD**YYD**
2C9F1	ASGRISSSYD	SWSGGTKD	KWRPLRYSDYPSNSD**YYD**
2C9H5	ASGRISSSYD	SWSSGTTD	KWRPLRYSDYPSNSD**YYD**
2C7G11	ASGRISSSYD	SWSGGTTD	KWRPLRYSDSPSNSD**YYD**
2C9D9	ASGRISSMYD	SWSGGTTA	KWRPLRYSDDPSNSD**YYD**
2C9E4	ASGRISSMYD	SWSGGTTA	KWRPLRYSDDPSNSD**YYD**
A12	ASGRISSSYD	SWSGGTTD	KWRPLRYSDYPSNSD**YYD**
Intermediate Neutralisers			
2C7H5	ASGRISSSHD	SWSGGTTD	KWRPLRYSDDPSNSD**YND**
2C9E2	ASGRISSSHD	SWSGGTTD	KWRPLRYSDDPSNSD**YYY**
2C7F7	ASGRTSSSFD	SWSGGTTD	KWRPLRYSDNPSNSD**YYD**
Narrow Neutralisers			
2B10E8	VSGRISSSHD	SWSGGTTD	KWRPLRYSDNPSNSD**YND**
2C7E11	ASGRVSSSHD	SWSGGTTD	KWRPLRYSENPSNSD**YND**
2B10E2	ASGRISSSYD	SWSGGTTD	KWRPLRYSDYPSNSD**YNY**
2C9F6	ASGRISSSYD	SWSGGTTD	KWRPLRYSDYPSNSD**YYY**
2B10B10	ASGRISSSYD	SWSGGTTD	KWRPLRYSDYPSNSD**YNY**
2C7D1	ASGRISSSHD	SWSGGTTD	KWRPLRYSDDPSNSD**YNY**
2C7D5	ASGRLSSMYD	SWGGGSTT	KWRPLRYSDNPSNSD**YND**
D7	VSGRISSSHD	SWSGGTTN	KWRPLRYSDNPSNSD**YNY**

[a]Amino acid sequences of the CDRs are grouped according to their three different neutralisation categories. Grey highlights refer to regions with sequence variations. The YYD motifs at the end of the CDR3 are bolded and underlined. Variations of this motif are bolded.

[b]The numbers in brackets refer to the amino acid positions of the CDRs within the VHH sequence.

[A]

```
2C7D2           EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY 60
2C9E2           EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY 60

2C7D2           ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYYDWGQ 119
2C9E2           ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYYYWGQ 119
```

[B]

```
2B10G5          EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY 60
2C7D1           EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY 60

2B10G5          ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYYDWGQ 119
2C7D1           ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYNYWGQ 119
```

[C]

```
2B10G5          EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY 60
2C7H5           EVQLVESGGGLVQAGGSLRLSCTASGRISSSHDMGWFRQAPGKEREFVAAISWSGGTTDY 60
2C7E11          EVQLVESGGGLVQAGGSLRLSCTASGRVSSSHDMGWFRQAPGKEREFVAAISWSGGTTDY 60

2B10G5          ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYYDWGQ 119
2C7H5           ADSVKGRFAISKDNAKSAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDDPSNSDYNDWGQ 119
2C7E11          ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSENPSNSDYNDWGQ 119
```

[D]

```
2B10D7          EVQLVESGGGLVQAGGSLRLSCTASGRISSSYDMGWFRQAPGKEREFVAAISWSGGTTDY 60
2C9F6           EVQLVESGGGLVQAGGSLRLSCTASGRISSSYDMGWFRQAPGKEREFVAAISWSGGTTDY 60

2B10D7          ADSVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDYPSNSDYYDWGE 119
2C9F6           ADFVKGRFAISKDNAKNAVSLQMNSLKPEDTAVYYCAAKWRPLRYSDYPSNSDYYYWGQ 119
```

Figure 5-10. Full sequences of relevant VHH illustrating important mutants

The full sequences of relevant VHH are shown above, with mutations highlighted in grey. [A] A YYD to YYY mutation resulted in 2C9E2 turning into an intermediate neutraliser. [B] A YYD to YNY resulted in 2C7D1 turning into a narrow neutraliser. [C] A YYD to YND mutation resulted in 2C7H5 and 2C7E11 turning into an intermediate and narrow neutraliser respectively. The other mutations highlighted in its sequence are also present in other YYD variants, and do not contribute to the neutralisation category as defined in this study. [D] A YYD to YYY mutation resulted in 2C9F6 turning into a narrow neutraliser. The presence of an S63F mutation in the FR is unlikely to affect neutralisation breadth.

5.7 VHH show diverse kinetic measurements

To determine if there was a relationship between the binding kinetics of the VHH to gp120 and the neutralisation breadth of the VHH against HIV-1, the affinities of the VHH to recombinant gp120 IIIB were measured using surface plasmon resonance techniques. Surface plasmon resonance measures the angle by which polarised light is reflected from a gold-covered glass surface, to which molecules of interest are immobilised usually through a dextran layer linked to the gold. The angle by which the polarised light is reflected is used to calculate the density above the glass surface, enabling real-time analysis of the interactions between the immobilised molecule and molecules added to the fluid-phase. Kinetic measurements were performed on a BIAcore machine by Bart Hoorelbeke at Ablynx NV, who is our collaborator on the EMPRO project.

Recombinant gp120 IIIB was coupled to the biosensor chip using standard amine coupling techniques, and the periplasmic extracts containing VHH from all the clones of 2B10 were measured for their dissociation rate constants (k_d). Due to time constraints and limitations in access to the BIAcore facility, we were only able to test the VHH outputs from 2B10 only. A remarkably high proportion of the VHH clones (98%) from 2B10 were able to bind to recombinant gp120 IIIB. The k_d or off-rates ranged from 5.91×10^{-4} s^{-1} to 2.47×10^{-5} s^{-1}, representing a range of over 20-fold. This showed that the biological active members within the D7 sub-family library of VHH were fairly diverse, despite such variations not being evident in the DNA fingerprint using *Hin*FI digestion.

Twelve VHH were subsequently chosen, based on their wide range of off-rates, to be purified and further analysed in the BIAcore. The association and dissociation rate

constants (k_a and k_d) were determined and used to calculate the equilibrium dissociation constants (K_D). These are shown in Table 5-3, and the D7 and A12 VHH were included for comparison.

Interestingly, VHH with low off-rates of $1x10^{-4}$ s^{-1} and below possess a YYD116 motif, which is associated with a broad neutralising ability. VHH with high off-rates of $1x10^{-4}$ s^{-1} and above do not possess the YYD116 motif and display a corresponding lack in broad neutralising ability. A lower off-rate would mean that the VHH 'sticks' longer onto the antigen, hence VHH with the YYD116 motif were able to 'stick' longer onto gp120 IIIB compared to those VHH without the YYD116 motif. These also have a corresponding greater affinity (a lower dissociation equilibrium constant or K_D) for gp120 IIIB. A greater affinity for gp120 in the VHH with the YYD116 motif might therefore have a direct correlation with it having a broader neutralisation potential.

However, the kinetics of the D7 VHH seems to contravene the above observation. The D7 VHH has a YNY motif that is associated with a narrow neutralising ability, but yet its off-rates were in the range of those VHH in the broad neutralising category. A greater sampling of the VHH needs to be carried out to confirm this observation.

Table 5-3. VHH kinetic constants and affinities

VHH	k_D (1/s) [a]	K_D (nM)[b]	$Motif_{116}$[c]	Neut. Category[d]
2B10D11	2.47E-05	0.22	YYD	Broad
2B10H1	2.52E-05	0.17	YYD	Broad
2B10C2	2.76E-05	0.23	YYD	Broad
2B10G5	3.09E-05	0.21	YYD	Broad
2B10H3	3.12E-05	0.19	YYD	Broad
2B10A2	6.80E-05	ND	YYD	Broad
2B10D7	7.37E-05	0.57	YYD	Broad
2B10D5	7.61E-05	0.66	YYD	Broad
2B10B4	8.56E-05	0.52	YYD	Broad
2B10E8	1.12E-04	1.1	YND	Narrow
2B10E2	3.18E-04	1.9	YNY	Narrow
2B10B10	3.39E-04	1.7	YNY	Narrow
A12	2.98E-05	0.1	YYD	Broad
D7	6.88E-05	0.1	YNY	Narrow

[a] Dissociation rate constant, [b] Equilibrium dissociation constant, [c] Amino acid motif at position 116, [d] Neutralisation category as shown in Table 5-1.

5.8 Inhibition of sCD4 binding

The parental D7 and A12 VHH was previously determined to compete with sCD4 for binding to gp120 in both ELISA and surface plasmon resonance (Forsman et al., 2008). Even though a general trypsin elution was employed during the panning of the VHH from the family-specific libraries, the selected VHH from this study were all assumed to have similar recognition properties and recognise a CD4bs-related epitope of gp120. A competition ELISA was therefore carried out to determine if the selected VHH behaved in a similar manner.

A fixed amount of gp120 IIIB was first allowed to bind to titrated amounts of VHH, and the ability of the bound VHH to inhibit the binding of gp120 to sCD4-precoated plates was measured. Therefore, greater amounts of a CD4bs recognising VHH will prevent gp120 from binding to the plates and lead to lower levels of gp120 detection. A sample of 15 different VHH from the different outputs representing a range of different affinities was tested in this assay. The parental D7 VHH and the CD4bs recognising mAb b12 were also included in this assay, together with an irrelevant negative control VHH (VHH #3). The results are shown in a graph in Figure 5-11.

VHH which possess a YYD116 motif are show in solid lines and those without the motif are in dashed lines. The negative control VHH showed no effect in affecting gp120 binding to sCD4. Together with the D7 VHH and positive control mAb b12, the 15 different VHH isolated in this study were all found to inhibit binding of gp120 to sCD4 in a dose dependent manner. This is possible if the VHH bind to an epitope which sterically overlaps the CD4bs and hinders the binding of sCD4, or if VHH binding locks gp120 in a conformation that prevents binding of sCD4. It is quite likely that this family of VHH all recognise related epitopes on gp120 but bind with

different off-rates. Unliganded D7 VHH has been crystallised and a high-resolution structure of D7 was obtained by W. Weissenhorn's laboratory, and a crystal structure of D7 liganded to gp120 is being sought.

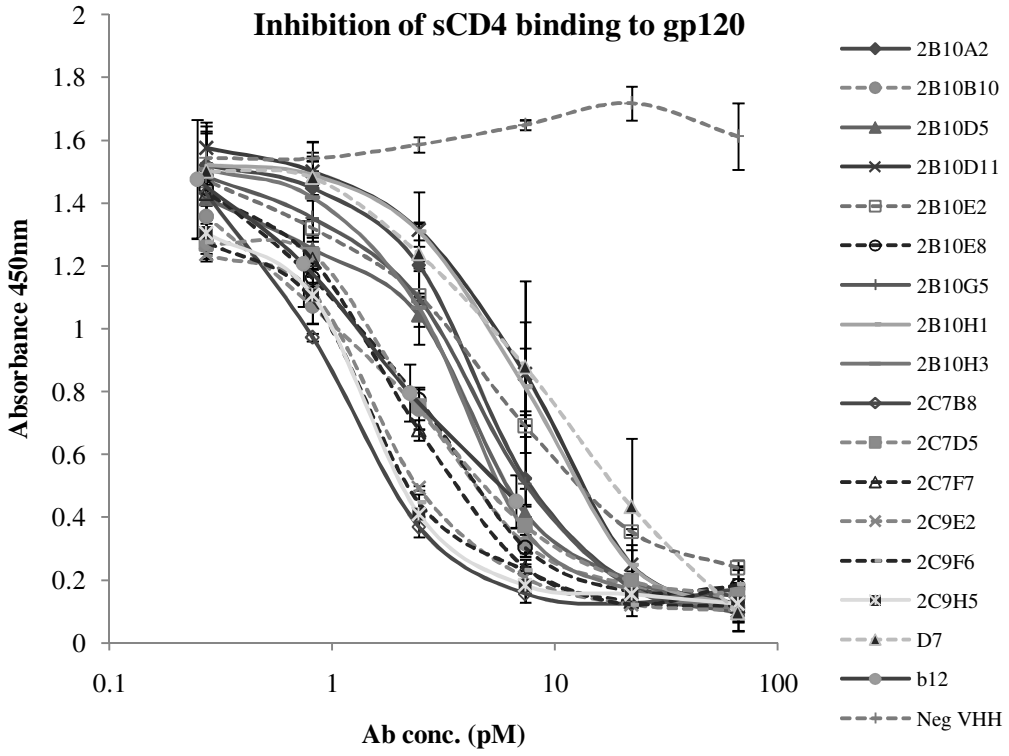

Figure 5-11. VHH inhibition of sCD4 binding to gp120 IIIB in ELISA

A range of VHH were tested for their ability to inhibit sCD4 binding to gp120 IIIB in ELISA. Dashed lines refer to VHH without the YYD motif, and solid lines refer to VHH with the YYD motif. All the VHH from the D7 subfamily were able to inhibit sCD4 binding to gp120 IIIB, in a manner similar to the mAb b12. The presence of the YYD motif did not produce a clear difference in the inhibition pattern that can be determined in this ELISA. The negative VHH does not inhibit gp120 binding to sCD4 even at high concentrations.

5.9 Discussion

In this study, I have successfully created a D7 family-specific library of HIV-1 neutralising VHH, although the C8-family only produced identical clones. This was achieved through the use of primers that target the tail of the CDR3 sequence, thereby isolating VHH with homologous sequences from the diverse repertoire naturally present in the immunised llama. The design of the primer was based on the sequence of the D7/A12 VHH, which was previously found to be able to neutralise various HIV-1 isolates of subtypes B and C. The D7 family-specific library was then panned on picomolar concentrations of recombinant gp120 in solution form to reduce potential avidity effects. In yet another indication that the library creation and panning had worked, a very high proportion (98%) of the selected VHH was specific for gp120, as determined with a high-throughput BIAcore machine. Although the main objective was to isolate VHH members that were able to target the CD4bs of gp120, a general trypsin elution method was employed to elute out all bound phages to gp120. It was assumed that all members of the family will be able to recognise an epitope overlapping the CD4bs, in a manner similar to the parental D7 VHH. This property of the family-specific library was confirmed with a competition ELISA experiment where all of isolated VHH tested were able to prevent soluble CD4 from binding to gp120.

Through this technique, 49 unique VHH belonging to the same family were isolated from a single llama. However, the members of this VHH subfamily do not show identical properties. Even though they share the ability to recognise gp120 at an epitope overlapping the CD4bs, they do so with different off-rates. From the subset of clones tested in surface plasmon resonance, the VHH within the D7-family show a 20-fold variation in off-rate. The upper peak in off-rate (k_d) reached was $2.47x10^{-05}$

s^{-1} for the 2B10D11 VHH, which was twice lower than the parental D7 value of 6.88 $x10^{-05}$ s^{-1}. Interestingly, the affinity (K_D) of D7 was twice better at 0.1nM, compared to 0.22nM for 2B10D11. It is worth noting that antibody affinities in this range are close to the suggested affinity ceiling for *in vivo* maturated antibodies (Batista and Neuberger, 1998; Foote and Eisen, 1995), which might explain the absence of VHH with even better affinities in the original immune library. Camelid VHH also usually bind to their target with affinities comparable to those of conventional antibodies (Harmsen and De Haard, 2007; Muyldermans et al., 2001). Nevertheless, the affinity and avidity of these VHH can often be enhanced by reconstructing them as a conventional bivalent antibody or as multimers (Power and Hudson, 2000).

The VHH within the family also showed variations in their ability to neutralise different strains of HIV-1. Although most of the VHH possessed similar potencies as the parental D7 VHH against some of the HIV-1 strains tested, 3 primary isolates of HIV-1 stood out. The selected VHH displayed different neutralisation potencies (IC_{50} values) when tested on C27d, C222 and 92BR025, and the VHH were grouped accordingly. When the sequences were analysed according to the neutralisation categories, it was interesting to find that certain amino acid residues were important for potent neutralising ability. An YYD116 motif at the tail of the CDR3 was found to correlate with good neutralising activity. Changes in the YYD motif changed the neutralising activity to 'poor' against the viruses tested in this study, whereas mutations in the other CDRs or the FRs did not affect neutralising ability. This motif is also directly related to its off-rates in BIAcore studies and is therefore crucial to its function. More viruses will be tested to determine if additional differentiation in neutralisation ability can be observed. We have thus exploited the naturally occurring pool of related sequences to help elucidate this correlation between

sequence and antigen-antibody interactions. Back mutations to the YYD motif via site-specific mutagenesis are currently being carried out on the VHH that are narrow neutralisers to determine if this changes their neutralisation breadth. Unfortunately, time constraints do not permit inclusion of this analysis in my thesis. Structural studies on VHH and its binding to antigen are currently in progress to further elucidate antigen-binding reactions.

In conclusion, we have used a novel approach to create a family-specific VHH library whereby members of the family showed similarity in its ability to recognise a particular antigen, eliminating the need for competitive elutions. However, variations exist within the members of the family in terms of their binding affinities, as well as neutralisation ability across different strains of viruses. The creation of a diverse pool of VHH from a single parental clone has enabled the broadening in neutralisation breadth and potencies. This approach can also be used on other llamas or be based on new sequences once new broadly neutralising antibodies have been identified. By analysing the sequences of the VHH, we were able to associate certain amino acid residues as crucial for broad neutralisation ability. This will be useful for future engineering of potent heavy chain antibody fragments against HIV-1, as well as offering new tools for development of inhibitors, vaccines and research reagents.

6 Discussion and Summary

When this project was started in 2005, subtype B viruses were the most studied subtype even though subtype C viruses were the cause of the majority of the new infections in the world. Since HIV-1 envelopes of different subtypes are very diverse and show different neutralisation patterns, we decided to study subtype C HIV-1 envelopes and to characterise new antibodies that can neutralise them.

To this end, I have described in Chapter 3 on the cloning of 18 subtype C HIV-1 *env* genes directly from patients and their insertion into vectors to create replication-competent chimeric viruses. The genotypes of these envelopes were then phylogenetically characterised and variations in their variable loops and positions of potential N-linked glycosylation sites were analysed. The sequences of these envelopes were uploaded onto Genbank.

I have also determined that these cloned subtype C envelopes have a much lower incidence in ability to use CCR3 as a coreceptor than in the commonly studied subtype B envelopes. Envelopes from the subtype B, C, CRF07_BC and CRF02_AG standard reference panels were then studied for their ability to use other coreceptors for virus entry, and we found that subtype B envelopes have a 100-fold higher incidence of using CCR3 as a coreceptor compared to the other subtype and recombinant forms. Unfortunately, similar findings were recently reported by another group (Nedellec et al., 2009).

Although some of the determinants in gp120 for CCR3 coreceptor usage were previously mapped for subtype B viruses (Aasa-Chapman et al., 2006b), these were found to be inapplicable for envelopes from other subtypes. As the sequences for the

cloned subtype C envelopes are known, future work could be developed to determine the amino acids that are important for CCR3 binding.

The patient samples used in this study were mostly obtained from Africa and represent current replicating strains. The creation of this panel of subtype C viruses adds to the other subtype C panels (Brown et al., 2005; Li et al., 2006b) that were created after the start of this project, and the panel described in this study will be a useful resource for antibody neutralisation studies as some of the envelopes are genetically and phenotypically diverse from the standard panel strains. The subtype C envelopes created here were used to test the neutralisation breadth of the VHH that were created in Chapters 4 and 5, as well as in other studies by Forsman *et al.* and McKnight *et al.* (Forsman et al., 2008; McKnight, personal communication). The characterisation of these subtype C envelopes for their sensitivity to antibody neutralisation might also have implications in the design and assessment of vaccine candidates. For instance, novel broadly neutralising antibodies or novel entry inhibitors to be used in clinical trials can be tested against these subtype C primary isolates to determine the candidates' neutralisation potency and breadth.

In this thesis, the unique properties of the llama single-chain antibodies were exploited to produce VHH that retain all the antigen-binding properties of conventional antibodies. Advantages of using such VHH include their longer than average CDR3 loops that can bind into clefts, ease of cloning and expression, as well as being robust and stable (refer to Section 1.3.2).

Chapter 4 described the isolation of four different anti-gp41 VHH from a llama that was immunised with recombinant trimeric gp140 CN54. These were found to neutralise subtype C viruses better than subtype B viruses and recognised a

conformational dependent epitope on gp41. Although the neutralisation potency of these VHH were not as strong as the well characterised mAbs 2F5 and 4E10, these VHH can be modified into bivalent or multivalent forms and engineered to give higher binding affinities. All the anti-gp41 neutralising antibodies described to date were isolated from humans naturally infected with HIV-1, and this may be the first instance of isolating anti-gp41 neutralising antibodies from an immunised animal (Haynes and Montefiori, 2006; Pantophlet and Burton, 2006; Zwick and Burton, 2007).

The VHH produced in Chapter 5 were based on our earlier success in isolating neutralising VHH that target the CD4 binding site of gp120 (Forsman et al., 2008). Using a novel cloning strategy, a D7/A12 family-specific library of VHH was successfully produced and described in this chapter. This newly isolated family of VHH were all able to compete with sCD4 for binding to gp120, and displayed a range of binding affinities to gp120. Based on their ability to neutralise different strains of HIV-1, this family of VHH can be classified into "Broad", "Intermediate" and "Narrow" neutralisers. The last three amino acid residues of the CDR3 are implicated in these differences in affinities and neutralisation abilities. Mutational studies are currently being carried out to verify this, as well as to determine the binding epitope on gp120.

The creation of homologue antibodies in the family-specific library of VHH has allowed the mining of antibodies of interest from a diverse pool that has undergone affinity maturation *in vivo*, removing the painstaking task of antibody engineering. This panel of VHH can also be used for the identification and characterization of conserved epitopes on HIV-1 envelope proteins that can serve as templates for the

design of new immunogens in reverse immunology, and also as research reagents for potential use as therapeutics in combination with other antiretroviral drugs.

As mentioned earlier, these VHH can be easily manipulated to make them better candidates for microbicide or vaccine development. For example, these VHH can be humanised and reconstructed into conventional antibodies to remove any potential immunogenicity in humans (Desmyter et al., 2001; Men et al., 2004). VHH can also undergo *in vitro* affinity maturation to produce VHH with greater affinities to their antigens. Such methods include the use of ribosome display, which is a cell-free system for the in vitro selection of proteins through the formation of protein-ribosome-mRNA (PRM) complexes, and then harvesting the captured RNA for further rounds of selections. When used in combination with low-fidelity DNA polymerase to generate random mutations, *in vitro* protein evolution can be achieved (Zahnd et al., 2007).

In Chapters 4 and 5 of this thesis, the isolated VHH were first selected by panning on recombinant antigens and individual colonies were then picked for further screening. Due to the hundreds (sometimes thousands) of clones selected, the individual screening of the selected clones were often limited to testing for binding in ELISA. Although it would be desirable to screen for neutralisation instead of binding, since binding to recombinant envelope does not always correlate with neutralisation of virions (Pantophlet and Burton, 2006; Zanetti et al., 2006; Zhu et al., 2006), the testing of hundreds of clones in neutralisation assays against a panel of various viruses would involve thousands of assays and prove to be impractical. Although the TZM-bl assay offers medium-throughput in 96-well formats, a higher-throughput

neutralisation assay is needed to effectively screen the hundreds of selected VHH against a variety of HIV-1 isolates to identify interesting VHH.

Thus far, VHH have been utilised to target gp41 and the CD4bs on gp120 in HIV-1 research. For future work, VHH can also be employed to target CD4-induced sites (including the coreceptor binding site) on gp120. Although the antibodies targeting the CD4-induced sites are usually non-neutralising, presumably due to steric hindrance by conventional immunoglobulins which are too bulky to bind onto virus-associated functional spike, several Fab fragments to CD4-induced epitopes have been shown to be able to neutralise primary isolates (Burton et al., 2004; Labrijn et al., 2003). VHH, which are even smaller than Fabs, might be ideal candidates to interrogate the coreceptor binding site.

In conclusion, this study has investigated and characterised subtype C HIV-1 envelopes from primary isolates, which are responsible for the majority of new infections in the world today. The unique properties of llama antibody fragments were exploited in this study to isolated VHH that can neutralise HIV-1 infection derived from these subtype C envelopes, as well as envelopes from other isolates and subtypes. These anti-gp41 and anti-CD4 binding site VHH may be the first reported incidence of neutralising antibody isolation from an immunised animal instead of a natural infection in humans. These neutralising VHH can be utilised for further use in microbicide and vaccine research.

References

Aasa-Chapman, M. M., Aubin, K., Williams, I. and McKnight, A. (2006a) Primary CCR5 only using HIV-1 isolates does not accurately represent the in vivo replicating quasi-species. *Virology* **351,** 489-96.

Aasa-Chapman, M. M., Hayman, A., Newton, P., Cornforth, D., Williams, I., Borrow, P., Balfe, P. and McKnight, A. (2004) Development of the antibody response in acute HIV-1 infection. *AIDS* **18,** 371-81.

Aasa-Chapman, M. M., Seymour, C. R., Williams, I. and McKnight, A. (2006b) Novel envelope determinants for CCR3 use by human immunodeficiency virus. *J Virol* **80,** 10884-9.

Agrawal, L., Maxwell, C. R., Peters, P. J., Clapham, P. R., Liu, S. M., Mackay, C. R. and Strayer, D. S. (2009) Complexity in human immunodeficiency virus type 1 (HIV-1) co-receptor usage: roles of CCR3 and CCR5 in HIV-1 infection of monocyte-derived macrophages and brain microglia. *J Gen Virol* **90,** 710-22.

Aiken, C. (2006) Viral and cellular factors that regulate HIV-1 uncoating. *Curr. Opin. HIV AIDS* **1,** 194-199.

Alam, S. M., McAdams, M., Boren, D., Rak, M., Scearce, R. M., Gao, F., Camacho, Z. T., Gewirth, D., Kelsoe, G., Chen, P. and Haynes, B. F. (2007) The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes. *J Immunol* **178,** 4424-35.

Albert, J., Abrahamsson, B., Nagy, K., Aurelius, E., Gaines, H., Nystrom, G. and Fenyo, E. M. (1990) Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. *Aids* **4,** 107-12.

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M. and Berger, E. A. (1996) CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272,** 1955-8.

Allan, J. S., Coligan, J. E., Barin, F., McLane, M. F., Sodroski, J. G., Rosen, C. A., Haseltine, W. A., Lee, T. H. and Essex, M. (1985) Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science* **228,** 1091-4.

Allen, S. J., Crown, S. E. and Handel, T. M. (2007) Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* **25,** 787-820.

Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., Earl, P. L., Smith, J. M., Ma, H. L., Grimm, B. D., Hulsey, M. L., Miller, J., McClure, H. M., McNicholl, J. M., Moss, B. and Robinson, H. L. (2001) Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292,** 69-74.

Ambrose, Z., Compton, L., Piatak, M., Jr., Lu, D., Alvord, W. G., Lubomirski, M. S., Hildreth, J. E., Lifson, J. D., Miller, C. J. and KewalRamani, V. N. (2008) Incomplete protection against simian immunodeficiency virus vaginal transmission in rhesus macaques by a topical antiviral agent revealed by repeat challenges. *J Virol* **82,** 6591-9.

Anderson, R. A., Feathergill, K. A., Diao, X. H., Cooper, M. D., Kirkpatrick, R., Herold, B. C., Doncel, G. F., Chany, C. J., Waller, D. P., Rencher, W. F. and Zaneveld, L. J. (2002) Preclinical evaluation of sodium cellulose sulfate (Ushercell) as a contraceptive antimicrobial agent. *J Androl* **23,** 426-38.

Ariyoshi, K., Harwood, E., Chiengsong-Popov, R. and Weber, J. (1992) Is clearance of HIV-1 viraemia at seroconversion mediated by neutralising antibodies? *Lancet* **340,** 1257-8.

Asjo, B., Morfeldt-Manson, L., Albert, J., Biberfeld, G., Karlsson, A., Lidman, K. and Fenyo, E. M. (1986) Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. *Lancet* **2,** 660-2.

Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., Cavacini, L. A., Posner, M. R., Katinger, H., Stiegler, G., Bernacky, B. J., Rizvi, T. A., Schmidt, R., Hill, L. R., Keeling, M. E., Lu, Y., Wright, J. E., Chou, T. C. and Ruprecht, R. M. (2000) Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med* **6,** 200-6.

Baba, T. W., Liska, V., Khimani, A. H., Ray, N. B., Dailey, P. J., Penninck, D., Bronson, R., Greene, M. F., McClure, H. M., Martin, L. N. and Ruprecht, R. M. (1999) Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. *Nat Med* **5,** 194-203.

Back, N. K., Smit, L., De Jong, J. J., Keulen, W., Schutten, M., Goudsmit, J. and Tersmette, M. (1994) An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization. *Virology* **199,** 431-8.

Baleta, A. (1998) Concern voiced over "dry sex" practices in South Africa. *Lancet* **352,** 1292.

Balzarini, J. and Van Damme, L. (2007) Microbicide drug candidates to prevent HIV infection. *Lancet* **369,** 787-97.

Bandres, J. C., Wang, Q. F., O'Leary, J., Baleaux, F., Amara, A., Hoxie, J. A., Zolla-Pazner, S. and Gorny, M. K. (1998) Human immunodeficiency virus (HIV) envelope binds to CXCR4 independently of CD4, and binding can be enhanced by interaction with soluble CD4 or by HIV envelope deglycosylation. *J Virol* **72,** 2500-4.

Baral, T. N., Magez, S., Stijlemans, B., Conrath, K., Vanhollebeke, B., Pays, E., Muyldermans, S. and De Baetselier, P. (2006) Experimental therapy of African trypanosomiasis with a nanobody-conjugated human trypanolytic factor. *Nat Med* **12,** 580-4.

Barbas, C. F., 3rd, Bjorling, E., Chiodi, F., Dunlop, N., Cababa, D., Jones, T. M., Zebedee, S. L., Persson, M. A., Nara, P. L., Norrby, E. and et al. (1992) Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. *Proc Natl Acad Sci U S A* **89,** 9339-43.

Barin, F., M'Boup, S., Denis, F., Kanki, P., Allan, J. S., Lee, T. H. and Essex, M. (1985) Serological evidence for virus related to simian T-lymphotropic retrovirus III in residents of west Africa. *Lancet* **2,** 1387-9.

Barnett, S. W., Lu, S., Srivastava, I., Cherpelis, S., Gettie, A., Blanchard, J., Wang, S., Mboudjeka, I., Leung, L., Lian, Y., Fong, A., Buckner, C., Ly, A., Hilt, S., Ulmer, J., Wild, C. T., Mascola, J. R. and Stamatatos, L. (2001) The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. *J Virol* **75,** 5526-40.

Barré-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. and Montagnier, L. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220,** 868-71.

Batista, F. D. and Neuberger, M. S. (1998) Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. *Immunity* **8,** 751-9.

Beddows, S., Schulke, N., Kirschner, M., Barnes, K., Franti, M., Michael, E., Ketas, T., Sanders, R. W., Maddon, P. J., Olson, W. C. and Moore, J. P. (2005) Evaluating the immunogenicity of a disulfide-stabilized, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J Virol* **79,** 8812-27.

Belyakov, I. M., Hel, Z., Kelsall, B., Kuznetsov, V. A., Ahlers, J. D., Nacsa, J., Watkins, D. I., Allen, T. M., Sette, A., Altman, J., Woodward, R., Markham, P. D., Clements, J. D., Franchini, G., Strober, W. and Berzofsky, J. A. (2001) Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. *Nat Med* **7,** 1320-6.

Berger, E. A., Doms, R. W., Fenyo, E. M., Korber, B. T., Littman, D. R., Moore, J. P., Sattentau, Q. J., Schuitemaker, H., Sodroski, J. and Weiss, R. A. (1998) A new classification for HIV-1. *Nature* **391,** 240.

Berkhout, B., Silverman, R. H. and Jeang, K. T. (1989) Tat trans-activates the human immunodeficiency virus through a nascent RNA target. *Cell* **59,** 273-82.

Berkley, S. F. and Koff, W. C. (2007) Scientific and policy challenges to development of an AIDS vaccine. *Lancet* **370,** 94-101.

Berman, P. W., Gregory, T. J., Riddle, L., Nakamura, G. R., Champe, M. A., Porter, J. P., Wurm, F. M., Hershberg, R. D., Cobb, E. K. and Eichberg, J. W. (1990) Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* **345,** 622-5.

Berman, P. W., Nunes, W. M. and Haffar, O. K. (1988) Expression of membrane-associated and secreted variants of gp160 of human immunodeficiency virus type 1 in vitro and in continuous cell lines. *J Virol* **62,** 3135-42.

Bertley, F. M., Kozlowski, P. A., Wang, S. W., Chappelle, J., Patel, J., Sonuyi, O., Mazzara, G., Montefiori, D., Carville, A., Mansfield, K. G. and Aldovini, A. (2004) Control of simian/human immunodeficiency virus viremia and disease progression after IL-2-augmented DNA-modified vaccinia virus Ankara nasal vaccination in nonhuman primates. *J Immunol* **172,** 3745-57.

Bieniasz, P. D. (2004) Intrinsic immunity: a front-line defense against viral attack. *Nat Immunol* **5,** 1109-15.

Binley, J. M., Ditzel, H. J., Barbas, C. F., 3rd, Sullivan, N., Sodroski, J., Parren, P. W. and Burton, D. R. (1996) Human antibody responses to HIV type 1 glycoprotein 41 cloned in phage display libraries suggest three major epitopes are recognized and give evidence for conserved antibody motifs in antigen binding. *AIDS Res Hum Retroviruses* **12,** 911-24.

Binley, J. M., Wrin, T., Korber, B., Zwick, M. B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C. J. and Burton, D. R. (2004) Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. *J Virol* **78,** 13232-52.

Borrow, P., Lewicki, H., Wei, X., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Oldstone, M. B. and Shaw, G. M. (1997) Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med* **3,** 205-11.

Boyd, M. T., Simpson, G. R., Cann, A. J., Johnson, M. A. and Weiss, R. A. (1993) A single amino acid substitution in the V1 loop of human immunodeficiency virus type 1 gp120 alters cellular tropism. *J Virol* **67,** 3649-52.

Braibant, M., Brunet, S., Costagliola, D., Rouzioux, C., Agut, H., Katinger, H., Autran, B. and Barin, F. (2006) Antibodies to conserved epitopes of the HIV-1 envelope in sera from long-term non-progressors: prevalence and association with neutralizing activity. *AIDS* **20,** 1923-30.

Brelot, A., Heveker, N., Pleskoff, O., Sol, N. and Alizon, M. (1997) Role of the first and third extracellular domains of CXCR-4 in human immunodeficiency virus coreceptor activity. *J Virol* **71,** 4744-51.

Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., Nguyen, P. L., Khoruts, A., Larson, M., Haase, A. T. and Douek, D. C. (2004) CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* **200,** 749-59.

Briggs, J. A., Wilk, T., Welker, R., Krausslich, H. G. and Fuller, S. D. (2003) Structural organization of authentic, mature HIV-1 virions and cores. *Embo J* **22,** 1707-15.

Brown, B. K., Darden, J. M., Tovanabutra, S., Oblander, T., Frost, J., Sanders-Buell, E., de Souza, M. S., Birx, D. L., McCutchan, F. E. and Polonis, V. R. (2005) Biologic and genetic characterization of a panel of 60 human immunodeficiency virus type 1 isolates, representing clades A, B, C, D, CRF01_AE, and CRF02_AG, for the development and assessment of candidate vaccines. *J Virol* **79,** 6089-101.

Brown, P. O. (1997) Integration. In *Retroviruses*, (ed. H. S. H. Coffin J. M., Varmus H. E.), pp. 161-203, Cold Spring Harbor Laboratory Press, New York, USA.

Buchacher, A., Predl, R., Strutzenberger, K., Steinfellner, W., Trkola, A., Purtscher, M., Gruber, G., Tauer, C., Steindl, F., Jungbauer, A. and et al. (1994) Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. *AIDS Res Hum Retroviruses* **10,** 359-69.

Bukrinskaya, A., Brichacek, B., Mann, A. and Stevenson, M. (1998) Establishment of a functional human immunodeficiency virus type 1 (HIV-1) reverse transcription complex involves the cytoskeleton. *J Exp Med* **188,** 2113-25.

Bukrinsky, M. I., Sharova, N., Dempsey, M. P., Stanwick, T. L., Bukrinskaya, A. G., Haggerty, S. and Stevenson, M. (1992) Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc Natl Acad Sci U S A* **89,** 6580-4.

Burton, D. R., Barbas, C. F., 3rd, Persson, M. A., Koenig, S., Chanock, R. M. and Lerner, R. A. (1991) A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci U S A* **88,** 10134-7.

Burton, D. R., Desrosiers, R. C., Doms, R. W., Koff, W. C., Kwong, P. D., Moore, J. P., Nabel, G. J., Sodroski, J., Wilson, I. A. and Wyatt, R. T. (2004) HIV vaccine design and the neutralizing antibody problem. *Nat Immunol* **5,** 233–236.

Burton, D. R., Pyati, J., Koduri, R., Sharp, S. J., Thornton, G. B., Parren, P. W., Sawyer, L. S., Hendry, R. M., Dunlop, N., Nara, P. L. and et al. (1994) Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. *Science* **266,** 1024-7.

Burton, D. R., Stanfield, R. L. and Wilson, I. A. (2005) Antibody vs. HIV in a clash of evolutionary titans. *Proc Natl Acad Sci U S A* **102,** 14943-8.

Bushman, F. D., Fujiwara, T. and Craigie, R. (1990) Retroviral DNA integration directed by HIV integration protein in vitro. *Science* **249,** 1555-8.

Cao, J., Sullivan, N., Desjardin, E., Parolin, C., Robinson, J., Wyatt, R. and Sodroski, J. (1997) Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope glycoprotein. *J Virol* **71,** 9808-12.

Cardoso, R. M., Zwick, M. B., Stanfield, R. L., Kunert, R., Binley, J. M., Katinger, H., Burton, D. R. and Wilson, I. A. (2005) Broadly neutralizing anti-HIV antibody

4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. *Immunity* **22,** 163-73.

Cecilia, D., Kulkarni, S. S., Tripathy, S. P., Gangakhedkar, R. R., Paranjape, R. S. and Gadkari, D. A. (2000) Absence of coreceptor switch with disease progression in human immunodeficiency virus infections in India. *Virology* **271,** 253-8.

Centers for Disease Control. (1981a) Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. *MMWR Morb Mortal Wkly Rep* **30,** 305-8.

Centers for Disease Control. (1981b) Pneumocystis pneumonia--Los Angeles. *MMWR Morb Mortal Wkly Rep* **30,** 250-2.

Centers for Disease Control. (1982) Update on acquired immune deficiency syndrome (AIDS)--United States. *MMWR Morb Mortal Wkly Rep* **31,** 507-8, 513-4.

Centers for Disease Control. (1992) Extension of public comment period for revision of HIV infection classification system and expansion of AIDS surveillance case definition. *MMWR Morb Mortal Wkly Rep* **40,** 891.

Chakrabarti, B. K., Kong, W. P., Wu, B. Y., Yang, Z. Y., Friborg, J., Ling, X., King, S. R., Montefiori, D. C. and Nabel, G. J. (2002) Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. *J Virol* **76,** 5357-68.

Chakrabarti, B. K., Ling, X., Yang, Z. Y., Montefiori, D. C., Panet, A., Kong, W. P., Welcher, B., Louder, M. K., Mascola, J. R. and Nabel, G. J. (2005) Expanded breadth of virus neutralization after immunization with a multiclade envelope HIV vaccine candidate. *Vaccine* **23,** 3434-45.

Chan, D. C., Fass, D., Berger, J. M. and Kim, P. S. (1997) Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89,** 263-73.

Chan, D. C. and Kim, P. S. (1998) HIV entry and its inhibition. *Cell* **93,** 681-4.

Chang, T. L., Chang, C. H., Simpson, D. A., Xu, Q., Martin, P. K., Lagenaur, L. A., Schoolnik, G. K., Ho, D. D., Hillier, S. L., Holodniy, M., Lewicki, J. A. and Lee, P. P. (2003) Inhibition of HIV infectivity by a natural human isolate of Lactobacillus jensenii engineered to express functional two-domain CD4. *Proc Natl Acad Sci U S A* **100,** 11672-7.

Chen, B., Vogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C. and Harrison, S. C. (2005a) Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433,** 834-41.

Chen, H., Xu, X., Bishop, A. and Jones, I. M. (2005b) Reintroduction of the 2G12 epitope in an HIV-1 clade C gp120. *AIDS* **19,** 833-5.

Cheng-Mayer, C., Brown, A., Harouse, J., Luciw, P. A. and Mayer, A. J. (1999) Selection for neutralization resistance of the simian/human immunodeficiency virus

SHIVSF33A variant in vivo by virtue of sequence changes in the extracellular envelope glycoprotein that modify N-linked glycosylation. *J Virol* **73,** 5294-300.

Cheng-Mayer, C. and Levy, J. A. (1988) Distinct biological and serological properties of human immunodeficiency viruses from the brain. *Ann Neurol* **23 Suppl,** S58-61.

Cheng-Mayer, C., Seto, D., Tateno, M. and Levy, J. A. (1988) Biologic features of HIV-1 that correlate with virulence in the host. *Science* **240,** 80-2.

Cho, M. W., Kim, Y. B., Lee, M. K., Gupta, K. C., Ross, W., Plishka, R., Buckler-White, A., Igarashi, T., Theodore, T., Byrum, R., Kemp, C., Montefiori, D. C. and Martin, M. A. (2001) Polyvalent envelope glycoprotein vaccine elicits a broader neutralizing antibody response but is unable to provide sterilizing protection against heterologous Simian/human immunodeficiency virus infection in pigtailed macaques. *J Virol* **75,** 2224-34.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C. and Sodroski, J. (1996) The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85,** 1135-48.

Christensen, N. D., Reed, C. A., Culp, T. D., Hermonat, P. L., Howett, M. K., Anderson, R. A. and Zaneveld, L. J. (2001) Papillomavirus microbicidal activities of high-molecular-weight cellulose sulfate, dextran sulfate, and polystyrene sulfonate. *Antimicrob Agents Chemother* **45,** 3427-32.

Clapham, P. R. and McKnight, A. (2002) Cell surface receptors, virus entry and tropism of primate lentiviruses. *J Gen Virol* **83,** 1809-29.

Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M. A., Santos-Ferreira, M. O., Laurent, A. G., Dauguet, C., Katlama, C., Rouzioux, C. and et al. (1986) Isolation of a new human retrovirus from West African patients with AIDS. *Science* **233,** 343-6.

Clayton, R., Ohagen, A., Goethals, O., Smets, A., Van Loock, M., Michiels, L., Kennedy-Johnston, E., Cunningham, M., Jiang, H., Bola, S., Gutshall, L., Gunn, G., Del Vecchio, A., Sarisky, R., Hallenberger, S. and Hertogs, K. (2007) Binding kinetics, uptake and intracellular accumulation of F105, an anti-gp120 human IgG1kappa monoclonal antibody, in HIV-1 infected cells. *J Virol Methods* **139,** 17-23.

Coffin, J., Haase, A., Levy, J. A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P. and Weiss, R. A. (1986) What to call the AIDS virus? *Nature* **321,** 10.

Coffin, J. M., Hughes, S. H. and Varmus, H. E. (1997) Retroviruses, (eds. J. M. Coffin, S. H. Hughes and H. E. Varmus), Cold Spring Harbour Laboratory Press, New York, USA.

Cohen, J. (2003) Public health. AIDS vaccine trial produces disappointment and confusion. *Science* **299,** 1290-1.

Cohen, J. (2007a) AIDS research. Did Merck's failed HIV vaccine cause harm? *Science* **318,** 1048-9.

Cohen, J. (2007b) AIDS research. Promising AIDS vaccine's failure leaves field reeling. *Science* **318,** 28-9.

Collman, R., Hassan, N. F., Walker, R., Godfrey, B., Cutilli, J., Hastings, J. C., Friedman, H., Douglas, S. D. and Nathanson, N. (1989) Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. *J Exp Med* **170,** 1149-63.

Connor, R. I. and Ho, D. D. (1994) Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J Virol* **68,** 4400-8.

Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S. and Landau, N. R. (1997a) Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. *J Exp Med* **185,** 621-8.

Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S. and Landau, N. R. (1997b) Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. *J Exp Med* **185,** 621-8.

Coppieters, K., Dreier, T., Silence, K., de Haard, H., Lauwereys, M., Casteels, P., Beirnaert, E., Jonckheere, H., Van de Wiele, C., Staelens, L., Hostens, J., Revets, H., Remaut, E., Elewaut, D. and Rottiers, P. (2006) Formatted anti-tumor necrosis factor alpha VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. *Arthritis Rheum* **54,** 1856-66.

Cordonnier, A., Montagnier, L. and Emerman, M. (1989) Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. *Nature* **340,** 571-4.

Cortez-Retamozo, V., Backmann, N., Senter, P. D., Wernery, U., De Baetselier, P., Muyldermans, S. and Revets, H. (2004) Efficient cancer therapy with a nanobody-based conjugate. *Cancer Res* **64,** 2853-7.

Cosson, P. (1996) Direct interaction between the envelope and matrix proteins of HIV-1. *Embo J* **15,** 5783-8.

Cranage, M., Sharpe, S., Herrera, C., Cope, A., Dennis, M., Berry, N., Ham, C., Heeney, J., Rezk, N., Kashuba, A., Anton, P., McGowan, I. and Shattock, R. (2008) Prevention of SIV rectal transmission and priming of T cell responses in macaques after local pre-exposure application of tenofovir gel. *PLoS Med* **5,** e157; discussion e157.

Crooks, E. T., Jiang, P., Franti, M., Wong, S., Zwick, M. B., Hoxie, J. A., Robinson, J. E., Moore, P. L. and Binley, J. M. (2008) Relationship of HIV-1 and SIV envelope glycoprotein trimer occupation and neutralization. *Virology* **377,** 364-78.

Crooks, E. T., Moore, P. L., Franti, M., Cayanan, C. S., Zhu, P., Jiang, P., de Vries, R. P., Wiley, C., Zharkikh, I., Schulke, N., Roux, K. H., Montefiori, D. C., Burton, D. R. and Binley, J. M. (2007) A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. *Virology* **366,** 245-62.

Cullen, B. R. (1991) Regulation of HIV-1 gene expression. *Faseb J* **5,** 2361-8.

Cummins, J. E., Jr., Guarner, J., Flowers, L., Guenthner, P. C., Bartlett, J., Morken, T., Grohskopf, L. A., Paxton, L. and Dezzutti, C. S. (2007) Preclinical testing of candidate topical microbicides for anti-human immunodeficiency virus type 1 activity and tissue toxicity in a human cervical explant culture. *Antimicrob Agents Chemother* **51,** 1770-9.

Curran, J. W., Morgan, W. M., Hardy, A. M., Jaffe, H. W., Darrow, W. W. and Dowdle, W. R. (1985) The epidemiology of AIDS: current status and future prospects. *Science* **229,** 1352-7.

Daar, E. S., Kesler, K. L., Petropoulos, C. J., Huang, W., Bates, M., Lail, A. E., Coakley, E. P., Gomperts, E. D. and Donfield, S. M. (2007) Baseline HIV type 1 coreceptor tropism predicts disease progression. *Clin Infect Dis* **45,** 643-9.

Daar, E. S., Li, X. L., Moudgil, T. and Ho, D. D. (1990) High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. *Proc Natl Acad Sci U S A* **87,** 6574-8.

Dalgleish, A. G., Beverley, P. C., Clapham, P. R., Crawford, D. H., Greaves, M. F. and Weiss, R. A. (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312,** 763-7.

Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K. and Desrosiers, R. C. (1992) Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258,** 1938-41.

De Genst, E., Silence, K., Decanniere, K., Conrath, K., Loris, R., Kinne, J., Muyldermans, S. and Wyns, L. (2006) Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc Natl Acad Sci U S A* **103,** 4586-91.

de Witte, L., Nabatov, A. and Geijtenbeek, T. B. (2008) Distinct roles for DC-SIGN+-dendritic cells and Langerhans cells in HIV-1 transmission. *Trends Mol Med* **14,** 12-9.

Deeks, S. G., Schweighardt, B., Wrin, T., Galovich, J., Hoh, R., Sinclair, E., Hunt, P., McCune, J. M., Martin, J. N., Petropoulos, C. J. and Hecht, F. M. (2006) Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a

constraint on the ability of HIV to completely evade neutralizing antibody responses. *J Virol* **80,** 6155-64.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R. and Landau, N. R. (1996) Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381,** 661-6.

Denton, P. W., Estes, J. D., Sun, Z., Othieno, F. A., Wei, B. L., Wege, A. K., Powell, D. A., Payne, D., Haase, A. T. and Garcia, J. V. (2008) Antiretroviral pre-exposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT mice. *PLoS Med* **5,** e16.

Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Wu, X., O'Brien, W. A., Ratner, L., Kappes, J. C., Shaw, G. M. and Hunter, E. (2000) Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J Virol* **74,** 8358-67.

Desmyter, A., Decanniere, K., Muyldermans, S. and Wyns, L. (2001) Antigen specificity and high affinity binding provided by one single loop of a camel single-domain antibody. *J Biol Chem* **276,** 26285-90.

Dey, A. K., Khati, M., Tang, M., Wyatt, R., Lea, S. M. and James, W. (2005) An aptamer that neutralizes R5 strains of human immunodeficiency virus type 1 blocks gp120-CCR5 interaction. *J Virol* **79,** 13806-10.

Dey, B., Pancera, M., Svehla, K., Shu, Y., Xiang, S. H., Vainshtein, J., Li, Y., Sodroski, J., Kwong, P. D., Mascola, J. R. and Wyatt, R. (2007) Characterization of human immunodeficiency virus type 1 monomeric and trimeric gp120 glycoproteins stabilized in the CD4-bound state: antigenicity, biophysics, and immunogenicity. *J Virol* **81,** 5579-93.

Dhillon, A. K., Donners, H., Pantophlet, R., Johnson, W. E., Decker, J. M., Shaw, G. M., Lee, F. H., Richman, D. D., Doms, R. W., Vanham, G. and Burton, D. R. (2007) Dissecting the neutralizing antibody specificities of broadly neutralizing sera from human immunodeficiency virus type 1-infected donors. *J Virol* **81,** 6548-62.

di Marzo Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C. and Sarngadharan, M. G. (1986) Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* **231,** 1289-91.

Dittmar, M. T., McKnight, A., Simmons, G., Clapham, P. R., Weiss, R. A. and Simmonds, P. (1997) HIV-1 tropism and co-receptor use. *Nature* **385,** 495-6.

Dolk, E., van der Vaart, M., Lutje Hulsik, D., Vriend, G., de Haard, H., Spinelli, S., Cambillau, C., Frenken, L. and Verrips, T. (2005) Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo. *Appl Environ Microbiol* **71,** 442-50.

Doms, R. W. and Moore, J. P. (2000) HIV-1 membrane fusion: targets of opportunity. *J Cell Biol* **151,** F9-14.

Doranz, B. J., Lu, Z. H., Rucker, J., Zhang, T. Y., Sharron, M., Cen, Y. H., Wang, Z. X., Guo, H. H., Du, J. G., Accavitti, M. A., Doms, R. W. and Peiper, S. C. (1997) Two distinct CCR5 domains can mediate coreceptor usage by human immunodeficiency virus type 1. *J Virol* **71,** 6305-14.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G. and Doms, R. W. (1996) A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85,** 1149-58.

Draenert, R., Allen, T. M., Liu, Y., Wrin, T., Chappey, C., Verrill, C. L., Sirera, G., Eldridge, R. L., Lahaie, M. P., Ruiz, L., Clotet, B., Petropoulos, C. J., Walker, B. D. and Martinez-Picado, J. (2006) Constraints on HIV-1 evolution and immunodominance revealed in monozygotic adult twins infected with the same virus. *J Exp Med* **203,** 529-39.

Dragic, T. (2001) An overview of the determinants of CCR5 and CXCR4 co-receptor function. *J Gen Virol* **82,** 1807-14.

Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. and Paxton, W. A. (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381,** 667-73.

du Bois, R. M., Branthwaite, M. A., Mikhail, J. R. and Batten, J. C. (1981) Primary Pneumocystis carinii and cytomegalovirus infections. *Lancet* **2,** 1339.

Dumoulin, M., Conrath, K., Van Meirhaeghe, A., Meersman, F., Heremans, K., Frenken, L. G., Muyldermans, S., Wyns, L. and Matagne, A. (2002) Single-domain antibody fragments with high conformational stability. *Protein Sci* **11,** 500-15.

Dumoulin, M., Last, A. M., Desmyter, A., Decanniere, K., Canet, D., Larsson, G., Spencer, A., Archer, D. B., Sasse, J., Muyldermans, S., Wyns, L., Redfield, C., Matagne, A., Robinson, C. V. and Dobson, C. M. (2003) A camelid antibody fragment inhibits the formation of amyloid fibrils by human lysozyme. *Nature* **424,** 783-8.

Earl, P. L., Doms, R. W. and Moss, B. (1990) Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc Natl Acad Sci U S A* **87,** 648-52.

Earl, P. L., Sugiura, W., Montefiori, D. C., Broder, C. C., Lee, S. A., Wild, C., Lifson, J. and Moss, B. (2001) Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. *J Virol* **75,** 645-53.

Els Conrath, K., Lauwereys, M., Wyns, L. and Muyldermans, S. (2001) Camel single-domain antibodies as modular building units in bispecific and bivalent antibody constructs. *J Biol Chem* **276,** 7346-50.

Engelbrecht, S., de Villiers, T., Sampson, C. C., zur Megede, J., Barnett, S. W. and van Rensburg, E. J. (2001) Genetic analysis of the complete gag and env genes of HIV type 1 subtype C primary isolates from South Africa. *AIDS Res Hum Retroviruses* **17,** 1533-47.

Etemad-Moghadam, B., Karlsson, G. B., Halloran, M., Sun, Y., Schenten, D., Fernandes, M., Letvin, N. L. and Sodroski, J. (1998) Characterization of simian-human immunodeficiency virus envelope glycoprotein epitopes recognized by neutralizing antibodies from infected monkeys. *J Virol* **72,** 8437-45.

Farnet, C. M. and Haseltine, W. A. (1991) Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. *J Virol* **65,** 1910-5.

Fassati, A. and Goff, S. P. (2001) Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1. *J Virol* **75,** 3626-35.

Fatkenheuer, G., Pozniak, A. L., Johnson, M. A., Plettenberg, A., Staszewski, S., Hoepelman, A. I., Saag, M. S., Goebel, F. D., Rockstroh, J. K., Dezube, B. J., Jenkins, T. M., Medhurst, C., Sullivan, J. F., Ridgway, C., Abel, S., James, I. T., Youle, M. and van der Ryst, E. (2005) Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1. *Nat Med* **11,** 1170-2.

Fauci, A. S., Johnston, M. I., Dieffenbach, C. W., Burton, D. R., Hammer, S. M., Hoxie, J. A., Martin, M., Overbaugh, J., Watkins, D. I., Mahmoud, A. and Greene, W. C. (2008) HIV vaccine research: the way forward. *Science* **321,** 530-2.

Feldblum, P. J., Adeiga, A., Bakare, R., Wevill, S., Lendvay, A., Obadaki, F., Olayemi, M. O., Wang, L., Nanda, K. and Rountree, W. (2008) SAVVY vaginal gel (C31G) for prevention of HIV infection: a randomized controlled trial in Nigeria. *PLoS ONE* **3,** e1474.

Feng, Y., Broder, C. C., Kennedy, P. E. and Berger, E. A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272,** 872-7.

Fichorova, R. N., Tucker, L. D. and Anderson, D. J. (2001) The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. *J Infect Dis* **184,** 418-28.

Flynn, N. M., Forthal, D. N., Harro, C. D., Judson, F. N., Mayer, K. H. and Para, M. F. (2005) Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis* **191,** 654-65.

Foote, J. and Eisen, H. N. (1995) Kinetic and affinity limits on antibodies produced during immune responses. *Proc Natl Acad Sci U S A* **92,** 1254-6.

Forsman, A., Beirnaert, E., Aasa-Chapman, M. M., Hoorelbeke, B., Hijazi, K., Koh, W., Tack, V., Szynol, A., Kelly, C., McKnight, A., Verrips, T., de Haard, H. and

Weiss, R. A. (2008) Llama antibody fragments with cross-subtype human immunodeficiency virus type 1 (HIV-1)-neutralizing properties and high affinity for HIV-1 gp120. *J Virol* **82,** 12069-81.

Fouts, T. R., Binley, J. M., Trkola, A., Robinson, J. E. and Moore, J. P. (1997) Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex. *J Virol* **71,** 2779-85.

Franke, E. K., Yuan, H. E. and Luban, J. (1994) Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* **372,** 359-62.

Frankel, A. D. and Young, J. A. (1998) HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem* **67,** 1-25.

Freed, E. O. (1998) HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology* **251,** 1-15.

Freed, E. O. (2001) HIV-1 replication. *Somat Cell Mol Genet* **26,** 13-33.

Freed, E. O. and Martin, M. A. (1995) The role of human immunodeficiency virus type 1 envelope glycoproteins in virus infection. *J Biol Chem* **270,** 23883-6.

Frenken, L. G., van der Linden, R. H., Hermans, P. W., Bos, J. W., Ruuls, R. C., de Geus, B. and Verrips, C. T. (2000) Isolation of antigen specific llama VHH antibody fragments and their high level secretion by Saccharomyces cerevisiae. *J Biotechnol* **78,** 11-21.

Friedrich, T. C. and Watkins, D. I. (2008) Wanted: correlates of vaccine-induced protection against simian immunodeficiency virus. *Curr Opin HIV AIDS* **3,** 393-398.

Frost, S. D., Trkola, A., Gunthard, H. F. and Richman, D. D. (2008) Antibody responses in primary HIV-1 infection. *Curr Opin HIV AIDS* **3,** 45-51.

Frost, S. D., Wrin, T., Smith, D. M., Kosakovsky Pond, S. L., Liu, Y., Paxinos, E., Chappey, C., Galovich, J., Beauchaine, J., Petropoulos, C. J., Little, S. J. and Richman, D. D. (2005) Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection. *Proc Natl Acad Sci U S A* **102,** 18514-9.

Fuerst, T. R., Niles, E. G., Studier, F. W. and Moss, B. (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc Natl Acad Sci U S A* **83,** 8122-6.

Fuller, D. H., Rajakumar, P. A., Wilson, L. A., Trichel, A. M., Fuller, J. T., Shipley, T., Wu, M. S., Weis, K., Rinaldo, C. R., Haynes, J. R. and Murphey-Corb, M. (2002) Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. *J Virol* **76,** 3309-17.

Gallo, R. C. and Montagnier, L. (2003) The discovery of HIV as the cause of AIDS. *N Engl J Med* **349,** 2283-5.

Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Oleske, J., Safai, B. and et al. (1984) Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224,** 500-3.

Gallo, S. A., Finnegan, C. M., Viard, M., Raviv, Y., Dimitrov, A., Rawat, S. S., Puri, A., Durell, S. and Blumenthal, R. (2003) The HIV Env-mediated fusion reaction. *Biochim Biophys Acta* **1614,** 36-50.

Ganser-Pornillos, B. K., Yeager, M. and Sundquist, W. I. (2008) The structural biology of HIV assembly. *Curr Opin Struct Biol* **18,** 203-17.

Gao, F., Morrison, S. G., Robertson, D. L., Thornton, C. L., Craig, S., Karlsson, G., Sodroski, J., Morgado, M., Galvao-Castro, B., von Briesen, H., Beddows, S., Weber, J., Sharp, P. M., Shaw, G. M. and Hahn, B. H. (1996) Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. The WHO and NIAID Networks for HIV Isolation and Characterization. *J Virol* **70,** 1651-67.

Gao, F., Yue, L., Craig, S., Thornton, C. L., Robertson, D. L., McCutchan, F. E., Bradac, J. A., Sharp, P. M. and Hahn, B. H. (1994) Genetic variation of HIV type 1 in four World Health Organization-sponsored vaccine evaluation sites: generation of functional envelope (glycoprotein 160) clones representative of sequence subtypes A, B, C, and E. WHO Network for HIV Isolation and Characterization. *AIDS Res Hum Retroviruses* **10,** 1359-68.

Garaicoechea, L., Olichon, A., Marcoppido, G., Wigdorovitz, A., Mozgovoj, M., Saif, L., Surrey, T. and Parreno, V. (2008) Llama-derived single-chain antibody fragments directed to rotavirus VP6 protein possess broad neutralizing activity in vitro and confer protection against diarrhea in mice. *J Virol* **82,** 9753-64.

Gartner, S., Markovits, P., Markovitz, D. M., Kaplan, M. H., Gallo, R. C. and Popovic, M. (1986) The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science* **233,** 215-9.

Gauduin, M. C., Parren, P. W., Weir, R., Barbas, C. F., Burton, D. R. and Koup, R. A. (1997) Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1. *Nat Med* **3,** 1389-93.

Gauduin, M. C., Yu, Y., Barabasz, A., Carville, A., Piatak, M., Lifson, J. D., Desrosiers, R. C. and Johnson, R. P. (2006) Induction of a virus-specific effector-memory CD4+ T cell response by attenuated SIV infection. *J Exp Med* **203,** 2661-72.

Germain, R. N. (2002) T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* **2,** 309-22.

Gnanakaran, S., Lang, D., Daniels, M., Bhattacharya, T., Derdeyn, C. A. and Korber, B. (2007) Clade-specific differences between human immunodeficiency virus type 1

clades B and C: diversity and correlations in C3-V4 regions of gp120. *J Virol* **81,** 4886-91.

Gnann, J. W., Jr., Nelson, J. A. and Oldstone, M. B. (1987) Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus. *J Virol* **61,** 2639-41.

Gonda, M. A., Wong-Staal, F., Gallo, R. C., Clements, J. E., Narayan, O. and Gilden, R. V. (1985) Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus. *Science* **227,** 173-7.

Gottlieb, M. S., Schroff, R., Schanker, H. M., Weisman, J. D., Fan, P. T., Wolf, R. A. and Saxon, A. (1981) Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* **305,** 1425-31.

Gottlinger, H. G., Dorfman, T., Sodroski, J. G. and Haseltine, W. A. (1991) Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. *Proc Natl Acad Sci U S A* **88,** 3195-9.

Graham, F. L., Smiley, J., Russell, W. C. and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36,** 59-74.

Grant, R. M., Hamer, D., Hope, T., Johnston, R., Lange, J., Lederman, M. M., Lieberman, J., Miller, C. J., Moore, J. P., Mosier, D. E., Richman, D. D., Schooley, R. T., Springer, M. S., Veazey, R. S. and Wainberg, M. A. (2008) Whither or wither microbicides? *Science* **321,** 532-4.

Gray, E. S., Meyers, T., Gray, G., Montefiori, D. C. and Morris, L. (2006) Insensitivity of paediatric HIV-1 subtype C viruses to broadly neutralising monoclonal antibodies raised against subtype B. *PLoS Med* **3,** e255.

Gray, E. S., Moore, P. L., Choge, I. A., Decker, J. M., Bibollet-Ruche, F., Li, H., Leseka, N., Treurnicht, F., Mlisana, K., Shaw, G. M., Karim, S. S., Williamson, C. and Morris, L. (2007a) Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. *J Virol* **81,** 6187-96.

Gray, R. H., Kigozi, G., Serwadda, D., Makumbi, F., Watya, S., Nalugoda, F., Kiwanuka, N., Moulton, L. H., Chaudhary, M. A., Chen, M. Z., Sewankambo, N. K., Wabwire-Mangen, F., Bacon, M. C., Williams, C. F., Opendi, P., Reynolds, S. J., Laeyendecker, O., Quinn, T. C. and Wawer, M. J. (2007b) Male circumcision for HIV prevention in men in Rakai, Uganda: a randomised trial. *Lancet* **369,** 657-66.

Grundner, C., Li, Y., Louder, M., Mascola, J., Yang, X., Sodroski, J. and Wyatt, R. (2005) Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. *Virology* **331,** 33-46.

Guindon, S. and Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52,** 696-704.

Haase, A. T. (2005) Perils at mucosal front lines for HIV and SIV and their hosts. *Nat Rev Immunol* **5,** 783-92.

Hahn, B. H., Shaw, G. M., De Cock, K. M. and Sharp, P. M. (2000) AIDS as a zoonosis: scientific and public health implications. *Science* **287,** 607-14.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E. B., Bendahman, N. and Hamers, R. (1993) Naturally occurring antibodies devoid of light chains. *Nature* **363,** 446-8.

Hanke, T., Samuel, R. V., Blanchard, T. J., Neumann, V. C., Allen, T. M., Boyson, J. E., Sharpe, S. A., Cook, N., Smith, G. L., Watkins, D. I., Cranage, M. P. and McMichael, A. J. (1999) Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. *J Virol* **73,** 7524-32.

Harmsen, M. M. and De Haard, H. J. (2007) Properties, production, and applications of camelid single-domain antibody fragments. *Appl Microbiol Biotechnol* **77,** 13-22.

Harmsen, M. M., Ruuls, R. C., Nijman, I. J., Niewold, T. A., Frenken, L. G. and de Geus, B. (2000) Llama heavy-chain V regions consist of at least four distinct subfamilies revealing novel sequence features. *Mol Immunol* **37,** 579-590.

Harmsen, M. M., van Solt, C. B., Fijten, H. P. D., van Keulen, L., Rosalia, R. A., Weerdmeester, K., Cornelissen, A. H. M., de Bruin, M. G. M., Eblé, P. L. and Dekker, A. (2007) Passive immunization of guinea-pigs with llama single-domain antibody fragments against foot-and-mouth disease. *Vet Microbiol* **120,** 193-206.

Harouse, J. M., Gettie, A., Eshetu, T., Tan, R. C., Bohm, R., Blanchard, J., Baskin, G. and Cheng-Mayer, C. (2001) Mucosal transmission and induction of simian AIDS by CCR5-specific simian/human immunodeficiency virus SHIV(SF162P3). *J Virol* **75,** 1990-5.

Hartley, O., Klasse, P. J., Sattentau, Q. J. and Moore, J. P. (2005) V3: HIV's switch-hitter. *AIDS Res Hum Retroviruses* **21,** 171-89.

Haynes, B. F., Fleming, J., St Clair, E. W., Katinger, H., Stiegler, G., Kunert, R., Robinson, J., Scearce, R. M., Plonk, K., Staats, H. F., Ortel, T. L., Liao, H. X. and Alam, S. M. (2005) Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. *Science* **308,** 1906-8.

Haynes, B. F. and Montefiori, D. C. (2006) Aiming to induce broadly reactive neutralizing antibody responses with HIV-1 vaccine candidates. *Expert Rev Vaccines* **5,** 347-63.

He, J., Chen, Y., Farzan, M., Choe, H., Ohagen, A., Gartner, S., Busciglio, J., Yang, X., Hofmann, W., Newman, W., Mackay, C. R., Sodroski, J. and Gabuzda, D. (1997) CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* **385,** 645-9.

Helseth, E., Olshevsky, U., Furman, C. and Sodroski, J. (1991) Human immunodeficiency virus type 1 gp120 envelope glycoprotein regions important for association with the gp41 transmembrane glycoprotein. *J Virol* **65,** 2119-23.

Hemelaar, J., Gouws, E., Ghys, P. D. and Osmanov, S. (2006) Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *Aids* **20,** W13-23.

Henderson, L. E., Bowers, M. A., Sowder, R. C., 2nd, Serabyn, S. A., Johnson, D. G., Bess, J. W., Jr., Arthur, L. O., Bryant, D. K. and Fenselau, C. (1992) Gag proteins of the highly replicative MN strain of human immunodeficiency virus type 1: posttranslational modifications, proteolytic processings, and complete amino acid sequences. *J Virol* **66,** 1856-65.

Herrera, C., Klasse, P. J., Kibler, C. W., Michael, E., Moore, J. P. and Beddows, S. (2006) Dominant-negative effect of hetero-oligomerization on the function of the human immunodeficiency virus type 1 envelope glycoprotein complex. *Virology* **351,** 121-32.

Hessell, A. J., Hangartner, L., Hunter, M., Havenith, C. E., Beurskens, F. J., Bakker, J. M., Lanigan, C. M., Landucci, G., Forthal, D. N., Parren, P. W., Marx, P. A. and Burton, D. R. (2007) Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* **449,** 101-4.

Hicks, D. R., Martin, L. S., Getchell, J. P., Heath, J. L., Francis, D. P., McDougal, J. S., Curran, J. W. and Voeller, B. (1985) Inactivation of HTLV-III/LAV-infected cultures of normal human lymphocytes by nonoxynol-9 in vitro. *Lancet* **2,** 1422-3.

Hill, C. P., Worthylake, D., Bancroft, D. P., Christensen, A. M. and Sundquist, W. I. (1996) Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. *Proc Natl Acad Sci U S A* **93,** 3099-104.

Hillier, S. L., Moench, T., Shattock, R., Black, R., Reichelderfer, P. and Veronese, F. (2005) In vitro and in vivo: the story of nonoxynol 9. *J Acquir Immune Defic Syndr* **39,** 1-8.

Hinz, A., Schoehn, G., Quendler, H., Hulsik, D. L., Stiegler, G., Katinger, H., Seaman, M. S., Montefiori, D. and Weissenhorn, W. (2009) Characterization of a trimeric MPER containing HIV-1 gp41 antigen. *Virology* **390,** 221-7.

Hladik, F. and McElrath, M. J. (2008) Setting the stage: host invasion by HIV. *Nat Rev Immunol* **8,** 447-57.

Hladik, F., Sakchalathorn, P., Ballweber, L., Lentz, G., Fialkow, M., Eschenbach, D. and McElrath, M. J. (2007) Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. *Immunity* **26,** 257-70.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M. and Markowitz, M. (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373,** 123-6.

Hofmann-Lehmann, R., Vlasak, J., Rasmussen, R. A., Smith, B. A., Baba, T. W., Liska, V., Ferrantelli, F., Montefiori, D. C., McClure, H. M., Anderson, D. C., Bernacky, B. J., Rizvi, T. A., Schmidt, R., Hill, L. R., Keeling, M. E., Katinger, H., Stiegler, G., Cavacini, L. A., Posner, M. R., Chou, T. C., Andersen, J. and Ruprecht, R. M. (2001) Postnatal passive immunization of neonatal macaques with a triple combination of human monoclonal antibodies against oral simian-human immunodeficiency virus challenge. *J Virol* **75,** 7470-80.

Hogg, R. S., Heath, K. V., Yip, B., Craib, K. J., O'Shaughnessy, M. V., Schechter, M. T. and Montaner, J. S. (1998) Improved survival among HIV-infected individuals following initiation of antiretroviral therapy. *Jama* **279,** 450-4.

Hoglund, S., Ohagen, A., Goncalves, J., Panganiban, A. T. and Gabuzda, D. (1997) Ultrastructure of HIV-1 genomic RNA. *Virology* **233,** 271-9.

Huang, C. C., Lam, S. N., Acharya, P., Tang, M., Xiang, S. H., Hussan, S. S., Stanfield, R. L., Robinson, J., Sodroski, J., Wilson, I. A., Wyatt, R., Bewley, C. A. and Kwong, P. D. (2007a) Structures of the CCR5 N terminus and of a tyrosine-sulfated antibody with HIV-1 gp120 and CD4. *Science* **317,** 1930-4.

Huang, C. C., Tang, M., Zhang, M. Y., Majeed, S., Montabana, E., Stanfield, R. L., Dimitrov, D. S., Korber, B., Sodroski, J., Wilson, I. A., Wyatt, R. and Kwong, P. D. (2005) Structure of a V3-containing HIV-1 gp120 core. *Science* **310,** 1025-8.

Huang, W., Eshleman, S. H., Toma, J., Fransen, S., Stawiski, E., Paxinos, E. E., Whitcomb, J. M., Young, A. M., Donnell, D., Mmiro, F., Musoke, P., Guay, L. A., Jackson, J. B., Parkin, N. T. and Petropoulos, C. J. (2007b) Coreceptor tropism in human immunodeficiency virus type 1 subtype D: high prevalence of CXCR4 tropism and heterogeneous composition of viral populations. *J Virol* **81,** 7885-93.

Huang, W., Toma, J., Fransen, S., Stawiski, E., Reeves, J. D., Whitcomb, J. M., Parkin, N. and Petropoulos, C. J. (2008) Coreceptor tropism can be influenced by amino acid substitutions in the gp41 transmembrane subunit of human immunodeficiency virus type 1 envelope protein. *J Virol* **82,** 5584-93.

Huber, M. and Trkola, A. (2007) Humoral immunity to HIV-1: neutralization and beyond. *J Intern Med* **262,** 5-25.

Hwang, S. S., Boyle, T. J., Lyerly, H. K. and Cullen, B. R. (1991) Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253,** 71-4.

Hymes, K. B., Cheung, T., Greene, J. B., Prose, N. S., Marcus, A., Ballard, H., William, D. C. and Laubenstein, L. J. (1981) Kaposi's sarcoma in homosexual men-a report of eight cases. *Lancet* **2,** 598-600.

Jeffs, S. A., Goriup, S., Kebble, B., Crane, D., Bolgiano, B., Sattentau, Q., Jones, S. and Holmes, H. (2004) Expression and characterisation of recombinant oligomeric envelope glycoproteins derived from primary isolates of HIV-1. *Vaccine* **22,** 1032-46.

Jolly, C., Kashefi, K., Hollinshead, M. and Sattentau, Q. J. (2004) HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. *J Exp Med* **199,** 283-93.

Jouvenet, N., Bieniasz, P. D. and Simon, S. M. (2008) Imaging the biogenesis of individual HIV-1 virions in live cells. *Nature* **454,** 236-40.

Keele, B. F., Jones, J. H., Terio, K. A., Estes, J. D., Rudicell, R. S., Wilson, M. L., Li, Y., Learn, G. H., Beasley, T. M., Schumacher-Stankey, J., Wroblewski, E., Mosser, A., Raphael, J., Kamenya, S., Lonsdorf, E. V., Travis, D. A., Mlengeya, T., Kinsel, M. J., Else, J. G., Silvestri, G., Goodall, J., Sharp, P. M., Shaw, G. M., Pusey, A. E. and Hahn, B. H. (2009) Increased mortality and AIDS-like immunopathology in wild chimpanzees infected with SIVcpz. *Nature* **460,** 515-9.

Keele, B. F., Van Heuverswyn, F., Li, Y., Bailes, E., Takehisa, J., Santiago, M. L., Bibollet-Ruche, F., Chen, Y., Wain, L. V., Liegeois, F., Loul, S., Ngole, E. M., Bienvenue, Y., Delaporte, E., Brookfield, J. F., Sharp, P. M., Shaw, G. M., Peeters, M. and Hahn, B. H. (2006) Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. *Science* **313,** 523-6.

Keller, M. J., Zerhouni-Layachi, B., Cheshenko, N., John, M., Hogarty, K., Kasowitz, A., Goldberg, C. L., Wallenstein, S., Profy, A. T., Klotman, M. E. and Herold, B. C. (2006) PRO 2000 gel inhibits HIV and herpes simplex virus infection following vaginal application: a double-blind placebo-controlled trial. *J Infect Dis* **193,** 27-35.

Kiepiela, P., Leslie, A. J., Honeyborne, I., Ramduth, D., Thobakgale, C., Chetty, S., Rathnavalu, P., Moore, C., Pfafferott, K. J., Hilton, L., Zimbwa, P., Moore, S., Allen, T., Brander, C., Addo, M. M., Altfeld, M., James, I., Mallal, S., Bunce, M., Barber, L. D., Szinger, J., Day, C., Klenerman, P., Mullins, J., Korber, B., Coovadia, H. M., Walker, B. D. and Goulder, P. J. (2004) Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* **432,** 769-75.

Kim, M., Qiao, Z. S., Montefiori, D. C., Haynes, B. F., Reinherz, E. L. and Liao, H. X. (2005) Comparison of HIV Type 1 ADA gp120 monomers versus gp140 trimers as immunogens for the induction of neutralizing antibodies. *AIDS Res Hum Retroviruses* **21,** 58-67.

Kim, Y. B., Han, D. P., Cao, C. and Cho, M. W. (2003) Immunogenicity and ability of variable loop-deleted human immunodeficiency virus type 1 envelope glycoproteins to elicit neutralizing antibodies. *Virology* **305,** 124-37.

Klasse, P. J., Shattock, R. and Moore, J. P. (2008) Antiretroviral drug-based microbicides to prevent HIV-1 sexual transmission. *Annu Rev Med* **59,** 455-71.

Klasse, P. J., Shattock, R. J. and Moore, J. P. (2006) Which topical microbicides for blocking HIV-1 transmission will work in the real world? *PLoS Med* **3,** e351.

Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J. C. and Montagnier, L. (1984) T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* **312,** 767-8.

Koff, W. C., Johnson, P. R., Watkins, D. I., Burton, D. R., Lifson, J. D., Hasenkrug, K. J., McDermott, A. B., Schultz, A., Zamb, T. J., Boyle, R. and Desrosiers, R. C. (2006) HIV vaccine design: insights from live attenuated SIV vaccines. *Nat Immunol* **7,** 19-23.

Kozarsky, K., Penman, M., Basiripour, L., Haseltine, W., Sodroski, J. and Krieger, M. (1989) Glycosylation and processing of the human immunodeficiency virus type 1 envelope protein. *J Acquir Immune Defic Syndr* **2,** 163-9.

Kruger, C., Hultberg, A., Marcotte, H., Hermans, P., Bezemer, S., Frenken, L. and Hammarstrom, L. (2006) Therapeutic effect of llama derived VHH fragments against Streptococcus mutans on the development of dental caries. *Appl Microbiol Biotechnol* **72,** 732–737.

Kwong, P. D., Wyatt, R., Majeed, S., Robinson, J., Sweet, R. W., Sodroski, J. and Hendrickson, W. A. (2000) Structures of HIV-1 gp120 envelope glycoproteins from laboratory-adapted and primary isolates. *Structure* **8,** 1329-39.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. and Hendrickson, W. A. (1998) Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393,** 648-59.

Labrijn, A. F., Poignard, P., Raja, A., Zwick, M. B., Delgado, K., Franti, M., Binley, J., Vivona, V., Grundner, C., Huang, C. C., Venturi, M., Petropoulos, C. J., Wrin, T., Dimitrov, D. S., Robinson, J., Kwong, P. D., Wyatt, R. T., Sodroski, J. and Burton, D. R. (2003) Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. *J Virol* **77,** 10557-65.

Lama, J. and Planelles, V. (2007) Host factors influencing susceptibility to HIV infection and AIDS progression. *Retrovirology* **4,** 52.

Land, A., Zonneveld, D. and Braakman, I. (2003) Folding of HIV-1 envelope glycoprotein involves extensive isomerization of disulfide bonds and conformation-dependent leader peptide cleavage. *Faseb J* **17,** 1058-67.

Lapham, C. K., Ouyang, J., Chandrasekhar, B., Nguyen, N. Y., Dimitrov, D. S. and Golding, H. (1996) Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. *Science* **274,** 602-5.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23,** 2947-8.

Lasky, L. A., Nakamura, G., Smith, D. H., Fennie, C., Shimasaki, C., Patzer, E., Berman, P., Gregory, T. and Capon, D. J. (1987) Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell* **50,** 975-85.

Laspia, M. F., Rice, A. P. and Mathews, M. B. (1989) HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell* **59,** 283-92.

Lathey, J. L., Pratt, R. D. and Spector, S. A. (1997) Appearance of autologous neutralizing antibody correlates with reduction in virus load and phenotype switch during primary infection with human immunodeficiency virus type 1. *J Infect Dis* **175,** 231-2.

Lauwereys, M., Arbabi Ghahroudi, M., Desmyter, A., Kinne, J., Holzer, W., De Genst, E., Wyns, L. and Muyldermans, S. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *Embo J* **17,** 3512-20.

Law, M., Cardoso, R. M., Wilson, I. A. and Burton, D. R. (2007) Antigenic and immunogenic study of membrane-proximal external region-grafted gp120 antigens by a DNA prime-protein boost immunization strategy. *J Virol* **81,** 4272-85.

Lederman, M. M., Offord, R. E. and Hartley, O. (2006) Microbicides and other topical strategies to prevent vaginal transmission of HIV. *Nat Rev Immunol* **6,** 371-82.

Lederman, M. M., Veazey, R. S., Offord, R., Mosier, D. E., Dufour, J., Mefford, M., Piatak, M., Jr., Lifson, J. D., Salkowitz, J. R., Rodriguez, B., Blauvelt, A. and Hartley, O. (2004) Prevention of vaginal SHIV transmission in rhesus macaques through inhibition of CCR5. *Science* **306,** 485-7.

Ledford, H. (2007) HIV vaccine may raise risk. *Nature* **450,** 325.

Lee, B., Sharron, M., Montaner, L. J., Weissman, D. and Doms, R. W. (1999) Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proc Natl Acad Sci U S A* **96,** 5215-20.

Lee, D., Graham, B. S., Chiu, Y. L., Gilbert, P. B., McElrath, M. J., Belshe, R. B., Buchbinder, S. P., Sheppard, H. W., Koblin, B. A., Mayer, K. H., Keefer, M. C., Mulligan, M. J. and Celum, C. L. (2004) Breakthrough infections during phase 1 and 2 prime-boost HIV-1 vaccine trials with canarypox vectors (ALVAC) and booster dose of recombinant gp120 or gp160. *J Infect Dis* **190,** 903-7.

Leitner, T., Korber, B., Daniels, M., Calef, C. and Foley, B. (2005) HIV-1 subtype and circulating recombinant form (CRF) reference sequences, Los Alamos National Laboratory, Los Alamos, NM.

Letvin, N. L. (2006) Progress and obstacles in the development of an AIDS vaccine. *Nat Rev Immunol* **6,** 930-9.

Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A., Shimabukuro, J. M. and Oshiro, L. S. (1984) Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225,** 840-2.

Li, B., Decker, J. M., Johnson, R. W., Bibollet-Ruche, F., Wei, X., Mulenga, J., Allen, S., Hunter, E., Hahn, B. H., Shaw, G. M., Blackwell, J. L. and Derdeyn, C. A.

(2006a) Evidence for potent autologous neutralizing antibody titers and compact envelopes in early infection with subtype C human immunodeficiency virus type 1. *J Virol* **80,** 5211-8.

Li, M., Gao, F., Mascola, J. R., Stamatatos, L., Polonis, V. R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P., Greene, K. M., Bilska, M., Kothe, D. L., Salazar-Gonzalez, J. F., Wei, X., Decker, J. M., Hahn, B. H. and Montefiori, D. C. (2005) Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. *J Virol* **79,** 10108-25.

Li, M., Salazar-Gonzalez, J. F., Derdeyn, C. A., Morris, L., Williamson, C., Robinson, J. E., Decker, J. M., Li, Y., Salazar, M. G., Polonis, V. R., Mlisana, K., Karim, S. A., Hong, K., Greene, K. M., Bilska, M., Zhou, J., Allen, S., Chomba, E., Mulenga, J., Vwalika, C., Gao, F., Zhang, M., Korber, B. T., Hunter, E., Hahn, B. H. and Montefiori, D. C. (2006b) Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. *J Virol* **80,** 11776-90.

Li, S., Hill, C. P., Sundquist, W. I. and Finch, J. T. (2000) Image reconstructions of helical assemblies of the HIV-1 CA protein. *Nature* **407,** 409-13.

Li, Y., Migueles, S. A., Welcher, B., Svehla, K., Phogat, A., Louder, M. K., Wu, X., Shaw, G. M., Connors, M., Wyatt, R. T. and Mascola, J. R. (2007) Broad HIV-1 neutralization mediated by CD4-binding site antibodies. *Nat Med* **13,** 1032-4.

Li, Y., Svehla, K., Louder, M. K., Wycuff, D., Phogat, S., Tang, M., Migueles, S. A., Wu, X., Phogat, A., Shaw, G. M., Connors, M., Hoxie, J., Mascola, J. R. and Wyatt, R. (2009) Analysis of neutralization specificities in polyclonal sera derived from human immunodeficiency virus type 1-infected individuals. *J Virol* **83,** 1045-59.

Li, Y., Svehla, K., Mathy, N. L., Voss, G., Mascola, J. R. and Wyatt, R. (2006c) Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolate trimeric and monomeric envelope glycoproteins in selected adjuvants. *J Virol* **80,** 1414-26.

Lifson, J. D., Rossio, J. L., Piatak, M., Jr., Bess, J., Jr., Chertova, E., Schneider, D. K., Coalter, V. J., Poore, B., Kiser, R. F., Imming, R. J., Scarzello, A. J., Henderson, L. E., Alvord, W. G., Hirsch, V. M., Benveniste, R. E. and Arthur, L. O. (2004) Evaluation of the safety, immunogenicity, and protective efficacy of whole inactivated simian immunodeficiency virus (SIV) vaccines with conformationally and functionally intact envelope glycoproteins. *AIDS Res Hum Retroviruses* **20,** 772-87.

Liu, J., Bartesaghi, A., Borgnia, M. J., Sapiro, G. and Subramaniam, S. (2008) Molecular architecture of native HIV-1 gp120 trimers. *Nature* **455,** 109-13.

Lores, P., Boucher, V., Mackay, C., Pla, M., Von Boehmer, H., Jami, J., Barre-Sinoussi, F. and Weill, J. C. (1992) Expression of human CD4 in transgenic mice does not confer sensitivity to human immunodeficiency virus infection. *AIDS Res Hum Retroviruses* **8,** 2063-71.

Lu, M., Blacklow, S. C. and Kim, P. S. (1995) A trimeric structural domain of the HIV-1 transmembrane glycoprotein. *Nat Struct Biol* **2,** 1075-82.

Lu, Y. L., Spearman, P. and Ratner, L. (1993) Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J Virol* **67,** 6542-50.

Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V. and Goff, S. P. (1993) Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73,** 1067-78.

Lucey, D. R., Dorsky, D. I., Nicholson-Weller, A. and Weller, P. F. (1989) Human eosinophils express CD4 protein and bind human immunodeficiency virus 1 gp120. *J Exp Med* **169,** 327-32.

Lusso, P. (2006) HIV and the chemokine system: 10 years later. *Embo J* **25,** 447-56.

Maass, D. R., Sepulveda, J., Pernthaner, A. and Shoemaker, C. B. (2007) Alpaca (Lama pacos) as a convenient source of recombinant camelid heavy chain antibodies (VHHs). *J Immunol Methods* **324,** 13-25.

Maddon, P. J., Littman, D. R., Godfrey, M., Maddon, D. E., Chess, L. and Axel, R. (1985) The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell* **42,** 93-104.

Maggioni, C. and Braakman, I. (2005) Synthesis and quality control of viral membrane proteins. *Curr Top Microbiol Immunol* **285,** 175-98.

Maher, D., Wu, X., Schacker, T., Horbul, J. and Southern, P. (2005) HIV binding, penetration, and primary infection in human cervicovaginal tissue. *Proc Natl Acad Sci U S A* **102,** 11504-9.

Malim, M. H. and Emerman, M. (2008) HIV-1 accessory proteins--ensuring viral survival in a hostile environment. *Cell Host Microbe* **3,** 388-98.

Malim, M. H., Hauber, J., Le, S. Y., Maizel, J. V. and Cullen, B. R. (1989) The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* **338,** 254-7.

Malonza, I. M., Mirembe, F., Nakabiito, C., Odusoga, L. O., Osinupebi, O. A., Hazari, K., Chitlange, S., Ali, M. M., Callahan, M. and Van Damme, L. (2005) Expanded Phase I safety and acceptability study of 6% cellulose sulfate vaginal gel. *Aids* **19,** 2157-63.

Mann, D. L., O'Brien, S. J., Gilbert, D. A., Reid, Y., Popovic, M., Read-Connole, E., Gallo, R. C. and Gazdar, A. F. (1989) Origin of the HIV-susceptible human CD4+ cell line H9. *AIDS Res Hum Retroviruses* **5,** 253-5.

Mansfield, K., Lang, S. M., Gauduin, M. C., Sanford, H. B., Lifson, J. D., Johnson, R. P. and Desrosiers, R. C. (2008) Vaccine protection by live, attenuated simian immunodeficiency virus in the absence of high-titer antibody responses and high-frequency cellular immune responses measurable in the periphery. *J Virol* **82,** 4135-48.

Mansky, L. M. and Temin, H. M. (1995) Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J Virol* **69,** 5087-94.

Margolis, L. and Shattock, R. (2006) Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved? *Nat Rev Microbiol* **4,** 312-7.

Margottin, F., Bour, S. P., Durand, H., Selig, L., Benichou, S., Richard, V., Thomas, D., Strebel, K. and Benarous, R. (1998) A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. *Mol Cell* **1,** 565-74.

Martin, L., Stricher, F., Misse, D., Sironi, F., Pugniere, M., Barthe, P., Prado-Gotor, R., Freulon, I., Magne, X., Roumestand, C., Menez, A., Lusso, P., Veas, F. and Vita, C. (2003) Rational design of a CD4 mimic that inhibits HIV-1 entry and exposes cryptic neutralization epitopes. *Nat Biotechnol* **21,** 71-6.

Mascola, J. R., D'Souza, P., Gilbert, P., Hahn, B. H., Haigwood, N. L., Morris, L., Petropoulos, C. J., Polonis, V. R., Sarzotti, M. and Montefiori, D. C. (2005) Recommendations for the design and use of standard virus panels to assess neutralizing antibody responses elicited by candidate human immunodeficiency virus type 1 vaccines. *J Virol* **79,** 10103-7.

Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S., Birx, D. L. and Lewis, M. G. (2000) Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* **6,** 207-10.

Masur, H., Michelis, M. A., Greene, J. B., Onorato, I., Stouwe, R. A., Holzman, R. S., Wormser, G., Brettman, L., Lange, M., Murray, H. W. and Cunningham-Rundles, S. (1981) An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. *N Engl J Med* **305,** 1431-8.

McBurney, S. P., Young, K. R. and Ross, T. M. (2007) Membrane embedded HIV-1 envelope on the surface of a virus-like particle elicits broader immune responses than soluble envelopes. *Virology* **358,** 334-46.

McCoombe, S. G. and Short, R. V. (2006) Potential HIV-1 target cells in the human penis. *Aids* **20,** 1491-5.

McCune, J. M., Rabin, L. B., Feinberg, M. B., Lieberman, M., Kosek, J. C., Reyes, G. R. and Weissman, I. L. (1988) Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* **53,** 55-67.

McCutchan, F. E. (2006) Global epidemiology of HIV. *J Med Virol* **78 Suppl 1,** S7-S12.

McDonald, D., Wu, L., Bohks, S. M., KewalRamani, V. N., Unutmaz, D. and Hope, T. J. (2003) Recruitment of HIV and its receptors to dendritic cell-T cell junctions. *Science* **300,** 1295-7.

McKeating, J. A., Bennett, J., Zolla-Pazner, S., Schutten, M., Ashelford, S., Brown, A. L. and Balfe, P. (1993) Resistance of a human serum-selected human immunodeficiency virus type 1 escape mutant to neutralization by CD4 binding site monoclonal antibodies is conferred by a single amino acid change in gp120. *J Virol* **67,** 5216-25.

McKeating, J. A., McKnight, A., McIntosh, K., Clapham, P. R., Mulder, C. and Weiss, R. A. (1989) Evaluation of human and simian immunodeficiency virus plaque and neutralization assays. *J Gen Virol* **70 (Pt 12),** 3327-33.

McKeating, J. A., Zhang, Y. J., Arnold, C., Frederiksson, R., Fenyo, E. M. and Balfe, P. (1996) Chimeric viruses expressing primary envelope glycoproteins of human immunodeficiency virus type I show increased sensitivity to neutralization by human sera. *Virology* **220,** 450-60.

McMichael, A. J. (2006) HIV vaccines. *Annu Rev Immunol* **24,** 227-55.

Melikyan, G. B., Markosyan, R. M., Hemmati, H., Delmedico, M. K., Lambert, D. M. and Cohen, F. S. (2000) Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J Cell Biol* **151,** 413-23.

Men, R., Yamashiro, T., Goncalvez, A. P., Wernly, C., Schofield, D. J., Emerson, S. U., Purcell, R. H. and Lai, C. J. (2004) Identification of chimpanzee Fab fragments by repertoire cloning and production of a full-length humanized immunoglobulin G1 antibody that is highly efficient for neutralization of dengue type 4 virus. *J Virol* **78,** 4665-74.

Milich, L., Margolin, B. H. and Swanstrom, R. (1997) Patterns of amino acid variability in NSI-like and SI-like V3 sequences and a linked change in the CD4-binding domain of the HIV-1 Env protein. *Virology* **239,** 108-18.

Miller, C. J., Alexander, N. J., Gettie, A., Hendrickx, A. G. and Marx, P. A. (1992a) The effect of contraceptives containing nonoxynol-9 on the genital transmission of simian immunodeficiency virus in rhesus macaques. *Fertil Steril* **57,** 1126-8.

Miller, C. J., Alexander, N. J., Vogel, P., Anderson, J. and Marx, P. A. (1992b) Mechanism of genital transmission of SIV: a hypothesis based on transmission studies and the location of SIV in the genital tract of chronically infected female rhesus macaques. *J Med Primatol* **21,** 64-8.

Miller, C. J. and Shattock, R. J. (2003) Target cells in vaginal HIV transmission. *Microbes Infect* **5,** 59-67.

Miyauchi, K., Kim, Y., Latinovic, O., Morozov, V. and Melikyan, G. B. (2009) HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. *Cell* **137,** 433-44.

Montefiori, D. C. (2004) Evaluating neutralizing antibodies against HIV, SIV and SHIV in luciferase reporter gene assays. In *Current protocols in immunology*, (ed. A.

M. K. J. E. Coligan, D. H. Margulies, E. M. Shevach, W. Strober, and R. Coico), pp. 12.11.1-12.11.15, John Wiley & Sons, New York, NY.

Montefiori, D. C., Morris, L., Ferrari, G. and Mascola, J. R. (2007) Neutralizing and other antiviral antibodies in HIV-1 infection and vaccination. *Curr Opin HIV AIDS* **2,** 169-176.

Moog, C., Fleury, H. J., Pellegrin, I., Kirn, A. and Aubertin, A. M. (1997) Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficiency virus type 1-infected individuals. *J Virol* **71,** 3734-41.

Moore, J. P., Burkly, L. C., Connor, R. I., Cao, Y., Tizard, R., Ho, D. D. and Fisher, R. A. (1993) Adaptation of two primary human immunodeficiency virus type 1 isolates to growth in transformed T cell lines correlates with alterations in the responses of their envelope glycoproteins to soluble CD4. *AIDS Res Hum Retroviruses* **9,** 529-39.

Moore, J. P., Cao, Y., Qing, L., Sattentau, Q. J., Pyati, J., Koduri, R., Robinson, J., Barbas, C. F., 3rd, Burton, D. R. and Ho, D. D. (1995) Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. *J Virol* **69,** 101-9.

Moore, J. P., Sattentau, Q. J., Wyatt, R. and Sodroski, J. (1994a) Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies. *J Virol* **68,** 469-84.

Moore, J. P. and Sodroski, J. (1996) Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. *J Virol* **70,** 1863-72.

Moore, J. P., Willey, R. L., Lewis, G. K., Robinson, J. and Sodroski, J. (1994b) Immunological evidence for interactions between the first, second, and fifth conserved domains of the gp120 surface glycoprotein of human immunodeficiency virus type 1. *J Virol* **68,** 6836-47.

Moore, P. L., Crooks, E. T., Porter, L., Zhu, P., Cayanan, C. S., Grise, H., Corcoran, P., Zwick, M. B., Franti, M., Morris, L., Roux, K. H., Burton, D. R. and Binley, J. M. (2006) Nature of nonfunctional envelope proteins on the surface of human immunodeficiency virus type 1. *J Virol* **80,** 2515-28.

Mortuza, G. B., Haire, L. F., Stevens, A., Smerdon, S. J., Stoye, J. P. and Taylor, I. A. (2004) High-resolution structure of a retroviral capsid hexameric amino-terminal domain. *Nature* **431,** 481-5.

Moulard, M., Lortat-Jacob, H., Mondor, I., Roca, G., Wyatt, R., Sodroski, J., Zhao, L., Olson, W., Kwong, P. D. and Sattentau, Q. J. (2000) Selective interactions of polyanions with basic surfaces on human immunodeficiency virus type 1 gp120. *J Virol* **74,** 1948-60.

Moulard, M., Phogat, S. K., Shu, Y., Labrijn, A. F., Xiao, X., Binley, J. M., Zhang, M. Y., Sidorov, I. A., Broder, C. C., Robinson, J., Parren, P. W., Burton, D. R. and Dimitrov, D. S. (2002) Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes. *Proc Natl Acad Sci U S A* **99,** 6913-8.

Moyle, G. J., Wildfire, A., Mandalia, S., Mayer, H., Goodrich, J., Whitcomb, J. and Gazzard, B. G. (2005) Epidemiology and predictive factors for chemokine receptor use in HIV-1 infection. *J Infect Dis* **191,** 866-72.

Muesing, M. A., Smith, D. H., Cabradilla, C. D., Benton, C. V., Lasky, L. A. and Capon, D. J. (1985) Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. *Nature* **313,** 450-8.

Mujeeb, A., Clever, J. L., Billeci, T. M., James, T. L. and Parslow, T. G. (1998) Structure of the dimer initiation complex of HIV-1 genomic RNA. *Nat Struct Biol* **5,** 432-6.

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., Ruker, F. and Katinger, H. (1993) A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J Virol* **67,** 6642-7.

Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J. A. and Hamers, R. (1994) Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. *Protein Eng* **7,** 1129-1135.

Muyldermans, S., Cambillau, C. and Wyns, L. (2001) Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains. *Trends Biochem Sci* **26,** 230-5.

Myszka, D. G., Sweet, R. W., Hensley, P., Brigham-Burke, M., Kwong, P. D., Hendrickson, W. A., Wyatt, R., Sodroski, J. and Doyle, M. L. (2000) Energetics of the HIV gp120-CD4 binding reaction. *Proc Natl Acad Sci U S A* **97,** 9026-31.

Narayan, O. and Clements, J. E. (1989) Biology and pathogenesis of lentiviruses. *J Gen Virol* **70 (Pt 7),** 1617-39.

Nedellec, R., Coetzer, M., Shimizu, N., Hoshino, H., Polonis, V. R., Morris, L., Martensson, U. E., Binley, J., Overbaugh, J. and Mosier, D. E. (2009) Virus entry via the alternative coreceptors CCR3 and FPRL1 differs by human immunodeficiency virus type 1 subtype. *J Virol* **83,** 8353-63.

Neil, S. J., Aasa-Chapman, M. M., Clapham, P. R., Nibbs, R. J., McKnight, A. and Weiss, R. A. (2005) The promiscuous CC chemokine receptor D6 is a functional coreceptor for primary isolates of human immunodeficiency virus type 1 (HIV-1) and HIV-2 on astrocytes. *J Virol* **79,** 9618-24.

Neil, S. J., Zang, T. and Bieniasz, P. D. (2008) Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* **451,** 425-30.

Nermut, M. V. and Fassati, A. (2003) Structural analyses of purified human immunodeficiency virus type 1 intracellular reverse transcription complexes. *J Virol* **77,** 8196-206.

Neurath, A. R., Strick, N. and Li, Y. Y. (2006) Role of seminal plasma in the anti-HIV-1 activity of candidate microbicides. *BMC Infect Dis* **6,** 150.

Nguyen, V. K., Desmyter, A. and Muyldermans, S. (2001) Functional heavychain antibodies in Camelidae. *Adv Immunol* **79,** 261–296.

Nguyen, V. K., Hamers, R., Wyns, L. and Muyldermans, S. (2000) Camel heavy-chain antibodies: diverse germline VHH and specific mechanisms enlarge the antigen-binding repertoire. *EMBO J* **19,** 921-930.

Nilsson, C., Makitalo, B., Thorstensson, R., Norley, S., Binninger-Schinzel, D., Cranage, M., Rud, E., Biberfeld, G. and Putkonen, P. (1998) Live attenuated simian immunodeficiency virus (SIV)mac in macaques can induce protection against mucosal infection with SIVsm. *Aids* **12,** 2261-70.

Nisole, S. and Saib, A. (2004) Early steps of retrovirus replicative cycle. *Retrovirology* **1,** 9.

Nitayaphan, S., Pitisuttithum, P., Karnasuta, C., Eamsila, C., de Souza, M., Morgan, P., Polonis, V., Benenson, M., VanCott, T., Ratto-Kim, S., Kim, J., Thapinta, D., Garner, R., Bussaratid, V., Singharaj, P., el-Habib, R., Gurunathan, S., Heyward, W., Birx, D., McNeil, J. and Brown, A. E. (2004) Safety and immunogenicity of an HIV subtype B and E prime-boost vaccine combination in HIV-negative Thai adults. *J Infect Dis* **190,** 702-6.

Nuttall, S. D., Irving, R. A. and Hudson, P. J. (2000) Immunoglobulin VH domains and beyond: design and selection of single-domain binding and targeting reagents. *Curr Pharm Biotechnol* **1,** 253-63.

Nyambi, P. N., Mbah, H. A., Burda, S., Williams, C., Gorny, M. K., Nadas, A. and Zolla-Pazner, S. (2000) Conserved and exposed epitopes on intact, native, primary human immunodeficiency virus type 1 virions of group M. *J Virol* **74,** 7096-107.

Ofek, G., Tang, M., Sambor, A., Katinger, H., Mascola, J. R., Wyatt, R. and Kwong, P. D. (2004) Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with its gp41 epitope. *J Virol* **78,** 10724-37.

Olshevsky, U., Helseth, E., Furman, C., Li, J., Haseltine, W. and Sodroski, J. (1990) Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. *J Virol* **64,** 5701-7.

Ono, A. and Freed, E. O. (2004) Cell-type-dependent targeting of human immunodeficiency virus type 1 assembly to the plasma membrane and the multivesicular body. *J Virol* **78,** 1552-63.

Paillart, J. C., Shehu-Xhilaga, M., Marquet, R. and Mak, J. (2004) Dimerization of retroviral RNA genomes: an inseparable pair. *Nat Rev Microbiol* **2,** 461-72.

Paillart, J. C., Skripkin, E., Ehresmann, B., Ehresmann, C. and Marquet, R. (1996) A loop-loop "kissing" complex is the essential part of the dimer linkage of genomic HIV-1 RNA. *Proc Natl Acad Sci U S A* **93,** 5572-7.

Palker, T. J., Matthews, T. J., Clark, M. E., Cianciolo, G. J., Randall, R. R., Langlois, A. J., White, G. C., Safai, B., Snyderman, R., Bolognesi, D. P. and et al. (1987) A conserved region at the COOH terminus of human immunodeficiency virus gp120 envelope protein contains an immunodominant epitope. *Proc Natl Acad Sci U S A* **84,** 2479-83.

Pancera, M. and Wyatt, R. (2005) Selective recognition of oligomeric HIV-1 primary isolate envelope glycoproteins by potently neutralizing ligands requires efficient precursor cleavage. *Virology* **332,** 145-56.

Pantaleo, G., Demarest, J. F., Soudeyns, H., Graziosi, C., Denis, F., Adelsberger, J. W., Borrow, P., Saag, M. S., Shaw, G. M., Sekaly, R. P. and et al. (1994) Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature* **370,** 463-7.

Pantophlet, R. and Burton, D. R. (2003) Immunofocusing: antigen engineering to promote the induction of HIV-neutralizing antibodies. *Trends Mol Med* **9,** 468-73.

Pantophlet, R. and Burton, D. R. (2006) GP120: target for neutralizing HIV-1 antibodies. *Annu Rev Immunol* **24,** 739-69.

Parren, P. W., Burton, D. R. and Sattentau, Q. J. (1997) HIV-1 antibody--debris or virion? *Nat Med* **3,** 366-7.

Parren, P. W., Marx, P. A., Hessell, A. J., Luckay, A., Harouse, J., Cheng-Mayer, C., Moore, J. P. and Burton, D. R. (2001) Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. *J Virol* **75,** 8340-7.

Parren, P. W., Mondor, I., Naniche, D., Ditzel, H. J., Klasse, P. J., Burton, D. R. and Sattentau, Q. J. (1998) Neutralization of human immunodeficiency virus type 1 by antibody to gp120 is determined primarily by occupancy of sites on the virion irrespective of epitope specificity. *J Virol* **72,** 3512-9.

Patterson, B. K., Landay, A., Siegel, J. N., Flener, Z., Pessis, D., Chaviano, A. and Bailey, R. C. (2002) Susceptibility to human immunodeficiency virus-1 infection of human foreskin and cervical tissue grown in explant culture. *Am J Pathol* **161,** 867-73.

Peeters, M., Vincent, R., Perret, J. L., Lasky, M., Patrel, D., Liegeois, F., Courgnaud, V., Seng, R., Matton, T., Molinier, S. and Delaporte, E. (1999) Evidence for differences in MT2 cell tropism according to genetic subtypes of HIV-1: syncytium-inducing variants seem rare among subtype C HIV-1 viruses. *J Acquir Immune Defic Syndr Hum Retrovirol* **20,** 115-21.

Pellegrin, I., Legrand, E., Neau, D., Bonot, P., Masquelier, B., Pellegrin, J. L., Ragnaud, J. M., Bernard, N. and Fleury, H. J. (1996) Kinetics of appearance of

neutralizing antibodies in 12 patients with primary or recent HIV-1 infection and relationship with plasma and cellular viral loads. *J Acquir Immune Defic Syndr Hum Retrovirol* **11,** 438-47.

Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M. and Ho, D. D. (1996) HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. *Science* **271,** 1582-6.

Perez, J. M., Renisio, J. G., Prompers, J. J., van Platerink, C. J., Cambillau, C., Darbon, H. and Frenken, L. G. (2001) Thermal unfolding of a llama antibody fragment: a two-state reversible process. *Biochemistry* **40,** 74-83.

Perez, L. G., Costa, M. R., Todd, C. A., Haynes, B. F. and Montefiori, D. C. (2009) Utilization of immunoglobulin G Fc receptors by human immunodeficiency virus type 1: a specific role for antibodies against the membrane-proximal external region of gp41. *J Virol* **83,** 7397-410.

Peters, P. J., Bhattacharya, J., Hibbitts, S., Dittmar, M. T., Simmons, G., Bell, J., Simmonds, P. and Clapham, P. R. (2004) Biological analysis of human immunodeficiency virus type 1 R5 envelopes amplified from brain and lymph node tissues of AIDS patients with neuropathology reveals two distinct tropism phenotypes and identifies envelopes in the brain that confer an enhanced tropism and fusigenicity for macrophages. *J Virol* **78,** 6915-26.

Phogat, S. and Wyatt, R. (2007) Rational modifications of HIV-1 envelope glycoproteins for immunogen design. *Curr Pharm Des* **13,** 213-27.

Pilgrim, A. K., Pantaleo, G., Cohen, O. J., Fink, L. M., Zhou, J. Y., Zhou, J. T., Bolognesi, D. P., Fauci, A. S. and Montefiori, D. C. (1997) Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection. *J Infect Dis* **176,** 924-32.

Ping, L. H., Nelson, J. A., Hoffman, I. F., Schock, J., Lamers, S. L., Goodman, M., Vernazza, P., Kazembe, P., Maida, M., Zimba, D., Goodenow, M. M., Eron, J. J., Jr., Fiscus, S. A., Cohen, M. S. and Swanstrom, R. (1999) Characterization of V3 sequence heterogeneity in subtype C human immunodeficiency virus type 1 isolates from Malawi: underrepresentation of X4 variants. *J Virol* **73,** 6271-81.

Pinter, A., Honnen, W. J., Tilley, S. A., Bona, C., Zaghouani, H., Gorny, M. K. and Zolla-Pazner, S. (1989) Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. *J Virol* **63,** 2674-9.

Pitisuttithum, P., Gilbert, P., Gurwith, M., Heyward, W., Martin, M., van Griensven, F., Hu, D., Tappero, J. W. and Choopanya, K. (2006) Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis* **194,** 1661-71.

Plantier, J. C., Leoz, M., Dickerson, J. E., De Oliveira, F., Cordonnier, F., Lemee, V., Damond, F., Robertson, D. L. and Simon, F. (2009) A new human immunodeficiency virus derived from gorillas. *Nat Med* **15,** 871-2.

Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B. and Kabat, D. (1998) Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol* **72,** 2855-64.

Poignard, P., Moulard, M., Golez, E., Vivona, V., Franti, M., Venturini, S., Wang, M., Parren, P. W. and Burton, D. R. (2003) Heterogeneity of envelope molecules expressed on primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies. *J Virol* **77,** 353-65.

Pollard, S. R., Rosa, M. D., Rosa, J. J. and Wiley, D. C. (1992) Truncated variants of gp120 bind CD4 with high affinity and suggest a minimum CD4 binding region. *Embo J* **11,** 585-91.

Polonis, V. R., Brown, B. K., Rosa Borges, A., Zolla-Pazner, S., Dimitrov, D. S., Zhang, M. Y., Barnett, S. W., Ruprecht, R. M., Scarlatti, G., Fenyo, E. M., Montefiori, D. C., McCutchan, F. E. and Michael, N. L. (2008) Recent advances in the characterization of HIV-1 neutralization assays for standardized evaluation of the antibody response to infection and vaccination. *Virology* **375,** 315-20.

Popovic, M., Sarngadharan, M. G., Read, E. and Gallo, R. C. (1984) Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224,** 497-500.

Power, B. E. and Hudson, P. J. (2000) Synthesis of high avidity antibody fragments (scFv multimers) for cancer imaging. *J Immunol Methods* **242,** 193-204.

Pugach, P., Kuhmann, S. E., Taylor, J., Marozsan, A. J., Snyder, A., Ketas, T., Wolinsky, S. M., Korber, B. T. and Moore, J. P. (2004) The prolonged culture of human immunodeficiency virus type 1 in primary lymphocytes increases its sensitivity to neutralization by soluble CD4. *Virology* **321,** 8-22.

Purtscher, M., Trkola, A., Gruber, G., Buchacher, A., Predl, R., Steindl, F., Tauer, C., Berger, R., Barrett, N., Jungbauer, A. and et al. (1994) A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* **10,** 1651-8.

Pusch, O., Kalyanaraman, R., Tucker, L. D., Wells, J. M., Ramratnam, B. and Boden, D. (2006) An anti-HIV microbicide engineered in commensal bacteria: secretion of HIV-1 fusion inhibitors by lactobacilli. *Aids* **20,** 1917-22.

Qiao, Z. S., Kim, M., Reinhold, B., Montefiori, D., Wang, J. H. and Reinherz, E. L. (2005) Design, expression, and immunogenicity of a soluble HIV trimeric envelope fragment adopting a prefusion gp41 configuration. *J Biol Chem* **280,** 23138-46.

Rabson, A. B. and Martin, M. A. (1985) Molecular organization of the AIDS retrovirus. *Cell* **40,** 477-80.

Ratner, L., Gallo, R. C. and Wong-Staal, F. (1985a) HTLV-III, LAV, ARV are variants of same AIDS virus. *Nature* **313,** 636-7.

Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K. and et al. (1985b) Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313,** 277-84.

Reeves, J. D. and Doms, R. W. (2002) Human immunodeficiency virus type 2. *J Gen Virol* **83,** 1253-65.

Reitter, J. N., Means, R. E. and Desrosiers, R. C. (1998) A role for carbohydrates in immune evasion in AIDS. *Nat Med* **4,** 679-84.

Richman, D. D., Wrin, T., Little, S. J. and Petropoulos, C. J. (2003) Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A* **100,** 4144-9.

Robert-Guroff, M., Popovic, M., Gartner, S., Markham, P., Gallo, R. C. and Reitz, M. S. (1990) Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. *J Virol* **64,** 3391-8.

Robertson, D. L., Anderson, J. P., Bradac, J. A., Carr, J. K., Foley, B., Funkhouser, R. K., Gao, F., Hahn, B. H., Kalish, M. L., Kuiken, C., Learn, G. H., Leitner, T., McCutchan, F., Osmanov, S., Peeters, M., Pieniazek, D., Salminen, M., Sharp, P. M., Wolinsky, S. and Korber, B. (2000) HIV-1 nomenclature proposal. *Science* **288,** 55-6.

Robertson, D. L., Sharp, P. M., McCutchan, F. E. and Hahn, B. H. (1995) Recombination in HIV-1. *Nature* **374,** 124-6.

Robey, W. G., Safai, B., Oroszlan, S., Arthur, L. O., Gonda, M. A., Gallo, R. C. and Fischinger, P. J. (1985) Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. *Science* **228,** 593-5.

Roddy, R. E., Zekeng, L., Ryan, K. A., Tamoufe, U., Weir, S. S. and Wong, E. L. (1998) A controlled trial of nonoxynol 9 film to reduce male-to-female transmission of sexually transmitted diseases. *N Engl J Med* **339,** 504-10.

Rodenburg, C. M., Li, Y., Trask, S. A., Chen, Y., Decker, J., Robertson, D. L., Kalish, M. L., Shaw, G. M., Allen, S., Hahn, B. H. and Gao, F. (2001) Near full-length clones and reference sequences for subtype C isolates of HIV type 1 from three different continents. *AIDS Res Hum Retroviruses* **17,** 161-8.

Roovers, R. C., Laeremans, T., Huang, L., De Taeye, S., Verkleij, A. J., Revets, H., de Haard, H. J. and van Bergen en Henegouwen, P. M. (2007) Efficient inhibition of EGFR signaling and of tumour growth by antagonistic anti-EFGR Nanobodies. *Cancer Immunol Immunother* **56,** 303-317.

Rothbauer, U., Zolghadr, K., Tillib, S., Nowak, D., Schermelleh, L., Gahl, A., Backmann, N., Conrath, K., Muyldermans, S., Cardoso, M. C. and Leonhardt, H. (2006) Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nat Methods* **3,** 887-9.

Roux, K. H. and Taylor, K. A. (2007) AIDS virus envelope spike structure. *Curr Opin Struct Biol* **17,** 244-52.

Royce, R. A., Sena, A., Cates, W., Jr. and Cohen, M. S. (1997) Sexual transmission of HIV. *N Engl J Med* **336,** 1072-8.

Rozenbaum, W., Coulaud, J. P., Saimot, A. G., Klatzmann, D., Mayaud, C. and Carette, M. F. (1982) Multiple opportunistic infection in a male homosexual in France. *Lancet* **1,** 572-3.

Ryu, S. E., Kwong, P. D., Truneh, A., Porter, T. G., Arthos, J., Rosenberg, M., Dai, X. P., Xuong, N. H., Axel, R., Sweet, R. W. and et al. (1990) Crystal structure of an HIV-binding recombinant fragment of human CD4. *Nature* **348,** 419-26.

Sanchez-Martinez, S., Lorizate, M., Katinger, H., Kunert, R. and Nieva, J. L. (2006) Membrane association and epitope recognition by HIV-1 neutralizing anti-gp41 2F5 and 4E10 antibodies. *AIDS Res Hum Retroviruses* **22,** 998-1006.

Sanchez-Pescador, R., Power, M. D., Barr, P. J., Steimer, K. S., Stempien, M. M., Brown-Shimer, S. L., Gee, W. W., Renard, A., Randolph, A., Levy, J. A. and et al. (1985) Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). *Science* **227,** 484-92.

Sanders, R. W., Venturi, M., Schiffner, L., Kalyanaraman, R., Katinger, H., Lloyd, K. O., Kwong, P. D. and Moore, J. P. (2002a) The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. *J Virol* **76,** 7293-305.

Sanders, R. W., Vesanen, M., Schuelke, N., Master, A., Schiffner, L., Kalyanaraman, R., Paluch, M., Berkhout, B., Maddon, P. J., Olson, W. C., Lu, M. and Moore, J. P. (2002b) Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *J Virol* **76,** 8875-89.

Sarngadharan, M. G., Popovic, M., Bruch, L., Schupbach, J. and Gallo, R. C. (1984) Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. *Science* **224,** 506-8.

Sattentau, Q. (2008) Correlates of antibody-mediated protection against HIV infection. *Curr Opin HIV AIDS* **3,** 368-374.

Sattentau, Q. J. and Moore, J. P. (1991) Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *J Exp Med* **174,** 407-15.

Sattentau, Q. J. and Moore, J. P. (1995) Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. *J Exp Med* **182,** 185-96.

Sattentau, Q. J., Moore, J. P., Vignaux, F., Traincard, F. and Poignard, P. (1993) Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. *J Virol* **67,** 7383-93.

Sattentau, Q. J., Zolla-Pazner, S. and Poignard, P. (1995) Epitope exposure on functional, oligomeric HIV-1 gp41 molecules. *Virology* **206,** 713-7.

Scanlan, C. N., Pantophlet, R., Wormald, M. R., Ollmann Saphire, E., Stanfield, R., Wilson, I. A., Katinger, H., Dwek, R. A., Rudd, P. M. and Burton, D. R. (2002) The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120. *J Virol* **76,** 7306-21.

Scarlatti, G., Tresoldi, E., Bjorndal, A., Fredriksson, R., Colognesi, C., Deng, H. K., Malnati, M. S., Plebani, A., Siccardi, A. G., Littman, D. R., Fenyo, E. M. and Lusso, P. (1997) In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. *Nat Med* **3,** 1259-65.

Schaeffer, D. J. and Krylov, V. S. (2000) Anti-HIV activity of extracts and compounds from algae and cyanobacteria. *Ecotoxicol Environ Saf* **45,** 208-27.

Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L. and Reimann, K. A. (1999) Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283,** 857-60.

Schonning, K., Jansson, B., Olofsson, S., Nielsen, J. O. and Hansen, J. S. (1996) Resistance to V3-directed neutralization caused by an N-linked oligosaccharide depends on the quaternary structure of the HIV-1 envelope oligomer. *Virology* **218,** 134-40.

Schroder, A. R., Shinn, P., Chen, H., Berry, C., Ecker, J. R. and Bushman, F. (2002) HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110,** 521-9.

Schuitemaker, H., Koot, M., Kootstra, N. A., Dercksen, M. W., de Goede, R. E., van Steenwijk, R. P., Lange, J. M., Schattenkerk, J. K., Miedema, F. and Tersmette, M. (1992) Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J Virol* **66,** 1354-60.

Schulke, N., Vesanen, M. S., Sanders, R. W., Zhu, P., Lu, M., Anselma, D. J., Villa, A. R., Parren, P. W., Binley, J. M., Roux, K. H., Maddon, P. J., Moore, J. P. and Olson, W. C. (2002) Oligomeric and conformational properties of a proteolytically mature, disulfide-stabilized human immunodeficiency virus type 1 gp140 envelope glycoprotein. *J Virol* **76,** 7760-76.

Schupbach, J., Popovic, M., Gilden, R. V., Gonda, M. A., Sarngadharan, M. G. and Gallo, R. C. (1984) Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. *Science* **224,** 503-5.

Schwartz, S., Felber, B. K., Benko, D. M., Fenyo, E. M. and Pavlakis, G. N. (1990) Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. *J Virol* **64,** 2519-29.

Selvarajah, S., Puffer, B., Pantophlet, R., Law, M., Doms, R. W. and Burton, D. R. (2005) Comparing antigenicity and immunogenicity of engineered gp120. *J Virol* **79,** 12148-63.

Sewankambo, N., Gray, R. H., Wawer, M. J., Paxton, L., McNaim, D., Wabwire-Mangen, F., Serwadda, D., Li, C., Kiwanuka, N., Hillier, S. L., Rabe, L., Gaydos, C. A., Quinn, T. C. and Konde-Lule, J. (1997) HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet* **350,** 546-50.

Shattock, R. J. and Moore, J. P. (2003) Inhibiting sexual transmission of HIV-1 infection. *Nat Rev Microbiol* **1,** 25-34.

Sheehy, A. M., Gaddis, N. C., Choi, J. D. and Malim, M. H. (2002) Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418,** 646-50.

Sheehy, A. M., Gaddis, N. C. and Malim, M. H. (2003) The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat Med* **9,** 1404-7.

Sherer, N. M., Lehmann, M. J., Jimenez-Soto, L. F., Horensavitz, C., Pypaert, M. and Mothes, W. (2007) Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission. *Nat Cell Biol* **9,** 310-5.

Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., Willey, R., Cho, M. W. and Martin, M. A. (1999) Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat Med* **5,** 204-10.

Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z. Q., Simon, A. J., Trigona, W. L., Dubey, S. A., Huang, L., Harris, V. A., Long, R. S., Liang, X., Handt, L., Schleif, W. A., Zhu, L., Freed, D. C., Persaud, N. V., Guan, L., Punt, K. S., Tang, A., Chen, M., Wilson, K. A., Collins, K. B., Heidecker, G. J., Fernandez, V. R., Perry, H. C., Joyce, J. G., Grimm, K. M., Cook, J. C., Keller, P. M., Kresock, D. S., Mach, H., Troutman, R. D., Isopi, L. A., Williams, D. M., Xu, Z., Bohannon, K. E., Volkin, D. B., Montefiori, D. C., Miura, A., Krivulka, G. R., Lifton, M. A., Kuroda, M. J., Schmitz, J. E., Letvin, N. L., Caulfield, M. J., Bett, A. J., Youil, R., Kaslow, D. C. and Emini, E. A. (2002) Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415,** 331-5.

Simoes, J. A., Citron, D. M., Aroutcheva, A., Anderson, R. A., Jr., Chany, C. J., 2nd, Waller, D. P., Faro, S. and Zaneveld, L. J. (2002) Two novel vaginal microbicides (polystyrene sulfonate and cellulose sulfate) inhibit Gardnerella vaginalis and anaerobes commonly associated with bacterial vaginosis. *Antimicrob Agents Chemother* **46,** 2692-5.

Soda, Y., Shimizu, N., Jinno, A., Liu, H. Y., Kanbe, K., Kitamura, T. and Hoshino, H. (1999) Establishment of a new system for determination of coreceptor usages of HIV based on the human glioma NP-2 cell line. *Biochem Biophys Res Commun* **258,** 313-21.

Sodroski, J., Rosen, C., Wong-Staal, F., Salahuddin, S. Z., Popovic, M., Arya, S., Gallo, R. C. and Haseltine, W. A. (1985) Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. *Science* **227,** 171-3.

Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A. and Wain-Hobson, S. (1985) Nucleotide sequence of the visna lentivirus: relationship to the AIDS virus. *Cell* **42,** 369-82.

Sonza, S., Burgess, S. H. and Crowe, S. M. (1991) Direct quantification of HIV-1 infectivity for monocyte--macrophages using an infectious focus assay. *AIDS* **5,** 571-4.

Sougrat, R., Bartesaghi, A., Lifson, J. D., Bennett, A. E., Bess, J. W., Zabransky, D. J. and Subramaniam, S. (2007) Electron tomography of the contact between T cells and SIV/HIV-1: implications for viral entry. *PLoS Pathog* **3,** e63.

Sowinski, S., Jolly, C., Berninghausen, O., Purbhoo, M. A., Chauveau, A., Kohler, K., Oddos, S., Eissmann, P., Brodsky, F. M., Hopkins, C., Onfelt, B., Sattentau, Q. and Davis, D. M. (2008) Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nat Cell Biol* **10,** 211-9.

Srivastava, I. K., Stamatatos, L., Legg, H., Kan, E., Fong, A., Coates, S. R., Leung, L., Wininger, M., Donnelly, J. J., Ulmer, J. B. and Barnett, S. W. (2002) Purification and characterization of oligomeric envelope glycoprotein from a primary R5 subtype B human immunodeficiency virus. *J Virol* **76,** 2835-47.

Stafford, M. K., Ward, H., Flanagan, A., Rosenstein, I. J., Taylor-Robinson, D., Smith, J. R., Weber, J. and Kitchen, V. S. (1998) Safety study of nonoxynol-9 as a vaginal microbicide: evidence of adverse effects. *J Acquir Immune Defic Syndr Hum Retrovirol* **17,** 327-31.

Stahl, R. E., Friedman-Kien, A., Dubin, R., Marmor, M. and Zolla-Pazner, S. (1982) Immunologic abnormalities in homosexual men. Relationship to Kaposi's sarcoma. *Am J Med* **73,** 171-8.

Starcich, B., Ratner, L., Josephs, S. F., Okamoto, T., Gallo, R. C. and Wong-Staal, F. (1985) Characterization of long terminal repeat sequences of HTLV-III. *Science* **227,** 538-40.

Starcich, B. R., Hahn, B. H., Shaw, G. M., McNeely, P. D., Modrow, S., Wolf, H., Parks, E. S., Parks, W. P., Josephs, S. F., Gallo, R. C. and et al. (1986) Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. *Cell* **45,** 637-48.

Stein, B. S. and Engleman, E. G. (1990) Intracellular processing of the gp160 HIV-1 envelope precursor. Endoproteolytic cleavage occurs in a cis or medial compartment of the Golgi complex. *J Biol Chem* **265,** 2640-9.

Steinbrook, R. (2007) One step forward, two steps back--will there ever be an AIDS vaccine? *N Engl J Med* **357,** 2653-5.

Stiegler, G., Kunert, R., Purtscher, M., Wolbank, S., Voglauer, R., Steindl, F. and Katinger, H. (2001) A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* **17,** 1757-65.

Stijlemans, B., Conrath, K., Cortez-Retamozo, V., Van Xong, H., Wyns, L., Senter, P., Revets, H., De Baetselier, P., Muyldermans, S. and Magez, S. (2004) Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies. African trypanosomes as paradigm. *J Biol Chem* **279,** 1256–1261.

Stremlau, M., Owens, C. M., Perron, M. J., Kiessling, M., Autissier, P. and Sodroski, J. (2004) The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* **427,** 848-53.

Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D. J., Sundquist, W. I. and Sodroski, J. (2006) Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. *Proc Natl Acad Sci U S A* **103,** 5514-9.

Su, L., Graf, M., Zhang, Y., von Briesen, H., Xing, H., Kostler, J., Melzl, H., Wolf, H., Shao, Y. and Wagner, R. (2000) Characterization of a virtually full-length human immunodeficiency virus type 1 genome of a prevalent intersubtype (C/B') recombinant strain in China. *J Virol* **74,** 11367-76.

Subramaniam, S. (2006) The SIV surface spike imaged by electron tomography: one leg or three? *PLoS Pathog* **2,** e91.

Sullivan, N., Sun, Y., Sattentau, Q., Thali, M., Wu, D., Denisova, G., Gershoni, J., Robinson, J., Moore, J. and Sodroski, J. (1998) CD4-Induced conformational changes in the human immunodeficiency virus type 1 gp120 glycoprotein: consequences for virus entry and neutralization. *J Virol* **72,** 4694-703.

Swanstrom, R., and Wills, J.W. (1997) Synthesis, assembly, and processing of viral proteins. In *Retroviruses*, (ed. H. S. H. Coffin J. M., Varmus H. E.), pp. 263-334, Cold Spring Harbor Laboratory Press, New York, USA.

Taylor, B. S., Sobieszczyk, M. E., McCutchan, F. E. and Hammer, S. M. (2008) The challenge of HIV-1 subtype diversity. *N Engl J Med* **358,** 1590-602.

Temin, H. M. (1993) Retrovirus variation and reverse transcription: abnormal strand transfers result in retrovirus genetic variation. *Proc Natl Acad Sci U S A* **90,** 6900-3.

Terhorst, C., van Agthoven, A., Reinherz, E. and Schlossman, S. (1980) Biochemical analysis of human T lymphocyte differentiation antigens T4 and T5. *Science* **209,** 520-1.

Tersmette, M., de Goede, R. E., Al, B. J., Winkel, I. N., Gruters, R. A., Cuypers, H. T., Huisman, H. G. and Miedema, F. (1988) Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J Virol* **62,** 2026-32.

Terwilliger, E. F., Cohen, E. A., Lu, Y. C., Sodroski, J. G. and Haseltine, W. A. (1989) Functional role of human immunodeficiency virus type 1 vpu. *Proc Natl Acad Sci U S A* **86,** 5163-7.

Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C. T., Sodroski, J. and Gottlinger, H. G. (1994) Functional association of cyclophilin A with HIV-1 virions. *Nature* **372,** 363-5.

The Population Council. (2008) Trial shows anti-HIV microbicide is safe, but does not prove it effective. In *Population Council Media Center News Release 18 Feb 2008.*

Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. J. and Moore, J. P. (1996a) CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* **384,** 184-7.

Trkola, A., Kuster, H., Rusert, P., Joos, B., Fischer, M., Leemann, C., Manrique, A., Huber, M., Rehr, M., Oxenius, A., Weber, R., Stiegler, G., Vcelar, B., Katinger, H., Aceto, L. and Gunthard, H. F. (2005) Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. *Nat Med* **11,** 615-22.

Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J. P. and Katinger, H. (1996b) Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J Virol* **70,** 1100-8.

Tsai, C. C., Emau, P., Jiang, Y., Agy, M. B., Shattock, R. J., Schmidt, A., Morton, W. R., Gustafson, K. R. and Boyd, M. R. (2004) Cyanovirin-N inhibits AIDS virus infections in vaginal transmission models. *AIDS Res Hum Retroviruses* **20,** 11-8.

Tscherning, C., Alaeus, A., Fredriksson, R., Bjorndal, A., Deng, H., Littman, D. R., Fenyo, E. M. and Albert, J. (1998) Differences in chemokine coreceptor usage between genetic subtypes of HIV-1. *Virology* **241,** 181-8.

UNAIDS/WHO. (2007) AIDS epidemic update : December 2007.

Van Damme, L., Govinden, R., Mirembe, F. M., Guedou, F., Solomon, S., Becker, M. L., Pradeep, B. S., Krishnan, A. K., Alary, M., Pande, B., Ramjee, G., Deese, J., Crucitti, T. and Taylor, D. (2008) Lack of effectiveness of cellulose sulfate gel for the prevention of vaginal HIV transmission. *N Engl J Med* **359,** 463-72.

Van Damme, L., Ramjee, G., Alary, M., Vuylsteke, B., Chandeying, V., Rees, H., Sirivongrangson, P., Mukenge-Tshibaka, L., Ettiegne-Traore, V., Uaheowitchai, C., Karim, S. S., Masse, B., Perriens, J. and Laga, M. (2002) Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. *Lancet* **360,** 971-7.

van de Laar, T., Visser, C., Holster, M., Lopez, C. G., Kreuning, D., Sierkstra, L., Lindner, N. and Verrips, T. (2007) Increased heterologous protein production by

Saccharomyces cerevisiae growing on ethanol as sole carbon source. *Biotechnol Bioeng* **96,** 483-94.

van de Wijgert, J. H. and Shattock, R. J. (2007) Vaginal microbicides: moving ahead after an unexpected setback. *Aids* **21,** 2369-76.

van der Linden, R. H., Frenken, L. G., de Geus, B., Harmsen, M. M., Ruuls, R. C., Stok, W., de Ron, L., Wilson, S., Davis, P. and Verrips, C. T. (1999) Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. *Biochim Biophys Acta* **1431,** 37-46.

van der Loeff, M. F., Awasana, A. A., Sarge-Njie, R., van der Sande, M., Jaye, A., Sabally, S., Corrah, T., McConkey, S. J. and Whittle, H. C. (2006) Sixteen years of HIV surveillance in a West African research clinic reveals divergent epidemic trends of HIV-1 and HIV-2. *Int J Epidemiol* **35,** 1322-8.

van der Vaart, J. M., Pant, N., Wolvers, D., Bezemer, S., Hermans, P. W., Bellamy, K., Sarker, S. A., van der Logt, C. P. E., Svensson, L., Verrips, C. T., Hammarstrom, L. and van Klinken, B. J. W. (2006) Reduction in morbidity of rotavirus induced diarrhoea in mice by yeast produced monovalent llama-derived antibody fragments. *Vaccine* **24,** 4130-4137.

Van Heuverswyn, F., Li, Y., Neel, C., Bailes, E., Keele, B. F., Liu, W., Loul, S., Butel, C., Liegeois, F., Bienvenue, Y., Ngolle, E. M., Sharp, P. M., Shaw, G. M., Delaporte, E., Hahn, B. H. and Peeters, M. (2006) Human immunodeficiency viruses: SIV infection in wild gorillas. *Nature* **444,** 164.

Van Rompay, K. K., Berardi, C. J., Dillard-Telm, S., Tarara, R. P., Canfield, D. R., Valverde, C. R., Montefiori, D. C., Cole, K. S., Montelaro, R. C., Miller, C. J. and Marthas, M. L. (1998) Passive immunization of newborn rhesus macaques prevents oral simian immunodeficiency virus infection. *J Infect Dis* **177,** 1247-59.

van't Wout, A. B., Kootstra, N. A., Mulder-Kampinga, G. A., Albrecht-van Lent, N., Scherpbier, H. J., Veenstra, J., Boer, K., Coutinho, R. A., Miedema, F. and Schuitemaker, H. (1994) Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. *J Clin Invest* **94,** 2060-7.

Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley, D. R., Knight, H. L., Rosenzweig, M., Johnson, R. P., Desrosiers, R. C. and Lackner, A. A. (1998) Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* **280,** 427-31.

Veazey, R. S., Shattock, R. J., Pope, M., Kirijan, J. C., Jones, J., Hu, Q., Ketas, T., Marx, P. A., Klasse, P. J., Burton, D. R. and Moore, J. P. (2003) Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. *Nat Med* **9,** 343-6.

Verheesen, P., ten Haaft, M. R., Lindner, N., Verrips, C. T. and de Haard, J. J. (2003) Beneficial properties of single-domain antibody fragments for application in

immunoaffinity purification and immuno-perfusion chromatography. *Biochim Biophys Acta* **1624,** 21-8.

Veronese, F. D., DeVico, A. L., Copeland, T. D., Oroszlan, S., Gallo, R. C. and Sarngadharan, M. G. (1985) Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. *Science* **229,** 1402-5.

Vilaseca, J., Arnau, J. M., Bacardi, R., Mieras, C., Serrano, A. and Navarro, C. (1982) Kaposi's sarcoma and toxoplasma gondii brain abscess in a Spanish homosexual. *Lancet* **1,** 572.

Vilmer, E., Barré-Sinoussi, F., Rouzioux, C., Gazengel, C., Brun, F. V., Dauguet, C., Fischer, A., Manigne, P., Chermann, J. C., Griscelli, C. and et al. (1984) Isolation of new lymphotropic retrovirus from two siblings with haemophilia B, one with AIDS. *Lancet* **1,** 753-7.

Vogt, V. M. (1997) Retroviral virions and genomes. In *Retroviruses*, (ed. H. S. H. Coffin J. M., Varmus H. E.), pp. 27-69, Cold Spring Harbor Laboratory Press, New York, USA.

Vu, K. B., Ghahroudi, M. A., Wyns, L. and Muyldermans, S. (1997) Comparison of llama VH sequences from conventional and heavy chain antibodies. *Mol Immunol* **34,** 1121-31.

Wain-Hobson, S., Alizon, M. and Montagnier, L. (1985a) Relationship of AIDS to other retroviruses. *Nature* **313,** 743.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. and Alizon, M. (1985b) Nucleotide sequence of the AIDS virus, LAV. *Cell* **40,** 9-17.

Walker, B. D. and Burton, D. R. (2008) Toward an AIDS vaccine. *Science* **320,** 760-4.

Wang, J. H., Meijers, R., Xiong, Y., Liu, J. H., Sakihama, T., Zhang, R., Joachimiak, A. and Reinherz, E. L. (2001) Crystal structure of the human CD4 N-terminal two-domain fragment complexed to a class II MHC molecule. *Proc Natl Acad Sci U S A* **98,** 10799-804.

Wang, S., Pal, R., Mascola, J. R., Chou, T. H., Mboudjeka, I., Shen, S., Liu, Q., Whitney, S., Keen, T., Nair, B. C., Kalyanaraman, V. S., Markham, P. and Lu, S. (2006) Polyvalent HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. *Virology* **350,** 34-47.

Wang, T., Zhang, Z., Wallace, O. B., Deshpande, M., Fang, H., Yang, Z., Zadjura, L. M., Tweedie, D. L., Huang, S., Zhao, F., Ranadive, S., Robinson, B. S., Gong, Y. F., Ricarrdi, K., Spicer, T. P., Deminie, C., Rose, R., Wang, H. G., Blair, W. S., Shi, P. Y., Lin, P. F., Colonno, R. J. and Meanwell, N. A. (2003) Discovery of 4-benzoyl-1-[(4-methoxy-1H- pyrrolo[2,3-b]pyridin-3-yl)oxoacetyl]-2- (R)-methylpiperazine

(BMS-378806): a novel HIV-1 attachment inhibitor that interferes with CD4-gp120 interactions. *J Med Chem* **46,** 4236-9.

Watkins, D. I., Burton, D. R., Kallas, E. G., Moore, J. P. and Koff, W. C. (2008) Nonhuman primate models and the failure of the Merck HIV-1 vaccine in humans. *Nat Med* **14,** 617-21.

Watts, J. M., Dang, K. K., Gorelick, R. J., Leonard, C. W., Bess, J. W., Jr., Swanstrom, R., Burch, C. L. and Weeks, K. M. (2009) Architecture and secondary structure of an entire HIV-1 RNA genome. *Nature* **460,** 711-6.

Weber, J., Nunn, A., O'Connor, T., Jeffries, D., Kitchen, V., McCormack, S., Stott, J., Almond, N., Stone, A. and Darbyshire, J. (2001) 'Chemical condoms' for the prevention of HIV infection: evaluation of novel agents against SHIV(89.6PD) in vitro and in vivo. *Aids* **15,** 1563-8.

Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., Saag, M. S., Wu, X., Shaw, G. M. and Kappes, J. C. (2002) Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother* **46,** 1896-905.

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., Hahn, B. H., Kwong, P. D. and Shaw, G. M. (2003) Antibody neutralization and escape by HIV-1. *Nature* **422,** 307-12.

Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H. and et al. (1995) Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373,** 117-22.

Weiler, A. M., Li, Q., Duan, L., Kaizu, M., Weisgrau, K. L., Friedrich, T. C., Reynolds, M. R., Haase, A. T. and Rakasz, E. G. (2008) Genital ulcers facilitate rapid viral entry and dissemination following intravaginal inoculation with cell-associated simian immunodeficiency virus SIVmac239. *J Virol* **82,** 4154-8.

Weiner, D. B., Huebner, K., Williams, W. V. and Greene, M. I. (1991) Human genes other than CD4 facilitate HIV-1 infection of murine cells. *Pathobiology* **59,** 361-71.

Weiss, C. D., Levy, J. A. and White, J. M. (1990) Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. *J Virol* **64,** 5674-7.

Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J. and Wiley, D. C. (1997) Atomic structure of the ectodomain from HIV-1 gp41. *Nature* **387,** 426-30.

Whatmore, A. M., Cook, N., Hall, G. A., Sharpe, S., Rud, E. W. and Cranage, M. P. (1995) Repair and evolution of nef in vivo modulates simian immunodeficiency virus virulence. *J Virol* **69,** 5117-23.

Willey, R. L., Bonifacino, J. S., Potts, B. J., Martin, M. A. and Klausner, R. D. (1988) Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. *Proc Natl Acad Sci U S A* **85,** 9580-4.

Willey, R. L., Rutledge, R. A., Dias, S., Folks, T., Theodore, T., Buckler, C. E. and Martin, M. A. (1986) Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. *Proc Natl Acad Sci U S A* **83,** 5038-42.

Williamson, C., Morris, L., Maughan, M. F., Ping, L. H., Dryga, S. A., Thomas, R., Reap, E. A., Cilliers, T., van Harmelen, J., Pascual, A., Ramjee, G., Gray, G., Johnston, R., Karim, S. A. and Swanstrom, R. (2003) Characterization and selection of HIV-1 subtype C isolates for use in vaccine development. *AIDS Res Hum Retroviruses* **19,** 133-44.

Wilson, D. P., Coplan, P. M., Wainberg, M. A. and Blower, S. M. (2008) The paradoxical effects of using antiretroviral-based microbicides to control HIV epidemics. *Proc Natl Acad Sci U S A* **105,** 9835-40.

Wood, G. S., Warner, N. L. and Warnke, R. A. (1983) Anti-Leu-3/T4 antibodies react with cells of monocyte/macrophage and Langerhans lineage. *J Immunol* **131,** 212-6.

World Health Organisation. (1983) 'Acquired Immune Deficiency Syndrome Emergencies', Report of a WHO Meeting, Geneva, 22-25, November 1983.

Wrin, T., Loh, T. P., Vennari, J. C., Schuitemaker, H. and Nunberg, J. H. (1995) Adaptation to persistent growth in the H9 cell line renders a primary isolate of human immunodeficiency virus type 1 sensitive to neutralization by vaccine sera. *J Virol* **69,** 39-48.

Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A. A., Desjardin, E., Newman, W., Gerard, C. and Sodroski, J. (1996) CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* **384,** 179-83.

Wyand, M. S., Manson, K., Montefiori, D. C., Lifson, J. D., Johnson, R. P. and Desrosiers, R. C. (1999) Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J Virol* **73,** 8356-63.

Wyand, M. S., Manson, K. H., Garcia-Moll, M., Montefiori, D. and Desrosiers, R. C. (1996) Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J Virol* **70,** 3724-33.

Wyatt, R., Kwong, P. D., Desjardins, E., Sweet, R. W., Robinson, J., Hendrickson, W. A. and Sodroski, J. G. (1998) The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393,** 705-11.

Wyatt, R., Moore, J., Accola, M., Desjardin, E., Robinson, J. and Sodroski, J. (1995) Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. *J Virol* **69,** 5723-33.

Wyatt, R. and Sodroski, J. (1998) The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280,** 1884-8.

Wyatt, R., Sullivan, N., Thali, M., Repke, H., Ho, D., Robinson, J., Posner, M. and Sodroski, J. (1993) Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. *J Virol* **67,** 4557-65.

Xu, J. Y., Gorny, M. K., Palker, T., Karwowska, S. and Zolla-Pazner, S. (1991) Epitope mapping of two immunodominant domains of gp41, the transmembrane protein of human immunodeficiency virus type 1, using ten human monoclonal antibodies. *J Virol* **65,** 4832-8.

Yamamoto, H., Kawada, M., Takeda, A., Igarashi, H. and Matano, T. (2007) Post-infection immunodeficiency virus control by neutralizing antibodies. *PLoS ONE* **2,** e540.

Yang, X., Florin, L., Farzan, M., Kolchinsky, P., Kwong, P. D., Sodroski, J. and Wyatt, R. (2000) Modifications that stabilize human immunodeficiency virus envelope glycoprotein trimers in solution. *J Virol* **74,** 4746-54.

Yang, X., Kurteva, S., Ren, X., Lee, S. and Sodroski, J. (2005) Stoichiometry of envelope glycoprotein trimers in the entry of human immunodeficiency virus type 1. *J Virol* **79,** 12132-47.

Yang, X., Wyatt, R. and Sodroski, J. (2001) Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. *J Virol* **75,** 1165-71.

Yang, Z. N., Mueser, T. C., Kaufman, J., Stahl, S. J., Wingfield, P. T. and Hyde, C. C. (1999) The crystal structure of the SIV gp41 ectodomain at 1.47 A resolution. *J Struct Biol* **126,** 131-44.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33,** 103-19.

Yirrell, D. L., Shaw, L., Burns, S. M., Cameron, S. O., Quigg, M., Campbell, E. and Goldberg, D. (2004) HIV-1 subtype in Scotland: the establishment of a national surveillance system. *Epidemiol Infect* **132,** 693-8.

Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P. and Yu, X. F. (2003) Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* **302,** 1056-60.

Yuan, W., Bazick, J. and Sodroski, J. (2006) Characterization of the multiple conformational States of free monomeric and trimeric human immunodeficiency virus envelope glycoproteins after fixation by cross-linker. *J Virol* **80,** 6725-37.

Yuste, E., Sanford, H. B., Carmody, J., Bixby, J., Little, S., Zwick, M. B., Greenough, T., Burton, D. R., Richman, D. D., Desrosiers, R. C. and Johnson, W. E. (2006) Simian immunodeficiency virus engrafted with human immunodeficiency virus type 1 (HIV-1)-specific epitopes: replication, neutralization, and survey of HIV-1-positive plasma. *J Virol* **80,** 3030-41.

Zahnd, C., Amstutz, P. and Pluckthun, A. (2007) Ribosome display: selecting and evolving proteins in vitro that specifically bind to a target. *Nat Methods* **4,** 269-79.

Zaitseva, L., Cherepanov, P., Leyens, L., Wilson, S. J., Rasaiyaah, J. and Fassati, A. (2009) HIV-1 exploits importin 7 to maximize nuclear import of its DNA genome. *Retrovirology* **6,** 11.

Zanetti, G., Briggs, J. A., Grunewald, K., Sattentau, Q. J. and Fuller, S. D. (2006) Cryo-electron tomographic structure of an immunodeficiency virus envelope complex in situ. *PLoS Pathog* **2,** e83.

Zapp, M. L. and Green, M. R. (1989) Sequence-specific RNA binding by the HIV-1 Rev protein. *Nature* **342,** 714-6.

Zhang, C. W., Chishti, Y., Hussey, R. E. and Reinherz, E. L. (2001) Expression, purification, and characterization of recombinant HIV gp140. The gp41 ectodomain of HIV or simian immunodeficiency virus is sufficient to maintain the retroviral envelope glycoprotein as a trimer. *J Biol Chem* **276,** 39577-85.

Zhang, L., Huang, Y., He, T., Cao, Y. and Ho, D. D. (1996) HIV-1 subtype and second-receptor use. *Nature* **383,** 768.

Zhang, L. Q., MacKenzie, P., Cleland, A., Holmes, E. C., Brown, A. J. and Simmonds, P. (1993) Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection. *J Virol* **67,** 3345-56.

Zhang, M., Gaschen, B., Blay, W., Foley, B., Haigwood, N., Kuiken, C. and Korber, B. (2004a) Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. *Glycobiology* **14,** 1229-46.

Zhang, M. Y., Xiao, X., Sidorov, I. A., Choudhry, V., Cham, F., Zhang, P. F., Bouma, P., Zwick, M., Choudhary, A., Montefiori, D. C., Broder, C. C., Burton, D. R., Quinnan, G. V., Jr. and Dimitrov, D. S. (2004b) Identification and characterization of a new cross-reactive human immunodeficiency virus type 1-neutralizing human monoclonal antibody. *J Virol* **78,** 9233-42.

Zhang, P. F., Cham, F., Dong, M., Choudhary, A., Bouma, P., Zhang, Z., Shao, Y., Feng, Y. R., Wang, L., Mathy, N., Voss, G., Broder, C. C. and Quinnan, G. V., Jr. (2007) Extensively cross-reactive anti-HIV-1 neutralizing antibodies induced by gp140 immunization. *Proc Natl Acad Sci U S A* **104,** 10193-8.

Zheng, N. N. and Daniels, R. S. (2001) Maintenance of glycoprotein-determined phenotype in an HIV type 1 (pNL43) env gene-cassetting system. *AIDS Res Hum Retroviruses* **17,** 1501-6.

Zhou, T., Xu, L., Dey, B., Hessell, A. J., Van Ryk, D., Xiang, S. H., Yang, X., Zhang, M. Y., Zwick, M. B., Arthos, J., Burton, D. R., Dimitrov, D. S., Sodroski, J., Wyatt, R., Nabel, G. J. and Kwong, P. D. (2007) Structural definition of a conserved neutralization epitope on HIV-1 gp120. *Nature* **445,** 732-7.

Zhu, P., Chertova, E., Bess, J., Jr., Lifson, J. D., Arthur, L. O., Liu, J., Taylor, K. A. and Roux, K. H. (2003) Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. *Proc Natl Acad Sci U S A* **100,** 15812-7.

Zhu, P., Liu, J., Bess, J., Jr., Chertova, E., Lifson, J. D., Grise, H., Ofek, G. A., Taylor, K. A. and Roux, K. H. (2006) Distribution and three-dimensional structure of AIDS virus envelope spikes. *Nature* **441,** 847-52.

Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A. and Ho, D. D. (1993) Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* **261,** 1179-81.

Zolla-Pazner, S. (2004) Identifying epitopes of HIV-1 that induce protective antibodies. *Nat Rev Immunol* **4,** 199-210.

Zwick, M. B. (2005) The membrane-proximal external region of HIV-1 gp41: a vaccine target worth exploring. *Aids* **19,** 1725-37.

Zwick, M. B. and Burton, D. R. (2007) HIV-1 neutralization: mechanisms and relevance to vaccine design. *Curr HIV Res* **5,** 608-24.

Zwick, M. B., Labrijn, A. F., Wang, M., Spenlehauer, C., Saphire, E. O., Binley, J. M., Moore, J. P., Stiegler, G., Katinger, H., Burton, D. R. and Parren, P. W. (2001) Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *J Virol* **75,** 10892-905.