Characterisation of llama antibody fragments able to act as HIV-1 entry inhibitors

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I, Anna Maria Matilda Forsman, 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

Human immunodeficiency virus type 1 (HIV-1) entry into cells is mediated by the functional envelope spike, which consists of trimers of gp120 bound to gp41. Most variants of HIV-1 enter cells through attachment of the envelope spike to the main cellular receptor CD4, allowing interaction with a co-receptor and eventually fusion of viral and cellular membranes. Neutralising antibodies inhibit HIV-1 entry by targeting epitopes on the functional spike. HIV-1 has, however, evolved several ways to evade recognition by antibodies, including variable regions, carbohydrates, and conformational masking. As a result, the neutralising antibody response in HIV-1 infection and post-immunisation is generally narrow, and only a handful of broadly neutralising monoclonal antibodies have been reported. In this thesis, the isolation and characterisation of novel, broadly neutralising antibody fragments derived from llamas is described. Llamas produce antibodies devoid of light chains, which have their antigen-binding properties confined to a single fragment, the VHH, and a preference for cleft-recognition. VHH were isolated from llamas immunised with recombinant gp120 using phage display-based methods. In order increase the chances of isolating neutralising VHH, a functional selection strategy was employed, involving a competitive elution with soluble CD4. Three VHH able to neutralise HIV-1 primary isolates of subtype B and C were characterised. These VHH bound to gp120 with high affinities and competed with soluble CD4 and antibodies to the CD4-binding site for this binding, indicating that their mechanism of neutralisation involves interacting with the functional envelope spike prior to binding to CD4. These results indicate that llama VHH can be potent HIV-1 entry inhibitors. Since VHH are stable and can be produced at a relatively low cost, they may be considered for HIV-1 microbicide development. Anti-gp120 VHH might also prove useful in defining neutralising and non-neutralising epitopes on HIV-1 envelope proteins, with implications for HIV-1 vaccine design.

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Abbreviations

ABCE1	ATP-binding cassette, sub-family E, member 1
ADCC	antibody-dependent cellular toxicity
ADCVI	antibody-dependent cell-mediated virus inactivation
AIDS	acquired immunodeficiency syndrome
Alix	apoptosis-linked gene 2 interacting protein X
ALV	avian leukosis virus
AP	adaptor protein
APOBEC	apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like
AZT	azidothymidine
BiP	Ig heavy-chain binding protein
BLV	bovine leukaemia virus
bp	base pairs
BSA	bovine serum albumin
C	constant region of gp120
CA	capsid protein, p24
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
ccPBMC	co-cultured PBMC
CCR5	CC-chemokine receptor 5
CD	cluster of differentiation
CD4bs	CD4-binding site
CDK9	cyclin-dependent kinase 9
cDNA	complementary DNA
CDR	complementarity determining region
CH	constant domain of an Ig heavy chain
C-HR	C-terminal heptad repeat
CL	constant domain of an Ig light chain
CNS	central nervous system
cPPT	central polypurine tract
CREB	cAMP response element binding

CRF	circulating recombinant form
CRM1	chromosomal region maintenance 1
C-terminal	carboxy-terminal
CTL	cytotoxic T lymphocyte
CTLA-4	CTL antigen 4
CXCR4	CXC-chemokine receptor 4
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule 3-grabbing non-integrin
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EGF	epidermal growth factor
EGFR	EGF receptor
ELISA	enzyme-linked immunosorbent assay
Env	envelope
ER	endoplasmatic reticulum
ESCRT	endosomal sorting complexes required for transport
Fab	fragment antigen-binding
Fc	fragment crystallisable
FcR	Fc receptor
FeLV	feline leukaemia virus
FFU	focus-forming units
FR	framework region
Fv	fragment variable
Fv1	Friend virus susceptibility factor 1
Gag	group-specific antigen
GALT	gut-associated lymphoid tissue
GALV	gibbon ape leukaemia virus
GFP	green fluorescent protein
gp120	glycoprotein, 120 kDa
gp160	glycoprotein, 160 kDa
gp41	glycoprotein, 41 kDa
GPCR	G-protein-coupled receptor
GRP78	78-kDa glucose-regulated protein

HAART	highly active antiretroviral therapy
HEWL	hen egg white lysosyme
hGCN5	human general control of amino acid synthesis protein 5
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HLA	human leukocyte antigen
hNAP-1	human nucleosome assembly protein 1
HPV	human papilloma virus
hRIP	human Rev-interacting protein
HSV	herpes simplex virus
HTLV-1	human leukaemia virus type 1
HTLV-2	human leukaemia virus type 2
IC_{50}	50% inhibitory concentration
IC_{90}	90% inhibitory concentration
ICAM-1	intracellular adhesion molecule 1
IFN	interferon
Ig	immunoglobulin
IN	integrase
JSRV	jaagsiekte sheep retrovirus
k_a	association rate constant
k_d	dissociation rate constant
K_D	equilibrium dissociation constant
LEDGF	lens epithelium-derived growth factor
LFA-1	lymphocyte function-associated molecule 1
LTNP	long-term non-progressor
LTR	long terminal repeat
M	major
MA	matrix protein, p17
mAb	monoclonal antibody
MC	molecular clone
MHC	major histocompatibility complex
MIP	macrophage inflammatory proteins
MLV	murine leukaemia virus
MMLV	Moloney MLV

MMTV	mouse mammary tumour virus
MPER	membrane-proximal external region
MPMV	Mason-Pfizer monkey virus
mRNA	messenger RNA
N	non-major, non-outlier
NAR	novel antigen receptor
NC	nucleocapsid protein, p7
Nef	negative factor
NFκB	nuclear factor κB
N-HR	N-terminal heptad repeat
NMR	nuclear magnetic resonance
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside/nucleotide analogue reverse transcriptase inhibitor
NSI	non-syncytium-inducing
N-terminal	amino-terminal
O	outlier
PBMC	peripheral blood mononuclear cells
PBS	primer binding site
P/CAF	p300/CBP-associating factor
PCR	polymerase chain reaction
PDI	protein disulphide isomerise
PFU	plaque-forming units
PHA	phytohaemagglutinin
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PIC	pre-integration complex
Pol	polymerase
PR	protease
$Pr160^{Gag\text{-}Pol}$	Gag-Pol precursor polyprotein, 160 kDa
$Pr55^{Gag}$	Gag precursor polyprotein, 55 kDa
P-TEFb	positive transcription elongation factor b
PV	envelope pseudotyped virus
R	repeat
RANTES	regulated upon activation, normal T cell expressed and secreted
Rev	regulator of virion

RLU	relative light units
RNA	ribonucleic acid
RNase H	ribonuclease H
RSS	recombination signal sequence
RSV	Rous sarcoma virus
RT	reverse transcriptase
RTC	reverse transcription complex
RU	response units
sCD4	soluble CD4
scFv	single-chain Fv
SCID	severe combined immunodeficient
SI	syncytium-inducing
siRNA	small interfering RNA
SIRT1	silent mating type information regulation 2 homologue 1
SIV	simian immunodeficiency virus
SIV_{cpz}	SIV from chimpanzee (*Pan troglodytes*)
SIV_{cpzPtt}	SIV from the *Pan t. troglodytes* subspecies of chimpanzees
SIV_{gor}	SIV from gorilla
SIV_{sm}	SIV from sooty mangabey
SL	stem loop
SP	spacer peptide
SRP	signal recognition particle
SU	surface glycoprotein, gp120
TAR	transactivation response region
Tat	transactivator of transcription
TCID50	50% tissue culture infectious dose
TCLA	T cell line-adapted
TIP47	tail-interacting protein of 47 kDa
TM	transmembrane glycoprotein, gp41
TNFα	tumour necrosis factor α
TRIM5α	tripartite motif protein 5α
tRNA	transfer RNA
Tsg101	tumour susceptibility gene 101

U3	unique 3′
U5	unique 5′
ucPBMC	uncultured PBMC
V	variable region of gp120
VH	variable domain of an Ig heavy chain
VHH	VH of a camelid heavy-chain antibody
Vif	viral infectivity factor
VL	variable domain of an Ig light chain
VLP	virus-like particle
Vpr	viral protein R
Vpu	viral protein U
VSV	vesicular stomatitis virus
WDSV	Walleye dermal sarcoma virus

Chapter 1

Introduction

In this thesis, the isolation and characterisation of llama antibody fragments able to inhibit human immunodeficiency virus type 1 (HIV-1) infection will be described. This chapter will provide a scientific background to the data presented. The first part of the chapter will focus on the research fields of HIV-1 and acquired immunodeficiency syndrome (AIDS) and the second part on the humoral immune system of camelids.

1.1 HIV-1 and AIDS

In the first part of this chapter, an introduction to the research fields of HIV-1 and AIDS will be given, with a particular focus on HIV-1 envelope proteins, neutralising antibodies to HIV-1 envelope, and HIV-1 vaccine and microbicide development.

1.1.1 A historical introduction to HIV-1 and AIDS

In June 1981, the Centres for Disease Control and Prevention (CDC) in the USA published a report in the Morbidity and Mortality Weekly Report of *Pneumocystis carinii* pneumonia, a disease usually only seen in immunosuppressed individuals, in five previously healthy, young, homosexual men in Los Angeles (1). This report would later be referred to as the beginning of AIDS. The CDC report was followed by two publications in the New England Journal of Medicine a few months later, where the *Pneumocystis carinii* pneumonia was recognised as being associated with a depletion of T helper cells (2, 3). The same year, there were reports of an aggressive form of the usually relatively benign cancer Kaposi's sarcoma in young homosexual men in New York (4, 5). These cases were also associated with a loss of T helper cells (6). A number of reports on the newly described immune deficiency were to follow, including reports of cases outside of the USA (7-9). At the end of 1982, the

condition was officially named acquired immunodeficiency syndrome, or AIDS (10). Although it was initially associated with the homosexual community, it was soon detected in intravenous drug users, individuals from Haiti that had recently entered the USA, and individuals who had received blood transfusions (3, 11, 12). These observations, together with the observation of a possible mother-to-child transmission, and disease among a group of homosexual men in California, led to the proposal that the immunodeficiency syndrome was caused by an infectious agent (13-16).

In 1980, Poiesz *et al*. (17), in the group of Robert Gallo, had isolated the first infectious human retrovirus, human leukaemia virus type 1 (HTLV-1). HTLV-1 had been shown to have a tropism for T cells and was assumed to be transmitted along the same routes as what had been suggested for the AIDS agent. In addition, retroviruses, for example the feline leukaemia virus (FeLV), had previously been associated with immune deficiencies (18, 19). Because of these notions, and since AIDS had been associated with a loss of T helper cells, it was suggested that AIDS might be caused by a retrovirus and more specifically by a member of the HTLV family (20). In 1983, HTLV-1 was also reported to be present in some individuals with AIDS and suggested as a possible aetiological agent of AIDS (21-23). However, the frequency by which HTLV-1 was detected in patients with AIDS was low. In addition, antibodies to HTLV were not often detected in individuals with AIDS symptoms (24, 25). In conclusion, no evidence for an aetiological role was presented, and it would later become apparent that the reported cases were merely co-infections.

Meanwhile, at the end of 1982, the group of Luc Montagnier at the Pasteur Institute in France had set out to discover whether a retrovirus might be the cause of AIDS (26). Their results were published in May 1983, when Barré-Sinoussi *et al*. (27) described the isolation of a novel retrovirus from a lymph node of a patient with AIDS-like illness. Reverse transcriptase activity was detected in the culture supernatant after 15 days of culturing the lymph node lymphocytes, indicating the presence of a retrovirus. Electron microscopy as well as sucrose gradient experiments further supported this notion. The virus could be transmitted to lymphocytes from healthy adult donors as well as to lymphocytes

from umbilical cord blood in two subsequent propagation steps. Antisera to HTLV-1 did not react with cells infected with the novel virus, indicating that the new virus was distinct from HTLV. Serum from the patient did, however, show reactivity with HTLV-1-infected cells. 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, that is was related to but distinct from HTLV, and finally that its role in the aetiology of AIDS remained unclear. The virus was subsequently called lymphadenopathy virus, or LAV.

In September 1983, at the Cold Spring Harbor Meeting in New York, a few months after the first publication on LAV, Luc Montagnier presented data on further LAV-like isolates from other individuals with AIDS, including additional electron microscopy data on the virus morphology. These data were later published, along with data on a selective tropism for T helper cells and further evidence for an aetiological role in AIDS (28-30, reviewed in 31).

One year after Barré-Sinoussi and colleagues published their first set of data on LAV (27), the group of Robert Gallo, in May 1984, published four reports describing the isolation of a novel retrovirus from individuals with AIDS, the establishment of a cell line continuously producing the virus, and extensive evidence for a role in the aetiology of AIDS (32-35). The virus was called human T lymphotropic virus type 3, or HTLV-3. In August 1984, Jay Levy *et al*. (36) reported an additional isolation of retroviruses from patients with AIDS. This virus was called AIDS-associated retrovirus, or ARV. Molecular cloning (37-39) and sequencing (40-43) of the LAV, HTLV-3 and ARV genomes soon made it clear that these viruses belonged to the same species (44, 45). Moreover, the reported nucleotide sequences along with additional genetic and morphological studies (45-48), suggested the AIDS virus to be more related to the *Lentivirus* genus of the *Retroviridae* family than to the *Deltavirus* genus to which HTLV belongs. In 1986, the virus was named human immunodeficiency virus, HIV (49). A series of important findings were to follow. Antibody and antigen tests had already been made available (50, 51), the main cellular receptor was

discovered (52, 53) and the first anti-HIV drug was developed and proven efficient in clinical trials (54). Moreover, by the mid-1980s the severity of the epidemic had started to show, as it had become apparent that HIV and AIDS were widespread in several regions of Africa, including Zambia, Zaire, Uganda and Rwanda (55-61). Evidence of seropositivity in individuals in central Africa was even found from as far back as 1959 (62). In the south-western parts of Uganda, a disease referred to as slim disease, which would later be identified as AIDS, had been observed with increasing frequency since 1982 (61).

Another novel lentivirus, related to but distinct from the HIV strains already described, was isolated from individuals with AIDS in 1986 by Clavel *et al.* (63). Serological evidence for a novel lentivirus distinct from previous HIV isolates had, however, already been presented the year before by Barin *et al*. (64). This virus was eventually termed HIV-2, while the HIV isolated by Barré-Sinoussi and colleagues was called HIV-1. HIV-2 would later turn out to cause a more confined epidemic in the western parts of Africa, whereas HIV-1 is the cause of the worldwide AIDS pandemic (65, 66).

Now, almost exactly 25 years after the identification of HIV as the cause of AIDS, there are estimated to be 33.2 million people infected worldwide (65). Despite the acquisition of a vast amount of knowledge of the biology of HIV, and considerable advances in addressing the pandemic, there is still no cure or vaccine and the pandemic continues to have a devastating impact on society. Even though antiretroviral therapy has proven effective in slowing disease progression, these drugs are expensive and not readily available to the majority of HIV infected individuals. Hence, there is a need for effective preventive methods to control the HIV pandemic, such as an HIV vaccine or alternative preventive strategies such as a topically applied HIV microbicide. All attempts to design a vaccine have, however, so far failed. The latest major drawback came at the end of 2007, when the second vaccine candidate to be tested in efficacy studies failed to slow down or protect against infection (67).

1.1.2 Epidemiology of HIV-1

The total number of people living with HIV continues to increase. Among the estimated 33.2 million people living with HIV worldwide, 2.1 million are children under 15 years (65). During 2007, there was an estimated 2.1 million deaths due to AIDS, and an estimated 2.5 million new HIV infections, the majority of these acquired through heterosexual transmission (65). The global HIV incidence is, however, estimated to be on the decline, having peaked at the end of the 1990s. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS) 2007 AIDS epidemic update (65), there are currently two main trends in pandemic, that of a widespread epidemic in the general population in regions of sub-Saharan Africa, as well as the epidemics in the rest of the world, which are concentrated to high-risk populations, such as sex workers, men who have sex with men, and intravenous drug users and their partners. 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 (65, 66, 68).

Sub-Saharan Africa continues to be the worst affected region (Fig. 1.1.2.1), with an estimated 68% of the total number of infections and almost 90% of the infected children. In contrast to in the rest of the world, the majority (61%) of the infected people are women. In this region, AIDS remains the major cause of death, with 76% of the global annual AIDS deaths (65). The estimated number of annual new infections has, however, decreased between 2001 and 2007. In the western, central and eastern parts of sub-Saharan Africa, the adult HIV prevalence seems to have stabilised or declined. In most countries in southern Africa, however, the adult prevalence has just reached, or is still approaching, a plateau (65). In Mozambique, the prevalence is even increasing. In eight sub-Saharan countries (Botswana, Lesotho, Mozambique, Namibia, South Africa, Swaziland, Zambia and Zimbabwe) the adult prevalence is more than 15%. Adult prevalence is highest in Botswana, Lesotho and Swaziland, where recent estimates suggest that around one in four people is infected (65). South Africa remains the country with the largest number of HIV infections in the world.

In Southeast Asia, the HIV prevalence in Vietnam and Indonesia continues to increase, while it is on the decline in Thailand, Burma and Cambodia. In China, the epidemic is continuing to grow, with slightly less than half of all new infections thought to be acquired during intravenous drug use. In India, the country with the second largest number of HIV infections, an extended HIV surveillance system has estimated the proportion of people living with HIV to be lower than what was previously thought. In Eastern Europe and Central Asia, the epidemics continue to grow, with a 150% increase in the total number of infected people between 2001 and 2007. More than 90% of the new infections in this region occurred in Russia and Ukraine (65). Almost two thirds of the estimated new infections in 2006 were thought to be acquired during intravenous drug use.

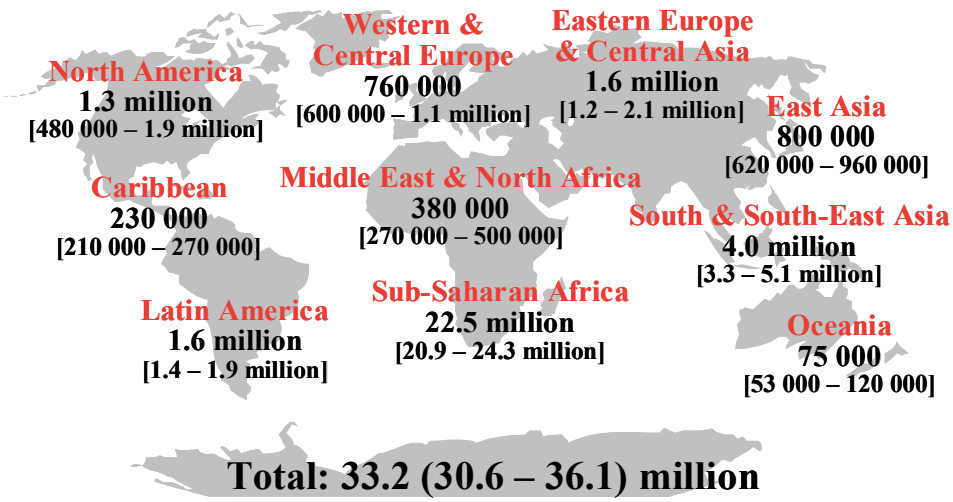


Figure 1.1.2.1. Number of people estimated to be living with HIV in 2007. Figure obtained from the '2007 AIDS epidemic update', Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organisation (WHO), http://www.unaids.org/.

In Latin America, the HIV epidemic remains stable, with the majority of new infections occurring in high-risk populations, including sex workers and their partners, injecting drug users and men who have sex with men (65). In the Caribbean, the adult prevalence is around 1%, with Haiti and the Dominican Republic being the worst affected (65). Transmission occurs primarily through the heterosexual route. The annual incidence has stabilised but HIV remains one of the leading causes of death among people aged 25-44. In North America,

Western and Central Europe, the total number of people infected with HIV continues to increase. In North America, around half of all new infections are estimated to occur among men who have sex with men, whereas in Western Europe, the majority of new infections are thought to be acquired through heterosexual transmission (65).

1.1.3 Biology of HIV-1

The virus that Barré-Sinoussi *et al*. (27) isolated from an individual with AIDS symptoms, and which was subsequently named HIV-1, belongs to the *Lentivirus* genus of the *Retroviridae* family. In this section, a general introduction to retroviruses will be given, as well as an overview of the origin and virology of HIV-1.

1.1.3.1 Retroviruses

The *Retroviridae* is a large and diverse family of viruses able to infect a wide range of species. It has been associated with a number of diseases such as cancers and immunological disorders. Its 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. A typical retroviral provirus is 6-11 kb and contains three main open reading frames, the *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) genes (69). Retroviruses containing these three genes only are referred to as simple retroviruses, whereas retroviruses with several additional, accessory or regulatory genes are referred to as complex.

The first retroviruses to be described were the avian leukosis virus (ALV) and the Rous sarcoma virus (RSV), which were isolated when Ellermann and Bang, in 1908, and Rous, in 1911, showed that neoplastic diseases in chicken could be transmitted experimentally by cell-free filtrates (70). The transmission of a disease caused by a retrovirus had, however, already been observed a few years earlier, in 1904, when Vallée and Carré showed that equine anaemia was

experimentally transmissible by a filtrate (70). These discoveries were followed by the isolation of other viruses causing tumours in additional hosts such as mice (71-73). The particles of these tumour-causing viruses were eventually shown to contain RNA and the viruses were subsequently called RNA tumour viruses (69, 74). After the formulation of the DNA provirus hypothesis by Temin in 1964 (75, 76), the Nobel prize-awarded discovery of the reverse transcriptase by Baltimore (77) and Temin and Mizutani (78) in 1970, and the transfection of an infectious DNA provirus from RSV-infected cells by Hill and Hillova in 1972 (79), more became known about the biology of these RNA tumour viruses and they were eventually named retroviruses in 1974 (80). Retroviruses have since been detected in a variety of species, including mammals, reptiles and fish (69). The first human-to-human transmissible retrovirus to be described was HTLV-1, which was detected and isolated from a patient with cutaneous T cell lymphoma in 1980 by Poiesz *et al*. (17). The same virus was independently detected in Japan in 1981, using serology and electron microscopy (81, 82) and subsequently molecular approaches (83), and reported as adult T cell leukaemia virus (ATLV).

Historically, retroviruses have been divided into three subfamilies, the onco-, lenti- and spumaviruses, according to their biological properties and the type of disease they caused, and further classified into genera according to the morphology of the mature virion, as observed by electron microscopy. Present-day classification is, however, based mainly on the sequence of the *pol* gene. According to current taxonomy, the *Retroviridae* family can be divided into two subfamilies, the *Orthoretrovirinae* and the *Spumaretrovirinae* (84). The *Orthoretrovirinae* is divided into six genera, the *Alpha*-, *Beta*-, *Gamma*-, *Delta*- and *Epsilonretrovirus* genera as well as the *Lentivirus* genus, whereas the *Spumaretrovirinae* consists of the *Spumavirus* genus only (84).

Viruses belonging to the *Alpharetrovirus* genus have simple genomes and have so far only been isolated in birds. ALV is the type species. Viruses belonging to the *Betaretrovirus* genus, on the other hand, have been detected mainly in mammals. They include the jaagsiekte sheep retrovirus (JSRV), which causes ovine pulmonary adenocarcinoma in sheep (85), the mouse mammary tumour virus (MMTV), which causes mammary carcinoma in mice (72) and the Mason-

Pfizer monkey virus (MPMV), which was the first retrovirus to be isolated from primates (86, 87). Viruses of the *Gammaretrovirus* genus have been detected in a variety of vertebrate species and includes the gibbon ape leukaemia virus (GALV) and the type species murine leukaemia virus (MLV), which cause leukaemia in their respective hosts (73, 88). All retroviruses in this genus have simple genomes. Unlike the gammaretroviruses, viruses in the *Deltaretrovirus* genus have complex genomes (69). The type species is the bovine leukaemia virus (BLV). The genus also includes the first transmissible retroviruses to be detected in humans, HTLV-1 and -2 (17, 89). The *Epsilonretrovirus* genus consists of viruses with complex genomes that are primarily found in fish. The type species is the walleye dermal sarcoma virus (WDSV), which was first described in the 1960s (90, 91). The *Lentivirus* genus, to which HIV belongs, consists of retroviruses with complex genomes. They have so far only been isolated from mammals, are known to cause slow disease syndromes, and can induce immunodeficiency in their hosts (69, 92). The term lentivirus was coined by Björn Sigurdsson in Iceland in the mid-1950s from the Latin word *lentus*, meaning slow, after his description of the visna and maedi diseases in sheep and the isolation of the virus that causes them, the visna/maedi lentivirus (93, 94). The visna/maedi virus causes paralysis (visna) or pneumonia (maedi) in sheep and goats and gave rise to epidemics in sheep in Iceland between 1930 and 1950. Its genome was first sequenced in 1985, around the same time as the HIV genome, helping to classify HIV to the *Lentivirus* genus (48). Like the lentiviruses, the spumaviruses have complex genomes. The type species of the *Spumavirus* genus is the simian foamy virus (84). Spumavirus infections are widespread among mammals but have still not been observed to cause disease, despite having cytopathic effects *in vitro* (95).

Retroviruses have been linked to a range of diseases, such as cancers and immunodeficiencies, but have also been associated with persistent infections without any apparent pathogenic effects. Retroviruses that have integrated into the germ-line of their hosts, and which replicate in a Mendelian fashion, were first discovered in the late 1960s (96-101). Such so-called endogenous retroviruses have since been detected in all vertebrate species in which they have been searched for, and endogenous counterparts have been found for all genera

of the *Retroviridae*, with the exception of the *Deltavirus* genus. Endogenous lentiviral sequences were only recently described, in one species of rabbit (102). These lentiviral sequences were determined to be more than seven million years old, providing evidence for an ancient origin of the *Lentivirus* genus. The first human endogenous retrovirus was detected in 1981 (103). Whether expression of endogenous retroviruses have any pathogenic effect in humans remains disputed, although association with autoimmune disease, endometriosis and some cancers have been suggested (104). There are, however, some examples where retroviral genes have become important for physiological processes in their hosts. The envelope protein of a human endogenous retrovirus has, for example, been shown to be essential in the human placental morphogenesis (105). In addition, a murine protein of retroviral origin, Friend virus susceptibility factor 1 (Fv1), has been shown to confer resistance to exogenous retroviruses in mice as well as in other mammals (106, 107). Since the complete sequence of the human genome was made available at the beginning of this century, it has been estimated that endogenous retroviral sequences make up approximately 8% of the human genome (108-113). In light of these notions, it is apparent that retroviruses have played a significant role in the biological history of several species, not only as disease-causing agents and as the cause of global burdens such as the HIV pandemic, but also as contributors to, for example, the plasticity of vertebrate genomes and thus to the overall evolution of the vertebrate species (108, 110, 114-116).

1.1.3.2 Origin and diversity of HIV

Lentiviruses have been detected in more than 30 non-human primate species in sub-Saharan Africa (117, 118). These simian immunodeficiency viruses (SIVs) form host-specific clades and appear to be non-pathogenic in their hosts. HIV is thought to have been brought into the human population by several cross-species transmissions of SIVs. HIV-1 is most closely related to SIV_{cpz}, which is present in the chimpanzee (*Pan troglodytes*) population. In 2006, Keele *et al*. (119) reported data confirming SIV_{cpz} from the *Pan t. troglodytes* subspecies of chimpanzees (SIV_{cpzPtt}) as the progenitor of HIV-1. They showed that HIV-1 of group M (major) and N (non-major, non-outlier) arose from two distinct SIV_{cpzPtt}

strains, circulating in two geographically separated chimpanzee populations present in west central Africa. Other studies have indicated that SIV_{cpz} itself is a result of a recombination event between SIVs from red capped mangabey and greater spot-nosed monkey (120). 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 (121). HIV-2 is thought to have its origin in SIV_{sm}, which naturally infects sooty mangabeys (118). From here onwards, this thesis will focus on HIV-1.

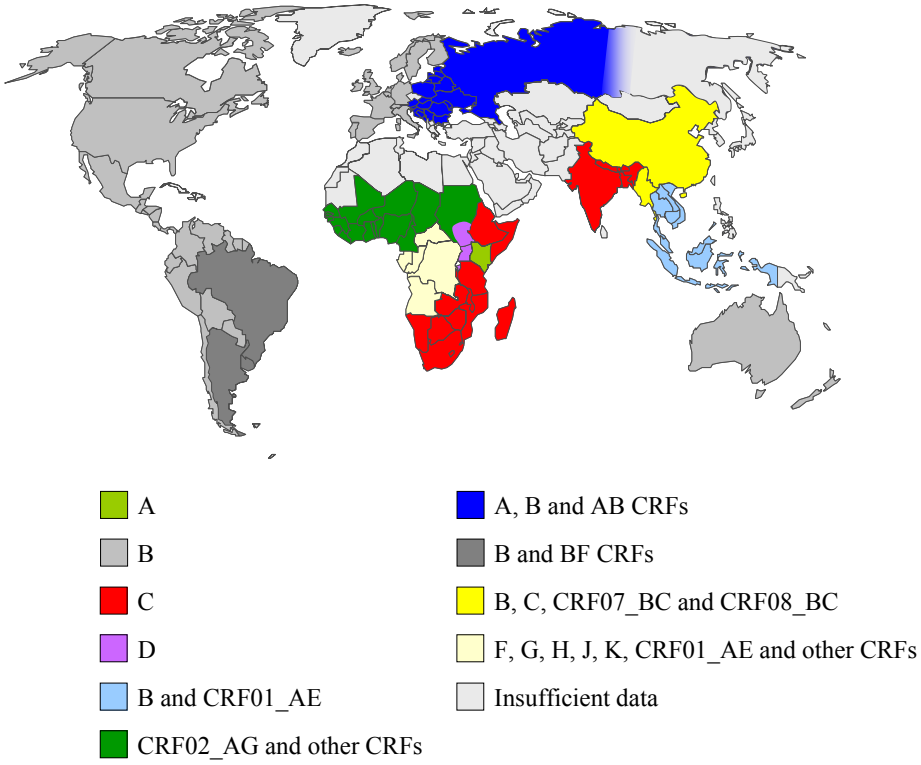


Figure 1.1.3.2.1. Global distribution of HIV-1 group M subtypes and CRFs. Data obtained from Taylor, B. S., M. E. Sobieszczyk, F. E. McCutchan, and S. M. Hammer, 2008, 'The challenge of HIV-1 subtype diversity', N Engl J Med 358:1590-602.

Phylogenetically, HIV-1 can be classified into group M (major), N (non-major, non-outlier) and O (outlier). Group N infections have been detected in only a few individuals in Cameroon, whereas group O is present in Cameroon and a few

surrounding countries (122). Group M, on the other hand, accounts for over 99% of infections and is the most variable, with extraordinary diversity in the envelope sequence between isolates. Group M can be divided into subtypes A-D, F-H, J and K, plus a number of circulating recombinant forms (CRFs; defined as circulating when isolated from at least three individuals without epidemiologic linkage), with subtype C accounting for around 50% of infections worldwide and currently infecting more people than any other subtype (122-124). Genetic variation between subtypes is usually around 25-35% (123). The geographical distribution of HIV-1 group M genetic subtypes and CRFs is shown in Fig. 1.1.3.2.1.

1.1.3.3 The HIV-1 genome and gene products

Like all retroviruses, HIV-1 has a diploid genome composed of two identical positive-sense RNA molecules of approximately 9 kb. The genome was first sequenced in 1985 (40-43) and found to resemble the genomes of other complex retroviruses (40). Like all retroviruses, it contains the *gag*, *pol* and *env* genes (125). The *gag* gene encodes a 55 kDa Gag precursor polyprotein ($Pr55^{Gag}$), which is cleaved by the viral protease into the 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, located between CA and NC, and NC and p6 in $Pr55^{Gag}$, respectively (126). 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 ($Pr160^{Gag\text{-}Pol}$) but cleaved into individual enzymes by the viral protease (125, 127, 128). The *env* gene encodes an Env precursor glycoprotein (gp160), which, like for all retroviruses, is cleaved and processed by cellular enzymes into a surface glycoprotein, SU or gp120, and a transmembrane glycoprotein, TM or gp41 (128-131).

In addition to the *gag*, *pol* and *env* genes, the HIV-1 genome contains genes encoding regulatory and accessory proteins, located downstream of the *pol* gene (125, 127). 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). The *gag*, *vif* and the second exon of *tat* are in the first reading frame, whereas the first exon of *tat*, *vpu* and the second exon of *rev* are in the second reading frame, and *pol*, *vpr* and the first exon of *rev* in the third reading frame (125). A schematic representation of the HIV-1 proviral genome is shown in Fig. 1.1.3.3.1.

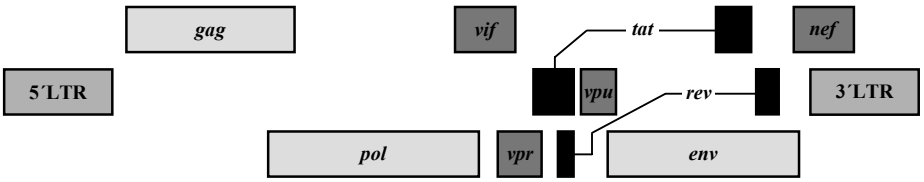


Figure 1.1.3.3.1. Schematic representation of the HIV-1 proviral genome. Open reading frames are represented as rectangles. The *gag*, *pol* and *env* open reading frames encode structural proteins and enzymes, whereas *rev* and *tat* encode gene regulatory proteins and *vif*, *vpr*, *vpu* and *nef* encode accessory proteins. The HIV-1 genome is flanked by two long terminals repeats (LTRs). Details are given in the text. The vertical position of each box in the diagram represents the reading frame of the gene.

The HIV-1 RNA genome is flanked by two untranslated repeat regions (R). Internal of the repeat regions are a unique 5′ (U5) region at the 5′ end and a unique 3′ (U3) region at the 3′ end. During reverse transcription of the RNA genome into double-stranded DNA, longer repeat regions are generated. Thus, the proviral DNA genome is flanked by two identical long terminals repeats (LTRs) containing the U3, R and U5 regions. The HIV-1 LTRs were first analysed in 1985 and found to be 630-640 bp long (40, 132). The U3 region contains the viral promoter and enhancer sequences, whereas the R region contains the polyadenylation signal and the transactivation response element (TAR) that serves as the binding site for the viral Tat protein (133, 134). Differences in the LTR sequence and in the enhancer/promoter configuration have been observed for different HIV-1 subtypes and have been linked to *in vitro* replication rate (135, 136).

1.1.3.4 The mature HIV-1 virion

The structure of HIV-1 virions has been extensively studied using electron microscopy and cryo-electron microscopy techniques. The diameter of mature HIV-1 virions vary but is on average around 145 nm (137). A schematic representation and a cryo-electron micrograph of a mature HIV-1 particle are shown in Fig. 1.1.3.4.1.

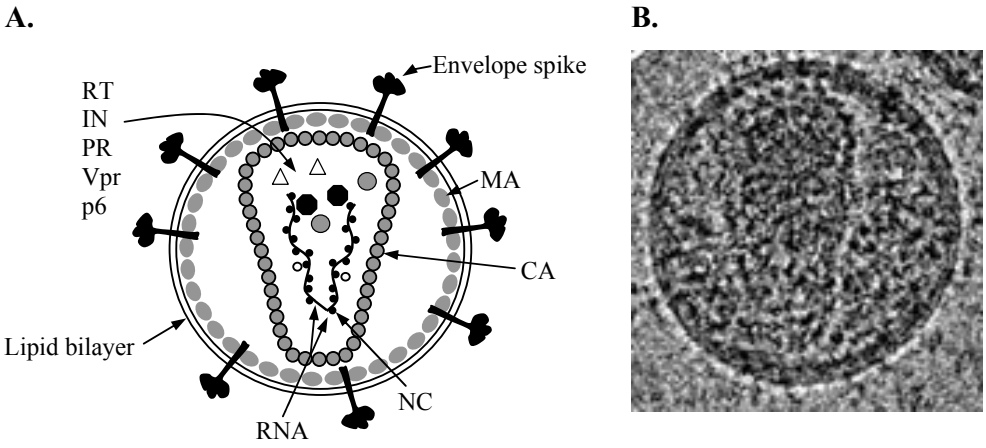


Figure 1.1.3.4.1. Structure of the mature HIV-1 virion. A. Schematic representation of a mature HIV-1 particle. The diploid viral RNA genome is associated with the nucleocapsid protein (NC) and surrounded by a conical core made up of the capsid protein (CA). Within the core are several viral proteins such as the reverse transcriptase (RT), integrase (IN), protease (PR), viral protein R (Vpr) and p6. Surrounding the conical core is a lipid bilayer, which is derived from the cellular plasma membrane. The viral matrix protein (MA) forms a layer beneath the membrane. The lipid bilayer membrane contains viral envelope spikes made up of the surface glycoprotein gp120 non-covalently bound to the transmembrane glycoprotein gp41. **B.** Cryo-electron micrograph of a mature HIV-1 particle obtained from infected T cells. The cryo-electron micrograph is obtained from Briggs, J. A., T. Wilk, R. Welker, H. G. Kräusslich, and S. D. Fuller, 2003, 'Structural organization of authentic, mature HIV-1 virions and cores', Embo J 22:1707-15. Reprinted by permission from Macmillan Publishers Ltd.

The exterior of an HIV-1 particle is made up of an outer, host cell-derived lipid bilayer, the envelope. Embedded in the envelope bilayer are virus-encoded envelope spikes, made up of the surface glycoprotein gp120 non-covalently linked to the transmembrane glycoprotein gp41, as well as host-cell specific proteins. The functional viral envelope spike exists as a trimer of gp120/gp41 on

the virus surface (138-140), although there is evidence for the presence of non-functional envelope spikes such as gp41 stumps and gp120/gp41 monomers (141, 142). Viral envelope spikes will be further discussed in section 1.1.6.

Beneath and associated with the envelope bilayer is a layer of trimeric MA proteins, which interact with the lipid bilayer via amino (N)-terminal myristoyl groups (143). The MA shell surrounds the characteristic cone-shaped core, consisting of hexameric CA proteins forming a hexagonal lattice (137, 144, 145). The conical core, in turn, surrounds the viral nucleocapsid, or ribonucleoprotein complex, which is made up of the viral genomic RNA closely associated with the NC protein. The NC protein is a small, 55 amino acid residue-protein, which contains zinc-finger motifs common to many proteins that bind nucleic acids (127). 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 (146-149). Also contained in the viral core are PR, RT and IN, as well as Vpr.

1.1.3.5 The HIV-1 life-cycle

The HIV-1 life-cycle is initiated by interaction of the viral envelope spike with the main cellular receptor, the cluster of differentiation 4 (CD4) antigen, as well as a co-receptor, typically the CC-chemokine receptor 5 (CCR5) or the CXC-chemokine receptor 4 (CXCR4). Interaction with the receptors eventually leads to the fusion of the viral and cellular membranes, allowing for entry of the conical viral core. HIV-1 entry into host cells will be further discussed in section 1.1.6. A schematic overview of the HIV-1 life-cycle is shown in Fig. 1.1.3.5.1 and described below.

Entry into the cytoplasm is followed by reverse transcription of the viral genomic RNA into DNA, as well as partial disassembly and rearrangement of the viral core in a process called uncoating, leading to the formation of a reverse transcription complex, RTC (150). The viral uncoating process, the co-ordination and initiation of the reverse transcription, and the mechanisms behind RTC

formation and are not well understood and little is known about the viral and cellular factors involved (150, 151).

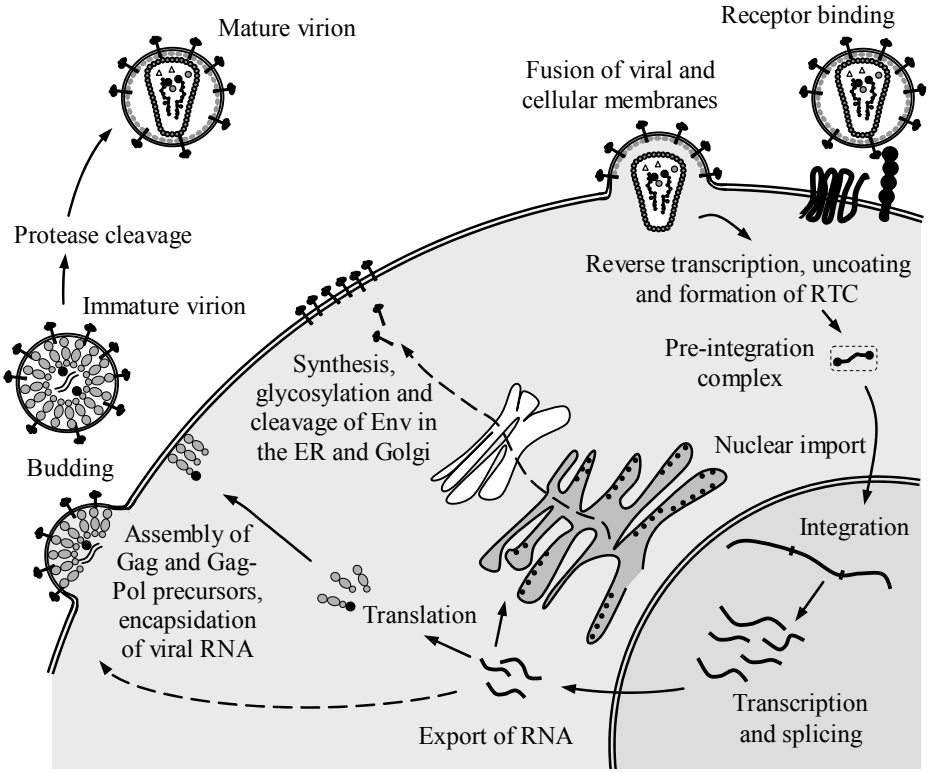


Figure 1.1.3.5.1. Schematic representation of the HIV-1 life-cycle. The HIV-1 life-cycle is similar to that of other retroviruses and consists, in brief, of virus entry, uncoating, reverse transcription of the viral RNA into DNA, nuclear import of a pre-integration complex, integration of viral DNA into the cellular genomic DNA, transcription and splicing of RNA transcripts, nuclear export of mRNAs, translation, multimerisation and assembly of Gag and Gag-Pol precursors at the cellular plasma membrane, encapsidation of viral RNA, synthesis, glycosylation, folding, oligomerisation and cleavage of the gp160 precursor in the ER and Golgi, incorporation of viral envelope glycoproteins into virions, budding and release of immature virions followed by maturation into infectious mature HIV-1 particles. Each step is described in more detail in the text.

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 (151). Studies using CA mutants have indicated that the amount of CA in the viral core is important for

core stability and proper uncoating in host cells. A deviation in the amount of CA led to changes in core stability and in impaired HIV-1 infectivity (151). In addition to CA, MA and NC may also affect uncoating (151). Alongside viral factors, cellular factors may also be required. One study has suggested the involvement of cell cycle-dependent host factors in the uncoating process (152). Cellular proteins, such as cyclophilin A, which binds to CA and is incorporated into virions (153-155), may also affect uncoating (151). In addition, the cellular tripartite motif protein TRIM5α restricts retroviral infection in a species-specific manner by blocking an early, post-entry event in the retroviral replication (156). This block to infection is mediated by TRIM5α associating with CA and promoting its degradation, leading to accelerated core disassembly and uncoating (157).

The uncoating process leads to the formation of an RTC. Sedimentation velocity and electron and confocal microscopy studies have indicated that the RTCs are large nucleoprotein structures consisting of packed filaments containing IN and Vpr, where the viral genome is covered with proteins that are not NC (158, 159). CA has, however, been shown to be absent from functional RTCs, although trace amounts have also been detected, indicating that most of the CA proteins disassociate soon after entry of the viral core (158, 160). The extent to which cellular factors are part of or important for the formation of RTCs remains unclear, but studies have shown that RTCs interact with the cytoskeleton (160, 161). This interaction has been suggested to occur via association of MA with actin (161). In addition, NC has been shown to interact with actin (162, 163), as has the p66 subunit of RT (164). Other viral proteins such as IN, Rev, Vpr and Vif have also been suggested to have an effect on the cytoskeleton, although the role of these effects and interactions in the viral replication remain unresolved (150).

Reverse transcription of the viral genomic RNA into DNA is thought to take place essentially within the RTC. The active form of the viral RT is a heterodimer consisting of the p66 and p51 subunits. The p51 subunit contains the domain responsible for the DNA polymerase activity, whereas the p66 subunit

contains a domain with RNase H activity in addition to the DNA polymerase domain (165). Reverse transcription is initiated using a cellular $tRNA^{Lys-3}$, which is packaged into virions and which anneals to the primer binding site (PBS) located downstream of the U5 region at the 5′-end of the viral RNA genome (166). DNA is synthesised from the 3′-end of the $tRNA^{Lys-3}$ towards the 5′-end of the RNA genome. The 5′-end of the viral genome, the R and U5 regions, are then removed by the RNase H domain of RT. The newly synthesised DNA segment does then transfer and anneal to the R region at the 3'-end of the RNA genome, by means of sequence homology, in a process called first strand transfer. This DNA segment is then extended and most of the genomic RNA strand is degraded by the RNase H domain of RT. A second strand of DNA is subsequently synthesised using the remaining parts of the genomic RNA as primers. After removal of the $tRNA^{Lys-3}$ and the remaining genomic RNA by the RNase H domain, a second strand transfer occurs, when the PBS region of the second strand hybridises with the PBS region of the first strand. Both DNA strands are then extended, forming a double-stranded DNA genome flanked by two identical LTRs.

The RT polymerase lacks the proof-reading ability seen in cellular DNA polymerases. Because of this characteristic, the RT is highly error-prone, making approximately 3.4×10^{-5} errors per base pair per cycle (167). This error rate contributes to the extreme sequence variation observed among HIV-1 isolates. 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 (168). Vpr has been suggested to have an effect on the accuracy of the reverse transcription, thus modulating the *in vivo* mutation rate of HIV-1 (169).

While the biochemical basis for reverse transcription is well described, the mechanism that triggers it is poorly understood. Again, little is known about the importance of cellular factors for the formation of the reverse transcriptase complex and the reverse transcription process. Recent studies have indicated that

cellular factors may be important for efficient reverse transcription (170, 171). Importantly, cellular factors can modulate reverse transcription in a way that inhibits viral replication. In 2002, Sheehy *et al*. (172) identified a novel mechanism by which a member of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) protein family of cellular cytidine deaminases, APOBEC3G, interferes with HIV-1 reverse transcription, hence restricting viral replication. APOBEC3G restricts retroviral replication by deaminating cytosine residues in the first retroviral DNA strand that is synthesised during reverse transcription. Deamination of cytosine leads to the formation of uracil, resulting in guanine-to-adenine hypermutation of the viral DNA and impaired viral replication. APOBEC proteins are present throughout the vertebrate lineage, highlighting the conservation of this innate restriction mechanism (173). In HIV-1, this restriction mechanism is overcome by the viral Vif protein. Vif mediates polyubiquitylation and proteasomal degradation of APOBEC3G, thus preventing APOBEC3G incorporation into virions and its modulation of the reverse transcription process (174-176).

Once the reverse transcription is complete, the RTCs are gradually transformed into integration-competent pre-integration complexes (PICs). The mechanism behind this transformation is unknown. As for RTCs, the definite composition of PICs remains to be determined, but includes the viral genomic DNA and IN (177, 178). PR, RT and Vpr are also thought to be included, whereas the presence of CA, MA and NC has been debated (150).

HIV-1 replication requires that the virus enters the nucleus. The viral genome, both in the context of the RTC and the PIC, is thought to continuously migrate towards the nucleus, utilising the cytoskeleton. Once at the nuclear membrane, the integration-competent PIC has to be imported into the nucleus. Most retroviruses, such as the gammaretrovirus MLV, rely on cell proliferation for their replication, as they are thought to require the partial breakdown of the nuclear membrane that occurs during mitosis for their genomes to enter into the nucleus (179). Unlike these retroviruses, HIV-1 and other members of the *Lentivirus* genus can infect terminally differentiated, non-dividing cells, such as macrophages (180), or cells arrested in the G_1 or G_2 phase of the cell cycle

through treatment with DNA synthesis inhibitors or irradiation (181, 182), indicating that their genomes are actively imported into the nucleus. The precise mechanism behind this nuclear import is unknown. It is thought to be mediated by engagement of the viral PIC with the cellular nuclear import machinery, although the precise cellular and viral elements that participate in this process remain to be identified (183). It is, however, thought that viral Vpr enhances nuclear import of the PIC (169, 184). Vpr contains nuclear localisation signals and has been shown to localise to the nucleus both in infected cells and when expressed in cells on its own (185). In addition to Vpr, it has been suggested that MA contains nuclear localisation signals, although this notion remains debated (183). Viral IN and Rev as well as the central polypurine tract (cPPT) present in the *pol* gene have also been proposed to take part in the nuclear localisation process, along with cellular factors such as importins (nuclear import receptors) and tRNAs (183, 186).

In addition to promoting nuclear import, Vpr has been shown to cause G_2 cell cycle arrest in infected cells (169). The relevance of this function is not known, but it is thought that the HIV-1 LTRs are more active in the G_2 phase. This arrest does not require *de novo* synthesis of Vpr but only Vpr from the infecting virus particles, suggesting that the arrest occurs prior to integration.

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 (187). Integration is mediated by the viral IN, which has three distinct enzymatic functions (188). In the initial phase of the integration process, an exonuclease activity of IN hydrolyses a dinucleotide from the 3´-end of both strands of the viral DNA (189). This reaction is known as 3´-end processing and takes place in the cytoplasm. Following the 3´-end processing and nuclear import of the PIC, a double-stranded endonuclease activity of IN cuts the cellular DNA in a staggered fashion and a ligase activity connects the generated 5´-overhangs to the recessed 3´-ends of the viral DNA in a reaction known as strand transfer (190). Cellular repair enzymes then fill in any gaps between viral and cellular DNA.

HIV-1 has been shown to specifically integrate into transcriptionally active regions of the host genome (191). Recent three-dimensional confocal microscopy studies has shown that HIV-1 PICs preferentially target regions of lightly packed euchromatin in the nuclear periphery as opposed to the dense heterochromatin (192). Even though the integration process has been extensively studied *in vitro* using recombinant IN, the *in vivo* process, including PIC and IN targeting to chromatin, is not fully understood, nor is the extent to which cellular components and viral factors other than IN play a role. In addition to viral IN, Gag proteins have been shown to play a role in the selection of target sites (193).

Cherepanov *et al*. (194) have shown that IN isolated from the nucleus of IN-expressing 293T cells exists as homotetramers associated with the cellular lens epithelium-derived growth factor (LEDGF/p75), a transcriptional co-activator and a ubiquitous nuclear protein that is tightly associated with chromatin throughout the cell-cycle. In the same study, the catalytic activity of IN was shown to be enhanced by LEDGF/p75. Subsequent studies have found LEDGF/p75 to be important for the targeting of IN to chromatin and for directing viral integration into transcription units, suggesting that chromatin-associated LEDGF/p75 may function as a receptor for HIV-1 PICs (195-198). However, while LEDGF/p75 is required for efficient viral integration and replication, it is not essential, as some level of integration takes place in cells where LEDGF/p75 expression has been knocked out or silenced (199, 200). The frequency and distribution of integration was, however, significantly altered (201).

Once integrated into the host genome, the provirus can stay latent or be transcribed by the cellular machinery. The HIV-1 promoter is located in the 5´LTR and contains a number of regulatory elements essential for transcription (202). Proviral transcription is initiated from the U3/R junction and mediated by the cellular RNA polymerase II. Upstream of the transcription start site is a TATA box to which the cellular transcription factor IID binds. In addition to the TATA box, the U3 region also contains three binding sites for transcription factor Sp1 and two binding sites for transcription factor nuclear factor κB

(NFκB). Upstream of these sites are binding sites for additional cellular transcription factors (202).

Initially, the early viral transcripts, encoding for Tat, Rev and Nef, are produced. Prior to the production of the viral transactivator Tat, the LTR is activated by cellular transcription factors alone, resulting in low transcriptional levels and short transcripts. Once Tat is present, it strongly enhances transcriptional activation and elongation in a positive feedback loop (203). Tat binds to an RNA stem-loop structure in the nascent viral RNA, the transactivation response region, TAR (133, 134). This interaction leads to the subsequent recruitment of the cellular co-factor positive transcription elongation factor b (P-TEFb) complex to the HIV-1 promoter. The P-TEFb complex phosphorylates the carboxy (C)-terminal domain of RNA polymerase II, leading to increased polymerase processivity and enhanced elongation. Tat recruits the P-TEFb complex to the viral promoter by interacting with the cellular protein cyclin T1, which forms a heterodimer with the cyclin-dependent kinase CDK9 and makes up part of the P-TEFb complex (204).

In addition to TAR-dependent transcriptional activation, Tat has been shown to activate transcription in a TAR-independent manner (205). A direct interaction between Tat and the NFκB enhancer sequence could explain the TAR-independent transactivation (206). Tat is also thought to recruit cellular acetylating enzymes able to acetylate histones, such as the histone acetyltransferase cyclic adenosine monophosphate (cAMP) response element binding (CREB)-binding protein p300/CBP, the p300/CBP-associating factor P/CAF, and the histone acetyltransferase human general control of amino acid synthesis protein 5 (hGCN5), leading to modification of the chromatin structure and improved access for polymerases (207-210). Moreover, cycles of acetylation and deacetylation of Tat itself by acetylating and deacetylating cellular enzymes, like p300/CBP and the class III histone deacetylase SIRT 1 (silent mating type information regulation 2 homologue 1), have been shown to regulate the disassociation and association of Tat from the TAR element, thus having an important role in regulating HIV-1 transcription (211, 212). In addition to recruiting acetylating enzymes to modify chromatin structure and improve

polymerase access to proviral DNA, a recent report from Vardabasso *et al*. (213) has suggested that Tat interacts with the human nucleosome assembly protein 1 (hNAP-1), a histone chaperone that promotes chromatin fluidity and thus gene transcription.

In addition to regulating transcription, Tat is thought to have additional effects, which may influence HIV-1 pathogenesis (214). Extracellular Tat secreted from HIV-1-infected cells is believed to affect uninfected bystander cells in various ways, such as being able to induce apoptosis or to have immunomodulatory abilities, including being able to up- or down-regulate the expression of immunoregulatory cytokines and activate uninfected T cells in an antigen-independent way (214). Because of its immunomodulatory properties, Tat has been suggested to contribute to T cell hyperactivation, a mechanism thought to be important in HIV-1 pathogenesis. Kwon *et al*. (215) have proposed a model where Tat contributes to T cell hyperactivation by inhibiting the normal function of the SIRT1 deacetylase as a negative regulator of T cell activation. On a final note regarding Tat, it should be remembered that, as with HIV-1 research in general, most Tat-related research is carried out using HIV-1 of subtype B and that differences in Tat *in vitro* properties have been observed between different HIV-1 subtypes (216, 217).

After transcription, the viral RNAs are polyadenylated, spliced and exported from the nucleus. The primary HIV-1 RNA transcript is 9 kb and contains at least four splice donors and eight splice acceptors, enabling alternative splicing and generation of more than 40 different viral mRNAs (218-220). The obtained transcripts are either unspliced (9 kb), incompletely spliced (around 4 kb), or fully spliced RNAs (around 2 kb). Unspliced RNAs are used for expression of Gag and Gag-Pol precursors or packaged into virions as genomic RNA. Incompletely spliced mRNAs, on the other hand, have had the *gag-pol* region spliced out and can be used for expression of Env, Vif, Vpr and Vpu, whereas fully spliced mRNAs have had the *gag*, *pol* and most of *env* removed, contain no intron sequences, and are used to express Tat, Rev and Nef (218, 220).

Typically, mRNAs containing introns are retained in the nucleus and not exported into the cytoplasm for translation, meaning that only the fully spliced viral mRNAs encoding for Tat, Rev and Nef can be exported from the nucleus by means of the cellular RNA export machinery. These proteins are referred to as the early HIV-1 proteins, whereas Gag, Pol, Env, Vif, Vpr and Vpu are referred to as the late HIV-1 proteins. To facilitate nuclear export of the intron-containing mRNAs encoding the late proteins, HIV-1 produces the Rev protein (221). When Rev accumulates in the nucleus, it promotes nuclear export of unspliced and incompletely spliced viral RNAs, thus inducing and regulating the transition from early to late HIV-1 gene expression in a negative feedback loop (222, 223). Rev binds to a *cis*-acting, highly structured RNA region called the Rev responsive element (RRE) (222, 224). The RRE, which is around 250 bp and consists of a series of stem-loop structures, is present in the *env* region in all unspliced or incompletely spliced viral RNAs. Rev is a 19-kDa phosphoprotein containing at least two functional domains as well as sequences responsible for multimerisation. The N-terminal domain is rich in arginine and responsible for nuclear localisation and interaction with the RRE (225), while the leucine-rich C-terminal domain contains a nuclear export signal, thus mediating nuclear export (226, 227). Rev has been shown to bind to one of the stem-loops of the RRE and to multimerise with other Rev molecules until around eight Rev molecules bind to the RRE. Once associated with the RRE, Rev recruits the cellular chromosomal region maintenance 1 (CRM1) protein, which is an export receptor for leucine-rich nuclear export signals (228-230). Interaction of the Rev-mRNA complex with CRM1 leads to nuclear export by means of the cellular nuclear export machinery via a pathway that is typically used for small nuclear and ribosomal RNAs rather than cellular mRNAs (221). Additional cellular proteins, such as the human Rev-interacting protein (hRIP) or Sam68, are important for Rev function and viral replication (231, 232).

The Gag ($Pr55^{Gag}$) and Gag-Pol ($Pr160^{Gag\text{-}Pol}$) polyproteins are synthesised on polyribosomes in the cytoplasm. These two polyproteins are produced at an estimated 20:1 ratio as a result of a ribosomal frameshift event mediated by a *cis*-acting RNA motif (166). The Gag polyprotein contains most of the viral determinants required for viral assembly, since the expression of the Gag

polyprotein alone leads to the generation and release of virus-like particles (233). Still, the precise mechanism behind Gag assembly into virus particles, in particular the involvement of cellular factors, is poorly understood. By and large, HIV-1 assembly involves the targeting of Gag and Gag-Pol polyproteins to the cellular plasma membrane, association of Gag to the membrane, multimerisation of Gag, encapsidation of viral genomic RNA and association with viral envelope glycoproteins anchored to the plasma membrane (128, 234). Interestingly, the function of each of the four main structural domains of the Gag polyprotein (MA, CA, NC and p6) in virus assembly, as part of the polyprotein, differs to the roles they have as individual proteins after virus maturation. The Gag polyprotein contains at least three functional elements shown to be important for assembly and budding. The M domain in MA is largely responsible for membrane targeting and binding, the I domain in NC mediates Gag-Gag interactions, and L domains in p6 are important for particle budding and release (128, 234).

The M domain is localised in the N-terminus of MA, and includes a cluster of basic residues and a myristoyl group on the first glycine residue. Mutations and deletions within this region abolish membrane binding and virus budding and release (235). Myristoylation occurs during translation and is required for virus replication (236). The myristoyl group is thought to insert into the lipid bilayer, thus anchoring MA to plasma membrane (143). It has been suggested that a 'myristoyl switch' is responsible for targeting of Gag to the membrane, where the myristoyl group is thought to be largely concealed in the context of monomeric Gag but that multimerisation of Gag leads to exposure of the myristoyl, resulting in membrane binding (237, 238). In addition, recent studies carried out by Ono *et al*. (239) have indicated that Gag localisation and assembly is regulated by the cellular factor phosphatidylinositol 4,5-bisphosphate, $PI(4,5)P_2$, as altering the levels and localisation of $PI(4,5)P_2$ could redirect localisation of Gag. Saad *et al*. (240) subsequently used NMR techniques to show that MA interacts directly with $PI(4,5)P_2$ and that this interaction exposes the myristoyl group of MA, hence enhancing membrane binding. As $PI(4,5)P_2$ is present in lipid raft-like structures in the plasma membrane, interaction of MA with $PI(4,5)P_2$ would thereby target Gag to lipid raft-like microdomains.

Gag multimerisation is mediated by the I domain, which is localised in the NC. Several regions in Gag are, however, important for mediating Gag-Gag interactions, including the C-terminal region of CA (241). Cellular proteins such the ATP-binding cassette, sub-family E, member 1 (ABCE1 or HP68), have been shown to be required for Gag assembly (242), possibly through interacting with NC and promoting multimerisation of Gag (243). Some multimerisation is thought to take place soon after Gag synthesis, during trafficking of Gag to the plasma membrane. At the plasma membrane, high-order multimerisation occurs and a layer of Gag-particles associated with the inner layer of the plasma membrane will eventually form a spherical immature virus particle (128, 234, 241).

In addition to mediating Gag-Gag interactions, NC is thought to be responsible for encapsidation of viral genomic RNA by interacting with the packaging signal in the RNA dimer (128, 234, 241). The HIV-1 genome packaging signal, or the Ψ-site, is composed of four stem loop structures (SL1-SL4) present in the 5′-region of the genome. SL3 seems to be the major signal.

The HIV-1 envelope glycoprotein is synthesised as a precursor polyprotein designated gp160, based on its molecular mass (128-131). Unlike the Gag and Gag-Pol polyproteins, gp160 is synthesised and post-translationally processed on the rough endoplasmatic reticulum (ER) and in the Golgi, following the same route as other glycoproteins destined for the plasma membrane and cellular proteins directed into the secretory pathway (139, 244, 245). It is initially synthesised as a 90-kDa polypeptide that is co-translationally translocated into the lumen of the ER where it undergoes glycosylation, folding and oligomerisation. The ER is specialised for protein glycosylation and folding, containing a range of cellular folding factors such as folding enzymes and molecular chaperones, needed to prevent aggregation of newly synthesised proteins in the protein-rich milieu of the ER.

Translocation into the ER is initiated when the N-terminal signal peptide of the nascent gp160 polypeptide chain, as soon as it emerges from the ribosome,

interacts with the signal recognition particle (SRP). The SRP-nascent peptide chain-ribosome complex then associates with the SRP receptor, targeting the complex to the ER, where the nascent peptide is transferred into the lumen of the ER through the Sec61 translocation channel (244). In addition to the Sec61 complex, a range of cellular factors including the molecular chaperone 78-kDa glucose-regulated protein/immunoglobulin binding protein (GRP78/BiP) are thought to take part in the translocation process (244).

Once in the lumen of the ER, the gp160 polypeptide is co-translationally glycosylated (128). The gp160 contains around 30 potential N-linked glycosylation sites (40-43), which are made up of asparagine-X-serine/threonine motifs where X is any amino acid except proline. Initially, N-linked glycan precursors ($glucose_3$-$mannose_9$-N-$acetylglucosamine_2$) are added to the amide groups of the asparagine residue (128). The first two glucoses are then removed by ER glucosidases. At this stage, bearing N-linked glycan precursors with only one glucose residue, the gp160 precursor is a substrate for the molecular chaperones calnexin and calreticulin. These chaperones are found throughout the eukaryotic kingdom and bind different but overlapping sets of glycoproteins carrying N-linked glycans with only one glucose residue. Newly synthesised HIV-1 gp160 has been shown to interact with both calnexin and calreticulin (246, 247). Calnexin and calreticulin bind and release glycoproteins in cycles, thereby prolonging localisation in the ER and allowing for correct folding (244). The final glucose residue of the N-linked glycan precursor is removed by an ER glucosidase, releasing the glycoprotein from calnexin/calreticulin and allowing it to exit the ER. Glycoproteins, which have had the final glucose removed, are checked for correct folding in a process involving an ER glycosyltransferase. If the glycoprotein is not correctly folded, the glycosyltransferase adds a new glucose to the N-linked glycan precursors of the glycoprotein, again making it a substrate for calnexin and calreticulin, thus prolonging ER localisation. Glycoproteins which are correctly folded, hence not recognised by the glycosyltransferase, are allowed to exit the ER and proceed to the Golgi. Whilst bound to calnexin and calreticulin, the gp160 precursor interacts with other ER molecular chaperones such as the GRP78/BiP (244, 248).

Folding of HIV-1 gp160, and retroviral glycoproteins in general, has been shown to be a slow process compared to the folding of many other glycoproteins, and depends not only on amino acid sequence and glycosylation but also on the formation and isomerisation of disulphide bonds (128, 244, 246, 248-253). Disulphide bond formation is catalysed by protein thiol-disulphide oxidoreductases, such as ER protein 57 (ERp57) and protein disulphide isomerise (PDI), and favoured by the oxidising milieu that the ER constitutes (244). For HIV-1 gp160, disulphide bonds start forming during translation but undergo extensive isomerisation during the folding process until correct folding is obtained. Land *et al*. (252) have suggested that there is a relationship between disulphide bond isomerisation and cleavage of the HIV-1 signal peptide, which may be responsible for the slow maturation kinetics of HIV-1 gp160, whilst at the same time ensuring a highly productive folding process where little gp160 is subject to degradation. Unlike the signal peptides of many other proteins produced in the ER, the gp160 signal peptide is not cleaved off until protein synthesis has been completed (244, 249, 252, 254). Replacement of the gp160 signal peptide with other signal peptides has led to increased secretion of gp120, indicating that release from ER is linked to the rate of signal peptide removal (249).

In addition to being subjected to modifications such as attachment of glycans and formation of disulphide bonds, gp160 undergoes oligomerisation in the ER (128, 255-258). This step is thought to be required for exit from the ER and entry into the Golgi. Although dimers and tetramers have been observed, the functional spike exists as a trimer (138-140, 259-261).

In the ER, the HIV-1 glycoprotein precursor may interact prematurely with the main cellular receptor utilised by HIV-1, CD4 (128). This premature interaction may inhibit transport of HIV-1 envelope glycoproteins to the cell surface, but is prevented by the viral protein Vpu, which has been shown to mediate CD4 degradation through ubiquitin-mediated proteolysis (262). CD4 cell-surface expression is also down-regulated by the viral protein Nef. Nef interacts with the CD4 cytoplasmic tail and recruits the clathrin adaptor protein 2 (AP-2) complex, which mediates internalisation and eventually degradation of CD4 via clathrin-

coated pits and lysosomes (263). Nef is also responsible for down-regulating cell-surface expression of major histocompatibility complex (MHC) class I molecules on infected cells, limiting recognition of infected cells by cytotoxic T cells (264). Moreover, the observation that deletions in the *nef* gene of HIV-1 or SIV have an effect on virus replication and viral load in humans and macaques, leading to delayed onset of disease and long-term survival (265, 266), has led to the conclusion that Nef is important for viral pathogenicity.

After synthesis, folding and glycosylation in the ER, the oligomeric gp160 is 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 carbohydrate glycans and eventually leaving only three mannose residues (128, 267, 268). This allows for the addition of a variety of different monosaccharides including fucose, galactose, sialic acid, N-acetylglucosamine and N-acetylgalactosamine, resulting in the formation of complex N-linked glycans (128, 267, 268). However, only the high-mannose glycans that are accessible to the cellular modification enzymes will be processed in the Golgi. For HIV-1 envelope glycoproteins, around half of all the N-linked glycans are inaccessible and will remain in the high-mannose form (269, 270). The variability in glycosylation and processing of glycans contributes to the heterogeneity of the HIV-1 envelope.

In the Golgi, the trimeric gp160 is proteolytically cleaved into the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 by cellular subtilisin-like serine proteinases also known as protein convertases, like furin or other furin-like enzymes (268, 271-273). 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.

Following modification of glycans and proteolytic cleavage in the Golgi, the envelope glycoprotein complex traffics to the plasma membrane (128). This process is not fully understood (274). For example, it is not known whether the HIV-1 glycoproteins traffic to the cell surface via the constitutive or regulated

secretory pathway. While it has been proposed that around 5-15% of envelope glycoproteins traffic directly from the Golgi to the plasma membrane (271), studies in T cells have suggested that a proportion transit to intracellular granules containing the cytotoxic T lymphocyte antigen 4, CTLA-4 (275). CTLA-4 is a T cell regulatory protein whose cell surface expression is controlled through the regulated secretory pathway. It has been shown to traffic to the cell surface where it is rapidly internalised by endocytosis and delivered to intracellular regulated secretory granules. The co-localisation of HIV-1 envelope glycoproteins with CTLA-4 may indicate that HIV-1 envelope glycoproteins traffic to the plasma membrane via the regulated secretory pathway, rather than being constitutively delivered to the cell surface. Regulated delivery of envelope glycoproteins to the cell surface may offer advantages for the virus, such as limited or delayed exposure of envelope spike to the immune system, and has implications for the timing and co-ordination of virus assembly (275).

At the plasma membrane, viral envelope spikes are incorporated into virions. Again, the molecular mechanism behind this process remains largely unknown (274). The envelope spikes are anchored to the plasma membrane by gp41. 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 (235, 274, 276-282). For example, mutations in either MA and the cytoplasmic tail of gp41 have been shown to abolish incorporation of the envelope glycoproteins into virions, without having an effect on virus particle release (235, 274, 276-282). In addition, gp41 and MA have been shown to interact *in vitro* (283). However, envelope spikes lacking the gp41 cytoplasmic tail can still be incorporated into virions (276-278) and it is widely known that HIV-1 can be pseudotyped with heterologous envelope proteins (284), as used for lentiviral vectors (285, 286). Incorporation of envelope spikes containing full-length gp41 has also been shown to be cell-type dependent, indicating that cellular factors influence this process (287). This notion was verified by the identification of the cellular factor TIP47 (tail-interacting protein of 47 kDa) as required for HIV-1 envelope glycoprotein incorporation into virions (288, 289). TIP47, which has previously been shown to be required for intracellular transport of cellular proteins such as the mannose 6-phosphate receptor, was shown to

serve as a bridge between MA and the gp41 cytoplasmic tail (289). In addition, it was shown to influence trafficking by re-cycling cell surface expressed HIV-1 envelope glycoproteins to the Golgi network (288).

In addition to TIP47, other cellular factors are thought to affect trafficking of HIV-1 glycoproteins. For example, several studies have shown that HIV-1 envelope spikes undergo clathrin-mediated endocytosis after reaching the cell surface (274, 290, 291). So-called endocytosis motifs in the cytoplasmic tail of gp41 are believed to initiate endocytosis by interacting with clathrin-associated adaptor protein complexes AP-1 or AP-2 as a first step in the formation of clathrin-coated vesicles (274, 290, 291). Endocytosis of envelope glycoproteins has been suggested to be important for immune evasion. Indeed, immunisation of mice with a DNA vaccine encoding an HIV-1 envelope glycoprotein with mutations in a highly-conserved tyrosine-based gp41 endocytosis motif resulted in enhanced antibody responses (292). The same tyrosine-based endocytosis motif has also been shown to be important for basolateral targeting of virions during budding from polarised cells (293). Furthermore, SIV_{mac239} carrying mutations in the same motif displayed reduced *in vivo* pathogenicity in macaques despite ongoing viral replication, suggesting that this motif is important not only for endocytosis of envelope glycoproteins but also for viral pathogenesis (294).

Additional understanding of the mechanism behind envelope glycoprotein incorporation into virions was obtained in a recent study by Leung *et al*. (295), who showed that co-expression of the HIV-1 and Ebola envelope glycoproteins with HIV-1 Gag in cells gave rise to two distinct populations of pseudotyped viruses, bearing one, but not both, of the two glycoproteins. In addition, confocal microscopy experiments showed that the HIV-1 and Ebola glycoproteins segregated to separate lipid raft-like microdomains in the plasma membrane, despite being expressed from the same vector, indicating that each virus particle is formed from a single lipid raft domain.

It has been generally believed that HIV-1, like all lentiviruses, assembles at and buds from the plasma membrane of infected cells (128, 234). Since the discovery of HIV-1, this notion has been supported by a vast number of studies (128, 234).

However, in the last decade the location of HIV-1 assembly and budding has been subject to debate and suggested to be cell-type dependent (296-310). In T cells, it has been largely established that HIV-1 assembles at the plasma membrane. This assembly has been suggested to take place at glycolipid-enriched membrane domains or lipid rafts (306, 311, 312). In contrast, several studies have shown that in macrophages, HIV-1 assembly and budding take place in intracellular compartments thought to be late endosomes or intracellular multivesicular bodies because of the presence of the tetraspanin and late endosomal marker CD63 (296-298, 313, 314). In fact, cytoplasmic assembly of HIV-1 in monocytes and macrophages was reported already in the late 1980s (313, 314). In addition to intracellular assembly in macrophages, several studies have reported HIV-1 virions and Gag in intracellular compartments in epithelial-derived cell lines and, controversial to what has been observed in previous studies, in T cells, suggesting HIV-1 assembly and budding on endosomal membranes also in these cell types (299-302). The observation that MA interacts with AP-3, an adaptor protein that normally functions in the sorting of proteins to late endosomes, was suggested to support the notion that trafficking of Gag to the late endosome is part of a productive virus pathway (315).

These studies, along with the studies on HIV-1 assembly into late endosomes in macrophages, gave rise to the suggestion that, in most cells, Gag is initially targeted to late endosomal membranes, where it can either assemble and bud or assemble and traffic to the plasma membrane via vesicles. Alternatively, the Gag polyprotein could be targeted to late endosomal membranes and then traffic unassembled to the plasma membrane via vesicles. In addition to being controversial to early studies of HIV-1 assembly and budding, these suggestions have also been contradicted by several recent reports. For example, a study carried out by Deneka *et al*. (316) has shown that, in macrophages, HIV-1 assembles and buds into intracellular compartments that are in fact not late endosomes but surface-connected, internally sequestered extensions of the plasma membrane. These compartments were found to contain markers not present in late endosomes, and to lack the late endosome marker CD63. CD63 was, however, recruited to the compartment in HIV-1-infected cells post-

infection, explaining previous reports and assumptions of HIV-1 assembly into endosomes.

A study by Welsch *et al*. (305) has supported these findings, detecting HIV-1 assembly at seemingly intracellular plasma membrane structures in macrophages but not at endosomal membranes. In addition, Jouvenet *et al*. (303) have shown that in macrophages and in a cell line of epithelial origin, HIV-1 does not assemble on endosomes. Instead, Gag is directly targeted to the plasma membrane. Moreover, a small proportion of Gag was internalised as a result of endocytosis or phagocytosis, suggesting that virus particles only reaches endosomes after endocytosis of virions formed at the plasma membrane. This notion has been supported by the observation that release of already assembled virions at the plasma membrane is inhibited by endocytosis in certain cells (304, 308).

In addition to the above models, some reports have indicated that the plasma membrane contains microdomains derived from late endosomes as a result of fusion of plasma and endosome membranes (307, 310). Furthermore, it has been shown that Gag co-localises with tetraspanins such as CD63 and CD81, both intracellularly and at the membrane of T cells, with the majority of viral proteins present on the plasma membrane, indicating that HIV-1 assembly and budding takes place in tetraspanin-enriched plasma membrane domains (317). The cytoskeleton has also been proposed to have a role in HIV-1 assembly and budding (318). Real-time imaging of Gag assembly has further supported the notion that HIV-1 assembles at the plasma membrane (319).

After virus assembly, a series of partially unknown mechanisms leads to virus budding and release of immature virus particles. Since the beginning of the 1990s, it has been clear that 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 (320). The domains in p6 responsible for this function are referred to as the late (L) domains. There is now a growing body of evidence indicating that HIV-1 employs the cellular endosomal budding machinery, in particular the endosomal sorting complexes

required for transport (ESCRT) machinery, for virus release and budding, as the late domains of p6 have been shown to interact with the ESCRT factors Tsg101 (tumour susceptibility gene 101) and Alix (apoptosis-linked gene 2 interacting protein X), interactions shown to be essential for virus release (321-324). In addition to the p6 protein, the Vpu protein is also important for virus release. It has long been known that Vpu enhances virus release and that budding from certain cell types requires Vpu (325). Early in 2008, it was reported that a human membrane protein with previously unknown function called CD317, and renamed tetherin, was responsible for tethering virus particles to the cell surface prior to endocytosis into tetherin-containing compartments (326). Tetherin was present only in cells requiring Vpu for virus release and depletion of tetherin abolished the need for Vpu, indicating that Vpu somehow inhibits tetherin-mediated retention of virus on the cell surface.

HIV-1 buds from the membrane of infected cells in an immature, non-infectious form (128). In an immature virus particle, the Gag precursor polyprotein $Pr55^{Gag}$ (MA-CA-SP1-NC-SP2-p6) is arranged radially, with the MA anchored to the lipid bilayer, and p6 in the centre. Vpr is incorporated into the virus particle via its association with p6 (327). 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 from the Pol part. The viral PR is an aspartyl protease that functions as a homodimer with the active site located in a cleft formed in between the two monomers (328).

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. The spacer peptide SP1 has been suggested to have a role in the maturation process, as Gag constructs have been shown to assemble into immature cores if SP1 is present and mature cores if SP1 is absent (329, 330). In addition, recent electron cryotomography studies of immature virions have indicated that the spacer peptide SP1 stabilises the CA hexamers in an immature lattice formation, whereas cleavage and separation of the CA and SP1 units destabilises the CA hexamers and allow them to rearrange

and form the mature hexagonal lattice (331). One mature core has been estimated to be made up by 1000-1500 CA molecules (137, 145). In contrast, an immature virus particle has been estimated to contain around 5000 copies of Gag, meaning that less than half of the CA proteins present in the immature virion forms the mature core (332). Furthermore, a third of viral particles produced *in vitro* by T cells have been observed to contain two cores (137).

Overall, despite encoding for only 15 proteins, the HIV-1 replication cycle is extraordinary complex. Several aspects of the HIV-1 life-cycle remain poorly understood, especially the way the virus exploits cellular processes (333). A recent study (334) has shed further light on this matter. Brass *et al*. (334) used a genome-wide siRNA screening experiment, targeting over 21000 genes, to identify more than 250 host factors required for HIV-1 replication, the majority of which had not previously been associated with HIV-1. These findings are likely to be important for furthering the understanding of the HIV-1 life-cycle.

1.1.4 Immunopathology of HIV-1

HIV-1 infection is characterised by a progressive loss of CD4+ T cells. Host immune responses generally fail to control infection, and the decline in circulating CD4+ T cells eventually culminates in the development of AIDS. However, the reason why HIV-1 infection is pathogenic remains unclear. In this section, a brief overview of the cellular tropism, pathogenesis and natural course of HIV-1 infection will be given, as well as an introduction to the host immune responses.

1.1.4.1 HIV-1 cellular receptors and tropism

In 1984, HIV-1 was shown to have a selective tropism for CD4+ T cells (30). This finding was soon followed by the identification of CD4 as a cellular receptor for HIV-1 by Klatzmann *et al*. (52) and Dalgleish *et al*. (53), making CD4 the first receptor to be discovered for any human or animal retrovirus. Klatzmann *et al*. (52) reported of the inhibition of HIV-1 infection by three mAbs to CD4, but not by antibodies to four other lymphocyte markers. Dalgleish

et al. (53) showed that only cells expressing CD4 were sensitive to syncytium-induction when mixed with an HIV-1-producing T cell line. In addition, only CD4+ cells were susceptible to infection by vesicular stomatitis virus (VSV) particles pseudotyped with the HIV-1 envelope (53). Dalgleish and colleagues furthermore showed that this syncytium-induction and pseudotype infection was blocked by antibodies to CD4. To come to this conclusion, they screened 155 different monoclonal antibodies (mAbs) to T cell surface antigens, showing that 14 out of 14 antibodies to CD4 could inhibit infection, whereas the majority of the remaining 141 antibodies could not (53). Two years later, Maddon *et al*. (335) further confirmed the role of CD4 as a receptor for HIV-1 by showing that engineering human cell lines to express CD4 rendered them susceptible to HIV-1 infection. In conclusion, the reports by Klatzmann *et al*. (30, 52), Dalgleish *et al*. (53) and Maddon *et al*. (335), together with the early recognition of the association of AIDS with a selective depletion of CD4+ T cells (2, 3, 6), suggested CD4 to be an essential part of the HIV-1 receptor and the CD4+ subset of T cells to be a major target for HIV-1 infection.

The CD4+ T helper cell and CD8+ cytotoxic T cell subsets were first discriminated in 1979 by Reinherz *et al*. (336), using mAbs to what would later be known as the CD4 and CD8 antigens. Biochemical analysis of the CD4 protein (337) and the subsequent cloning and sequencing of a CD4-encoding cDNA (338) later revealed that CD4 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily of receptors. It is expressed predominantly on T helper cells as well as on most thymocytes but also, to lower levels, on several other cell types including monocytes, macrophages, dendritic cells, eosinophils and mast cells (339-344). CD4 on T helper cells binds to MHC class II molecules on antigen-presenting cells. While the T cell antigen receptor binds to the polymorphic region of the MHC class II, CD4 acts as a co-receptor, binding to the non-polymorphic regions (345). Its binding restricts the T helper cell to recognising peptides presented on MHC class II only and enhances the interaction between the T helper cell and the antigen-presenting cell. In addition, the CD4-MHC class II interaction mediates intracellular T helper cell signalling through the tyrosine protein kinase $p56^{LCK}$, which is associated with the CD4 cytoplasmic domain, leading to activation of the T helper cell and enhancement

of antigen-driven T cell responses (339, 343). CD4 is also thought to play a role in T cell maturation (346). 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. Domains D1 and D3 are antibody variable domain-like, whereas D2 and D4 are antibody constant domain-like (338, 347). Binding to the MHC class II molecule extends over the D1 and D2 domains (345). HIV-1 gp120, however, binds to the D1 domain only, in its initiation of the HIV-1 entry process (348). HIV-1 entry into cells will be further discussed in section 1.1.6.

Soon after the identification of CD4 as a receptor for HIV-1, it became apparent that CD4 alone was not sufficient for virus entry into cells. While Maddon *et al*. (335) had showed that human cell lines engineered to express CD4 were susceptible to HIV-1 infection, a range of mouse cell lines made to express CD4 were not. Efficient binding of HIV-1 to the CD4+ mouse cells was, however, demonstrated, as was production of infectious virus following transfection of the cells with HIV-1 DNA, indicating that the block to infection resided in the viral entry step but after the initial binding event. Susceptibility of human cell lines engineered to express CD4, but not of a range of non-human cell lines, was later also reported in other studies (349-351). Cell hybrid experiments subsequently revealed that the absence of a human factor in the non-human cell lines, rather than the presence of an inhibitory factor, was responsible for the block to infection (352-354).

Around the same time, it became evident that two distinct HIV-1 phenotypes could be recognised, with different tropism for CD4+ cells. Åsjö *et al*. (355) propagated viruses from 31 HIV-1 seropositive individuals in peripheral blood mononuclear cells (PBMC) from healthy donors whilst assaying RT activity, observing two patterns of virus production, rapid/high or slow/low. They further found that viruses with the rapid/high phenotype in PBMC could replicate continuously and induce syncytia in immortalised CD4+ T cell lines, whereas isolates with the slow/low phenotype were less likely to. In addition, they observed that the viruses with the rapid/high phenotype were all isolated from individuals with low CD4+ cell counts and AIDS symptoms. In contrast, the

majority of individuals with no or mild symptoms and normal CD4+ cell counts had viruses with the slow/low phenotype, indicating a relation between the clinical severity of HIV-1 infection and the *in vitro* replication properties of the virus. Similar findings were made by other groups (356-358). Viruses with the rapid/high phenotype were later referred to as syncytium-inducing (SI), whereas viruses with the slow/low phenotype were referred to as non-syncytium-inducing (NSI), based on their ability to induce syncytia in immortalised T cell lines (357, 359, 360). The SI isolates were also referred to as T cell line-tropic. The NSI isolates, on the other hand, whilst being able to replicate in CD4+ PBMC, were unable to replicate in T cell lines. Instead, their tropism extended to macrophages; hence they were referred to as macrophage-tropic (180, 361). Macrophage-tropic viruses could, however, be adapted to growth in immortalised T cell lines through extensive passaging (362). Cell hybrid experiments subsequently indicated this selective cellular tropism was due to the existence of cell type-specific fusion co-factors, rather than the presence of inhibitory factors (363).

In 1996, ten years after it first became evident that presence of CD4 alone was not enough to support HIV-1 entry into cells, light was finally shed on the missing fusion co-factor, when Feng *et al*. (364) used a cDNA library approach to identify a member of the superfamily of seven-transmembrane chemokine receptors as a co-receptor for HIV-1. This receptor was named fusin or LESTR, but would later be called CXCR4 according to an agreed chemokine receptor classification. Feng *et al*. showed that co-expression of CD4 and CXCR4, but not CD4 alone, rendered previously non-permissive cells susceptible to infection. Co-expression of CXCR4 did, however, only support infection with so-called T cell line-tropic isolates and not with macrophage-tropic strains, indicating that yet another co-factor was responsible for mediating entry of the macrophage-tropic isolates, offering an explanation into the different tropisms. Shortly after the identification of CXCR4 as a co-receptor for HIV-1, Bleul *et al*. (365) and Oberlin *et al*. (366) identified the stromal cell-derived factor 1 (SDF-1) as the natural ligand for CXCR4. In the same studies, SDF-1 was also shown to inhibit infection by T cell line-tropic but not macrophage-tropic HIV-1 isolates, further

confirming the role of CXCR4 as an essential co-receptor for the so-called T cell line-tropic viruses.

Meanwhile, a report from Cocchi *et al*. (367) had shown that the chemokines RANTES (regulated upon activation, normal T cell expressed and secreted), MIP-1α and MIP-1β (macrophage inflammatory proteins) displayed HIV-inhibitory properties. It was already known that CD8+ T cells secreted HIV-suppressive factors. Cocchi *et al*. used fractionation and chromatography techniques to identify RANTES, MIP-1α and MIP-1β as the proteins responsible for the inhibition. The chemokines were able to inhibit infection by HIV-1 macrophage-tropic isolates but not infection by T cell line-tropic HIV-1 isolates.

Shortly after the identification of CXCR4 as an essential co-receptor for the so-called T cell line-tropic isolates and the observation that RANTES, MIP-1α and MIP-1β could inhibit infection by macrophage-tropic viruses, several groups simultaneously identified CCR5, the receptor for RANTES, MIP-1α and MIP-1β, as a co-receptor for macrophage-tropic HIV-1 (368-372). The reports from these groups showed that expression of CCR5 in previously non-permissive CD4+ cells rendered them selectively susceptible to infection by macrophage-tropic viruses.

CXCR4 and CCR5 are chemokine receptors. Chemokines are small cytokines primarily involved in the chemoattraction of lymphocytes, monocytes and neutrophils. They are secreted by a variety of cell types, including activated lymphocytes and monocytes/macrophages as well as endothelial cells, platelets, neutrophils and fibroblasts. Chemokines are grouped into four different classes, the CC, CXC, C and CX_3C chemokines, based on the number and spacing of conserved cysteine residues in their amino acid sequence. Chemokine receptors, in turn, are classified according to the group of chemokines that they bind. 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. They are coupled to heterotrimeric G (guanine nucleotide-binding) proteins, which act as the signal moiety of the receptor, and are thought to form a rod spanning the membrane, with a central hole surrounded by the seven transmembrane domains. There is, however, little structural information available for chemokine receptors, as no structure has been resolved to date (373). Instead, structural models of CCR5 and CXCR4 have been based on the crystal structure of bovine rhodopsin. Rhodopsin, a seven-transmembrane GPCR present in the retina, was until recently the only GPCR for which a high-resolution structure was available (374). However, the first structure of a human GPCR, the adrenergic receptor β_2AR, which was solved at the end of 2007, might give further insight into the structures of CCR5 and CXCR4 (375). In the absence of three-dimensional structural data, the binding site for gp120 has been mapped to the N-terminus and the second extracellular loop of CCR5 or CXCR4 (376-378). This notion have been supported by recent nuclear magnetic resonance (NMR) data (379).

The identification of CXCR4 and CCR5 as co-receptors for HIV-1 provided an explanation for the two distinct HIV-1 phenotypes that had been observed. In addition, it became clear that the old classification system and the assumed relationship between replication kinetics (rapid/high or slow/low), ability to induce syncytia (SI or NSI) and tropism (T cell line- or macrophage-tropic) was not completely clear-cut (380). The terms SI/NSI and T cell line-/macrophage-tropic had been used interchangeably, despite not being synonymous (381). It is now recognised that while only the SI or T cell line-tropic viruses can replicate and induce syncytia in immortalised CD4+ T cell lines (which usually express CXCR4 but not CCR5), all primary viruses can replicate in primary CD4+ T cells and induce syncytia in cells expressing the right co-receptor. In addition, some CXCR4-using isolates have been found to infect macrophages (382-384). A new classification system based on co-receptor usage was therefore suggested to replace the old nomenclature (381). Virus isolates selectively able to use CCR5 or CXCR4 are now referred to as CCR5- or CXCR4-using, or R5 or X4 viruses, respectively, whereas virus isolates able to use both receptors are referred to as R5X4- or dual-tropic. In addition to the nomenclature based on co-

receptor usage, virus isolates are distinguished on the basis of how they have been propagated. 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.

All primary HIV-1 isolates use one or both of CCR5 and CXCR4 as co-receptor (385). 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* (386, 387). However, the *in vivo* relevance of these alternative receptors remains to be determined. The frequency by which viruses use receptors other than CXCR4 and CCR5 on primary target cells has been questioned, as infection is usually blocked by inhibitors to CXCR4 or CCR5 (388).

While most HIV-1 strains depend on CD4 for initial attachment and entry into cells, some reports have shown that certain HIV-1 isolates can infect cells lacking CD4 *in vitro* (386). These strains are able to infect cells through direct contact with the co-receptor. However, it remains uncertain whether such CD4-independent infection is of any relevance *in vivo*.

In spite of the relative promiscuity of some HIV-1 isolates *in vitro*, epidemiological studies indicate that the number of co-receptors used *in vivo* is rather limited. The first evidence for the importance of CCR5 as a co-receptor *in vivo* came from genetic studies of HIV-1-exposed uninfected individuals. It was found that individuals homozygous for a defective CCR5 allele (CCR5-Δ32) were strongly protected from HIV-1 infection (389-392). The CCR5-Δ32 allele contains a 32-bp deletion in the CCR5 gene, which results in a truncated receptor that is retained and degraded in the endoplasmatic reticulum. While homozygousity for the CCR5-Δ32 allele confers strong protection, heterozygousity is associated with a slower progression to AIDS (389, 390). The distribution of the CCR5-Δ32 polymorphism is largely restricted to Northern Europe and populations of Northern European descent (393). Its prevalence

decreases in the southern and Mediterranean regions of Europe and almost disappears in Asia and Africa. It is estimated that around 1% and 20% of Caucasians are homozygous and heterozygous for the allele, respectively.

In addition to the CCR5-Δ32 allele, several other polymorphisms in the CCR5 gene, or in the genes encoding its ligands, have been associated with susceptibility to HIV-1 infection or disease progression, further highlighting the importance of CCR5 as a co-receptor *in vivo* (394). For example, plasma levels of the CCR5 ligands MIP-1α, MIP-1β and RANTES have been shown to influence disease progression (395, 396). Moreover, a low copy number of the gene encoding MIP-1α has been associated with an increased susceptibility to HIV-1 infection and a more rapid progression to AIDS, especially in combination with other known disease-accelerating CCR5 genotypes (397). Polymorphisms in the gene encoding the CXCR4 ligand SDF-1 have also been suggested to influence HIV-1 resistance and disease progression (394). Further evidence for the importance CCR5 and CXCR4 as co-receptors *in vivo* has come from the antiviral effects observed in clinical trials of several CCR5-binding agents and one CXCR4 antagonist (398).

The protective effects of these CCR5-related polymorphisms, especially the CCR5-Δ32 allele, suggest a major role for CCR5 in HIV-1 transmission. Indeed, CCR5-using viruses have been suggested to be preferentially transmitted over CXCR4-using viruses (399). The reason behind the selective transmission is unclear, although several mechanisms that restrict transmission of CXCR4-using viruses have been suggested (400). In addition to being preferentially transmitted, early studies (some of which are mentioned above) have shown that CCR5-using viruses predominate during the acute and asymptotic phase of natural HIV-1 infection, whereas CXCR4-using viruses emerge at a later stage in around 50% of individuals infected with subtype B viruses (355, 401-405). The emergence of CXCR4-using viruses have been associated with lower CD4+ T cell counts and faster progression to AIDS (355, 401-405), although loss of CD4+ T cells and AIDS have also been reported in individuals harbouring CCR5-using viruses only (406, 407).

Most studies looking at co-receptor usage have, however, been carried out in relatively small cohorts, as methods for determining HIV-1 co-receptor use have typically been relatively low-throughput and cost-intensive. Recent technological advances have allowed for large-scale studies of the prevalence of co-receptor usage. These studies have confirmed the association of lower CD4+ T cell count with the appearance of CXCR4-using viruses but have detected CXCR4 usage in a lower percentage of individuals (408-412). For example, Moyle *et al*. (412) assessed co-receptor use in 563 treatment-naïve and treatment-experienced HIV-1-infected individuals, with known viral load and CD4+ T cell counts, and found that approximately 80% of the individuals had viruses exclusively using CCR5. The remaining 20% had CXCR4-using, dual-tropic or mixed virus populations. However, only 0.7% of the samples showed exclusive CXCR4-usage. CCR5-usage was significantly associated with a lower viral load and higher CD4+ T cell count and the presence of CXCR4-using, dual-tropic or mixed populations were more common at higher viral load and lower CD4+ cell count. No significant association was found between co-receptor use and exposure to antiretroviral therapy. Similarly, Brumme *et al*. (411) found CXCR4-using, dual-tropic or mixed virus populations viruses in 18% of almost a thousand treatment-naïve HIV-1-infected individuals. Viruses that exclusively used CXCR4 were, however, only detected in less than 1% of the individuals. Presence of CXCR4-using virus was again associated with lower CD4+ T cell levels. Whether the appearance of CXCR4-using viruses is a cause or a consequence of disease progression remains to be determined.

The viral determinants for co-receptor usage have, unsurprisingly, been mapped to the viral envelope proteins. HIV-1 cellular tropism and co-receptor usage was first shown to be determined by the envelope protein (413-416) and in particular by the V3 region of gp120 (417) in the early 1990s. Several studies have since shown that the V3 region is a major determinant of co-receptor tropism, but sequence changes in the V1, V2, C3 and C4 regions of gp120 and in gp41 have also been shown to modulate co-receptor use (418-422).

As for most HIV-1 research, the majority of studies on HIV-1 co-receptor usage and tropism have been carried out using HIV-1 of subtype B. As subtype B

infections only account for around a tenth of infection worldwide (124), co-receptor usage among non-subtype B viruses is of interest and has been addressed by some studies. Although some studies have found no significant difference in phenotype among different genetic subtypes (412, 423, 424), others have reported of subtype-specific differences. For example, 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 (407, 425-430). In addition, CXCR4 usage has been reported to be more prevalent among subtype D viruses compared to among subtype A viruses (431), which is consistent with other reports observing a more rapid progression to AIDS among individuals infected with subtype D isolates compared to individuals infected with subtype A isolates (432-434). Further large-scale studies are needed to assess co-receptor use in non-subtype B isolates, especially in light of the recent developments of CCR5 antagonists as antiretroviral drugs.

So which cells are infected by HIV-1 *in vivo*? As HIV-1 tropism is largely determined by the distribution of the cellular receptors, HIV-1 should be able to infect all cells expressing CD4 and either of the identified co-receptors. CCR5 is expressed to high levels on activated memory CD4+ T cells (435, 436). These cells are the main target cells of HIV-1 infection *in vivo* (437). Activated memory CD4+ T cells are especially abundant in the gut-associated lymphoid tissue (GALT), which has been shown to be a major site of HIV and SIV replication (438, 439). Compared to CCR5, CXCR4 is expressed on a wider range of CD4+ T cells, including antigen naïve T cells, providing CXCR4-using viruses with a wider tropism (435, 436). Consistent with this notion, an increased proportion of the viral load has been found in antigen naïve CD4+ T cells in individuals harbouring CXCR4-using viruses (440).

However, CD4+ T cells are not the only target cells for HIV-1 *in vivo*. Like other members of the *Lentivirus* genus, HIV-1 is able to infect cells of the mononuclear phagocyte lineage. Macrophages express CD4, CCR5 and CXCR4 and were first shown to be infected with HIV-1 in 1986 (180). Latent as well as productive infection of tissue macrophages, such as brain macrophages or microglia, has since been frequently observed *in vivo*. The viral dynamics of

HIV-1 infection of macrophages has been found to be different to that in CD4+ T cells. Compared to HIV-1-infected CD4+ T cells, the turnover of infected macrophages is rather slow (441). Instead, there seem to be an absence of cytopathic effects and production of virus over a prolonged period of time, for up to 60 days after infection *in vitro* (442). These notions, along with the tendency of viruses to accumulate in what was initially thought to be intracellular multivesicular bodies or late endosomes (297, 298) but subsequently shown to be internally sequestered, surface-connected extensions of the plasma membrane (305, 316), has led to the suggestion that cells of the mononuclear phagocyte lineage serve as reservoirs for HIV-1 (443). Importantly, perivascular macrophages are also the main target for HIV-1 infection in the central nervous system (CNS) and may have a role in the progressive encephalopathy often seen in AIDS patients (444, 445).

In addition of CD4+ T cells and macrophages, the distribution of the HIV-1 cellular receptors also allows for infection of dendritic cells (DCs). DCs are antigen-presenting cells of haematopoietic origin crucial to the activation of T cells and the initiation of primary immune responses (446). The DC population is highly heterogeneous and several DC subsets have been described, based on their lineage, anatomical localisation and cell surface expression markers (447). Anatomically, DCs can be divided into lymphoid-tissue resident DCs and migratory DCs (447, 448). The lymphoid-tissue resident DCs can be further divided into the conventional lymphoid-tissue resident DCs and the relatively recently described plasmacytoid DCs (447, 448). The conventional lymphoid-tissue resident DCs have the typical dendritic form and capture and present antigens within the lymphoid organs, whereas the plasmacytoid DCs are round, non-dendritic and have been shown to produce interferon following viral infection (447, 448). The migratory DCs are conventional DCs that reside in an immature form in the peripheral tissues, such as the skin and mucosal tissues. Immature DCs in the periphery capture and process antigens, develop into mature DCs as a result of antigen deposition and inflammation, and migrate to lymph nodes where they stimulate T helper cells (446). DCs that reside in tissues include the Langerhans cells in the epidermis and mucosal epithelia, and the dermal and interstitial DCs in the dermis, submucosal epithelia and rectal

epithelia. Langerhans cells, characterised by the surface expression of the C-type lectin Langerin and containing the Langerhans cell-specific organelles Birbeck granules, were the first DCs to be described, by Langerhans in 1868. The next description of DCs did not come until a hundred years later, when Steinman and Cohn reported data on a novel cell type with dendritic morphology isolated from spleen (449). Initially, conventional DCs were thought to be of myeloid lineage. This notion was, however, contradicted by reports showing that fractions of both myeloid and lymphoid precursors can give rise to conventional DCs, *in vitro* and *in vivo* (450, 451). Even though the precise ontogeny and differentiation pathways of the various DC subsets remain unclear, two recent reports have identified a common progenitor that can give rise to both conventional and plasmacytoid DCs (452, 453). Many DC subsets express CD4, CCR5 and CXCR4, although the expression levels may differ between subsets and at different maturation stages. DC subset not expressing CD4 have also been observed (454).

Because of their expression of the HIV-1 cellular receptors, their location in mucosal tissues and their antigen-presenting role, DCs have been suggested to be the first cells to encounter HIV-1 during mucosal transmission and to mediate spread to CD4+ T cells (455). *In vitro* infection of human peripheral blood dendritic cells was first demonstrated in the 1980s (456). Subsequent *in vitro* experiments as well as *in vivo* studies in SIV and rhesus macaque models have, however, shown that the frequency by which DCs are infected is low (457-460). One study showed that, on average, only a few percent of DCs isolated from healthy donors can be productively infected by HIV-1 (461). In addition, infection of DCs has been shown to be less productive than infection of CD4+ T cells (462). These notions have, in part, been explained by observations indicating that different DC subsets show different susceptibility to productive HIV-1 infection (461, 463). This differential susceptibility may be due to different expression levels of the HIV-1 cellular receptors but also to intracellular restriction factors such as APOBEC3G (464). In addition to depending on phenotype, the susceptibility of DCs to HIV-1 infection depends on the maturation stage (458, 465). Langerhans cells have been shown to be susceptible to productive HIV-1 infection (466, 467).

In addition to being able to support infection, thus aiding in transmitting infection to CD4+ T cells, DCs are thought to mediate HIV-1 infection of CD4+ T cells *in trans*, i.e. without becoming infected themselves. Transfer of HIV-1 from DCs to CD4+ T cells was first reported in the early 1990s, when infection of CD4+ T cells was observed after co-culture with DCs that had been exposed to HIV-1 and washed (468, 469). Since then, several studies have shown that DCs can capture and keep virus in an infectious state even for several days before transfer to CD4+ T cells (462). Several mechanisms underlying this transfer have been proposed. DCs possess unique pathways which facilitate capture and internalisation of pathogens. In addition to CD4, DCs express other receptors that can capture HIV-1. For example, DCs have been shown to capture HIV-1 via C-type lectins, a family of cell surface receptors that recognise carbohydrate structures. Capture by C-type lectins leads to internalisation of HIV-1 into endosomes. Transfer to CD4+ T cells can then occur via an infectious synapse formed between DCs and CD4+ T cells (470, 471). In addition, to being transferred across an infectious synapse, HIV-1 captured by C-type lectins and internalised into endosomes has been shown to be targeted to multivesicular bodies and subsequently transferred to CD4+ T cells via exosomes (472). Alternatively, upon internalisation, HIV-1 can be targeted to proteasomes or lysosomes, leading to degradation and presentation on MHC class I or II. Capture by C-type lectins can also enhance infection of the DC *in cis* (473).

The C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), was identified in 2000 and shown to be able to capture HIV-1 and promote efficient infection of CD4+ T cells *in trans* (474, 475). DC-SIGN binds to gp120. It is expressed on a subset of immature DCs, including DCs in the submucosal epithelia and the rectal epithelia. Other receptors present on DCs and able to capture HIV-1 include the mannose receptor and galactosyl ceramide (476, 477). In spite of the identification of DC-SIGN and other attachment receptors, the initial interactions between HIV-1 and DCs remain unclear and debated. Some studies have reported of DC-SIGN-independent transfer of HIV-1 infection from DCs to CD4+ T cells, indicating that additional, unknown receptors are involved (478, 479). A recent study has demonstrated the

DC-specific heparan sulphate proteoglycan syndecan-3 as a major HIV-1 attachment factor on DCs (480).

Unlike other DCs, Langerhans cells have been shown to bind HIV-1 via the C-type lectin Langerin (481). It has previously been though that Langerin on Langerhans cells function in a manner similar to that of DC-SIGN (476). This notion has, however, been contradicted by a recent study carried out by de Witte *et al*. (481), showing that capture of HIV-1 particles by Langerin leads to internalisation of the virus and degradation in the Birbeck granules, thus preventing infection rather than mediating it to CD4+ T cells. Blocking Langerin with antibodies or saturating it with high concentrations of virus did, however, result in infection of the Langerhans cells and transfer of HIV-1 to CD4+ T cells.

Due to the low abundance of DCs *in vivo*, many studies on HIV-1 infection of DCs are carried out using monocyte-derived DCs, which are cells with DC-like properties generated *in vitro* from human monocytes. While these cells provide a good model, they may not accurately represent DCs *in vivo*. For example, it has been shown that monocyte-derived DCs mainly interact with HIV-1 gp120 via C-type lectins, whereas primary DCs isolated from blood were found to bind gp120 mainly via CD4 (482). Studies on primary DCs isolated from blood have furthermore shown that DC ability to transfer infection to CD4+ T cells varies with DC subset and with maturation stage. While conventional DCs can efficiently mediate infection of CD4+ T cells, plasmacytoid DCs have been shown to inhibit HIV-1 replication in CD4+ T cells, probably because of their interferon (IFN)-α production (483). This notion has, however, been contradicted by another study reporting efficient transfer of HIV-1 to CD4+ T cells by plasmacytoid DCs (484). In light of these observations, it is clear that further studies using primary DCs are needed to elucidate the mechanisms by which DCs interact with HIV-1 *in vivo*. Increased understanding of how HIV-1 interacts with DCs would shed light on the events involved in sexual transmission of HIV-1, with obvious implications for the development of strategies to prevent HIV-1 transmission. Transmission of HIV-1 will be further discussed in the next section (section 1.1.4.2).

In conclusion, it is the distribution of the cellular receptors that is the main determinant for HIV-1 tropism. Even so, cellular tropism, in particular the species-specific aspect, is in part determined by cellular restriction factors that act post-entry (485).

1.1.4.2 Transmission of HIV-1

HIV-1 can be transmitted along three major routes, either through exposure to HIV-1 positive blood or blood products, via mother-to-child transmission, or through sexual contact. For all routes of transmission, the probability of transmission depends on a number of factors, including host susceptibility and infectiousness, viral properties and environmental factors (486).

Transfusion of infected blood or blood products poses the greatest risk of infection based on a single exposure, as it leads to infection in more than 90% of cases (487). The risk of acquiring HIV-1 through the use of shared, contaminated needles is lower, as the probability of infection per injection with a contaminated syringe is less than 1% (488, 489). Exposure to HIV-1 positive blood, blood products, and the use of contaminated needles accounts for around 5-10% of HIV-1 infections worldwide (490). The majority of these infections are, however, acquired through intravenous drug use, as routine screening of blood and blood-derived products has reduced the spread of HIV-1 via blood and blood product transfusions (65, 490, 491).

Mother-to-child transmission of HIV-1 can occur *in utero* via the placenta, during delivery or via breastfeeding. While a few cases may be the result of true vertical transmission, the majority are believed to represent mucosal transmission due to exposure of the small intestine or the oral mucosa to maternal body fluids (492). This notion is, in part, supported by studies carried out in macaque models, which have indicated that neonates are more susceptible to oral transmission than adults (493, 494). In the absence of antiretroviral treatment, 30-45% of infants born to HIV-1 positive mothers become infected (495). Risk of transmission can, however, be reduced to less than 10% with a short course of

antiretroviral therapy. Still, an estimated half a million infants acquire HIV-1 annually through mother-to-child transmission (65, 495).

Sexual transmission of HIV-1 accounts for the vast majority of HIV-1 infections worldwide and occurs primarily at the genital and rectal mucosa (65). The probability of sexual transmission varies with anatomical site. The risk of acquiring HIV-1 during oral sex appears to be almost negligible, whereas anal sex confers the highest risk, with an estimated transmission probability per coital act of around 0.001-0.01, and vaginal sex an intermediate risk with an estimated transmission probability of around 0.0001-0.001 (486, 496-500). Transmission can be facilitated by breaks in the epithelium but can also occur across intact mucosal surfaces (501, 502). Both cell-free and cell-associated virus can be mucosally transmitted, although cell-free virus seem to be more infectious in the macaque model (503, 504). Most HIV-1 infections worldwide result from genital exposure to semen of HIV-1-infected men (486). Semen can contain both cell-free virus and infected cells (505, 506). Recently, Münch *et al*. (507) have reported that amyloid fibrils, composed of fragments of prostatic acidic phosphatase and naturally present in semen, strongly enhance HIV-1 infection of T cells, macrophages and human tonsillar explants.

Transmission to women during vaginal intercourse can occur through the vaginal, ectocervical, or endocervical mucosa (490). 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. It is not known whether most women are infected via the vagina or via the cervix. However, studies of macaques, who lack the cervix after surgical removal of the uterus, have shown that the vaginal mucosa is sufficient for SIV transmission (508). In addition, treating macaques with progestins, which leads to thinning of the vaginal mucosa without having an effect on the cervix, increases the risk of SIV infection, suggesting the vaginal mucosa as the primary site of transmission (509). Moreover, the risk of acquiring HIV-1 infection has been suggested to be higher during pregnancy, when progesterone levels are higher (510). In contrast, treating macaques with oestrogen thickens the vaginal mucosa and decreases the risk of HIV-1 transmission (511). Disruption of the

mucosal integrity as a result of physical abrasion induced by intercourse (512-514), abnormalities in the vaginal flora caused by e.g. bacterial vaginosis (515, 516), or inflammation or genital ulcers caused by sexually transmitted diseases or chemical agents such as a topical microbicide (517, 518), all lead to increased risk of HIV-1 transmission. The single-layer epithelium of the endocervix is believed to support viral transmission, but is protected by a mucus plug thought to acts as a barrier to infection (519).

In men, HIV-1 transmission across the genital mucosa occurs most frequently through the inner foreskin and perhaps also through the penile urethra. An estimated 70-75% of all male infections are acquired through vaginal intercourse (490). The inner foreskin is lined by a multilayer stratified squamous epithelium, and has been shown to contain target cells for HIV-1 infection and to be susceptible to HIV-1 infection in an explant model (520-522). The urethra is lined by some layers of columnar epithelial cells. Circumcision confers a reduced risk of HIV-1 infection, highlighting the important role of the foreskin in HIV-1 transmission (523-525).

While much understanding has come from epidemiological studies, human explant studies, and *in vivo* studies of SIV- or SHIV-infected macaques (437, 486, 490, 501, 502, 526, 527), the biology of mucosal transmission of HIV-1 is still poorly understood, and the events from when HIV-1 reaches the epithelium and until there is an established infection remain unclear. For example, it is not known which cells are initially infected, although potential target cells include Langerhans cells, subepithelial DCs, macrophages and CD4+ T cells (490, 501, 528). According to current dogma and as discussed in section 1.1.4.1, HIV-1 is believed to hijack DCs to reach CD4+ T cells (462). However, the exact role of DCs in HIV-1 transmission remains obscure. DCs in the mucosal tissues include the subepithelial DC-SIGN+ DCs and Langerhans cells in the epithelia. Langerhans cells have long been thought to be an initial target for HIV-1 infection because of their susceptibility to infection and the observation that Langerhans cells harbour virus after vaginal inoculation of macaques with SIV (466, 467, 501), as well as the location of Langerhans cells in the mucosal epithelium and the fact that they can reach into and sample antigen in the lumen

of for example the vagina (529, 530). Recent studies by de Witte *et al*. (481) have, however, indicated that Langerhans cells, instead of becoming infected or transferring infection to other target cells, trap and degrade incoming virus.

CD4+ T cells have been found beneath the epithelia of the vagina and cervix, as well as dispersed within in the epithelia (531, 532). These CD4+ T cells are primarily memory T cells. Several studies carried out in female genital tissue explant models (533-535) have shown that HIV-1 productively infects CD4+ T cells in the genital mucosa (536-538). A study carried out in a vaginal explant model by Hladik *et al*. (536), showed that the initial infection of CD4+ T cells in the vaginal mucosa is likely to be independent of Langerhans cells. Instead, virus was found to be endocytosed by Langerhans cells, a notion which is in concordance with the observations made by de Witte *et al*. (481), discussed above. The important role of genital CD4+ T cells in SIV transmission has also been supported by data obtained in the macaque model (539, 540).

In addition to CD4+ T cells, macrophages have been shown to be infected in human genital explant models (533, 534, 541). Infected macrophages could, however, not be detected, or were rarely detected, in genital compartments in macaques infected with SIV (459, 460, 540). The precise role of macrophages in HIV-1 transmission is therefore unclear.

Little is also known about the nature of the virus that is transmitted (542). The characteristics of the transmitted virus and the early diversification of the virus population have been difficult to study, since there is a delay of at least a week before viral RNA can be isolated from blood of infected individuals. Several studies of newly transmitted viruses have, however, indicated that the virus passes through a genetic bottleneck during mucosal transmission, since viral diversity is limited in the early stages of infection compared to later on or in the donor (401, 542-548). In contrast to the above reports, several studies have reported heterogeneous virus populations soon in early infection and also that there are gender differences (549-551). In addition, it has been suggested that transmission of multiple virus variants occurs frequently but that the population is homogenised soon after transmission (552). Furthermore, it is not known

whether compartmentalisation of virus between the blood and genital compartments of the transmitting individual contributes to the reduced viral diversity frequently observed soon after transmission, or whether selection takes place primarily at the mucosal membrane of the receiver (542). Miller *et al*. (519) have shown that in macaques, the mucosal barrier greatly limits the initial infection of target cells present in the vaginal and cervical tissues, leading to a small founder population of viruses, suggesting that the mucosa is responsible for the initial, limited viral diversity. These observations has been further supported by Chomont *et al*. (548), who suggested that virus variants from the male donor are selected in the female mucosa during HIV-1 transmission, since HIV-1 gp120 sequences from the vaginal and blood compartments were virtually identical soon after infection, but not in chronic disease.

Salazar-Gonzalez *et al*. (553) have described an experimental strategy using single-genome amplification and sequencing of viral genes, which facilitates identification of the transmitted virus, or at least of early founder viruses that have initiated productive clinical infection, as well as analysis of early virus diversification. Using this approach and phylogenetic techniques, Salazar-Gonzalez and colleagues found that out of twelve Zambian individuals infected with HIV-1, eight were originally infected with a single virus, or at least that a single founder virus that gave rise to the clinical infection, whereas the remaining four were originally infected with several viruses. This approach is likely to be useful in furthering the knowledge of the properties of the transmitted virus.

Several studies have indicated that there may be differences in the biology of sexual transmission between HIV-1 subtypes. For example, for HIV-1 of subtype A and C, transmission of viruses bearing envelope glycoproteins with shorter and more compact variable loops seems to be favoured (547, 554), whereas among subtype B viruses, this observation has not been made (555, 556).

Several factors contribute to the probability of transmission (486). Plasma viral load has, for example, been associated with sexual transmission of HIV-1 (557-560). Higher plasma loads are found in the early stages of HIV-1 infection, and several phylogenetic studies have highlighted the importance of primary HIV-1

infection in onward transmission (505, 561-564). Wawer *et al*. (497) were first to present empirical data on the variation in HIV-1 transmission by stage of infection. Using data from studies of 235 HIV-1-discordant couples in the Rakai district of Uganda, Wawer and colleagues estimated that the probability of HIV-1 transmission within the first 2.5 months after seroconversion of the infected partner (0.0082 per coital act) was almost twelve-fold higher than that observed during asymptomatic infection (0.0007 per coital act). During the last two years before death, the probability of transmission increased again to 0.0031 per coital act. Subsequent studies carried out in the Rakai district of Uganda have estimated primary and late-stage infection to be around 26 and 7 times more infectious than asymptomatic infection, respectively (565). Transmission does, however, occur throughout the course of HIV-1 infection. Fraser *et al*. (566) used epidemiological tools to quantify the transmission potential of HIV-1 as a function of the viral load set-point, and found that the most commonly observed viral load set-points resulted in nearly optimal transmissibility over the life-time of the infected host, which may indicate that HIV-1 has evolved to have a maximised transmission potential in humans.

1.1.4.3 Natural course of HIV-1 infection

Within the first hours and days after mucosal transmission of HIV-1, infection is established in target cells located in the lamina propria of the mucosal tissue. Infection then spreads to the draining lymph nodes and subsequently to other lymphoid tissues via the circulation (502). Miller *et al*. (519) have shown that a small founder population of infected cells is developed at the point of entry in macaques following intravaginal challenge with SIV, and that local proliferation and continued seeding of distal lymphoid tissues was needed to establish systemic infection.

Once established, natural HIV-1 infection is characterised by three phases. The first phase is the primary or acute infection, which is associated with intense viral replication, a rapid increase in viral load and a decline in CD4+ T cells, especially the effector memory CD4+ T cells at mucosal sites. Over the following weeks, as antiviral immune responses are developed, the number of

circulating CD4+ T cells start to recover and plasma viral load decreases to a set-point. This viral load set-point has been proposed to be a strong predictor of disease progression (567, 568).

The second phase is the chronic, and in general clinically asymptomatic, infection, which is characterised by a gradual increase in plasma viral load and a progressive decrease in circulating CD4+ T cells. Despite the lack of clinical symptoms, persistent viral replication occurs throughout this phase (569, 570). The duration of the chronic phase varies and can last from a few to fifteen or more years.

The third phase is characterised by the terminal failure of the immune system, symptomatic infection and onset of AIDS. AIDS is defined as the occurrence of any of more than twenty opportunistic infections or HIV-related cancers, as defined by the United States Centres for Disease Control and Prevention (CDC) and the European Centre for the Epidemiological Monitoring of AIDS (EuroHIV). The CDC also includes a circulating CD4+ T cell count below 200 cells/μl of blood in the definition of AIDS (571). A schematic representation of the natural course of pathogenic HIV-1 infection is shown in Fig. 1.1.4.3.1.

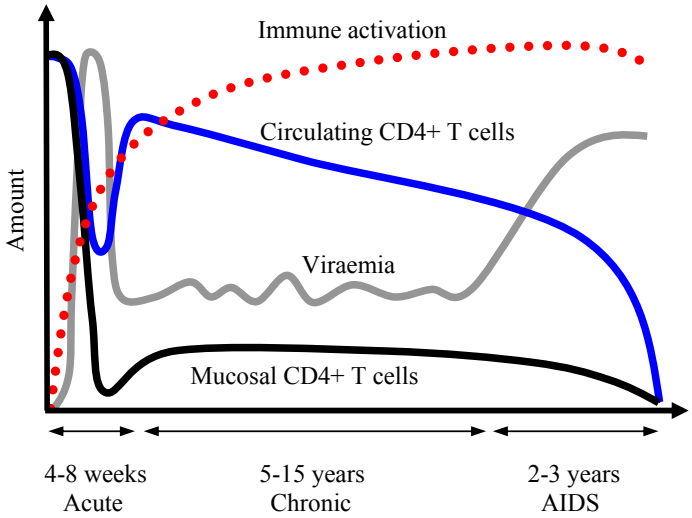


Figure 1.1.4.3.1. Schematic representation of the natural course of HIV-1 infection. Figure obtained from Forsman, A. and Weiss, R. A., 'Why is HIV a pathogen?', Trends in microbiology, *in press*.

The course of HIV-1 infection and the rate of disease progression vary between individuals. A proportion of HIV-1-infected individuals do not seem to develop AIDS, but have low viral loads and stable circulating CD4+ T cell levels (572, 573). These individuals are referred to as long-term non-progressors, or LTNP. Several host genetic factors such as the CCR5-Δ32 allele and some human leukocyte antigen (HLA) genotypes have been associated with the rate of disease progression and the LTNP phenotype (574-577). In addition to host factors, viral elements have also been implied to have a role in the rate of disease progression in humans (578). Deletions in the viral *nef* gene have, for example, been associated with slower disease progression (265, 266). Differences have also been observed between different HIV-1 genetic subtypes (432-434).

The major characteristic of AIDS is a selective depletion of CD4+ T cells followed by the onset of immune deficiency. Loss of CD4+ T cells is particularly striking in mucosal tissues, especially in the GALT of HIV-infected humans (439, 579, 580) and SIV-infected macaques (438, 581-585). Containing the majority of the T cells of the body, the GALT is a major site for HIV-1 replication. In the GALT, most CD4+ T cells are depleted within the first few weeks of infection, resulting in potentially irreversible tissue damage, which is thought to determine further disease progression (586). The mechanism behind the rapid depletion of CD4+ T cells in the GALT remains obscure. A recent report by Arthos *et al*. (587) has, however, shown that HIV-1 gp120 can bind to the integrin $\alpha_4\beta_7$, which is a receptor that mediates migration of peripheral CD4+ T cells to the GALT, possibly indicating that HIV-1 preferentially attaches to and infects cells in the GALT.

Loss of CD4+ T cells can occur through direct infection of CD4+ T cells but also through indirect effects, such as immune activation, apoptosis and killing of bystander CD4+ T cells by CD8+ T cells (588). In addition, certain viral proteins can be toxic to CD4+ T cells and the virus has also been suggested to impair thymic regeneration of CD4+ T cells (588). Overall, the mechanisms behind CD4+ T cell depletion and development of AIDS remain poorly understood. Some understanding has, however, come from studies of SIV infection of non-

human primates. Unlike in non-natural or recent hosts, such as macaques or humans, SIV does not cause immunodeficiency in its natural hosts. African monkey species that are naturally infected with SIVs, including chimpanzee, sooty mangabey and African green monkey, do not develop disease and generally retain normal levels of peripheral CD4+ T cells despite high levels of viral replication and high plasma viral loads (589). Depletion of mucosal CD4+ T cells does, however, occur in non-pathogenic SIV infection of sooty mangabeys, with a kinetics similar to that observed in pathogenic SIV infection of macaques, indicating that mucosal CD4+ T cell depletion is not sufficient for, or predictive of, disease progression (590, 591).

Studies of SIV infection of natural and non-natural hosts have, instead, highlighted the importance of immune activation in pathogenic SIV/HIV infection. Several studies have shown that in their natural monkey hosts, SIV infections are not associated with elevated levels of immune inactivation, whereas elevated levels of immune activation is observed in non-natural hosts such as macaques (589, 592). Elevated levels of immune activation have also been associated with HIV-1 disease progression in humans, and markers of immune activation have been shown to have prognostic value (593-601). For example, cell-surface expression of CD38 on CD8+ T cells, a measurement of CD8+ T cell activation, strongly correlates with disease progression to AIDS and death in HIV-1-infected individuals, and appears to be a better predictor of disease progression than CD4+ cell count or viral load (595-597, 599). The importance of immune activation in HIV-1 and AIDS pathogenesis is further supported by the observations that in HIV-2 infected individuals, the majority of the non-progressors show limited levels of immune activation, whereas elevated levels are seen in individuals who progress to AIDS (602).

Even though it is clear that systemic immune activation is a major characteristic of pathogenic HIV/SIV infection, the aetiology behind it and the mechanisms behind its association with disease progression remain unclear. It has, however, been suggested that bystander activation of T cells may play a role (603). Circulating microbial products such as lipopolysaccharide, thought to have translocated across a damaged gastrointestinal mucosa, have also been proposed

to contribute to systemic immune activation, as elevated levels have been observed in HIV-1-infected individuals and in SIV-infected macaques, but not in SIV-infected sooty mangabeys (604).

The observation that natural hosts for SIV infection, such as chimpanzees and sooty mangabeys, but not non-natural and recent hosts, such as humans and macaques, express lower levels of CCR5 on their blood, lymph node and mucosal CD4+ T cells, may indicate that this receptor is important in pathogenic SIV and HIV infection and perhaps in immune activation (605). Differences in the ability of SIV, HIV-1 and HIV-2 Nef to suppress T cell activation have, furthermore, been suggested to have a role (606). Recently, it has been proposed that self-reactive CD8+ T cells, recognising caspase-generated protein fragments released by dying CD4+ T cells, may contribute to the systemic immune activation seen in chronic HIV-1 infection (607).

1.1.4.4 Host immune responses to HIV-1 infection

In the natural course of HIV-1 infection, the host immune responses fail to clear infection and to contain virus replication, allowing for persistent viraemia (608). While virus-specific CD8+ T cell responses are thought to suppress primary HIV-1 replication, the contribution of anti-HIV-1 antibodies in clearance of primary infection has been debated. Although the majority of research on host immune responses to HIV-1 infection has focused on the adaptive immune response, attention has also been given to the role of the innate immune response in HIV-1 pathogenesis (609, 610). In addition, there has been a growing interest in so-called 'intrinsic immunity' to retroviral infection, conferred by cellular factors such as TRIM5α, APOBEC3G and Fv1, which restrict retroviral replication in post-entry events (485). The discussion in this section, however, will focus on the adaptive host immune responses to HIV-1 infection, in particular the humoral responses.

HIV-1-specific CD8+ T cells emerge within weeks of the initial infection, coinciding with the decline in viral load and restoration of circulating CD4+ T cells, suggesting that CD8+ T cell responses have a role in controlling viraemia

(611-613). CD8+ T cells have been shown to be important in the control of SIV infection of macaques, as a rapid increase and decrease in viral load was observed in macaques after the elimination and reappearance of CD8+ T cells during chronic SIV infection (614, 615). Further support for a role of the adaptive cellular immune response in the control of HIV-1 infection has come from studies demonstrating the selection of cytotoxic T Lymphocyte (CTL) epitope escape mutants of the virus in primary SIV infection of macaques (616) and in primary HIV-1 infection of humans (617, 618).

The antibody response to HIV-1 infection is generally vigorous and primarily directed against the gp120, gp41, CA and MA viral proteins (608, 619). The neutralising antibody response, however, appears relatively poor. It varies widely in terms of kinetics, magnitude and neutralisation breadth between individuals, and is restricted to antibodies recognising gp120 and gp41 epitopes (139, 619-622). 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 (141, 142, 623-627). The presence of antibodies able to neutralise HIV-1 *in vitro* in sera from HIV-1-infected individuals was first demonstrated in 1985 (628-630).

While serum anti-envelope antibodies can be detected within in weeks of HIV-1 infection, neutralising antibodies have been observed to appear at a later stage, typically around 3-12 months after acute infection, after the decline in primary infection viral load (631-643). Because of these observations, it has been assumed that neutralising antibodies do not contribute to the control of primary viraemia. A number of studies have, however, demonstrated the development of neutralising activity within weeks of infection in some study subjects (555, 631, 641, 643-646), although this observation was sometimes found to depend on assay stringency (631). These reports indicate a possible role for neutralising antibodies in the control of primary viraemia. The initial neutralising antibody response is, in general, highly specific for the autologous early virus variants and show limited cross-neutralisation properties (631, 636, 638, 643, 645, 647). The specificities of anti-envelope antibodies in sera of HIV-1-infected individuals

and the antigenic properties of the envelope glycoproteins will be further discussed in section 1.1.6.

Soon after the emergence of a neutralising antibody response, neutralisation escape mutants of the virus can be isolated, as shown in longitudinal studies of HIV-1 infection (622, 632, 643, 644). The appearance of neutralisation-resistant viruses prompts the generation of new antibodies able to neutralise the escape mutants, which in turn drives the evolution of new escape mutants, the mounting of new antibody responses, and so on (620, 622, 648). The average HIV-1 generation time, i.e. the time from the release of a virus particle until it has infected a new target cell and caused the release of a new round of virus particles is as short as 2.6 days (441). This high replication rate, together with a mutation rate of 3.4×10^{-5} mutations per bp per cycle (167), contributes to the ability of HIV-1 to escape neutralising antibodies faster than new antibodies can be generated, contributing to the low titres of neutralising antibodies observed in sera from infected individuals. The observations that serum samples from late in infection can often neutralise early virus isolates (632, 638, 643, 645, 649-651), suggest that the neutralising antibody response may well be vigorous, although not able to keep up with virus evolution.

The selective pressure of the neutralising antibody response thus drives virus evolution (648), contributing to the extreme genetic diversity observed in the *env* gene and the variation in neutralisation sensitivity observed among HIV-1 variants, even within single plasma samples (652). Similarly, viral evolution and escape from autologous antibody responses is likely to drive the gradual broadening of serum neutralising activity and the slow increase in heterologous neutralising responses that have been observed throughout the course of HIV-1 infection (555, 641, 643). Moderate titres of broadly neutralising antibodies can, however, only be detected in around a tenth of chronically infected individuals (641, 653, 654). In spite of the rapid rate of evolution, recent data suggest that there may be limits to the ability of HIV-1 to continuously evolve to escape neutralising antibody responses in chronic infection (655). In further support of this notion, studies of twins infected with the same HIV-1 isolate through needle-

sharing have shown similar neutralising antibody responses and similar changes in the patterns of evolution in the *env* gene, suggesting that there are constraints on the mutational pathways HIV-1 can follow to evade antibody responses (656).

The *in vivo* role of the neutralising antibody response remains unclear. The emergence of neutralisation escape mutants in HIV-1 infection, however, provides indirect evidence for *in vivo* activity of neutralising antibodies. Direct evidence has come from several passive immunisation studies in animal models and in humans. Systemic infusion of neutralising antibodies has protected macaques against systemic and mucosal SIV or SHIV challenge in several studies (657-665). However, the serum titres required to achieve protection are high. In one study, the serum titre (90% *in vitro* neutralisation) required for sterilising protection was estimated to be 1:400 (662), whereas another study estimated that a titre of 1:38 (100% *in vitro* neutralisation) in plasma would protect 99% of macaques (666). Passive immunisation with the broadly neutralising mAb b12 has also been shown to protect severe combined immunodeficient (SCID) mice populated with human PBMCs against HIV-1 challenge (667). In addition, vaginal application of mAb b12 has been shown to protect macaques against mucosal challenge with SHIV (668).

A role for neutralising antibodies in established infection has also come from studies in the macaque model. Haigwood *et al*. (669, 670) have in two subsequent reports demonstrated that passive immunisation of SIV-infected macaques, early in infection, led to reduced viraemia and delayed disease progression. In addition, in 2007 Yamamoto *et al*. (671) showed that passive immunisation of macaques with pooled neutralising sera from chronically infected animals, a week post-challenge with SIV, resulted in a significant reduction of set-point plasma viral loads and preservation of central memory CD4+ T cell levels. Additional support for a possible role of neutralising antibodies in partial control of viraemia *in vivo* has come from studies of acutely and chronically HIV-1-infected humans undergoing interruption of antiretroviral treatment. The study showed that acutely infected individuals who received a cocktail of three neutralising mAbs had a delay in viral rebound compared to study subjects not receiving the mAbs or chronically infected individuals (672).

In contrast, it has been shown that passive immunisation of HIV-1-infected SCID mice populated with human PBMC, with a cocktail of neutralising antibodies, has little effect on viral load and furthermore that virus escape occurs within a few days (673). The observation that less potent and cross-reactive antibody responses predisposes to HIV-1 superinfection further points to a protective role for neutralising antibodies against HIV-1 infection (674).

The presence and role of mucosal IgA in HIV-1 infection has been debated (675). While low levels of HIV-1-specific IgA antibodies has been demonstrated in female and male genital tract secretions of HIV-1-infected patients, the frequency of detection and the levels were much lower than for HIV-1-specific IgG, indicating that unlike many other mucosally encountered microbes, HIV-1 does not induce vigorous specific IgA responses (676). The presence of HIV-1-specific IgA in mucosal secretions of HIV-1-exposed but seronegative individuals has been controversial (677, 678).

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 (679). Such antiviral immune functions can be mediated by neutralising as well as non-neutralising antibodies specific for the HIV-1 envelope glycoproteins (679). The so-called non-neutralising antibodies fail to recognise functional envelope spike but have the potential to bind to non-functional spikes present on the virus surface (142). The non-neutralising anti-envelope glycoprotein antibodies detected early in infection, prior to the appearance of neutralising antibodies, may induce such FcR- or complement-mediated immune effector functions, possibly contributing to the decline in plasma viral load seen at the interface of acute and chronic infection (680-682).

FcR-mediated effector functions include antibody-dependent cellular toxicity, ADCC. In ADCC, FcRs expressed on effector cells such as natural killer cells, granulocytes, monocytes, macrophages and subsets of γδ T cells interact with the Fc portion of antibodies bound to infected cells, leading to the release of

granzymes and perforins and subsequent lysis of the infected cell. In HIV-1-infected individuals, anti-envelope glycoprotein antibodies able to mediate ADCC have been demonstrated early in infection (681, 683-686). In addition to mediating lysis of HIV-1-infected cells, interaction of FcR-bearing effector cells with antibody-virus complexes can lead to the release of cytokines and chemokines that inhibit virus replication in a mechanism referred to as antibody-dependent cell-mediated virus inactivation, or ADCVI (687). ADCVI activity has also been demonstrated in non-neutralising serum that prevents SIV infection of neonatal macaques after oral challenge, possibly suggesting that the mechanism has relevance *in vivo* (688). Serum with potent ADCVI activity has, however, failed to protect against SIV infection of macaques in other studies (689).

Although ADCC and ADCVI have been suggested to influence disease progression in HIV-1-infected individuals, the importance of these mechanisms *in vivo* remain uncertain (679). Antibody-dependent FcR-mediated effector functions also include phagocytosis of antibody-coated viruses by phagocytic cells. Non-neutralising or poorly neutralising antibodies from HIV-1-infected individuals have been shown to inhibit HIV-1 infection of macrophages and immature dendritic cells in an FcR-dependent manner that did not require natural killer cells (690, 691). 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 (692). Further support for a possible role of FcR-mediated immune functions in HIV-1 pathogenesis has come from the observation that polymorphisms in certain FcR genes have been associated with progression of HIV-1 infection (693).

Complement can be activated either by direct interaction with HIV-1 envelope glycoproteins on the virus surface or by interaction with antibody bound to virus particles. Activation of the complement system initiates a cascade of events leading to the formation of a membrane attack complex and lysis of the virus or of infected cells. Several studies have indicated that HIV-1 uses complement

regulatory proteins, present in its host cell-derived membrane, to resist complement-mediated lysis (679). In addition to mediating lysis, the complement system can induce opsonisation and phagocytosis through binding of complement-opsonised virus to complement receptors on phagocytic cells. Aasa-Chapman *et al*. (682) have shown that non-neutralising anti-envelope antibodies detected during peak viral load in acute HIV-1 infection, before the appearance of neutralising antibodies, can induce complement-mediated inactivation of autologous and heterologous virus *in vitro*. Similarly, Huber *et al*. (680) showed that antibodies present in plasma of HIV-1-infected individuals induced complement-mediated inactivation of autologous virus *in vitro*, and that acute infection plasma virus load correlated inversely with complement lysis activity. The observations made in these two studies may indicate a possible role for complement-mediated viral inactivation in control of HIV-1 viraemia.

In addition to mediating effector functions that cause viral inactivation, anti-envelope antibodies have been shown to mediate enhancement of HIV-1 infection *in vitro* (679, 694, 695). Antibody-dependent enhancement of HIV-1 infection can be mediated by binding of antibody-virus complexes to FcRs or by binding of antibody and complement-opsonised virus to complement receptors present on target or bystander cells. Binding to target cells can enhance infection *in cis*, whereas binding to bystander cells can result in enhancement of infection *in trans*. Up to 300-fold complement-mediated antibody-dependent enhancement of infection of target cells bearing complement receptors has been observed using early primary HIV-1 isolates and autologous sera (695). The role of these antibody-mediated effector functions in HIV-1 pathogenesis remains unclear, but is of importance to HIV-1 vaccine design.

As touched upon above, HIV-1, like many viruses, has evolved a number of ways to evade immune responses. For example, the integration of proviral DNA into the host cell genome provides a reservoir of latency since virus is only recognised by the immune system when it is actively replicating. In addition, HIV-1 is able to down-regulate cell surface expression of MHC class I molecules, making infected cells less recognisable by CTLs (263). The main reason for the immune evasive properties of HIV-1 might, however, be credited

to the extreme sequence heterogeneity and the characteristics of the HIV-1 envelope glycoproteins. The immune evasive properties conferred by the HIV-1 envelope glycoproteins will be briefly discussed in section 1.1.6.

1.1.5 Treatment and preventive strategies

In the absence of host immune control of HIV-1 infection, antiretroviral treatment has reduced HIV-1 mortality substantially. However, while current HIV-1 therapy can control viral load and prevent progression to AIDS, it is not available to the majority of the infected population, nor can it completely clear infection. Therefore, in order to control the HIV-1 pandemic, there is an urgent need for preventive measures, such as an effective HIV-1 vaccine or alternative preventive strategies such as HIV-1 microbicides. In this section, antiretroviral therapy, HIV-1 vaccine development, and HIV-1 microbicides will be briefly discussed.

1.1.5.1 Antiretroviral therapy

Available antiretroviral therapy targets several steps in the HIV-1 lifecycle. The first antiretroviral drug to be tested and proven efficient in clinical trials was azidothymidine, or AZT (54). AZT is a nucleoside analogue, which inhibits the viral reverse transcriptase process by terminating DNA chain elongation when incorporated into the nascent DNA chain. It was the most effective of about a hundred nucleoside analogues screened for antiretroviral activity at the Burroughs Wellcome laboratories (now GlaxoSmithKline) in the UK and the National Cancer Institute in the USA. AZT was approved and released into the market in 1987 as Zidovudine, as the first in a series of nucleoside or nucleotide analogue reverse transcriptase inhibitors (NRTIs). Despite the initial success, it soon became apparent that NRTI monotherapy led to the development of HIV-1 resistance and would not have long-lasting efficacy (696). Combination therapy, using more than one NRTIs would, however, prove more efficient (697, 698).

Within ten years after the introduction of NRTI therapy, additional drugs such as the non-nucleoside reverse transcriptase inhibitors (NNRTIs), specifically

targeting the HIV-1 reverse transcriptase, as well as protease inhibitors, binding to the HIV-1 protease hence inhibiting virion maturation, had been developed and approved (699). The introduction of highly active antiretroviral therapy (HAART) in 1996, using a combination of at least three antiretroviral compounds from at least two classes of antiretrovirals, resulted in a massive drop in the number of AIDS deaths in the industrialised part of the world (700-702). Today, there are a total of 24 approved antiretroviral drugs, including eight NRTIs, three NNRTIs, and ten protease inhibitors (699). In addition, there are two approved entry inhibitors, the fusion inhibitor Enfuvirtide, also known as T-20, which targets gp41 (703), and the CCR5 antagonist Maraviroc (704). The latest antiretroviral drug to be approved, in 2007, was an integrase inhibitor (705). Additional antiretroviral drugs are in advanced clinical trials, including further integrase and reverse transcriptase inhibitors, as well as a range of entry inhibitors such as CCR5 and CXCR4 antagonists and compounds targeting CD4 and the HIV-1 envelope glycoproteins (398, 706). Also in clinical trials is the first of the so-called maturation inhibitors, which bind to the Gag precursor polyprotein, thus inhibiting polyprotein cleavage, resulting in the release of defective virions (707). Strategies involving gene therapy are also being investigated (708), as are therapeutic vaccines.

Despite the advances and successes in the development of antiretroviral drugs, the cost and toxicity of antiretroviral therapy remains substantial, as does the problem with drug resistance in circulating and transmitted viruses (709, 710). Moreover, antiretroviral therapy remains unavailable to the vast majority of infected people. To expand the access to antiretroviral therapy in low-income countries, the WHO/UNAIDS launched its ‘3 by 5’ initiative in 2003, aiming at having 3 million people in resource-poor settings on HAART by the end of 2005. While this target was not met in 2005, it is estimated that at the end of 2007 around 3 million people in low- and middle-income countries were receiving antiretroviral therapy, nearly a million more than at the end of 2006 (711). In spite of the progress, the treatment coverage remains low, with less than a third of the people who would need it receiving therapy (711).

1.1.5.2 HIV-1 vaccine development

The field of vaccinology is said to have started with the experiments carried out by Edward Jenner in 1796, showing that inoculation with cowpox virus could protect humans against smallpox infection (712). Since then, smallpox has been declared eradicated following a mass vaccination programme (713, 714), and vaccines have been effective in preventing human disease caused by a range of viruses, including influenza, rabies, hepatitis A and B, rubella, measles, mumps and poliovirus (712, 715). For these and most other successful vaccines, protection has typically been achieved by mimicking infection with the pathogen itself, using live attenuated or inactivated pathogen preparations or subunit vaccines (715). Although antibody titre appears to be the primary immune correlate of protection in most cases, the relative contributions of different antibody functions are unclear and the precise mechanisms by which vaccines prevent infection or disease remain undefined (716). For many pathogens, it is likely that both the humoral and the cellular arms of the immune system are important for long-lasting protection (716).

Despite the overall success in developing vaccines against a range of other viral diseases, all attempts to develop a successful HIV-1 vaccine have so far failed (717). Several features of HIV-1 make it a challenging target for vaccine design, in particular its high mutation rate and remarkable genetic diversity, which by far exceeds that of other entire virus families. The immune evasive properties of the HIV-1 envelope glycoproteins (discussed in section 1.1.6) are other major obstacles in vaccine development, as is the ability of HIV-1 to remain latent in cells and to down-regulate cell surface expression of MHC.

Moreover, the correlates of immune protection in HIV-1 infection remain undefined, further contributing to the difficulties in developing an HIV-1 vaccine (718-720). However, even though the role of antibodies in natural HIV-1 infection is unclear, the observation that passively transferred neutralising antibodies can protect macaques against SHIV and SIV challenge (657-665) indicates that if elicited, antibodies could potentially prevent infection. Similarly, evidence for a role of T cells in the control of viraemia in HIV and SIV infection,

has provided a rationale for the development of vaccine aimed at inducing T cell responses (717, 721, 722). Overall, 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 (717, 723, 724).

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 (717, 718, 725-727). Although the immune responses in vaccinees and infected individuals can be evaluated in *in vitro* assays, such as neutralisation and IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays, data obtained *in vitro* do not necessarily correspond to the *in vivo* situation in humans, nor do they necessarily correlate with results obtained in animal models (718, 727). In addition, data from animal models do not necessarily predict outcome in human trials (725). Virus transmission in the best available animal model, the SHIV/SIV macaque model, is likely to differ substantially from HIV-1 transmission in humans (725, 726). 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.

The vaccine candidates that has so far provided the best protection in the macaque model are live attenuated SIVs (726, 728, 729). A live attenuated viral vaccine contains live virus that have been attenuated, for example through extensive cell-culture passaging or direct genetic modification. This kind of vaccine typically elicits long-lasting humoral and cellular immune responses and has been highly successful when developing vaccines against measles, rubella, mumps, yellow fever and poliovirus (716). 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 (730-733). Interestingly, strong protection of macaques immunised with a live attenuated SIV have been observed even in the absence of high-titre anti-SIV antibodies and high-frequency anti-SIV cellular immune responses, as measured by neutralisation and IFN-γ ELISPOT assays, high-lighting the lack of

knowledge of what correlates with immune protection (734). Even though immunisation with live attenuated SIV provides protection against homologous challenge, protection against heterologous SIV isolates has been less effective (735-737). In addition, there are safety issues with this approach, 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. The *in vivo* repair of an attenuating deletion in the *nef* gene of a SIV molecular clone, associated with a reversion to virulence, has been reported in macaques (738). In another study, carried out by Baba *et al*. (739), macaques were infected with an SIV isolate with large deletions in the *nef*, *vpr* and negative regulatory element genes. Adult monkeys that were infected with this attenuated virus were protected from subsequent infection with pathogenic SIV but did develop disease with a delayed onset from the attenuated virus. Moreover, the attenuated virus caused disease soon after infection in newborn monkeys (739), albeit in some studies only after challenge with high viral doses (740).

Immunising with inactivated or killed virus preparations may be safer, and have proven effective when developing vaccines against hepatitis A, rabies, influenza and poliovirus (715). However, even though inactivated SIV has conferred some short-lived protection in the macaque model, it has been mainly against identical virus (741). Virus-like particles (VLPs) are also being explored as vaccine candidates (742).

Subunits vaccines can be purified from crude viral extractions, or made from recombinant proteins. Recombinant viral proteins are easily produced and generally thought to be safe to administer. This approach has been used to develop a vaccine against hepatitis B virus. The recombinant hepatitis B vaccine was licensed in 1986 and contains recombinant viral surface antigen expressed in yeast (743, 744). Two recombinant vaccines against certain strains of human papilloma virus (HPV-6, -11, -16 and -18 or HPV-16 and -18 only) have recently been approved (745-748). These vaccines are based on yeast- or baculovirus-expressed recombinant HPV capsid protein L1, which self-assembles into virus-like particles, VLPs (749).

The first HIV-1 vaccine candidates to enter clinical efficacy trials in humans were two vaccine candidates developed by VaxGen (San Francisco, USA), containing recombinant gp120 derived from HIV-1 of subtype B and A/E. The subtype B gp120 had previously been shown to protect chimpanzees against challenge with homologous virus (750). Both candidates were taken through clinical safety and efficacy trials in the USA and/or in Thailand, but 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* (751-753).

Given what is now known about the immune evasive mechanisms of the HIV-1 envelope glycoproteins and the extreme HIV-1 genetic diversity, the results of the VaxGen trials were perhaps not surprising (721). Great efforts are currently focused on designing immunogens that can elicit a broader and more potent neutralising antibody response (259, 754). 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 (section 1.1.6). However, so far, no immunogen have been developed that elicit a neutralising antibody response of the breadth and potency that will be required of a vaccine (717, 754). To solve the problem of how to elicit broadly neutralising antibodies, further knowledge about the antigenicity and immunogenicity of the HIV-1 envelope glycoproteins is needed.

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 (Fig. 1.1.4.3.1) and delayed onset of disease (717, 721, 722). One of these strategies involves the evaluation of naked DNA encoding HIV-1 proteins. However, even though intramuscular administration of plasmid DNA encoding HIV-1 proteins can elicit cellular responses in mice, these DNA vaccines have been shown to be less immunogenic in non-human primates (722, 755) and even less immunogenic in humans (756).

Instead, the use of DNA vaccines as priming vaccines in various prime-boost strategies has been more successful, eliciting better responses than the boosting vaccine alone (757-762). Co-expression with cytokines may also increase immunogenicity (763).

HIV-1 genes can also be incorporated into the genome of microorganisms that are not pathogenic in humans, which may be a way of mimicking the efficacy of live attenuated vaccines but without the safety concerns associated with live attenuated HIV-1 (755). For this purpose, the use of adenoviruses, adeno-associated viruses and poxviruses, including modified vaccinia virus Ankara, canarypox and fowlpox virus, have been explored (755). These vectors have been engineered to express various HIV-1 antigens, such as Gag, Pol and the envelope glycoproteins. A vaccine candidate developed by Merck, based on a replication-defective adenovirus type 5 vector engineered to express HIV-1 Gag, Pol and Nef proteins, was taken forward to clinical efficacy testing in 2004 in the so-called STEP trial conducted by Merck and the HIV Vaccine Trials Network (764). 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. Moreover, results obtained in the macaque model had indicated some level of protection, as immunisation with a replication-defective adenovirus type 5 vector expressing SIV proteins could partially protect against subsequent challenge with pathogenic SHIV89.6P (758). Whereas control animals showed rapid disease progression, the immunised animals were generally able to contain virus replication and rarely developed disease, showing a 1-3 log decrease in viral load (758). However, when tested in humans, the Merck vaccine candidate failed to prevent infection and did not reduce the viral load in subsequently infected individuals (67, 764). In addition, it may even have enhanced infection in vaccinated individuals with pre-existing immunity to adenovirus of serotype 5 (765, 766).

While results obtained in the SHIV89.6 macaque model did not predict the outcome of the STEP trial, the failure of the STEP trial has been mirrored by results observed in other macaque experiments (725, 767). Although the vaccine strategy used in the STEP trial, along with a number of other vector-based

vaccine candidates, has been able to partially protect macaques against pathogenic SHIV89.6 challenge (758, 759, 763, 768), vector-based vaccine regimens have rarely led to reduced levels of disease or viral load in macaques challenged with various SIV isolates, including SIV_{mac239} (767, 769-771). In addition, in some cases protection has only been observed in macaques carrying a specific HLA allele (767, 772). It has been argued that infection of macaques with CCR5-using SIV isolates represents a better model of HIV-1 infection in humans than infection of macaques with the CXCR4-using SHIV89.6 (773-776).

In addition to the Merck vaccine candidate, one canarypox vector developed by Sanofi-Pasteur is currently in clinical efficacy trials in Thailand in around 16000 volunteers, in a prime-boost regime together with the recombinant gp120 vaccine candidate developed by VaxGen (722). 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 (777, 778). The results of the large-scale efficacy trial are expected in 2009.

Since HIV-1 is predominantly spread across mucosal surfaces, efforts to develop mucosal vaccine candidates have also been made (779). It has been shown that mucosal administration of a vaccine often induces more potent mucosal immune responses than administration by injection (780, 781). Mucosal administration of several vaccine candidates have been shown to reduce viral loads in macaques after mucosal challenge with SHIV (782-786).

Overall, a large number of HIV-1 vaccine candidates have been evaluated in phase I or II clinical safety and immunogenicity trials (717, 721, 724, 755). None of these vaccine strategies have, however, been able to protect macaques against SIV challenge (725, 726). 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 (717). In light of these notions, an HIV-1 vaccine remains elusive. It is clear that further

knowledge is needed about the correlates of immune protection in HIV-1 and SIV infection, and how to elicit such protective immune responses.

1.1.5.3 Topical preventive strategies

Without a promising HIV-1 vaccine candidate in sight, there is a need for alternative preventive strategies to stop the rapid spread of the HIV-1 pandemic. While the use of condoms can prevent transmission, it is largely male-controlled and may not be compatible with marriage, nor can it be combined with reproduction. Therefore, alternative prevention strategies are being explored, such as the development of topical microbicides aimed at inhibiting sexual transmission of HIV-1 (787-794). Microbicides are agents that can be applied topically in the vagina or rectum. As microbicides could be potentially inexpensive, readily available and socially acceptable, they could offer a promising preventive intervention for sexual transmission of HIV-1.

A useful microbicide must fulfil several criteria (787-794). First, self-evidently, it must show good efficacy against relevant HIV-1 variants. The extraordinary genetic heterogeneity observed among HIV-1 isolates may hence be a problem (123). Evaluating efficacy prior to clinical efficacy testing in humans may also be problematic, as results obtained in *in vitro* experiments and animal models may not necessarily predict outcome in human trials (791, 794-797). Currently, *in vitro* cell culture systems, human explant models (533, 541), and animal models are being used, including humanised mice models (798, 799) and the SHIV (or SIV) macaque vaginal transmission model (509, 668, 800-804). While the macaque transmission model is the most relevant animal model available, it has drawbacks. For example, the limited number of SHIVs available may not be representative of the majority of the genetically diverse circulating HIV-1 strains. In addition, it is not clear which of the currently used protocols, involving low or high doses of challenge virus administered on single or multiple occasions, that better represents human HIV-1 transmission (509, 668, 800-812). Some (805) but not all (806, 811) microbicide candidates have failed to protect macaques against repeated vaginal virus challenges, despite being able to protect against a single challenge.

In addition to being efficacious, it must be ensured that microbicides do not reduce the natural defences against HIV-1 infection. A potential microbicide must not cause inflammation or have adverse effects on the resident microflora or the structural integrity of the mucosal epithelia, as this might increase the risk of transmission (788, 789, 793, 794, 796). Microbicide safety is initially assessed in *in vitro* systems as well as in animal models, and eventually in clinical safety trials in humans. Moreover, a useful microbicide should retain its potency in the presence of semen (813). It would also need to be stable at a range of temperatures, easy to use, inexpensive, readily accessible, compatible with condom use, and acceptable to users and sexual partners (787-794).

A large number of compounds are currently being evaluated as microbicides (787, 788). Many of them aim at inactivating the incoming virus before it has the chance to infect (788). Compounds such as detergents or surfactants 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 (788). However, many of these compounds show little selectivity, and could potentially be toxic for host cells and for resident microflora. As a result, rather than providing protection, such agents could actually increase the risk of HIV-1 transmission, as observed for nonoxynol-9, the first compound to be evaluated as an HIV-1 microbicide in clinical efficacy trials (Table 1.1.5.3.1).

Nonoxynol-9 is a membrane-disruptive non-ionic surfactant originally developed in the 1960s as a spermicide used in lubricants and to coat condoms. Anti-HIV-1 activity was first demonstrated *in vitro* in 1985 (814) and *in vivo* in animal models in 1992 (815). Safety studies conducted in women predicted low doses of nonoxynol-9 to be safe, although signs of inflammation were also reported (796, 816, 817). Two large-scale clinical efficacy trials were carried out (Table 1.1.5.3.1). In one of them, nonoxynol-9 was found to have no effect on HIV-1 incidence (818), whereas in the other, it increased the risk of transmission when used more than three times a day (819). Studies carried out after the clinical efficacy trials indicated that nonoxynol-9 increased the risk of transmission by disrupting the membranes of the cells in the epithelial layer of the vagina and cervix, thereby damaging the protective barrier of the epithelium and causing

inflammation, leading to the recruitment of target cells to the mucosa (796, 820, 821). A second surfactant, 31G or Savvy (Cellegy Pharmaceuticals, Huntingdon Valley, USA), was also entered into large-scale clinical efficacy trials (Table 1.1.5.3.1). These trials were, however, prematurely discontinued, as the observed HIV-1 incidence at the trial sites were determined to be too low (822). In a total of 2153 women, 33 seroconversions were observed, 21 in the group receiving Savvy and 12 in the placebo group (822).

So-called pH-modifying or buffering agents are also being evaluated as microbicides. These agents aim at inactivating virus particles by maintaining a low vaginal pH, in particular in presence of the alkaline semen. The low pH of the vagina, normally around 3.5-4.5, is part of the innate defence against microbial pathogens, and is predominantly due to lactic acid and H_2O_2 production by vaginal lactobacilli (823). HIV-1 is typically inactivated at pH below 4.5. One buffering agent, the carbopol polymer Buffergel (ReProtect, Baltimore, USA), is currently being evaluated in clinical efficacy trials (Table 1.1.5.3.1). In addition to inhibiting HIV-1 transmission, it has been shown to inhibit pregnancy, herpes simplex virus (HSV), HPV, and *Chlamydia trachomatis* infections in preclinical studies carried out in animal models, without damaging the resident microflora or the reproductive epithelium (824). Furthermore, data from clinical safety trials have indicated that it is well tolerated and safe to use in humans (825, 826), although some changes in the vaginal microflora have been observed (827). Lime juice has also been suggested as a candidate microbicide aimed at keeping the vaginal environment acidic. However, studies have indicated that the concentrations needed for effective HIV-1 prevention would potentially cause damage to the mucosa, increasing the risk of infection (828).

Other microbicide candidates under development aim at blocking steps in the early stages of the HIV-1 life-cycle, such as virus entry into cells. Candidates aimed at non-specifically inhibiting HIV-1 entry include a variety of anionic polymers or polyanions, which are thought to bind to positively charged regions of gp120, such as the V3 region and the co-receptor binding site (829, 830). Polyanions are often more active against CXCR4-using viruses (830-834), as the

V3 regions of CXCR4-using viruses tend to have a higher net positive charge than those of CCR5-using viruses (421). Three polyanions have so far entered large-scale clinical efficacy trials, cellulose sulphate, Carraguard, and PRO 2000/5 (Table 1.1.5.3.1).

Cellulose sulphate, also known as Ushercell (Polydex, Toronto, Canada), had shown good activity against HIV-1, HSV, HPV, *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Gardnerella vaginalis* in preclinical studies, without having an effect against lactobacilli (835-838). Moreover, it was found to be safe in three different clinical safety trials and one contraceptive efficacy trial (837, 839-841). Nevertheless, in two clinical efficacy trials carried out in a total of 4716 women in high-risk populations, cellulose sulphate failed to protect against HIV-1 infection (842). In one of the trials there seemed to be a trend towards an enhanced risk of transmission, leading to the premature halting of both trials in January 2007 (797, 842). The cause of the apparent enhanced risk of infection remains unclear (797).

The second polyanion to enter clinical trials was Carraguard (Population Council, New York, USA), which contains a carrageenan extracted from the red seaweed *Gigartina skottsbergii* (793, 843). Carrageenans are sulphated polysaccharides that have been widely used as gelling agents in food, cosmetics and lubricants. In addition to having *in vitro* anti-HIV-1 activity (831, 843, 844), they have also been shown to be active against a range of sexually transmitted pathogens (845-848). As Carraguard appeared safe in clinical safety studies (849, 850) it was taken forward to clinical efficacy trials carried out in 6270 women in South Africa (Table 1.1.5.3.1). The results of the trials were announced in February 2008, revealing that no effect on HIV-1 incidence had been observed (851). Hence, Carraguard became the fourth microbicide candidate to fail clinical efficacy trials.

According to some researchers (795), the failure of two polyanions to protect against HIV-1 transmission in clinical efficacy trials is not surprising, as the compounds show limited potency *in vitro*, particularly against CCR5-using viruses. Moreover, in addition to being able to inhibit HIV-1 infection,

polyanions have been shown to enhance *in vitro* infection of CCR5-using viruses in some studies (852-854). Preclinical studies carried out by Neurath *et al*. (813) have also shown that the *in vitro* antiviral activity of polyanions, including cellulose sulphate, carrageenans and PRO 2000/5 (discussed below), is reduced in the presence of semen. Up to 70-fold reductions in potency were observed in some cases. The reduced potency may be due to binding of the polyanions to the semen polyamines spermine, spermidine and putrescine, which are positively charged at neutral pH (813).

A third polyanion, PRO 2000/5 (Indevus Pharmaceuticals, Lexington, USA), a synthetic naphthalene sulphonate polymer, is still in clinical efficacy trials (Table 1.1.5.3.1). PRO 2000/5 is active against HIV-1 *in vitro* (533, 541, 832, 834, 855-857) and against SHIV in the macaque model (812). Two large-scale efficacy trials of PRO 2000/5 were started. The first trial, funded by the USA National Institute of Health, is carried out in 3220 women using PRO 2000/5, Buffergel, a placebo or condom. The second trial, sponsored by the UK Medical Research Council, aimed to compare two different concentrations of PRO 2000/5. In February 2008, it was announced that one arm of the second trial was going to be discontinued due to lack of evidence of a protective effect in women (858). This arm of the clinical trial tested the efficacy of a 2% formulation of the PRO 2000/5 gel compared to a placebo gel. The remaining arm of the trial, testing the efficacy of a 0.5% formulation of PRO 2000/5 in a total of 9395 women, will, however, be continued (859).

HIV-1 entry inhibitors that specifically bind to gp120 or gp41 are also being developed and evaluated as candidate microbicides (787, 788). These compounds include lectins, which recognise glycans on the viral envelope glycoproteins. Several plant lectins have been reported to have HIV-1 inhibitory activity (860-863). One of the most extensively studied lectin candidates is cyanovirin-N, which is originally isolated from the cyanobacterium *Nostoc ellipsosporum*. It recognises α1,2-mannose glycans on gp120 (864) and inhibits HIV-1 infection *in vitro* (865). Topical application of cyanovirin-N has been shown to prevent infection of macaques after vaginal SHIV challenge (808, 809).

In addition to lectins, mAbs to gp120 and gp41 are also being considered for microbicides use (787, 788). These mAbs include anti-gp120 mAbs b12 and 2G12 and anti-gp41 mAb 2F5, which will be discussed in further detail in section 1.1.6. Vaginal application of the anti-gp120 mAb b12 has been shown to protect macaques against vaginal SHIV challenge (668). PRO 542, which is a tetravalent construct consisting of CD4 fused to an IgG2 immunoglobulin has been shown to inhibit HIV-1 infection in cervical explant models (538, 866) and could be a potential microbicide candidate.

Small molecules may offer advantages in terms of cost and large-scale production over large proteins such as full immunoglobulins (794). The small molecule BMS-378806 binds to gp120 and inhibits HIV-1 entry *in vitro* (867-870) and in the macaque model (871) and may be useful in microbicide development. Peptides based on the second heptad repeat in gp41 (section 1.1.6), which can inhibit fusion of viral end cellular membranes, are other small molecules being considered as candidate microbicides (787). The gp41 peptide and fusion inhibitor Enfuvirtide (T-20) is already approved as a therapeutic for HIV-1 infection (703, 872) and could be a potential microbicide candidate (787). The gp41 peptides T-1249 and C52L can inhibit SHIV infection of macaques after vaginal challenge (787, 871). Small molecules such as CD4 mimics (873) and RNA aptamers binding to gp120 and able to inhibit HIV-1 infection (874, 875) might also be potential microbicide candidates.

Other entry inhibitors considered as microbicide candidates target the HIV-1 cellular receptors, CD4, CXCR4 or CCR5. Vaginal application of PSC-RANTES, a modified form of the CCR5 ligand RANTES, has been shown to protect macaques against vaginal SHIV challenge (804). PSC-RANTES causes internalisation and down-regulation of CCR5, which is thought to contribute to its anti-HIV-1 activity (804, 876-878). The small CCR5-binding molecule CMPD167 can also inhibit vaginal transmission of CCR5-using SHIVs to macaques (871). PSC-RANTES and CMPD167 may hence be potential microbicide candidates, as may the CCR5 antagonist Maraviroc (704), which has been licensed for treatment of HIV-1 infection, and the anti-CCR5 mAb PRO 140, which has been tested in clinical trials as an injectable therapeutic (879). In

addition, a range of other CCR5-binding compounds have been tested for antiviral and microbicide activity in *in vitro* studies and animal models (787, 788). Compounds targeting CXCR4 are also being developed. The CXCR4-binding small-molecule AMD3465 (880) is, for example, currently being evaluated in the macaque transmission model (787). Small molecules (881) and mAbs (882) that inhibit HIV-1 infection by binding to CD4 on target cells are also of potential interest, as are certain cyclotriazadisulfonamide (CADA) molecules, which have been shown to downregulate CD4 in a reversible fashion (883).

In summary, a large number of HIV-1 entry inhibitors have been assessed for microbicide activity in preclinical studies. However, apart from the polyanions, no entry inhibitors have so far been evaluated as microbicides in clinical trials in humans.

With disappointing results in all clinical efficacy trials completed to date (Table 1.1.3.5.1), increasing efforts are being made to develop microbicides containing existing antiretroviral drugs such as the NRTIs and NNRTIs (787, 795). One candidate, a gel containing the NRTI Tenofovir, has so far advanced to large-scale clinical efficacy trials (Table 1.1.5.3.1). In preclinical studies, rectal application of Tenofovir has been shown to protect macaques from SIV infection following rectal challenge (884). Microbicides containing the NNRTIs Dapivirine (TMC120) and UC-781 are currently being evaluated in safety trials (787). Combination microbicides, such as microbicides containing a mix of carrageenan and NNRTIs, are also being explored (885). Whether the use of NRTIs and NNRTIs for HIV-1 prevention will contribute to the spread of HIV-1 resistance to the same drugs remains unclear (886, 887).

Since the mucosal layer contains dendritic cells that can bind HIV-1 and possibly mediate transport of HIV-1 to lymph nodes, agents that are able to block binding of DC-SIGN to HIV-1 have also been under evaluation as microbicides (788). These agents include mannan, an oligosaccharide rich in mannose, which has been shown to bind to DC-SIGN and to block DC binding to HIV-1 gp120 (474). However, when tested in the macaque model, topical application of mannan

failed to protect against vaginal SHIV challenge (871), highlighting the notion that the role of DC-SIGN in HIV-1 transmission remains unclear and that a better understanding of the exact mechanism of HIV-1 transmission is important for microbicide design.

Table 1.1.5.3.1. Microbicide candidates that have entered efficacy trials[a].

Candidate	Type[b]	Status/outcome
Nonyxol-9 film	S	Completed, no effect on incidence
Nonyxol-9 gel	S	Completed, increased incidence
Savvy™ (C31G)	S	Halted due to too low incidence
Ushercell™ (cellulose sulphate)	PA	Halted due to increased incidence
Carraguard™	PA	Completed, no effect on incidence
2% PRO 2000/5	PA	Halted due to lack of effect
0.5% PRO 2000/5	PA	Ongoing
Buffergel and 0.5% PRO2000/5	BA, PA	Ongoing
Tenofovir	NRTI	Ongoing

[a]Microbicide candidates that have entered large-scale clinical efficacy trials, as of September 2008, based on information compiled and presented by the Alliance for microbicide development (888) and van de Wijgert *et al*. (797).

[b]S, Surfactant; PA, polyanion; BA, buffering agent; NRTI, nucleotide analogue reverse transcriptase inhibitor.

In addition to developing compounds with anti-HIV-1 activity, emphasis has also been put on the development of microbicide formulations and delivery systems (788). Most of the microbicides evaluated in clinical trials to date have been formulated as gels to be applied intravaginally or intrarectally. However, films, foams and tablets are also under development, as are sustained-release devices like vaginal rings (889). Vaginal rings slowly releasing the NNRTI TMC-120 have been developed and evaluated in preclinical systems (890), and clinical safety studies are underway.

Live commensal bacteria engineered to express HIV-1 inhibitors and able to colonise the vagina or rectum may also offer a promising delivery system. For example, Chang *et al*. (891) engineered a human vaginal isolate of *Lactobacillus*

jensenii to secrete a two-domain version of CD4, which showed HIV-1 inhibitory effects *in vitro*. Lactobacilli expressing surface-associated two-domain CD4 have also been generated (892). Similarly, Rao *et al*. (893) engineered a probiotic *Escherichia coli* (*E. coli*) strain to express HIV-1 fusion inhibitors, whereas Pusch *et al*. (894, 895) developed lactobacilli strains engineered to express either the plant lectin cyanovirin-N or HIV-1 fusion inhibitors. The use of live lactobacilli as microbicides may also enhance the natural vaginal defences against HIV-1 and other pathogens (823). Using genetically engineered organisms as microbicides might, however, cause controversy, similar to the controversy caused by genetically modified food.

1.1.6 A closer look at HIV-1 envelope, entry and neutralisation

The HIV-1 envelope glycoproteins are the main targets for neutralising antibodies. The work in this thesis will focus on camelid antibodies to the CD4-binding site (CD4bs) of the HIV-1 envelope glycoprotein gp120. The structure and properties of the HIV-1 envelope spike as well as the mechanism behind HIV-1 entry and neutralisation will therefore be further reviewed in this section.

1.1.6.1 Structure and distribution of the HIV-1 envelope spike

The HIV-1 envelope spike is made up of the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 (128-131). The surface glycoprotein gp120 is weakly associated with the membrane-spanning gp41 via non-covalent interactions between discontinuous structures in its N- and C-terminal regions and the ectodomain of gp41 (896). The functional HIV-1 envelope spike exist as a trimer of the gp120/gp41 heterodimers (138-140, 260, 261). In addition to functional trimeric spikes, there is evidence for non-functional spikes on the virus surface, such as gp120/gp41 monomers and gp41 stumps formed after shedding of gp120 (141, 142, 897). Non-functional spikes could also include different isoforms of gp120/gp41 trimers as well as trimers or other oligomeric forms of uncleaved gp160 precursor (142).

The transmembrane protein gp41 contains a cytoplasmic tail, a membrane-spanning domain and an ectodomain (139). 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). The ectodomain is thought to be responsible for oligomerisation (898, 899). High-resolution 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 (261, 900). Formation of the six-helix bundle is essential for fusion of the cellular and viral membranes. Little is known, however, about the pre-fusion structure of gp41 in the native spike, prior to interaction of the spike with cellular receptors. Antibody binding studies have indicated that in the context of the functional spike, gp41 is relatively occluded from recognition by antibodies (901-904). 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 (905). 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 it (906-908). There are two atomic structures available of fragments of the MPER in complex with two neutralising antibodies, 2F5 and 4E10 (909, 910). The fusion inhibitor Enfuvirtide (T-20) is based on the C-HR and binds to the N-HR (703, 872).

The surface glycoprotein gp120 consists of five conserved (C1-C5) and five variable (V1-V5) regions (911-913). Prior to any high-resolution structural knowledge, antibody competition and mutational studies provided insight into the function and spatial location of the various gp120 regions (896, 914-919). 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 (917, 919). 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 (917, 918) and since mutations in these

regions could abolish gp120/gp41 interactions (896). Similarly, observations made in mutational studies also indicated that the variable regions mask more conserved sites of gp120, such as the CD4bs (914, 915). 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 (914, 917, 920-922). Deletions in the V4 region has been observed to result in impaired folding and processing of gp160 into gp120 and gp41 subunits (914). In early studies, CD4 binding was observed to be dependent of sequences within the C3 and C4 regions (414, 923, 924).

The first high-resolution structural data came in 1998, when Kwong *et al*. (348) used X-ray crystallography to solve the atomic structure of a deglycosylated gp120 core (lacking the more flexible V1, V2 and V3 regions as well as the N- and C-termini) from the TCLA isolate HXB2 and liganded to soluble CD4 (sCD4) and an antibody to a CD4-induced epitope, 17b. The crystal structure of a primary isolate gp120 core liganded to sCD4 has since been solved and found to be more or less superimposable with the HXB2 structure (925). In 2005, the atomic structure of a fully glycosylated and unliganded SIV gp120 core was solved by Chen *et al*. (926), providing further valuable insight into the conformational features of gp120. Structural data on the V3 region in the context of gp120 was also obtained in 2005, when Huang *et al*. (927) reported the structure of a V3-containing gp120 core liganded to sCD4 and X5, an antibody to a CD4-indiced epitope, revealing that the V3 forms a 50 Å long and 15 Å wide structure that protrudes from the core, confirming observations made in mutagenesis and antibody binding studies and further explaining its immunodominant properties. The tip of the V3 region, consisting of a β-hairpin structure, was found to be structurally conserved, as it displayed similar conformations in the settings of the gp120 core and as a free peptide or in the context of V3 peptide-antibody complexes (927).

The first structure reported by Kwong *et al*. (348) revealed that the core of monomeric, CD4-liganded gp120 has the approximate dimension of 50 × 50 × 25 Å and 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 (Fig. 1.1.6.1.1). 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 (348, 928), as well as the above mentioned antibody binding and mutagenesis studies (917, 919). The bridging sheet consists of four anti-parallel β-strands organised into the β2-β3 and β20-β21 ribbons (348). The V1 and V2 regions stem from the β2-β3 ribbon. The outer domain is thought to be located on the outside of the envelope spike and contains the V3, V4 and V5 variable regions (348, 927). It is heavily glycosylated, making it less visible to the host immune system (348, 928).

The CD4bs lies at the interface of the outer domain, the bridging sheet and the inner domain (Fig. 1.1.6.1.1), and covers more than 800 $Å^2$ of the gp120 surface (348). Twenty-six amino acid residues in gp120 make contact with twenty-two residues in the D1 domain of CD4 (348). In gp120, these residues are located in the stem of the V1/V2 region (one residue), the β20-β21 ribbon of the bridging sheet (six residues), the connection between an α-helix in the inner domain and a β-strand in the outer domain (four residues), and a loop (four residues), a β–strand (five residues) and an excursion made up by an α-helix and a β-strand, referred to as the CD4-binding loop (six residues), in the outer domain (Fig. 1.1.6.1.1.). The CD4-gp120 interaction is mainly electrostatic but also results from 219 van der Waals contacts and twelve hydrogen bonds (348). Around 60% of the contacts are made by main-chain atoms in gp120, allowing for gp120 sequence variability.

The interface of gp120 and CD4 contains two large cavities (348). The larger cavity is 279 $Å^3$ in size, lined primarily by hydrophilic residues and water-filled. The gp120 residues lining this cavity are highly variable, probably aiding the ability of HIV-1 to escape recognition by antibodies to CD4bs-related epitopes. The smaller cavity is 152 $Å^3$, extends approximately 10 Å into the centre of gp120 and is lined mainly by highly conserved, hydrophobic residues. The phenylalanine-43 (Phe43) residue of CD4, responsible for 23% of the interatomic

contacts CD4 makes with gp120, reaches in to and interacts with this cavity, forming a lid at one end.

Comparison of the CD4-liganded HIV-1 gp120 and the unliganded SIV gp120 structures (Fig. 1.1.6.1.1) has demonstrated that binding of CD4 to gp120 results in substantial conformational changes in gp120 (926). This observation is in agreement with antibody binding experiments using gp120 complexed to sCD4 (901), and the unusually large reduction in entropy that have been observed for gp120 upon binding to CD4 in thermodynamic studies carried out by Myszka *et al*. (929). The high entropy of the unliganded gp120 indicates conformational flexibility in the absence of CD4 (929), in keeping with observations made in mathematical modelling experiments, which have indicated that free gp120 may oscillate to assume a conformation resembling the CD4-bound state (930). In further support of these notions, Ashish *et al*. (931) have reported a set of solution small-angle X-ray scattering experiments of a fully glycosylated, full-length gp120 in its unliganded state and bound to sCD4, demonstrating a gp120 structure with several protrusions, some of which undergo substantial conformational rearrangements upon CD4 binding.

According to the atomic structural data reported by Kwong *et al*. (348) and Chen *et al*. (926), it is predominantly the inner domain of gp120 that has undergone extensive rearrangements, whereas the outer domains of both the unliganded and the CD4-liganded gp120 structure have essentially the same conformation. Moreover, in the unliganded structure, the bridging sheet does not exist; even though its two β-ribbons are present, they are separated by up to 25 Å (926). The CD4bs, as seen in the CD4-liganded state, is not present in the unliganded gp120 (Fig. 1.1.6.1.1). Instead, in the unliganded gp120, 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. The position of the V1 and V2 stem indicates that the V1 and V2 regions may cover part of the inner domain, including this cavity, whereas the V3 region may

cover the β20-β21 ribbon (926). The V1 and V2 regions have been suggested to make contact the V3 region of another gp120 subunit in the spike (926, 932).

The co-receptor binding site is less well-defined than the CD4bs, as there is no available crystal structure of gp120 bound to any of the co-receptors. Instead, insight into the gp120-co-receptor interaction has come from mutagenesis studies and from studies of the interaction of gp120 with antibodies to CD4-induced epitopes, as well as from recent NMR data. The V3 region of gp120 has been shown to be critical for co-receptor binding (933-937) and is thought to interact with the second extracellular loop of the co-receptor (376-378). In addition to the V3 region, several highly conserved amino acid residues in the gp120 core have been shown to have a role in co-receptor binding (938, 939) and to interact with the N-terminus of the co-receptor (376-378). A recent NMR structure of the N-terminus of CCR5 bound to CD4-liganded gp120, reported by Huang *et al*. (379), have supported these data, demonstrating that the CCR5 N-terminus binds to a site at the interface of the bridging sheet and the stem of the V3 region in gp120.

In the CD4-liganded gp120 core structure, the residues implied to be involved in co-receptor binding are located close to each other (Fig. 1.1.6.1.1), in the region connecting the gp120 bridging sheet and the outer domain and close to the V3 region (938, 939). In the unliganded structure, however, they are separated, in keeping with the notion that the co-receptor binding site is only assembled and presented after CD4 binding (139, 259, 926, 934-936). The structures of the liganded and unliganded gp120 core constructs, solved by Kwong *et al*. (348) and Chen *et al*. (926), are shown in Fig. 1.1.6.1.1.

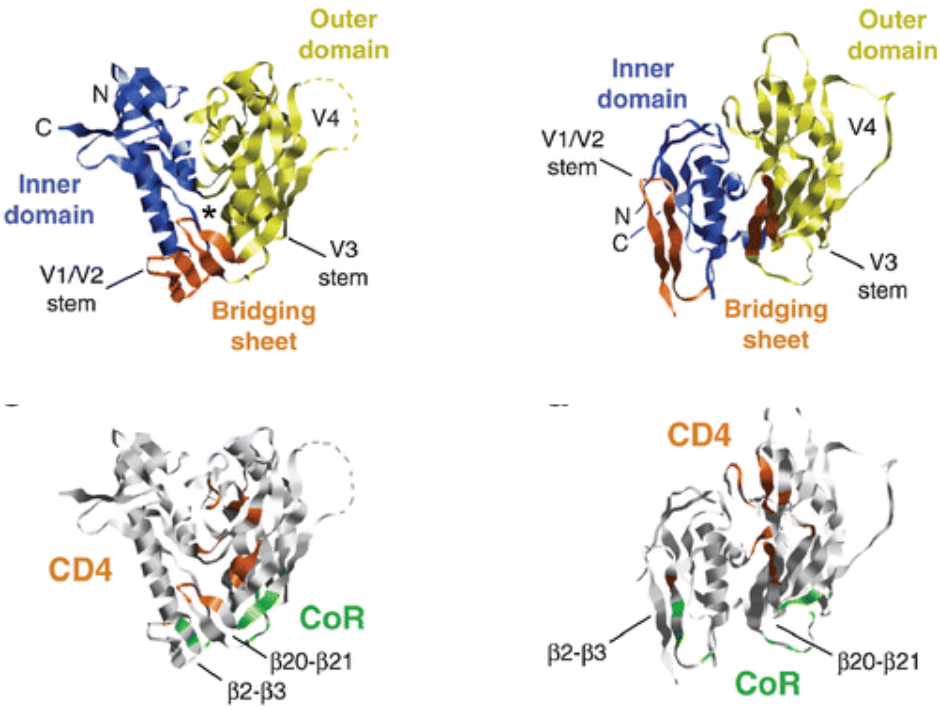


Figure 1.1.6.1.1. Ribbon diagrams of HIV-1 and SIV gp120 cores. The left panel shows the structure of a deglycosylated HIV-1 HXB2 gp120 core (lacking the V1/V2 and V3 variable regions and with truncated N- and C-termini), liganded to sCD4 and an antibody to the CD4-induced site, solved by Kwong *et al*. (348) The structure is shown from the perspective of CD4. The right panel shows the structure of a fully glycosylated, unliganded SIV gp120 core solved by Chen *et al*. (926). In the top panel, the bridging sheet is shown in orange, and the outer and inner domains are shown in yellow and blue, respectively. The positions of the variable regions and the N- and C-termini are also indicated. The location of the cavity into which the CD4 Phe43 residue binds is indicated by an asterisk. In the lower panel, the amino acid residues in contact with CD4 in the liganded state are shown in orange and residues that have been shown to influence co-receptor binding are shown in green. The figure is obtained from Pantophlet, R., and D. R. Burton, 2006, 'GP120: target for neutralizing HIV-1 antibodies', Annual Review of Immunology, 24:739-69.

All high-resolution structures of HIV-1 envelope glycoproteins solved so far are of recombinant monomeric gp120, gp41 peptides or gp41 in its fusogenic state. While these structures give invaluable insight into the mechanisms behind HIV-1 entry into host cells and interaction with and evasion of neutralising antibodies, they might not be good representatives of the envelope glycoproteins as they

appear in the functional envelope spike, as the conformation and accessibility to antibodies of gp120 and gp41 in the context of the spike are likely to be affected by intra-spike interactions between gp120 and gp41 subunits, and by the proximity to the viral membrane. This notion is illustrated by the observation that antibody ability to bind to recombinant proteins does not necessarily correlate with ability to neutralise the corresponding virus (141, 142, 623-626, 897, 940). Instead, neutralisation has been shown to depend on the ability to recognise functional envelope spike (141, 142, 624-626, 897). Obtaining a high-resolution structure of the functional envelope spike would have obvious implications for understanding HIV-1 entry into cells, recognition by antibodies, and vaccine design. However, due to the labile and flexible nature of the spike and the high carbohydrate content, obtaining X-ray crystallographic data has been shown to be difficult.

In the absence of high-resolution structures, electron microscopy and electron microscopy tomography experiments have provided some insight into the three-dimensional nature of native HIV-1 spikes. Initial data came from Center *et al*. (941, 942), who used scanning electron microscopy to show that the majority of detergent-solubilised SIV and HIV-1 spikes are trimeric. Subsequently, in 2003, Zhu *et al*. (943) reported data from electron microscopy tomography studies, demonstrating trimeric spikes on the surface of chemically fixed HIV-1 and SIV virions, providing the first conclusive evidence for the presence of trimers on actual virions. Both HIV-1 and SIV had an average of 8-10 trimers per virion on their surface, correlating with the envelope glycoprotein content as analysed by biochemical assays (943). Heterogeneity was observed among different spikes, possible reflecting the heterogeneity and the existence of non-functional spikes predicted by observations made using biochemical assays (142).

However, due to artefacts associated with the chemical fixing procedure, the envelope spikes could not be viewed in much detail. Zhu and colleagues therefore went on to using cryo-electron microscopy tomography to study the three-dimensional structure of envelope spikes on frozen, unfixed, unstained and hydrated HIV-1 and SIV particles. Their results were published in 2006 (140), showing that wild-type HIV-1 virions are around 110 nm in diameter and have

an estimated 14 ± 7 spikes per virion (ranging from 4 to 35, n = 40). Interestingly, the spikes were observed to cluster together rather than being uniformly dispersed on the virion surface. To facilitate the analysis of individual spikes, they used a mutant form of SIV, containing gp41 with a truncated cytoplasmic tail lacking endocytosis signals, resulting in higher levels of spikes on the virus surface. Cryo-electron tomography data for a total of 6175 spikes were used to generate an average three-dimensional density map. The results show a multi-lobed structure with a height of 13.7 nm, a diameter of 10.5 nm, and a tripod-like foot consisting of three well-separated legs (Fig. 1.1.6.1.2 A). The tripod-like feature has also been observed in cryo-electron tomography studies of the MMLV envelope spike (944).

Three months after the report by Zhu *et al*. (140), Zanetti *et al*. (138) reported a second three-dimensional, low-resolution structure of a virion-associated SIV envelope spike based on cryo-electron tomography data (Fig. 1.1.6.1.2 B). Data from a total of 2986 spikes from 77 virions were used to create the three-dimensional reconstruction, revealing a structure made up of three distinct gp120 subunits on top of a narrow gp41 stalk (138), of similar height and width but rather different in shape compared to the tripod-like structure predicted by Zhu and colleagues (Fig. 1.1.6.1.2 A and B). The structure suggested by Zanetti *et al*. (138) is 120 Å in height and 110 Å in diameter, and, interestingly, has a cavity at the interface of gp120 and gp41. Whether the differences between the three suggested structures are a result of differences in methodology or limitations of the electron tomography technique, or whether they represent true spike heterogeneity remains unclear (945, 946).

In 2008, Liu *et al*. (947) reported a third three-dimensional spike structure based on a density map average of cryo-electron tomographic data for 4741 HIV-1 envelope spikes. This structure is similar in dimension to the previous SIV structures, with a height of 120 Å and a maximal width of 150 Å, but is rather different in shape, being held together by contacts at the gp41 base and at the tip of the trimer (Fig. 1.1.6.1.2 C). It does, however, share the narrow gp41 stalk feature predicted by Zanetti *et al*. (138). Fitting of gp120 atomic structures into

the density map provides further insight into the spike structure. In the report by Liu *et al*. (947), it indicates that the variable regions and the glycosylated residues of gp120 are located on the outside of the spike in solvent accessible regions, in keeping with previous predictions (932). The CD4bs is located about 20Å from the top of the spike in a recessed cavity, covered by the V1 and V2 regions (947). The V3 region is also suggested to be located towards the top of the spike.

Liu *et al*. (947) also carried out cryo-electron microscopy tomography analysis for an HIV-1 spike liganded to sCD4 and mAb 17b. The obtained average density map shows that the spike undergoes drastic quaternary conformational rearrangements upon CD4 binding (Fig. 1.1.6.1.2 D). This observation is consistent with previous reports of conformational changes in recombinant HIV-1 envelope glycoproteins upon binding to CD4 (901, 902, 926, 929-931), as discussed above. The presence of sCD4 and mAb 17b allows for an unambiguous fit of the atomic structure of gp120 bound to sCD4 and 17b into the spike density map, providing valuable insight into the nature of the conformational changes (947). This fit indicates that, upon CD4 binding, each gp120 monomer undergoes considerable rotation by up to 45°, resulting in a vertical upward displacement of gp120, a more open spike structure, and exposure of the gp41 stalk (Fig. 1.1.6.1.2 D). Furthermore, the V1, V2 and V3 regions are suggested to move so that the V1 and V2 regions point away from the centre of the spike, whereas the V3 region point towards the target cell membrane (947). 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.

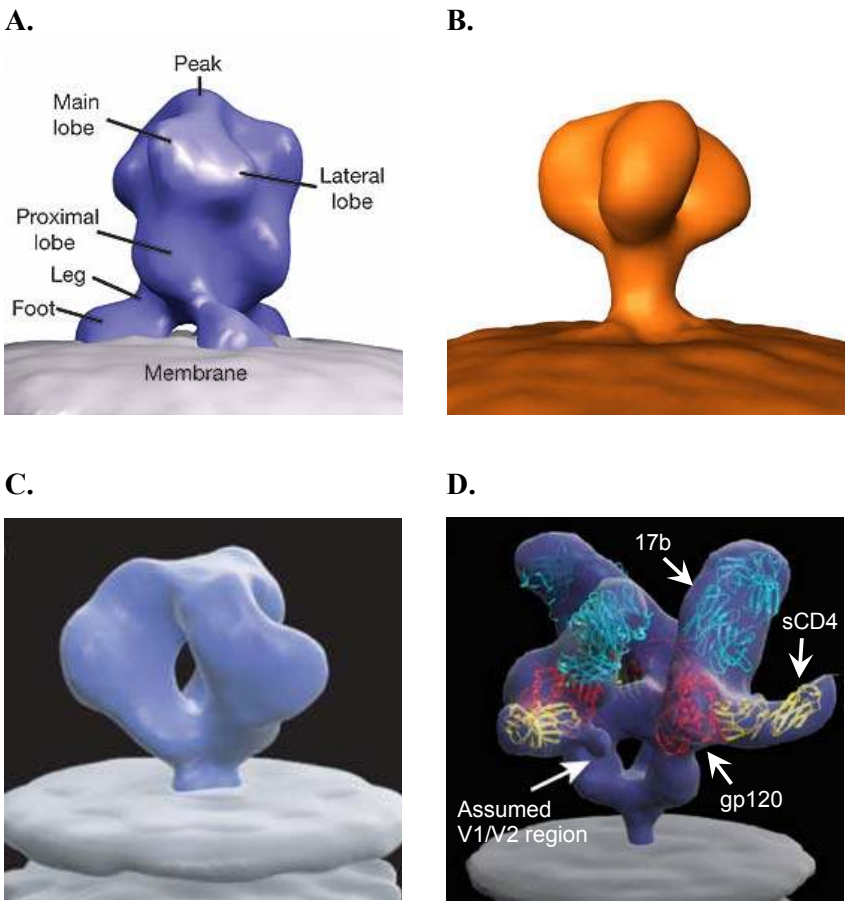


Figure 1.1.6.1.2. Three-dimensional models of the SIV and HIV-1 envelope spikes based on cryo-electron tomography studies. A. Density map of an unliganded SIV envelope spike constructed by Zhu *et al*. (140). Image obtained from Zhu, P., J. Liu, J. Bess, Jr., E. Chertova, J. D. Lifson, H. Grise, G. A. Ofek, K. A. Taylor, and K. H. Roux, 2006, 'Distribution and three-dimensional structure of AIDS virus envelope spikes', Nature, 441:847-52. Reprinted by permission from Macmillan Publishers Ltd. **B.** Density map of an unliganded SIV envelope spike constructed by Zanetti *et al*. (138). Image obtained from Zanetti, G., J. A. Briggs, K. Grunewald, Q. J. Sattentau, and S. D. Fuller, 2006, 'Cryo-electron tomographic structure of an immunodeficiency virus envelope complex in situ', PLoS Pathogens, 2:e83. **C-D.** Density maps of an unliganded HIV-1 envelope spike **(C)** and an HIV-1 envelope spike complexed with sCD4 and mAb 17b **(D)**, constructed by Liu *et al*. (947), with the atomic structure of gp120 liganded to sCD4 and mAb 17b fitted into the model. Images obtained from Liu, J., A. Bartesaghi, M. J. Borgnia, G. Sapiro, and S. Subramaniam, 2008, 'Molecular architecture of native HIV-1 gp120 trimers', Nature 455:109-13. Reprinted by permission from Macmillan Publishers Ltd.

1.1.6.2 HIV-1 entry into cells

Most variants of HIV-1 enter cells through interaction with the main cellular receptor CD4, followed by interaction with a co-receptor, usually CCR5 or CXCR4. Binding to the cellular receptors eventually leads to gp41-mediated fusion of the viral and cellular membranes. A schematic representation of the main viral and cellular elements involved in HIV-1 entry into target cells is shown in Fig. 1.1.6.2.1. The exact mechanisms behind HIV-1 entry remain unclear. Insight into the entry process has, however, come from several studies, some of which are discussed below.

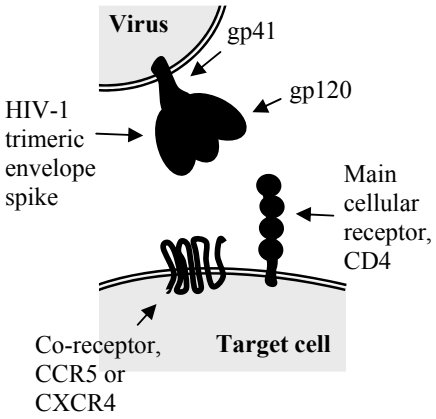


Figure 1.1.6.2.1. Schematic representation of the main viral and cellular elements involved in HIV-1 entry into target cells. The functional HIV-1 envelope spike consists of trimers of the surface glycoprotein g120 non-covalently bound to the membrane-spanning gp41. HIV-1 entry into target cells is initiated by association of gp120 with the main cellular receptor CD4. This engagement triggers conformational changes in the envelope spike, exposing the co-receptor binding site. CD4-bound gp120 then engages with a cellular co-receptor, typically CCR5 or CXCR4, resulting in a series of events eventually leading to the fusion of viral and cellular membranes. Details are given in the text.

The HIV-1 entry process is initiated by attachment of the gp120 subunit of the viral envelope spike to the primary cellular receptor CD4 (52, 53). This interaction is thought to be one of the main rate-limiting events in the HIV-1 entry process (948-951). The initial HIV-1 attachment step can be influenced by interactions between host-derived molecules incorporated into the viral membrane and their natural ligands on target cells (952). These interactions,

although not necessary for virus entry, can enhance the rate of adhesion and the efficiency of gp120 binding to CD4. For example, it has been suggested that interaction of host-derived intracellular adhesion molecule 1 (ICAM-1), present on HIV-1 particles, with its ligand lymphocyte function-associated molecule 1 (LFA-1) can enhance infectivity (953-955).

Binding of gp120 to CD4 triggers a series of conformational rearrangements in gp120 and in the envelope spike as a whole (348, 901, 902, 926, 929, 947), as discussed in section 1.1.6.1, allowing for exposure of the co-receptor binding site and interaction with either of the main cellular co-receptors CCR5 or CXCR4 (933-937). The V3 loop determines co-receptor usage and thereby cell tropism, as initially demonstrated by Hwang *et al*. (417) in 1991. More specifically, co-receptor use seems to be associated with the net charge of the V3 region, as the V3 regions of CXCR4-using viruses seem to have a higher net positive charge than those of CCR5-using viruses (421). However, even though the V3 region is the major determinant, sequence changes in the V1, V2, C3 and C4 regions of gp120 and in gp41 have also been shown to have a role (418, 420-422).

Binding of gp120 to CD4 and subsequently to a co-receptor results in conformational changes in gp41, leading to the formation of a pre-hairpin intermediate, insertion of the gp41 fusion peptide into the host cell membrane, and finally fusion of viral and cellular membranes (951, 956). The details of this fusion reaction remain unclear. However, the pre-hairpin intermediate conformation is believed to be in the form of an energetically unstable three-helix rod (945, 956). The formation of this 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 (957). This six-helical bundle is made up of the N-HR and C-HR of gp41, is energetically stable, and forms when the three C-HR domains fall back in a hairpin configuration into the hydrophobic grooves formed by the three N-HR domains (261, 900).

Melikyan *et al*. (958) have shown, using suboptimal and optimal temperatures to modulate fusion rate, in combination with fusion inhibitory peptides such as the T-20 (872) and membrane-damaging agents such as lysophosphatidylcholine,

that the formation of the six-helix bundle coincides with fusion of the viral and cellular membranes. Based on these observations, it has been suggested that it is the actual formation of the six-helix bundle, and not the configuration of the bundle itself, that induces fusion by bringing the viral and cellular membranes in close proximity to each other, aided by the energy release associated with bundle formation (956, 958).

The number of spikes needed for HIV-1 entry into target cells is unknown. Yang *et al*. (959, 960), using the methodology described by Schønning *et al*. (961), produced viruses bearing mixed heterotrimers containing different ratios of wild-type and dominant-negative envelope glycoproteins to estimate the minimal number of envelope spikes required for infection, and found that one functional spike per virion was sufficient. In contrast, influenza virus infection required eight to nine spikes per virion. In a subsequent study, Yang *et al*. (962) observed that only two out of the three gp120/gp41 subunits needed to be functional in order for infection to take place. In a set of similar experiments, using virus bearing mixed heterotrimers of cleavage-incompetent gp160 and wild-type gp160, Herrera *et al*. (963) estimated that at least four to five functional spikes are needed to support infection. More work is needed to elucidate the number of spikes required and the precise stochiometry of HIV-1 entry into target cells.

Further insight into the HIV-1 entry process is given by a recent report by Sougrat *et al*. (964), who used electron tomography to determine the three-dimensional architectures of SIV and HIV-1 particles in contact with T cells. Their results show that the contact region between virus and target cell is around 400 Å and contains five to seven rod-like structures, assumed to be the viral spikes (Fig. 1.1.6.2.2). This observation is in keeping with previous observations indicating that multiple envelope spikes and receptors are present at the HIV-1 fusion pore (965, 966). Sougrat and colleagues term this contact region the ‘entry claw’. Interestingly, the viral surface not in contact with the target cell seems to be devoid of viral spikes, even for SIV particles observed to have up to a hundred viral spikes pre-contact with target cells. Further work is needed to elucidate the *in vivo* relevance and mechanisms of the formation of the ‘entry claw’, as well as the stage of viral entry that it represents.

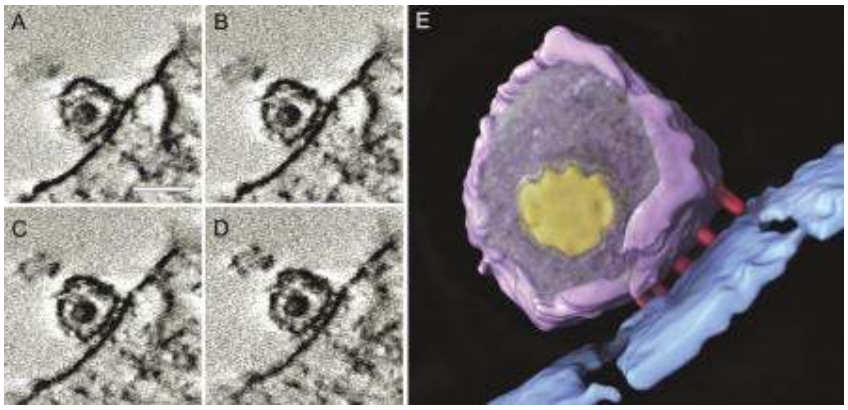


Figure 1.1.6.2.2. Electron tomography of an HIV-1 particle in contact with a CD4+ T cell. **A-D.** Single slices of an electron tomogram of the contact between a virus particle and a CD4+ T cell. **E.** Three-dimensional model of an HIV-1 particle in contact with a T cells, showing the viral core in yellow, the viral membrane in magenta, the T cell membrane in blue and the contact rods in red. Image obtained from Sougrat, R., A. Bartesaghi, J. D. Lifson, A. E. Bennett, J. W. Bess, D. J. Zabransky, and S. Subramaniam, 2007, 'Electron tomography of the contact between T cells and SIV/HIV-1: implications for viral entry', PLoS Pathogens, 3:e63.

In addition to the cell-free virus dissemination and entry into target cells, intercellular spread of HIV-1 is also thought to take place. This means of transmission has been suggested to be predominant mode of HIV-1 spread, and moreover to be up to a thousand times more efficient than cell-free transfer (967, 968). For example, HIV-1 has been observed to transfer from infected to uninfected cells via a so-called virological synapse (470, 969, 970). This transfer has been shown to be dependent on HIV-1 envelope glycoprotein and CD4. Cell-cell transfer has also been suggested to occur across filopodial bridges formed between a viral envelope spike on the infected cell and a cellular receptor on the target cell (971). Furthermore, data reported by Sowinski *et al*. (972) have indicated that HIV-1 can transfer from infected to uninfected CD4+ T cells across so-called nanotubes in a CD4-dependent manner. While it has been suggested that cell-cell spread allows for more rapid replication kinetics and helps the virus to evade host antibody responses, more work is needed to elucidate the relevance of these mechanisms and the relative contributions of cell-cell and cell-free spread *in vivo* (972, 973).

1.1.6.3 Antigenic properties of the HIV-1 envelope glycoproteins

In the mid-1990s, antibody competition analysis revealed that, antigenically, gp120 possesses a non-neutralising and a neutralising face (919), a notion which was later supported by high-resolution structural data (348, 928). The non-neutralising face, which is located on the inner domain of gp120, contains a number of epitopes for non-neutralising antibodies (919) and consists of regions that are buried within the trimer and that interact with gp41 or other gp120 protomers. These regions, which are accessible on non-functional spikes on the virus surface (141, 142) and on shed gp120, are highly immunogenic (974-976) but do not elicit neutralising antibodies (919, 928). The neutralising face of gp120 includes parts of the bridging sheet and the inner and outer domains, including the V2 and V3 regions (928). It contains the receptor-binding sites and epitopes for a number of neutralising antibodies (259, 919). However, conserved and functionally important regions of gp120, such as the CD4bs, are often occluded from antibody recognition (259). Moreover, as a result of the extraordinary genetic diversity and the immune evasive properties of gp120, neutralising antibodies to gp120 are typically strain-specific (259). The V3 region is particularly immunogenic but typically elicits isolate-specific antibodies (259, 421). In addition to the non-neutralising and neutralising faces of gp120 initially identified through antibody competition analyses, high-resolution structural data revealed an immunologically silent face of gp120, consisting of the highly glycosylated outer surface of the outer domain (348, 928).

As discussed in section 1.1.6.1, antibody binding studies have indicated that 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 (901-905). Several regions of gp41 have, however, been found to be highly immunogenic (977-980), and a large number of anti-gp41 antibodies have been isolated from infected individuals (977, 980-982). Competition analysis has revealed that the epitopes recognised by these anti-gp41 antibodies can be grouped into three clusters, termed cluster I, II and III (977, 980). 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. A few broadly neutralising antibodies to gp41 have, however, been isolated (section 1.1.6.6). These antibodies are directed at the MPER, located between the viral membrane and the gp120-gp41 interface, which although not well-exposed on virus or virus-infected cells is accessible to antibodies on a fusion intermediate form of gp41 (906-908). Analyses of the specificity of sera from individuals with a broadly neutralising antibody response do, however, indicate that the contribution of anti-MPER antibodies is relatively small (654, 983-986).

1.1.6.4 Antibody evasive properties of the HIV-1 envelope spike

HIV-1 has evolved a number of ways to evade the humoral immune response. For example, as discussed in section 1.1.4.4, the high mutation rate of HIV-1 allows for rapid escape from neutralising antibodies, and contributes to the extreme sequence diversity that is observed among HIV-1 isolates, particularly in the *env* gene. In addition to the high mutation rate, several other features of HIV-1 confer protection against recognition by antibodies, the majority of which can be attributed to the HIV-1 envelope glycoproteins. These features, which are summarised in Table 1.1.6.4.1, include conformational and entropic masking, glycan shields, and variable regions that occlude conserved and functionally important receptor-binding sites.

The variable regions are thought to mask receptor-binding sites (914, 915), as antibodies to the receptor-binding sites have been observed to bind with higher affinity to gp120 with deleted V1, V2 and V3 regions (914, 917, 920-922). The co-receptor binding site has been suggested to be masked by the V1 and V2 variable regions (921, 922), perhaps from a neighbouring gp120 protomer (932).

The glycan shield renders the outside of the envelope spike less visible to the immune system. It has been suggested that the glycan shield is evolving, and HIV-1 has been shown to reposition its glycans in order to escape neutralising antibodies (644, 987, 988).

Table 1.1.6.4.1. Antibody evasive properties of the HIV-1 envelope spike.

Feature	Comment
Sequence heterogeneity	The RT polymerase is error prone (167), giving rise to a high mutation rate and rapid antibody escape (622, 632, 643, 644).
Variable regions	Conserved and functionally important receptor-binding sites are masked by variable regions on the surface of gp120 (911-913). High sequence variability in the variable regions allows for rapid antibody escape.
Glycan shield	Around half of gp120 is covered by complex and high-mannose carbohydrates, which are added by the host cell machinery and render the underlying surface less immunogenic (139, 267, 270).
Entropic and conformational masking	The HIV-1 envelope spike is thought to exhibit a high degree of entropy and conformational flexibility, creating entropic barriers to antibody binding and giving the appearance of heterogeneity (929, 989, 990). The entry process and the conformational changes associated with receptor binding limit the exposure of functionally important and conserved regions to antibodies (901, 922, 928).
Non-functional spikes	The presence of non-functional spikes on the virus surface (gp41 stumps, gp120/gp41 monomers and uncleaved gp160) and shed g120 may divert the antibody response (141, 142, 754).

Thermodynamic studies carried out by Myszka *et al*. (929) and Kwong *et al*. (989) have shown that unliganded gp120 exhibits a high degree of entropy, indicating that it is conformationally flexible. Kwong *et al*. (989) also showed that binding of many non-neutralising or weakly neutralising antibodies to receptor-binding sites on the unliganded gp120 is accompanied by a large

decrease in entropy, whereas potently neutralising antibodies can bind with small changes in entropy. The high degree of conformational flexibility in the unliganded gp120 has been suggested to contribute to masking the envelope spike from antibodies that recognise the receptor-binding regions. A high degree of conformational flexibility of gp120 was also demonstrated by Yuan *et al*. (990), who fixed monomeric and trimeric gp120 constructs and probed them with neutralising and non-neutralising antibodies, showing that unliganded gp120 can adopt multiple conformations, and that potent neutralising antibodies were able to recognise the greatest proportion of fixed gp120 conformations.

Based on these and similar observations, it has been suggested that HIV-1 uses entropic and conformational masking of functional sites, such as the receptor-binding sites, to minimise recognition by antibodies (259, 754, 989, 990). Targeting the least conformationally variable parts of the functional envelope spike may be the valuable in vaccine design. Such parts could be identified by studying broadly and potently neutralising antibodies to the HIV-1 envelope glycoproteins.

1.1.6.5 Specificities of anti-envelope antibodies in HIV-1 infection

As discussed in section 1.1.6.3, a large proportion of the antibody response to HIV-1 in natural infection is non-neutralising and directed towards highly immunogenic regions accessible on non-functional or disassembled envelope spikes or viral debris (139, 141, 142, 623-627). The regions of gp120 and gp41 that take part in the gp120-gp41 interaction have been found to be especially immunodominant (974-979).

Neutralising antibodies are thought to make up only a fraction of the total antibody response and are often strain-specific (619, 631, 636, 638, 643-645, 647). Many of the strain-restricted neutralising antibodies that have been isolated are directed at the V3 region of gp120 (991). Anti-V3 antibodies are abundantly elicited after immunisation as well as during natural HIV-1 infection but do rarely exhibit broad cross-subtype neutralisation activity (421, 992-996). Antibodies with relatively potent and broad neutralising activity across HIV-1

subtypes can, however, be detected in a subset of HIV-1-infected individuals, although little is known about the specificities that mediate this broad neutralisation activity (621, 641, 653, 654, 984, 985, 997-1000). In addition, it is not known whether broadly neutralising sera contain antibodies that target conserved regions on the envelope glycoproteins, or whether they contain polyclonal antibodies to a variety of neutralising epitopes. Further knowledge about the epitope specificities present in broadly neutralising sera is of obvious value for HIV-1 vaccine design.

A recent study carried out by Li *et al*. (985), using adsorption and elution on a set of wild-type gp120 and gp120 constructs carrying mutations in the CD4bs followed by neutralisation experiments, has indicated that the neutralisation activity in broadly neutralising sera is mediated by antibodies to the CD4bs. No neutralising anti-MPER antibodies could be detected (985). In another study, Dhillon *et al*. (984) dissected the neutralising specificities in broadly neutralising sera from three HIV-1-infected but asymptomatic individuals and found that the neutralising activity was mediated by the IgG fraction of the sera. By using chimeric SIV and HIV-2 viruses grafted with HIV-1 MPER neutralising epitopes, they found that the specificities did not map to the MPER of gp41. Moreover, by evaluating the neutralisation activity of the sera in the presence and absence of competing V3 region peptides, they concluded that the neutralisation activity was not due to anti-V3 region antibodies. The same conclusion was made for the V1 and V2 regions. Instead, by evaluating the neutralisation activity after depletion on monomeric gp120, they suggest that the neutralising activities are due to multiple specificities and may be mediated by antibodies to the CD4bs of gp120, to gp120 epitopes not present on monomeric gp120, or to carbohydrate epitopes on gp120 (984).

Similarly, Yuste *et al*. (986) failed to detect neutralising antibodies to the MPER of gp41 in serum samples from 92 HIV-1-infected individuals, by testing the neutralisation activity against SIV carrying HIV-1 MPER neutralising epitopes. Moreover, Crooks *et al*. (983) analysed plasma from HIV-1-infected individuals of varying neutralisation potencies and found that the neutralisation activity was

not mediated by antibodies to the MPER, as the sera were not able to inhibit infection of target cells post-binding of viral gp120 to CD4 and CCR5.

In another study, Braibant *et al*. (654) used competition ELISAs to analyse the specificities of the antibodies in sera from LTNP, and found that all LTNP had antibodies that competed with the anti-CD4bs mAb b12 (section 1.1.6.6). Moreover, they detected higher levels of antibodies that compete with mAbs 2F5 (anti-MPER mAb; section 1.1.6.6) and 2G12 (mAb recognising a carbohydrate motif on gp120; section 1.1.6.6) in LTNP sera with broad neutralising activity than in other LTNP sera. Some studies have also indicated that neutralising antibodies to CD4-induced epitopes are frequent in HIV-1-infected individuals. For example, Decker *et al*. (1001) detected antibodies that cold neutralise virus that had been pre-incubated with sCD4 in sera from individuals infected with clade A, B, C, D, F, G, and H as well as CRF01, CRF02, and CRF11. Sera from 178 out of 189 HIV-1-infected individuals contained antibodies that could even neutralise HIV-2 (pre-incubated with sCD4).

1.1.6.6 Neutralising antibodies to HIV-1

Antibody-mediated virus neutralisation can be defined as a quantifiable reduction in virus infectivity *in vitro* due to the dose-dependent activity of antibodies (754, 1002, 1003). Neutralising antibodies are thought to recognise functional spikes on the virus surface, whereas non-neutralising antibodies are believed to recognise epitopes that are exposed only on non-functional or disassembled envelope species, including shed gp120, gp41 stumps, gp120/gp41 monomers, and unprocessed trimers (141, 142, 623-626, 897, 940).

The main mechanism behind antibody-mediated neutralisation of HIV-1 is considered to be antibody occupancy of functional viral envelope spikes (139, 624-626, 754, 1002), with epitope specificity being less important. This notion is supported by the observation that the incorporation of an unrelated epitope into gp120 rendered a number of HIV-1 variants sensitive to neutralisation by an antibody to the unrelated epitope (1004). Although little is known about the

stochiometry behind neutralisation, it has been suggested that one antibody per functional envelope spike is sufficient for neutralisation (960).

Some studies, however, indicate that the mechanism behind neutralisation may be more complex. For example, incorporation of an MPER-neutralising epitope into the MLV envelope spike rendered the virus sensitive to neutralisation (1005). Incorporation of an influenza virus neutralising epitope into the same region of the MLV envelope spike did, however, not render the virus sensitive to neutralisation. It is also possible that antibody binding may induce shedding of gp120, thus inactivating the viral spike (1006). A recent study has also indicated that binding of gp120-specific molecules to viral spikes can result in virus rupture. Using cryo-electron tomography techniques, Bennett *et al*. (1007) have visualised binding of D1D2-IgP, a multimeric construct consisting of CD4 fused to an immunoglobulin, to virus particles, showing that binding of D1D2-IgP correlated with virus rupture.

Antibodies to HIV-1 gp120 and gp41 have provided valuable insight into the antigenic structure of the HIV-1 envelope spike. Studies using broadly neutralising mAbs have helped identifying vulnerable and conserved regions on the HIV-1 envelope glycoproteins. However, although many mAbs to HIV-1 envelope have been isolated from animals post-immunisation and from humans following HIV-1 infection, only a handful has been found to be broadly neutralising across HIV-1 subtypes (754). Two of these broadly neutralising mAbs are directed against gp120: mAb b12, which binds to an epitope that in part overlaps a subset the CD4bs (1008-1012), and 2G12, which recognises a carbohydrate motif (981, 1013-1015). Two broadly neutralising mAbs, mAbs 4E10 and 2F5, recognise the MPER of gp41 (906-908, 981). The Fab fragment X5 (1016), which recognises an epitope on gp120 that is better exposed after CD4 binding also display some neutralising activity across HIV-1 subtypes. All of the broadly neutralising mAbs reported in the literature to date are from individuals infected with HIV-1 of subtype B (754), although broadly neutralising mAbs from individuals infected with subtype C are currently being characterised (Davide Corti, Institute for Research in Biomedicine, Bellinzona, Switzerland, *personal communication*).

The anti-CD4bs mAb b12, also referred to as IgG1b12, is one of the most extensively studied mAbs to HIV-1 envelope. It was initially isolated in 1991 as a Fab fragment, through panning of a combinatorial phage library displaying monoclonal Fab fragments from an asymptomatic HIV-1-infected individual on recombinant monomeric gp120 (1011), and has since been expressed as a full immunoglobulin and shown to neutralise a large proportion of HIV-1 isolates from a wide range of subtypes (259, 1009, 1010, 1012, 1017) and to protect macaques against SHIV challenge (662, 668). For example, in a study carried out by Binley *et al*. (1017), mAb b12 has been shown to neutralise (to at least 50%) 45 out of 90 viruses of various subtypes. Competition studies with sCD4 along with mutational studies initially indicated that b12 targets a region that is over-lapping or near to the CD4bs (1009-1011, 1018). These observations were confirmed in 2007, when Zhou *et al*. (1008) reported high-resolution structural data on mAb b12 in complex with a gp120 construct that had been engineered to remain in an CD4-liganded conformation, revealing that mAb b12 binds to an epitope that overlaps part of the CD4bs (Fig 1.1.6.6.1).

The structural data reported by to Zhou *et al*. (1008) revealed that mAb b12 binds gp120 only with its heavy-chain. This unusual feature may be due to the combinatorial library approach by which mAb b12 was isolated, where the cloned heavy- and light-chain repertoires were randomly recombined in the selection process. Three b12 residues, an asparagine in the first complementarity determining region (CDR1), a tyrosine in CDR2, and a tryptophan in CDR3, were found to interact with the CD4-binding loop of gp120, and to make up around 40% of the total b12 contact surface (1008). The gp120 contact surface was found to be confined largely to the outer domain on gp120, with the CD4-binding loop making up more than 50% of the total interface. Most of the overlap between the b12 epitope and the CD4bs was found to be located on the outer domain of gp120, in particular around the CD4-binding loop. However, the b12 epitope overlaps only part of the CD4bs (Fig. 1.1.6.6.1). For example, the unusually long and protruding heavy-chain CDR3 of mAb b12 does not reach into the Phe43-binding cavity of the CD4bs, as had been predicted by molecular modelling experiments using the unbound mAb b12 structure (1019). Instead, it interacts with residues that are peripheral to this site. According to the data

presented by Zhou *et al*. (1008), mAb b12 is able to neutralise a wide range of HIV-1 isolates by interacting with the functionally conserved and vulnerable initial contact surface of CD4 without introducing conformational changes. This notion is consistent with the observation made by Kwong *et al*. (989), showing that mAb b12 is able to bind gp120 without a large change in entropy.

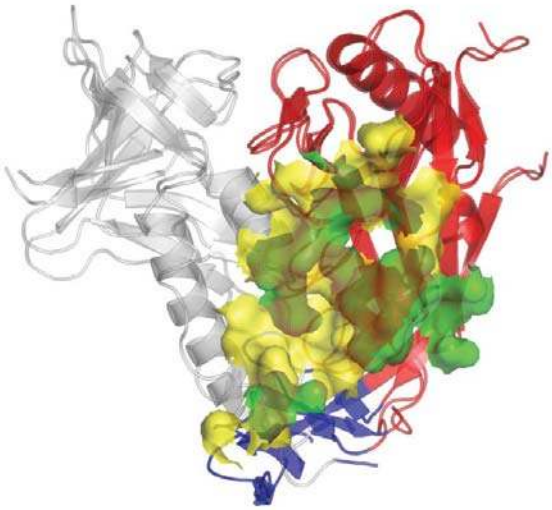


Figure 1.1.6.6.1. The mAb b12 epitope and the CD4bs of gp120. Superposition of gp120 bound to mAb b12 and gp120 bound to CD4. The outer domain of gp120 is shown in red, the inner domain in gray, and the bridging sheet in blue. Amino acid residues in contact with mAb b12 are shown in green and the CD4bs in depicted in yellow. The figure is obtained from Zhou, T., L. Xu, B. Dey, A. J. Hessell, D. Van Ryk, S. H. Xiang, X. Yang, M. Y. Zhang, M. B. Zwick, J. Arthos, D. R. Burton, D. S. Dimitrov, J. Sodroski, R. Wyatt, G. J. Nabel, and P. D. Kwong, 'Structural definition of a conserved neutralization epitope on HIV-1 gp120', 2007, Nature 445:732-7 (1008). Reprinted by permission from Macmillan Publishers Ltd.

In addition to mAb b12, there is a number of other mAbs to the CD4bs that exhibit some neutralisation activity across subtypes. None, however, exhibits the cross-reactive neutralisation breadth exhibited by mAb b12. These antibodies include mAb m14 (1020) and mAb F105 (1021), which have both been shown to compete with CD4 for binding to gp120. A large number of non-neutralising anti-CD4bs mAbs and anti-CD4bs mAbs able to neutralise only neutralisation-sensitive TCLA isolates have also been isolated. These are thought to recognise epitopes that are not accessible on functional spikes. For example, the non-

neutralising human mAb b6, which was derived from the same phage library as mAb b12, compete with sCD4 and with mAb b12 for binding to recombinant gp120. Furthermore, it can capture virus particles and inhibit capture by mAb b12, but has no effect on the neutralisation activity of b12, suggesting it fails to recognise functional spike, but can bind to non-functional envelope spikes present on the virus surface (142, 897, 1018).

The second broadly neutralising mAb that targets gp120 is 2G12 (981, 1015). This antibody has been shown to neutralise (to 50%) 37 out of 90 viruses of different subtypes in the study carried out by Binley *et al*. (1017). Notably, it failed to neutralise HIV-1 of subtype C. It recognises a cluster of oligomannose residues on gp120 and 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 (981, 1013-1015). This antibody has shown efficacy *in vivo*, in monkey models in combination with other mAbs (657-659), but also in humans, as intravenous administration delayed the viral rebound in individuals undergoing interrupted antiviral therapy (672).

The remaining broadly neutralising antibodies, 4E10 (906, 908) and 2F5 (907, 981), target neighbouring continuous epitopes within the conserved MPER of gp41. Antibody 4E10 is the broadest neutralising mAb to HIV-1 described to date. In many studies, it has shown a neutralisation activity of extraordinary breadth (1017, 1022, 1023). In the study carried out by Binley *et al*. (1017), mAb 4E10 was able to neutralise all of the 90 viruses, when assayed in engineered cell lines. It has, however, been observed to be less broadly reactive in PBMC-based neutralisation assays (906, 908, 1017). It recognises an epitope that is located C-terminal to the 2F5 epitope, overlapping the highly conserved sequence NWF(D/N)IT (906, 910). Crystal structures of mAb 4E10 with this peptide sequence has shown that the peptide adopts a helical conformation (910). Antibody 2F5 binds to an epitope overlapping the conserved sequence ELDKWA (907, 909). In the study carried out by Binley *et al*. (1017), this mAb was able to neutralise 60 out of 90 viruses (to more than 50%). Notably, it did not neutralise any of the subtype C viruses, explained by the observation that

most subtype C viruses lack the DKW motif present in the 2F5 recognition sequence (1017). Instead, many of them display a DSW motif. The epitopes for mAbs 4E10 and 2F5 have been shown be slightly hydrophobic and possibly membrane-bound (1024-1026). In addition to 4E10 and 2F5, anti-MPER mAb Z13 also displays some neutralisation activity, although less broad and potent compared to that of 4E10 and 2F5 (906). A human mAb recognising the N-HR of gp41 and selected from a naïve library, mAb D5, has also been shown to display some neutralisation activity (1027).

Antibodies to the CD4-induced sites (including the co-receptor binding site) are generally unable to neutralise virus *in vitro*, or able to neutralise only weakly, unless the virus is pre-incubated with sCD4 (1001, 1028, 1029). The mechanism of neutralisation has been suggested to be interference with co-receptor binding (139, 934). This inability to neutralise in the absence of sCD4 is thought to be due to kinetic and steric constraints, as these epitopes are typically not exposed until after interaction with CD4 (921, 1030). In contrast, several Fab fragments to CD4-induced epitopes have been shown to be able to neutralise primary isolates (1016, 1030). Based on these notions, it has been suggested that full immunoglobulins are too bulky to be able to bind to CD4-induced epitopes in the context of the virus-associated functional spike, as there may be limited and short-lived access to these epitopes in the presence of viral and cellular membranes (997). Viruses with deleted V1 and V2 variable regions have been shown to be more susceptible to neutralisation by mAbs to CD4-induced epitopes, supporting the notion that the co-receptor binding site is masked by V1 and V2 (920). An interesting feature of some of the isolated antibodies to the co-receptor site is that they have sulphated tyrosines in their heavy-chain CDR3s, possibly mimicking the sulphated tyrosines present on CCR5 and CXCR4 and critical for CCR5 or CXCR4 interaction with gp120 (1031). One of the most broadly reactive Fab fragments to CD4-induced epitopes is Fab X5 (1016), which have been shown to neutralise viruses of subtype A, B, C, D, F and G. However, when used as a full immunoglobulin in the study carried out by Binley *et al*. (1017), mAb X5 was only able to neutralise 3 out of 90 viruses.

Several mAbs to the V3 region have also been described. Although most neutralising antibodies to the V3 region appear to be isolate-specific, a minority of the anti-V3 antibodies have been shown to neutralise a relatively large proportion of mainly subtype B isolates (991, 1032). These anti-V3 mAbs have been reported to recognise conformation-sensitive rather than linear V3 epitopes (1032). The broadest of the anti-V3 mAbs isolated to date is mAb 447-52D, which recognises an epitope that includes the GPXR (where X is any amino acid residue but often a G) motif on the tip of the V3 that is conserved among subtype B isolates (991, 1032). In the study carried out by Binley *et al*. (1017), mentioned above, 447-52D was able to neutralise (to at least 50%) 17 out of 90 viruses, 13 of which were of subtype B. A small number of isolate-specific anti-V2 mAbs have been also isolated (1033, 1034).

All of the neutralising anti-HIV-1 mAbs described to date, with one exception, are able to bind recombinant monomeric gp120 or gp41 constructs in addition to recognising functional spikes. The only reported trimer-specific mAb is mAb 2909 (1035), which recognises an epitope involving the V2 and V3 variable regions and the CD4bs that is not present on monomeric gp120. Even though it can only neutralise neutralisation-sensitive HIV-1 isolates that have been cultured *in vivo*, it is able to do so at very low (pM) concentrations.

1.1.6.7 HIV-1 envelope glycoproteins as immunogens

Immunisation with recombinant HIV-1 envelope glycoprotein constructs typically elicits antibodies with weak neutralisation activity against heterologous isolates (1036-1043). In addition to antibodies to non-neutralising epitopes, anti-V3 region antibodies are frequently elicited after immunisation (421, 992-996). In some studies, it has also been shown that vaccine-elicited autologous virus neutralisation can be mediated by antibodies against the V1 region of gp120, in particular a hypervariable region at the tip of the V1 (1042, 1044, 1045). Antibodies with cross-subtype reactive neutralising activity of moderate potency have recently been detected in rabbits immunised with a recombinant gp140 capable of mediating CD4-independent infection and derived from an HIV-1-infected individual whose serum was found to be broadly neutralising (1046).

Further knowledge about the antibody specificities present in these sera would be useful in vaccine design.

In order to develop an immunogen able to elicit broadly neutralising antibodies, efforts are being made to design envelope constructs that mimic the functional envelope spike (259, 1047). As discussed in section 1.1.5.2, recombinant gp120 was initially considered as an immunogen and vaccine candidate, but failed to protect against infection in clinical trials. To better mimic the functional spike, recombinant oligomeric gp140 molecules, which are gp160 molecules that have been truncated just before the transmembrane region of gp41, have been developed (1038, 1048-1053). To stabilise the typically labile gp120-gp41 interaction, cysteine residues have been introduced in gp120 and gp41, resulting in the formation of disulphide bridges that prevent gp120-gp41 disassociation (1054-1056). Other attempts to prevent gp120-gp41 disassociation involve mutation of the protease cleavage site that is located between gp120 and gp41, resulting in the generation of unprocessed gp160 molecules (1049-1053, 1057). Uncleaved gp160 has, however, been shown to be antigenically different from fully cleaved envelope glycoproteins constructs (1056, 1058-1060). To increase the efficiency of cleavage in disulphide bridge-stabilised gp140 constructs, the cleavage site has been modified (1061). Attempts have also been made to improve trimerisation levels and the stability of trimers by introducing heterologous trimerisation domains at the C-terminus of envelope glycoproteins (1041, 1049, 1057). While trimeric envelope constructs have been shown to be somewhat better at eliciting cross-reactive neutralising antibodies compared to monomeric envelope glycoproteins (1036-1041), the majority of these oligomeric gp140 constructs have been shown to be recognised by non-neutralising antibodies, indicating that they do not fully mimic the functional viral spike (259, 1056, 1060).

Envelope glycoprotein constructs engineered to better present known functionally conserved regions and neutralising epitopes, such as the receptor-binding sites and the epitope to which mAb b12 binds, are also being generated (1062). These constructs include deglycosylated gp120 molecules, gp120 with deletions in the immunodominant variable regions (1063, 1064), and gp120

locked in a CD4-bound conformation (1065, 1066). Other strategies involve masking of immunodominant regions, like the V3 and non-neutralising regions, with glycans or through mutation (1062, 1067). Masking immunodominant regions with glycans has, however, been shown not to improve the neutralisation breadth in immunisation studies in rabbits (1043).

Attempts to design an immunogen that elicits 2G12-like antibodies have also been made based on the crystal structure of the Fab fragment of 2G12 with high-mannose glycans (1068). No 2G12-like antibodies have, however, been reported. Various scaffolds that hold the MPER neutralising epitopes are also under evaluation (1069, 1070), as is presenting envelope glycoproteins in the context of viral like particles, VLPs (1071). Polyvalent vaccine approaches, which may be a way of dealing with HIV-1 sequence and conformational heterogeneity, are also being considered (754, 1072-1074).

In summary, all attempts to design an immunogen able to elicit broadly neutralising and potent antibodies have so far been unsuccessful (754). This task represents one of the major challenges in HIV-1 vaccine design. To solve this problem, increased knowledge about the structure of the functional envelope spike and the way it interacts with broadly neutralising antibodies is likely to be needed.

1.2 Camelid antibodies

In this thesis, the use of a camelid immune system to generate antibodies to HIV-1 envelope will be described. In this section, the unique features of the camelid immune system will be reviewed and an introduction to the research field of camelid antibodies will be given.

1.2.1 The antibody repertoire in camelids

The antibodies of all jawed vertebrates studied so far are composed of paired heavy and light chains (1075, 1076). In 1993, Hamers-Casterman *et al*. (1077) reported that members of the *Camelidae* family, in addition to these conventional

antibodies, produce functional antibodies devoid of light chains, so-called heavy-chain antibodies. The *Camelidae* family includes the camel (*Camelus bactrianus*), dromedary (*Camelus dromedarius*), llama (*Lama glama*), alpaca (*Lama pacos*), guanaco (*Lama guanicoe*) and vicuna (*Lama vicugna*), and is the only remaining family within the *Tylopoda* suborder of the *Artiodactyla* order, and the only mammalian family in which heavy-chain antibodies have been detected.

Analyses of the molecular mass and the protein A and G binding properties of camelid antibodies have revealed that camelids possess one fraction of conventional IgG antibodies and two fractions of heavy-chain IgG antibodies. The conventional IgG1 fraction binds to protein A and G, whereas the heavy-chain IgG2 and IgG3 fractions bind to protein A, and protein A and G, respectively (1077). The heavy-chain antibody fraction has been estimated to constitute up to 50% of the total serum IgG content in camelids (1078, 1079), although a lack of reliable reagents makes exact quantification of the different immunoglobulin isotypes and subclasses difficult. Immunisation experiments have indicated that the kind of antibody response, consisting of heavy-chain or conventional antibodies, depend on the immunogen (1080).

Based on germline and cDNA analysis, more than one isotype has been suggested to exist within the IgG1 and IgG2 fractions. The camelid genomes contain separate germline genes for the constant regions of the conventional and heavy-chain antibodies (1081). The dromedary genome has been reported to contain five functional γ genes, encoding for different hinge regions, therefore suggested to encode for different IgG subclasses (1082). Two of these genes are reported to encode for the constant region of the conventional IgG1 antibody fraction, whereas two genes encode for the constant region of the heavy-chain IgG2 antibodies and one gene for the constant region of the heavy-chain IgG3 antibodies (1082). In llama, six γ genes have been reported, five of which correspond to the IgG1, IgG2 and IgG3 subclasses reported in dromedary, and the sixth giving rise to a third subclass of the heavy-chain IgG2 antibodies (1083, 1084).

In addition to IgG, camelids have also been reported to produce IgA, IgD and IgM immunoglobulins (1085). However, so far there is no evidence for the presence of IgA, IgD or IgM molecules lacking light chains. In contrast, studies of the dromedary IgM fraction have revealed conventional antibody structures composed of heavy and light chains (1086).

Antibodies lacking light chains have also been observed in some species of cartilaginous fish (1087, 1088), such as nurse shark *Ginglymostoma cirratum* and wobbegong shark (*Orectobulus maculates*). These antibodies are referred to as IgNAR (where NAR stands for novel antigen receptor) and differ in structure from the camelid heavy-chain antibodies (1089). For example, the constant region of the IgNAR contains five domains, whereas the constant region of camelid heavy-chain antibodies contains two domains only. Furthermore, the variable regions of the IgNAR show only 25% sequence identity with mammalian heavy-chain variable regions. In addition, they are not closely related to the variable regions of the heavy chain of other immunoglobulin isotypes in cartilaginous fish (1088, 1090, 1091). Instead, they have been reported to cluster with the variable regions of T cell receptors and immunoglobulin light chains (1088, 1090). Since cartilaginous fish were the first jawed vertebrate group to emerge, it has been suggested that the IgNAR may represent an evolutionary intermediate between a putative primordial antigen receptor and the conventional antibodies (1087, 1092).

The ratfish (*Hydrolagus colliei*), another species of cartilaginous fish, have also been shown to produce antibodies devoid of light chains (1093). These antibodies are believed to be derived from IgM and, unlike shark IgNAR, their variable regions cluster with the variable regions of the heavy chains of other vertebrates (1091, 1093).

Thus, heavy-chain antibodies have been detected in species from three unrelated taxa, in camelids and in two species of cartilaginous fish. Phylogenetic studies have, however, indicated that these heavy-chain antibodies have evolved independently from each other, reflecting an evolutionary convergence (1081, 1089, 1091). In other words, camelid antibodies are not thought to have

originated in an ancestral dormant gene. Instead, phylogenetic studies have indicated that they evolved from conventional antibodies within the *Camelidae*, appearing after the divergence of the *Tylopoda* suborder from the other *Artiodactyla* suborders, but prior to the *Camelus* and *Lama* split, meaning that they may have emerged in the *Tylopoda* between 11 and 83 million years ago (1082, 1084, 1091, 1094).

Antibodies devoid of light chains also appear in the so-called heavy-chain disease in humans (1095). These antibodies are somatically generated and are unlikely to interact with antigen.

1.2.2 Basic features of camelid heavy-chain antibodies

Conventional IgG consist of two heavy chains and two light chains (Fig. 1.2.2.1). The heavy chains consist of a constant region (CH), organised into three constant domains (CH1-CH3) and one variable domain (VH). The CH1 and CH2 domains are linked by a hinge region. The light chains are composed of one constant (CL) and one variable domain (VL). The paired VH and VL domains are responsible for antigen binding.

The overall structure of the camelid heavy-chain antibodies resembles that of the typical mammalian immunoglobulins, with a number of important deviations. First, they lack the light chains (Fig. 1.2.2.1). As a result, and in contrast to conventional antibodies, the antigen-binding properties of the heavy-chain antibodies are provided by one single fragment, the variable region of the heavy-chain (1077), which has been termed the VHH or Nanobody®. In addition to lacking light chains, the heavy-chain antibodies differ from conventional antibodies in that they lack the first constant domain (CH1) of the constant region of the heavy chain (1077), meaning that the VHH is joined directly to the CH2 domain via the hinge region of the antibody (Fig. 1.2.2.1). However, despite the absence of the CH1 domain in the actual protein, all genes encoding the heavy-chain antibody constant regions contain sequences encoding the CH1 domain, further indicating that camelid heavy-chain antibodies are derived from

conventional antibodies (1082, 1084, 1091). The absence of the CH1 domain is instead due to a point mutation in the 5´-end of the CH1-hinge region intron, resulting in the removal of the CH1 sequence in an unconventional splicing event during mRNA maturation, confirmed by the absence of the CH1 region in all analysed cDNAs (1082, 1084).

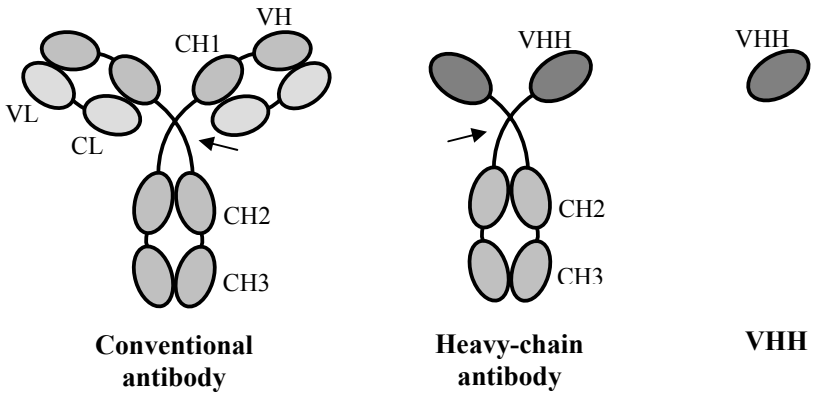


Figure 1.2.2.1. Schematic representation of conventional antibodies and camelid heavy-chain antibodies. Conventional mammalian IgG consist of two heavy chains, made up of three constant domains (CH1-CH3) and one variable domain (VH), and two light chains, made up of a constant (CL) and a variable (VL) 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. The hinge regions, connecting the Fc and the Fab portions of conventional antibodies, and the VHH and the Fc portion of heavy-chain antibodies, are indicated by arrows.

The acquisition of a mechanism to remove the CH1 domain from the H-chain is likely to have played a key role in the evolution and emergence of heavy-chain antibodies, as the CH1 domain is the binding site for the chaperone protein BiP. Binding of BiP to the CH1 leads to retention of the heavy-chain in the ER. Association of the light chain with the heavy chain releases it from BiP, allowing for completion of the folding process and continued processing along the secretory pathway. Indeed, the absence or modification of the CH1 domain is a feature camelid heavy-chain antibodies share with both the IgNAR of sharks and the heavy-chain antibodies of ratfish, highlighting its importance in the emergence of antibodies devoid of light chains (1077, 1088, 1093).

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 (1082-1084).

1.2.3 Properties of VHH

The antigen-recognition properties of the heavy-chain antibodies are confined to the VHH. Sequence and structural analysis of the VHH reveal that they share many features with conventional VH domains (1077, 1083, 1096-1098). Like the VH, the VHH are organised into four relatively conserved framework regions, FR1-FR4, and three hypervariable complementarity-determining regions, CDR1-CDR3 (Fig. 1.2.3.1).

Figure 1.2.3.1. Schematic representation of a VHH domain. Like conventional VH domains, the VHH domain of heavy-chain antibodies contains four framework regions (FR1-FR4) and three complementarity-determining regions (CDR1-CDR3). The position of the two cysteine residues forming an intradomain disulphide bond in conventional VH as well as in heavy-chain antibody VHH are indicated by a C. The positions of the four amino acid residues conserved in VH but constitutively substituted in VHH are indicated by an asterisk.

The amino acid residues that determine the typical immunoglobulin variable domain fold are conserved in the VHH, including the two cysteine residues in FR1 and FR3 that form an intradomain disulphide bond, and the three-dimensional structures of VHH can be superimposed onto those of conventional VH (1096, 1099-1102). Furthermore, the VHH show high sequence similarity, around 80%, to the human VH of family III (1083), the human VH family most commonly incorporated into antibodies (1103).

Some notable differences between VHH and VH domains have, however, been observed (1077, 1083, 1098, 1104). For example, four amino acid residues in the FR2 region are constitutively substituted in the VHH (Table 1.2.3.1). These amino acid residues are highly conserved in the VH and form a hydrophobic

patch that participates in the interaction with the VL (1076). The VHH hallmark substitutions, on the other hand, render the region more hydrophilic. Even the hydrophobic phenylalanine residue at position 37 (Table 1.2.3.1) has been suggested to increase VHH hydrophilicity, as structural studies have shown that it fills a hydrophobic pocket created by hydrophobic residues in positions 47, 91 and 103 (1096). In keeping with the above notions, grafting of the VHH hallmark residues onto a human VH domain rendered it more soluble when expressed in the absence of a VL (1105).

Table 1.2.3.1. VHH hallmark amino acid residue substitutions.

Position[a]	Amino acid residue in VH	Amino acid residue in VHH
37	Valine	Phenylalanine, tyrosine
44	Glycine	Glutamic acid, glutamine
45	Leucine	Arginine, cysteine
47	Tryptophan	Glycine

[a]Kabat numbering.

In addition to the VHH hallmark substitutions, the tryptophan residue in position 103 is sometimes also substituted for a hydrophilic residue. The CDR3 of VHH is also thought to fold over part of the region involved in interaction with the VL in conventional VH, covering hydrophobic patches (1096). Overall, the above-mentioned features of the VHH lead to increased hydrophilicity of the VHH compared to the VH, which tend to aggregate when expressed on their own in the absence of a VL (1106), and explain how VHH base-pairing with VL domains is avoided. Some residues in contact with the CH1 in VH are also exchanged for hydrophilic residues in VHH (1097, 1098).

There are also differences in the CDRs of VHH and VH. On average, camelid VHH display longer CDRs compared to the VH counterparts (1083, 1097, 1098). This notion is especially true for dromedaries, where the average length of the CDR3 of VHH and VH is 17 and 11 amino acids, respectively (1094). The CDR3 loops of llama VHH are on average slightly shorter (15 amino acids) than those of dromedaries but slightly longer than the average human (12 amino

acids) and mouse (9 amino acids) CDR3 (1083). In addition, the VHH of camels and dromedaries, and sometimes llamas, often include non-canonical cysteine residues, leading to the formation of extra disulphide bonds, typically between the CDR1 and FR2, resulting in a stabilised CDR3 and possibly to alternative loop structures (1094, 1098). Moreover, the extent of somatic hypermutation is much higher in VHH compared to in VH. As a result, VHH show an overall increased sequence diversity compared to VH, including in the otherwise relatively conserved FRs (1104). The N-terminal part of the CDR1, in particular, is more variable than its VH counterpart (1083, 1097, 1104). In addition, the CDR1 and CDR2 are thought to be able to adopt alternative canonical structures not observed in VH (1104, 1107).

The VHH and VH are encoded for by separate germline genes (1108). Like the VH domain, the VHH domain is obtained during B cell differentiation after DNA recombination between a dedicated VHH germline gene with one diversity gene (D) and one heavy joining (JH) minigene in an event referred to as V-D-J rearrangement. In dromedary, 42 different germline VHH genes and 50 different germline VH genes have been reported (1104). In llama, five VHH genes and one VH gene have been reported so far (1081). The VHH and VH genes show high sequence homology, but are distinguished by the four hallmark VHH substitutions. In addition, the VHH seem to have more hotspots for mutation in the region upstream of the CDR1 (1091), in keeping with the increased sequence diversity observed in the CDR1 (1083, 1097, 1104). At the 3'-end, the VHH genes are flanked by conserved recombination signal sequences (RSS), essential for the somatic V-D-J rearrangement, supporting the notion that the VHH genes take part in V-D-J rearrangement. It is thought that the VHH and VH genes recombine with the same D and JH genes (1091).

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 (1109). Moreover, they exhibit diverse antigen-binding properties (1094, 1099, 1100). The increased level of sequence diversity and somatic hypermutation, and the longer CDRs able to adopt alternative canonical structures, are likely to contribute to these features and to compensate

for the lack of interaction surface and combinatorial diversity provided by the VH-VL base-pairing (1094).

VHH-antigen interface areas of up to 1700 $Å^2$ have been reported (1110), which equals the size of the interaction surface typically observed between a VH-VL pair and its antigen (1111). Similar interface sizes have been observed for the interaction of VHH and conventional antibodies with the same antigen (1112). The lack of interaction surface provided by the VL domain of the light chains could also be compensated for by the involvement of framework residues in the interaction with antigen, as interactions of VHH framework residues with both a hapten (1101) and a proteinaceous antigen have been observed (1113).

Conventional antibodies typically interact with protein antigens via planar paratopes and with haptens or peptides via concave paratopes (1114, 1115). Unlike conventional antibodies, the VHH of heavy-chain antibodies have been shown to have a preference for binding into active sites (1116) and to be able to recognise cryptic epitopes typically occluded to conventional antibodies (1117). To further investigate this feature of VHH, de Genst *et al*. (1112) generated high-resolution structural data for eight VHH in complex with hen egg white lysosyme (HEWL), isolated from dromedaries immunised with HEWL, showing that six out of eight VHH bound into the HEWL active site cleft. In contrast, previous observations have shown that conventional antibodies typically bind to planar epitopes of HEWL. Unlike conventional antibodies, which often used all their six CDRs to interact with HEWL, the paratopes of the anti-HEWL VHH were made up predominantly by the CDR3. The majority (85%) of heavy-chain antibodies present in polyclonal sera from the immunised dromedaries were also found to target the active site. These observations confirm the notion that VHH have a preference for recognising clefts and binding into active sites of proteins, and that this feature tends to be conferred by a protruding CDR3 loop.

1.2.4 Use of VHH in biotechnological and therapeutical applications

VHH have several features that make them potentially suitable for use in biotechnological and therapeutical applications (1100). For example, the VHH

domain can be easily cloned from immunised camelids and expressed to high levels in bacteria or yeast (1118, 1119). Individual VHH can be isolated using well-established selection technologies such as phage display. When constructing libraries for phage display, the single-domain nature of VHH offers an advantage over conventional antibody Fab (fragment antigen-binding) or scFv (single-chain Fv) fragments, since there is no need for random recombination of the VH and VL domains. Initially, VHH domains were isolated from camels and dromedaries but has since been successfully generated from llamas (1100), which may be more readily available and easier to keep. Recently, alpacas have also been used (1079, 1120).

Furthermore, VHH have been shown to be thermally and conformationally stable, exhibiting thermodynamic stabilities that have not been observed for functional conventional antibody fragments (1109, 1121, 1122). For example, unlike conventional antibodies, some VHH have been shown to retain their binding properties at temperatures up to 90°C (1109, 1121). In addition, chemically induced unfolding has been shown to be fully reversible (1122).

The stability and solubility of VHH also allows for engineering of multivalent and bispecific molecules (1123). Engineered multivalent VHH to the epidermal growth factor receptor (EGFR) and tumour necrosis factor α (TNFα) have been shown to exhibit better binding and neutralising properties than their monovalent counterparts (1124, 1125).

The unique properties of camelid VHH have proven them potential in a number of applications against a wide range of biological targets. For example, VHH targeting the Malf1 surface protein of the fungus *Malassezia furfur*, implicated in the formation of dandruff, has been isolated from llamas immunised with extracts of the fungus (1126). Remarkably, the stability of the VHH allowed for phage display-based selections carried out in the presence of shampoo. Furthermore, VHH targeting human lysosyme has been reported to inhibit *in vitro* aggregation of an amyloidogenic lysosyme variant, with implications of amyloid disease (1127). In addition, intracellularly expressed VHH binding to nuclear poly(A)-

binding protein 1 (implicated in the protein aggregation disorder Oculopharyngeal muscular dystrophy), isolated from a VHH library derived from a naïve llama, has been shown to reduce protein aggregation in an *in vitro* model (1128).

Moreover, VHH against EGFR have been isolated from llamas immunised with EGFR-containing cell preparations (1125). These VHH were able to inhibit EGF-induced signalling and EGF-induced cell proliferation, as well as being able to delay tumour outgrowth in an *in vivo* model for solid tumours (1125). In addition, VHH targeting TNFα, isolated from llamas immunised with recombinant TNFα, have been shown to block interaction of TNFα with its receptor, and to show therapeutic effects against collagen-induced arthritis in a mice model (1124). VHH against the human tumour-associated carcinoembryonic antigen, conjugated to an anti-cancer prodrug (*Enterobacter cloacae* beta-lactamase) have shown to be effective *in vitro* as well as in *in vivo* models (1129).

VHH raised against a range of pathogens have also been isolated. These pathogens include *Streptococcus mutans* causing dental caries (1130), *Trypanosoma brucei rhodesiense* causing sleeping sickness (1131), and foot and mouth virus (1132). In addition, VHH targeting rotavirus have been shown to reduce the severity of rotavirus-induced diarrhoea in an *in vivo* mice model, when administered orally as recombinant proteins (1133) as well as when expressed by orally administered lactobacilli (1134). In 2007, these VHH were the first to enter a phase I clinical trial. VHH to the rotavirus VP6 protein has also been shown to confer protection in a mice model (1135).

Because of their properties in terms of stability, VHH are suitable for intracellular expression (1120, 1128). They can also be used in biotechnical applications, such as immunoaffinity chromatography (1136). In addition, intracellular expression of VHH against a desired antigen, fused with fluorescent proteins, can enable targeting and tracing of antigenic structures within living cells (1120).

In summary, the unique properties of camelid VHH in terms of size, non-conventional binding characteristics, protruding and non-canonical CDRs, stability, solubility, ease of cloning and production yield, make them potentially valuable reagents in many therapeutical and biotechnical applications.

1.3 Scope of this thesis

Monoclonal antibodies 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. Monoclonal antibodies 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. Broadly neutralising mAbs may also have potential use as HIV-1 microbicides. Only a handful of broadly neutralising mAbs have, however, been described, all a result of infection. To gain further insight into the antigenic structure of the HIV-1 envelope spike and to further define conserved neutralising epitopes, there is a need for additional broadly neutralising antibodies. The aim of the work presented in this thesis was to use the non-conventional immune system of llamas to generate novel, neutralising antibody fragments, VHH, to the HIV-1 envelope surface glycoprotein gp120, and to characterise these antibody fragments in biological and biochemical assays. The methodology behind this work will be described in chapter 2, and the results will be presented and discussed in chapters 3-5, followed by a final discussion in chapter 6.

Chapter 2

Materials and methods

In this thesis, the isolation and characterisation of neutralising llama VHH will be described. This chapter provides details of the materials and methodology used to generate the data presented in chapter 3-5.

2.1 Materials

This section provides details of the buffers, solutions, human antibodies, and recombinant proteins used in this study. Cell lines and viruses will be described in section 2.3 and 2.4.

2.1.1 Buffers and solutions

The buffers and solutions used in this study are listed in Table 2.1.1.1.

2.1.2 Recombinant HIV-1 envelope glycoproteins

Recombinant gp120 derived from the HIV-1 subtype B'/C (CRF07_BC) primary isolate CN54 (1137, 1138), produced in a baculovirus expression system, was kindly provided by I. Jones, Reading University, UK, through the European Microbicide Project (EMPRO). Additional batches of recombinant gp120 from HIV-1 CN54 were expressed as described in section 2.7. Recombinant CN54 gp140 and gp140 derived from the HIV-1 subtype A primary isolate 92UG037 (1048, 1139), 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 (32, 1140), produced in a baculovirus expression system, was obtained from the Centralised Facility for AIDS Reagents (catalogue number EVA607) at the National Institute for Biological Standards and Control (NIBSC), Herts, UK. Recombinant gp120 derived from the HIV-1 subtype A primary isolate 92UG037 (1139), the subtype

C primary isolate 92BR025 (1139), and the molecular clones CB7, which expresses gp120 cloned from plasma of an individual infected with HIV-1 of subtype C, were cloned, expressed and purified as detailed in section 2.5 and 2.7.

2.1.3 Monoclonal antibodies to HIV-1 gp120 and gp41

Monoclonal antibodies b12 (1011) and b6 (1011) were kindly provided by D. Burton, Scripps Institute, La Jolla, USA. Monoclonal antibody GP68 (1141) was obtained through the Centralised Facility for AIDS Reagents, NIBSC (original source A. Osterhaus and M. Schutten). Monoclonal antibodies 654-D (975, 1034, 1142) and 447-52D (991, 1032) were obtained through the Centralised Facility for AIDS Reagents, NIBSC (original source S. Zolla-Pazner). Monoclonal antibody 17b (1143) was obtained from the NIH AIDS Research and Reference Reagent Programme, Division of AIDS, National Institute of Allergies and Infectious Diseases (NIAID), National Institutes of Health (NIH), USA (original source James E. Robinson). Monoclonal antibodies 2G12 (981) and 4E10 (908) were obtained from Polymun Scientific GmbH, Vienna, Austria, as part of the Collaboration for AIDS Vaccine Discovery (CAVD).

2.1.4 Sera and plasma from HIV-1-seropositive individuals

Quality control (QC) sera 2, 5 and 6 from HIV-1-seropositive individuals have been described previously (1144). Plasma from individuals infected with HIV-1 subtype C was kindly provided by D. Yirrel, Glasgow, UK.

2.1.5 Recombinant sCD4

Recombinant sCD4 consisting of the extracellular domains D1-D4 was available and has been previously described (1145). In addition, recombinant sCD4 (D1-D4) was obtained from R&D Systems (Minneapolis, USA, catalogue number 514-CD-050/CF).

Table 2.1.1.1. Buffers and solutions[a].

Buffer/solution	Composition
2 × tryptone/yeast (TY) medium	1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl
Lysogeny broth or 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
VHH elution buffer	50 mM Na_2HPO_4, 300 mM NaCl, 150 mM imidazole, pH 7.0
VHH equilibrium buffer	50 mM Na_2HPO_4, 300 mM NaCl, pH 8.0
VHH pre-elution buffer	50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 7.0

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

2.2 Molecular biological techniques

This section provides details on the general molecular biological techniques used in this study.

2.2.1 Isolation of genomic DNA

Total DNA was isolated from HIV-1-infected PBMC using the QIAamp DNA Blood Mini Kit (QIAGEN, Crawley, UK), according to the manufacturer's instructions. Briefly, 5×10^6 pelleted cells were resuspended in PBS to a final volume of 200 µl. Cells were lysed by the addition of QIAGEN protease and AL lysis buffer and incubated at 56°C for 10 minutes. To ensure complete lysis and inactivation of virus, samples were subjected to an additional incubation step of 30 minutes at 56°C. Total DNA was then purified using QIAamp Mini Spin columns and eluted in 200 µl of QIAGEN AE elution buffer.

2.2.2 Isolation of RNA

RNA was isolated from plasma of individuals infected with HIV-1 of subtype C, or from cell-free viral stocks of HIV-1 CN54 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 incubation at room temperature for 10 minutes, in the presence of polyA carrier RNA. RNA was then purified on QIAamp Mini Spin columns and eluted in QIAGEN AVE elution buffer.

2.2.3 Isolation of plasmid DNA

LB medium (5 ml) 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 containing vectors with full-length HIV-1 molecular clones or gp160 clones) with orbital shaking at 250 revolutions per minute (rpm) and bacteria were pelleted by centrifugation at 3000 × *g* for 10 minutes 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, which contains RNase A, and subsequently lysed under alkaline conditions using buffer P2, which contains NaOH/sodium dodecyl sulphate (SDS), and 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 17900 × *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).

2.2.4 Restriction enzyme digestion of plasmid DNA

Plasmid DNA was digested using restriction enzymes obtained from Promega (Southampton, UK) for 1 hour under conditions specified by the manufacturer. Partial digests were carried out by shortening the incubation time and/or diluting the enzyme. Digested DNA was separated using agarose gel electrophoresis (section 2.2.5).

2.2.5 Agarose gel electrophoresis of DNA

DNA fragments were separated according to size by agarose gel electrophoresis. Gels contained 0.5 µg/ml of ethidium bromide (Sigma-Aldrich, Poole, UK) and were made with 1% (DNA fragments >500 bp) or 1.5-2% (DNA fragments <500 bp) agarose (w/v; Roche Diagnostics, Lewes, UK) in 1 × TAE buffer. 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 1 × TAE buffer at 80-100 V for an appropriate amount of time. DNA was visualised by illumination with ultraviolet light.

2.2.6 Extraction of DNA fragments from agarose gels

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

2.2.7 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 minutes. The bacteria were subsequently subjected to heat-shock at 42°C for 30 seconds, followed by incubation on ice for 1 minute and addition of 250 µl of SOC medium (Invitrogen). The bacteria were then incubated for 1 hour at 37°C (or 30°C for bacteria transformed with vectors containing full-length HIV-1 molecular clones or gp160 clones) and 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 SOC medium, the bacteria were incubated for 1 hour at 37°C and 250 rpm and subsequently plated onto LB agar plates containing 100 µg/ml of ampicillin (or carbenicillin) and 2% (w/v) glucose. Plates were then incubated overnight at 37°C.

2.2.8 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 pmol of sequencing primer and 1 × CEQ DTCS Quick Start master mix. Thermal cycling conditions consisted of 30 cycles of 96°C for 20 seconds, followed by 50°C for 20 seconds and 60°C for 4 minutes. 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; contains deionised formamide). 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).

Some DNA sequencing was also carried 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.

2.2.9 Determination of nucleic acid concentration

Nucleic acid concentration and purity was determined spectrophotometrically using the NanoDrop 1000 Spectrophotometer (NanoDrop products, Thermo Fisher Scientific, Wilmington, USA).

2.2.10 Determination of protein concentration

Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories), which is based on the method developed by Bradford (1146), according to the manufacturer's instructions. Three dilutions of each sample were assayed in triplicate or quadruplicate on two separate occasions.

2.2.11 SDS-PAGE and Coomassie blue staining

Proteins were resolved using SDS-polyacrylamide gel electrophoresis (PAGE). Separating polyacrylamide gels were made with 15% (w/v) AA-Bis polyacrylamide (Bio-Rad Laboratories) and 0.1% (w/v) SDS in 375 mM Tris-HCl (pH 8.8), with 100 µl of 10% (w/v) ammonium persulphate (APS) and 10 µl of N, N, N', N'-tetramethylethylenediamine (TEMED) per 10 ml of gel, to polymerise. Stacking gels were made with 4% (w/v) AA-Bis polyacrylamide and 0.1% (w/v) 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 minutes, 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 1 × NuPAGE LDS Sample Buffer and 1 × NuPAGE Reducing Agent and heated to 70°C for 10 minutes 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% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) in 10% (v/v) acetic acid and 40% (v/v) methanol and subsequently destained in 10% (v/v) acetic acid and 40% (v/v) ethanol.

2.3 Cell culture

This section describes the cell lines and the general cell culture techniques used in this study.

2.3.1 Cell lines and culture media

The TZM-bl cell line (1147-1149), previously designated JC53-bl (clone 13), is a HeLa cell line, hence derived from a human cervical carcinoma (1150), which stably expresses human CD4 and CCR5 and contains 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 (1151) are human glioma cell lines expressing human CD4 and either of the HIV-1 main co-receptors, CXCR4 (NP2/CD4/CXCR4) or CCR5 (NP2/CD4/CCR5). The CCC/CCR5 and CCC/CD4/CCR5 cell lines (349, 382, 1152, 1153) are feline kidney cells expressing human CCR5, and human CD4 and CCR5, respectively. NP2 and CCC cells were cultured in DMEM (Invitrogen) containing 5% (v/v) FCS. The 293T cell line, of human embryonic kidney origin (1154), 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 (1155), was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% (v/v) FCS. Baby hamster kidney (BHK) cells (1156) were cultured in DMEM supplemented with 10% (v/v) FCS.

2.3.2 Maintenance of cell lines

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 1:3-1:20 depending of the cell line. For adherent cell lines, cell monolayers were disrupted and removed by treatment with trypsin-versene (0.5% (w/v) trypsin and 0.02% (w/v) versene in PBS) or trypsin-EDTA (0.25% (w/v) trypsin, 1 mM EDTA; Invitrogen) at confluency, prior to passaging. Trypsinisation was performed by washing cells once in trypsin-versene, trypsin-

EDTA, or sterile PBS, followed by incubation with approximately 2 ml trypsin-versene or trypsin-EDTA 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. Cells engineered to express CD4 were occasionally passaged in selective medium containing 1 mg/ml Geneticin G418 sulphate (Invitrogen), in order to maintain stable CD4 expression, whereas cells engineered to express CXCR4 or CCR5 were occasionally passaged in the presence of 1 μg/ml puromycin for selection.

2.3.3 Freezing and thawing of cells

Cells were pelleted by centrifugation at 325 × *g* for 10 minutes and resuspended at approximately 10^7 cells/ml in the appropriate cell culture medium (section 2.3.1) containing 20% (v/v) FCS and 10% (v/v) dimethyl sulphoxide (DMSO). Adherent cells were trypsinised as described in section 2.3.2 prior to being pelleted. Resuspended cells were aliquoted into cryovials and subsequently transferred to -80°C. To allow for slow freezing, vials were wrapped in tissue and 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 minutes. Pelleted cells were resuspended in growth medium, transferred to cell culture flasks and kept at 37°C.

2.3.4 Preparation of peripheral blood mononuclear cells

PBMC were prepared from fresh blood. Blood was drawn from healthy volunteers by me, S. Willey, or K. Aubin, under ethical approval obtained from the UCL Committee for the Ethics of non-NHS Human Research (Ethical Approval Number 0335/001), using S-Monovette collection tubes containing EDTA (Sarstedt, Nümbrecht, Germany). Fresh blood was diluted 1:1 in PBS, and a total of 35 ml was layered onto 15 ml of Lymphoprep (Axis-Shield, Oslo, Norway) in a 50 ml Falcon tube (BD Biosciences, Oxford, UK) and subsequently

centrifuged at 700 × *g* for 30 minutes. After centrifugation with Lymphoprep, the PBMC form a layer at the Lymphoprep-plasma interface. The PBMC layer was carefully collected and diluted in PBS to a total volume of 50 ml, followed by centrifugation at 325 × *g* for 10 minutes. Cells were washed twice more in PBS and finally resuspended at a density of approximately 5×10^6 cells/ml in RPMI 1640 supplemented with 10% (v/v) FCS and 2.5 μg/ml of phytohaemagglutinin (PHA). The cells were ready to be infected after 3-4 days of culture. After 3 days, the growth medium was changed to RPMI 1640 supplemented with 10% (v/v) FCS and 20 U/ml of interleukin-2 (Roche Diagnostics) and cultured for up to another 4 days.

2.3.5 Transfection of 293T cells

Exponentially dividing 293T cells were transfected with plasmid DNA using FuGENE 6 (Roche Diagnostics), according to the manufacturer's instructions. Briefly, the 293T cells were seeded in 10 cm cell culture dishes at a density of 2.4×10^6 cells per dish and incubated overnight at 37°C. The next day, the growth medium was replaced with 12 ml fresh growth medium per dish. For transfection, 1.2 ml of Opti-MEM (Invitrogen) was mixed with 36 μl of FuGENE 6 and a total of 12 μg of plasmid DNA, incubated for 15 minutes at room temperature, and subsequently added dropwise to cells. After 24 hours, the transfection medium was aspirated and replaced with fresh growth medium. For smaller-scale transfections, 293T cells were seeded in a 6-well cell culture plate at a density of approximately 4×10^5 cells per well and transfection volumes adjusted accordingly.

2.4 HIV-1 strains and preparation of virus stocks

In this section, details of the HIV-1 strains used in this study will be given, along with information on preparation of virus stocks.

2.4.1 HIV-1 strains

HIV-1 TCLA isolates, primary PBMC-propagated isolates, and infectious molecular clones used in this study are detailed in Table 2.4.1.1. TCLA, CXCR4-using isolates HIV-1 IIIB (32, 1140) and HIV-1 MN (33), which are subtype B isolates originally isolated from PBMC of HIV-1 seropositive individuals in the 1980s, were obtained from the Centralised Facility for AIDS Reagents (NIBSC) as cell-free cell-culture supernatant from infected H9 cells.

Cell-free stocks of PBMC-propagated primary isolates HIV-1 BaL (CCR5-using), which was originally isolated from human infant lung tissue in 1986 (180), and HIV-1 SF162 (CCR5-using), which was originally isolated from brain tissue in 1988 (1157) were also obtained from the Centralised Facility for AIDS Reagents (NIBSC). PBMC-propagated HIV-1 CRF07_BC primary isolate CN54 (1137, 1138) was obtained from the European Microbicides Project (EMPRO). PBMC-propagated HIV-1 primary isolates 92UG037, 92UG001, 92BR025, 97IN003 and ZA97001 were obtained from the WHO-UNAIDS collection of primary isolates (1139). Obtained HIV-1 TCLA and primary isolates were propagated as described in section 2.4.2.

The replication-competent, full-length HIV-1 molecular clone YU2 (CCR5-using), cloned directly from brain tissue from an individual with AIDS dementia by Li *et al*. (1158, 1159), was obtained from the NIH AIDS Research and Reference Reagent Programme, as was the replication-competent, full-length HIV-1 molecular clone JRFL (CCR5-using), which is derived from frontal lobe brain tissue of a patient with AIDS dementia (416, 1160, 1161).

Replication-competent chimeric molecular clones 4.10.3 (GenBank accession number DQ425070), 8.8.8 (GenBank accession number DQ425074), 23.8.12 (GenBank accession number DQ425081) and 23.2.E (GenBank accession number DQ425078) were recently reported by Aasa-Chapman *et al*. (1162), and contain subtype B gp120 cloned from plasma of HIV-1-infected individuals held in the pHXB2Δ*env* vector (1163). Replication-competent chimeric molecular

clones CA6 and CB7, which contain subtype C gp120 held in the pHXB2Δ*env* vector (1163), were kindly provided by M. Aasa-Chapman (UCL, London, UK).

Chimeric molecular clones C222, C261, 27b and 27d were cloned by Willie Koh as described in section 2.6. Chimeric molecular clones 92UG037.A9, 92BR025.C1, ZA97001.1, 97IN003.4.2, 92UG001.D8, 37.4.2 and 38.2.2 were cloned by me as described in section 2.6. Stocks of HIV-1 molecular clones were prepared as described in section 2.4.3.

HIV-1 envelope pseudotyped viruses were generated using the gp160 clones listed in Table 2.4.1.2. The HIV-1 subtype B and subtype C reference panels of envelope clones, generated by Li *et al*. (1022, 1023), consists of clones from acute and early HIV-1 infections and were obtained through the NIH AIDS Research and Reference Reagent Programme. 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.

HIV-1 subtype CRF07_BC gp160 clones CH181.12, CH064.20, CH091.9, CH117.4, CH119.10, CH110.2, CH114.8, CH120.6, CH115.12, CH070.1, CH111.8 and CH038.12 are derived from chronic HIV-1 infections and 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, as were subtype CRF02_AG gp160 clones T257-31 (derived from acute/early infection) and T33-7 (derived from chronic infection), and the subtype C gp160 clones 93MW965.26 and 96ZM651.02. HIV-1 envelope pseudotyped viruses were produced in 293T cells by co-transfection with the pSG3Δ*env* plasmid as described in section 2.4.3.

Table 2.4.1.1. HIV-1 isolates and molecular clones included in this study.

Virus	Subtype	Type[a]	Source[b]	Geographical origin[c]
92UG037.A9	A	MC (gp120/HXB2)	ccPBMC	Uganda
IIIB	B	TCLA	PBMC	USA
MN	B	TCLA	PBMC	USA
BaL	B	PBMC	Lung	USA
SF162	B	PBMC	Brain	USA
JRFL	B	MC (full-length)	Brain	USA
YU2	B	MC (full-length)	Brain	USA
4.10.3	B	MC (gp120/HXB2)	Plasma	UK
8.8.8	B	MC (gp120/HXB2)	Plasma	UK
23.2.E	B	MC (gp120/HXB2)	Plasma	UK
23.8.12	B	MC (gp120/HXB2)	Plasma	UK
CN54	B′/C	PBMC	PBMC	China
97IN003	C	PBMC	PBMC	India
ZA97001	C	PBMC	PBMC	South Africa
92BR025.C1	C	MC (gp120/HXB2)	ccPBMC	Brazil
CA6	C	MC (gp120/HXB2)	Plasma	No info.
CB7	C	MC (gp120/HXB2)	Plasma	No info.
C222	C	MC (gp120/HXB2)	Plasma	No info.
C261	C	MC (gp120/HXB2)	Plasma	No info.
97IN003.4.2	C	MC (gp160/NL43)	ccPBMC	India
ZA97001.1	C	MC (gp160/NL43)	ccPBMC	South Africa
37.4.2	C	MC (gp160/NL43)	Plasma	No info.
38.2.2	C	MC (gp160/NL43)	Plasma	No info.
27b	C	MC (gp160/NL43)	Plasma	No info.
27d	C	MC (gp160/NL43)	Plasma	No info.
92UG001.D8	D	MC (gp160/NL43)	ccPBMC	Uganda

[a]TCLA, T cell line-adapted isolate; PBMC, Primary PBMC-propagated isolate; MC, molecular clone. Molecular clones were either full-length infectious HIV-1 clones, chimeric clones consisting of heterologous gp120 slotted into the HIV-1 HXB2-based pHXB2Δ*env* backbone (1163), or heterologous gp160 slotted into the HIV-1 NL43-based C2 cassette (1164).

[b]Source of original isolate. For molecular clones, this indicates the source of the *env* (gp120 or gp160) gene; ccPBMC, co-cultured PBMC.

[c]No info., information not available.

Table 2.4.1.2. HIV-1 envelope pseudotyped viruses included in this study.

Virus	Subtype	Source[a]	Geographical origin	Accession no.[b]
T257-31	A/G	No info.	West Africa	n/a
T33-7	A/G	No info.	West Africa	n/a
6535.3	B	ccPBMC	USA	AY835438
QH0692.42	B	ccPBMC	Trinidad	AY835439
SC422661.8	B	Plasma	Trinidad	AY835441
PVO.4	B	ccPBMC	Italy	AY835444
TRO.11	B	ccPBMC	Italy	AY835445
AC10.0.29	B	ccPBMC	USA	AY835446
RHPA4259.7	B	Plasma	USA	AY835447
THRO4156.18	B	Plasma	USA	AY835448
REJO4541.67	B	Plasma	USA	AY835449
TRJO4551.58	B	Plasma	USA	AY835450
WITO4160.33	B	Plasma	USA	AY835451
CAAN5342.A2	B	Plasma	USA	AY835452
CH181.12	B′/C	ccPBMC	China	EF117259
CH064.20	B′/C	ccPBMC	China	EF117254
CH091.9	B′/C	ccPBMC	China	EF117256
CH117.4	B′/C	ccPBMC	China	EF117262
CH119.10	B′/C	ccPBMC	China	EF117261
CH110.2	B′/C	ccPBMC	China	EF117257
CH114.8	B′/C	ccPBMC	China	EF117264
CH120.6	B′/C	ccPBMC	China	EF117260
CH115.12	B′/C	ccPBMC	China	EF117263
CH070.1	B′/C	ccPBMC	China	EF117255
CH038.12	B′/C	ccPBMC	China	EF042692
93MW965.26	C	No info.	Malawi	n/a
96ZM651.02	C	No info.	Zambia	n/a
Du156.12	C	ccPBMC	South Africa	DQ411852
Du172.17	C	ccPBMC	South Africa	DQ411853
Du422.1	C	ccPBMC	South Africa	DQ411854
ZM197M.PB7	C	ucPBMC	Zambia	DQ388515
ZM214M.PL15	C	Plasma	Zambia	DQ388516
ZM233M.PB6	C	ucPBMC	Zambia	DQ388517
ZM249M.PL1	C	Plasma	Zambia	DQ388514
ZM53M.PB12	C	ucPBMC	Zambia	AY423984
ZM109F.PB4	C	ucPBMC	Zambia	AY424138
ZM135M.PL10a	C	Plasma	Zambia	AY424079
CAP45.2.00.G3	C	Plasma	South Africa	DQ435682
CAP210.2.00.E8	C	Plasma	South Africa	DQ435683

[a]Source of *env* gene; no info., information not available; ccPBMC, co-cultured PBMC; ucPBMC, uncultured PBMC.

[b]GenBank accession number; n/a, not applicable.

2.4.2 Propagation of viruses in T cell lines or PBMC

HIV-1 primary isolates were propagated in PHA-stimulated PBMC. Approximately 1-2 × 10^7 PBMC (pooled from two donors) were incubated with virus at 37°C in a total volume of 1-2 ml. After 2 hours 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). After 6-7 days, the cells were pelleted by centrifugation at 325 × g for 10 minutes 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 × 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 minutes. The supernatant was subsequently filtered (0.45 μm) and transferred to the vapour phase of liquid nitrogen in 0.5 ml aliquots. HIV-1 TCLA isolates were propagated in H9 cells in a similar manner, but harvested on day 5 after infection.

2.4.3 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 *env*, as described by Li *et al*. (1022). The pSG3Δ*env* plasmid is based on the full-length replication-competent HIV-1 pSG3.1 clone (GenBank accession number L02317), but with deleted *env*. 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 2.3.5. The virus-containing cell culture supernatant was harvested at 48 and/or 72 hours after transfection, filtered through a 0.45 μm

filter, transferred to cryovials in 0.5 ml aliquots, and stored in the vapour phase of liquid nitrogen.

2.5 Amplification and cloning of *env* genes

In this section, the PCR amplification of gp120 and gp160 gene segments and the subsequent cloning of these fragments into expression and cloning vectors will be detailed.

2.5.1 Sources of DNA and RNA

HIV-1 gp120 and gp160 were amplified from viral RNA and from proviral DNA isolated from infected PBMC. PBMC were prepared from blood of healthy volunteers, as described in section 2.3.4, and were subsequently infected with HIV-1 primary isolates CN54, 92UG037, 92BR025, 97IN003, ZA97001 and 92UG001, as described in section 2.4.2. After 5 days of culture, genomic DNA was isolated from infected cells as described in section 2.2.1. RNA was isolated from plasma of infected individuals or from cell-free virus stocks as described in section 2.2.2. HIV-1 CN54 gp120 was also amplified from plasmids holding a full-length proviral CN54 molecular clone or from plasmids containing a synthetic CN54 gp160 gene (obtained from EMPRO). HIV-1 CB7 gp120 was amplified from the chimeric CB7 molecular clone (section 2.4.1).

2.5.2 Primers and primer design

The primers used to amplify HIV-1 gp120 and gp160 are listed in Table 2.5.2.1. Primers o_envf and o_envr were designed using the Primer3 software (1165) and analysed for secondary structures using the IDT Oligo Analyzer software (available at http://www.idtdna.com). Furthermore, primers o_envf and o_envr were designed to target nucleotide stretches conserved among most HIV-1 isolates, and generated an amplicon of expected size from 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 *Xho*I and *Eco*RI to facilitate subsequent sub-cloning, were modified from primers described by

Zheng and Daniels (1164). Primers 988L, 943S, 609RE, 628L, 125Y and 626L, which amplify gp120, have been described by Aasa-Chapman *et al*. (1162), as has primer 944S, which contains a natural Kozak sequence and a start codon, to facilitate subsequent protein expression. Antisense primer E400010_His was based on primer E400010, which has been described previously (1162), but encoded a 6-histidine-tag in addition to the stop codon present in E400010. Primer E400010_His (and E400010) also encodes part of 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).

Table 2.5.2.1. Primers used for amplification of HIV-1 gp160 and gp120[a].

Primer	Sequence (5′to 3′)[b]	Position[c]
o_envf	TYTCCTATGGCAGGAAGAAGC	5964
o_envr	TAACCCWTCCAGTCCCCCCTTTT	9096
988L	GTAGCATTAGCGGCCGCAATAATAATAGCAATAG	6089
943S	CAATAGYAGCATTAGTAGTAG	6084
609RE	CCCATAGTGCTTCCGGCCGCTCCCAAG	7816
628L	TCATCTAGAGATTTATTACTCC	8086
fenv3	AGCTCGAGCAGAAGACAGTGGCAATGA	6202
renv3	CTATGGAATTCTTCGACCACTTGCCCCCCATTT	8827
944S	AGAAAGAGCGGCCGCCAGTGGCAATG	6202
E400010_His	TTAATGATGATGATGATGATGTCTTTTTTCTCTCTGCACCACT	7778
626L	GTGGGTCACCGTCTATTATGGG	6326
125Y	CACCACGCGTCTCTTTGCCTTGGTGGG	7742

[a]Primers 944S, 626Y and 125Y have been described previously (1162). Primer E400010_His was based on primer E400010 described previously (1162). Primers fenv3 and renv3 were modified from primers described previously (1164).

[b]Restriction sites (*Xho*I in fenv3, *Eco*RI in renv3, *BstE*II in 626L, and *Mlu*I in 125Y) are underlined. Y equals C or T and W equals A or T according to standard International Union of Pure and Applied Chemistry (IUPAC) nomenclature.

[c]Nucleotide position of primer 5′-end in the HIV-1 HXB2 genome (GenBank accession number K03455).

Additional primers used for DNA sequencing of gp120 and gp160 clones are shown in Table 2.5.2.2. Primers A589-, E80+, 621L-, 617L-, and 015S+ were provided by M. Aasa-Chapman. Primers gp160 +1, gp160 +298, gp160 -317,

gp160 +580, gp160 -600, gp160 +901, gp160 +1198, gp160 +1581, gp160 +1881, gp160 +2191, and gp160 +2431 had been designed by K. Aubin.

Table 2.5.2.2. Primers used for sequencing of HIV-1 gp160 and gp120.

Primer	Sequence (5´to 3´)	Position[a]
A589-	CAGAGTGGGGTTAATTTTACAC	6600
E80+	CCAATTCCCATACATTATTGTG	6858
621L-	GTACATTGTACTGTGCTGACATT	6967
617L-	CTGGGTCCCCTCCTGAGG	7332
015S+	ACATTGTAACATTAGTAGAG	7211
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

[c]Nucleotide position in the HIV-1 HXB2 genome (GenBank accession number K03455).

2.5.3 RT-PCR

Specific first-strand cDNA was synthesised from RNA (section 2.5.1) 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 2.5.2.1) 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 1 × 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 minutes. The reverse transcription step was

immediately followed by the PCR step, which consisted of initial denaturation at 96°C for 5 minutes, followed by 35 cycles of denaturation at 96°C for 1.5 minutes, annealing at 60°C for 1 minute and elongation at 68°C for 10 minutes, followed by a final elongation step of 20 minutes 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, as described in section 2.5.4.

2.5.4 PCR amplification of gp120 and gp160

Full-length HIV-1 gp120 and gp160 was amplified from proviral DNA isolated from infected PBMC (section 2.5.1) 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 1× 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 minutes at 96°C, followed by 35 cycles of denaturation at 96°C for 1.5 minutes, annealing at 60°C for 1 minute and elongation at 68°C for 10 minutes, followed by a final elongation step of 20 minutes at 68°C. Alternatively, for amplification of gp120 from some of the viruses, the first round of PCR was performed as described by Aasa-Chapman *et al*. (1162), using a mix of forward primers 988L and 943S and either of reverse primers 609RE and 628L and an annealing temperature of 44°C.

A 1-5 µl aliquot from the first round of PCR amplification (or RT-PCR amplification; section 2.5.3) 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 2.5.5) for expression in a T7 RNA polymerase-recombinant vaccinia virus system, gp120 was amplified as described by Aasa-Chapman *et al*. (1162) using primers 944S and E400010_His. For HIV-1 CN54 and CB7, gp120 was amplified from molecular clones using primers 944S and E400010_His. If the gp120 was going to be slotted into cloning vectors (section 2.5.6), 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 minutes at 96°C, followed by 35 cycles of denaturation at 96°C for 1.5 minutes, annealing at 50°C (primers fenv3/env3), 55°C (primers 944S/E400010_His), or 57°C (primers 125Y/626L) for 1 minute, and elongation at 68°C for 5 minutes (gp120 amplification) or 10 minutes (gp160 amplification), followed by a final elongation step of 20 minutes 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, as described in section 2.2.5, and DNA fragments of the expected size were purified from the gel as described in section 2.2.6.

2.5.5 Cloning of amplified gp120 into expression vectors

Gel-purified gp120 amplified using primers 944S/E400010_His was ligated into the expression vector pcDNA3.1-TOPO (Invitrogen) according to the manufacturer's instructions. The ligation reactions were subsequently transformed into competent TOP10 bacteria (section 2.2.7). Screening of pcDNA3.1-TOPO plasmids carrying an insert of the expected size was carried out by restriction enzyme digestion (section 2.2.4) followed by agarose gel electrophoresis (section 2.2.5). Clones producing functional gp120 when

transfected into T7 RNA polymerase-recombinant vaccinia virus-infected 293T cells (section 2.7) were sequenced (section 2.2.8).

2.5.6 Cloning of amplified gp120 and gp160 into cloning vectors

Gel-purified gp120 and gp160 clones were ligated into the pGEM-T Easy (Promega) or the pCR2.1-TOPO (Invitrogen) cloning vectors according to the manufacturers' instructions. Competent bacteria were transformed with the ligation reactions (section 2.2.7) and plated onto LB/ampicillin plates onto which 40 µl of 40 mg/ml of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) in N,N´-dimethylformamide (Promega) had been spread. As successful cloning of an insert into the pGEM-T Easy and 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 2.2.4) and agarose gel electrophoresis (section 2.2.5).

2.6 Generation of HIV-1 infectious recombinant molecular clones

Chimeric, infectious HIV-1 molecular clones containing heterologous *env* genes in subtype B-based backbones were generated as described below.

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

Full-length gp120 was sub-cloned from the pGEM-T Easy and pCR2.1-TOPO vectors into the pHXB2Δ*env* vector constructed by McKeating *et al*. (1163), by means of the *BstE*II recognition site present in primer 626L and the *Mlu*I recognition site present in primer 125Y. 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 pGEM-T Easy or pCR2.1-TOPO cloning vectors by double digestion with *BstE*II and *Mlu*I (section 2.2.4). Digested plasmid DNA was separated using agarose gel electrophoresis (section 2.2.5) and the gel-purified DNA fragment of the

expected size was then ligated (overnight at 4°C) 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) and the Rapid Ligation Buffer (Promega), according to the manufacturer's instructions. For some virus isolates, the gp120 contained an internal *BstE*II recognition site. Partial restriction digests were then carried out (section 2.2.4) and bands of the expected size were purified from the gel and ligated into pHXB2Δ*env*.

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

The C2 cassette, which is constructed by Zheng and Daniels (1164), is based on the infectious HIV-1 NL43 (subtype B) molecular clone and allows incorporation of heterologous full-length *env* genes. Full-length gp160 was extracted from the pGEM-T Easy and pCR2.1-TOPO vectors by double digestion with enzymes *Xho*I and *Eco*RI, whose recognition sites were present in primers fenv3 and renv3. Digested gp160 fragments were then gel-purified and transferred into the similarly digested and gel-purified C2 cassette using the T4 DNA ligase (Promega) and the Rapid Ligation Buffer (Promega), according to the manufacturer's instructions.

2.6.3 Identification of infectious gp120/HXB2 and gp160/NL43 chimeras

The gp120-pHXB2Δ*env* and gp160-C2 ligation reactions were transformed into competent bacteria (section 2.2.7). Clones containing inserts of the correct sizes were identified and used for virus production in 293T cells as described in section 2.4.3. 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 2.9.1. Clones producing infectious virus were sequenced (section 2.2.8).

2.7 Expression of recombinant gp120

Recombinant gp120 derived from HIV-1 CN54, 92UG037, 92BR025 and CB7 was expressed as described by Aasa-Chapman *et al*. (631) using a bacteriophage

T7 RNA polymerase-recombinant vaccinia virus system developed by Fuerst *et al*. (1166) in 1986. 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 hours 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 generated as described in section 2.5.5. Transfections were carried out as described in section 2.3.5 using 4 μg of plasmid DNA and 12 μl of FuGENE 6 (Roche Diagnostics). Cell culture media was not changed at 24 hours after transfection, as the monolayers of vaccinia virus-infected cells were easily disrupted. Cell culture supernatants were harvested 72 hours after transfection, treated with 1% (v/v) Igepal CA-630 (octylphenyl-polyethylene glycol; 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 (1167) 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 2.16. Alternatively, gp120 was purified using Ni Sepharose 6 Fast Flow (GE Healthcare, Little Chalfont, UK) by means of the 6-histidine-tag encoded by primer E400010_His, according to the manufacturer's instructions.

2.8 Virus detection assays

This section details the virus detection assays used in this study.

2.8.1 Intracellular p24 immunostaining

HIV-1 infection of NP2 cells (and CCC cells) were detected using *in situ* intracellular p24 immunostaining as previously described (1168). The cell culture medium was removed and the cells were fixed for 10 minutes at room

temperature using 200 µl per well of a mixture of methanol and acetone (at a 1:1 ratio) that had been pre-cooled at -40°C, and subsequently washed twice in PBS containing 1% (v/v) FCS (Invitrogen). Fixed cells were then incubated with 200 µl per well of an equal mixture of mouse anti-p24 mAbs ADP365 and ADP366 (Centralised Facility for AIDS Reagents, NIBSC) at 1:40 dilution in PBS containing 1% (v/v) FCS, for 1 hour at room temperature. After another two washes in PBS containing 1% (v/v) 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 containing 1% (v/v) FCS, for 1 hour at room temperature. The stained cells were then washed twice in PBS containing 1% (v/v) 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. Infected cells turned blue, and focus-forming units (FFU) were counted under visible light microscopy.

2.8.2 Detection of infection of TZM-bl cells

TZM-bl cells contain a Tat-inducible, LTR-driven luciferase gene. 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 minutes and luminescence (in relative light units; RLU) was then detected using a GloMax 96 Luminometer (Promega).

2.8.3 HIV-1 p24 ELISA

Cell-free virus in cell culture supernatants were detected using the HIV-1 p24 antigen capture assay kit developed by the NCI-Frederick Cancer Research and Development Centre AIDS Vaccine Programme (SAIC-Frederick, Frederick,

USA) according to the manufacturer's instructions. Briefly, samples were lysed by incubation with 1% (v/v) Triton X-100 (Sigma-Aldrich) at 37°C for 1 hour, and subsequently diluted in sample diluent buffer consisting of 1% (w/v) bovine serum albumin (BSA; Kirkegaard & Perry Laboratories, Gaithersburg, USA) and 0.2% (v/v) Tween 20 (Sigma-Aldrich) in RPMI 1640 (Invitrogen). Two-fold serial dilutions of a HIV-1 p24 standard (provided with the kit) ranging from 40000 pg/ml to 100 pg/ml was prepared using the same sample diluent. HIV-1 p24 antigen capture assay plates (96-well microtitre plates pre-coated with an anti-p24 mAb and blocked with a casein solution; SAIC-Frederick) were washed ten times with 1 × wash buffer (0.002 M imidazole, 0.02% (v/v) Tween 20, 0.5 mM EDTA, 160 mM NaCl; Kirkegaard & Perry Laboratories) using a MultiWash III automatic plate washer (TriContinent, Grass Valley, USA), turning the plates 180° after five washes.

Samples (100 µl per well) and p24 standard (100 µl per well of each dilution, in duplicate) were then added to the plates. Two wells per plate were left empty (blank wells) and two wells per plate were left with only sample diluent, for measurement of background absorbance levels. After incubation at 37°C for 2 hours, the plates were washed as described above, and 100 µl per well of a rabbit anti-p24 antibody (provided with the kit; SAIC-Frederick) diluted 1:200 in 10% (v/v) FCS and 2% (v/v) normal mouse serum (Sigma-Aldrich) in RPMI 1640 (Invitrogen) were subsequently added. After incubation at 37°C for 1 hour, the plates were washed as before, and 100 µl per well of a horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (provided with the kit; SAIC-Frederick) diluted 1:50 in 2% (v/v) normal mouse serum (Sigma-Aldrich), 5% (v/v) GS (Invitrogen) and 0.01% (v/v) Tween 20 (Sigma-Aldrich) in RPMI 1640 were added to the plates. The plates were then incubated for 1 hour at 37°C. After another washing step as before, 100 µl per well of TMB MicroWell Peroxidase Substrate solution (Kirkegaard & Perry Laboratories) was added and the plates were incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 100 µl per well of 1 M HCl. The absorbance at 450 nm (with the absorbance at 650 nm subtracted) was then measured. Absorbance

readings for the standard were plotted against concentration and fitted to a sigmoidal equation using the XLFit 4 software (IDBS, Guildford, UK).

2.9 Titration of virus stocks

Virus stocks were titrated onto the different cell types as detailed below.

2.9.1 Titration on NP2 (and CCC) cells

NP2/CD4/CXCR4 and NP2/CD4/CCR5 (or CCC cells) were seeded in 48-well cell culture plates at a density of 2×10^4 cells per well and incubated at 37°C. The day after, ten-fold serial dilutions of viral stocks were prepared using fresh cell culture medium, and the cells were incubated with 100 µl per well of the serially diluted virus for 2 hours at 37°C. Each dilution of virus was assayed in duplicate or triplicate wells. The cells were then washed once in cell culture medium, and subsequently overlaid with 0.5 ml of fresh medium. After 72 hours at 37°C, the cells were fixed and infection was detected by intracellular HIV-1 p24 immunostaining as described in section 2.8.1. FFU were counted microscopically and the virus titre determined as FFU/ml.

2.9.2 Titration on TZM-bl cells

Viral stocks were five-fold serially diluted in cell culture medium in quadruplicate wells of opaque 96-well cell culture plates, starting at a 1:5 dilution, in a total of 100 µl per well. Eight wells were left with 100 µl of cell culture medium only. Approximately 1×10^4 newly trypsinised TZM-bl cells in 100 µl of DMEM supplemented with 10% (v/v) 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. DEAE-dextran and other polycations have been frequently used to enhance retroviral, including HIV-1, infection *in vitro* (1169). The optimal concentration range of DEAE-dextran was determined by titrating DEAE-dextran into the assay (Fig. 2.9.2.1).

The plates, containing serially diluted virus and TZM-bl cells, were subsequently incubated at 37°C. After 48 hours of incubation, luminescence was detected as described in section 2.8.2, 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 according to the method developed by Reed and Muench (1170) using an Excel macro set up by D. Montefiori at the Duke University Medical Centre, Durham, USA.

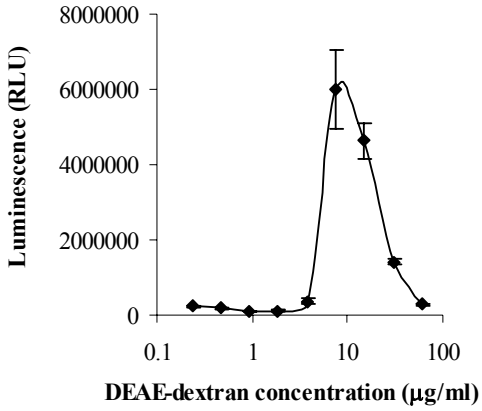


Figure 2.9.2.1. Determination of optimal concentration of DEAE-dextran. TZM-bl cells were infected with HIV-1 SF162 in the presence of two-fold serial dilutions of DEAE-dextran. After 48 hours of incubation, luminescence was detected as described in the text. Background-subtracted RLU were plotted against DEAE-dextran concentration. Data points represent the mean and bars the standard deviation of three replicates.

2.9.3 Titration on PBMC

Viral stocks were five-fold serially diluted in RPMI 1640 supplemented with 10% (v/v) FCS and 20 U/ml of interleukin-2 (Roche Diagnostics) in quadruplicate wells of a V-bottomed 96-well plate in a total of 100 µl per well. PHA-stimulated PBMC were counted and 1×10^5 cells in 100 µl of fresh growth medium (containing interleukin-2) were added to each well. After 24 hours at 37°C the cells were washed twice in growth medium by centrifugation at $210 \times g$ for 5 minutes, followed by the removal of 190 µl of cell culture supernatant and the addition of 190 µl of fresh growth medium. Cells were then incubated at 37°C and productive infection was monitored by removing 25 µl of cell culture

supernatant and replacing with fresh growth medium on day 1, 3, 5 and 8 after infection. Virus production was measured by p24 ELISA (section 2.8.3).

2.10 Neutralisation assays

This section details the neutralisation assays used in this study.

2.10.1 HIV-1 neutralisation in NP2 cells

VHH, mAb b12 or sCD4 neutralisation activities were assessed in NP2/CD4/CXCR4 or NP2/CD4/CCR5 cells as described previously (631). NP2 cells were seeded in 48-well cell culture plates at a density of 2×10^4 cells per well. The day after, 100 FFU of virus were pre-incubated with two-fold serial dilutions of VHH (or mAb b12 or sCD4) in growth medium for 1 hour at 37°C, in a total of 100 µl per well, and subsequently added to the NP2 cells seeded the day before. The cells were incubated with the virus-antibody mixture for 2 hours and thereafter washed gently in growth medium. After 72 hours at 37°C, the cells were fixed and infection was detected by intracellular p24 immunostaining (section 2.8.1). FFU were counted microscopically and neutralisation titres were determined as the VHH (or mAb b12 or sCD4) concentration required to give 90% reduction of infection (IC_{90}) compared to a concentration-matched irrelevant (negative control) VHH/mAb. The neutralisation activity of each VHH (or mAb b12 or sCD4) was assayed in triplicate and on a minimum of two separate occasions. Control wells containing virus and cells but no VHH or mAb were included on each plate.

2.10.2 HIV-1 neutralisation in TZM-bl cells

HIV-1 neutralisation in TZM-bl cells was evaluated using an assay developed by Derdeyn *et al*. (1148), Wei *et al*. (1149) and Li *et al*. (1022). Serial three-fold dilutions of VHH and mAb b12 (starting at 50 µg/ml) 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 hour of incubation, 1×10^4 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 hours, after which the cells were lysed and luminescence was detected (section 2.8.2). For each plate, eight wells containing cells and growth medium only, and eight wells containing virus and cells only, were included. Neutralisation was measured as the reduction in RLU in test wells compared to in virus control wells after subtraction of background luminescence. The lowest VHH or antibody concentration required to give 50% and 90% reduction in RLU (IC_{50} and IC_{90}) were determined by fitting the data to a sigmoidal equation using the XLFit 4 software (IDBS). The neutralisation activity of each VHH and mAb b12 was assayed in duplicate and on a minimum of two separate occasions, apart from the CRF07_BC pseudovirus panel, against which the VHH and mAb b12 were assayed once. A concentration-matched negative control VHH (VHH #3; kindly provided by A. Gorlani, Utrecht University, the Netherlands) was tested in parallel. The HIV-1 neutralisation potencies of llama sera and plasma were also evaluated in TZM-bl cells. Serum and plasma samples were heat-inactivated in order to destroy complement by incubation at 56°C for 1 hour prior to use in neutralisation assays. Three-fold serial dilutions of llama sera were then tested, starting at a 1:5 dilution.

2.10.3 HIV-1 neutralisation in PBMC

Serial three-fold dilutions of VHH (and mAb b12) were prepared in RPMI 1640 supplemented with 10% (v/v) FCS and 20 U/ml of interleukin-2 (Roche Diagnostics) and subsequently incubated with the appropriate dilution of virus at 37°C in a total volume of 100 µl per well. After 1 hour of incubation, 1×10^5 PHA-stimulated PBMC in 100 µl of fresh growth medium (containing interleukin-2) were added to each well and the plates were incubated at 37°C. After 24 hours of incubation, the cells were washed twice in growth medium as described in section 2.9.3. Cells were then incubated at 37°C and productive infection was monitored by removing 25 µl of cell culture supernatant and replacing with fresh growth medium on day 1, 3, 5 and 8 after infection. For each

plate, eight wells containing only virus and cells were included (virus only controls). Virus production was measured by p24 ELISA (section 2.8.3). Initially, the p24 content of virus only controls from day 1, 3, 5 and 8 post infection were determined. The p24 content of remaining assay wells were determined only for the assay day for which a sufficient amount of p24 could be detected in the virus only controls. Neutralisation was measured as reduction in p24 content in test wells compared to in virus only controls. VHH and mAb b12 IC_{50}, IC_{75} and IC_{90} values were calculated using curve-fitting software XLfit4 (IDBS). All VHH, including a negative control VHH (and mAb b12), were assayed in triplicate and in PBMC from two separate donors.

2.10.4 Neutralisation of an irrelevant virus in BHK cells

The VHH were tested against a pseudovirus bearing the rabies virus G protein, CVS-11 (1171). This virus was kindly provided by E. Wright, UCL, London, UK. Neutralisation of virus CVS-11, which contains a luciferase reporter gene, was evaluated in BHK target cells, as described previously (1171). Briefly, 200 TCID50 of virus were pre-incubated with VHH and a neutralising human anti-rabies serum (as a positive control; kindly provided by E. Wright, UCL, London, UK), and subsequently added to 2×10^4 BHK cells. Luminescence was detected after 48 hours. No specific reduction of CVS-11 infection was observed.

2.11 Immunisation of *Llama glama* and construction of phage library

Immunisation of llamas and construction of phage libraries expressing the VHH repertoires of the immunised llamas were carried out by staff at Ablynx, NV, Ghent, Belgium. Two llamas (numbered L40 and L44) were immunised with recombinant CN54 gp120. One llama (L48) was immunised with recombinant CN54 gp140. Immunisations and VHH library construction were carried out as described previously (1172). In brief, the llamas 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. On days 0 and 7, the llamas received 100 μg of antigen, and on days 14, 21, 28 and 35, they received 50 μg of antigen. 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.

For each llama, total RNA was isolated from peripheral blood lymphocytes and lymph node biopsies collected post-immunisation by acid guanidinium thiocyanate-phenol-chloroform extraction, and RNA isolated from the two different tissues were subsequently pooled. Oligo-dT primed cDNA was prepared from total RNA using the 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 (chapter 1.2), 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 introduce 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 (Ablynx NV), followed by transformation into electrocompetent *E. coli* TG1 cells. To obtain recombinant bacteriophages expressing the VHH as fusion proteins with the bacteriophage geneIII 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 (PEG; Sigma-Aldrich)

to remove free VHH as described in section 2.12.3. From each library, a colony PCR using an M13 reverse primer and a geneIII primer was carried out on 24 randomly picked colonies, and the proportion of clones containing an insert of the correct size was estimated.

2.12 Selection of anti-CD4bs VHH through panning on gp120 followed by competitive elution with sCD4

VHH targeting the CD4bs of gp120 were selected through panning of the phage libraries described in section 2.11 on recombinant gp120, using the functional selection approach described below. The first set of selections and isolation of 148 VHH clones were carried out by Els Beirnaert at the Ablynx NV selection laboratory in Porto, Portugal, although I took part in the planning of the experiments. The second round of selections and isolation of 672 VHH clones were carried out by me at the Ablynx NV selection laboratory in Porto, Portugal.

2.12.1 Panning on recombinant gp120

Recombinant IIIB or CN54 gp120 (section 2.1.2), or recombinant 92BR025 gp120 (expressed and purified as described in section 2.7), was coated onto 96-well MaxiSorp plates (Nalgene, NUNC International, Hereford, UK) at 2.5 and 0.5 μg/ml in PBS in a total volume of 100 μl per well. Negative control wells containing only PBS were 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 hour at room temperature and orbital shaking at 200 rpm. After the blocking step, the plates were washed four times with PBS-T. Alternatively, plates were coated with 10 μg/ml of sheep 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 92UG037 gp120 in cell culture supernatant (section 2.7) at saturating and non-saturating dilutions in 0.2% (w/v) casein in PBS. Negative control wells containing PBS only, 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 hour at room temperature, the plates were washed four times with PBS-T.

Phages, expressing the cloned VHH repertoires, prepared as described in section 2.11, were then diluted 1:10 in 0.2% (w/v) casein in PBS and subsequently pre-incubated at room temperature for 10 minutes to allow for unspecific binding. Wells containing immobilised gp120 (and negative control wells) were then incubated with 100 µl per well of diluted phage, at room temperature and orbital shaking at 200 rpm. After 1 hour of incubation, wells were washed 20 times in PBS-T to remove unbound or unspecifically bound phage. To increase the stringency of the washing step, every five washes the plates were incubated with PBS-T for 15 minutes at room temperature and orbital shaking at 200 rpm. Plates were then washed twice in PBS. In order to elute phage targeting the CD4bs, the wells were then incubated with 100 µl per well of 30 and 200 µg/ml of sCD4 in 0.2% (w/v) casein in PBS. Control wells were incubated with 100 µl per well of 30 or 200 µg/ml of BSA in 0.2% (w/v) casein in PBS. Elutions were carried out at room temperature for 0.5-3 hours and orbital shaking at 200 rpm. Additional control wells were incubated with 100 µl per well of 0.1 M glycine, for elution of all phages through low pH shock. After for 20 minutes at room temperature and orbital shaking at 200 rpm, the glycine-eluted phage (100 µl), were transferred to 50 µl of 1 M Tris-HCl, pH 7.5, for neutralisation. Each concentration of immobilised gp120, and negative controls, were incubated with both concentrations of sCD4 and BSA, and with glycine.

2.12.2 Titration of eluted phage onto *E. coli* TG1 cells

To determine the success of the competitive sCD4 elution and the level of unspecific background binding in the panning step, eluted phages were titrated onto *E. coli* TG1 cells. After the first round of panning, the eluted phages were diluted 1:5, 1:25 and 1:125 in PBS, whereas after the second round of panning, the eluted phages were diluted 1:10 and 1:100 in PBS. A 5 µl aliquot of each dilution (including the undiluted eluted phage) was subsequently used to infect

100 µl of *E. coli* TG1 cells at an optical density at 600 nm (OD_{600}) of approximately 0.5, hence exponentially growing, in V-bottomed 96-well microtitre plates. After incubation at 37°C for 30 minutes, without shaking, 5 µl of bacteria were spotted onto LB agar plates containing 100 µg/ml of ampicillin and 2% (w/v) glucose. Each dilution of eluted phage was tested in duplicate. The LB agar plates were then incubated overnight at 37°C.

2.12.3 Amplification and rescue of eluted phage

Phages eluted by sCD4 were multiplied in TG1 cells and rescued by superinfection with helper phage. 40 µl of eluted phage were used to infect 0.5 ml of exponentially growing *E. coli* TG1 cells (OD_{600} of around 0.5). After incubation for 30 minutes at 37°C without shaking, the cells were pelleted, resuspended in 5 ml of 2 × TY 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. A 0.5 ml aliquot of the overnight culture was then used to inoculate another 50 ml of 2 × TY medium containing 100 µg/ml of ampicillin and 2% (w/v) glucose. At an OD_{600} of around 0.5, 2.5 ml of this culture was infected with 2.5 µl of helper phage M13KO7 (corresponding to approximately 10^{11} plaque-forming units; PFU) at 37°C for 30 minutes without shaking. Cells were then pelleted by centrifugation at 3200 × *g* for 10 minutes, resuspended in 50 ml of 2 × TY medium containing 100 µg/ml of ampicillin and 25 µg/ml kanamycin and incubated at 37°C with 250 rpm. After an overnight incubation, cells were pelleted by centrifugation at 3200 × *g* for 10 minutes. 40 ml of the culture supernatant was then transferred to 10 ml of 20% (w/v) PEG (Sigma-Aldrich) and 2.5 M NaCl, incubated on ice for 30 minutes, and subsequently centrifuged for 20 minutes at 3200 × *g* at 4°C. The supernatant was then discarded and the pellet allowed to dry. Phages were then resuspended in 1 ml PBS, which was subsequently centrifuged at 17900 × *g* for 3 minutes in a tabletop centrifuge to remove cellular debris. The supernatant was collected, transferred to a new 1.5 ml tube containing 250 µl of 20% (w/v) PEG (Sigma-Aldrich) and 2.5 M NaCl, and incubated on ice. After ten minutes of incubation, precipitated phage were pelleted by centrifugation at 17900 × *g* for

10 minutes and the supernatant was discarded. To remove the final traces of PEG, the pellet was centrifuged for another 2 minutes at 17900 × *g* and the remaining supernatant was carefully removed. Phages were then resuspended in 1 ml of PBS and subjected to another 2 minutes of centrifugation at 17900 × *g* to remove cellular debris. The supernatant (new phage stock) was collected, titrated onto *E. coli* TG1 cells, and taken forward to a second round of panning.

2.13 Isolation and small-scale expression of individual VHH

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

2.13.1 Cloning of VHH repertoire into expression vectors

The DNA fragments coding for VHH from selections where a larger number of clones had been eluted by sCD4 than by BSA were digested using restriction enzymes *Sfi*I (or *Bgl*I) and *BstE*II, gel-purified, and ligated into the similarly digested expression vector pAX051 (Ablynx NV) using T4 DNA ligase (Promega), followed by transformation into electrocompetent *E. coli* TG1 cells (section 2.2.7). The pAX051 expression vector is a derivative of pUC19 (1173) and contains the LacZ promoter, which enables a controlled induction of expression using isopropyl-ß-D-thiogalactopyranosid (IPTG). Expression from the pAX051 vector incorporates a 6-histidine- and a c-myc-tag to the C-terminus of the VHH. Transformed bacteria were spread onto LB agar plates containing 100 µg/ml of ampicillin and 2% (w/v) glucose and incubated at 37°C overnight. Individual clones were then picked and used to inoculate 1 ml of LB medium containing 100 µg/ml of ampicillin (or carbenicillin) and 2% (w/v) 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. A subset of the isolated VHH was subjected to DNA fingerprint analysis using the restriction enzyme *HinfI*.

2.13.2 Small-scale expression of individual VHH in a 96-well format

10 µl of the overnight cultures of *E. coli* TG1 cells prepared as described in section 2.13.1 were used to inoculate 1 ml of 2 × TY medium containing 100 µg/ml carbenicillin and 0.1% (w/v) glucose in 96-well deep-well plates. Plates were then incubated at 37°C and 250 rpm for approximately 3 hours, after which VHH production was induced by the addition of 0.1 mM or 1 mM IPTG (Promega). Bacteria were then incubated for another 4 hours at 37°C with 250 rpm, and subsequently pelleted by centrifugation at 3200 × *g* for 10 minutes. The supernatants were discarded and the pellets frozen at -20°C overnight. The day after, the pelleted bacteria were thawed and resuspended in 100 µl of PBS, and subsequently centrifuged at 3200 × *g* for 10 minutes. The resulting supernatants (periplasmic extracts containing the expressed VHH) were then transferred to new 96-well plates and stored at -80°C.

2.14 Screening of individual VHH

Individual VHH in *E. coli* periplasmic extracts (prepared as described in section 2.13.2) or purified VHH (prepared as described in section 2.15.2) were screened for binding to recombinant gp120 in ELISA and/or for neutralisation of HIV-1.

2.14.1 Screening of individual VHH for binding to gp120

Individual VHH in *E. coli* periplasmic extracts (or purified VHH) were screened for ability to bind to recombinant CN54, IIIB, 92BR025, or 92UG037 gp120 using the ELISA described in section 2.16.5, using 5 µl of VHH-containing *E. coli* periplasmic extract per well. Irrelevant (negative control) *E. coli* periplasmic extracts were included in each ELISA.

2.14.2 Screening of individual VHH for neutralisation of HIV-1

Individual VHH in *E. coli* periplasmic extracts (or purified VHH) were screened for ability to neutralise HIV-1 CN54, IIIB, 92BR025, or 92UG037 in NP2 cells or TZM-bl cells, as described in section 2.10. In the NP2 cell-based

neutralisation assay, 40 µl of VHH-containing *E. coli* periplasmic extract was pre-incubated with 100 FFU of virus, per well. In the TZM-bl cell-based neutralisation assay, 50 µl of VHH-containing *E. coli* periplasmic extract was pre-incubated 200 TCID50 of virus, per well. Irrelevant (negative control) *E. coli* periplasmic extracts, and *E. coli* periplasmic extract containing neutralising VHH (if available), were included on each plate. When *E. coli* periplasmic extracts were used in neutralisation assays, the cell culture media were supplemented with 50 U/ml of penicillin and 50 µg/ml of streptomycin (penicillin-streptomycin, Invitrogen). Clones found to bind to gp120 in ELISA or to neutralise HIV-1 were sequenced. DNA sequencing of individual VHH clones was carried out at Ablynx NV, Porto, Portugal). Predicted amino acid sequences were aligned using the Clustal W multiple sequence alignment software (1174).

2.15 Large-scale expression and purification of selected VHH

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

2.15.1 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 (or carbenicillin) 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 l of 2 × TY medium containing 100 µg/ml of ampicillin (or carbenicillin) and 0.1% (w/v) glucose. This culture was incubated at 37°C and 250 rpm until an OD_{600} of 0.6-0.8 was reached. VHH expression was then induced with 0.1 or 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 2.2.11). After the overnight incubations, bacteria were harvested by centrifugation of 4 × 250 ml aliquots at 4000 × *g* for 30 minutes at 4°C. The pelleted bacteria were then frozen at -80°C overnight.

The day after, the bacteria were thawed, resuspended in 4 × 25 ml of PBS, incubated at 8°C for 1 hour at 250 rpm, and subsequently subjected to centrifugation at 4000 × *g* for 30 minutes at 4°C. The resulting supernatants, corresponding to the VHH-containing *E. coli* periplasmic extracts, were collected.

2.15.2 Purification of selected VHH

Expressed VHH were purified from *E. coli* periplasmic extracts by means of the attached 6-histidine-tag using TALON Metal Affinity Resin (Clontech, Mountain View, USA), according to the manufacturer's instructions. Briefly, the TALON Metal Affinity Resin suspension was washed twice in 50 ml of VHH equilibrium buffer (Table 2.1.1.1). 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 hour with head-over-head rotation. The resin was then pelleted by centrifugation at 700 × *g* for 2 minutes at 4°C. The supernatant was discarded and an aliquot of was saved for analysis by SDS-PAGE (unbound sample). The resin was then washed twice in 50 ml of VHH equilibrium buffer (Table 2.1.1.1), by incubating the resin with buffer at room temperature for 10 minutes with head-over-head rotation followed by centrifugation at 700 × *g* for 2 minutes 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 (Table 2.1.1.1) was then added and the eluate collected. Bound protein was then eluted with approximately 4 × 0.5 bed volumes of VHH elution buffer (Table 2.1.1.1) and the eluate collected in 4 fractions.

The eluted fractions were analysed by SDS-PAGE and Coomassie blue staining (section 2.2.11) and fractions containing the bulk of the eluted VHH were pooled and dialysed for 6-8 hours at 4°C against 0.5-1 l 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. The PBS was then changed and the samples dialysed for another 6-8 hours at 4°C. This procedure was repeated once more. After dialysis, the purified VHH were analysed by SDS-PAGE and Coomassie blue staining (section 2.2.11) and the protein concentration was determined (section 2.2.10).

2.16 Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISAs) were carried out as described below.

2.16.1 Detection of gp120 and gp140 in ELISA using anti-HIV-1 sera

Opaque 96-well Maxisorp plates (Nalgene) were coated overnight with 10 µg/ml of sheep antibody D7324 (Aalto Bio Reagents) 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% (w/v) milk powder in TBS for 1 hour at room temperature. Wells were then washed four times with TBS-T and subsequently incubated with 50 µl per well of serial dilutions of recombinant gp120 (purified or in cell culture supernatant) or gp140 in 1% (w/v) milk powder in TBS, for 1 hour at room temperature. Each dilution of gp120 was assayed in duplicate. Blank wells were included, as were wells lacking gp120, as negative controls, and wells containing serial dilutions of recombinant IIIB gp120, as positive controls. If gp120 in cell culture supernatant was used, serial dilutions of 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 also included. After another washing step, gp120 (or gp140) was detected using 50 µl per well of an equal mixture of QC sera 2, 5 and 6 (section 2.1.4) at a 1:100 dilution in TMT/GS and incubation for 1 hour at room temperature. After another four washes in TBS-T, wells were incubated with 100 µl per well of 0.5 µg/ml of an alkaline phosphatase (AP)-conjugated goat anti-human immunoglobulin antibody (Harlan SeraLab, Crawley Down, UK) in TMT/GS, for 1 hour at room temperature. After six washes with TBS-T,

100 µl per well of Lumi-Phos Plus substrate (Aureon Biosystems) was added and chemiluminescence was detected after 0.5 hours of incubation at 37°C. Blank-corrected RLU were plotted against gp120 (or gp140) dilution.

2.16.2 Binding of sCD4 to envelope proteins in ELISA

Opaque 96-well Maxisorp plates (Nalgene) were coated with antibody D7324 (Aalto Bio Reagents), blocked with 4% (w/v) milk powder in TBS, and washed, as described in section 2.16.1. Wells were then incubated with 50 µl per well of saturating concentrations of recombinant gp120 (or gp140) diluted in 1% (w/v) milk powder in TBS, for 1 hour at room temperature. After four washes in TBS-T, the wells were incubated with 50 µl per well of serial dilutions of sCD4 (section 2.1.5) in TMT/GS for 1 hour at room temperature. Each dilution of sCD4 was assayed in duplicate. Blank wells and the appropriate negative controls were included. After another washing step, wells were incubated with 50 µl per well of the mouse anti-CD4 mAb L120.3 (ARP359; Centralised Facility for AIDS Reagents, NIBSC) at 2.5 µg/ml in TMT/GS, for 1 hour at room temperature. Well were then washed as before and incubated with 100 µl per well of 0.5 µg/ml of an AP-conjugated goat anti-mouse IgG antibody (catalogue number 1030004, Oxford Biotechnology, Kidlington, UK) in TMT/GS, for 1 hour at room temperature. Luminescence was then detected as described in section 2.16.1, and blank-corrected RLU were plotted against sCD4 concentration.

Alternatively, opaque 96-well Maxisorp plates (Nalgene) were coated overnight with 10 µg/ml of sCD4 (section 2.1.5) in 0.1 M $NaHCO_3$, pH 8.5. Some wells were left with 0.1 M $NaHCO_3$, pH 8.5 only. Wells were then blocked and washed as above, and subsequently incubated with 50 µl per well of serial dilutions of purified gp120 (or gp140) in 1% (w/v) milk powder in TBS, for 1 hour at room temperature. After another washing step, gp120 (or gp140) binding to immobilised sCD4 was detected by incubation with 50 µl per well of 10 µg/ml of antibody D7324 (Aalto Bio Reagents) in TMT/BS for 1 hour at room temperature. Wells were then washed and subsequently incubated with 100 µl

per well 0.5 μg/ml of AP-conjugated rabbit anti-sheep IgG antibody (catalogue number. ab6748-1, Abcam, Cambridge, UK) in TMT/BS, for 1 hour at room temperature. After another six washes in TBS-T, luminescence was detected as above. Blank-corrected RLU were plotted against gp120 (or gp140) concentration.

2.16.3 Binding of human mAbs to envelope proteins in ELISA

Opaque 96-well Maxisorp plates (Nalgene) were coated with antibody D7324 (Aalto Bio Reagents), blocked with 4% (w/v) milk powder in TBS, incubated with gp120 (or gp140), and washed, as described in section 2.16.2. Wells were then incubated with 50 μl per well of serial dilutions of mAb in TMT/GS, for 1 hour at room temperature. Each mAb dilution was tested in duplicate. Blank wells and the appropriate negative controls were included. Binding of mAb to gp120 (or gp140) was then detected using 0.5 μg/ml of an AP-conjugated goat anti-human immunoglobulin antibody (Harlan SeraLab), as described in section 2.16.1. Luminescence was detected as above and blank-corrected RLU were plotted against mAb concentration.

2.16.4 Detection of anti-envelope antibodies in sera from immunised llamas

Recombinant HIV-1 CN54 gp120 and gp140 at 1 μg/ml in PBS and 50 μl per well was coated onto 96-well Maxisorp plates (Nalgene) and left overnight at 4°C. Wells were then washed three times with PBS-T and non-specific protein binding was blocked by incubation with 200 μl per well of 1% (w/v) casein in PBS for 1 hour at room temperature. After another washing step, wells were incubated at room temperature for 1 hour with 100 μl per well of three-fold serial dilutions of serum from immunised llamas collected on day 0 (pre-immunisation) and day 43 (post-immunisation). Bound immunoglobulins were detected using 100 μl per well of a rabbit anti-llama antiserum followed by 0.5 μg/ml of an AP-conjugated goat anti-rabbit immunoglobulin antibody (Sigma-Aldrich). Substrate (2 mg/ml p-nitrophenyl phosphate; 100 μl per well) was added to the wells and absorbance at 405 nm was measured. Absorbance readings were plotted against serum dilution.

2.16.5 VHH binding to envelope proteins in ELISA

Opaque 96-well Maxisorp plates (Nalgene) were coated with antibody D7324 (Aalto Bio Reagents), blocked with 4% (w/v) milk powder in TBS, incubated with saturating concentrations of recombinant gp120 (or gp140), and washed, as described in section 2.16.2. Wells were then incubated with 50 µl per well of serial dilutions of VHH (including an irrelevant, negative control VHH) in TMT/GS. Each dilution of VHH was added to duplicate wells. Blank wells were included. After 1 hour of incubation at room temperature, the plates were washed four times with TBS-T. The wells were then incubated, in two subsequent steps, with 0.5 µg/ml of a mouse anti-c-myc mAb (catalogue number 11667149001, Roche Diagnostics) in TMT/GS for 1 hour at room temperature, followed by four washes in TBS-T and then incubation with 100 µl per well of 0.5 µg/ml of an AP-conjugated goat anti-mouse IgG antibody (catalogue number 1030004, Oxford Biotechnology). After six washes with TBS-T, luminescence was detected as described in section 2.16.1, and blank-corrected RLU were plotted against VHH concentration. Alternatively, AP substrate p-nitrophenyl phosphate (2 mg/ml; 100 µl per well) was added to the wells. Absorbance at 405 nm was measured and absorbance readings were plotted against VHH concentration.

2.16.6 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 (Nalgene) were coated with sCD4, blocked and washed, as described in section 2.16.2. Serial dilutions of VHH in 1% (w/v) milk powder in TBS were pre-incubated with purified gp120 (or gp140) at 0.5 µg/ml in 1% (w/v) milk powder in TBS, for 1 hour at room temperature. The pre-incubated VHH-gp120 (or VHH-gp140) 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, as negative controls. After 1 hour at room temperature, the plates were washed four times with TBS-T and bound gp120 (or gp140) was then detected using antibody D7324 (Aalto Bio Reagents) and an AP-conjugated rabbit anti-sheep IgG antibody (Abcam), as

described in section 2.16.2. Alternatively, bound gp120 (or gp140) was detected using QC sera 2, 5 and 6 an AP-conjugated goat anti-human immunoglobulin antibody as described in section 2.16.1. Luminescence was detected as before and blank-corrected RLU were plotted against VHH concentration.

2.16.7 VHH competition with anti-CD4bs mAbs for binding to envelope proteins in ELISA

Opaque 96-well Maxisorp plates (Nalgene) were coated overnight with 50 µl per well of 10 µg/ml of mAb b12 and subsequently blocked and washed, as described above. VHH ability to inhibit gp120 binding to mAb b12 was assayed by pre-incubating serial dilutions of VHH with 0.5 µg/ml of gp120 for 1 hour at room temperature, followed by incubation with the immobilised mAb b12 in a total of 50 µl per well. Each dilution of VHH was tested in duplicate. Wells lacking mAb b12 were included as controls. Bound gp120 was then detected, using antibody D7324 (Aalto Bio Reagents) and an AP-conjugated rabbit anti-sheep IgG antibody (Abcam), as described in section 2.16.6. Blank-corrected RLU were plotted against VHH concentration.

VHH inhibition of human anti-gp120 mAbs b12, 2G12, 447-52D, 17b as well as an irrelevant mAb (4E10) was assayed by pre-incubating serial dilutions of the mAbs with 0.5 µg/ml of gp120 in 1% (w/v) milk powder in TBS for 1 hour at room temperature. The pre-incubated mAb-gp120 mixtures were then added to duplicate wells containing immobilised VHH, which had been pre-coated onto opaque 96-well Maxisorp plates as described in section 2.16.8. The mAb-gp120 mixtures (50 µl per well) were incubated with the immobilised VHH for 1 hour at room temperature. Bound gp120 was then detected, using antibody D7324 (Aalto Bio Reagents) and an AP-conjugated rabbit anti-sheep IgG antibody (Abcam), as described in section 2.16.2. Blank-corrected RLU were plotted against mAb concentration.

VHH inhibition of mAb b6, 654-D and GP68 binding to gp120 was assayed by incubating serial dilutions of VHH in TMT/GS (50 µl per well), with wells

containing gp120 that had been captured at saturating concentrations onto opaque 96-well Maxisorp plates (Nalgene) by the immobilised antibody D7324 (Aalto Bio Reagents), as described in section 2.16.2. After 1 hour at room temperature, the wells were then washed as above and subsequently incubated with 50 µl per well of mAbs b6, 654-D or GP68 in TMT/GS (at a concentration giving a signal in the linear range of a dose-response curve). Binding of the mAbs to gp120 was detected, using an AP-conjugated goat anti-human immunoglobulin antibody (Harlan SeraLab), as described in section 2.16.3. Blank-corrected RLU were plotted against VHH concentration.

2.16.8 VHH competition with each other for binding to envelope proteins in ELISA

VHH competition with each other and with fluid-phase sCD4 was also assayed by ELISA. Opaque 96-well Maxisorp plates (Nalgene) were coated overnight with 5 µg/ml of VHH 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% (w/v) milk powder in TBS for 1 hour at room temperature. Wells were then washed four times with TBS-T. Serial dilutions of VHH (or sCD4) in 1% (w/v) milk powder in TBS were then pre-incubated with 0.5 µg/ml of gp120 in 1% (w/v) milk powder in TBS for 1 hour at room temperature. The pre-incubated VHH-gp120 (or sCD4-gp120) mixtures were then added to duplicate wells of the VHH-coated plates (50 µl per well). Wells lacking immobilised VHH were included, as was an irrelevant VHH. After 1 hour at room temperature, the plates were washed four times with TBS-T and bound gp120 was then detected using antibody D7324 (Aalto Bio Reagents) and an AP-conjugated rabbit anti-sheep IgG antibody (Abcam), as described in section 2.16.2. Luminescence was detected as above and blank-corrected RLU were plotted against VHH concentration.

2.17 Surface plasmon 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). Briefly, approximately 2700 response units (RU) of recombinant IIIB gp120 were immobilised onto a CM5 chip (BIAcore) using standard amine-coupling techniques, as described by the manufacturer. VHH D7, serially diluted to 75, 50, 40, 30, and 20 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), was then injected at a flow rate of 45 µl/minute. The samples were allowed to dissociate for 1800 seconds. To measure the affinity of VHH A12 and C8, 2100 RU of biotinylated IIIB gp120 was non-covalently captured by streptavidin pre-immobilised onto a SA sensor chip (BIAcore). VHH A12 and C8, serially diluted to 80, 60, 40, 20 and 10 nM in HBS-EP buffer, were injected onto the chip at a flow rate of 45 µl/minute for 3 minutes and were allowed to dissociate for 1200 seconds. 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 $K_D = k_d/k_a$.

The ability of the sCD4 to bind to gp120, and the ability of VHH to inhibit binding of sCD4 to gp120, was assayed using a BIAcore X (BIAcore). This work was carried out by me together with Karolin Hijazi in the laboratory of Charles Kelly, King's College London, UK. Mouse anti-CD4 mAb L120.3 (ARP359; Centralised Facility for AIDS Reagents, NIBSC) in 10 mM sodium acetate, pH 4, was coupled to both flow cells of a CM5 chip (BIAcore), using standard amine-coupling chemistry as described by the manufacturer. Briefly, the carboxymethylated dextran-coated surface of the CM5 chip was activated for 7 minutes at a flow rate of 5 µl/minute, using a 1:1 ratio of 0.4 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS), to give reactive succinimide esters, which can react with amino groups on the protein to be coupled. Approximately 3100 RU of mAb L120.3 was coupled to each flow cell. Remaining activated groups were blocked by injecting 1 M ethanolamine, pH 8.5, for 7 minutes at a flow rate of 5 µl/minute. Approximately 1000 RU of sCD4 was then injected and subsequently cross-linked (to allow for several injections of gp120 without having to re-inject sCD4)

to the anti-CD4 mAb in flow cell 2 by the addition of EDC/NHS, while flow cell 1 was kept as a negative control reference cell. The ability of recombinant IIIB and CN54 gp120 to bind to this captured and cross-linked sCD4 was then evaluated. Three different concentrations of gp120 (100, 200 and 300 nM) in HBS-EP buffer were injected onto the chip over 4 minutes at a flow rate of 5 μl/minute, and the peak background-subtracted RU reading was recorded.

The same chip was used to evaluate the ability of the VHH to inhibit binding of CN54 gp120 to sCD4. Serial dilutions of VHH in HBS-EP buffer were pre-incubated for 10 minutes at room temperature with 300 nM of HIV-1 CN54 gp120 in HBS-EP buffer, and subsequently injected onto the chip at a flow rate of 5 μl/minute for 4 minutes. Data was analysed using the BIAevaluation software. Binding of HIV-1 CN54 gp120 to sCD4 was represented as the normalised difference in RU observed between flow cell 2 (with sCD4) and flow cell 1 (reference cell), plotted against time. Regeneration of the chip surfaces was achieved by injecting 2 M KSCN, 10 mM glycine-HCl, pH 1.5, or the Pierce Gentle Ag/Ab Elution Buffer (Pierce Protein Research Products, Thermo Fisher Scientific).

Chapter 3

Generation of VHH that can inhibit HIV-1 infection

In this chapter, the generation of llama VHH that can inhibit HIV-1 infection will be described.

3.1 Introduction

Monoclonal antibodies to HIV-1 envelope, whether obtained from natural HIV-1 infection of from immunisation, can help defining neutralising and non-neutralising epitopes on HIV-1 envelope proteins, and may be useful as tools in HIV-1 vaccine design and in the development of candidate HIV-1 microbicides. Many anti-envelope mAbs have been isolated from animals such as mice post-immunisation and from humans as a result of HIV-1 infection. Out of these, only a handful has been found to be broadly neutralising across HIV-1 subtypes and all are a result of HIV-1 infection rather than immunisation (259). Two of these are directed against gp120, b12 which binds to an epitope that overlaps a subset of the CD4bs of gp120 (1008-1012), and 2G12 which recognises a carbohydrate motif (981, 1013-1015). Two broadly neutralising mAbs, 4E10 and 2F5, recognise gp41 (906-908, 981).

All of the broadly neutralising mAbs reported to date are from individuals infected with HIV-1 of subtype B. While subtype B infections are predominant in Northern America and Western Europe, they account for only about a tenth of infections worldwide (124). HIV-1 of subtype C, on the other hand, accounts for about half of the infections in the world and is currently infecting more people than any other subtype (124). Therefore, there is an urgent need for greater focus on generating and characterising neutralising mAbs to non-subtype B viruses, and to subtype C viruses in particular. In this study, we have attempted to use the non-conventional immune system of camelids to generate novel antibodies against a subtype C envelope. We hypothesised that the small size of camelid VHH in combination with their protruding CDR3 loops and their preference for

cleft-recognition (1083, 1112) may render them able to recognise conserved motifs on gp120 that are occluded from conventional antibodies.

Traditionally, mAbs have been generated by immortalisation of lymphocytes through Epstein-Barr virus (EBV)-transformation or hybridoma technologies, followed by selection and cloning to obtain monoclonal cell lines secreting antibodies with desired specificities. Köhler and Milstein (1175) were first to describe a mAb-secreting cell line, the result of a fusion of mouse spleen and myeloma cells, in 1975. A few years later, a human EBV-transformed antibody-secreting cell line was reported, as well as a human hybridoma mAb-secreting cell line (1176, 1177). Since then, a vast number of mAbs have been generated using these techniques, from a variety of species and against a wide range of targets, and mAbs have become essential tools in scientific research, diagnostics as well as for medical and therapeutic use. The conventional hybridoma and EBV transformation technologies are, however, laborious and time-consuming and do not readily allow for rapid screening of larger numbers of mAbs. Instead, this challenge can be met by recombinant approaches such as the display of antibody libraries on filamentous bacteriophages, followed by the selection of individual antibodies with desired properties on immobilised target molecules, a technology known as phage display. Phage display was originally developed in 1985 by Smith (1178), and used to display recombinant antibody libraries for the first time a few years later (1179-1182). It has since then become a well-established and widely used technique. In 1991, the technique was used to isolate the broadly neutralising mAb b12 from an asymptotic, HIV-1 seropositive individual (1011).

In phage display, the antibody repertoire in the form of recombinant scFv or Fab fragments, derived from the lymphocytes of immune or non-immune animals or humans, is expressed on the surface of filamentous phages such as the M13 phage, typically fused to the phage pIII coat protein. Phage libraries are produced in *E. coli* and then panned on immobilised antigen in two or three sequential rounds of selection, to enrich for antibody fragments with wanted specificities. Individual antibody fragments are then isolated from enriched libraries, and screened for desired properties. All in all, phage display offers a versatile and

high-throughput tool for selection and screening of antibodies, where the selection methods can be adapted in a number of creative ways, to enrich for antibodies with very specific abilities (1183, 1184). For example, phage libraries have been panned on immobilised antigens in surface plasmon resonance settings (1185), on fixed or live cells (1186, 1187), and on tissue sections (1188). In an additional example, mutated variants of the CCR5 ligand RANTES, with improved anti-HIV-1 activities, were isolated using an extraordinarily elegant phage display strategy, involving panning on live CCR5 expressing cells and selection for internalisation (1189).

The phage display technology is ideal for the isolation of camelid VHH for a number of reasons (1100). The single-domain nature of VHH, for example, offers an advantage over conventional antibody Fab or scFv fragments in library construction, in that there is no need for randomly recombining the VH and VL domains, giving rise to a larger functional size of the library. Ever since the first description of camelid heavy-chain antibodies in 1993 (1077), the phage display technique has been widely used to select for VHH against a range of biological targets (1099, 1124-1129, 1131, 1134, 1136, 1190).

In this chapter, the use of phage display to isolate llama anti-HIV-1 envelope VHH will be described. First, llamas were immunised with recombinant envelope proteins, followed by the collection of peripheral blood and lymph node lymphocytes. The VHH repertoires were then cloned into a phagemid vector and the resulting phage libraries were panned on immobilised gp120. To increase the chances of isolating neutralising VHH, a functional selection approach was employed. This approach involved a competitive elution using sCD4, enabling the identification of VHH that compete with CD4 for binding to HIV-1 envelope. After two rounds of panning followed by competitive sCD4 elution, individual VHH were isolated and screened for binding to gp120 and/or neutralisation of HIV-1. A schematic overview of the experimental procedure is outlined in Fig. 3.1.1.

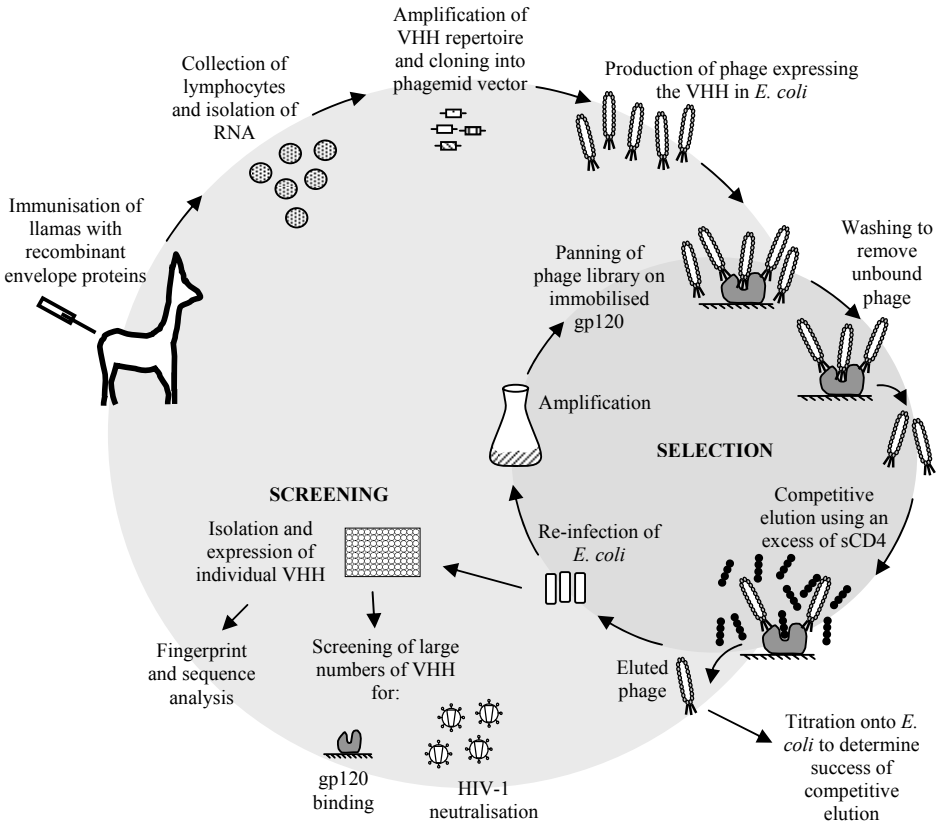


Figure 3.1.1. Schematic overview of the strategy for isolation of llama VHH targeting the CD4bs of gp120. Llamas were immunised with recombinant envelope proteins derived from an HIV-1 primary isolate of subtype B′/C (CN54 gp120 and gp140) over six weeks, and blood and lymph node lymphocytes were subsequently collected. Total RNA was isolated and the VHH repertoires were amplified and cloned into a phagemid vector. Phages expressing the VHH fused to their pIII coat protein were produced in *E. coli* (upon infection with a helper phage) and subjected to two rounds of selection. In the selection step, the phage libraries were first allowed to bind to immobilised gp120. Unbound phages were then washed away and phages expressing VHH targeting the CD4bs were eluted by an excess of sCD4. Elution by BSA was carried out in parallel. Eluted phages were titrated onto *E. coli*, to determine whether more phages had been eluted by sCD4 compared to by BSA, amplified and taken through a second round of panning. Individual VHH were isolated from the enriched phage pools and screened for ability to bind to gp120 and/or to neutralise HIV-1. Fingerprint and sequence analysis of isolated clones were carried out.

There were several reasons for focusing on VHH directed to the CD4bs. First, the CD4bs could be readily targeted using a competitive elution with sCD4. Meulemans *et al.* (1191) were first to describe a competitive elution approach in the panning step of phage display, in order to select for antibodies with desired specificities. This approach has since then been used extensively, also for the successful selection of camelid VHH able to inhibit receptor-ligand interactions (1125), making it an attractive option when trying to select for VHH targeting the CD4bs of gp120. An additional reason for focusing on VHH targeting the CD4bs is the existence of the extensively studied broadly neutralising mAb b12 and the recent definition of its CD4bs-related epitope (1008). Moreover, it has recently been shown that the neutralising ability of a number of broadly cross-subtype neutralising human anti-HIV-1 sera is largely mediated by antibodies to the CD4bs of gp120 (985).

3.2 Results

This section describes the results of this chapter.

3.2.1 Induction of a humoral anti-HIV-1 envelope response in llama, cloning of the VHH repertoires and construction of phage libraries

The work described in this section was carried out by staff at Ablynx NV, Ghent, Belgium. At the outset of this project, two llamas (L40 and L44) were immunised with recombinant gp120 derived from HIV-1 CN54, as part of the European Microbicide Project (EMPRO). One llama (L48) was later on in the project immunised with recombinant CN54 gp140. HIV-1 CN54 is a subtype B′/C recombinant (CRF07_BC) originally isolated in China, which is entirely subtype C in envelope apart from the very N-terminus (1137, 1138). The rationale behind choosing a CN54-derived immunogen was based on CN54 being a non-subtype B virus and the fact that recombinant baculovirus-expressed CN54 gp120 was already available in large amounts within the EMPRO consortium. This CN54 gp120 had been shown to bind to CD4 on cells in flow cytometry experiments (1192). In brief, each llama received six intramuscular injections at weekly intervals. The humoral immune response was evaluated in

ELISA on solid-phase gp120 or gp140, using serum samples from day 0 and 43 post immunisation. Anti-HIV-1 envelope antibodies appeared in all three llamas (Fig. 3.2.1.1).

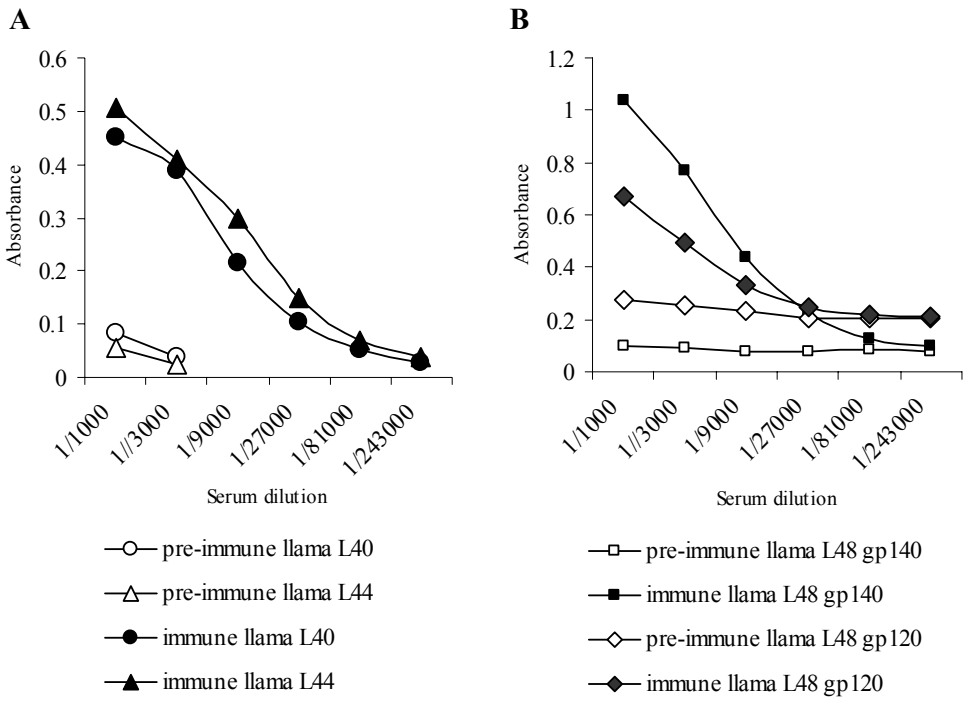


Figure 3.2.1.1. Anti-envelope antibody response in *Llama glama*. Two llamas (llama L40 and L44) were immunised with recombinant HIV-1 CN54 gp120. One llama (L48) was immunised with recombinant HIV-1 CN54 gp140. Each llama received six intramuscular doses at weekly intervals. Blood samples were taken at day 0 and 43 post immunisation and the serum anti-envelope response was evaluated in ELISA. A. Serum anti-gp120 response in llamas L40 and L44 evaluated on solid-phase CN54 gp120. B. Serum anti-gp140 and anti-gp120 response in llama L48 evaluated on solid-phase CN54 gp140 and gp120.

To enable the recovery of VHH repertoires of extensive diversity, both blood and lymph nodes from the immunised llamas were used as B cell sources. 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 pUC-derived pAX050 phagemid vector, followed by transformation into *E. coli* TG1 cells. The libraries derived

from llama L40, L44 and L48 were found to contain 3, 1 and 1.4×10^7 clones, respectively, out of which 58, 67 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 geneIII 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.

3.2.2 The antigenic profile of CN54 gp120

The ability of recombinant HIV-1 envelope proteins to bind to CD4 is an indicator of proper folding and biological function. To enable the selection of neutralising VHH able to block the gp120-CD4 interaction, it is especially important that the immunogen expresses an appropriately folded and structurally intact CD4bs. To characterise the antigenic profile and to confirm the structural integrity of the CN54-derived immunogen, its ability to bind to CD4 and to anti-CD4bs mAbs was therefore evaluated in biochemical assays.

First, the ability of sCD4 to bind to CN54 gp120 was evaluated in ELISA. Recombinant CN54 gp120, from the same batch as the immunogen for llama L40 and L44, was captured by sheep antibody D7324 coated onto plates. Antibody D7324 recognises a conserved motif in the C5 region of gp120. Captured gp120 was incubated with serial dilutions of sCD4 and binding was subsequently detected as described in section 2.16.2. Recombinant IIIB gp120 was included as a positive control. Recombinant sCD4 was shown to bind poorly, barely above background levels, to CN54 gp120 from the immunogen batch, whereas good binding was observed to IIIB gp120 (see below).

To investigate whether this observation was batch-dependent and perhaps due to partial denaturation of the gp120 in the immunogen batch, additional preparations of CN54 gp120 were produced. This was done ‘in-house’ by expressing CN54 gp120 in a T7 RNA polymerase-recombinant vaccinia virus system (1166), using the same molecular clone of CN54 that the immunogen had been derived from. Protein expression was confirmed in ELISA by capturing

gp120 in cell culture supernatant with immobilised antibody D7324 and detecting it with human anti-HIV-1 sera (Fig. 3.2.2.1).

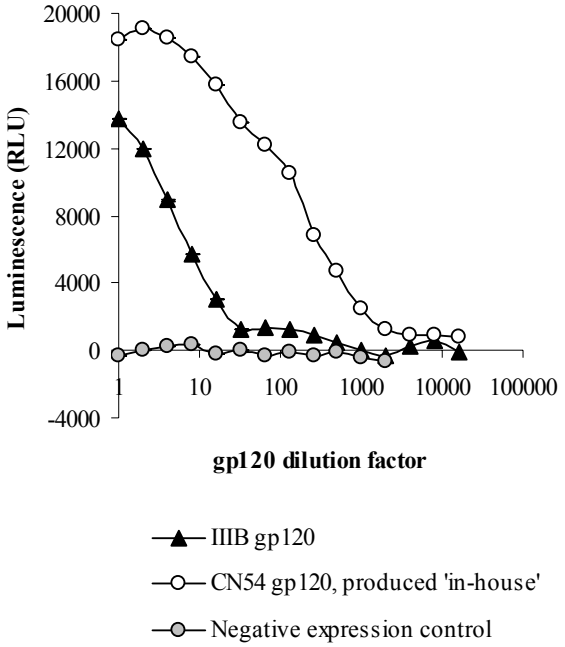


Figure 3.2.2.1. Detection of CN54 gp120 expressed in 293T cells infected with a T7 RNA polymerase-recombinant vaccinia virus. Serial dilutions of cell culture supernatant from the expression system were incubated with sheep antibody D7324 pre-coated onto plates. Captured gp120 was detected in ELISA using a mix of three human anti-HIV-1 sera followed by an AP-conjugated goat anti-human IgG antibody. A serial dilution of IIIB gp120, starting at 200 ng/ml, was included as a positive control. Cell culture supernatant from a mock expression was included as a negative control. Chemiluminescence was detected and RLU were plotted against gp120 dilution factor. Data points represent background-subtracted readings. Several CN54 gp120 preparations were produced; one representative experiment is shown.

CN54 gp120 produced in the vaccinia virus system was then tested for ability to bind to sCD4 in ELISA, alongside CN54 gp120 from the immunogen batch as well as IIIB gp120. Poor binding of sCD4 to all preparations of CN54 gp120 was observed (Fig. 3.2.2.2), indicating that the CD4bs of this recombinant CN54 gp120 is either not structurally intact, not accessible, or just not well exposed in this experimental setting. It should be noted that recombinant gp120 capable of binding to sCD4 in ELISA can be expressed in the T7 RNA polymerase-recombinant vaccinia virus system, as will be discussed in section 3.2.4.

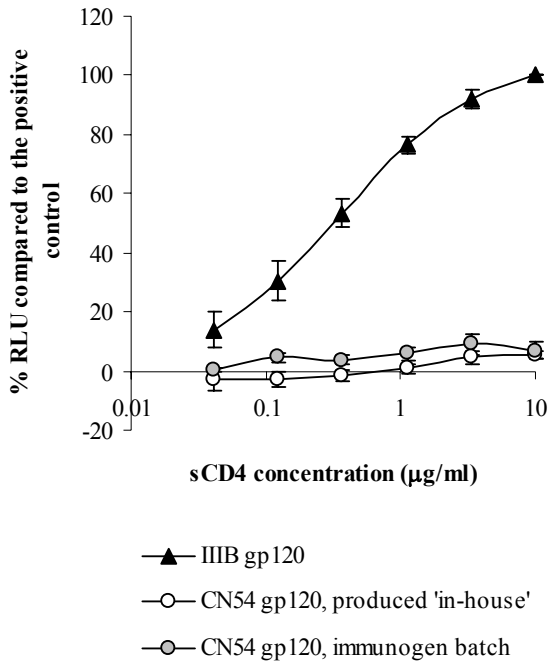


Figure 3.2.2.2. Binding of sCD4 to IIIB and CN54 gp120 in ELISA. Saturating levels of recombinant gp120 was captured by solid-phase sheep antibody D7324 followed by incubation with serial dilutions of sCD4 and subsequent detection of sCD4 using the mouse anti-CD4 mAb L120.3 and an AP-conjugated goat anti-mouse IgG antibody. Chemiluminescence was detected. Luminescence readings (in RLU) were normalised to readings obtained for 10 µg/ml of sCD4 binding to IIIB gp120 and plotted against sCD4 concentration. CN54 gp120 were either produced 'in-house' in a T7 RNA polymerase-recombinant vaccinia virus expression system, or from the same batch as the immunogen. Data points represent the mean and error bars the standard error of four independent experiments performed in duplicate.

To further confirm the poor binding of sCD4 to CN54 gp120 compared to IIIB gp120, an additional ELISA set up was tested. Instead of capturing the gp120 by immobilised C5-directed antibody D7324 followed by incubation with sCD4, sCD4 was coated onto wells of a microtitre plate and subsequently incubated with gp120 in fluid phase. Binding of gp120 to immobilised sCD4 was then detected using antibody D7324. Again, poor binding of sCD4 to CN54 gp120 was observed (Fig. 3.2.2.3).

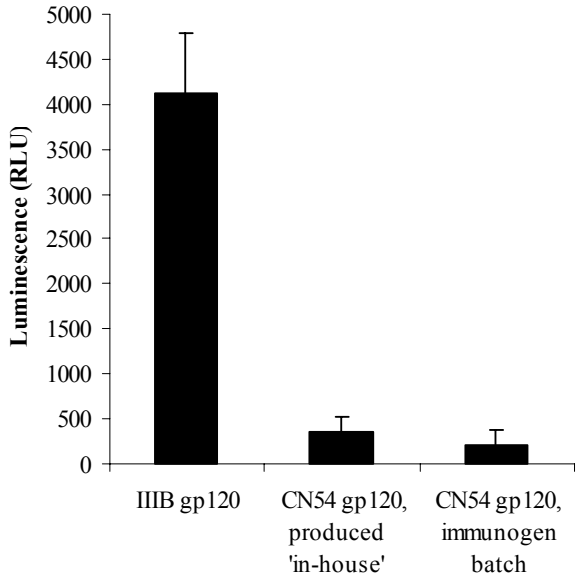


Figure 3.2.2.3. Binding of sCD4 to IIIB and CN54 gp120 in ELISA. Recombinant sCD4 (50 μg/ml) was coated onto wells of a microtitre plate. Wells were then incubated with IIIB gp120 (200 ng/ml), CN54 gp120 from the immunogen batch (200 ng/ml), and CN54 gp120 produced 'in-house' in the T7 RNA polymerase-recombinant vaccinia virus system (cell culture supernatant from the expression system, diluted 1:2). Binding of gp120 to sCD4 was detected using antibody D7324 followed by an AP-conjugated rabbit anti-sheep IgG antibody. Chemiluminescence was detected. Bars represent the mean and error bars the standard error of three independent experiments carried out in duplicate.

Sheep antibody D7324 is raised against a peptide (APTKAKRRVVQREKR) derived from the very C-terminus of IIIB gp120. This motif is highly conserved within HIV-1 of subtype B but a slight variation has been described among other HIV-1 subtypes (1193). D7324 has, however, been found to bind to gp120 derived from HIV-1 of subtype A, B, C, D and F (1048, 1193). The motif that D7324 recognises is present in CN54 gp120 (APTTAKRRMVEREKR), but with three amino acid residue differences compared to the IIIB sequence (underlined). In light of this notion, the ability of D7324 to bind to CN54 gp120 was tested in ELISA, to exclude the possibility that the poor reactivity of sCD4 with CN54 gp120 was not in fact due to poor capture or detection of CN54 gp120 by D7324. Despite the three amino acid-difference, D7324 was shown to bind almost equally well to both IIIB and CN54 gp120 in ELISA (Fig. 3.2.2.4 A). In addition,

the ability of sCD4 to bind to gp120 directly coated onto ELISA wells, thus not captured by D7324, was tested. Again, poor binding of sCD4 to CN54 gp120 was observed (Fig. 3.2.2.4 B). This observation suggested that the poor binding of sCD4 to CN54 gp120 observed in ELISA was not due to reduced ability of antibody D7324 to recognise CN54 gp120. This notion was further confirmed by the fact that part of the IIIB-derived D7324 target sequence (VVQREKR) had been introduced into the vaccinia virus-produced CN54 gp120 in the cloning step, by means of the antisense primer, as described in section 2.5, to ensure capture by D7324.

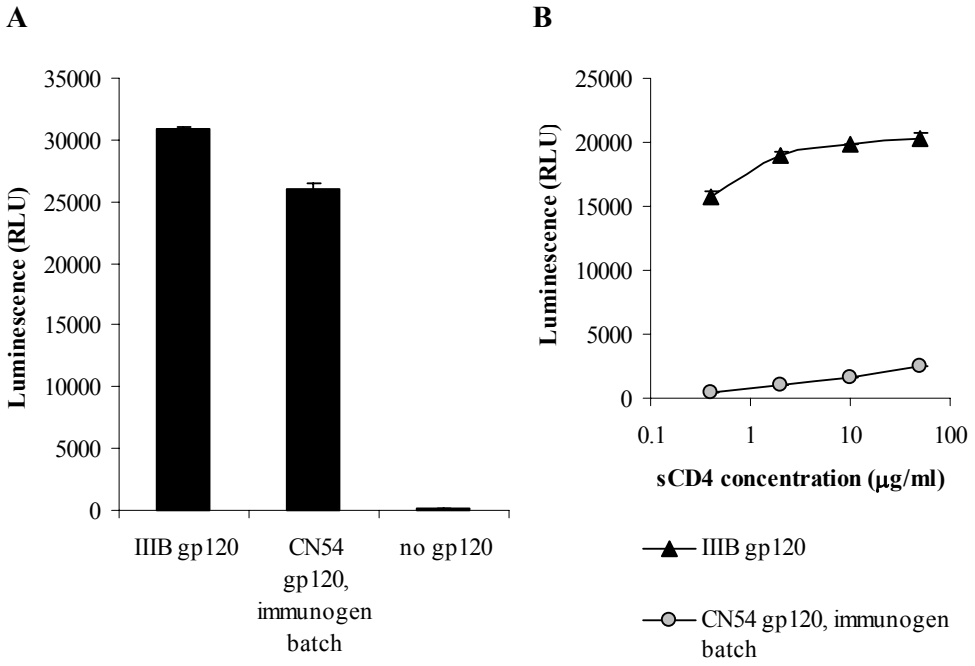


Figure 3.2.2.4. The poor binding of sCD4 to CN54 gp120 observed in ELISA is not due to reduced ability of sheep antibody D7324 to recognise CN54 gp120. Antibody D7324 recognises a motif in the C5 region of gp120. **A.** Binding of D7324 to immobilised gp120. Recombinant IIIB gp120 and CN54 gp120 (immunogen batch) was coated overnight onto microtitre plates at 10 µg/ml and subsequently incubated with 10 µg/ml of D7324 followed by detection using an AP-conjugated rabbit anti-sheep antibody. Chemiluminescence was detected. Bars represent the mean and error bars the standard deviation of duplicate reactions. **B.** Binding of sCD4 to gp120 coated straight onto microtitre plates. Recombinant IIIB gp120 and CN54 gp120 (immunogen batch) was coated overnight onto microtitre plates at 10 µg/ml and subsequently incubated with serial dilutions of sCD4 followed by detection of sCD4 using the mouse anti-CD4 mAb L120.3 and an AP-conjugated goat anti-mouse IgG antibody. Chemiluminescence was detected. Data points represent the mean and bars the standard deviation of duplicate reactions from one experiment.

In order to elicit a neutralising humoral response, an immunogen should favourably present epitopes that are recognised by neutralising antibodies, rather than exposing epitopes recognised by non-neutralising antibodies. To further assess the accessibility and structural integrity of the CD4bs of CN54 gp120, its reactivity with human anti-CD4bs mAbs b6 and b12 was evaluated. Anti-CD4bs mAb b12 showed poor binding to CN54 gp120 in ELISA, compared to IIIB gp120, whereas mAb b6 was observed to bind well (Fig. 3.2.2.5 A and B).

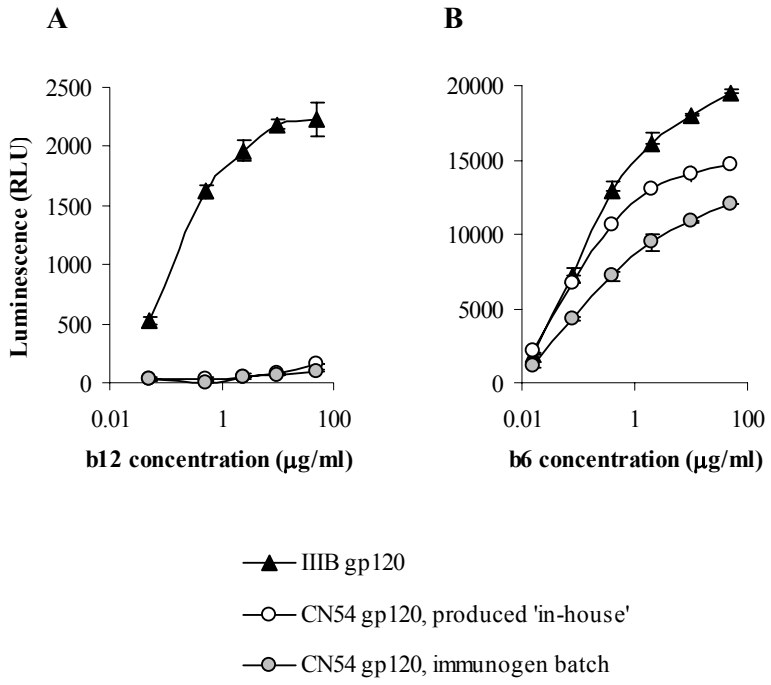


Figure 3.2.2.5. Binding of human anti-CD4bs mAbs to recombinant IIIB and CN54 gp120 in ELISA. Saturating levels of recombinant IIIB gp120 and CN54 gp120 was captured by solid-phase sheep antibody D7324 followed by incubation with serial dilutions of each mAb and subsequent detection of mAb binding to gp120 using an AP-conjugated goat anti-human immunoglobulin antibody. Chemiluminescence was detected and RLU were plotted against mAb concentration. Data points represent the mean and bars represent the standard deviation of duplicate reactions. **A.** Binding of broadly neutralising anti-CD4bs mAb b12 to IIIB and CN54 gp120. **B.** Binding of non-neutralising anti-CD4bs mAb b6 to IIIB and CN54 gp120.

While mAbs b12 and b6 both compete with sCD4 for binding to envelope glycoproteins, only b12 is able to neutralise virus (1009). The non-neutralising mAb b6 is thought to recognise a CD4bs-related epitope that is exposed on monomeric gp120, but not on gp120 in the context of the functional envelope

spike (142, 624, 897, 1018, 1194). The observation that mAb b12 binds poorly to CN54 gp120 compared to IIIB gp120 in ELISA suggests that the b12 epitope is either not present, not exposed or just not accessible in this experimental setup. The non-neutralising epitope recognised by b6 does, however, seem to be exposed.

Surface plasmon resonance techniques allow for real-time analysis of intermolecular interactions. This enables the detection of weak interactions that could remain undetected in ELISA. The ability of sCD4 to bind to CN54 gp120 (produced in a baculovirus system, like the immunogen) was therefore tested by surface plasmon resonance technology in the BIAcore system. Recombinant sCD4 was captured by, and cross-linked to, the anti-CD4 (recognising domain 4) mAb L120.3, which was coupled to the chip using standard amine-coupling techniques, as described in section 2.17, followed by subsequent injection of gp120 at three different concentrations. Good binding of fluid-phase IIIB gp120 to the immobilised sCD4 was observed (Fig. 3.2.2.6). Some binding of fluid-phase CN54 gp120 to the immobilised sCD4 was also observed, although less than for IIIB gp120 (Fig. 3.2.2.6), in concordance with ELISA results.

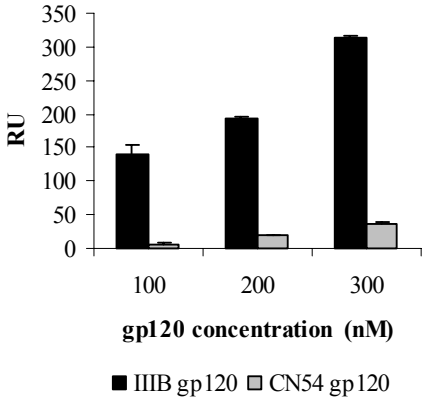


Figure. 3.2.2.6. Binding of IIIB and CN54 gp120 to immobilised sCD4 as detected by surface plasmon resonance techniques. The anti-CD4 mAb L120.3 (3100 RU) was coupled to a BIAcore CM5 ship using standard amine-coupling techniques, as described in chapter 2.17. Around 1000 RU of sCD4 was then bound and cross-linked to L120.3. Recombinant gp120 (100, 200 and 300 nM) was then injected over the chip over 4 minutes and the peak background-subtracted RU reading was recorded. Bars represent the mean of two separate injections.

The lack of sCD4 and b12 binding led to doubts regarding the structural integrity and functionality of CN54 gp120, at least the CN54 gp120 preparations included in this study, that is, the baculovirus-produced preparation used to immunise llamas, and the preparation produced 'in-house' in a vaccinia virus system but from the same CN54 clone as the immunogen. Results obtained in previous studies have indicated that sCD4 dissociates fast from CN54 gp140 in surface plasmon experiments (1195), giving a possible explanation for the poor binding observed for CN54 gp120 to sCD4 in ELISA, and the weak binding observed using surface plasmon resonance methods in this study. However, other previous studies have shown that CN54 gp120 binds to CD4 on cells in flow cytometry and to mAb b12 in ELISA (1192, 1196). It is unclear why a different result was obtained in this study.

To further evaluate whether CN54 gp120 derived from this clone was able to bind to its receptor, its ability to mediate infection when expressed on the virus surface in the context of an HIV-1 HXB2 backbone was tested. Chimeric molecular clones were generated by cloning of the CN54 gp120 sequence into an HIV-1 HXB2-based backbone and the resulting constructs were used to transfect 293T cells. Three days post-transfection, cell culture supernatant was titrated onto NP2/CD4/CCR5 and NP2/CD4/CXCR4 cells. After another 48 or 72 hours, cells were fixed and infection detected by immunostaining of HIV-1 p24. Ten different plasmid preparations were tested. The CN54 gp120/HXB2 chimeric constructs did not produce any infectious virus. Infectious chimeric virus using gp120 derived from other virus isolates have previously been produced in this system (422, 631, 1162, 1163). Generation of infectious chimeric viruses expressing envelopes derived from subtype A and C in the HXB2-derived backbone is described in section 3.2.5 of this chapter.

To exclude the possibility that CN54 gp120 together with an HXB2 gp41 did not result in a functional envelope clone, the CN54 gp160 sequence was cloned into an HIV-1 NL43-based backbone and the resulting chimeric construct was used to transfect 293T cells as described above. Ten plasmid preparations were tested and, again, no infectious virus was obtained. Generation of replication-competent chimeric viruses expressing envelopes from various subtypes has been described

for this system previously (1164). Production of infectious viruses expressing envelopes from subtype C and D in the NL43-derived backbone is described in section 4.2.1 of this thesis.

In conclusion, the CN54-derived immunogen displayed poor binding to sCD4 and to mAb b12 in biochemical assays and did not produce infectious virus when expressed on the virus surface in the context of an HIV-1 HXB2 or an HIV-1 NL43 backbone. Taken together, these data suggest that CN54 gp120 may not be an optimal immunogen, if the goal is to generate neutralising llama VHH that recognise the CD4bs. The notion that some binding of fluid-phase CN54 gp120 to immobilised sCD4 was observed in BIAcore studies could, however, be seen as encouraging. Based on the above data, it was decided to include IIIB gp120 as a control in the phage display panning step, alongside CN54 gp120.

3.2.3 First set of selections and screening - panning of phage libraries on gp120 from HIV-1 CN54 and IIIB

In this section, the first set of VHH selections and screening will be described, involving panning of the phage libraries on immobilised recombinant CN54 and IIIB gp120, followed by screening of individual VHH for ability to bind to gp120 and ability to neutralise HIV-1.

3.2.3.1 Panning of phage libraries on CN54 and IIIB gp120

The first set of selections, described in this section (3.2.3.1), was carried out by Els Beirnaert at the Ablynx NV selection laboratory in Porto, Portugal. However, I took part in the planning of the experiments. VHH targeting the CD4bs were selected for by panning of the phage libraries derived from llamas L40 and L44 on immobilised CN54 and IIIB gp120, directly coated onto microtitre plates. The CN54-derived immunogen displayed poor binding to sCD4 in ELISA and did not produce infectious virus when expressed on the virus surface in the context of an HIV-1 HXB2 or an HIV-1 NL43 backbone (described in section 3.2.2 of this thesis). Due to doubts regarding the functionality of the immunogen in terms of CD4 binding and ability to mediate infection, HIV-1 IIIB gp120 was included in

the selection protocol as a control, to enable selection of VHH targeting the CD4bs, while simultaneously allowing selection for VHH with cross-subtype recognition properties. Eluted phages were titrated onto *E. coli* TG1 cells. Selections where a larger number of clones were eluted by sCD4 compared to elution with BSA, possibly indicating enrichment of phage expressing VHH targeting the CD4bs, were taken forward to a second round of CD4bs-targeted selection. After two rounds of panning, the eluted phage output was again titrated onto *E. coli* TG1 cells, to determine whether more clones were eluted by sCD4 than by BSA. Selection outputs where a larger number of clones were eluted by sCD4 than by BSA, possibly indicating enrichment of phage expressing VHH targeting the CD4bs, were chosen and individual VHH were expressed.

A representative titration of eluted phage onto *E. coli* TG1 cells is shown in Fig. 3.2.3.1.1, resulting from two rounds of panning on IIIB gp120 followed by competitive sCD4 elution.

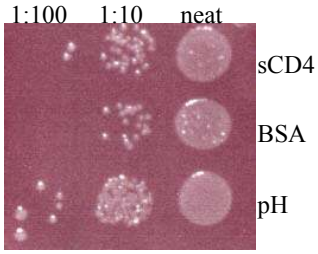


Figure 3.2.3.1.1. Titration of eluted phage onto *E. coli* TG1 cells. Phage libraries expressing the cloned VHH repertoires of llamas immunised with recombinant CN54 gp120 were panned on immobilised recombinant CN54 or IIIB gp120. To isolate phage expressing VHH targeting the CD4bs of gp120, a competitive elution with sCD4 was performed. Elution with BSA was performed in parallel, as was a general elution by low pH shock using glycine. The phage titration shown is from the second round of panning of the phage library expressing VHH derived from llama L44 on IIIB gp120, where more clones were eluted by sCD4 than by BSA, and from which individual clones were isolated and expressed.

The phage library derived from llama L40 was panned against CN54 gp120 only, whereas the phage library derived from llama L44 was panned against CN54 as well as IIIB gp120. From each selection route, 48 individual clones were isolated, as outlined in Fig. 3.2.3.1.2.

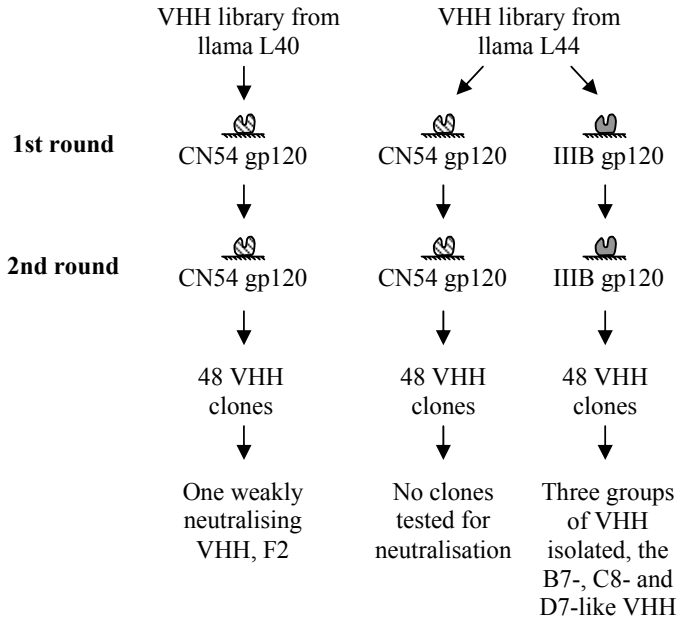


Figure 3.2.3.1.2. Overview of the first set of selections. Selections were carried out using phage libraries expressing VHH derived from llamas L40 and L44 on immobilised CN54 and IIIB gp120 as indicated. From each selection, 48 individual VHH were isolated and screened for binding and/or neutralisation of HIV-1, as described in sections 3.2.3.2 and 3.2.3.3.

3.2.3.2 Screening of selected VHH from llama L40

The phage libraries from llama L40 were panned on CN54 gp120. Forty-eight individual colonies were picked, grown in a 96-well plate and induced with IPTG. Culture supernatant was used in ELISA to identify clones reactive with solid-phase CN54 gp120. Weak binding to CN54 gp120 was observed for some of the 48 clones. However, as culture supernatant was used in the ELISA, the level of background binding was high and the results difficult to interpret. Even so, based on these binding data, as well as sequence and fingerprint analysis, four clones were selected. These four clones, designated C1, F2, F3 and F4 were re-cloned into an expression vector, expressed in *E. coli* and purified by means of the 6-histidine-tag. The VHH also carried a C-terminal c-myc-tag. This work was carried out by Els Beirnaert at Ablynx. The four purified VHH, and an irrelevant, negative control VHH, were then sent to UCL for further characterisation. At UCL, they were tested for binding to CN54 and IIIB gp120 in ELISA as well as

for neutralisation of IIIB and CN54 virus. This work was carried out by me. An infectious PBMC isolate of HIV-1 CN54 was obtained through EMPRO and propagated in PBMC, as described in section 2.4.1. Purified VHH F2, but not VHH C1, F3 and F4, was found to bind to CN54 gp120 in ELISA, whereas none of the selected VHH bound to IIIB gp120 (Fig. 3.2.3.2.1).

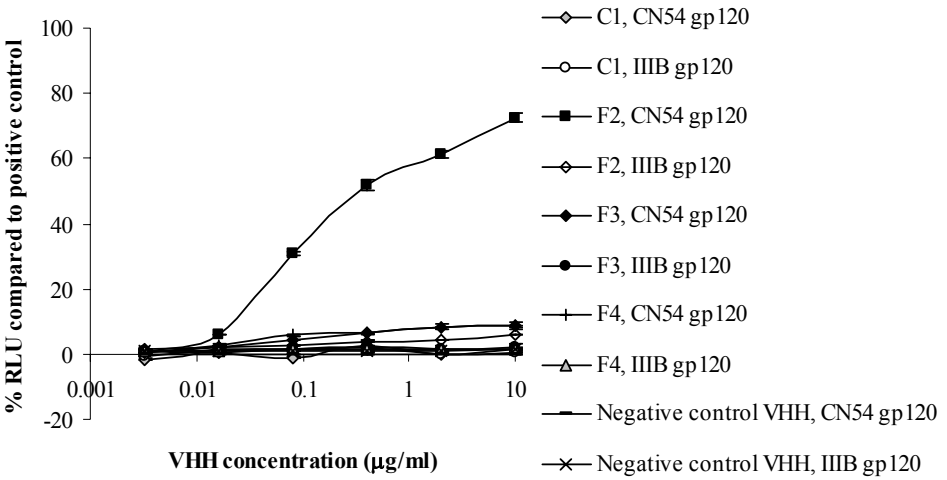


Figure 3.2.3.2.1. Reactivity of VHH C1, F2, F3 and F4, derived from llama L40, with CN54 and IIIB gp120 in ELISA. Recombinant envelope proteins were captured onto microtitre plates by the sheep polyclonal antibody D7324. Threefold serial dilutions of VHH were then added to the plate followed by detection using a mouse anti-c-myc antibody followed by an AP-conjugated goat anti-mouse IgG polyclonal antibody. Chemiluminescence was detected and background-subtracted luminescence readings (in RLU) were normalised to readings obtained for 200 ng/ml of VHH C8 binding to IIIB gp120 and plotted against VHH concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

Neutralisation was evaluated in a cell-based assay using a glioma cell line (NP2) expressing the HIV-1 receptor CD4 and either of the co-receptors CXCR4 or CCR5 (1151), followed by immunostaining of infected cells and manual counting of FFU. VHH F2 (at 100 µg/ml) was found to be weakly neutralising and reduced infection of the CN54 PBMC isolate with 47% compared to a negative control VHH, whereas VHH C1, F3 and F4 (at 100 µg/ml) had no apparent effect on CN54 infectivity (Fig. 3.2.3.2.2). None of the four VHH was

able to reduce IIIB infection by more than 20%, compared to a negative control VHH (Fig. 3.2.3.2.2).

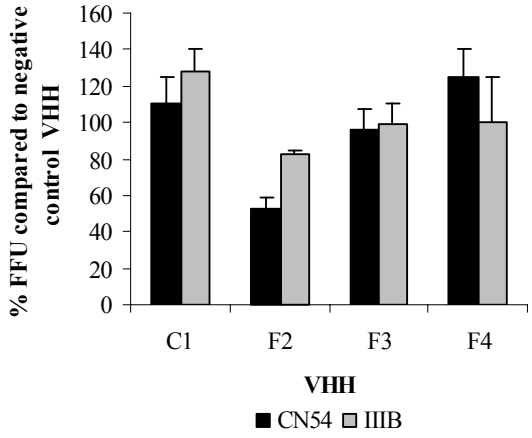


Figure 3.2.3.2.2. Neutralisation of HIV-1 CN54 and IIIB in NP2 cells by VHH C1, F2, F3 and F4, derived from llama L40. Neutralisation was assayed in NP2/CD4/CCR5 cells (CN54) or NP2/CD4/CXCR4 cells (IIIB) as described in section 2.10.1 of this thesis. Each VHH was tested at 100 μg/ml. At 72 hours post-infection, the cells were fixed and stained for p24. The number of FFU was counted microscopically and the percentage of FFU compared to the result obtained for the negative control VHH was determined. Bars represent the mean and error bars the standard error of two independent experiments carried out in duplicate.

VHH F2 was chosen for further characterisation; a more comprehensive analysis of its neutralisation properties will described in chapter 4 of this thesis. VHH C1, F3 and F4 were also tested against two additional subtype B viruses and one additional subtype C virus, but were not able to reduce infection by more than 20% (data not shown). The sequence of VHH F2 is shown in Fig. 3.2.3.2.3.

AVQLVESGGGLVQAGGSLRLSCAASGFTLDDYAICWFRQAPGKEREGVSCISSSDGSRYYADSVK
GRFTISSDNAKNTVYLQMISLKPEDTAVYYCAAXSKVMFYSDYMRYGMDYWGKGTLVTVSS

Figure 3.2.3.2.3. The amino acid sequence of VHH F2. VHH F2 was isolated through CD4bs-trageted panning on immobilised CN54 gp120 of the phage library expressing the VHH repertoire derived from llama L40. VHH CDR1, 2 and 3 are highlighted in grey. X, undetermined amino acid.

3.2.3.3 Screening of selected VHH from llama L44

The phage libraries from llama L44 were panned on CN54 and IIIB gp120, as outlined in Fig. 3.2.3.1.2. For each gp120, 48 individual colonies were picked, grown in a 96-well plate and induced with IPTG. Culture supernatant, or *E. coli* periplasmic extract, was used in ELISA to identify clones reactive with solid-phase CN54 or IIIB gp120. This work was carried out by staff at Ablynx NV, Porto, Portugal. The 48 VHH selected on CN54 gp120 were tested (in bacterial culture supernatant) for reactivity with CN54 gp120 in ELISA. However, as culture supernatants were used in the ELISA, there were high levels of background binding and the ELISA results were difficult to interpret, as discussed in section 3.2.3.2. Even so, it could be deduced that none of these 48 VHH reacted strongly with CN54 gp120. As a result, none of them was sent to UCL for test in neutralisation assays. Panning on IIIB gp120 was more successful. Out of the 48 VHH selected using IIIB gp120, 31 were found to bind to IIIB gp120 in ELISA, using VHH in *E. coli* periplasmic extracts. All the 48 VHH, in *E. coli* periplasmic extracts, were sent to UCL and tested for ability to neutralise HIV-1 IIIB in a TZM-bl cell-based neutralisation assay (1147-1149). TZM-bl cells express endogenous CXCR4 and transgenic CD4 and CCR5, and contain an LTR-driven, Tat-inducible firefly luciferase reporter gene (1147-1149).

First, it had to be established whether *E. coli* periplasmic extracts on its own would non-specifically decrease the level of infection. Ten different negative control periplasmic extracts were pre-incubated with HIV-1 IIIB and subsequently added to cells. After 48 hours, luciferase substrate and cell lysis reagent was added to the cells and luminescence detected. Reduction in RLU in test wells compared to in virus control wells after subtraction of background luminescence was determined. The mean reduction in RLU for the ten different negative control periplasmic extracts was 61%. The neutralisation cut off was set to 90% reduction in RLU, which corresponds to the mean reduction in RLU plus 2.1 standard deviations (Fig. 3.2.3.3.1).

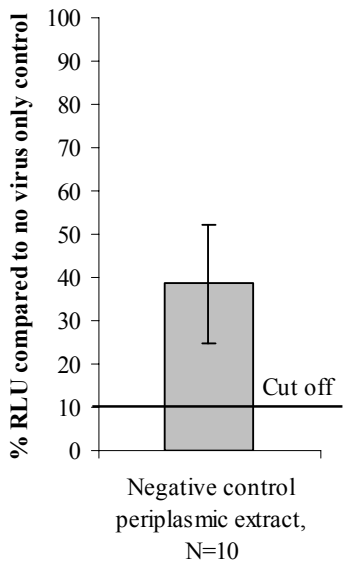


Figure 3.2.3.3.1. Determination of the neutralisation cut off when using VHH in *E. coli* periplasmic extracts in TZM-bl cells. Ten negative control periplasmic extracts were tested for neutralisation of HIV-1 IIIB in TZM-bl cells, as described in chapter 2.14.2. The % RLU compared to virus only control was determined and the mean ± the standard deviation for the ten extracts was plotted. The neutralisation cut off was set to 90% reduction of infection, which corresponds to the mean reduction in RLU plus 2.1 standard deviations.

The 48 periplasmic extracts containing the VHH selected using IIIB gp120 were then tested for neutralisation of HIV-1 IIIB in the TZM-bl assay. Out of the 48 clones, 24 were observed to neutralise HIV-1 IIIB in TZM-bl cells to more than 90%. Fingerprint analysis as well as sequencing of the 31 binding clones (including the 24 neutralising clones) revealed some identical sequences leaving 16 different clones that could be grouped into three families based on sequence similarity (Fig 3.2.3.3.2).

The first group, the B7-like VHH, consisted of seven different clones with 3-16 amino acid differences in framework 1 and 3 and in CDR1, 2 and 3 (Fig. 3.2.3.3.2). These clones, in *E. coli* periplasmic extracts, did not neutralise HIV-1 IIIB to more than 90% in TZM-bl cells, instead, 31-74% reduction of infection was observed. The second group, the D7-like VHH, consisted of three clones with 8-14 amino acid differences in the framework 1, 2 and 3 regions as well as in the CDR1, CDR2 and CDR3 (Fig. 3.2.3.3.2). They displayed a long CDR3 of 18 amino acids. These VHH clones, in *E. coli* periplasmic extracts, were found to neutralise HIV-1 IIIB to 99% in TZM-bl cells. The third group of identified VHH, the C8-like VHH, consisted of six clones with 1-3 amino acid differences only, in framework regions 1 and 3 (Fig. 3.2.3.3.2). They had identical CDRs and displayed a considerably shorter CDR3 of 10 amino acids. These VHH clones, in *E. coli* periplasmic extracts, were found to neutralise HIV-1 IIIB to 93-99% in

TZM-bl cells. A summary of the selected clones derived from llama L44 is shown in Table 3.2.3.3.1.

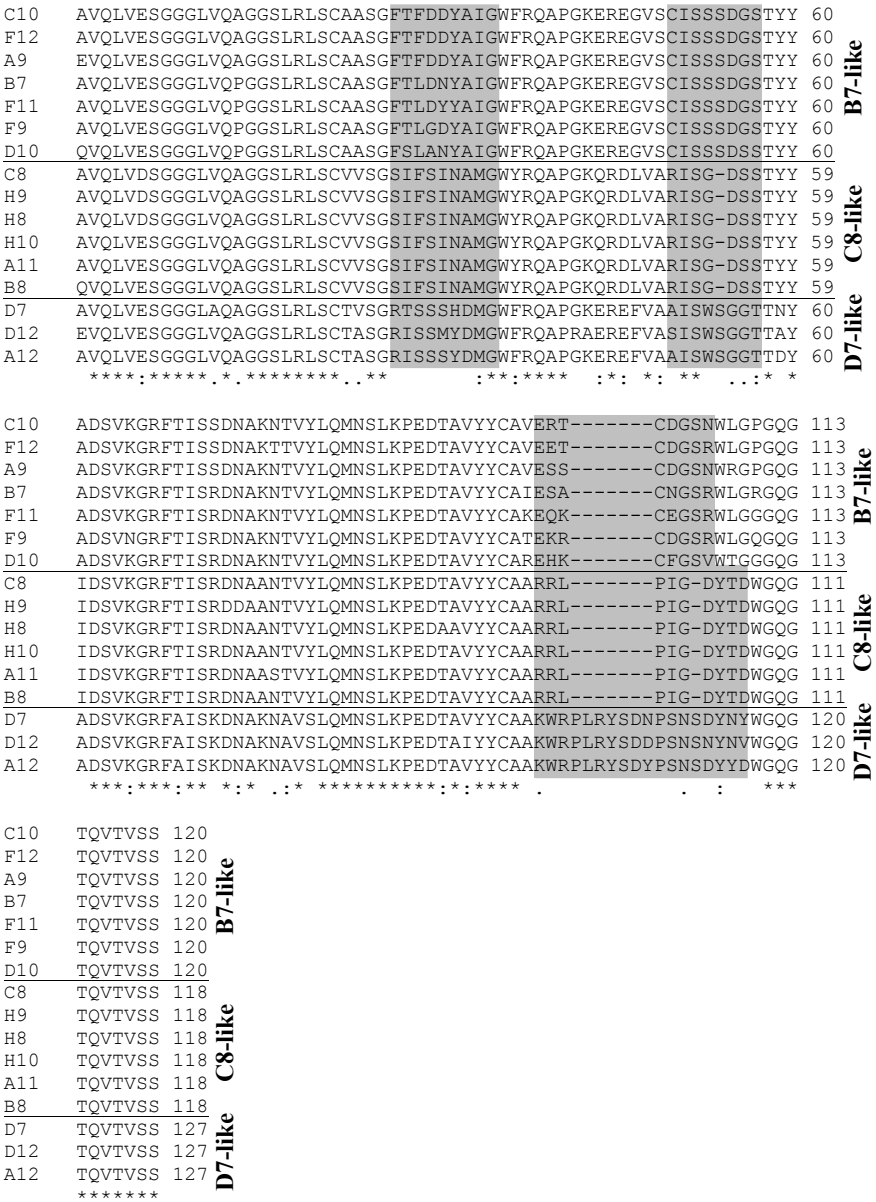


Figure 3.2.3.3.2. Amino acid sequence alignment of three groups of VHH derived from llama L44. VHH were selected by panning of phage libraries derived from llama L44 on immobilised HIV-1 IIIB gp120 followed by a competitive elution with sCD4. The three groups, the B7-, C8- and D7-like were identified by testing *E. coli* periplasmic extracts containing the VHH for reactivity with IIIB gp120 in ELISA and for neutralisation of HIV-1 IIIB in TZM-bl cells. All three groups bound to IIIB gp120 in ELISA, whereas only the C8- and D7-like VHH were able to neutralise HIV-1 IIIB. The VHH CDR1, 2 and 3 are shaded in grey. Alignment generated using the Clustal W multiple sequence alignment software (1174). Asterisks (*) indicate amino acid residues that are conserved among all the aligned sequences.

Table 3.2.3.3.1. Selected VHH derived from llama L44.

Clone	Group[a]	Total no. of clones isolated[b]	No. of amino acid differences[c]		Binding at Ablynx[d]	Neutralisation at UCL[e]
			FRs	CDRs		
B7	B7	7/48	0-4	2-12	++	-
A9	B7				++	-
F9	B7				++	-
C10	B7				+	-
D10	B7				++	-
F11	B7				++	-
F12	B7				++	-
B8	C8	19/48	1-3	0	+	+
C8	C8				+	+
H8	C8				+	+
H9	C8				+	+
H10	C8				+	+
A11	C8				+	+
D7	D7	5/48	3-7	5-7	+	+
A12	D7				+	+
D12	D7				+	+

[a]Based on amino acid sequence analysis.

[b]Proportion of individual VHH clones isolated from llama L44 belonging to each of the identified VHH groups (including duplicates).

[c]Number of amino acid differences within each of the identified VHH groups; FR, framework regions; CDR, complementarity determining region.

[d]Binding of unpurified VHH in E. coli periplasmic extracts to HIV-1 IIIB gp120 in ELISA carried out at Ablynx, NV, Porto, Portugal; - indicates less than 5-fold; + indicates 5 to 10-fold; ++ indicates more than 10-fold increase in absorbance signal compared to a negative control.

[e]Neutralisation of HIV-1 IIIB in TZM-bl cells using unpurified VHH in E. coli periplasmic extracts. Neutralisation cut off set to 90% reduction in RLU; - indicates 31-74% reduction in RLU; + indicates >90% reduction in RLU compared to virus only control.

Purified preparations of the VHH, as opposed to periplasmic extracts, were sent to UCL for characterisation. The VHH were re-cloned into the expression vector pAX051, which incorporates a 6-histidine- and a c-myc-tag to the C-terminus of the VHH, expressed in *E. coli* and purified by means of the 6-histidine-tag. This work was carried out by Els Beirnaert at Ablynx. Initially, only three clones were

sent. These were VHH B7, C8 and D7, representing the three identified VHH groups. The purified VHH were tested for reactivity with CN54 and IIIB gp120 in ELISA. VHH C8 and D7 were observed to bind to IIIB gp120, but not to CN54 gp120 (Fig. 3.2.3.3.3). Purified VHH B7, on the other hand, did not bind to either IIIB or CN54 gp120, in contrast to the result obtained using VHH B7 in periplasmic extracts (Fig. 3.2.3.3.3 and Table 3.2.3.3.1). The reason for this observed discrepancy is unclear.

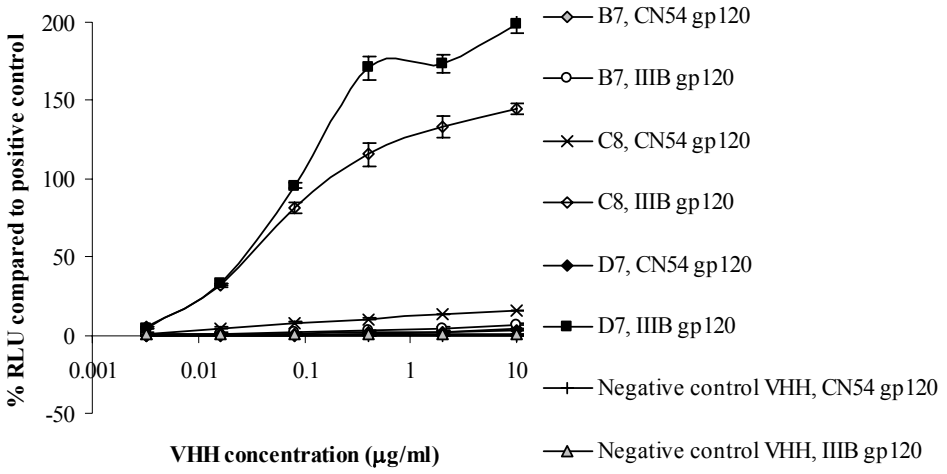


Figure 3.2.3.3.3. Reactivity of purified VHH B7, C8 and D7, derived from llama L44, with CN54 and IIIB gp120 in ELISA. Recombinant gp120 was captured onto microtitre plates by the sheep antibody D7324. Threefold serial dilutions of VHH were then added to the plate followed by detection using a mouse anti-c-myc mAb followed by an AP-conjugated goat anti-mouse IgG antibody. Chemiluminescence was detected and background-subtracted luminescence readings (in RLU) were normalised to readings obtained for 200 ng/ml of VHH C8 binding to IIIB gp120 and plotted against VHH concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

The purified VHH B7, C8 and D7 were then tested in the NP2 cell-based neutralisation assay. VHH C8 and D7 (at 100 µg/ml) reduced infection of CN54 virus with 52 and 36%, respectively, compared to a negative control VHH, whereas VHH B7 had no apparent effect on CN54 infectivity at 100 µg/ml (Fig. 3.2.3.3.4). VHH C8 and D7 potently neutralised IIIB infection at 100 µg/ml, reducing the number of FFUs by 99 and 100%, respectively, compared to a

negative control VHH. Again, VHH B7 had no apparent effect on infectivity (Fig. 3.2.3.3.4).

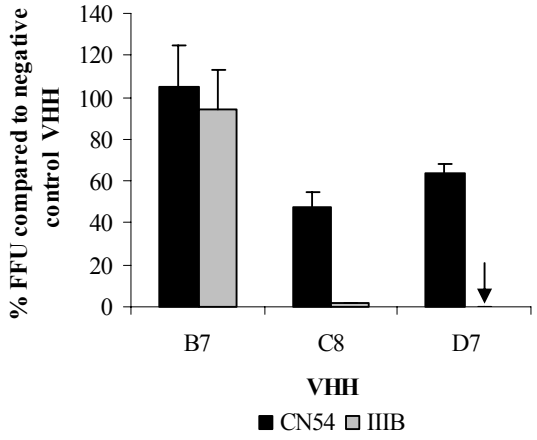


Figure 3.2.3.3.4. Neutralisation of HIV-1 CN54 and IIIB in NP2 cells by VHH B7, C8 and D7, derived from llama L44. Neutralisation was assayed in NP2/CD4/CCR5 cells (CN54) or NP2/CD4/CXCR4 cells (IIIB) as described in section 2.10.1 of this thesis. Each VHH was tested at a concentration of 100 μg/ml. The number of FFU was counted microscopically 72 hours post-infection and the percentage of FFU compared to the result obtained for the negative control VHH was determined. Bars represent the mean and error bars the standard error of two independent experiments carried out in duplicate.

The above observations made using purified VHH B7, C8 and D7 confirmed the results obtained with the periplasmic extracts, namely that the C8- and D7-like VHH were able to neutralise HIV-1 IIIB, whereas the B7-like VHH were not. Some weak neutralisation of HIV-1 CN54 was also observed for VHH C8 and D7. Based on this knowledge, purified preparations of additional members of the identified VHH groups were tested in neutralisation assays. In total, five clones from the B7, C8 and D7 groups were re-cloned, expressed and purified at Ablynx NV, Porto, and subsequently sent to UCL for characterisation. These clones were the B7-like VHH F12, the C8-like VHH H9 and A11, and the D7-like VHH A12 and D12 (Table 3.2.3.3.1).

The B7-like VHH F12 did not have any apparent effect on HIV-1 IIIB or CN54 infection, in concordance with the results observed using periplasmic extracts.

VHH F12 was also tested against two additional subtype B and one additional subtype C viruses, but was not able to reduce infection by more than 20%. No further neutralisation tests were done on the B7-like group of VHH. The two C8-like VHH H9 and A11 differed from VHH C8 in one or two amino acid positions in the framework 1 and 3 regions, respectively. As expected, they were found to behave like VHH C8 in neutralisation assays. Therefore, VHH C8 was chosen for further characterisation, as a representative for the C8-like group of VHH. As for the D7-like VHH, both remaining members (VHH A12 and D12) were chosen for further characterisation, as in addition to differences in the framework regions, these VHH also contained differences in the CDRs, which is more likely to affect antigen recognition.

3.2.3.4 Summary of the first set of selections and screening of VHH

In summary, 148 individual VHH clones were selected from llama L40 and L44 through panning of phage libraries on CN54 and IIIB gp120 (Fig. 3.2.3.1.2). These 148 clones were screened for binding to gp120 and/or neutralisation of HIV-1 CN54 and IIIB. Based on this screening as well as sequence analysis of the identified clones, five VHH were chosen for further characterisation (Table 3.2.3.4.1). A comprehensive analysis of the neutralisation properties of these will described in chapter 4 of this thesis.

Table 3.2.3.4.1. Chosen VHH from the first set of selections and screening[a].

VHH	Group[b]	Llama	Immunogen	Selection
D7	D7-like	L44	CN54 gp120	IIIB gp120
A12	D7-like	L44	CN54 gp120	IIIB gp120
D12	D7-like	L44	CN54 gp120	IIIB gp120
C8	C8-like	L44	CN54 gp120	IIIB gp120
F2	N/A	L40	CN54 gp120	CN54 gp120

[a]VHH libraries derived from llamas immunised with recombinant CN54 gp120 were panned on immobilised CN54 or IIIB gp120 followed by a competitive elution with sCD4. Individual VHH clones were isolated and screened for binding to recombinant gp120 and HIV-1 neutralisation activity. A number of similar VHH sequences were identified and representative clones were chosen for further characterisation.

[b]Based on amino acid sequence analysis; N/A, not applicable.

3.2.4 Production of recombinant gp120 from HIV-1 subtype of A and C to enable selection of VHH with cross-subtype reactivity

The two llamas immunised at the outset of this project (llama L40 and L44) had been immunised with CN54 gp120. It was therefore logical to carry out the first set of phage display selections using CN54 gp120. The immunogen did, however, show poor binding to sCD4 in biochemical assays and did not mediate infection when expressed on the surface of virus particles (as discussed in section 3.2.2). Hence, recombinant gp120 derived from HIV-1 IIIB was included in the selection protocol, due to its availability 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. Instead, HIV-1 TCLA isolates are widely known to be more sensitive to neutralisation by antibodies as well as by sCD4, compared to primary HIV-1 isolates. Using IIIB gp120 in the phage display panning step may therefore enrich for VHH that are able to bind to the more accessible CD4bs of a TCLA virus but less so to the CD4bs of primary HIV-1 isolates. In addition, IIIB is a subtype B virus; a subtype which accounts for only about 10% of infections worldwide. IIIB gp120 had been chosen mainly because of its availability. To enable selection of VHH with cross-subtype reactivity against primary HIV-1 isolates, it would be desirable to include recombinant envelope glycoproteins derived from non-subtype B in the panning protocol.

Recombinant envelope proteins from non-subtype B isolates were not readily available at the time of the first set of selections. Recombinant gp120 derived from primary isolates of non-subtype B viruses was therefore generated. To increase the chances of selecting for VHH able to recognise different HIV-1 subtypes, it was decided to include gp120 from both subtype A and subtype C isolates. The 92UG037 (subtype A) and 92BR025 (subtype C) isolates were obtained from the WHO-UNAIDS collection of primary isolates; they represent strains that were circulating in areas chosen for vaccine trials (1197).

Full-length gp120 was amplified from genomic DNA isolated from PBMC infected with HIV-1 92UG037 and 92BR025 in a nested PCR. The reverse nested primer incorporated part of the motif recognised by sheep antibody D7324 as well as a 6-histidine-tag. The amplified gp120 fragments were cloned into a mammalian expression vector. To ensure that only functional envelope clones were chosen, ten gp120 clones from each HIV-1 isolate were sub-cloned into an HIV-1 HXB2 backbone and screened for ability to mediate infection when expressed on the surface of virus particles, as will be described in section 3.2.5 of this thesis. Envelope clones giving rise to infectious virus were chosen (clones 92UG037.A9 and 92BR025.C1) and recombinant gp120 was expressed in 293T cells infected with a T7 RNA polymerase recombinant vaccinia virus, as described previously (631). Envelope protein was harvested 48 hours post-transfection and purified by means of the 6-histidine-tag (92BR025.C1 gp120) or kept in cell culture supernatant (92UG037.A9 gp120).

Expressed gp120 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 with broad neutralising activity, to ensure that a saturating level of each gp120 was applied for subsequent experiments (Fig. 3.2.4.1).

To examine the structural integrity and functionality of the expressed recombinant proteins, their reactivity with sCD4 was tested in ELISA. Recombinant sCD4 was observed to bind well to recombinant gp120 derived from the subtype A isolate 92UG037, giving a signal in the same range as for IIIB gp120 (Fig. 3.2.4.2 A). This observation is in concordance with a result obtained by Jeffs *et al.* where a recombinant gp140 derived from 92UG037 showed relatively strong binding to sCD4 in ELISA (1048).

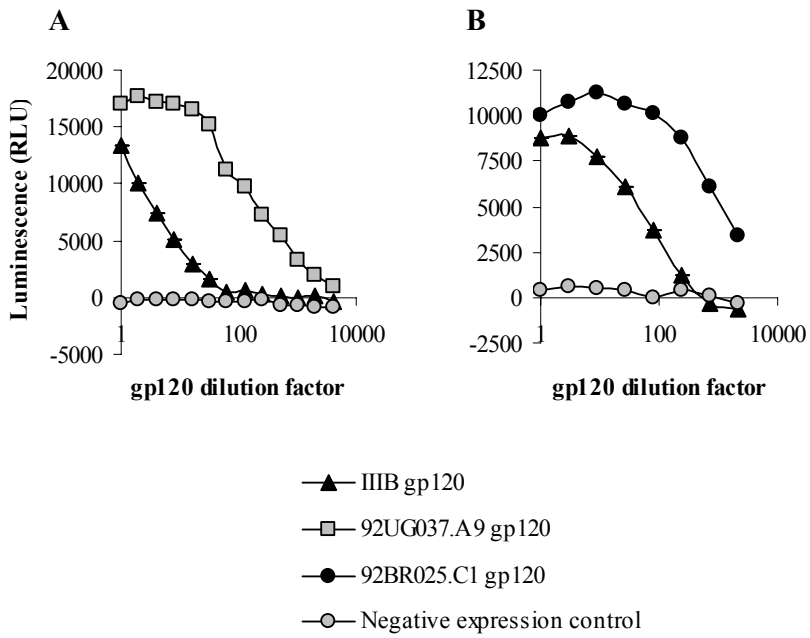


Figure 3.2.4.1. Detection of 92UG037.A9 and 92BR025.C1 gp120 expressed in 293T cells infected with a T7 RNA polymerase-recombinant vaccinia virus. Serial dilutions of gp120 were captured with antibody D7324 and detected in ELISA using a mix of three human anti-HIV-1 sera followed by an AP-conjugated goat anti-human IgG antibody. A serial dilution of IIIB gp120, starting at 0.2 or 1 μg/ml, was included as a positive control. A mock expression was included as a negative control. Chemiluminescence was detected and RLU were plotted against gp120 dilution factor. Data points represent the mean background-subtracted reading of duplicate reactions. **A.** Capture of 92UG037.A9 gp120 in cell culture supernatant. **B.** Capture of purified 92BR025.C1 gp120.

It is possible that the good binding to sCD4 makes the 92UG037.A9 gp120 suitable for selecting VHH that target the CD4bs using the selection methods described in section 3.2.3 of this thesis. Less good binding of sCD4 was observed to gp120 derived from the subtype C isolate 92BR025 (Fig. 3.2.4.2 B). This may be because of misfolding, a less accessible CD4bs or a lower affinity for CD4. Due to the observation that this gp120 produces infectious virus in the context of an HXB2 backbone (section 3.2.5) and the notion that a weakly neutralising VHH could be isolated using the poor sCD4-binder CN54 gp120 (Fig. 3.4.2), this gp120 was still included in the subsequent panning protocols, despite its relatively weak binding to sCD4. Recombinant gp120 from one additional

subtype C virus was also produced, but could not be used in selections due to the limited number of time slots available in the Ablynx selection laboratory.

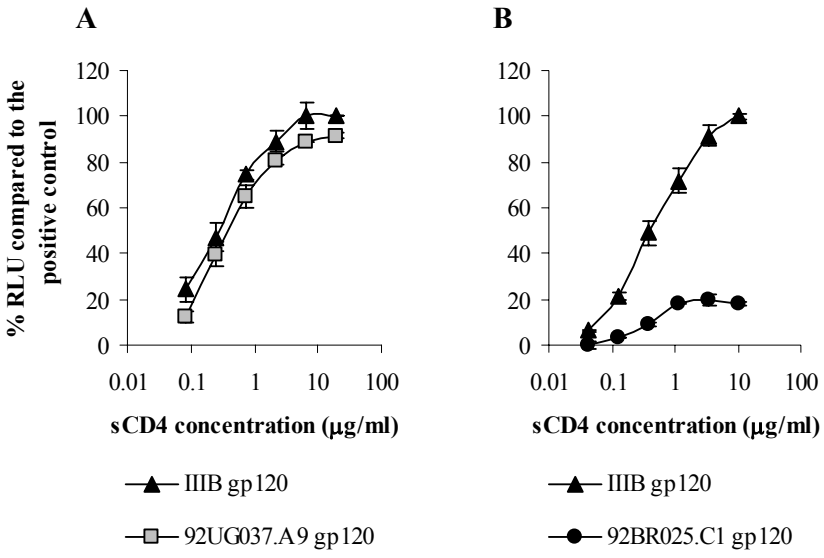


Figure 3.2.4.2. Binding of sCD4 to recombinant gp120 derived from HIV-1 92UG037 and 92BR025 in ELISA. Saturating levels of recombinant gp120 were captured by immobilised sheep antibody D7324 followed by incubation with serial dilutions of sCD4 and subsequent detection of sCD4 using the mouse anti-CD4 mAb L120.3 and an AP-conjugated goat anti-mouse IgG antibody. Chemiluminescence was detected and luminescence readings (in RLU) were normalised to readings obtained for 10 or 20 µg/ml of sCD4 binding to IIIB gp120 and plotted against sCD4 concentration. Data points represent the mean and error bars the standard error of two independent experiments performed in duplicate. **A.** Binding of sCD4 to 92UG037.A9 gp120. **B.** Binding of sCD4 to 92BR025.C1 gp120.

3.2.5 Production of chimeric viruses expressing gp120 from HIV-1 of subtype A and C for neutralisation screening of VHH selected using the corresponding envelopes

It would be desirable to screen individual selected VHH not only for binding to gp120 but also for *in vitro* neutralisation of HIV-1, as binding to monomeric gp120 does not necessarily correlate with neutralisation of the corresponding virus. The structure of soluble, recombinant envelope glycoproteins is likely to be different from the structure of the envelope glycoproteins in the context of the functional spike (138, 140, 259).

To enable screening of VHH selected through panning on recombinant 92UG037 and 92BR025 gp120 for neutralisation of the corresponding virus, the 92UG037 and 92BR025 envelopes were sub-cloned into an HXB2-based backbone and the resulting chimeric molecular clones were used to transfect 293T cells. Virus was harvested after 48 hours and titrated onto NP2/CD4/CCR5 and NP2/CD4/CXCR4 target cells. Ten different gp120/HXB2 chimeric constructs were screened for ability to produce infectious virus. One envelope clone giving rise to infectious virus was chosen for each of the two virus isolates. Both the molecular clones were predominantly R5-tropic as determined by titration on NP2 target cells (Table 3.2.5.1). The same gp120 clones (92UG037.A9 and 92BR025.C1) were used to produce recombinant gp120, for use in the panning of phage libraries, as well as for production of chimeric replication-competent virus, for use in the neutralisation screening of selected VHH.

Table 3.2.5.1. HIV-1 92UG037.A9 and 92BR025.C1 titres on NP2 cells[a].

	Titre on NP2 target cells (FFU/ml)	
	R5	**X4**
92UG037.A9	5×10^6	100
92BR025.C1	10^5	<10

[a]The envelope genes of HIV-1 92UG037 and 92BR025 were amplified and cloned into an HIV-1 pHXB2Δ*env* backbone to produce replication-competent chimeric molecular clones. Virus stocks were produced in 293T cells and titrated onto NP2/CD4/CCR5 and NP2/CD4/CXCR4 target cells. 72 hours post-infection cells were fixed and stained for HIV-1 p24 protein. FFU were counted microscopically and the virus titre on each cell line was determined.

Since the aim of the phage display selection strategy was to select for VHH targeting the CD4bs of HIV-1 envelope, the sensitivity of the chimeric viruses to neutralisation by sCD4 was tested. This was done in NP2/CD4/CCR5 cells. None of the viruses were neutralised by sCD4. Instead, a slight enhancement (2-2.5 times) of the number of FFUs was observed at high concentrations of sCD4 (Fig. 3.2.5.1). Enhancement of HIV-1 infection has been observed previously (1198). Resistance to neutralisation by sCD4 has been widely observed among primary isolates and has been shown not to correlate with sCD4 affinity for the corresponding monomeric gp120 (1199-1203).

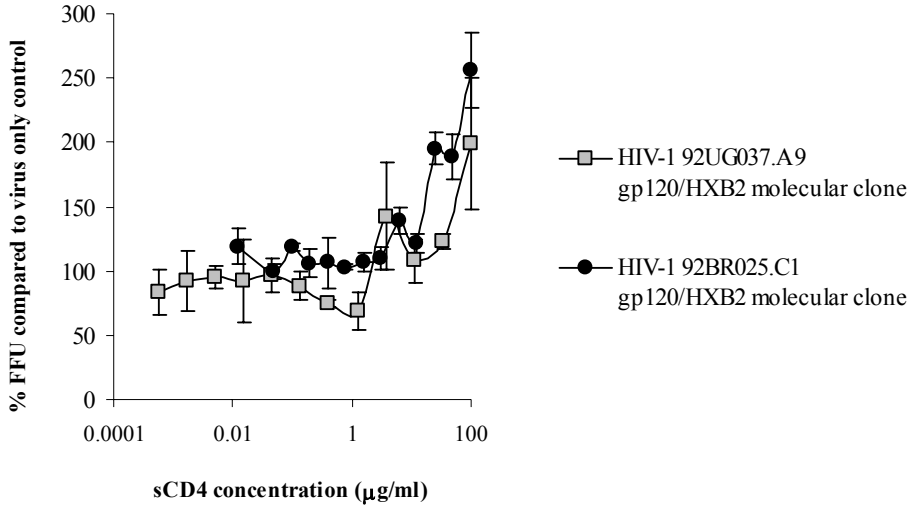


Figure 3.2.5.1. Effect of sCD4 on HIV-1 92UG037.A9 and 92BR025.C1 infection of NP2 cells. Virus was pre-incubated with serial dilutions of sCD4 and subsequently incubated with NP2/CD4/CCR5 cells. At 72 hours post-infection, the cells were fixed and stained for p24. The number of FFU was counted microscopically and the percentage of FFU compared to the result obtained for the virus only control was determined and plotted against sCD4 concentration. Data points represent the mean and error bars the standard error of three independent experiments carried out in triplicate.

There are several possible reasons for the resistance of primary isolates, including the 92UG037.A9 virus, to neutralisation by sCD4, despite a high affinity of sCD4 to monomeric gp120. It may be that the CD4bs in the context of the functional spike differs from the presentation of the CD4bs on monomeric gp120 (138, 140, 259). Recombinant sCD4 might hence be able to bind monomeric gp120 but not gp120 in the context of the trimeric spike. If this is the case, it may indicate that the 92UG037.A9 recombinant gp120 does not very well present the CD4bs as it is in the context of the functional spike and hence that this gp120 may be a bad choice for use in our selection strategies. Using similar reasoning, the 92BR025.C1 recombinant gp120 might better present the CD4bs, as the sCD4 binding and neutralisation results correlate. This notion is, however, speculative, and the weak binding of sCD4 to the soluble, recombinant protein may just as likely be due to a poor presentation of the CD4bs. An alternative explanation for the lack of correlation between the observed sCD4 neutralisation and binding data would be that sCD4 indeed binds to functional spikes on the

HIV-1 surface and that this triggers the same conformational changes that CD4 on cells do, leading to increased exposure of the co-receptor binding site on HIV-1 envelope and perhaps to increased level of infection. This mechanism is thought to be responsible for sCD4-mediated enhancement of SIV infection (1204). Such an event could possibly explain the slight enhancement of 92UG037.A9 and 92BR025.C1 infectivity observed at high concentrations of sCD4. This assumption is, however, speculative and further studies are needed to establish the mechanisms behind the resistance of the 92UG037.A9 and 92BR025.C1 virus to neutralisation by sCD4. Regardless of the mechanism, the observed results confirm that in order to increase the chances of identifying VHH able to inhibit cellular CD4 interaction with the functional envelope spike, it is important to screen selected VHH for neutralisation rather than for binding to recombinant envelope proteins. The generated 92UG037.A9 and 92BR025.C1 chimeric molecular viruses may therefore be of use.

The sensitivity of the 92UG037.A9 and 92BR025.C1 chimeric viruses to anti-CD4bs antibodies was evaluated using mAb b12 (Fig. 3.2.5.2).

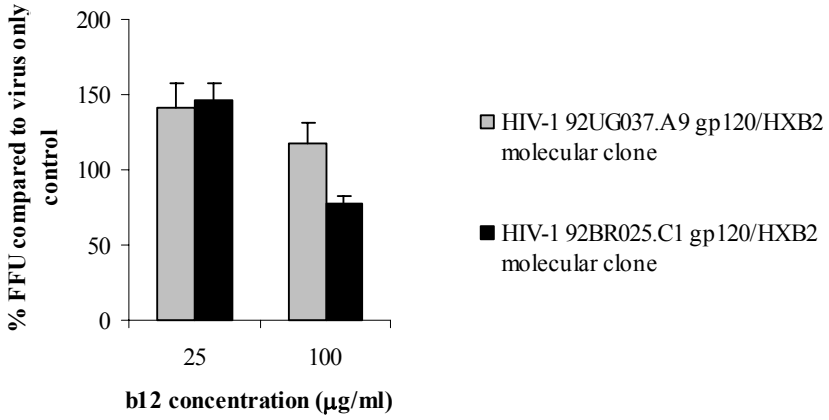


Figure 3.2.5.2. Effect of mAb b12 on HIV-1 92UG037.A9 and 92BR025.C1 infection of NP2 cells. Virus was pre-incubated with b12 (at 25 and 100 μg/ml) and subsequently incubated with NP2/CD4/CCR5 cells. At 72 hours post-infection, the cells were fixed and stained for HIV-1 p24 protein. The number FFU was counted and the percentage of FFU compared to the result obtained for the virus only control was determined and plotted. Bars represent the mean and error bars the standard error of three independent experiments carried out in duplicate.

Anti-CD4bs mAb b12 (at 25 and 100 μg/ml) did not reduce infection of the 92UG037.A9 virus and reduced infection of the 92BR025.C1 virus by only 22% (Fig. 3.2.5.2). This finding suggests that the b12 epitope is not present, or not accessible, on the functional spikes of 92UG037.A9 and 92BR025.C1 virus.

3.2.6 Second set of selections and screening - panning of phage libraries on gp120 from HIV-1 92UG037, 92BR025 and IIIB

In this section, the second set of selections and screening will be described.

3.2.6.1 Panning of phage libraries on 92UG037, 92BR025 and IIIB gp120

The first set of selections had been carried out using IIIB gp120 in addition to CN54 gp120. To facilitate selection of VHH that could recognise HIV-1 primary isolates, as opposed to the TCLA virus HIV-1 IIIB, the phage libraries expressing VHH derived from the immunised llamas were panned on recombinant gp120 derived from primary isolates of subtype A (92UG037) and C (92BR025). This work was carried out by me in the Ablynx NV selection laboratory in Porto, Portugal. In order to allow for selection of VHH recognising motifs conserved among HIV-1 subtypes, the 92UG037.A9, 92BR025.C1 and IIIB gp120 was alternated in different combinations in two subsequent rounds of panning. Selections were carried out as outlined in Fig. 3.1.1.

In a first round of panning, the phage libraries from llamas L40, L44 and L48 were panned on immobilised 92UG037.A9, 92BR025.C1 and IIIB gp120. The 92BR025.C1 and IIIB gp120 were purified and coated directly on microtitre plates, whereas the 92UG037.A9 gp120, which was in cell culture supernatant, was captured by antibody D7324 which had been pre-coated onto plates. Two different concentrations of each gp120 were used in parallel. The phages were allowed to bind to gp120 for 1 hour at room temperature, and unbound phages were then washed off in 20 cycles of washing. Again, an excess of sCD4 was used to competitively elute phage expressing VHH targeting the CD4bs, as described in section 3.2.3.1. This time, however, for each gp120 concentration, two different sCD4 concentrations were used in parallel (30 and 200 μg/ml). In

addition, for each combination, the elution was carried out for two different lengths of time (0.5 and 3 hours). Elution with BSA and low pH was carried out in parallel. Eluted phages were titrated onto *E. coli*. In general, no correlation was observed between the number of clones eluted by sCD4 and the concentration of sCD4 or length of incubation with sCD4 (Fig. 3.2.6.1.1). Selections where a larger number of clones were eluted by sCD4, compared to elution with BSA, were taken forward to a second round of CD4bs-targeted panning. The titrations from these chosen selections can be seen in Fig. 3.2.6.1.1.

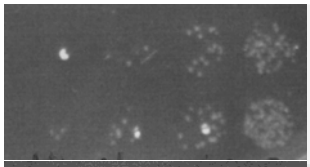
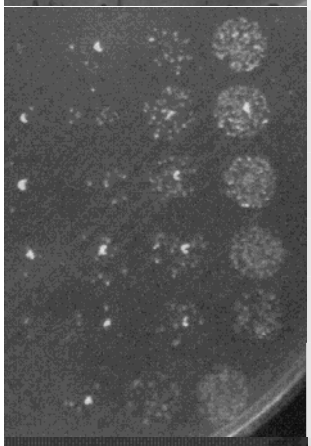
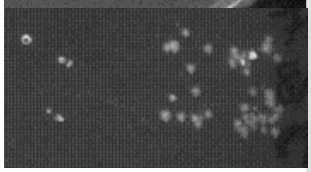
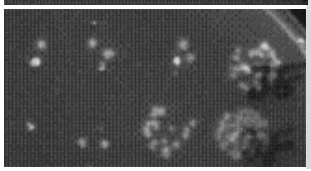
Titrations (1:125 1:25 1:5 neat)	Elution	Concentration sCD4	Concentration gp120	Library and gp120	Output
	BSA	Low	Low	**Llama L40 92UG037.A9 gp120**	**1**
	sCD4				
	BSA	Low	High		**2**
	sCD4				
	BSA	High	Low	**Llama L44 92UG037.A9 gp120**	**3**
	sCD4				
	BSA	Low	Low		**4**
	sCD4				
	BSA	Low	Low	**Llama L44 92BR025.C1 gp120**	**5**
	sCD4				
	BSA	High	Low	**Llama L48 IIIB gp120**	**6**
	sCD4				

Figure 3.2.6.1.1. Titrations of eluted phage from the first round of panning. Phage libraries from llamas L40, L44 and L48 were panned on 92UG037.A9, 92BR025.C1 and IIIB gp120. Phages were eluted with sCD4 and BSA. Only titrations from successful selections, where more clones were eluted by sCD4 than by BSA, are shown. Phages from these selections were taken forward to a second round of panning. No correlation was observed between gp120 or sCD4 concentration and number of eluted phage.

The second round of panning was carried out in the same way as the first round. Eluted phages were titrated onto *E. coli* and selections where a larger number of clones were eluted by sCD4 than by BSA were chosen (Fig. 3.2.6.1.2).

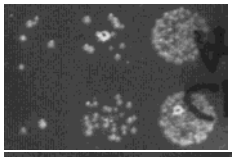
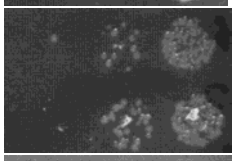
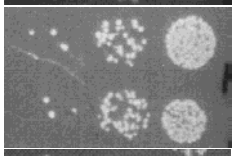
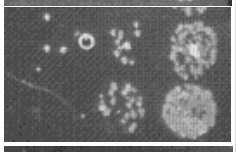
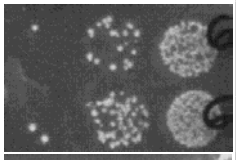
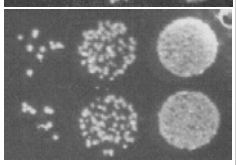
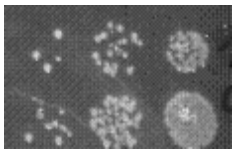
Titrations 1:100 1:10 neat	Elution	Concentration sCD4	Concentration gp120	Phage output and gp120	Output
	BSA sCD4	Low	High	**Output 2 92UG037.A9 gp120**	**A**
	BSA sCD4	Low	High	**Output 4 92UG037.A9 gp120**	**B**
	BSA sCD4	High	Low	**Output 4 92BR025.C1 gp120**	**C**
	BSA sCD4	Low	High	**Output 5 IIIB gp120**	**D**
	BSA sCD4	High	High	**Output 5 92BR025.C1 gp120**	**E**
	BSA sCD4	High	High	**Output 6 IIIB gp120**	**F**
	BSA sCD4	Low	Low	**Output 6 92BR025.C1 gp120**	**G**

Figure 3.2.6.1.2. Titrations of eluted phage from the second round of panning. Phage libraries from selections outputs 2, 4, 5 and 6 were panned on 92UG037.A9, 92BR025.C1 and IIIB gp120. Phages were eluted with sCD4 and BSA. Only titrations from successful selections, where more clones were eluted by sCD4 than by BSA, are shown. No correlation was observed between gp120 or sCD4 concentration and number of eluted phage. Individual clones were isolated from these selections outputs.

Larger numbers of sCD4-eluted clones, compared to the number of BSA-eluted clones, possibly indicating enrichment of phage expressing VHH targeting the CD4bs, could only be seen in phage outputs originating from llama L44 and L48, not from llama L40. Eluted phages from the chosen selection outputs were used to infect *E. coli* TG1 cells. The cells were grown overnight, after which DNA was isolated and the VHH fragments were cut and sub-cloned into an expression vector and the resulting plasmids were used to transfect electrocompetent *E. coli*.

Negative control wells without immobilised gp120 were also included in the panning steps. For the purified 92BR025.C1 and IIIB gp120, the negative control wells were empty, whereas for the 92UG037.A9 gp120, which was in cell culture supernatant, the negative control wells contained the capture antibody D7324 alone or D7324 plus cell culture supernatant from a mock expression. The empty negative control wells had a low background level of phage binding. In the D7324-coated wells, on the other hand, the level of phage eluted by low pH shock was just as high as the level eluted from wells containing gp120 (Fig. 3.2.6.1.3). This result suggested that some phage bound to the antibody D7324.

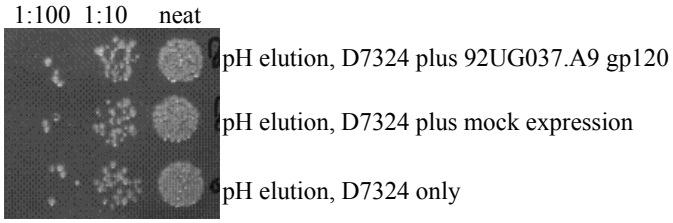


Figure 3.2.6.1.3. Titration of phage from low pH elutions in wells coated with D7324 with and without gp120. Phage libraries from llama L40, L44 and L48 were panned on 92UG037.A9 gp120 (in cell culture supernatant) captured by D7324 and eluted by glycine (low pH) as a control. Negative control wells containing only D7324, or D7324 plus cell culture supernatant from a mock expression, were included in the panning protocols. Phages were found to bind to negative control wells as well as to gp120 wells.

Attempts were made to reduce the level of phage binding sheep antibody D7324 by including goat serum in the panning steps. This strategy did, however, not reduce the level of background binding.

To investigate whether the high background binding of phage to D7324 had influenced the CD4bs-targeted selections carried out using D7324-captured gp120 (selection outputs 1-4 in Fig. 3.2.6.1.1 and A-B in Fig. 3.2.6.1.2), polyclonal VHH was tested for reactivity with gp120 and D7324 in ELISA. The polyclonal VHH were expressed from the sub-cloned VHH repertoire of selection output B (Fig. 3.2.6.1.2). As a positive control, polyclonal VHH were produced from the selection output giving rise to the C8- and D7-like VHH in the first set of selections, described in section 3.2.3. As a negative control, polyclonal VHH were produced from a phage output derived from llama L48 but selected using recombinant gp41. The gp41 selections were carried out by Willie Koh in a parallel project.

The three polyclonal VHH outputs were tested in ELISA. D7324 was coated onto the wells of a microtitre plate. Wells were blocked and then incubated with serial dilutions of 92UG037.A9 gp120 in cell culture supernatant or with cell culture supernatant from a mock expression (to exclude that the VHH reacted with anything in the cell culture supernatant). After a washing step, the wells were incubated with the three polyclonal VHH preparations. Bound VHH were detected by means of their anti-c-myc tag (Fig. 3.2.6.1.4).

The polyclonal VHH selected using D7324-captured 92UG037.A9 gp120 (selection output B) showed dose-dependent binding to 92UG037.A9 gp120 in this ELISA, as did the positive control polyclonal VHH, but not the negative control polyclonal VHH (Fig. 3.2.6.1.4 A). Some binding of the polyclonal VHH from selection output B was also observed in the wells (containing D7324) that had been incubated with cell culture supernatant from a mock expression (Fig. 3.2.6.1.4 B). This binding was, however, not dose-dependent, indicating that the polyclonal VHH from selection output B reacts with antibody D7324 rather than anything present in the cell culture supernatant, as was expected.

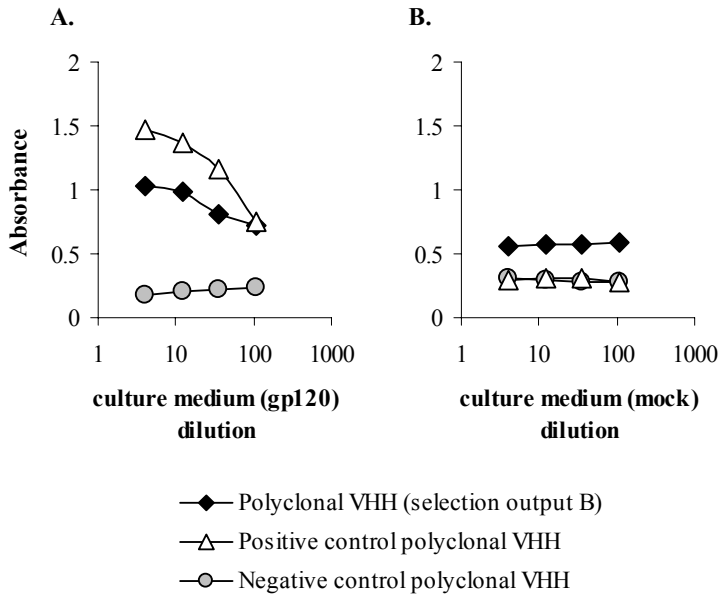


Figure 3.2.6.1.4. Reactivity of polyclonal VHH with unpurified 92UG037.A9 gp120 captured by antibody D7324 in ELISA. The wells of a microtitre plate were coated with a constant concentration of antibody D7324 and subsequently incubated with serial dilutions of **A.** 92UG037.A9 gp120 in cell culture supernatant, or **B.** cell culture supernatant from a mock expression. After a washing step, the wells were incubated with polyclonal from selection output B (selected through panning on 92UG037.A9 gp120 from cell culture supernatant captured by D7324). Positive and negative control polyclonal VHH were included, and bound VHH were detected by a mouse anti-c-myc mAb followed by an AP-conjugated rabbit anti-mouse antibody as described in the text. Absorbance readings were plotted against reciprocal culture medium dilution.

Since the 92UG037.A9 gp120 was in cell culture supernatant and could not be coated directly, without capture with D7324, the polyclonal VHH were tested for reactivity with directly coated IIIB gp120 as well as with D7324 alone. Serial dilutions of IIIB gp120 and D7324 in parallel were coated onto microtitre plate wells. Wells were blocked and then incubated with the polyclonal VHH which again were detected by means of their c-myc-tag. The polyclonal VHH selected using D7324-captured 92UG037.A9 gp120 showed dose-dependent binding to IIIB gp120 in ELISA (Fig. 3.2.6.1.5 A), although some binding to D7324 was also observed (Fig. 3.2.6.1.5 B).

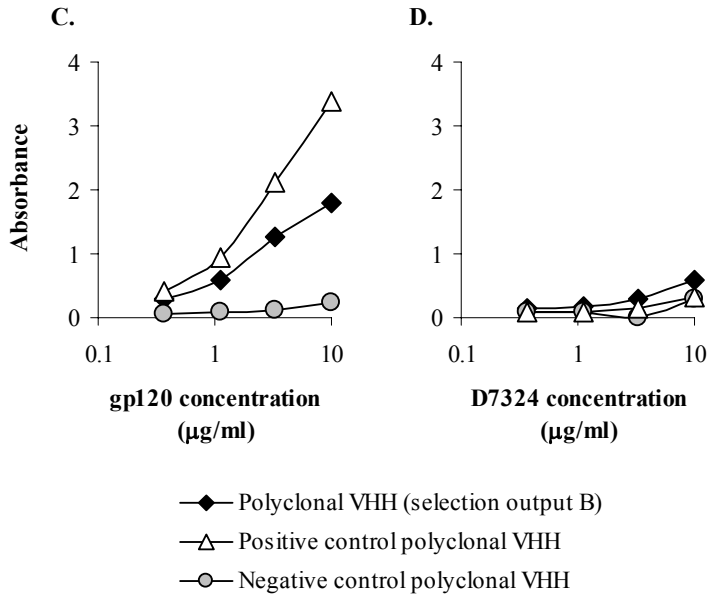


Figure 3.2.6.1.5. Reactivity of polyclonal VHH with IIIB gp120 and antibody D7324 in ELISA. Polyclonal VHH from selection output B (selected through panning on 92UG037.A9 gp120 from cell culture supernatant captured by D7324) were incubated with serial dilutions of IIIB gp120 or antibody D7324 pre-coated onto a microtitre plate. Bound VHH were then detected by a mouse anti-c-myc mAb followed by an AP-conjugated rabbit anti-mouse antibody. Positive and negative control polyclonal VHH were included, as described in the text. **A.** Polyclonal VHH reactivity with serial dilutions of IIIB gp120. **B.** Polyclonal VHH reactivity with serial dilutions of antibody D7324.

The above observations suggested that selections on D7324-captured gp120 results in some enrichment for VHH that react with the capture antibody D7324, but to a greater extent in enrichment for VHH that react with gp120. Individual clones were therefore picked from the re-cloned VHH repertoires of all the chosen selection outputs (Fig. 3.2.6.1.2), including those selected through panning on gp120 captured by D7324. For each output, 96 clones were isolated.

3.2.6.2 Screening of selected VHH for HIV-1 neutralisation and binding to gp120

The isolated monoclonal VHH were expressed overnight in *E. coli* TG1 cells in 1 ml of medium in deep-well 96-well plates. For each clone, approximately 90 µl of *E. coli* periplasmic extract containing the expressed VHH was prepared and tested for ability to neutralise HIV-1. A total of 672 VHH were expressed.

VHH selected through panning on 92UG037.A9 gp120 were screened for ability to neutralise HIV-1 92UG037.A9, whereas VHH selected using 92BR025.C1 gp120 were screened against HIV-1 92BR025.C1. In addition, all VHH were screened for ability to neutralise HIV-1 IIIB, based on the rationale that IIIB is generally sensitive to neutralisation, and the observation that both the 92UG037.A9 and the 92BR025.C1 viruses appeared to be resistant to CD4bs-targeted neutralisation (at least to sCD4 and the anti-CD4bs mAb b12). Each VHH was tested in one well only against each virus. HIV-1 CN54 was not included in the neutralisation screening, due to difficulties in propagating the isolate in PBMC to a high enough titre. In addition, the quasispecies that a PBMC-propagated primary isolate represents may also be more resistant to neutralisation and may not pick up neutralising VHH. Instead, VHH were screened for neutralisation of molecularly cloned chimeric viruses expressing the exact same envelope against which the phage libraries had been panned.

VHH neutralisation of HIV-1 was evaluated in NP2/CD4/CCR5 (HIV-1 92UG037.A9 and HIV-1 92BR025.C1) or NP2/CD4/CXCR4 (HIV-1 IIIB) target cells. Since the VHH were unpurified in *E. coli* periplasmic extracts, it had to be evaluated whether periplasmic extracts on its own would have any effect on HIV-1 infection of NP2 cells. Seven different negative control periplasmic extracts were therefore pre-incubated with HIV-1 92BR025.C1 gp120/HXB2 chimeric virus and subsequently added to cells. At 72 hours post infection, cells were fixed and stained for HIV-1 p24 protein in a β-galactosidase assay. FFU were counted microscopically and reduction in FFU in test wells compared to in virus control wells was determined. The mean reduction in FFU for the seven different negative control periplasmic extracts was 15%. The neutralisation cut

off was set to 70% reduction in FFU, which corresponds to the mean reduction in FFU plus 2.1 standard deviations (Fig. 3.2.6.2.1).

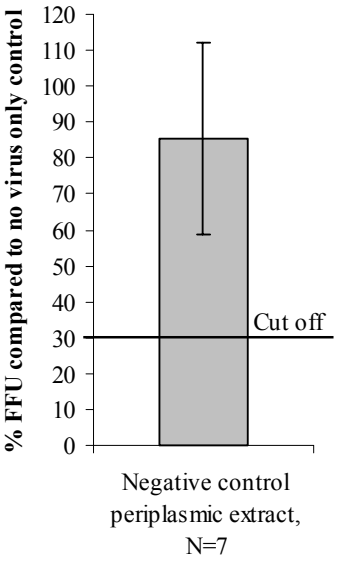


Figure 3.2.6.2.1. Effect of *E. coli* periplasmic extracts on HIV-1 infection of NP2 cells. Seven negative control periplasmic extracts were tested for effect of HIV-1 92BR025.C1 infection of NP2/CD4/CCR5 cells. The % FFU compared to virus only control was determined and the mean ± the standard deviation for the seven extracts was plotted.

The neutralisation screening revealed that out of the 192 clones isolated from llama L44 and selected using 92UG037.A9 gp120, none were able to neutralise the corresponding 92UG037.A9 virus to more than 20%. Two out of the 192 were, however, able to neutralise IIIB and 92BR025.C1 virus to more than 90%.

Out of the 96 clones derived from llama L44 and selected using 92UG037.A9 and 92BR025.C1 gp120, one clone was able to neutralise 92BR025.C1 and IIIB virus to more than 90%, whereas none of the others neutralised to more than 50%.

Out of the 192 clones derived from llama L44 and selected using IIIB and 92BR025.C1 gp120, or 92BR025.C1 gp120 alone, 16 and 24 clones, respectively, were able to neutralise 92BR025.C1 and IIIB virus to more than 80%. In contrast, none of the 192 VHH isolated from llama L48 were able to neutralise 92BR025.C1 or IIIB virus to more than 50%.

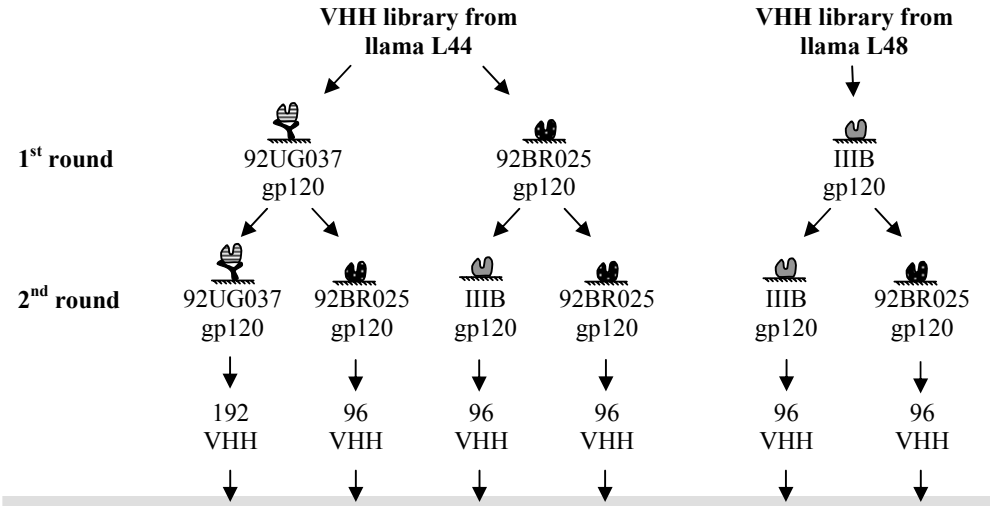
In summary, out of the 672 clones isolated in total, 43 were observed to neutralise HIV-1. All the 672 VHH were also screened for ability to bind to IIIB gp120 in ELISA. The results from the neutralisation and binding screening

showed complete correlation in that the same 43 clones were found to bind to IIIB gp120. The relatively low frequency of VHH able to bind to gp120 and to neutralise HIV-1 (43 out of 672, roughly corresponding to 6%) probably reflects the nature of the competitive elution method, as a significant number of clones were non-specifically eluted by BSA, as can be seen in Fig. 3.2.6.2.2. The fact that the antigen was varied in the panning protocol and that binding VHH therefore need to recognise motifs that are conserved between the different subtypes may also contribute.

Sequence analysis of the 43 neutralising clones revealed that they were all identical to VHH A12 (21 clones), which had already been selected from the same library through panning on HIV-1 IIIB gp120 as described in section 3.2.3, or identical to A12 with one amino acid difference in the framework 1 region (22 clones; a leucine to arginine in position 18). Characterisation of the latter VHH revealed a neutralisation profile similar to that of A12 (data not shown).

3.2.6.3 Summary of the second set of selections and screening of VHH

Phage libraries expressing the VHH repertoires derived from llamas L40, L44 and L48 were panned on gp120 from 92UG037.A9 (subtype A), 92BR025.C1 (subtype C) and IIIB gp120 followed by a competitive elution with sCD4. The antigen was alternated to allow for the enrichment of VHH able to recognise motifs conserved among HIV-1 subtypes. Larger numbers of sCD4-eluted clones, compared to the number of BSA-eluted clones, possibly indicating enrichment of phage expressing VHH targeting the CD4bs, were only observed for phage libraries derived from llama L44 and L48. In summary, 672 individual VHH clones were selected, out of which 43 were found to neutralise HIV-1 and bind to gp120. All 43 VHH turned out to be identical to the already identified VHH A12, or identical to A12 with one amino acid difference in the framework 1 region. A more thorough analysis of the neutralisation ability of VHH A12 will be described in chapter 4. The second set of selections as well as the neutralisation screening is summarised in Fig. 3.2.6.3.1.



Output	A, B	C	D	E	F	G
Neutralising 92UG037	0/192	0/96	NT	NT	NT	NT
Neutralising 92BR025	2/192	1/96	16/96	24/96	0/96	0/96
Neutralising IIIB	2/192	1/96	16/96	24/96	0/96	0/96
Binding to IIIB gp120	2/192	1/96	16/96	24/96	0/96	0/96

Figure 3.2.6.3.1. Schematic overview of the second set of selections and screening of VHH. Phage libraries expressing VHH derived from llamas immunised with CN54 envelope proteins were panned on immobilised gp120 followed by a competitive elution using an excess of sCD4. The antigen was altered in subsequent rounds of panning to possibly allow for the selection of VHH with cross-subtype neutralisation ability. Eluted phages were titrated onto *E. coli* cells and individual clones were isolated from selections where more phage had been eluted by sCD4 compared to by BSA. Only the panning routes where enrichment of clones could be observed are shown. A total of 672 VHH were screened for ability to neutralise HIV-1 as well as for binding to IIIB gp120. Out of the 672 clones, 43 were able to neutralise HIV-1; these were all identical to VHH A12 or identical to A12 with one amino acid difference.

3.3 Discussion

In this chapter, the generation of llama antibody fragments able to inhibit HIV-1 infection has been described. The antibody fragments, or VHH, were isolated from llamas immunised with recombinant envelope proteins derived from the HIV-1 primary isolate CN54. Phage libraries expressing the VHH repertoires were panned on immobilised gp120. To increase the chances of isolating neutralising VHH, a functional selection approach was employed, using a

competitive elution with sCD4, possibly allowing for the identification of VHH targeting the CD4bs. It can be argued that such an approach would by default only elute VHH with an affinity for gp120 that is lower than that of sCD4. However, by using a molar excess of sCD4, this problem may have been circumvented.

The efficiency of the competitive elution was not definitely evaluated. In order to assess whether the competitive elution actually lead to enrichment of phage expressing VHH targeting the CD4bs, the proportion of anti-CD4bs VHH eluted by each elution method could have been determined. Alternatively, panning on gp120 followed by sCD4, BSA and pH elution could have been carried out using a mini-library of phage expressing a set of VHH with known specificities, including anti-CD4bs VHH as well as VHH to other regions of gp120, followed by evaluation of the specificities of the VHH eluted using the different methods. In chapter 5 it will be evaluated whether the neutralising VHH isolated using the selection strategy described in this chapter actually target the CD4bs of gp120.

The phage libraries were panned on HIV-1 IIIB gp120 alone, subtype A gp120 alone, or subtype C gp120 alone, as well as on the same antigens alternated in various combinations in two subsequent rounds of panning, always using the competitive elution with sCD4. Interestingly, when selecting using HIV-1 IIIB gp120 alone, a range of neutralising VHH that could be grouped into two families (the C8-like and D7-like VHH) were isolated from one llama, whereas when the subtype A and C envelopes were included in the panning, only one of the VHH in the set were selected for (VHH A12, belonging to the D7-like group of VHH). This result may imply that VHH A12 is more broadly reactive across HIV-1 subtypes compared to the other VHH selected using IIIB gp120 only, but further studies are needed to determine whether this is true. Such studies will be described in chapter 4.

Further studies are also needed to determine the optimal panning protocol for selecting potent VHH with good cross-reactive properties. Previous studies have shown that panning of phage libraries expressing antibody fragments, derived from individuals infected with HIV-1, on a sequentially alternated antigen results

in enhanced identification of antibodies with broader neutralising ability, compared to panning on one single antigen (1020, 1205, 1206). In these studies, the phage libraries were panned on envelope proteins from different subtype B isolates as well as on envelope proteins in complex with sCD4. It would be interesting to see whether such an approach would be successful for isolating llama VHH.

The llamas used in this study were immunised with recombinant monomeric gp120 or trimeric gp140. Previous studies have shown that purified trimeric envelope proteins are somewhat better at eliciting cross-subtype-neutralising antibodies than monomeric recombinant gp120 (1036-1041). It is possible that immunising llamas with recombinant trimeric gp140, followed by panning of the resulting VHH libraries on trimeric gp140 as opposed to monomeric gp120, would result in VHH with more potent and cross-reactive neutralisation activity. However, in the study described here, immunising one llama with CN54 gp140 followed by panning of the VHH libraries on gp120 did not result in the identification of neutralising VHH. Furthermore, using the selection strategy described in this study, we could only isolate potently neutralising VHH from one out of three immunised llamas, despite all animals showing a significant antibody response, stressing that for future studies, more than one llama should be used for immunisation.

In conclusion, in this chapter it has been shown that the non-conventional immune system of llamas can be used to generate antibody fragments that are able to inhibit HIV-1 infection of target cells. Further studies are needed to evaluate the extent and breadth of this inhibitory effect. Such studies will be described in chapter 4.

Chapter 4

VHH neutralisation of HIV-1

In this chapter, the ability of the selected VHH to neutralise HIV-1 will be evaluated.

4.1 Introduction

Since the first reports in 1985 of *in vitro* neutralisation of HIV-1 by sera from individuals with suspected AIDS (628-630), various assays have been used to measure antibody neutralisation of HIV-1 *in vitro*. These assays differ in the source of virus used, the target cell and the way to measure infection. Initially, neutralisation was assessed in human T cell lines using virus propagated in T cell lines. In the mid-1990s, however, it became widely accepted that the adaptation of HIV-1 to replicate in T cell lines increases its sensitivity to antibody-mediated neutralisation and affects co-receptor usage, as most T cell lines express CXCR4 but not CCR5 (386, 1207-1211). Since then, HIV-1 neutralisation has commonly been measured in human PHA-stimulated PBMC, using virus produced in PBMC. While PBMCs offer the advantage of being natural target cells for HIV-1, their use carries problems of assay standardisation due to, for example, donor variability (1212, 1213). In addition, the PBMC assay is laborious and does not readily allow for high-throughput analysis of HIV-1 neutralisation. To overcome this problem, a number of neutralisation assays using engineered cell lines has been developed (1147, 1149, 1151, 1214-1217). These cell lines are typically engineered to express the HIV-1 receptors and sometimes contain a reporter gene, such as the luciferase or green fluorescent protein (GFP) genes, to allow for easy measurement of infection. To further increase reproducibility and allow for standardisation, the use of cloned viruses has been introduced. Cloned viruses, such as replication-competent molecular clones or envelope pseudotyped viruses, have been suggested to offer advantages in terms of reproducibility and well-defined envelope sequences, useful in for example epitope mapping studies, over the quasispecies-natured PBMC isolates (1022, 1023, 1215, 1218, 1219).

Moreover, the propagation of HIV-1 in PBMC has been shown to lead to increased neutralisation sensitivity, at least to anti-CD4bs-targeted neutralisation (1200, 1203). On the other hand, virus produced in PBMC may better represent the *in vivo* situation, and cloned viruses produced in 293T cells have in some studies been shown to differ in terms of neutralisation properties compared to the PBMC isolates they were derived from (1022, 1218). As a consequence, a range of neutralisation assays are currently being used, using T cell lines, engineered cell lines or primary cells, as well as using different sources of virus, including cloned virus produced in 293T cells and primary isolates propagated in PBMC. To be able to compare results obtained in different laboratories, there is a need for standardisation. However, a recent global consortium working on the standardisation of HIV neutralisation assays has come to the conclusion that, since there is little information available on which *in vitro* assay correlates with *in vivo* protection, it is necessary to use a range of neutralisation assays when assessing *in vitro* neutralisation of HIV-1 (1220). Assessing antibody ability to neutralise not only cell-free virus, but also their ability to inhibit virus spread through cell-cell fusion, may also be of importance (1221), as may activities such as complement-mediated antibody-dependent enhancement or inactivation of infection (682, 694).

In addition to the problem with which target cell line to use, and how to propagate or generate viruses, assessing antibody-mediated neutralisation of HIV-1 *in vitro* also involves the problem of the extreme genetic variation observed among HIV-1 isolates, in particular in the envelope region (discussed in chapter 1 of this thesis). Cloned viruses exhibiting a wide range of sensitivities to broadly neutralising mAbs, have been isolated from single infected individuals, even at single time-points (652, 1222). To deal with the extreme genetic variation, Mascola *et al*. (1219) has suggested the use of a tier 1, 2 and 3 approach for the assessment of neutralising antibodies to HIV-1, using standard panels of viruses belonging to different HIV-1 subtypes and with well-characterised and varying neutralisation sensitivities. This approach would involve initial testing against a tier 1 panel of viruses of different subtypes, shown to be sensitive to antibody-mediated neutralisation, followed by testing against a tier 2 panel of viruses, of the same subtype as the immunogen or

vaccine strain, shown to be of intermediate sensitivity to neutralisation. Tier 3 assessment would then be accomplished by testing against virus panels of additional subtypes.

Numerous mAbs to HIV-1 envelope have been isolated, from infected individuals as well as from immunised animals, and their abilities to neutralise HIV-1 have been characterised in a range of neutralisation assays. Many of these mAbs have been found to inhibit infection of neutralisation sensitive TCLA isolates of HIV-1, or homologous isolates post-immunisation. Only a handful have, however, been found to neutralise a broad range of primary HIV-1 subtypes. In chapter 3, a number of VHH were isolated from llamas immunised with recombinant HIV-1 CN54 gp120. These VHH were selected using phage display methods through panning on immobilised gp120 followed by a competitive elution using sCD4. Large numbers of VHH were screened for ability to neutralise HIV-1 *in vitro*. Five VHH were found to neutralise HIV-1 to various extents, when tested at one concentration only. These VHH were selected for further characterisation (Table 4.1.1). In this chapter, their neutralisation abilities will be further assessed, to characterise the breadth and potency by which they neutralise HIV-1 of different subtypes.

Table 4.1.1. VHH chosen for further characterisation[a].

VHH	Group[b]	Llama	Immunogen	Selection
A12	D7-like	L44	CN54 gp120	IIIB, 92UG037 & 92BR025 gp120
D7	D7-like	L44	CN54 gp120	IIIB gp120
D12	D7-like	L44	CN54 gp120	IIIB gp120
C8	C8-like	L44	CN54 gp120	IIIB gp120
F2	N/A	L40	CN54 gp120	CN54 gp120

[a]VHH derived from llamas immunised with recombinant CN54 gp120 were isolated using a functional selection approach allowing for the enrichment of VHH that compete with sCD4 for binding to gp120, as described in chapter 3 of this thesis. Large numbers of individual VHH were screened for binding to gp120 and/or in vitro neutralisation of HIV-1. A number of similar VHH sequences were identified and representative clones were chosen for further characterisation.

[b]Based on amino acid sequence analysis; N/A, not applicable.

4.2 Results

This section describes the results of this chapter.

4.2.1 Generation of chimeric viruses expressing envelopes from HIV-1 of subtype A, C and D

To evaluate the neutralisation breadth of the VHH, they needed to be tested against HIV-1 of different subtypes. HIV-1 isolates of subtype B have been extensively studied, and large numbers of isolates and clones are characterised and available. At the outset of this project, there were, however, very few non-subtype B viruses readily available. A small panel of non-subtype B viruses was therefore acquired, to be used alongside a panel of commonly used subtype B isolates, as well as recently described chimeric viruses expressing envelopes cloned from plasma of individuals infected with subtype B (1162). Two chimeric molecular clones (HIV-1 CA6 and CB7) containing R5-tropic gp120 cloned from plasma of individuals infected with HIV-1 of subtype C held in the pHXB2Δ*env* vector (1163), were kindly provided by M. Aasa-Chapman (UCL, London, UK).

A number of non-subtype B PBMC-propagated viruses were obtained from the WHO panel of primary isolates, which contains viruses representative of strains circulating in areas chosen for vaccine trials (1197). These included two subtype C isolates (IN97003 and ZA97001) as well as a subtype D isolate (92UG001). Some of the obtained isolates proved laborious to propagate to high titre in PBMCs. In addition, culturing primary isolates in PBMC has been shown to increase sensitivity to anti-CD4bs-targeted neutralisation (1200, 1203). For reasons of reproducibility, chimeric molecular clones, containing envelopes cloned from these primary isolates, were therefore generated. This was done by the isolation of genomic DNA from infected PBMC, followed by the amplification of the envelope gene from proviral DNA in a nested PCR. To ensure amplification of HIV-1 of all subtypes, the outer primers were designed to target conserved regions of the HIV-1 genome and were shown to amplify sequences from HIV-1 of subtype A, B′/C, C, D and F as well as from HIV-1 of group O. The nested primer pair was modified from Zheng and Daniels (1164).

The amplified gp160 gene was cloned into a cloning vector followed by sub-cloning into the HIV-1 NL43-based *env* gene cassetting system C2 (1164) to produce replication-competent chimeric molecular clones. Virus was produced in 293T cells. For each virus isolate, ten different gp160/NL43 chimeric constructs were screened for ability to infect NP2/CD4/CXCR4 and NP2/CD4/CCR5 target cells. One envelope clone giving rise to infectious virus was chosen for each of the virus isolates. The clones derived from the ZA97001 and 92UG001 isolates were found to be predominantly X4-tropic, whereas the clone derived from the 97IN003 isolate was predominantly R5-tropic, as determined by titration on NP2 target cells (Table 4.2.1.1). The generation and characterisation of chimeric viruses expressing envelopes derived from the subtype A (92UG037) and subtype C (92BR025) isolates has already been described in section 3.2.5 of this thesis.

To further extend the virus panel, envelope genes were also amplified from plasma of individuals infected with HIV-1 of subtype C. This work was carried out together with Willie Koh, who is a PhD student working on a related project. RNA was isolated from plasma and cDNA generated. Full-length gp160 was amplified from the cDNA and cloned into the C2 cassette as described above. Alternatively, gp120 was amplified and cloned into an HXB2-based backbone (1163). Chimeric virus was produced in 293T cells and titrated onto NP2 indicator cells. Chimeric viruses expressing envelopes derived from five different individuals were included in the study. The viruses were designated 37.4.2, 38.2.2, 27b, 27d, C222 and C261. Viruses 27b, 27d, C222 and C261 were cloned by Willie Koh. All viruses were found to be predominantly R5-tropic, apart from 27d which was predominantly X4-tropic (Table 4.2.1.1).

Again, attempts were made to generate a replication-competent chimeric molecular clone expressing an envelope derived from HIV-1 CN54. Previously, the same envelope clone from which the immunogen had been expressed was cloned into HXB2- or NL43-based backbones. This chimeric molecular clone did not, however, produce infectious virus, as described in section 3.2.2 of this thesis. This time, the CN54 envelope gene was amplified from cDNA generated from PBMC-propagated HIV-1 CN54 as well as from proviral DNA isolated

from infected PBMCs. More than twenty different clones were screened for ability to infect NP2 target cells, but again, no infectious virus could be generated. Failure to generate infectious pseudotyped virus expressing CN54 envelope has also been noted elsewhere (Neil Sheppard, DPhil thesis, University of Oxford, 2006).

Table 4.2.1.1. HIV-1 chimeric virus titres in NP2 cells[a].

				Titre on NP2 target cells (FFU/ml)	
Virus	**Source[b]**	**Chimera**	**Subtype**	**R5**	**X4**
ZA97001.1	ccPBMC	gp160/NL43	C	<10	2×10^5
97IN003.4.2	ccPBMC	gp160/NL43	C	1.8×10^5	<10
92UG001.D8	ccPBMC	gp160/NL43	D	<10	1.2×10^4
37.4.2	Plasma	gp160/NL43	C	4×10^3	<10
38.2.2	Plasma	gp160/NL43	C	1.4×10^5	<10
27b	Plasma	gp160/NL43	C	3×10^5	<10
27d	Plasma	gp160/NL43	C	<100	2.5×10^6
C222	Plasma	gp120/HXB2	C	2.5×10^5	<50
C261	Plasma	gp120/HXB2	C	2×10^5	<50

[a]The envelope genes were amplified and cloned into an HIV-1 pHXB2Δ*env* backbone (gp120) or the HIV-1 NL43-based *env* gene cassetting system C2 (gp160) to produce replication-competent chimeric molecular clones. Viruses 27b, 27d, C222, C261 were generated by Willie Koh. Virus stocks were produced in 293T cells and titrated onto NP2/CD4/CCR5 and NP2/CD4/CXCR4 target cells. At 48 or 72 hours post infection, the cells were fixed and stained for HIV-1 p24 protein. FFU were counted microscopically and the virus titre in each cell line was determined.

[b]Envelope genes were amplified from i) proviral DNA isolated from PBMCs infected with virus stocks obtained from the WHO-UNAIDS collection of primary isolates (1197), or ii) cDNA generated from RNA isolated from plasma obtained from individuals infected with HIV-1 of subtype C, as described in chapter 2 of this thesis. ccPBMC, co-cultured PBMC.

4.2.2 'Large-scale' expression of VHH

To be able to test the VHH against larger numbers of viruses, the VHH expression needed to be scaled up. Initial batches of recombinant VHH were produced at Ablynx. Later on, VHH A12, C8, D7 and negative control VHH were produced at UCL. The VHH DNA fragments were sent to UCL and re-cloned into an expression vector, followed by expression in *E. coli* TG1 cells and subsequent purification by means of the C-terminal 6-histidine-tag. The purification was monitored by SDS-PAGE (Fig. 4.2.2.1 A). The induction time was optimised. An overnight induction was shown to give the highest yield of VHH (Fig. 4.2.2.1 B).

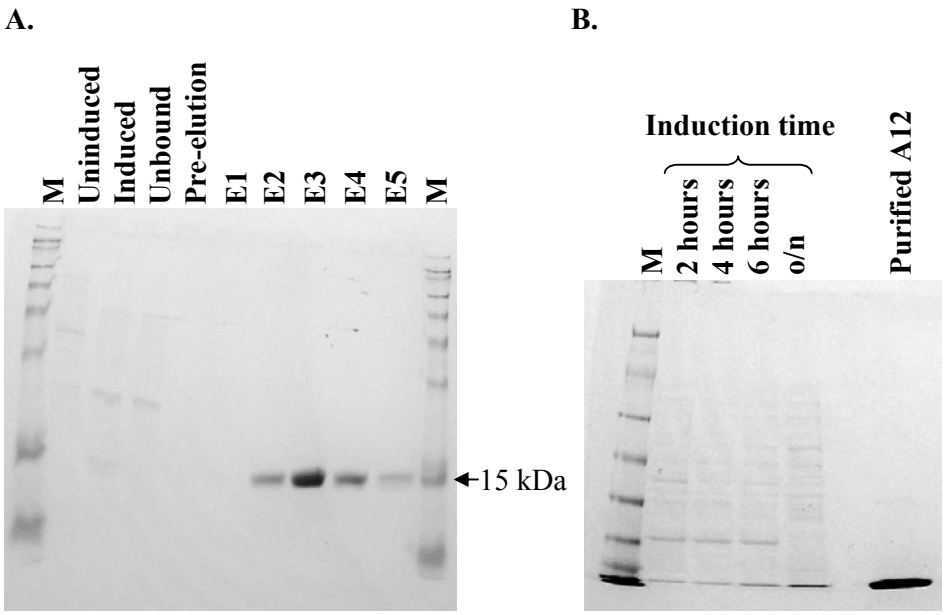


Figure 4.2.2.1. Expression and purification of VHH. Recombinant VHH were expressed in *E. coli* TG1 cells and purified by means of the C-terminal 6-histidine-tag, as described in chapter 2. **A.** The purification was monitored by SDS-PAGE followed by Coomassie blue staining. Shown is a representative analysis of the purification of VHH A12. The lanes contain *E. coli* periplasmic extract from the uninduced sample, *E. coli* periplasmic extract from the induced sample, the unbound fraction, the pre-elution fraction and elutions 1-5 (E1-E5). M, molecular weight marker. **B.** Optimisation of the induction time. The induction time was varied between 2, 4 or 6 hours or overnight (o/n). *E. coli* periplasmic extracts were prepared from the different inductions and analysed by SDS-PAGE and Coomassie blue staining. Shown is the expression of VHH A12.

4.2.3 Initial characterisation of VHH neutralisation of HIV-1 in NP2 cells

The neutralisation activities of the selected VHH were initially evaluated in a cell-based assay using a glioma cell line (NP2) engineered to express the HIV-1 main receptor CD4 and either of the co-receptors CXCR4 or CCR5. This cell line was established by Soda *et al*. (1151) and has since been used to assay antibody neutralisation of HIV-1 (631, 682).

4.2.3.1 Effect of negative control VHH on HIV-1 infection of NP2 cells and assay reproducibility

To exclude any non-specific effect of camelid VHH on the infectivity of HIV-1 in this assay format, the effect of two irrelevant VHH on HIV-1 infection of NP2/CD4/CXCR4 or NP2/CD4/CCR5 target cells was evaluated using 12 different X4- or R5-tropic HIV-1 isolates. Virus was incubated with irrelevant VHH (at 100 μg/ml) and subsequently incubated with target cells. At 48 or 72 hours post infection, the cells were fixed and infection detected by immunostaining of HIV-1 p24 protein. FFU were counted microscopically. The level of infection was determined as the percentage of FFUs in test wells compared to virus only control wells. Wells containing the irrelevant VHH were found to contain 96-156% FFU compared to in virus only control wells, showing that in this assay system, at least two irrelevant VHH do not cause any non-specific reduction of infection (Fig. 4.2.3.1.1).

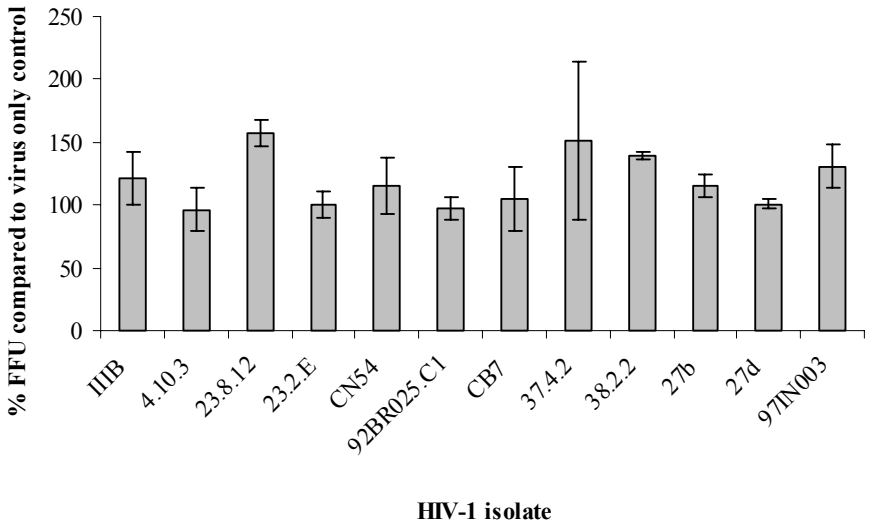


Figure 4.2.3.1.1. Negative control VHH effect on HIV-1 infection of NP2 cells. The effect of an irrelevant VHH on infection with 12 different X4- or R5-tropic HIV-1 isolates was evaluated in NP2 cells. Virus was incubated with the irrelevant VHH and subsequently incubated with NP2/CD4/CXCR4 or NP2/CD4/CCR5 indicator cells. At 48 or 72 hours post infection, the cells were fixed and stained for HIV-1 p24 protein. The number FFU was counted microscopically and the percentage of FFU compared to the result obtained for the virus only control was determined. Bars represent the mean and error bars the standard error of two or three independent experiments carried out in triplicate.

The reproducibility of the neutralisation assay was demonstrated by testing VHH C8 against the HIV-1 92BR025.C1 molecular clone on four separate occasions (Fig. 4.2.3.1.2). Serial dilutions of VHH C8 were incubated with HIV-1 92BR025.C1, in triplicate reactions, and subsequently incubated with NP2/CD4/CCR5 target cells for 72 hours, after which the cells were fixed and immunostained for HIV-1 p24 protein. FFU were counted microscopically and neutralisation titres were determined as the VHH concentration required to give 90% reduction of infection (IC_{90}) compared to a control VHH at 100 μg/ml. The variation between the four observed IC_{90} values was found to be 1.2-2.4-fold (Fig. 4.2.3.1.2 B). For the remaining VHH-virus isolate combinations, each VHH was assayed in duplicate or triplicate and on a minimum of two separate occasions, to ensure reproducibility.

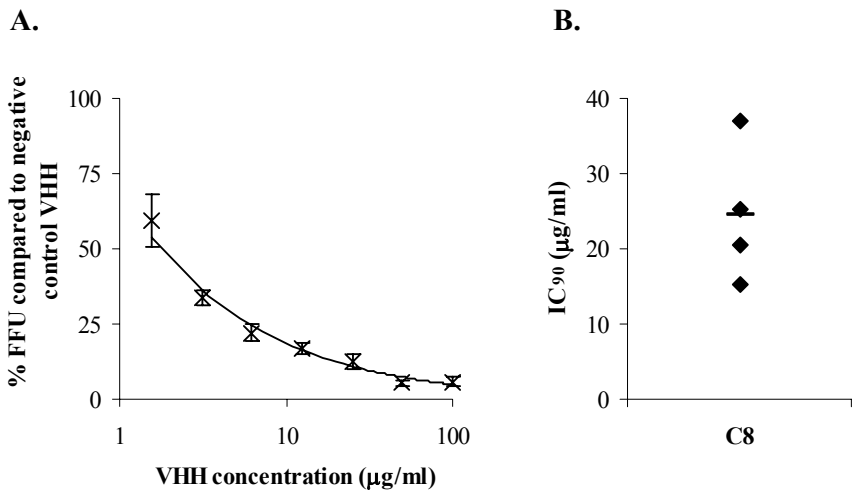


Figure 4.2.3.1.2. Evaluation of NP2 cell-based neutralisation assay reproducibility. HIV-1 92BR025.C1 was incubated with serial dilutions of VHH C8 and subsequently incubated with NP2/CD4/CCR5 target cells for 72 hours, after which the cells were fixed and stained for HIV-1 p24 protein. FFU were counted microscopically and neutralisation titres were determined as the VHH concentration required to give 90% reduction of infection (IC_{90}) compared to a control VHH at 100 µg/ml. IC_{90} titres were calculated using the curve-fitting software XLfit 4. **A.** Percentage FFU in wells containing VHH C8 compared to in wells containing a negative control VHH. Data points represent the mean and bars the standard error of four independent experiments carried out in triplicate. **B.** VHH C8 IC_{90} titres observed in four independent runs. The diamonds represent values from individual runs and the short horizontal bar represents the mean.

4.2.3.2 VHH neutralisation of the autologous virus HIV-1 CN54

VHH A12, D7, D12, C8 and F2 were tested for ability neutralise the autologous virus HIV-1 CN54. Each VHH was tested in duplicate at a final concentration of 100 µg/ml, in two independent experiments. The VHH were observed to weakly neutralise HIV-1 CN54 in NP2 cells, reducing infection by 29-62%, compared to a negative control VHH (Fig. 4.2.3.2.1). The lack of potent neutralisation of the autologous isolate may be due to the quasispecies nature of the HIV-1 CN54 PBMC isolate. The CN54 gp120 clone used as an immunogen may not be representative of the gp120s present on the majority of the infectious viruses within the swarm of the CN54 PBMC isolate.

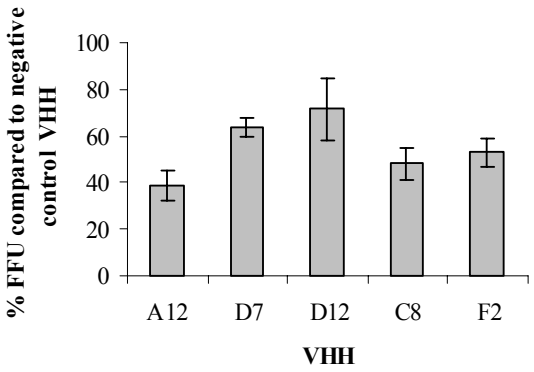


Figure 4.2.3.2.1. VHH ability to neutralise the autologous virus HIV-1 CN54 in NP2 cells. PBMC-propagated HIV-1 CN54 was incubated with VHH A12, D7, D12, C8 and F2 or an irrelevant VHH and subsequently incubated with NP2/CD4/CCR5 indicator cells. Each VHH was tested at 100 μg/ml. At 48 or 72 hours post infection, the cells were fixed and stained for HIV-1 p24 protein. The number FFU was counted microscopically and the percentage of FFU compared to the result obtained for the negative control VHH was determined. Bars represent the mean and error bars the standard error of two independent experiments carried out in duplicate.

4.2.3.3 VHH neutralisation of HIV-1 of subtype A, B, B′/C, C and D

As an initial assessment of neutralisation breadth and potency, the selected VHH (Table 4.1.1) were assayed against nine HIV-1 isolates of subtypes A, B, B′/C, C and D. In addition to the autologous PBMC isolate, HIV-1 CN54, this small panel of HIV-1 isolates included the viruses from which the envelopes used in the panning were derived. These were HIV-1 IIIB as well as the chimeric molecular clones 92UG037.A9 and 92BR025.C1, the generation of which were described in section 3.2.5. The TCLA isolate IIIB is known to be generally sensitive to antibody-mediated neutralisation.

Also included in the virus panel were the chimeric molecular clones 4.10.3, 8.8.8 and 23.2.E, expressing envelopes cloned from plasma of individuals infected with HIV-1 of subtype B (1162). These molecular clones have been shown to exhibit a varying degree of sensitivity to neutralisation by human anti-HIV-1 sera as well as anti-envelope mAbs. Virus 4.10.3 has been found to be sensitive, virus 8.8.8 has been found to be intermediately sensitive and virus 23.2.E has been found to be resistant to neutralisation by human anti-HIV-1 sera (M. Aasa-

Chapman, *et al*., in preparation). In addition, they show a varying degree of sensitivity to neutralisation by mAb b12 and sCD4, with 8.8.8 being sensitive and 23.2.E being resistant to both mAb b12 and sCD4, and 4.10.3 being sensitive to sCD4 but not to mAb b12 (M. Aasa-Chapman, *et al*., in preparation).

Finally, a chimeric molecular clone of subtype C was included, as was the chimeric subtype D virus described in section 4.2.1. The neutralisation activity of each VHH was assayed in triplicate and on a minimum of two separate occasions. The lowest VHH concentration required to achieve 90% reduction of infectivity (IC_{90}) compared to a negative control VHH was determined. Human anti-CD4bs mAb b12 as well as sCD4 was tested in parallel.

The results of this initial characterisation are summarised in Table 4.2.3.3.1. In summary, VHH A12, D7 and C8 were found to be more potent than VHH D12 and F2. VHH A12 and D7 could potently neutralise three out of the four subtype B viruses with IC_{90} values in the range of 0.01-1 μg/ml, whereas C8 potently neutralised two out of the four subtype B viruses with an IC_{90} of 0.4 and 12 μg/ml. VHH A12 and C8 also displayed some cross-subtype neutralisation ability, neutralising one out of the two subtype C viruses, 92BR025.C1 with IC_{90} titres of 25 μg/ml. Against this small virus panel, mAb b12 and sCD4 displayed a similar neutralisation breadth, neutralising two and three out of the four subtype B viruses, respectively, as well as one of the two subtype C viruses. None of the VHH, nor mAb b12 or sCD4, was able to neutralise the subtype A or D virus to 90%. VHH D12 and F2 did not neutralise any of the viruses included in this panel to 90% (Table 4.2.3.3.1), although weak neutralisation by up to 50% was observed against some isolates (data not shown).

Table 4.2.3.3.1. VHH, mAb b12 and sCD4 IC_{90} against HIV-1 in NP2 cells[a].

Virus[b]	Type[c]	Subtype	IC_{90} in NP2 cells (μg/ml)						
			A12	D7	D12	C8	F2	b12	sCD4
92UG037.A9	MC	A	•	•	•	•	•	•	•
IIIB	TCLA	B	0.8	0.8	•	12	•	<5	<5
4.10.3	MC	B	<1	0.01	•	0.4	•	•[d]	0.4[d]
8.8.8	MC	B	1	1	•	•	•	1.9[d]	20[d]
23.2.E	MC	B	•	•	•	•	•	•[d]	•[d]
CN54	PBMC	B′/C	•	•	•	•	•	•	nd
92BR025.C1	MC	C	25	•	•	25	•	•	•
CB7	MC	C	•	•	•	•	•	<50	50
92UG001.D8	MC	D	•	•	•	•	•	•	•

[a]VHH neutralisation activity was assessed in a cell-based neutralisation assay using a glioma cell line expressing CD4 and either of CXCR4 or CCR5, and immunostaining of p24 at 48 or 72 hours post-infection, as described in the text. • indicates >50 or >100 μg/ml; nd, not determined.

[b]Viruses are described in section 2.4.

[c]TCLA, T cell line adapted isolate; PBMC, PBMC-propagated primary isolate; MC, molecular clone.

[d]Data from M. Aasa-Chapman, *et al*., in preparation.

Based on the above observation, VHH A12, D7 and C8 were chosen for further characterisation and were tested against an extended virus panel consisting of an additional ten viruses, which were PBMC isolates or chimeric molecular clones of subtype B and C. Included were commonly used subtype B strains such as SF162, BaL and JRFL, PBMC-propagated primary isolates of subtype C, as well as chimeric molecular clones expressing envelopes of subtype B and C. VHH A12, D7 and C8 each neutralised three out of the four subtype B viruses included in this extended panel to at least 90% (Table 4.2.3.3.2). Human mAb b12 as well as sCD4 are known to neutralise all the four subtype B viruses (873, 1022, 1145, 1200, 1201, 1203). VHH A12, D7 and C8 as well as mAb b12 all neutralised one out of the six subtype C viruses included. Recombinant sCD4, on the other hand, could neutralise three out of the six subtype C viruses (Table 4.2.3.3.2).

Table 4.2.3.3.2. VHH, mAb b12 and sCD4 IC_{90} against HIV-1 in NP2 cells[a].

Virus[b]	Type[c]	Subtype	IC_{90} in NP2 cells (μg/ml)				
			A12	D7	C8	b12	sCD4
SF162	PBMC	B	12	6	50	S	S
BaL	PBMC	B	25	25	6	S	S
JRFL	MC	B	•	•	•	S	S
23.8.12	MC	B	<1	<1	<1	S	S
ZA97001	PBMC	C	•	•	•	•	•
97IN003	PBMC	C	•	•	•	•	•
37.4.2	MC	C	•	•	•	•	<50
38.2.2	MC	C	•	•	•	•	•
27b	MC	C	•	•	•	•	<50
27d	MC	C	1.2	1.2	<5	<5	<5

[a]VHH neutralisation activity was assessed in a cell-based neutralisation assay using a glioma cell line expressing CD4 and either of CXCR4 or CCR5, and immunostaining of p24 at 48 or 72 hours post-infection, as described in the text. • indicates >50 μg/ml (for mAb b12 and sCD4) or >100 μg/ml (for VHH); S indicates sensitive to neutralisation according to several reports (873, 1022, 1145, 1200, 1201, 1203) and M. Aasa-Chapman *et al*., in preparation.

[b]Viruses are described in section 2.4.

[c]PBMC, PBMC-propagated primary isolate; MC, molecular clone.

4.2.3.4 Summary of VHH neutralisation of HIV-1 in NP2 cells

The initial neutralisation characterisation, carried out against a panel of nine viruses of subtype A, B, B′/C, C and D of varying sensitivity to neutralisation by sCD4 and the anti-CD4bs mAb b12, revealed VHH A12, D7 and C8 as being more potent than VHH F2 and D12. VHH A12 could neutralise four and VHH D7 and C8 three out of the nine viruses to 90% or more. VHH D12 and F2, on the other hand, were not able to neutralise any of the nine viruses to 90% (Table 4.2.3.3.1). VHH A12, D7 and C8 were therefore tested against an extended panel of ten viruses of subtype B and C. Against this panel, the three VHH showed similar neutralisation profiles, neutralising four out of the ten viruses to 90% or more (Table 4.2.3.3.2).

Overall, VHH A12, D7 and C8 were tested against 19 viruses in this assay format. VHH A12 was able to neutralise 8 of the 19 viruses (42%), whereas D7

and C8 could neutralise 7 out of the 19 (37%). Against this virus panel, the broadly neutralising mAb b12 was of comparable breadth, also neutralising 8 out of the 19 viruses (42%).

In summary, the results observed in the NP2 assay suggested that three of the selected VHH could potently neutralise HIV-1 of subtype B and C. Moreover, the findings suggested that VHH A12, D7 and C8 may show a breadth of neutralisation that is comparable to that of the broadly neutralising anti-CD4bs mAb b12. Therefore, it was decided to subject VHH A12, D7 and C8 to a more comprehensive neutralisation characterisation, to better evaluate the extent of their cross-subtype reactivity.

4.2.4 Comprehensive characterisation of VHH neutralisation of HIV-1 in TZM-bl cells

The results observed in the NP2 cell assay indicated that three of the selected VHH may exhibit relatively broad cross-subtype neutralisation reactivities, possibly comparable to that of the broadly neutralising mAb b12. As no mAbs with broad neutralisation activities have previously, to our knowledge, been isolated from immunised animals, this encouraged us to test the VHH against a larger panel of viruses, preferably against viruses whose neutralisation susceptibilities had already been well-characterised. At this point of time in the study, the use of standard panels of viruses for tier 1, 2 and 3 assessment of neutralising antibody responses elicited by candidate HIV-1 vaccines, had been recommended by Moore and Burton (1215) and Mascola *et al*. (1219), and standard virus panels of envelope pseudotyped viruses for assessment of neutralising antibodies had been generated (1022, 1023, 1219, 1223). The tier 1, 2 and 3 classification is based on the sensitivity of the envelope pseudotyped viruses to neutralisation by sCD4, the broadly neutralising mAbs b12, 2G12, 2F5 and 4E10 as well as a set of human individual and pooled plasma samples (1022, 1023, 1223). Some of these envelope panels had been made available through the NIH AIDS Research and Reference Reagent Program, as well as from the pseudovirus repository of the CAVD Vaccine Immune Monitoring Centre, led by D. Montefiori (Duke University Medical Centre, Durham, USA). To further

evaluate the neutralisation breadth and potencies of the VHH, we decided to include some of these reference virus panels in this study.

The NP2 cell assay, especially the microscopical counting of FFUs, is, however, rather laborious and testing the VHH against large panels of viruses would therefore be very time-consuming. To enable a more high-throughput neutralisation characterisation, a neutralisation assay with a luciferase read-out was used. This neutralisation 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 was developed by the group of G. Shaw (1147-1149) and validated by the group of D. Montefiori (1022, 1214). It measures neutralisation as a reduction of Tat-induced luciferase expression.

VHH neutralisation potencies were assayed against HIV-1 of subtypes A, B, C, D, CRF07_BC and CRF02_AG using either PBMC-propagated primary isolates, TCLA isolates, or recombinant replication-competent chimeric viruses as well as envelope pseudotyped viruses. Included was the panel of 19 viruses against which the VHH had been tested in the NP2 cell assay format, as well as the additional chimeric molecular clones expressing envelopes cloned from plasma of infected individuals or PBMC isolates, which were described in section 4.2.1 of this thesis. Included were also the subtype B, C and CRF07_BC reference panels of envelope pseudotyped viruses and part of the CRF02_AG panel, as well as one tier 1 and an additional tier 2 virus of subtype C. For references and accession numbers, see section 2.4.

Virus was incubated with serial dilutions of VHH for 1 hour and subsequently incubated with TZM-bl cells. At 48 hours post infection, cells were lysed by the addition of a combined cell lysis and luciferase substrate reagent. Luminescence was measured and background luminescence subtracted. The percentage of RLU in test wells, compared to in virus only control wells, was then determined. The lowest VHH concentration required to achieve 50% and 90% reduction of infectivity (IC_{50} and IC_{90}) compared to a virus control was calculated using the XLfit 4 software. To ensure reproducible results, the neutralisation activity of

each VHH was assayed in duplicate and on a minimum of two separate occasions (apart from the HIV-1 subtype CRF07_BC pseudovirus panel, which was tested once). Anti-CD4bs mAb b12 was tested in parallel. Against each virus included in the study, a negative control VHH (VHH #3; at 50 μg/ml) was included in at least two wells, in at least one of the experiments.

The selected VHH were also tested against a pseudotyped virus expressing an irrelevant envelope (rabies virus G protein CVS-11; kindly provided by E. Wright, UCL, London, UK), to exclude non-specific virus inactivation. No dose-dependent, non-specific virus inactivation was observed (data not shown).

4.2.4.1 VHH neutralisation of the autologous virus

The VHH were first tested for ability to neutralise the autologous virus, HIV-1 CN54. VHH A12, D7 and C8 at 50 μg/ml were found to neutralise HIV-1 CN54 to 97, 88 and 87%, respectively (Fig. 4.2.4.1.1). This result represents more potent neutralisation compared to the results obtained in the NP2 cell assay, where VHH A12, D7 and C8 at 200 μg/ml could only neutralise HIV-1 CN54 to 62, 36 and 52%, respectively (Fig. 4.2.3.2.1). It is possible that the NP2 assay is more stringent than the TZM-bl assay. Discrepancies between results obtained in different neutralisation assays may be expected, taken the different target cells, differences in virus inputs and incubation times, as well as the use of the polycation DEAE-dextran to enhance the level of infection in some cell lines, including the TZM-bl cells. Differences between assays have been previously observed, for example in a global collaboration working on the standardisation of HIV-1 neutralisation assays (1220).

VHH IC_{50} and IC_{90} titres against HIV-1 CN54 in TZM-bl cells are shown in Tables 4.2.4.4.1 and 4.2.4.4.2. Against CN54 virus, VHH A12 was found to be slightly more potent than VHH D7 and C8. Anti-CD4bs mAb b12 could not reduce the infection of HIV-1 CN54 (Fig. 4.2.4.1.1).

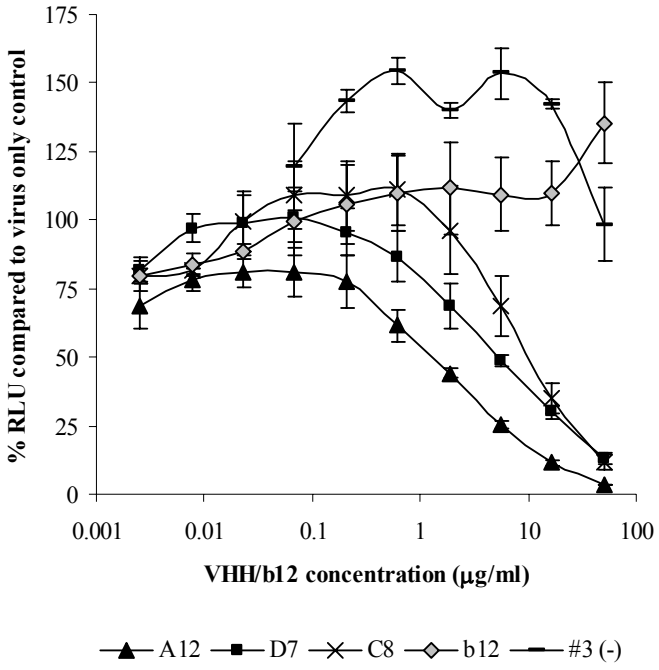


Figure 4.2.4.1.1. VHH and mAb b12 neutralisation of the autologous virus HIV-1 CN54 in TZM-bl cells. PBMC-propagated HIV-1 CN54 was pre-incubated with serial dilutions of VHH A12, D7, C8 and #3 (irrelevant negative control VHH) as well as mAb b12 and subsequently incubated with TZM-bl indicator cells. At 48 hours post infection, cell lysis and luciferase substrate reagent was added to the cells and luminescence detected. The percentage of RLU in test wells compared to in virus only control wells (after subtraction of background luminescence) was determined and plotted against VHH or mAb b12 concentration. Data points represent the mean and error bars the standard error of three independent experiments carried out in duplicate.

4.2.4.2 VHH neutralisation of HIV-1 of subtype B

The VHH and mAb b12 was assayed for ability to neutralise 22 HIV-1 viruses of subtype B. VHH IC_{50} and IC_{90} titres were determined. The results are shown in Fig. 4.2.4.2.1 and summarised in Tables 4.2.4.2.1 and 4.2.4.2.2.

In summary, VHH A12 showed the most potent and broad neutralisation reactivity of the VHH against the subtype B viruses included in this study. It neutralised 13 out of the 22 HIV-1 subtype B viruses, with IC_{50} values in the range of <0.003-27 µg/ml (Table 4.2.4.2.1). It was less potent on IC_{90} level, neutralising 8 out of 22 subtype B viruses with IC_{90} titres in the range of 0.005-27 µg/ml (Table 4.2.4.2.2). VHH D7 and C8 were slightly less reactive against

this panel, neutralising 10 and 9 out of the 22 viruses to 50%, respectively. Anti-CD4bs mAb b12 was found to be more broadly reactive against this panel, neutralising 17 out of the 22 viruses with IC_{50} titres in the range of 0.02-32 μg/ml.

Interestingly, the VHH and mAb b12 showed a different pattern of neutralisation, indicating that they recognise different epitopes. For example, the R5-tropic chimeric molecular clone 4.10.3 was shown to be very sensitive to neutralisation by the VHH, above all to VHH A12, which neutralised 4.10.3 with an IC_{90} of 0.005 μg/ml, whilst at the same time being completely resistant to neutralisation by mAb b12. This differential neutralisation pattern will be further discussed in section 4.3.4. A summary of the proportion of viruses neutralised by the VHH and mAb b12 will be given in section 4.2.4.7.

All of the VHH were able to neutralise the neutralisation sensitive TCLA isolates IIIB and MN, although A12 and D7 was found to be more potent than C8, especially against HIV-1 MN, which A12 neutralised with an IC_{50} of 0.004 μg/ml (Fig. 4.2.4.2.1 and Table 4.2.4.2.1). For HIV-1 IIIB, two different results were obtained for two different stocks of T cell line-propagated virus, with up to a 10-fold difference in observed IC_{90} titre. This difference may be due to the fact that these stocks may originate from stocks propagated in different T cell lines and for a different number of passages.

Against the envelope pseudotyped subtype B reference panel of viruses, mAb b12 was clearly more broadly reactive than the VHH, neutralising eight and six out of the twelve clones at IC_{50} and IC_{90} level, respectively. VHH A12, on the other hand, could neutralise five out of the twelve on IC_{50} level and only one virus on IC_{90} level (Fig. 4.2.4.2.1 and Tables 4.2.4.2.1 and 4.2.4.2.2).

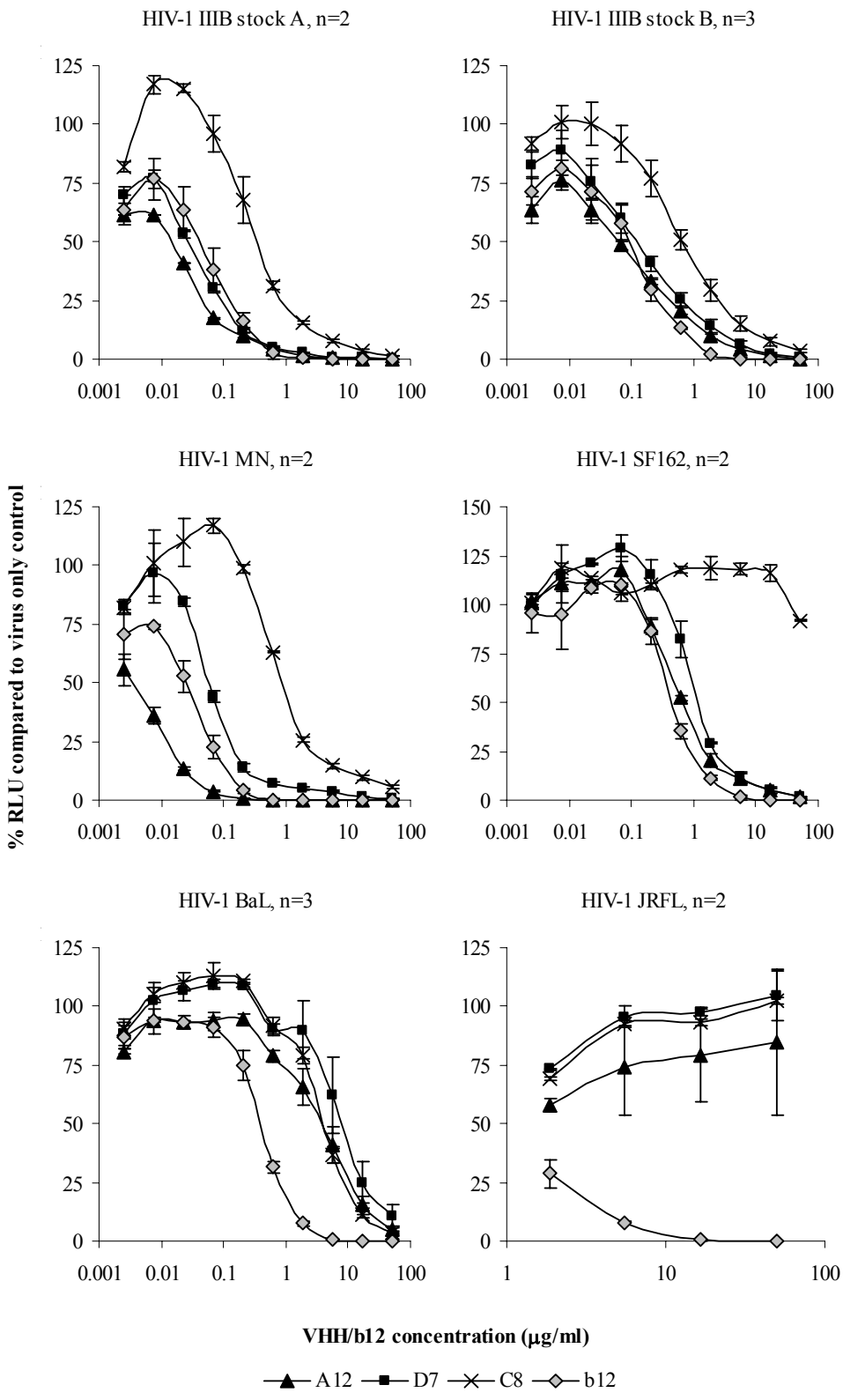


Figure 4.2.4.2.1 (continued on next page). VHH and mAb b12 neutralisation of HIV-1 of subtype B in TZM-bl cells.

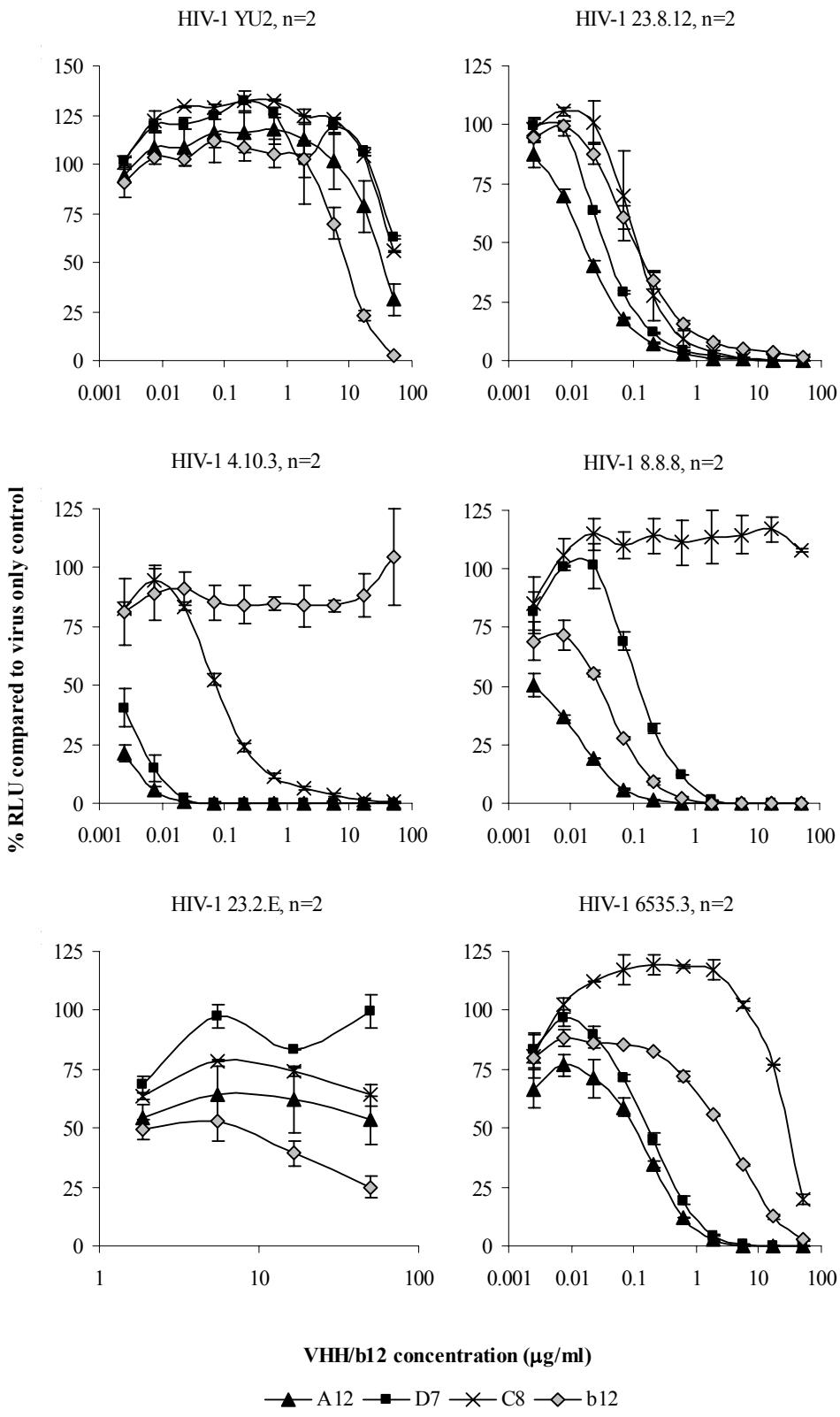


Figure 4.2.4.2.1 (continued from previous page and on next page). VHH and mAb b12 neutralisation of HIV-1 of subtype B in TZM-bl cells.

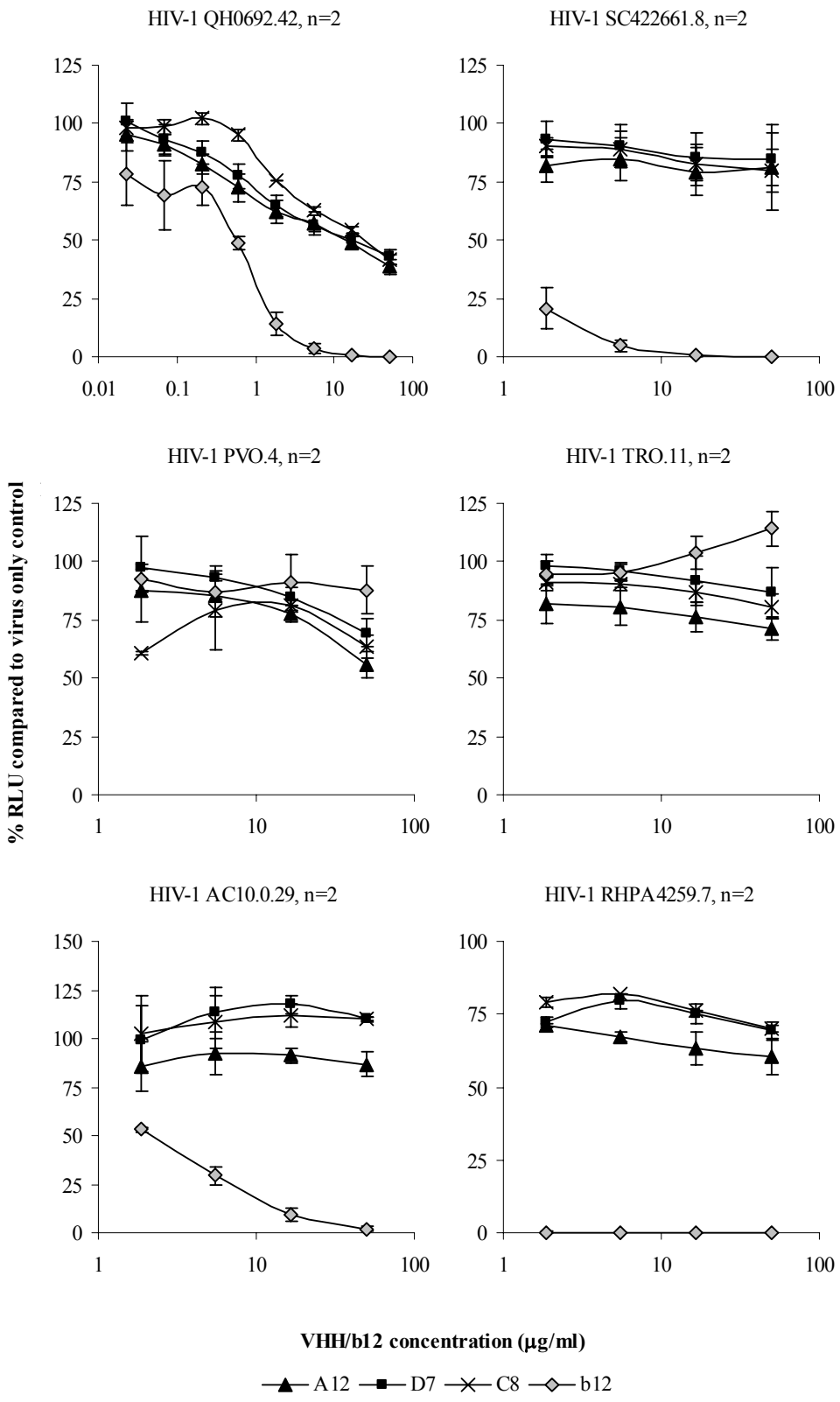


Figure 4.2.4.2.1 (continued from previous page and on next page). VHH and mAb b12 neutralisation of HIV-1 of subtype B in TZM-bl cells.

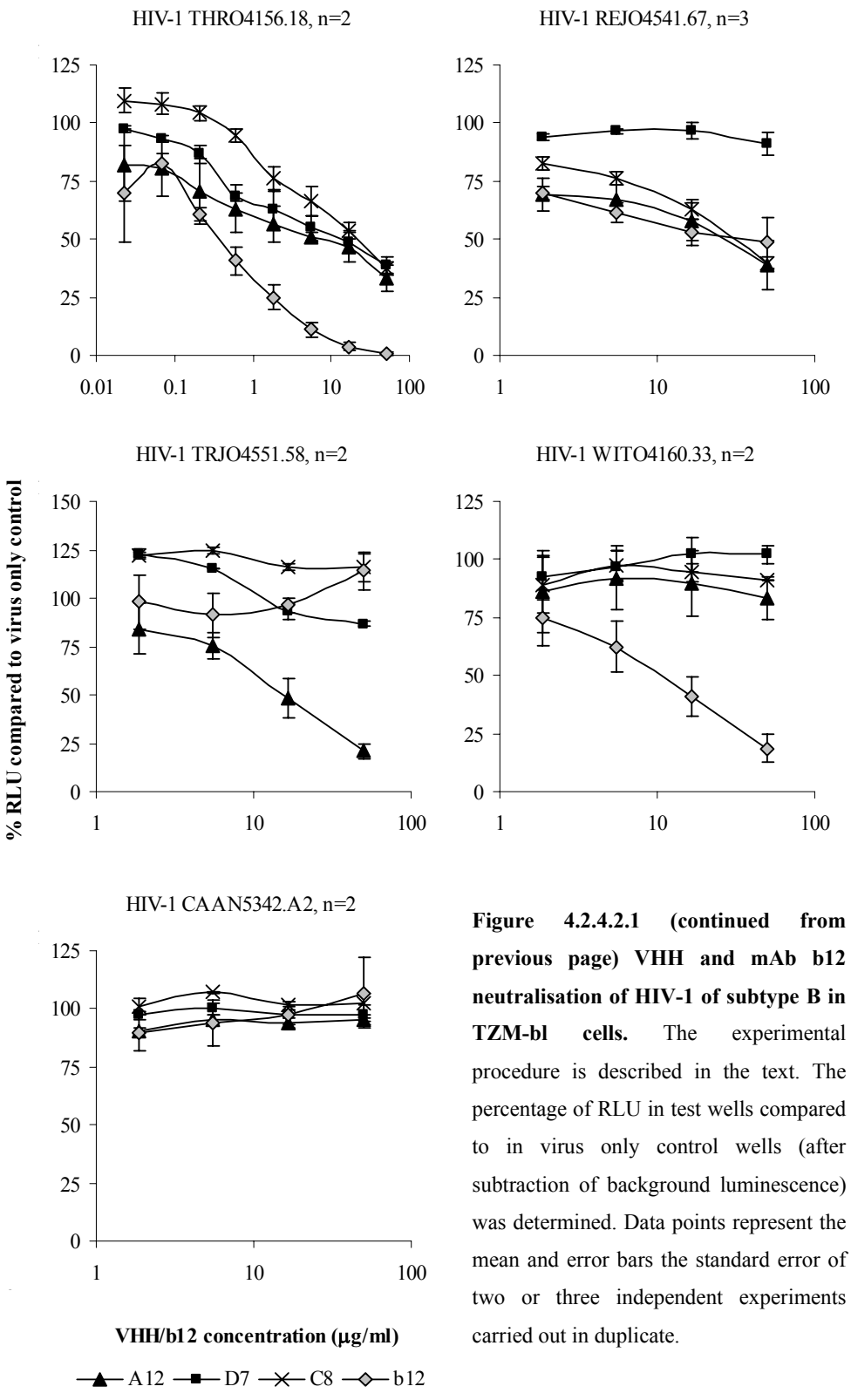


Figure 4.2.4.2.1 (continued from previous page) VHH and mAb b12 neutralisation of HIV-1 of subtype B in TZM-bl cells. The experimental procedure is described in the text. The percentage of RLU in test wells compared to in virus only control wells (after subtraction of background luminescence) was determined. Data points represent the mean and error bars the standard error of two or three independent experiments carried out in duplicate.

Table 4.2.4.2.1. VHH and mAb b12 IC_{50} titres against HIV-1 subtype B[a].

Virus[b]	Type[c]	Subtype	Tier[d]	IC_{50} in TZM-bl cells (μg/ml)			
				A12	D7	C8	b12
IIIB stock A	TCLA	B	1	0.01	0.03	0.3	0.04
IIIB stock B	TCLA	B	1	0.07	0.1	0.8	0.07
MN	TCLA	B	1	0.004	0.06	1	0.02
SF162	PBMC	B	1	1.3	2.3	•	0.4
BaL	PBMC	B	nd	3.6	8.1	3.9	0.4
JRFL	MC	B	nd	•	•	•	<1.9
YU2	MC	B	nd	34	•	•	8.6
23.8.12	MC	B	nd	0.02	0.03	0.1	0.1
4.10.3	MC	B	nd	<0.003	<0.003	0.07	•
8.8.8	MC	B	nd	0.003	0.1	•	0.03
23.2.E	MC	B	nd	•	•	•	7.4
6535.3	PV	B	2	0.1	0.2	28	2.5
QH0692.42	PV	B	2	13	17	21	0.7
SC422661.8	PV	B	2	•	•	•	<1.9
PVO.4	PV	B	2	•	•	•	•
TRO.11	PV	B	2	•	•	•	•
AC10.0.29	PV	B	2	•	•	•	2.2
RHPA4259.7	PV	B	2	•	•	•	<1.9
THRO4156.18	PV	B	2	6.2	7.2	18	0.5
REJO4541.67	PV	B	2	27	•	32	32
TRJO4551.58	PV	B	2	16	•	•	•
WITO4160.33	PV	B	2	•	•	•	11
CAAN5342.A2	PV	B	2	•	•	•	•

[a]VHH and mAb b12 neutralisation activity was assessed in TZM-bl cells, as described in the text, • indicates >50 μg/ml. To aid comprehension, the titres have been colour-coded so that the darker the colour, the more potent the neutralisation. Where b12 has not been titrated below 1.9 μg/ml, the IC_{50} is colour-coded as being 0.1-1 rather than 1-10 μg/ml.

[b]Viruses are described in section 2.4.

[c]TCLA, T cell line adapted isolate; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1, 2 or 3 assessment of neutralising antibodies (1219); nd, not determined.

Table 4.2.4.2.2. VHH and mAb b12 IC_{90} titres against HIV-1 subtype B[a].

Virus[b]	Type[c]	Subtype	Tier[d]	IC_{90} in TZM-bl cells (μg/ml)			
				A12	D7	C8	b12
IIIB stock A	TCLA	B	1	0.2	0.3	2.8	0.3
IIIB stock B	TCLA	B	1	1.7	3.3	11	0.5
MN	TCLA	B	1	0.03	0.4	11	0.1
SF162	PBMC	B	1	7.8	9.3	•	1.2
BaL	PBMC	B	nd	27	•	20	1.4
JRFL	MC	B	nd	•	•	•	5
YU2	MC	B	nd	•	•	•	28
23.8.12	MC	B	nd	0.1	0.3	0.6	1.4
4.10.3	MC	B	nd	0.005	0.01	0.8	•
8.8.8	MC	B	nd	0.05	0.6	•	0.2
23.2.E	MC	B	nd	•	•	•	•
6535.3	PV	B	2	0.7	1	•	21
QH0692.42	PV	B	2	•	•	•	3
SC422661.8	PV	B	2	•	•	•	3.5
PVO.4	PV	B	2	•	•	•	•
TRO.11	PV	B	2	•	•	•	•
AC10.0.29	PV	B	2	•	•	•	16
RHPA4259.7	PV	B	2	•	•	•	<1.9
THRO4156.18	PV	B	2	•	•	•	5
REJO4541.67	PV	B	2	•	•	•	•
TRJO4551.58	PV	B	2	•	•	•	•
WITO4160.33	PV	B	2	•	•	•	•
CAAN5342.A2	PV	B	2	•	•	•	•

[a]VHH and mAb b12 neutralisation activity was assessed in TZM-bl cells, as described in the text, • indicates >50 μg/ml. To aid comprehension, the titres have been colour-coded so that the darker the colour, the more potent the neutralisation. Where b12 has not been titrated below 1.9 μg/ml, the IC_{90} is colour-coded as being 0.1-1 rather than 1-10 μg/ml.

[b]Viruses are described in section 2.4.

[c]TCLA, T cell line adapted isolate; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1, 2 or 3 assessment of neutralising antibodies (1219); nd, not determined.

4.2.4.3 VHH neutralisation of HIV-1 of subtype C

The VHH and mAb b12 were then tested against 27 HIV-1 of subtype C. Again, IC_{50} and IC_{90} titres were determined. Results are shown in Fig. 4.2.4.3.1 and in Tables 4.2.4.3.1 and 4.2.4.3.2.

In summary, A12 showed the most potent neutralising activity of the VHH, being able to neutralise 13 out of the 27 viruses with IC_{50} values in the range of <0.003-38 μg/ml, again being less potent at the IC_{90} level, neutralising 6 out of the 27 viruses with IC_{90} titres of 0.04-50 μg/ml (Fig. 4.2.4.3.1 and Tables 4.2.4.3.1 and 4.2.4.3.2). VHH C8 was as broadly reactive as VHH A12 against these subtype C viruses, also neutralising 13 out of the 27 viruses to 50%, but generally with higher IC_{50} titres. Interestingly, VHH C8 neutralised a different pattern of viruses compared to VHH A12, indicating that they recognise different epitopes. VHH D7 was less reactive against these subtype C viruses, neutralising 9 out of the 27 viruses with IC_{50} titres in the range of 0.004-34 μg/ml.

Anti-CD4bs mAb b12 could neutralise 15 out of the 27 viruses, again a partially different set of viruses compared to the VHH. For example, VHH A12 showed strong neutralisation of the R5-tropic chimeric virus C261, which expresses an envelope cloned from plasma of an infected individual, with an IC_{50} of <0.003 μg/ml. In contrast, b12 was not able to reduce infection of this virus. The opposite was observed for several viruses such as, for example, the tier 1-classified pseudovirus 93MW965.26, which was neutralised by mAb b12 but not by VHH A12. Moreover, mAb b12 was more reactive than the VHH against the subtype C tier 2-classified envelope pseudotyped viruses included in the study, neutralising 6 out of the 13 viruses on IC_{90} level, compared to 0 out of 13 for VHH A12. Interestingly, viruses 27b and 27d, showed different sensitivities to neutralisation, despite their envelopes being cloned from a single plasma sample from the same individual, something which has been observed previously (652).

The observed IC_{90} titres were typically around 10-fold higher than the corresponding IC_{50} titres, an observation that has been made previously (1017). On a few occasions, however, this was not the case. Instead, the neutralisation

curve was found to plateau on a level of >10% of residual infection. This was the case for, for instance, mAb b12 against 93MW965.26 and VHH A12 against ZM109F.PB4 and 96ZM651.02 (Fig. 4.2.4.3.1). The IC_{50} titre of mAb b12 against 93MW965.26 was as low as 0.02 μg/ml. Still, 90% reduction of infection was not observed at the highest concentration tested (50 μg/ml). Instead, the residual infection (as measured by percentage RLU compared to virus only control) levelled out at approximately 20% (Fig. 4.2.4.3.1). This observation was reproducible in three independent experiments. A similar observation was made for VHH A12 against ZM109F.PB4 and 96ZM651.02, where 90% neutralisation could not be observed, despite IC_{50} titres at 0.8 and 0.1 μg/ml, respectively (Fig. 4.2.4.3.1 and Table 4.2.4.3.1). The reason for this observation is not known. When using PBMC isolates, neutralisation that levels out at levels below 90% have previously been observed and is thought to be due to the quasispecies nature of PBMC isolates, which may contain populations of virus which are more resistant to neutralisation (1224, 1225). This could, however, not be the explanation for the plateaus observed here, as they were seen using cloned envelope pseudotyped viruses.

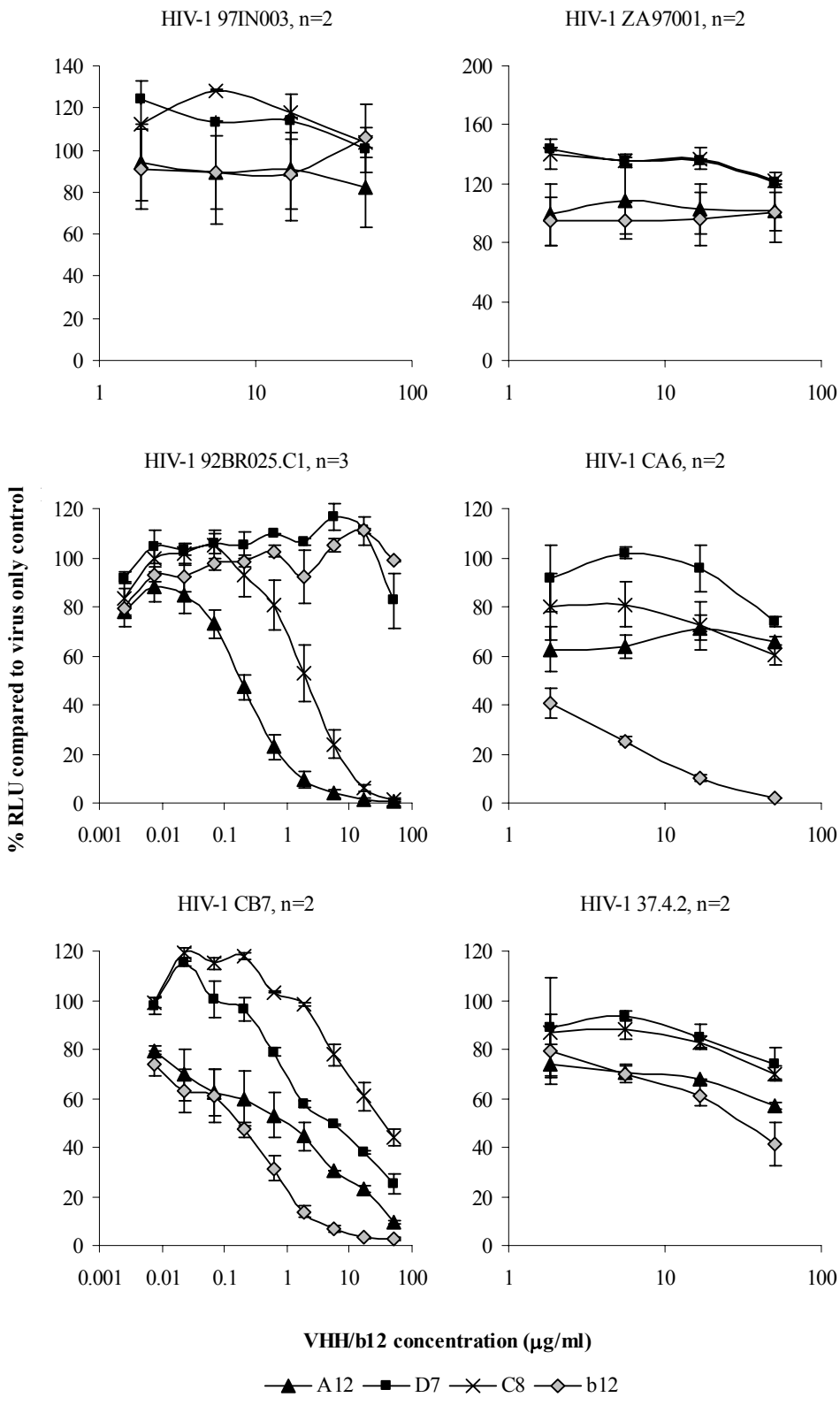


Figure 4.2.4.3.1 (continued on next page). VHH and mAb b12 neutralisation of HIV-1 of subtype C in TZM-bl cells.

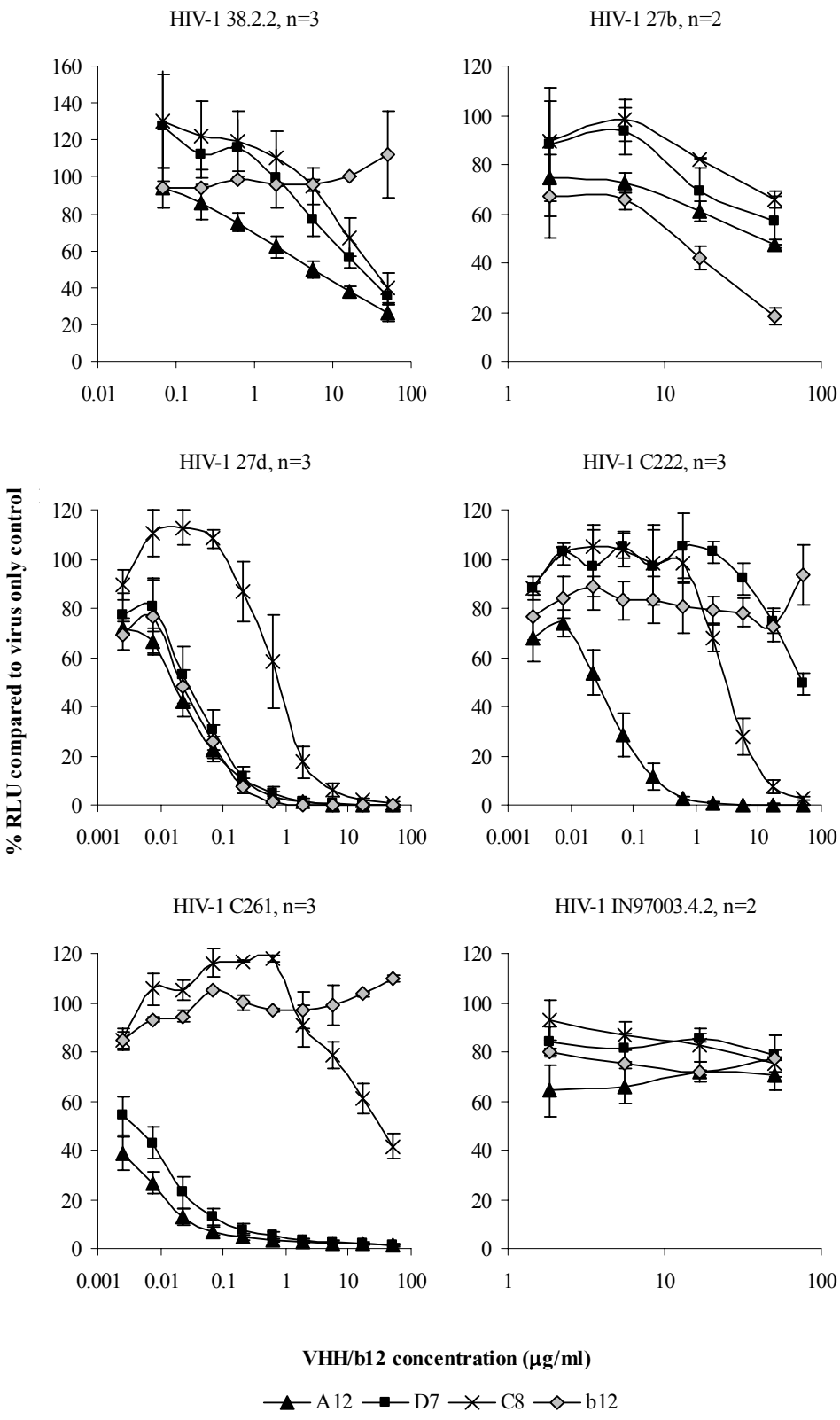


Figure 4.2.4.3.1 (continued from previous page and on next page). VHH and mAb b12 neutralisation of HIV-1 of subtype C in TZM-bl cells.

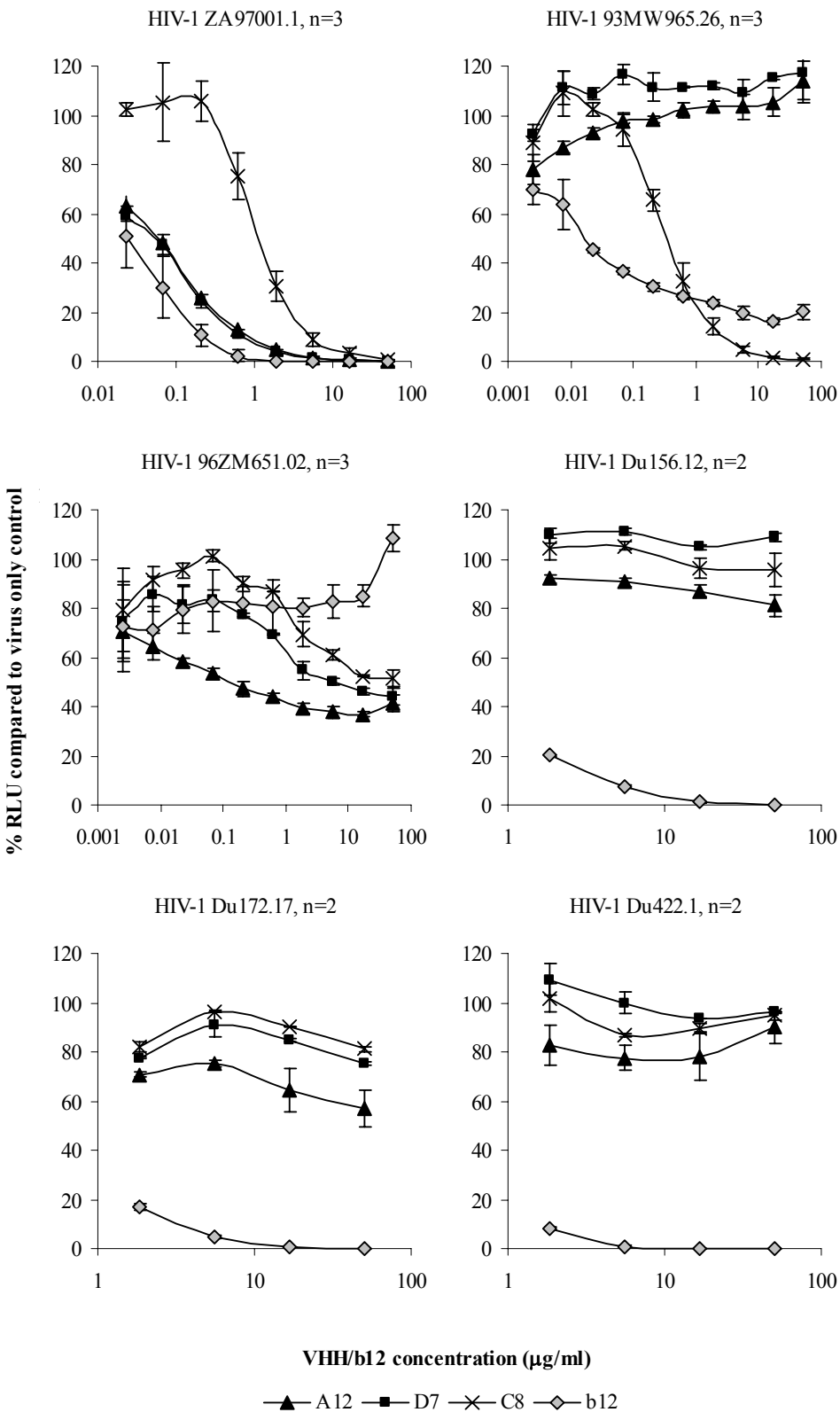


Figure 4.2.4.3.1 (continued from previous page and on next page). VHH and mAb b12 neutralisation of HIV-1 of subtype C in TZM-bl cells.

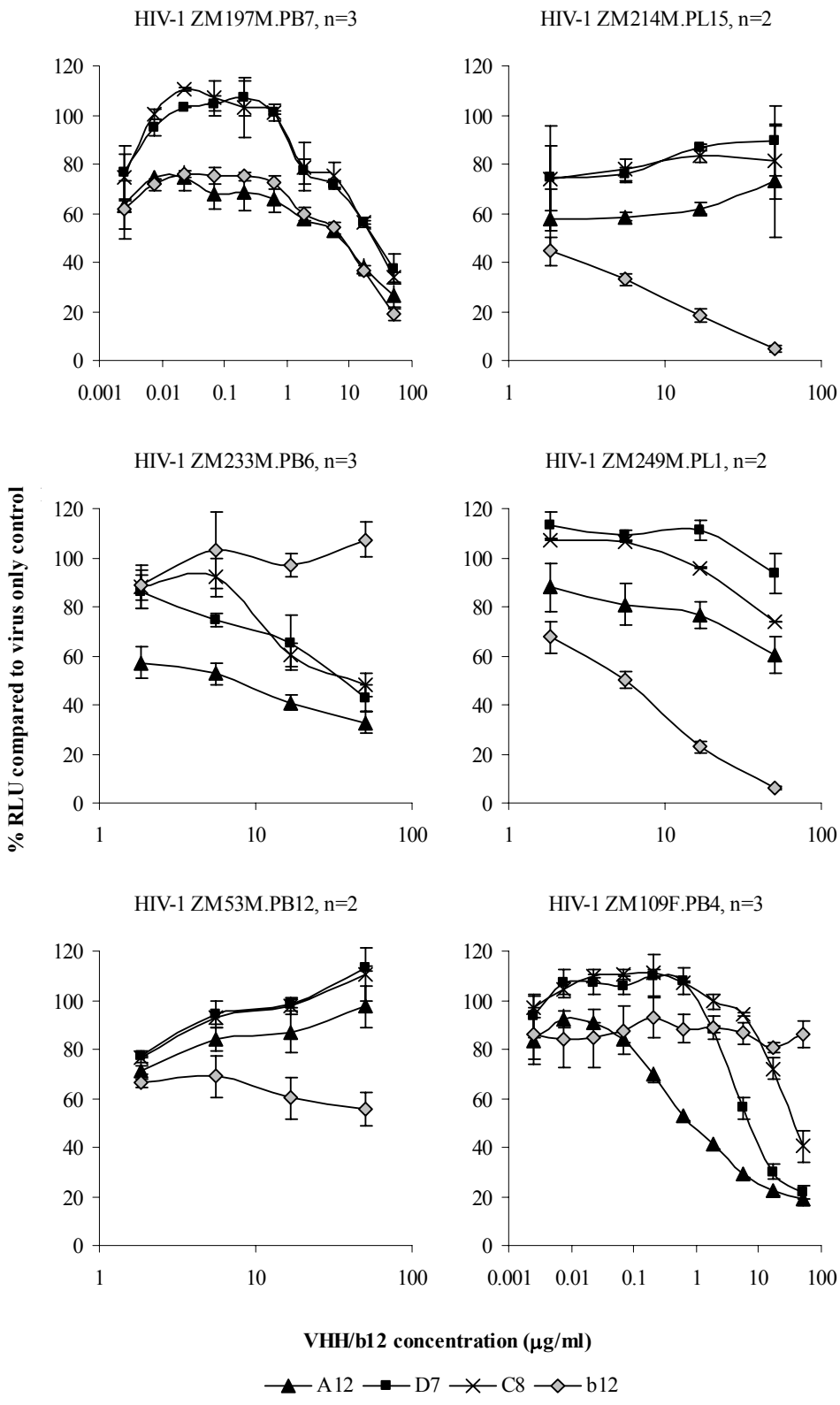


Figure 4.2.4.3.1 (continued from previous page and on next page). VHH and mAb b12 neutralisation of HIV-1 of subtype C in TZM-bl cells.

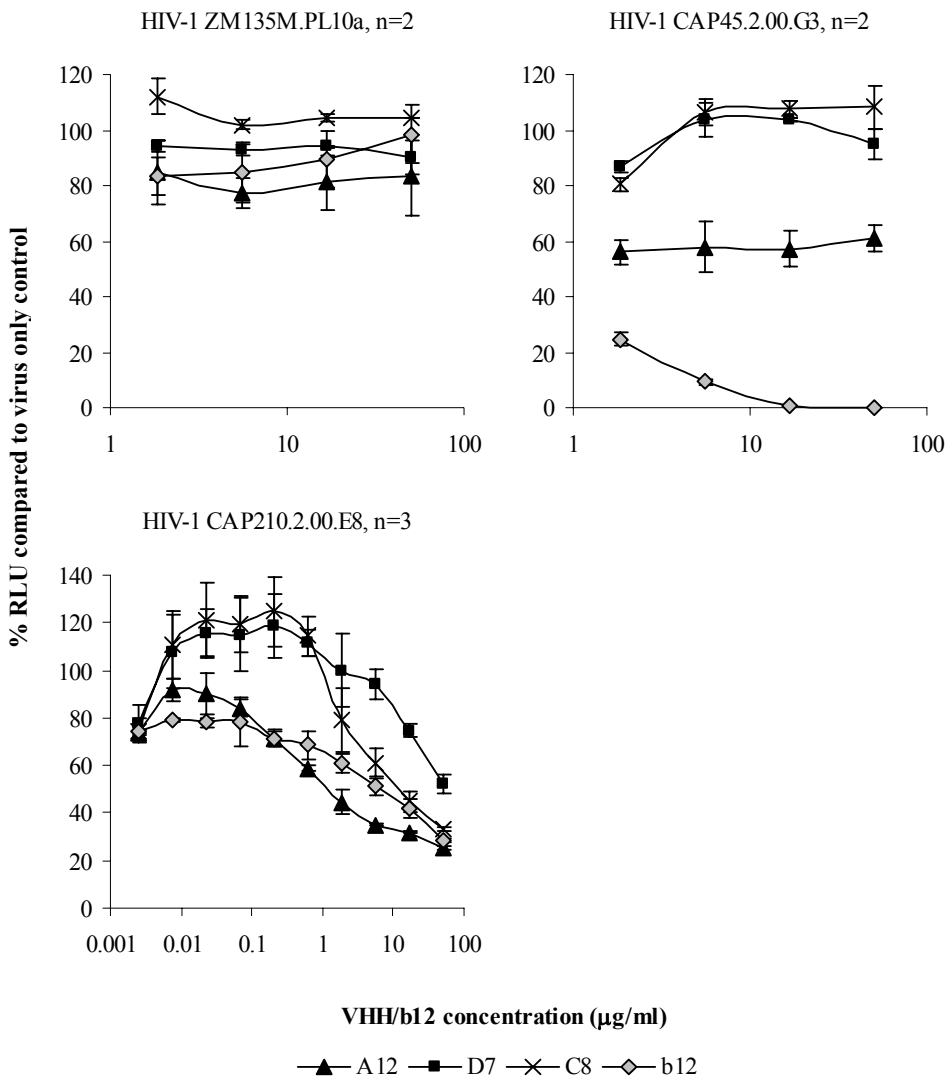


Figure 4.2.4.3.1 (continued from previous page). VHH and mAb b12 neutralisation of HIV-1 of subtype C in TZM-bl cells. Virus was incubated with serial dilutions of VHH A12, D7 and C8 and mAb b12 and subsequently incubated with TZM-bl cells. After 48 hours, cell lysis and luciferase substrate reagent was added to the cells and luminescence detected. The percentage of RLU in test wells compared to in virus only control wells (after subtraction of background luminescence) was determined. Data points represent the mean and error bars the standard error of two or three independent experiments carried out in duplicate. For each virus, a negative control VHH at 50 µg/ml was included in at least one of the runs in at least two wells.

Table 4.2.4.3.1. VHH and mAb b12 IC_{50} titres against HIV-1 subtype C[a].

Virus[b]	Type[c]	Subtype	Tier[d]	IC_{50} in TZM-bl cells (μg/ml)			
				A12	D7	C8	b12
97IN003	PBMC	C	nd	•	•	•	•
ZA97001	PBMC	C	nd	•	•	•	•
92BR025.C1	MC	C	nd	0.2	•	2	•
CA6	MC	C	nd	•	•	•	<1.9
CB7	MC	C	nd	0.7	4	36	0.17
37.4.2	MC	C	nd	•	•	•	36
38.2.2	MC	C	nd	5.4	22	33	•
27b	MC	C	nd	38	•	•	13
27d	MC	C	nd	0.02	0.03	0.7	0.02
C222	MC	C	nd	0.03	49	3	•
C261	MC	C	nd	<0.003	0.004	32	•
97IN003.4.2	MC	C	nd	•	•	•	•
ZA97001.1	MC	C	nd	0.05	0.05	1.1	0.02
93MW965.26	PV	C	1	•	•	0.3	0.02
96ZM651.02	PV	C	2	0.1	•	4.3	•
Du156.12	PV	C	2	•	•	•	<1.9
Du172.17	PV	C	2	•	•	•	<1.9
Du422.1	PV	C	2	•	•	•	<1.9
ZM197M.PB7	PV	C	2	6	22	24	7.4
ZM214M.PL15	PV	C	2	•	•	•	<1.9
ZM233M.PB6	PV	C	2	7	34	38	•
ZM249M.PL1	PV	C	2	•	•	•	5.6
ZM53M.PB12	PV	C	2	•	•	•	•
ZM109F.PB4	PV	C	2	0.8	6.6	38	•
ZM135M.PL10a	PV	C	2	•	•	•	•
CAP45.2.00.G3	PV	C	2	•	•	•	<1.9
CAP210.2.00.E8	PV	C	2	1.2	•	11	6.7

[a]VHH and mAb b12 neutralisation activity was assessed in TZM-bl cells, as described in the text, • indicates >50 μg/ml. To aid comprehension, the titres have been colour-coded so that the darker the colour, the more potent the neutralisation.

[b]Viruses are described in section 2.4.

[c]TCLA, T cell line adapted virus; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1, 2 or 3 assessment of neutralising antibodies (1219); nd, not determined.

Table 4.2.4.3.2. VHH and mAb b12 IC_{90} titres against HIV-1 subtype C[a].

Virus[b]	Type[c]	Subtype	Tier[d]	IC_{90} in TZM-bl cells (μg/ml)			
				A12	D7	C8	b12
97IN003	PBMC	C	nd	•	•	•	•
ZA97001	PBMC	C	nd	•	•	•	•
92BR025.C1	MC	C	nd	2.3	•	13	•
CA6	MC	C	nd	•	•	•	17
CB7	MC	C	nd	50	•	•	3.4
37.4.2	MC	C	nd	•	•	•	•
38.2.2	MC	C	nd	•	•	•	•
27b	MC	C	nd	•	•	•	•
27d	MC	C	nd	0.2	0.2	3.4	0.2
C222	MC	C	nd	0.2	•	13	•
C261	MC	C	nd	0.04	0.1	•	•
ZA97001.1	MC	C	nd	0.6	1	5.5	0.2
97IN003.4.2	MC	C	nd	•	•	•	•
93MW965.26	PV	C	1	•	•	2.4	•
96ZM651.02	PV	C	2	•	•	•	•
Du156.12	PV	C	2	•	•	•	4.5
Du172.17	PV	C	2	•	•	•	3.2
Du422.1	PV	C	2	•	•	•	<1.9
ZM197M.PB7	PV	C	2	•	•	•	•
ZM214M.PL15	PV	C	2	•	•	•	31
ZM233M.PB6	PV	C	2	•	•	•	•
ZM249M.PL1	PV	C	2	•	•	•	35
ZM53M.PB12	PV	C	2	•	•	•	•
ZM109F.PB4	PV	C	2	•	•	•	•
ZM135M.PL10a	PV	C	2	•	•	•	•
CAP45.2.00.G3	PV	C	2	•	•	•	5.3
CAP210.2.00.E8	PV	C	2	•	•	•	•

[a]VHH and mAb b12 neutralisation activity was assessed in TZM-bl cells, as described in the text, • indicates >50 μg/ml. To aid comprehension, the titres have been colour-coded so that the darker the colour, the more potent the neutralisation.

[b]Viruses are described in section 2.4.

[c]TCLA, T cell line adapted virus; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1, 2 or 3 assessment of neutralising antibodies (1219); nd, not determined.

4.2.4.4 VHH neutralisation of HIV-1 of subtype B′/C

The selected VHH were derived from llamas immunised with gp120 from HIV-1 CN54. Furthermore, they had been shown to neutralise the autologous CN54 isolate (Fig. 4.2.4.1.1). HIV-1 CN54 is a CRF07_BC recombinant which is mainly subtype C in envelope, apart from the very N-terminus of gp120. Because of this notion, it would be interesting to test whether the VHH would be reactive against other CRF07_BC viruses. A panel of CRF07_BC tier 2-classified envelopes to be used for pseudovirus production was therefore obtained from the pseudovirus repository of the CAVD Vaccine Immune Monitoring Centre and pseudoviruses were produced. The VHH were tested for ability to neutralise these viruses in TZM-bl cells. VHH and mAb b12 IC_{50} and IC_{90} titres were determined. Results are shown in Fig. 4.2.4.4.1 and Tables 4.2.4.4.1 and 4.2.4.4.2.

Apart from the autologous virus, the VHH were not able to neutralise any of the twelve CRF07_BC viruses tested (IC_{50} level). In contrast, mAb b12 was able to neutralise three out of the twelve viruses on an IC_{50} level, and two viruses on IC_{90} level, but did not reduce infection of HIV-1 CN54.

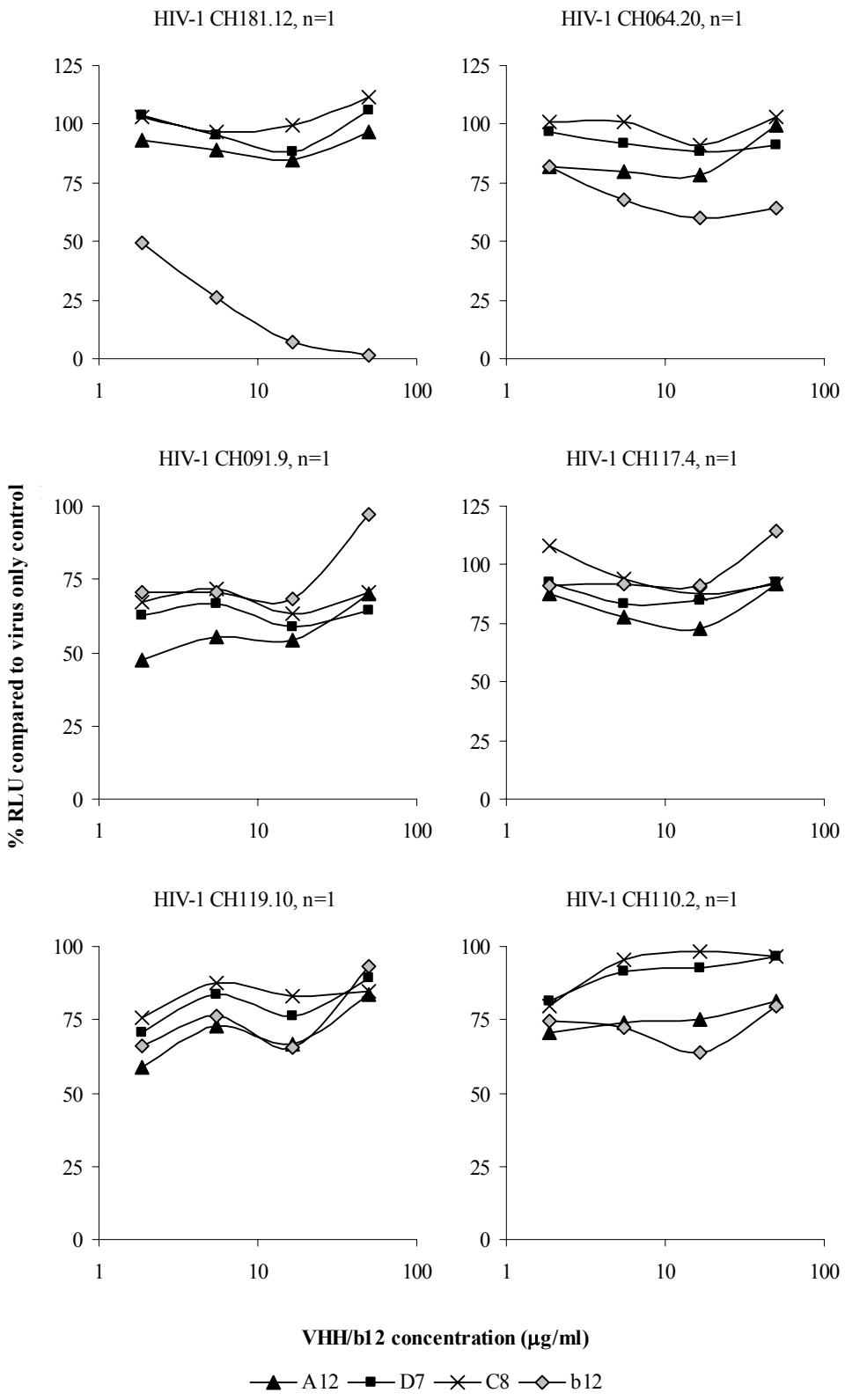


Figure 4.2.4.4.1 (continued on next page). VHH and mAb b12 neutralisation of HIV-1 of HIV-1 of subtype B′/C (CRF07_BC) in TZM-bl cells.

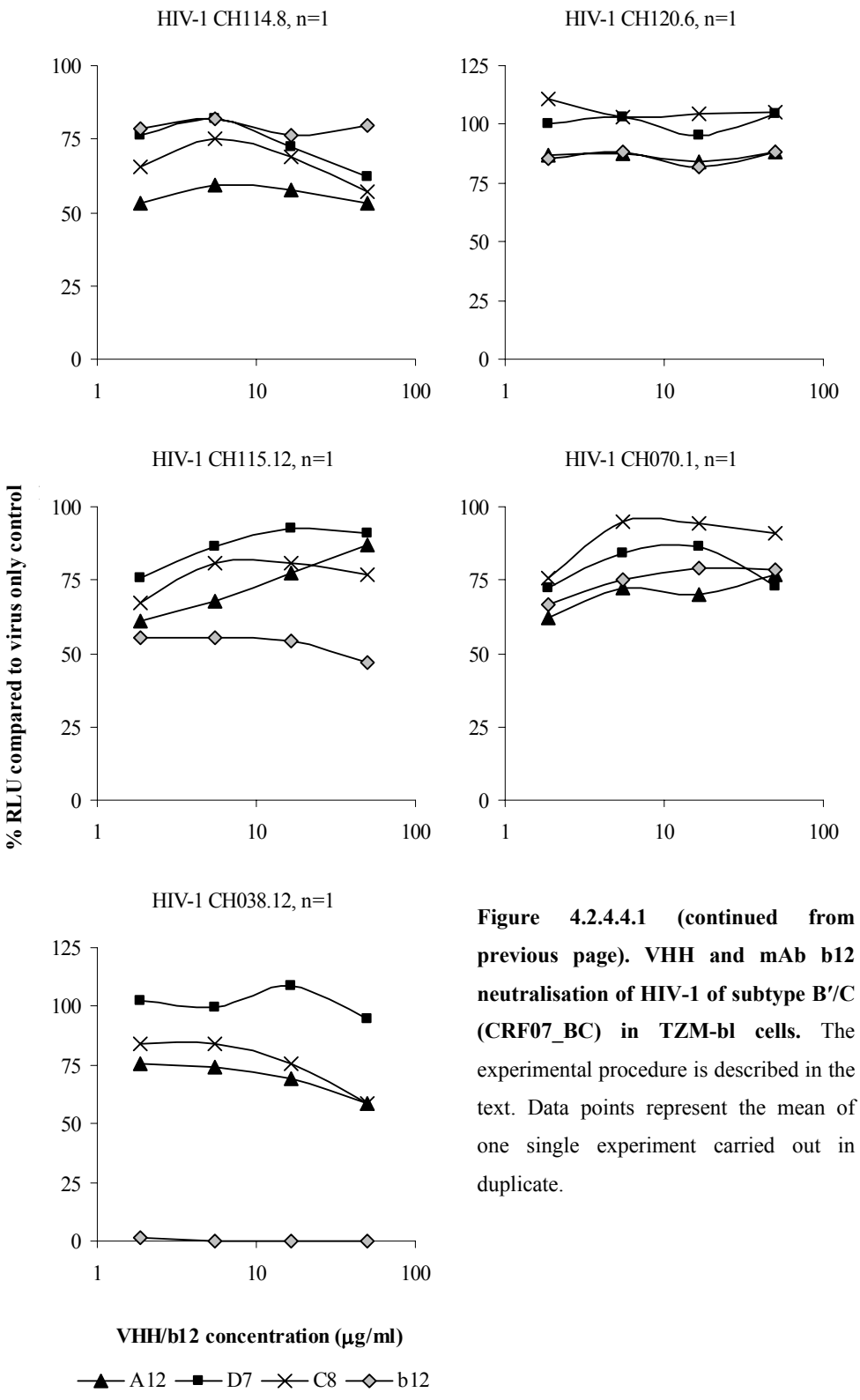


Figure 4.2.4.4.1 (continued from previous page). VHH and mAb b12 neutralisation of HIV-1 of subtype B′/C (CRF07_BC) in TZM-bl cells. The experimental procedure is described in the text. Data points represent the mean of one single experiment carried out in duplicate.

Table 4.2.4.4.1. VHH and mAb b12 IC_{50} titres against HIV-1 subtype B´/C[a].

Virus[b]	Type[c]	Subtype	Tier[d]	IC_{50} in TZM-bl cells (μg/ml)			
				A12	D7	C8	b12
CN54	PBMC	B'/C	nd	1.4	5.1	9.9	•
CH181.12	PV	B'/C	2	•	•	•	<1.9
CH064.20	PV	B'/C	2	•	•	•	•
CH091.9	PV	B'/C	2	•	•	•	•
CH117.4	PV	B'/C	2	•	•	•	•
CH119.10	PV	B'/C	2	•	•	•	•
CH110.2	PV	B'/C	2	•	•	•	•
CH114.8	PV	B'/C	2	•	•	•	•
CH120.6	PV	B'/C	2	•	•	•	•
CH115.12	PV	B'/C	2	•	•	•	36
CH070.1	PV	B'/C	2	•	•	•	•
CH038.12	PV	B'/C	2	•	•	•	<1.9

[a]VHH and mAb b12 neutralisation activity was assessed in TZM-bl cells, as described in the text, • indicates >50 μg/ml. To aid comprehension, the titres have been colour-coded so that the darker the colour, the more potent the neutralisation. Where b12 has not been titrated below 1.9 μg/ml, the IC_{90} is colour-coded as being 0.1-1 rather than 1-10 μg/ml.

[b]Viruses are described in section 2.4.

[c]TCLA, T cell line adapted isolate; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1, 2 or 3 assessment of neutralising antibodies (1219); nd, not determined.

Table 4.2.4.4.2. VHH and mAb b12 IC_{90} titres against HIV-1 subtype B´/C[a].

Virus[b]	Type[c]	Subtype	Tier[d]	IC_{90} in TZM-bl cells (μg/ml)			
				A12	D7	C8	b12
CN54	PBMC	B'/C	nd	18	•	•	•
CH181.12	PV	B'/C	2	•	•	•	13
CH064.20	PV	B'/C	2	•	•	•	•
CH091.9	PV	B'/C	2	•	•	•	•
CH117.4	PV	B'/C	2	•	•	•	•
CH119.10	PV	B'/C	2	•	•	•	•
CH110.2	PV	B'/C	2	•	•	•	•
CH114.8	PV	B'/C	2	•	•	•	•
CH120.6	PV	B'/C	2	•	•	•	•
CH115.12	PV	B'/C	2	•	•	•	•
CH070.1	PV	B'/C	2	•	•	•	•
CH038.12	PV	B'/C	2	•	•	•	<1.9

[a]VHH and mAb b12 neutralisation activity was assessed in TZM-bl cells, as described in the text, • indicates >50 μg/ml. To aid comprehension, the titres have been colour-coded so that the darker the colour, the more potent the neutralisation. Where b12 has not been titrated below 1.9 μg/ml, the IC_{90} is colour-coded as being 0.1-1 rather than 1-10 μg/ml.

[b]Viruses are described in section 2.4.

[c]TCLA, T cell line adapted isolate; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1, 2 or 3 assessment of neutralising antibodies (1219); nd, not determined.

4.2.4.5 VHH neutralisation of HIV-1 of subtype A, A/G and D

In the above sections, the selected VHH had been shown to potently neutralise a number of HIV-1 isolates of subtypes B and C. To elucidate whether the VHH would have any effect on infection of TZM-bl cells with HIV-1 from isolates outside subtype B or C, they were tested against a total of four HIV-1 isolates of subtype A and D, as well as CRF02_AG. The results are summarised in Fig. 4.2.4.5.1 and Table 4.2.4.5.1.

In summary, none of the VHH, nor mAb b12, were able to reduce infection of the subtype A, D or CRF02_AG viruses. Taken the small size of this panel, it is still possible the VHH would exhibit some subtype A, D, or CRF02_AG neutralisation reactivity. At least, this is true for mAb b12. In one study, mAb b12 was shown to neutralise eight out of eleven subtype D viruses to IC_{50} level (1017). It does, however, seem like mAb b12 has limited reactivity against subtype A viruses. In the same study, mAb b12 neutralised four out of twelve subtype A viruses to IC_{50} level (1017). In another study, mAb b12 was only able to neutralise one out of twelve subtype A pseudoviruses to IC_{50} level (1223). In the latter report, the viruses were also relatively resistant to neutralisation by sCD4 and the authors speculate whether this may be related to the observed relatively high affinity of subtype A envelopes for CD4 (1223).

Worth noting is that the subtype A virus included in this study, 92UG037.A9, is a chimeric virus expressing the same envelope that was used in the second set of phage display panning, leading to the selection of VHH A12 as described in section 3.2.6. It is interesting that even though VHH A12 was selected upon panning on recombinant 92UG037.A9 gp120, A12 was not able to neutralise the corresponding virus, expressing the exact same gp120. In addition, 92UG037.A9 was slightly enhanced, not neutralised, by sCD4, despite good binding of sCD4 to the recombinant 92UG037.A9 gp120, as shown and discussed in chapter 3. In chapter 5, it will be evaluated whether A12 actually binds to 92UG037.A9 gp120. The lack of correlation between binding and neutralisation will be further discussed in the final discussion of this thesis.

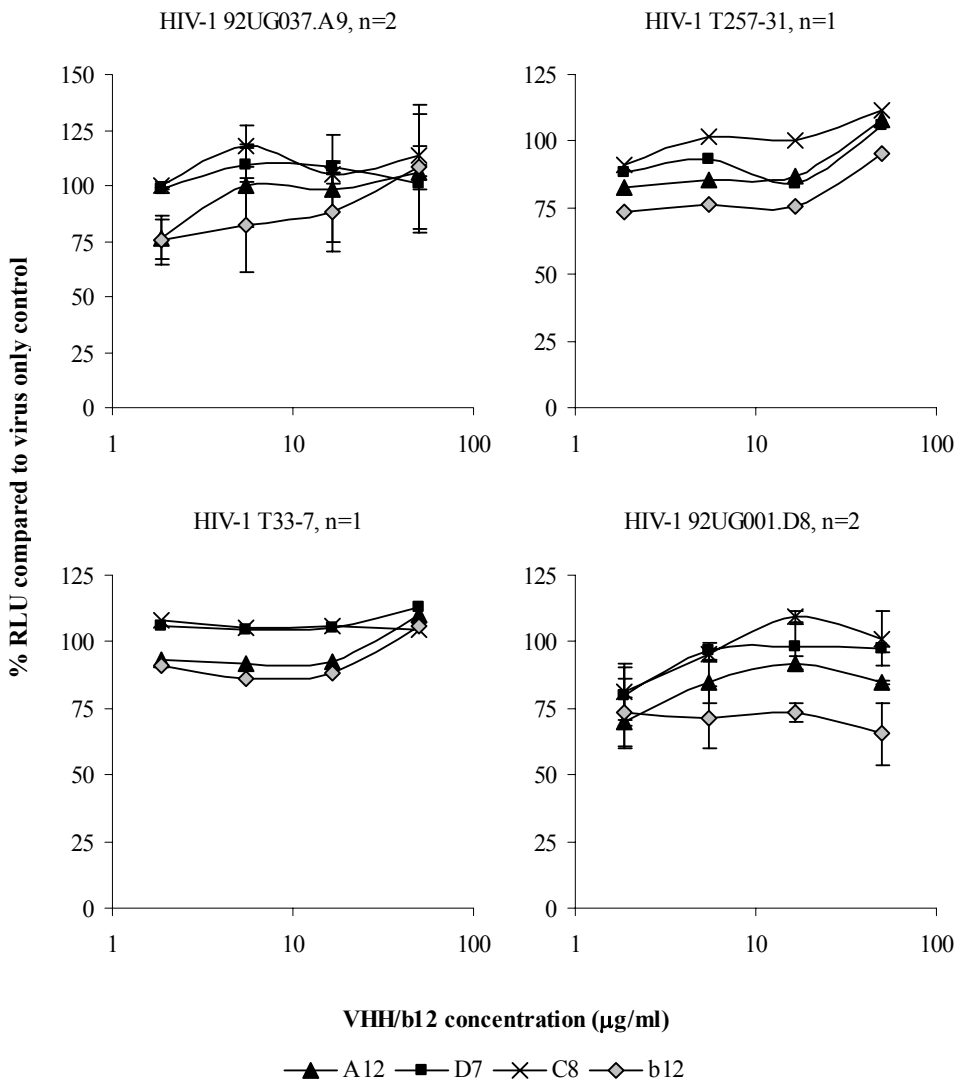


Figure 4.2.4.5.1. VHH and mAb b12 neutralisation of HIV-1 of subtype A, A/G (CRF02_AG) and D in TZM-bl cells. The experimental procedure is described in the text. For viruses T257-2 and T33-7 (subtype A/G), data points represent the mean of duplicate reactions in one single experiment. For viruses 92UG037.A9 (subtype A) and 92UG001.D8 (subtype D) data points represent the mean and bars the standard error of two independent experiments carried out in duplicate. For each virus, a negative control VHH at 50 μg/ml was included in at least one of the runs in at least two wells.

Table 4.2.4.5.1. VHH and b12 IC_{50} against HIV-1 subtype A, A/G and D[a].

Virus[b]	Type[c]	Subtype	Tier[d]	IC_{50} in TZM-bl cells (μg/ml)			
				A12	D7	C8	b12
92UG037.A9	MC	A	nd	•	•	•	•
T257-31	PV	A/G	2	•	•	•	•
T33-7	PV	A/G	2	•	•	•	•
92UG001.D8	MC	D	nd	•	•	•	•

[a]VHH and mAb b12 neutralisation activity was assessed in TZM-bl cells, as described in the text, • indicates >50 μg/ml.

[b]Viruses are described in section 2.4.

[c]TCLA, T cell line adapted isolate; PBMC, PBMC-propagated primary isolate; MC, molecular clone; PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1, 2 or 3 assessment of neutralising antibodies (1219); nd, not determined.

4.2.4.6 Test of VHH neutralisation of HIV-1 in an independent lab against a standard panel of viruses

As part of the Collaboration for AIDS Vaccine Discovery (CAVD), the VHH were sent to Michael Seaman, Harvard Medical School, Boston, USA, to be tested against some of the CAVD Vaccine Immune Monitoring Centre tier 1 and tier 2 pseudoviruses. The VHH were tested against one tier 1 subtype A envelope pseudotyped virus (DJ263), two tier 1 subtype B viruses (SF162.LS and BaL.26), one tier 1 subtype C virus (93MW965.26), as well as one tier 2 subtype B virus (SC422661.8). The results obtained by the CAVD Vaccine Immune Monitoring Centre are shown in Table 4.2.4.6.1.

According to the results obtained at the CAVD Vaccine Immune Monitoring Centre, VHH A12 and D7 neutralised SF162.LS and BaL.26 with IC_{50} titres of <0.02-0.21 μg/ml, whereas VHH C8 could neutralise BaL.26 but not SF162.LS. These results are in concordance with results obtained at UCL, where VHH A12 and D7 were able to neutralise uncloned PBMC-propagated SF162 and BaL, while VHH C8 was found to neutralise BaL only (Table 4.2.4.2.1).

Table 4.2.4.6.1. VHH IC_{50} titres in TZM-bl cells in an independent lab[a].

Virus[b]	Type[c]	Subtype	Tier[d]	IC_{50} in TZM-bl cells (μg/ml)			
				A12	D7	C8	#3 (-)
SF162.LS	PV	B	1	<0.02	0.03	•	•
BaL.26	PV	B	1	0.08	0.11	0.12	•
DJ263	PV	A	1	<0.02	0.09	0.32	•
93MW965.26	PV	C	1	•	•	0.2	•
SC422661.8	PV	B	2	•	•	25.1	•

[a]VHH neutralisation activity was assessed in TZM-bl cells in the lab of Michael Seaman (Harvard Medical School, Boston, USA) at the CAVD Vaccine Immune Monitoring Centre. VHH were tested in duplicate in one experiment; • indicates >50 μg/ml. VHH #3 is an irrelevant VHH. The titres are colour-coded to aid comprehensibility: the darker the colour, the more potent the neutralisation.

[b]Viruses are part of the CAVD Vaccine Immune Monitoring Centre standard panel of pseudoviruses.

[c]PV, envelope pseudotyped virus.

[d]Classified as suitable for tier 1 or 2 assessment of neutralising antibodies (1219).

VHH A12 and D7 neutralised the SF162 and BaL viruses more potently at the CAVD Vaccine Immune Monitoring Centre, compared to when tested against SF162 and BaL at UCL. The IC_{50} titres observed at UCL were 30 to 120-fold higher than the IC_{50} titres obtained by the CAVD Vaccine Immune Monitoring Centre (Tables 4.2.4.2.1 and 4.2.4.6.1). Such differences in IC_{50} titres may be due to the different sources of virus. PBMC isolates have previously been observed to be more neutralisation resistant compared to envelope pseudotyped viruses (1022). In contrast to a molecularly cloned envelope pseudotyped virus, an uncloned PBMC isolate represents a quasispecies which may contain viruses with different neutralisation properties. Several virus clones isolated from one patient sample have previously been shown to display a wide range of neutralisation sensitivities (652, 1222). In addition, the envelope content in viruses produced in PBMC compared to in, for example, 293T cells can differ, which can have an effect on neutralisation sensitivity (1226). Passage for 4-6 days only of a molecularly cloned virus in PBMC have been shown to give

increased neutralisation resistance to neutralising antibodies, an effect thought to be due to a higher level of envelope on the virus surface (1218).

Furthermore, according to the results obtained by the CAVD Vaccine Immune Monitoring Centre, VHH C8 was the only VHH able to neutralise the tier 1 subtype C virus, 93MW965.26. The observed IC_{50} was 0.2 μg/ml. This result is, again, in concordance with results obtained at UCL, where C8 could neutralise the 93MW965.26 virus with an IC_{50} of 0.3 μg/ml (Table 4.2.4.3.1). VHH C8 was, moreover, the only VHH able to neutralise the tier 2 subtype B virus SC422661.8, with an IC_{50} of 25.1 μg/ml. This result is not in concordance with results obtained at UCL, where VHH C8 could not neutralise SC422661.8 to 50%; instead, infection was reduced by 20% (Fig. 4.2.4.2.1 and Table 4.2.4.2.1). All of the tested VHH could neutralise the tier 1 subtype A virus.

4.2.4.7 Summary of VHH neutralisation of HIV-1 in TZM-bl cells

The selected VHH were tested against a panel of 65 viruses belonging to HIV-1 of subtypes A, A/G, B, B′/C, C and D. The broadly neutralising mAb b12 was tested in parallel. The percentage of viruses neutralised in each subtype by each of the VHH and mAb b12 are shown in Fig. 4.2.4.7.1. In brief, mAb b12 was more broadly reactive than any of the VHH against the subtype B viruses included in this study. It neutralised 77% and 64% of the subtype B viruses on an IC_{50} and IC_{90} level, respectively. VHH A12 was the most subtype B-reactive out of the three selected VHH, neutralising 59% and 36% on an IC_{50} and IC_{90} level, respectively.

As for subtype C viruses, VHH A12 and C8 displayed equally broad reactivity, neutralising 48% (IC_{50} level) and 22% (IC_{90} level) of the included viruses (Fig. 4.2.4.7.1). They did not, however, neutralise the exact same set of viruses. VHH D7 was less reactive against subtype C isolates; neutralising 33% and 7% on an IC_{50} and IC_{90} level, respectively. The broadly neutralising mAb b12 was slightly more reactive against subtype C viruses compared to the VHH. It could neutralise 55% (IC_{50} level) and 37% (IC_{90} level) of the subtype C viruses included in the study (Fig. 4.2.4.7.1).

None of the VHH, nor mAb b12, was particularly potent against the subtype B′/C viruses tested. The VHH neutralised one and mAb b12 three out of twelve viruses to 50%. Moreover, neither of the VHH or mAb b12 could neutralise the subtype A, A/G or D viruses that were part of this study (Fig. 4.2.4.7.1).

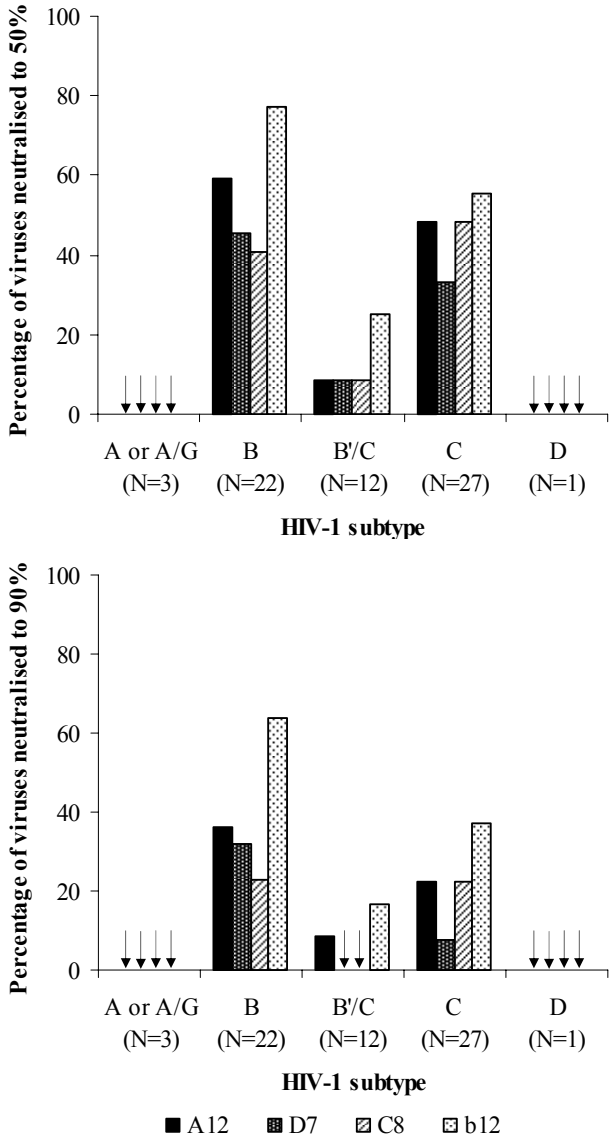


Figure 4.2.4.7.1. Percentage of HIV-1 isolates neutralised by the VHH and by mAb b12, according to HIV-1 subtype. Virus neutralisation was assayed in TZM-bl cells as described in the text. The percentage of viruses neutralised with an IC_{50} and IC_{90} of less than or equal to 50 μg/ml of antibody is shown.

4.2.5 VHH neutralisation of HIV-1 in primary cells

Despite the extensive use of engineered cell lines when assessing antibody neutralisation of HIV-1, they are not natural target cells for HIV-1 infection. The ability of the VHH to inhibit HIV-1 SF162 infection of primary cells was therefore tested in a PBMC-based assay. Serial dilutions of VHH A12, D7, C8, and a negative control VHH, as well as mAb b12, were incubated with virus for 1 hour at 37°C and subsequently added to PHA-stimulated PBMC obtained from blood donors. All VHH, including a negative control VHH (and mAb b12), were assayed in triplicate and in PBMC from two separate donors. After 24 hours at 37°C the cells were washed twice in growth medium. Cell culture supernatant was collected on day 1, 3, 5 and 8 after infection and assayed for HIV-1 p24 content to monitor virus replication. Virus replication in virus only control wells is shown in Fig. 4.2.5.1.

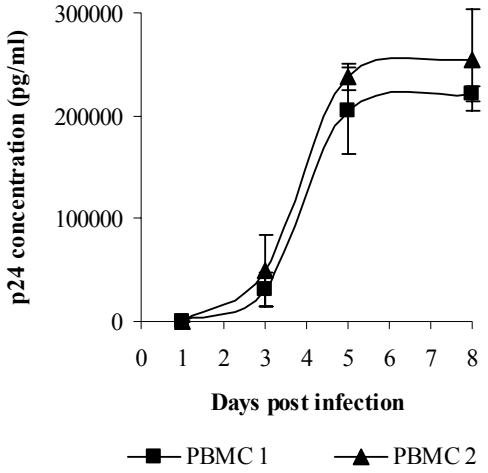


Figure 4.2.5.1. Replication of HIV-1 SF162 in PBMC from two donors in virus control wells of a PBMC neutralisation assay. For each donor, the replication of HIV-1 SF162 in one of the virus control wells was monitored by assaying the p24 content in the cell culture supernatant. PHA-stimulated PBMC (10^5 cells per well) were infected with HIV-1 SF162. After 24 hours at 37°C the cells were washed twice in growth medium. Cell culture supernatant was collected on day 1, 3, 5 and 8 after infection and assayed for HIV-1 p24 antigen in an antigen capture assay kit, as described in section 2.8.3. Data points represent the mean and bars the standard deviation of three replicates.

Neutralisation was measured on day 5 post infection as reduction in p24 content in test wells compared to in virus control wells (Fig. 4.2.5.2).

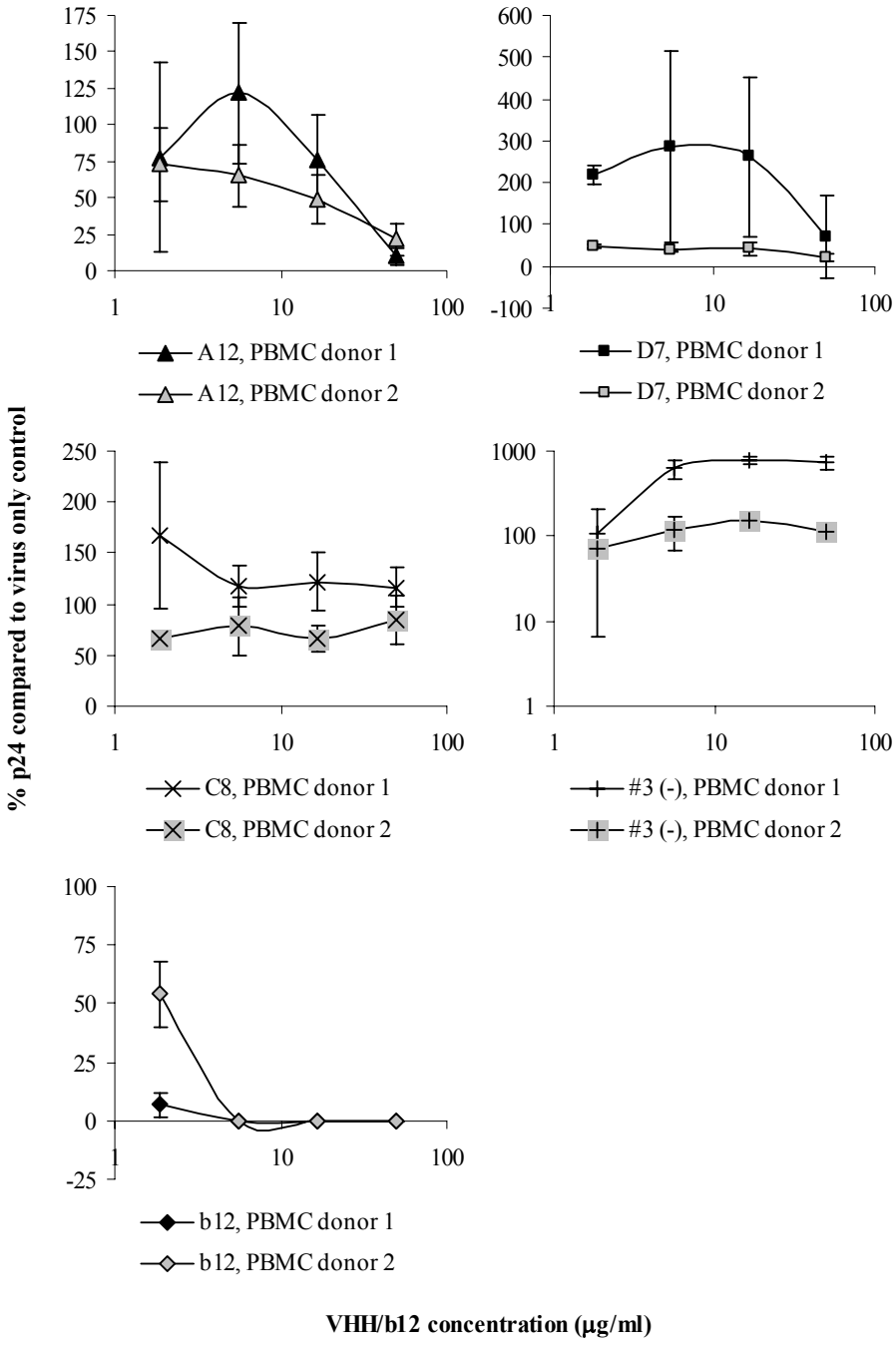


Figure 4.2.5.2. VHH and mAb b12 neutralisation of HIV-1 SF162 in PBMC. VHH A12, D7, C8 and #3 (negative control VHH) and mAb b12 was assayed for ability to neutralise HIV-1 SF162 in PBMC from two different donors as described in the text. HIV-1 p24 content on day 5 post infection in test wells compared to in virus only control wells was determined. Data points represent the mean and bars the standard deviation of the three replicates.

VHH and mAb b12 IC_{50}, IC_{75} and IC_{90} titres against SF162 in two different PBMC donors, on day 5 post infection, are shown in Table 4.2.5.1.

Table 4.2.5.1. VHH and mAb b12 neutralisation of HIV-1 SF162 in PBMC[a].

	IC_{50} (μg/ml)		**IC_{75} (μg/ml)**		**IC_{90} (μg/ml)**		**IC_{90} in TZM-bl cells (μg/ml)**
	PBMC donor 1	PBMC donor 2	PBMC donor 1	PBMC donor 2	PBMC donor 1	PBMC donor 2	
A12	24	16	35	44	50	•	7.8
D7	•	<1.9	•	46	•	•	9.3
C8	•	•	•	•	•	•	•
#3 (-)	•	•	•	•	•	•	•
b12	<1.9	1.9	<1.9	2	<1.9	2.1	1.2

[a]VHH A12, D7, C8 and #3 (negative control VHH) as well as mAb b12 was assayed in triplicate for ability to neutralise HIV-1 SF162 in PBMC from two different donors, as described in the text. HIV-1 p24 content on day 5 post infection in test wells compared to in virus only control wells was determined and the lowest concentration giving rise to 50%, 75% and 90% reduction in p24 content was calculated using XLfit 4 software, • indicates >50 μg/ml.

VHH A12 showed dose-dependent inhibition of infection of SF162 in PBMC from both of the two donors, with and IC_{75} of 35 and 44 μg/ml, respectively. VHH C8 could not inhibit SF162 infection in primary cells, which is in concordance with results obtained in the TZM-bl assay (Fig. 4.2.4.2.1 and Table 4.2.4.2.1). For VHH D7, the results were less clear, with D7 inhibiting infection in PBMC from just one of the two donors. Donor variability has, however, previously been observed to affect HIV-1 neutralisation (1212, 1213). The broadly neutralising mAb b12 potently inhibited SF162 infection in PBMC. It was more than twenty-fold more potent compared to VHH A12 against SF162 in PBMC, whereas in TZM-bl cells, mAb b12 was only about six-fold more potent (Fig. 4.2.4.2.1 and Table 4.2.4.2.1). The irrelevant VHH, which served as a negative control, did not reduce infection in any of the donors. Instead, there was up to approximately an eight-fold increase in p24 content in PBMC from one of the donors, in wells containing the irrelevant VHH (Fig. 4.2.5.2). The reason for this observed increase is unclear.

4.2.6 Neutralisation activity in sera and plasma from llama L44

In light of the relatively broad and sometimes potent neutralisation activity observed for VHH A12, D7 and C8, the neutralisation activities in heat-inactivated serum and plasma samples from llama L44, the llama from which the neutralising VHH were derived, were evaluated. Serum samples from day 0 (pre-immunisation) and day 28 (post-immunisation) and plasma samples from day 39 and 43 (post-immunisation) were available. The neutralisation activities in these samples were assayed against the autologous PBMC isolate, HIV-1 CN54, as well as against HIV-1 IIIB. HIV-1 IIIB was included since VHH A12, D7 and C8 had been selected through panning on IIIB gp120 (chapter 3) and had been observed to neutralise IIIB virus in with IC_{50} values of 0.07, 0.1, and 0.8 μg/ml in TZM-bl cells, respectively (Table 4.2.4.2.1). Viruses 27d and C261 were also included, as they had been found to be sensitive to neutralisation by the VHH (Tables 4.2.4.3.1 and 4.2.4.3.2). Neutralisation was assayed in the in the TZM-bl assay (Fig. 4.2.6.1).

Some virus inactivation was observed in the serum sample from day 28 (but not in plasma samples from day 39 and 43) against HIV-1 CN54 at high serum concentrations (1:5 dilution; Fig. 4.2.6.1). At a 1:15 and 1:45 serum dilution, a slight enhancement of infection was observed (around 2.5 times). No neutralisation (to more than 50%) was observed against HIV-1 IIIB and 27d in any of the samples. Against HIV-1 C261, relatively weak but dose-dependent neutralisation was observed in all samples obtained post-immunisation (Fig. 4.2.6.1). The serum or plasma dilutions giving 50% reduction in RLU were 1:21, 1:22, and 1:37, for the serum sample from day 28 and the plasma samples from day 39 and 43, respectively. HIV-1 C261 is more sensitive to neutralisation by VHH A12 and D7 than HIV-1 CN54, IIIB and 27d (Tables 4.2.4.3.1 and 4.2.4.3.2), suggesting it may be sensitive neutralisation by anti-CD4bs antibodies. It is, however, resistant to neutralisation by VHH C8 and mAb b12 (Tables 4.2.4.3.1 and 4.2.4.3.2). The IC_{50} of VHH A12 is <0.003 μg/ml against HIV-1 C261, and 1.4, 0.07 and 0.02 μg/ml against HIV-1 CN54, IIIB and 27d, respectively, possibly indicating that it is the presence of A12-like (or D7-like) antibodies in the llama sera and plasma that mediates neutralisation of HIV-1

C261 (but not HIV-1 CN54, IIIB and 27d). However, this notion is speculative, and more work would be needed to map the specificities of the weak neutralising activity observed in the llama serum and plasma samples.

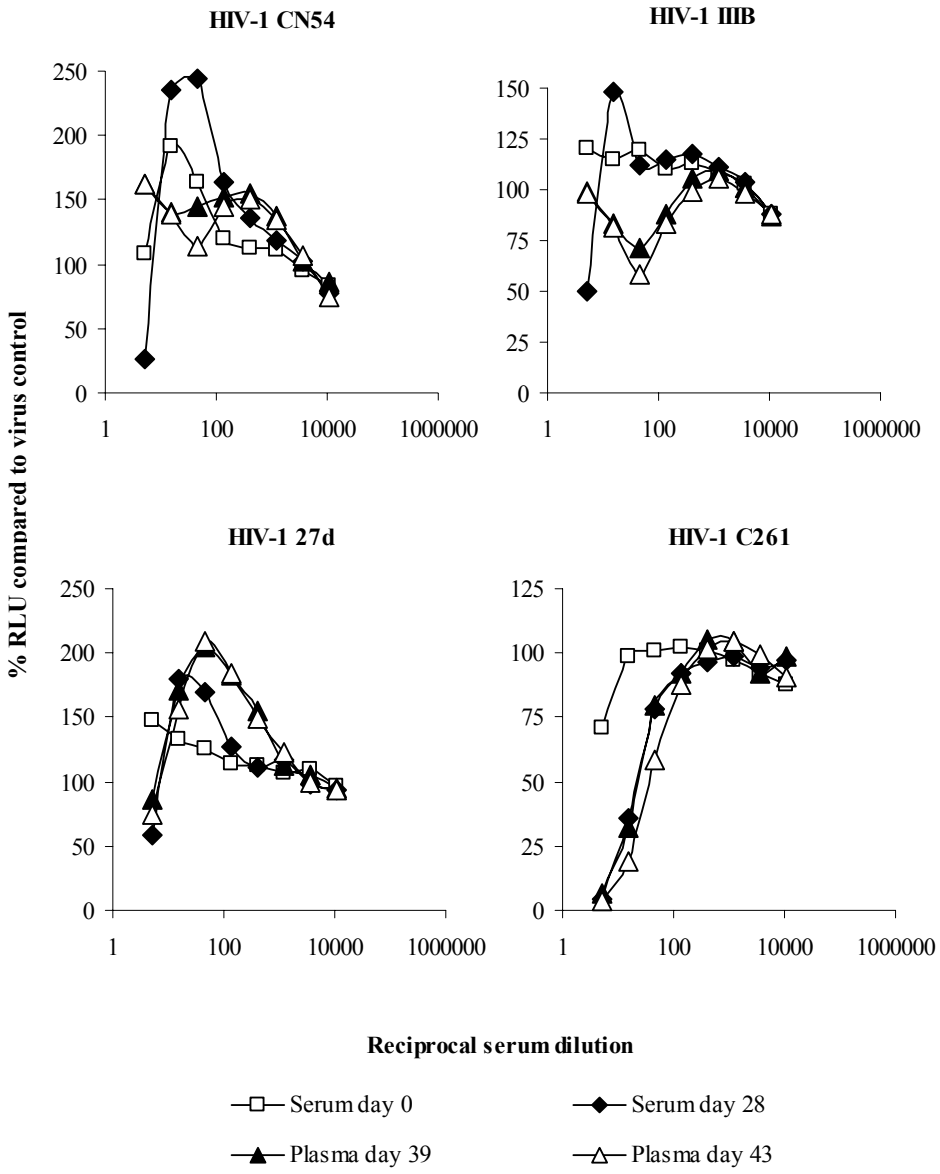


Figure 4.2.6.1. Neutralisation activity in sera and plasma from llama L44, immunised with HIV-1 CN54 gp120. The neutralisation activity in serum samples from day 0 (pre-immunisation) and 28 (post-immunisation) and plasma samples from day 39 and 43 (post-immunisation) was evaluated against HIV-1 CN54, IIIB (stock B in Table 4.2.4.2.1), 27d and C261 in TZM-bl cells as described in section 2.10.2. The percentage of RLU obtained in samples wells compared to the RLU obtained in wells containing virus and cells only is plotted against the reciprocal serum dilution. Data points represent the mean of duplicate reactions. Each serum or plasma sample was assayed against each virus on one occasion only, due to the limited amounts available.

In addition to not knowing the specificities of the weak neutralisation activity observed in the llama L44 post-immunisation sera and plasma, it should be noted that it remains unknown whether the neutralisation activity was mediated by conventional or heavy-chain antibodies. The ability of post-immunisation sera from llama L40 and L48 to neutralise HIV-1 was not evaluated, even though this would have been of interest.

4.3 Discussion

The results described in this chapter will be further discussed in the following sections.

4.3.1 Comparison of different neutralisation assays

Neutralisation results obtained in the NP2 and TZM-bl cell-based assays were overall fairly concordant. Nineteen of the viruses were tested in both assays for sensitivity to VHH A12, D7 and C8, making it 57 VHH-virus combinations. Results were qualitatively concordant for 51 out of the 57 VHH-virus combinations tested in both assays, in terms of whether or not 90% neutralisation could be achieved (section 4.2.3 and 4.2.4). For most virus isolates, the TZM-bl assay seemed slightly more sensitive, giving IC_{90} titres that were on average around 4-fold lower. The slight discrepancies observed may be due to differences between the two target cells, such as receptor expression levels, differences in infection kinetics, different incubation times, the use of DEAE-dextran to enhance infection of TZM-bl cells, or the different assay read-outs (1017, 1213, 1216, 1227).

In addition to in NP2 and TZM-bl cells, the ability of the VHH to neutralise HIV-1 SF162 was assessed in PBMC from two different donors. Unlike in the TZM-bl cell-based neutralisation assay, only VHH A12 was able to clearly neutralise HIV-1 SF162 in PBMCs. Furthermore, 90% neutralisation was only observed in PBMC from one of the two donors, and it required a 6-fold higher concentration of A12 compared to in TZM-bl cells (Table 4.2.5.1). Human anti-CD4bs mAb b12 was more than 20-fold more potent than VHH A12 at IC_{90} level

in the PBMC assay, whereas the difference between mAb IgG1 b12 and VHH A12 was only 6-fold in TZM-bl cells. Further studies will be needed to explain this observed discrepancy between the results obtained in the PBMC and the TZM-bl assays. Such discrepancies have, however, been reported previously. The broadly neutralising mAb 4E10 has, for example, been shown to be more broadly cross-subtype reactive and potent in engineered cell lines than in PBMCs (1017). It has been suggested that the choice of target cell affects HIV-1 neutralisation, and neutralisation assays using engineered cell lines have been shown to be more sensitive than PBMC assays (1017, 1213, 1216, 1218, 1227).

In addition, one study has shown that the presence of NK cells may contribute to the anti-HIV-1 activity when assaying HIV-1 neutralisation in PBMCs (1228). Neutralisation was shown to be more potent in non-adherent PBMCs than in purified CD4-positive lymphocytes and this increased neutralisation was reported to be due to Fc-FcR interactions. It is possible that the lack of the Fc domain on VHH might make them less potent in a PBMC neutralisation assay compared to a full immunoglobulin, although this notion is highly speculative. Further work, such as expressing the VHH in the context of a full immunoglobulin, is needed to determine whether this is a possibility.

4.3.2 VHH A12 is the most potent and broadly neutralising of the selected VHH

VHH A12 was shown to be the most broadly cross-subtype reactive out of the selected VHH. It neutralised 42% of the viruses included in this study on an IC_{50} level. On an IC_{90} level it was less potent, neutralising 23% of the viruses. The corresponding numbers for VHH D7 and C8 were 31% and 35% (IC_{50} level) and 14% and 17%, respectively. VHH A12 was also shown to be the most potent out of the VHH. It neutralised subtype B viruses with IC_{50} titres that were 1.2-33-fold lower than those of VHH D7 and 1.1-280-fold lower than those of VHH C8 (where IC_{50} for both VHH were $\leq$50 μg/ml). Against subtype C viruses, A12 required 1-1633-fold lower concentrations than D7 and 4-100-fold lower concentrations than C8. VHH A12 and C8 neutralised a partially different set of viruses, indicating that they recognise different epitopes. VHH D7, on the other

hand, neutralised the same, but smaller, set of viruses compared to VHH A12. This observation is in concordance with the fact that VHH A12 and D7 could be grouped together based on sequence similarity, as shown in section 3.2.3.3.

4.3.3 Comparing neutralisation data obtained for mAb b12 to data already published

The human anti-CD4bs mAb b12 is one of the most extensively studied mAbs to HIV-1 envelope and is one of only a handful mAbs that have been shown to neutralise a wide range if HIV-1 isolates of different subtypes. The neutralisation ability of mAb b12 has been tested in a large number of studies, using various assays and different virus panels of viruses. It is of interest to compare the results obtained in this study to results obtained in previous studies.

In this study, mAb b12 was tested in parallel to the VHH against a panel of 65 viruses. It was able to neutralise 54% of the viruses on an IC_{50} level, and 40% on an IC_{90} level. These findings are in concordance with a previous study carried out by Binley, *et al*. (1017), where a small panel of mAbs was tested against an extensive panel of 90 viruses using a high-throughput pseudovirus-based neutralisation assay and where mAb b12 was shown to neutralise 50% and 34% of viruses on an IC_{50} and IC_{90} level, respectively. In another study, Quakkelaar, *et al*. (1222), tested 149 viruses from ten individuals infected with HIV-1 of subtype B for neutralisation susceptibility to broadly neutralising mAbs in a PBMC-based assay, with b12 being able to neutralise 54% of viruses. Furthermore, mAb b12 was used in the characterisation of the subtype B and C reference panels of envelope pseudotyped viruses described by Li *et al*. (1022, 1023), which were included in this study. Neutralisation data obtained in this study are almost completely concordant with the data previously published (Table 4.3.3.1), demonstrating high reproducibility and low inter-laboratory variability of the assay.

Table 4.3.3.1. Comparing mAb b12 IC_{50} titres to those previously reported[a].

Subtype B reference panel	b12 IC_{50} (μg/ml)		Subtype C reference panel	b12 IC_{50} (μg/ml)	
	Li *et al.* 2005	This study		Li *et al.* 2006	This study
6535.3	1.4	2.5	Du156.12	0.8	<1.9
QH0692.42	0.3	0.7	Du172.17	1	<1.9
SC422661.8	0.2	<1.9	Du422.1	0.2	<1.9
PVO.4	•	•	ZM197M.PB7	19.9	7.4
TRO.11	•	•	ZM214M.PL15	3	<1.9
AC10.0.29	1.9	2.2	ZM233M.PB6	•	•
RHPA4259.7	0.1	<1.9	ZM249M.PL1	3.2	5.6
THRO4156.18	0.5	0.5	ZM53M.PB12	25.9	•
REJO4541.67	0.7	32	ZM109F.PB4	•	•
TRJO4551.58	•	•	ZM135M.PL10a	•	•
WITO4160.33	3.1	11	CAP45.2.00.G3	0.7	<1.9
CAAN5342.A2	•	•	CAP210.2.00.E8	20.4	6.7

[a]IC_{50} titres for mAb b12 against subtype B and C reference panels (1022, 1023) of envelope pseudotyped viruses in TZM-bl cells.

4.3.4 The VHH and mAb b12 show different patterns of neutralisation

The VHH neutralisation profiles were compared to that of broadly neutralising human anti-CD4bs mAb b12 (sections 4.2.3 and 4.2.4). Overall, mAb b12 was more reactive against the viruses included in this study, neutralising 54% of viruses included in this study, compared to 42% for A12, the most potent of the VHH (Fig. 4.2.4.7.1). Interestingly, the VHH displayed inhibitory profiles that were different to that of mAb b12. For example, mAb b12 was able to neutralise twelve subtype B'/C or C viruses that VHH A12 was not able to neutralise (IC_{50} level). At the same time, VHH A12 was able to neutralise eight subtype B'/C or C viruses that b12 was unable to neutralise (IC_{50} level). This observation may indicate that mAb b12 and VHH A12 recognise different epitopes. Additional studies are needed to determine whether the VHH and mAb b12 epitopes are related.

It should be noted that the VHH are approximately ten times smaller than mAb b12, and therefore less potent on a molar level, but that mAb b12, on the other hand, has two antigen-recognising sites per molecule, making accurate estimates of relative neutralisation potencies impossible. To be able to make a direct comparison, the VHH would need to be presented in the context of a complete camelid immunoglobulin.

4.3.5 Lack of potent neutralisation in serum and plasma from llama L44

As the selected VHH exhibited relatively broad neutralisation activity when assayed against a panel of 65 viruses in TZM-bl cells, it was of interest to see whether any neutralising activity could be detected in serum of the llama from which the VHH were derived. Three sequential serum and plasma samples from llama L44 were therefore assayed against the autologous HIV-1 CN54 PBMC isolate, as well as three viruses that had been shown to be sensitive to neutralisation by the VHH (HIV-1 IIIB, 27d and C261). Some weak, albeit dose-dependent, neutralisation was observed against HIV-1 C261, a virus that had been shown to be particularly sensitive to neutralisation by VHH A12 and D7 (IC_{50} of <0.003 and 0.004 μg/ml, respectively; Table 4.2.4.3.1). This finding may indicate that the neutralisation activity against C261 in the serum and plasma samples is due to A12- and D7-like antibodies, although this notion is speculative. However, overall, no potent neutralisation activity could be detected in the sera/plasma. As immunisation with recombinant envelope glycoproteins rarely results in potent and broad neutralising serum responses (754, 997, 1229), this finding is expected. Immunising llamas with an antigen that elicits a broad neutralising antibody response of high magnitude is likely to increase the chances of isolating more potent and broadly neutralising VHH. Identifying such an immunogen represents one of the major challenges in HIV-1 vaccine development.

4.3.6 Summary and conclusions

In this chapter, it has been shown that the non-conventional immune system of camelids can be used to generate antibody fragments to HIV-1 envelope with

relatively broad and potent cross-subtype neutralising ability. The VHH could potently neutralise HIV-1 in two different cell-based neutralisation assays, using two different target cell lines. They were, however, less potent at neutralising HIV-1 in primary cells.

The VHH were tested against HIV-1 from subtype A, B, C, D, CRF02_AG and CRF07_BC. The most potent of the characterised VHH was able to neutralise 59% of the subtype B viruses and 48% of the subtype C viruses included in the study on an IC_{50} level, a neutralisation breadth that is comparable to that of the broadly neutralising anti-CD4bs mAb b12. On an IC_{90} level, mAb b12 was, however, more broadly reactive. It should also be noted that mAb b12 was more reactive against the well-characterised subtype B and C reference panels of envelope pseudotyped viruses, which has been classified as suitable for tier 2 assessment of neutralising antibodies. Nevertheless, to our knowledge, this is the first description of a broadly neutralising mAb to HIV-1 envelope that is derived from an immunised animal, as all previously reported broadly neutralising anti-HIV-1 mAbs have been a result of natural infection rather than immunisation (754).

Despite being derived from a llama immunised with a recombinant gp120 from the CRF07_BC isolate CN54, the selected VHH were not able to neutralise any of the eleven CRF07_BC reference panel viruses that were included in this study. Taking the relatively broad neutralisation properties of the VHH against viruses from subtype B and C, this finding is a bit surprising. Further studies are needed to elucidate the extent to which these VHH-resistant CRF07_BC envelope clones differ from the CN54 envelope. Such studies would be especially interesting once more is known about the VHH epitopes. An initial attempt to characterise the VHH epitopes will be described in chapter 5 of this thesis.

Chapter 5

Characterisation of VHH epitope recognition

In this chapter, the way in which the VHH react with recombinant envelope glycoproteins will be evaluated using biochemical assays.

5.1 Introduction

In chapter 3, the isolation of HIV-1 neutralising anti-envelope VHH derived from llamas immunised with recombinant gp120 was described. The VHH were selected through panning of phage libraries, expressing the VHH repertoires of the immunised llamas, on immobilised gp120. To increase the chances of isolating neutralising VHH, a competitive elution with an excess of sCD4 was employed, possibly allowing for the elution of VHH targeting the CD4bs. Five of the selected VHH were chosen for further characterisation. These were VHH A12, D7, D12, C8 and F2. The neutralising abilities of these VHH were characterised in chapter 4, revealing VHH A12, D7 and C8 as the most potent. In this chapter, biochemical assays will be used to confirm that the neutralising VHH actually bind to gp120. Their cross-subtype binding reactivities will also be evaluated. In addition, an initial attempt to characterise the VHH epitopes will be carried out through competition studies.

5.2 Results

This section describes the results of this chapter.

5.2.1 VHH binding to recombinant envelope glycoproteins

In this section, the ability of the VHH to bind to HIV-1 recombinant envelope glycoproteins will be evaluated.

5.2.1.1 VHH binding to the immunogen in ELISA

All of the five VHH chosen for further characterisation were tested for their ability to bind to the immunogen, recombinant gp120 derived from HIV-1 CN54, in ELISA. VHH F2 was the only VHH found to bind well to CN54 gp120 (Fig. 5.2.1.1.1). This observation is in keeping with the notion that VHH F2 was the only VHH that had been selected through panning on CN54 gp120, as described in chapter 3. Nevertheless, it is surprising that the remaining VHH showed such poor binding to CN54 gp120, despite having been raised against the same protein. It is possible that the failure to detect any interaction between CN54 gp120 and the VHH is caused by the ELISA setting. The ability of the VHH to react with CN54 gp120 was therefore also evaluated in a surface plasmon resonance setting. This experiment is described in section 5.2.2.2.

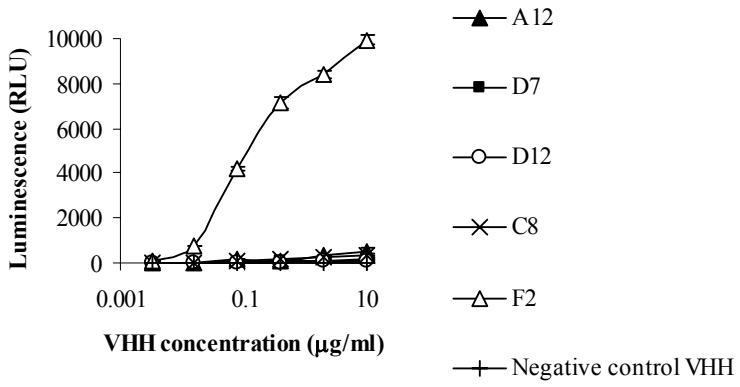


Figure 5.2.1.1.1. VHH binding to the immunogen in ELISA. The selected VHH and a negative control VHH were assayed for binding to the immunogen, recombinant gp120 derived from HIV-1 CN54, in ELISA. Recombinant CN54 gp120 was captured by antibody D7324 immobilised onto microtitre plates. Serial dilutions of VHH were added and binding detected using a mouse anti-c-myc antibody followed by an AP-conjugated goat anti-mouse IgG polyclonal antibody. Background-subtracted luminescence readings (in RLU) were plotted against VHH concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

5.2.1.2 The VHH bind to gp120 and gp140 of subtype A, B and C in ELISA

The neutralisation characterisation described in chapter 4 had revealed VHH A12, D7 and C8 as more potent and broadly cross-subtype neutralising than VHH D12 and F2. In the following sections, characterisation of envelope

reactivity will therefore only be carried out for VHH A12, D7 and C8. VHH D12 and F2 will be further discussed in section 5.2.2.8.

In spite of their ability to neutralise HIV-1 strains of subtype B and C, none of VHH A12, D7 and C8 was found to bind well to the protein against which they had been raised, CN54 gp120, in ELISA. This contradictory observation might to some extent be explained by doubts regarding the structural integrity of the immunogen, as discussed in chapter 3, and the notion that these VHH had been selected through panning on gp120 derived from HIV-1 IIIB, as opposed to on CN54 gp120. To evaluate whether that the VHH could actually bind to HIV-1 gp120, their reactivity with recombinant IIIB gp120 was assayed in an ELISA. All three VHH were observed to bind well to the subtype B HIV-1 IIIB gp120 in a dose-dependent manner, as shown in Fig. 5.2.1.2.1. The data were fitted to a four parameter logistics equation and the concentrations yielding half-maximal binding was determined using the XLfit 4 software. The concentrations yielding half-maximal binding, sometimes used as an estimate of the equilibrium dissociation constant, were determined to be 14.7, 13.4 and 12.1 ng/µl for A12, D7 and C8, respectively, corresponding to 0.92, 0.84 and 0.76 nM.

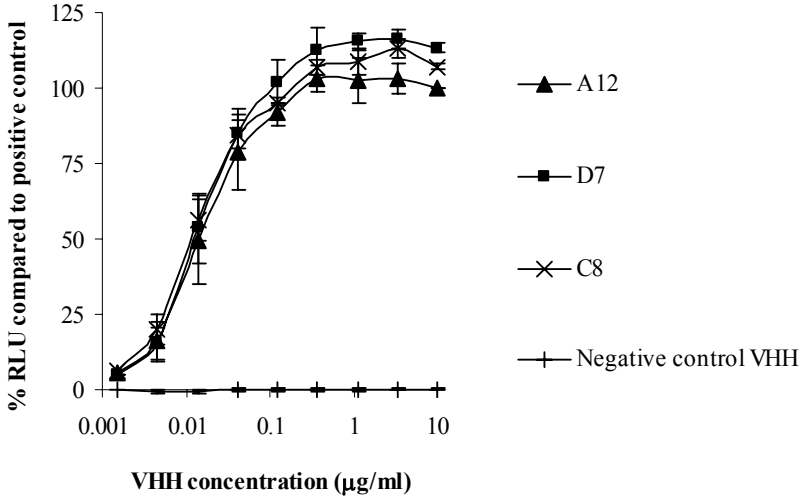


Figure 5.2.1.2.1. VHH binding to recombinant gp120 derived from HIV-1 IIIB in ELISA. VHH A12, D7, C8 and a negative control VHH were assayed for binding to HIV-1 IIIB gp120. Recombinant IIIB gp120 was captured by immobilised antibody D7324. Serial dilutions of VHH were added and binding was detected as described in chapter 2. Background-subtracted luminescence readings (in RLU) were normalised to a positive control reaction (VHH A12 at 10 µg/ml) and plotted against VHH concentration. Data points represent the mean and bars the standard error of two independent experiments carried out in duplicate.

The VHH had been shown to neutralise a fairly wide range of HIV-1 of subtype B and C. To further characterise their cross-subtype reactivity, their ability to bind to gp120 of subtype C was evaluated in ELISA. Dose-dependent binding of VHH A12, D7 and C8 to subtype C envelope proteins is shown in Fig. 5.2.1.2.2.

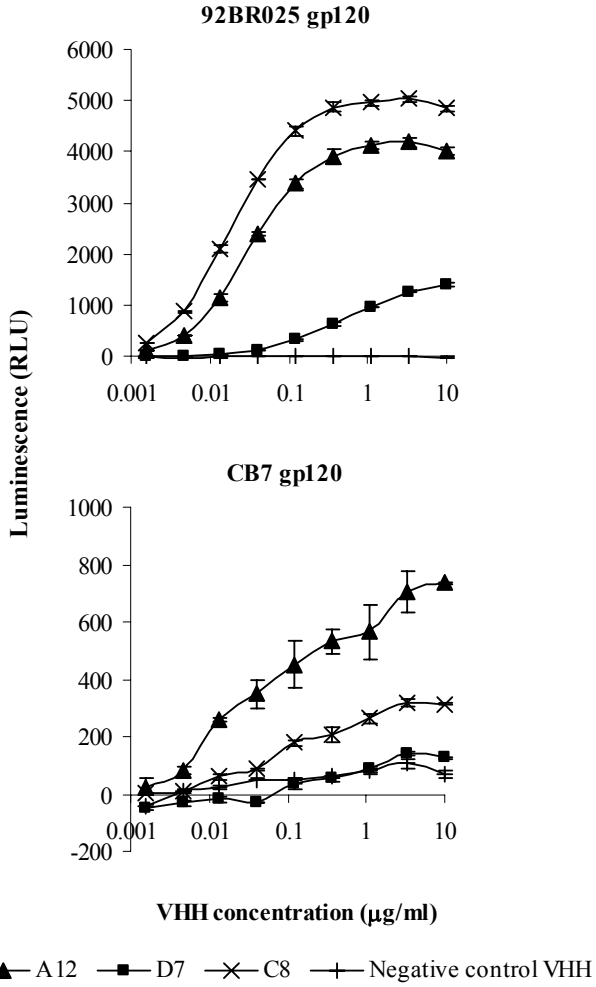


Figure 5.2.1.2.2. VHH binding to subtype C gp120 derived in ELISA. VHH A12, D7, C8 and a negative control VHH were assayed for binding to recombinant gp120 derived from HIV-1 92BR025 and HIV-1 CB7. Recombinant gp120 was captured by immobilised antibody D7324. Serial dilutions of VHH were added and binding was detected as described in chapter 2. Data points represent the mean and bars the standard deviation of background-subtracted luminescence readings (in RLU) from duplicate reactions.

As seen in Fig. 5.2.1.2.2, VHH A12 and C8 were able to bind well to the subtype C HIV-1 92BR025.C1 gp120 in ELISA, whereas D7 bound with a much weaker signal. This observation is in concordance with the finding that VHH A12 and

C8, but not D7, were able to neutralise the chimeric virus expressing the 92BR025.C1 envelope (Fig. 4.2.4.3.1 and Table 4.2.4.3.1). VHH C8, however, bound with a slightly greater signal strength to the 92BR025.C1 gp120, compared to VHH A12, which is not in complete concordance with the neutralisation results, where VHH A12 neutralised the 92BR025.C1 virus with an IC_{50} that was ten times lower than the IC_{50} of VHH C8 (Table 4.2.4.3.1). Of note is also that when the 92BR025.C1 gp120 was included in the phage display panning step, only VHH A12 was selected for, as described in chapter 3. VHH A12 was also found to show very weak reactivity with the CB7 gp120, whereas VHH D7 and C8 showed even poorer reactivity, barely above background level (Fig. 5.2.2.2). This finding is in agreement with the neutralisation data described in chapter 4, where VHH A12 was the only VHH able to just about neutralise CB7 virus to 90% with an IC_{90} of 50 μg/ml (Fig. 4.2.4.3.1 and Table 4. 2.4.3.2).

The VHH were also tested for reactivity with the subtype A 92UG037.A9 gp120. All three VHH showed dose-dependent binding with relatively high signal strength to this subtype A gp120 (Fig. 5.2.2.3).

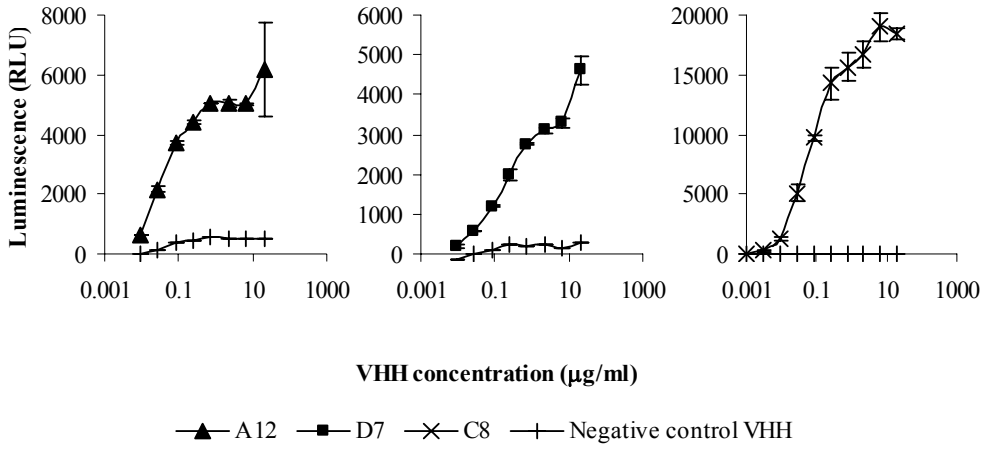


Figure 5.2.2.3. VHH binding to gp120 of subtype A in ELISA. VHH A12, D7, C8 and a negative control VHH were assayed for binding to 92UG037.A9 gp120. Recombinant gp120 was captured by immobilised antibody D7324. Serial dilutions of VHH were added and binding was detected as described in chapter 2. Data points represent the mean and bars the standard deviation of background-subtracted luminescence readings (in RLU) from duplicate reactions.

This observation is not in agreement with neutralisation results, where none of the VHH was able to neutralise the chimeric virus expressing the exact same gp120 (Fig. 4.2.4.5.1).

To investigate whether the VHH inability to neutralise the 92UG037.A9 virus was due to an ability to bind only to monomeric gp120, but not to gp120 in the context of the envelope trimer, the VHH were tested for reactivity with predominantly trimeric 92UG037 gp140. All three VHH showed dose-dependent reactivity with the trimeric 92UG037 gp140, indicating that the failure to neutralise 92UG037.A9 virus was not due to lack of reactivity with trimeric envelope (Fig. 5.2.2.4). It is still possible, though, that the lack of neutralisation is due to inability to bind to 92UG037.A9 gp120 in the context of the functional envelope spike, as opposed to the recombinant trimeric protein. Lack of correlation between antibody ability to bind recombinant envelope proteins and ability to neutralise the corresponding virus has been previously observed (141, 142, 623-626, 897, 940), and will be further discussed in section 5.2.3.

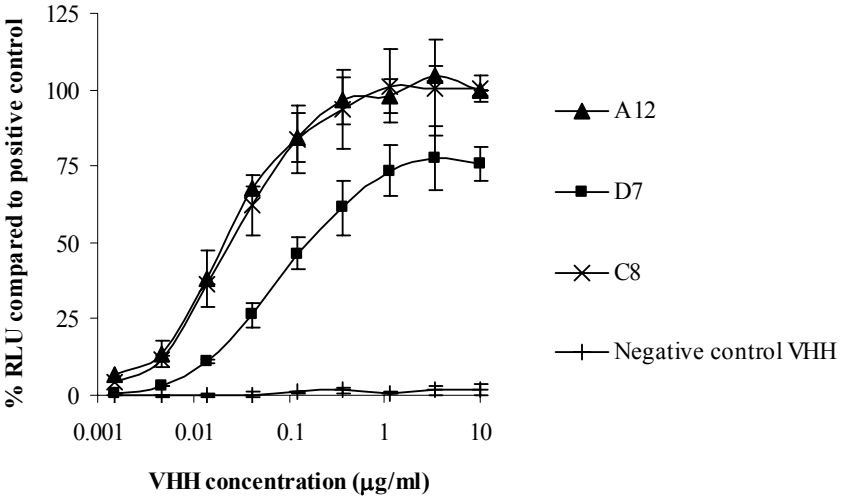


Figure 5.2.2.4. VHH binding to 92UG037 gp140 in ELISA. VHH A12, D7, C8 and a negative control VHH were assayed for binding to 92UG037 gp140 in ELISA. The experimental procedure is described in chapter 2. Background-subtracted luminescence readings were normalised to a positive control reaction (VHH A12 at 10 µg/ml) and plotted against VHH concentration. Data points represent the mean and bars the standard error of two independent experiments carried out in duplicate.

5.2.1.3 The VHH bind to recombinant gp120 with high affinity

To study the kinetics by which the VHH bound to gp120 and determine their affinities, their binding to gp120 was monitored using surface plasmon resonance techniques. Surface plasmon resonance techniques measure the angle by which polarised light is reflected from a gold-covered glass surface, to which molecules are immobilised through what is often a dextran layer linked to the gold. The technique was first used to measure real-time intermolecular interactions in 1991 (1230), and has since then been used extensively. 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.

Initially, attempts to detect VHH binding to gp120 were made together with Karolin Hijazi in the laboratory of Charles Kelly at the Department of Oral Immunology, King's College London, UK, as part of the EMPRO collaboration. Recombinant CN54 and IIIB gp120 was either coupled to one of the flow cells of a biosensor chip using standard amine coupling, or captured by sheep antibody D7324 coupled to the chip. VHH was then injected and the binding monitored. All the VHH tested, including the negative control VHH, did, however, bind to the control cell of the chip, i.e. the cell lacking gp120 (data not shown). Any specific binding to gp120 could not be distinguished from the background binding, and specific binding could hence not be detected. The reasons behind this non-specific binding are unclear.

Instead, the experiment was successfully repeated by Bart Hoorelbeke at Ablynx NV, Ghent, Belgium. Recombinant gp120 was coupled to a CM5 biosensor chip using standard amine coupling techniques, or captured by streptavidin on a SA biosensor chip, and VHH A12, D7 and C8 binding to the immobilised gp120 was subsequently monitored. The association and dissociation rate constants (k_a and k_d) were determined and used to calculate the equilibrium dissociation constants (K_D). Kinetic data are summarised in Table 5.2.1.3.1. In summary, VHH A12 and D7 showed affinities in the high picomolar range for recombinant IIIB gp120, with equilibrium dissociation constants of 100 and 97 pM, respectively.

VHH C8 was found to have a faster off-rate, leading to a more than eight-fold higher K_D.

Table 5.2.1.3.1. VHH kinetic constants and affinities for IIIB gp120[a].

VHH	K_D (nM)	k_a (1/(Ms))	k_d (1/s)
A12	0.1	2.73×10^5	2.98×10^{-5}
D7	0.097	4.00×10^5	3.89×10^{-5}
C8	0.85	2.05×10^5	1.74×10^{-4}

[a]VHH A12, D7 and C8 association rate constants (k_a), dissociation rate constants (k_d) and equilibrium dissociation constants ($K_D = k_d/k_a$) for recombinant IIIB gp120 were determined by surface plasmon resonance studies. Data were kindly provided by Bart Hoorelbeke, Ablynx NV, Ghent, Belgium.

Camelid VHH usually bind to their target with affinities comparable to those of conventional antibodies (1099, 1100). VHH with K_D values as low as those reported in this study (100 pM) have, however, been described on a few occasions, according to a recent review by Harmsen and de Haard (1100). Such low K_D values equate the affinity ceiling that has been suggested for antibodies generated through *in vivo* affinity maturation (1231-1233).

As a comparison, Fab b12 has been reported to bind to IIIB gp120 with a K_D of 6.3 nM (1234). Furthermore, sCD4 has been reported to bind to envelope proteins, including IIIB gp120, with K_D values of 22-35 nM (929, 1021, 1235). Another study reported K_D values in the range of 2.2-16 nM for range of anti-CD4bs Fab fragments (including Fab b12) to MN gp120 (624). Yet another human anti-CD4bs mAb, F105, has been shown to bind to IIIB gp120 with a K_D of 0.62 nM (1021).

5.2.1.4 Summary of VHH binding experiments

In summary, the VHH were shown to bind to gp120 of subtype A, B and C, indicating that they recognise a motif that is accessible on soluble recombinant envelope proteins and relatively conserved among HIV-1 subtypes. Furthermore, they were shown to bind to IIIB gp120 with a high affinity, perhaps lending

some explanation to the potency with which they can neutralise some viruses. Antibody neutralisation of virus has, however, been reported to show no significant correlation with ability to bind to monomeric gp120, but instead to correlate with affinity for oligomeric envelope (624).

5.2.2 VHH competition studies

In this section, an initial attempt to characterise the VHH epitopes through sCD4 and antibody competition studies will be described.

5.2.2.1 The VHH compete with sCD4 for binding to gp120 and gp140 in ELISA

The VHH had been specifically selected for ability to compete with sCD4, using the functional phage display panning method described in chapter 3. To investigate whether the VHH actually bound to gp120 in a way that prevented binding of CD4, and to possibly cast some light on the mechanism by which the VHH inhibit HIV-1 infection, the VHH capacity to inhibit sCD4 binding to recombinant envelope proteins was evaluated in ELISA.

An inhibition ELISA was set up. Titrations of VHH were pre-incubated with IIIB gp120 followed by subsequent incubation with solid-phase coated four-domain sCD4 after which gp120 binding to sCD4 was detected. VHH A12, D7 and C8 all inhibited binding of sCD4 to HIV-1 IIIB gp120 in a dose-dependent manner (Fig. 5.2.2.1.1, left panel). This finding indicates that the VHH bind to epitopes that overlap with the CD4bs, bind in a way that sterically hinders binding of sCD4, or that their binding locks gp120 in a conformation that prevents binding of sCD4.

The VHH were also tested for ability to inhibit sCD4 binding to the subtype A 92UG037 gp140. All three VHH were able to inhibit sCD4 binding to 92UG037 gp140 in a dose-dependent way (Fig. 5.2.2.1.1, right panel). VHH A12 was, however, able to inhibit sCD4 binding to recombinant 92UG037 gp140 at slightly lower concentrations than did the remaining VHH. This observation

indicates that the VHH failure to neutralise the 92UG037.A9 virus is not due to inability to bind to 92UG037.A9 envelope in a way that prevents CD4 binding. The reasons behind the VHH failure to neutralise 92UG037.A9 virus, despite being able to compete with sCD4 for binding to recombinant 92UG037 envelope proteins, will be further discussed in section 5.2.3.

In summary, the ability of the neutralising VHH to compete with sCD4 for binding to recombinant envelope proteins suggests that they inhibit HIV-1 infection by binding to the functional envelope spike prior to interaction with CD4. The ability of the selected VHH to inhibit sCD4 binding to the immunogen, CN54 gp120, could not be evaluated in ELISA, as no binding of sCD4 to CN54 gp120 could be observed (section 3.2.2).

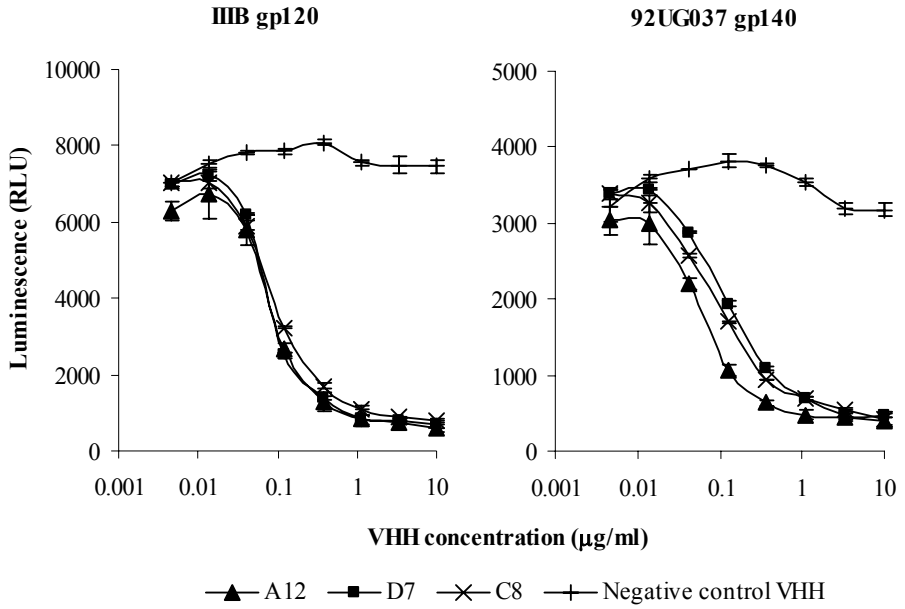


Figure 5.2.2.1.1. Dose-dependent competition of VHH A12, D7 and C8 with sCD4 for binding to recombinant envelope proteins in ELISA. Three-fold serial dilutions of VHH were pre-incubated with subtype B IIIB gp120 or subtype A 92UG037 gp140 and subsequently incubated with sCD4 pre-coated on microtitre plates. Envelope protein binding to sCD4 was detected as described in chapter 2. Background-subtracted luminescence readings were plotted against VHH concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

5.2.2.2 The VHH compete with sCD4 for binding to gp120 in a surface plasmon resonance assay

To further confirm that the selected VHH recognise an epitope over-lapping the CD4bs, or at least bind in a way that sterically hinders binding of sCD4, the VHH were assessed for their ability to inhibit sCD4-gp120 binding in a BIAcore surface plasmon resonance assay. Surface plasmon resonance techniques enables real-time monitoring of protein-protein interactions, which allows for the detection of weak interactions that can remain undetected in ELISA (1236-1238). This notion was supported by the results described in chapter 3.2.2 of this thesis, where weak binding of immobilised sCD4 to fluid-phase CN54 gp120 could be detected using BIAcore surface plasmon resonance techniques, but not using ELISA. This advantage of the surface plasmon resonance assay enabled testing whether the VHH could inhibit binding of sCD4 to CN54 gp120, something which could not be evaluated using conventional ELISA techniques. The surface plasmon resonance sCD4-gp120 inhibition assay was set up and optimised together with Karolin Hijazi in the laboratory of Charles Kelly at the Department of Oral Immunology, King's College London, UK, as part of the EMPRO collaboration. Initially, recombinant four-domain sCD4 was coupled straight to the biosensor chip using standard amine coupling techniques. No binding of this coupled sCD4 could, however, be observed to fluid-phase IIIB or CN54 gp120, but to an anti-CD4 antibody recognising domain 4 of CD4 (data not shown). This observation indicated that the amine coupling affects the structure and/or limits the accessibility of the gp120-binding site of this sCD4. The anti-CD4 antibody mentioned above was instead coupled to the chip and used to capture sCD4. Binding of the captured sCD4 could now be observed, both to IIIB and CN54 gp120, as described in section 3.2.2. To allow for several injections of gp120, without having to re-inject sCD4, sCD4 was cross-linked to the anti-CD4 antibody. This cross-linking had no apparent effect on the gp120-binding ability (data not shown).

To test VHH ability to inhibit sCD4 binding to gp120, titrations of VHH were pre-incubated with gp120 and then injected onto the chip. VHH C8 and D7 were tested. VHH A12 was not included in this experiment, as it had not yet been

isolated at the time of the BIAcore experiments. Both C8 and D7 showed dose-dependent inhibition of sCD4 binding to HIV-1 CN54 gp120, as can be seen in the BIAcore sensogram depicted in Fig. 5.2.2.2.1. Complete inhibition was observed, even at equimolar concentrations of VHH and gp120.

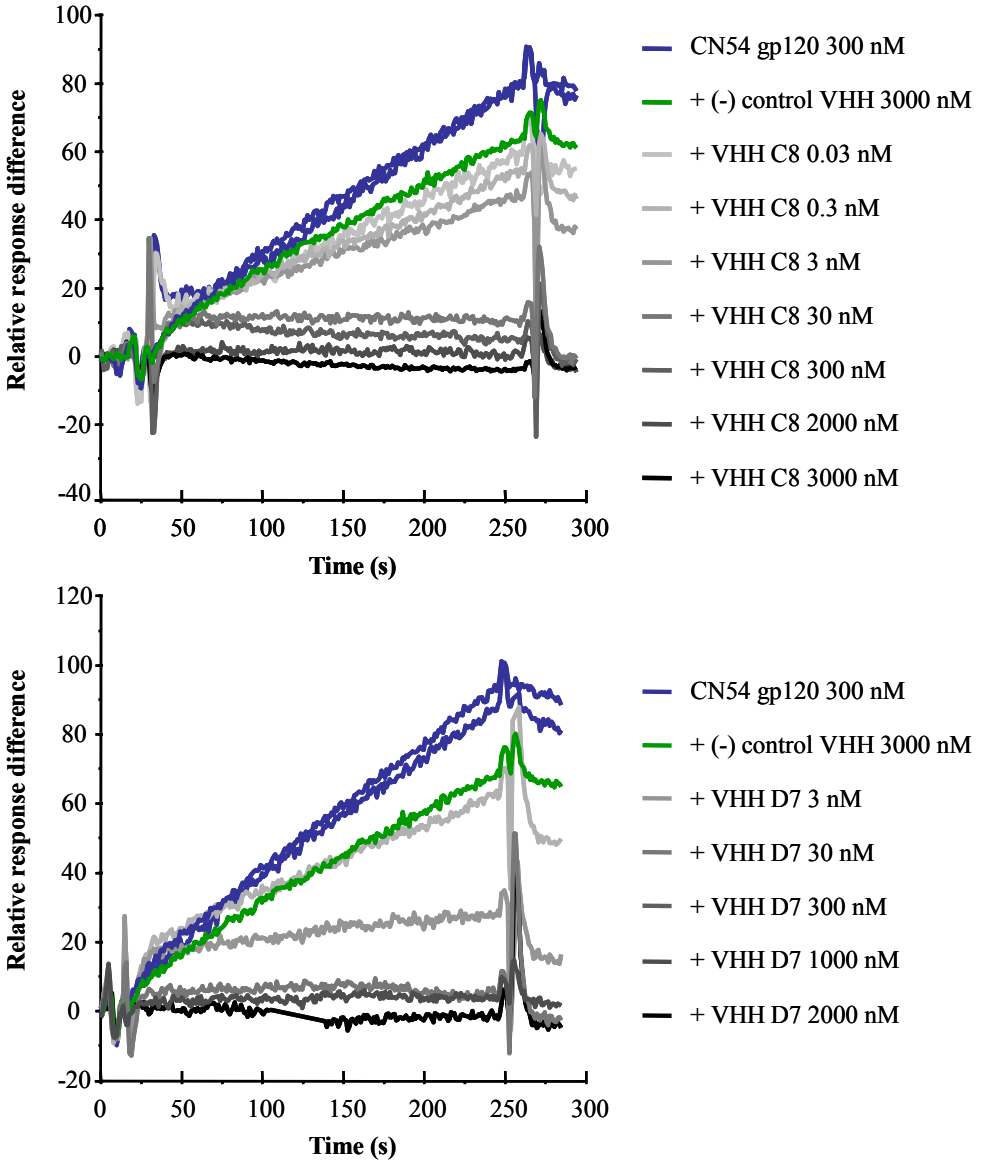


Figure 5.2.2.2.1. Dose-dependent inhibition of sCD4 binding to CN54 gp120 by VHH C8 and D7 as assayed by surface plasmon resonance. Recombinant sCD4 was cross-linked to an anti-CD4 mAb coupled to the chip. Serial dilutions of VHH were pre-incubated with HIV-1 CN54 gp120 and subsequently injected onto the chip. Data were analysed using the BIAevaluation software. Binding of HIV-1 CN54 gp120 to sCD4 is represented as the normalised difference between the response units (RU) observed in the cell containing the chip with sCD4 and the negative control flow cell, plotted versus time.

This finding confirms the observation described in section 5.2.2.1, that the VHH compete with sCD4 for binding to gp120, either through binding to an epitope that partly overlaps with the CD4bs, or by binding in a way that sterically hinders biding of sCD4, alternatively locks the gp120 in a conformation that hampers interaction with sCD4. Again, this finding may suggest that the VHH inhibit HIV-1 infection by interacting with gp120 prior to its engagement to CD4.

This observation furthermore suggests that the VHH are indeed able to interact with the immunogen, CN54 gp120, despite the failure to detect such an interaction in ELISA. This is in analogy with the observation that sCD4 binding to CN54 gp120 could not be detected in ELISA, whereas a weak interaction was observed in a surface plasmon resonance biosensor setting, as described in chapter 3. The ability of the surface plasmon resonance technique to detect weak protein-protein interactions that cannot be detected in ELISA is well-established (1236-1238). Therefore, it is possible that the failure to detect VHH binding to CN54 gp120 in ELISA is due to the weak nature of the interactions. Nonetheless, the interactions were potent enough to completely inhibit the (equally weak) binding of sCD4 to CN54 gp120 at an equimolar ratio, suggesting that this is the mechanism by which neutralisation occurs.

5.2.2.3 The VHH compete with mAb b12 for binding to gp120 in ELISA

In section 5.2.2.1 and 5.2.2.2, it had been shown that the VHH compete with sCD4 for binding to envelope proteins of subtype A, B and C, and hence are likely to recognise CD4bs-related epitopes. The broadly neutralising mAb b12 has been shown to bind to a relatively conserved epitope that overlaps a subset of the CD4bs (1008). In chapter 4, it was shown that the VHH and mAb b12 neutralise a different spectrum of viruses, indicating that they recognise different epitopes. To further map the epitopes of the selected VHH, they were tested for their ability to compete with mAb b12 in ELISA. VHH A12, D7 and C8 were all found to compete with mAb b12 for binding to recombinant IIIB gp120 (Fig. 5.2.2.3.1). This finding may indicate that the VHH bind to epitopes that overlap with the epitope recognised by mAb b12, or that they bind in such a way that they sterically hinder binding of mAb b12 to gp120. Alternatively, their binding

may induce conformational changes in gp120 which inhibits subsequent mAb b12 binding. VHH A12 was found to inhibit mAb b12 binding to IIIB gp120 at slightly lower concentrations than the other VHH. This observation may suggest that VHH A12 recognises an epitope that overlaps with the b12 epitope to a greater extent, which could be in line with the neutralisation results described in chapter 4, where VHH A12 showed a broader neutralising ability compared to the other VHH, indicating that it binds to an epitope that is more conserved. It may also imply that VHH A12 bind to IIIB gp120 with a higher affinity. VHH A12 and D7 were, however, shown to bind to IIIB gp120 with similar affinities, as described in section 5.2.1.3.

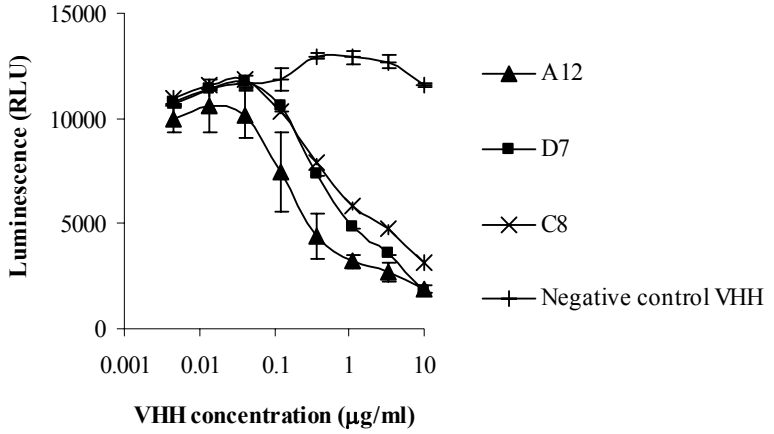


Figure 5.2.2.3.1. Dose-dependent competition of VHH A12, D7 and C8 with mAb b12 for binding to recombinant IIIB gp120 in ELISA. Serial dilutions of VHH were pre-incubated with IIIB gp120 and subsequently incubated with immobilised mAb b12. Envelope protein binding to mAb b12 was detected using the sheep polyclonal antibody D7324 followed by an AP-conjugated rabbit anti-sheep IgG antibody. Background-subtracted luminescence readings were plotted against VHH concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

5.2.2.4 The VHH compete with non-neutralising anti-CD4bs mAbs for binding to gp120 in ELISA

To further confirm that the VHH bind to CD4bs-related epitopes, they were tested for ability to inhibit the binding of additional anti-CD4bs mAbs to gp120. Human mAbs 654-D and GP68 have been shown to compete with sCD4 for binding to recombinant gp120 but can only neutralise some TCLA isolates and

not primary isolates (975, 1141, 1198, 1239). VHH A12, D7 and C8 were all able to completely inhibit binding of mAbs 654-D and GP68 to gp120, further confirming that the VHH recognise CD4bs-related epitopes (Fig. 5.2.2.4.1).

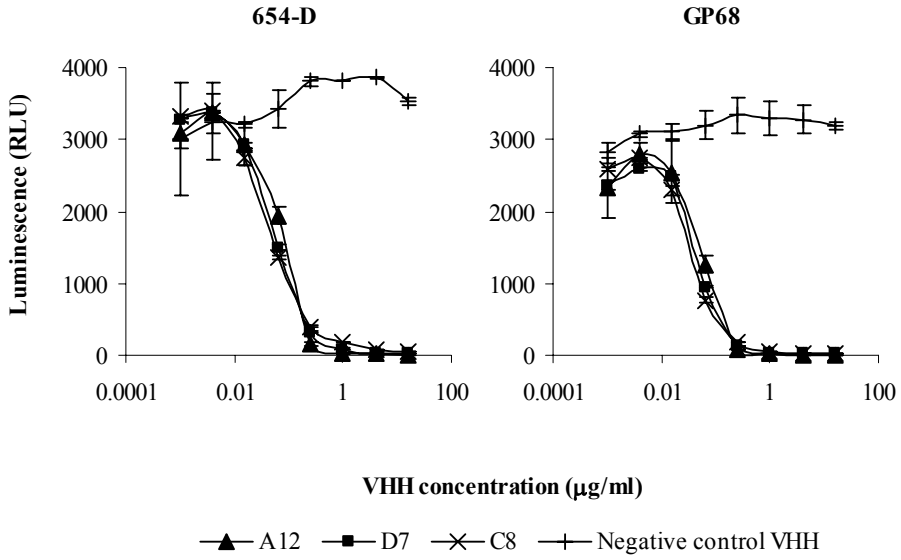


Figure 5.2.2.4.1. Dose-dependent inhibition of mAb 654-D and mAb GP68 binding to HIV-1 IIIB gp120 by VHH A12, D7 and C8. Serial dilutions of VHH were incubated with gp120 captured by antibody D7324 immobilised onto microtitre plates. Wells were washed and incubated with mAb 654-D or GP68. Binding of 654-D and GP68 to gp120 was detected using an AP-conjugated goat anti-human IgG antibody. Background-subtracted luminescence readings were plotted against VHH concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

VHH D7 and C8 were also tested for ability to inhibit binding of mAb b6 to gp120. Anti-CD4bs mAb b6 was isolated from the same individual and at the same point in time as mAb b12 (624). It competes with sCD4 for binding to envelope proteins, but can only neutralise some TCLA viruses (142, 897, 1009, 1018). VHH D7 inhibited binding of mAb b6 to HIV-1 IIIB gp120 (Fig. 5.2.2.4.2), possibly indicating that the VHH D7 and mAb b6 epitopes overlap or that VHH D7 inhibits mAb b6 binding through steric effects. VHH C8 was less potent at inhibiting mAb b6 binding to gp120, suggesting that its binding imposes less of a steric hinder for mAb b6 binding (Fig. 5.2.2.4.2). VHH A12 was not tested for ability to inhibit mAb b6 binding, as it had not been identified

at the time of the b6 experiments, and at the time of its identification, the source of mAb b6 was exhausted.

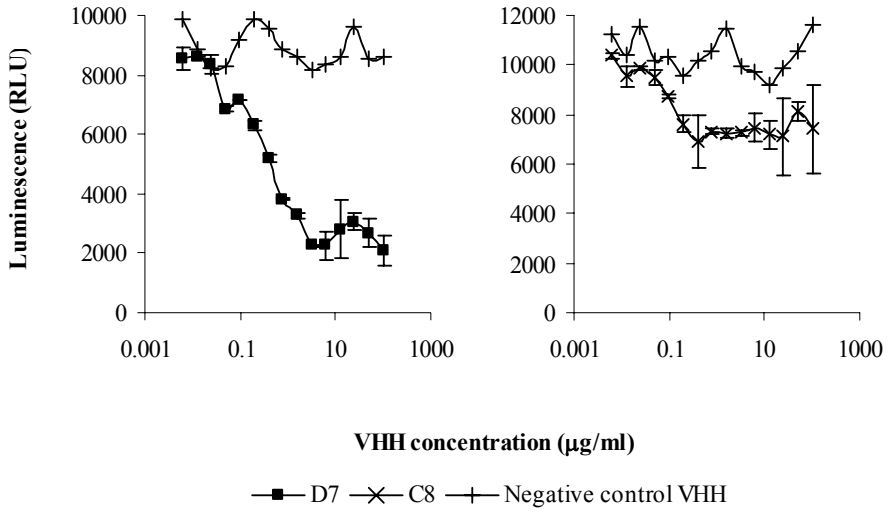


Figure 5.2.2.4.2. Dose-dependent inhibition of mAb b6 binding to HIV-1 IIIB gp120 by VHH D7 and C8. Serial dilutions of VHH were incubated with gp120 captured by immobilised antibody D7324. Wells were washed and incubated with mAb b6. Binding of mAb b6 to gp120 was detected using an AP-conjugated goat anti-human IgG antibody. Background-subtracted luminescence readings were plotted against VHH concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

5.2.2.5 The VHH compete to some extent with mAb 2G12, 447-52D and 17b for binding to gp120 in ELISA

In order to gain further understanding of the VHH epitopes, their ability to compete with mAbs to non-CD4bs epitopes of gp120 was evaluated. Included in the study was the broadly neutralising mAb 2G12, which recognises a carbohydrate motif on gp120 (981, 1013-1015), the anti-V3 mAb 447-52D, which neutralises primary isolates of subtype B (991, 1032), and mAb 17b, which recognises a CD4-induced epitope and which neutralises mainly TCLA isolates (1143). In addition, anti-CD4bs mAb b12 was included. The anti-gp41 mAb 4E10 (907, 981) was included as a control.

An inhibition ELISA was set up. Each of the VHH was coated onto microtitre plates. Titrations of the anti-gp120 mAbs were pre-incubated with IIIB gp120 and subsequently added to the VHH-coated microtitre plates. Binding of gp120

to the immobilised VHH was detected using the sheep antibody D7324, which recognises the very C-terminus of gp120, followed by an anti-sheep IgG antibody. The resulting graphs are shown in Fig. 5.2.2.5.1.

In summary, anti-CD4bs mAb b12 could clearly inhibit binding of VHH A12 and C8 to IIIB gp120. Binding VHH D7 to IIIB gp120 was inhibited to a slightly lesser extent, possible indicating that the epitopes recognised by VHH D7 and mAb b12 overlap to a lesser extent (provided that they overlap at all), or that mAb b12 binding to gp120 imposes less of a steric hinder for VHH D7 binding. However, overall, the results observed in this ELISA are in keeping with the results reported in section 5.2.2.3, where the VHH were found to inhibit binding of mAb b12 to IIIB gp120. This observation, again, indicates that the VHH bind to CD4bs-related epitopes. Some inhibition of VHH-gp120 binding was also observed for the three remaining non-CD4bs anti-gp120 mAbs, 2G12 (carbohydrate motif), 17b (CD4-induced epitope) and 447-52D (V3 epitope). It is possible that binding of these mAbs to gp120 imposes some steric hindrance, hence inhibiting VHH binding. The sheer bulk of an antibody binding to gp120 may reduce the ability of gp120 to bind the immobilised VHH. This is, however, not true for the antibody D7324, binding to the very C-terminal region of gp120, as it has been used both to capture and detect gp120 throughput this study. Including an irrelevant mAb in this competition ELISA, in this case the anti-gp41 mAb 4E10, did not lead to reduced VHH-gp120 binding, indicating that it is the binding, and not just the presence, of mAbs 2G12, 17b and 447-52D that slightly inhibits VHH-gp120 binding.

It is possible that reversing the ELISA, i.e. testing whether the VHH would inhibit binding of the mAbs to gp120, would cast further light on the extent to which these mAbs and the VHH compete for binding to gp120. It is possible that VHH binding to gp120 induces conformational changes that result in alteration of the exposure of other epitopes, such as for example the CD4-induced and V3 epitopes. Reversing the ELISA set up was, however, only carried out for mAb b12, as described in section 5.2.2.3. Strong reciprocal inhibition of binding to gp120 was observed for mAb b12 and the VHH. CD4-induced epitopes, such as the 17b epitope, have been found to be better exposed after sCD4 binding (1143),

but not after binding of anti-CD4bs mAbs (919). Whether the exposure of CD4-induced epitopes is affected by VHH binding to gp120 remains to be determined. In summary, the competition profiles of the VHH were found to be similar to competition profiles previously reported for anti-CD4bs mAbs (919, 1020), suggesting that the VHH recognise CD4bs-related epitopes.

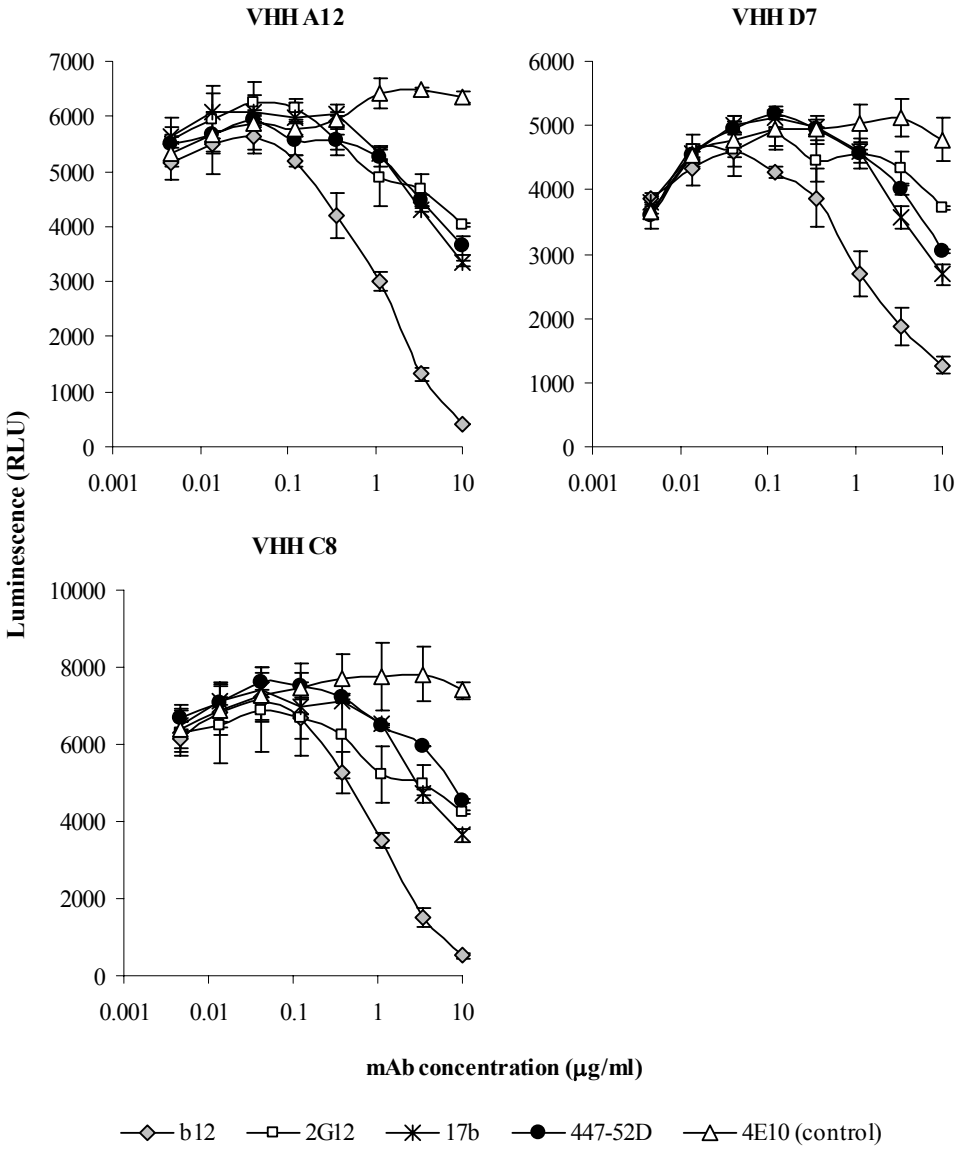


Figure 5.2.2.5.1. VHH competition with mAb b12 and mAbs to non-CD4bs epitopes of gp120 for binding to recombinant gp120 in ELISA. VHH A12, D7 and C8 were coated onto the wells of microtitre plates, as indicated above each graph. Serial dilutions of each mAb were pre-incubated with IIIB gp120 and subsequently incubated with the immobilised VHH. Envelope protein binding to VHH was detected as described in the text. Background-subtracted luminescence readings were plotted against mAb concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

5.2.2.6 The VHH compete with each other for binding to gp120 in ELISA

In sections 5.2.2.1-5.2.2.5, it was shown that VHH A12, D7 and C8 all compete with sCD4 and with anti-CD4bs mAbs for binding to gp120, indicating that they all bind to CD4bs-related regions. Earlier, in chapter 3, it was shown that VHH A12 and D7 showed high sequence similarity and could be grouped into the same sub-family, whereas VHH C8 could not. Hence, it is likely that A12 and D7, but perhaps not C8, recognise similar epitopes. In chapter 4, it was furthermore shown that VHH D7 neutralised a subset of the viruses neutralised by VHH A12, in line with the assumption that they bind to related epitopes. VHH C8, on the other hand, neutralised a different but overlapping spectrum of viruses, suggesting that it recognises a different epitope.

To further investigate whether the VHH bind to epitopes that are related, VHH A12, D7 and C8 were tested for their ability to compete with each other for binding to gp120. Each of the VHH was coated onto the wells of microtitre plates. Titrations of VHH were then pre-incubated with IIIB gp120 and subsequently incubated with the solid-phase VHH followed by detection of gp120 binding to the solid-phase VHH. The ability of fluid-phase sCD4 to inhibit binding of gp120 to each of the VHH was also assayed. An irrelevant VHH was included as a control. Results are shown in Fig. 5.2.2.6.1.

Each of the VHH was able to inhibit binding of IIIB gp120 to itself and to the other two VHH, indicating that the VHH either bind to epitopes that overlap, or that steric hindrance inhibits simultaneous binding. VHH C8 required higher concentrations than VHH A12 and D7 to completely inhibit gp120 binding to VHH A12 and D7, as well as to itself. This finding is in concordance with the higher affinity for IIIB gp120 observed for VHH A12 and D7 compared to for VHH C8 (section 5.2.1.3). VHH A12 and D7 showed similar inhibition curves, again in line with their similar affinities for IIIB gp120.

In section 5.2.2.1, it was shown that binding of the VHH to recombinant gp120 can completely inhibit sCD4 binding gp120. In this section the reverse was shown, as sCD4 was observed to completely inhibit VHH binding to gp120

binding in a dose-dependent manner (Fig. 5.2.2.6.1), further confirming that the VHH target CD4bs-related epitopes.

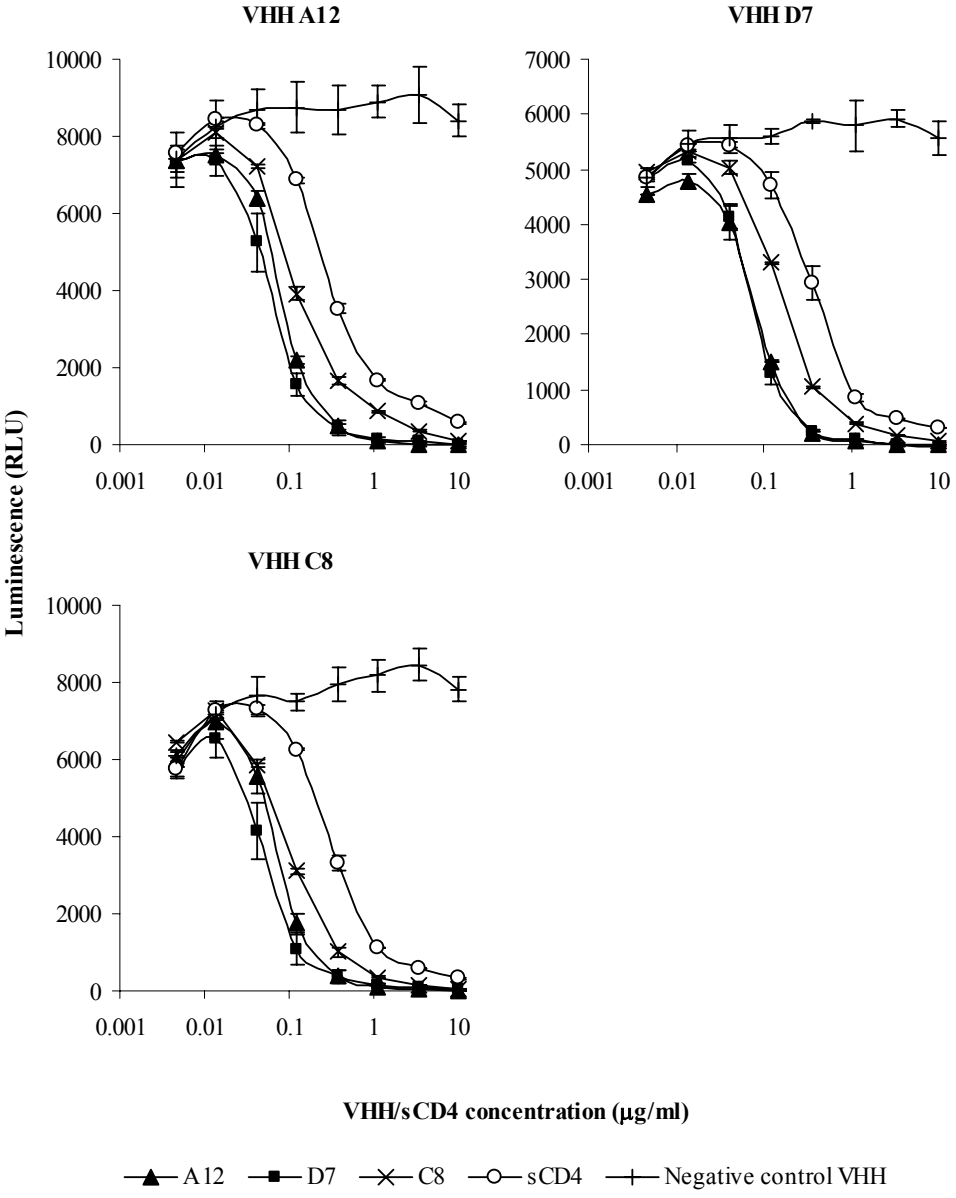


Figure 5.2.2.6.1. VHH competition with each other for binding to recombinant gp120 in ELISA. VHH A12, D7 and C8 were coated onto the wells of microtitre plates, as indicated above each graph. Serial dilutions of each VHH and sCD4 were pre-incubated with IIIB gp120 and subsequently incubated with the immobilised VHH. Envelope protein binding to VHH was detected as described in the text. Background-subtracted luminescence readings were plotted against mAb concentration. Data points represent the mean and bars the standard deviation of duplicate reactions.

5.2.2.7 Summary of VHH competition studies

In summary, the VHH were found to compete with sCD4 and four different anti-CD4bs mAbs for binding to gp120. Moreover, the VHH were found to compete with each other and to a lesser extent with three mAbs to non-CD4bs epitopes of gp120. Taken together, these observations indicate that the VHH bind to epitopes overlapping, or in proximity to, the CD4bs of gp120.

5.2.2.8 VHH D12 and F2 also compete with sCD4 for binding to gp120

The neutralisation characterisation described in chapter 4 had revealed VHH D12 and F2 as less potent than VHH A12, D7 and C8. VHH D12 and F2 had also been selected using the competitive sCD4 elution. To evaluate whether they also bound to CD4bs-related epitopes, their ability to inhibit sCD4 binding to gp120 was assayed.

VHH D12 had been selected through panning on IIIB gp120. Its ability to compete with sCD4 for binding to IIIB gp120 was therefore tested in ELISA. It was found to inhibit binding of sCD4 to gp120, indicating that it binds to a CD4bs-related epitope or binds in a way that sterically hinders binding of sCD4 (Fig. 5.2.2.8.1). This is not unexpected, since VHH D12 showed some sequence similarity to VHH A12 and D7, as discussed in chapter 3.

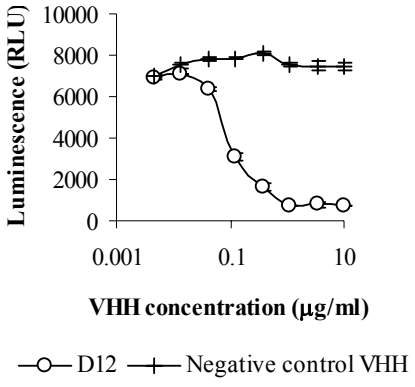


Figure 5.2.2.8.1. VHH D12 competition with sCD4 for binding to recombinant gp120 derived from HIV-1 IIIB in ELISA. Serial dilutions of VHH D12 were tested for ability to inhibit gp120 binding to solid-phase sCD4, using the experimental procedure described in section 5.2.2.1. Data points represent the mean and bars the standard deviation of duplicate reactions.

VHH F2 had been selected through panning on CN54 gp120 and had also been shown to bind to CN54 but not to IIIB gp120 in ELISA (Fig. 5.2.1.1.1 and chapter 3). Furthermore, as described in chapter 3, CN54 gp120 did not show good binding to sCD4 in ELISA, which is why the ability of VHH F2 to inhibit this sCD4-gp120 binding could not be evaluated in ELISA. Instead, it was tested in the BIAcore surface plasmon resonance assay developed in section 5.2.2.2. Unlike VHH D7 and C8, which could completely inhibit sCD4 binding to CN54 gp120, VHH F2 was only found to inhibit binding by approximately 50%, at a 10:1 VHH:gp120 molar ratio (Fig. 5.2.2.8.2). This lack of complete inhibition could indicate that VHH F2 binds to CN54 gp120 with a lower affinity compared to sCD4 and compared to VHH D7 and C8. Alternatively, VHH F2 recognises an epitope that overlaps with the CD4bs to a lesser extent or that its binding imposes less of a steric hinder for sCD4 binding.

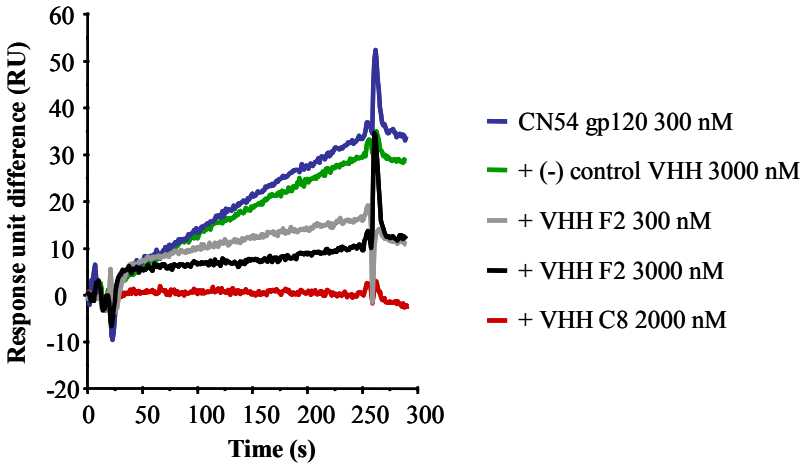


Figure 5.2.2.8.2. VHH F2 inhibition of sCD4 binding to CN54 gp120 as assayed by surface plasmon resonance. The experimental set up is described in section 5.2.2.2. Data were analysed using the BIAevaluation software. Binding of HIV-1 CN54 gp120 to sCD4 is represented as the difference between the response units (RU) observed in the cell containing the chip with sCD4 and the negative control flow cell, plotted versus time.

More work is needed to determine why VHH D12 and F2 were not as potent at neutralising HIV-1 as VHH A12, D7 and C8. It is possible that the epitopes which they recognise are not conserved or well-presented in the context of the functional envelope spike.

5.2.3 Lack of correlation between VHH reactivity with recombinant envelope glycoproteins and ability to neutralise the corresponding virus

The results described in this chapter confirm previous findings, that antibody binding to gp120 in biochemical assays does not necessarily correlate with ability to neutralise HIV-1 (141, 142, 623-626, 897, 940). This observation was made, for example, for HIV-1 CN54. As described in chapter 4, the VHH were found to neutralise HIV-1 CN54 infection of NP2/CD4/CCR5 cells to 29-62%. VHH F2 was, however, the only VHH found to bind well to CN54 gp120 in ELISA, but did not show more potent neutralisation than the other VHH, neutralising HIV-1 CN54 to 47% in the NP2 cell-based neutralisation assay. Observations made using BIAcore surface plasmon resonance studies did, however, reveal that the VHH were indeed able to interact with CN54 gp120 and inhibit its binding to sCD4. This finding is in line with the assumption that surface plasmon resonance techniques can detect weak interactions that may be undetectable by ELISA. Lack of correlation between VHH binding to CN54 gp120 and ability to neutralise CN54 virus may also be due to the CN54 gp120 construct not being a good representative of the gp120s expressed on the surface of the infectious particles present in the swarm of viruses that is the CN54 PBMC isolate.

Lack of correlation between reactivity with recombinant envelope and ability to neutralise the corresponding virus was furthermore observed for the subtype A 92UG037.A9 virus. The VHH were found to bind well to 92UG037 monomeric gp120 and trimeric gp140 in ELISA and to compete with sCD4 for this binding, but were still not able to neutralise 92UG037.A9 virus. As described in chapter 3, sCD4 was also found to bind to 92UG037.A9 envelope with high signal strength in ELISA, without being able to neutralise the 92UG037.A9 virus. There are at least two possible explanations for this observation. First, it is possible that the regions recognised by the VHH and by sCD4 are not accessible or not present on the 92UG037.A9 gp120 in the context of the functional envelope spike, despite being present on the recombinant gp120 and gp140 constructs. Second, it is possible, but perhaps less likely, that sCD4 and the VHH are indeed able to bind to the HIV-1 92UG037.A9 functional envelope spike, but that they do so in a

way that still allows for interaction of the functional spike with the receptor or the co-receptor. For instance, if HIV-1 92UG037.A9 was capable of CD4-independent infection, sCD4 and VHH binding to the functional spike might not be able to reduce virus infectivity. This notion is, however, speculative. In an initial attempt to investigate the reasons behind the 92UG037.A9 neutralisation resistance, 92UG037.A9 capability of CD4-independent infection was evaluated. HIV-1 92UG037.A9 was titrated onto CCC/CCR5 as well as CCC/CD4/CCR5 target cells. These cell lines are feline renal epithelial cell lines engineered to express human CD4 and/or human CCR5. HIV-1 SF162, known not to be capable of CD4-independent infection, was titrated in parallel as a control. HIV-1 92UG037.A9 and SF162 were able to infect CCC/CD4/CCR5 cells to a similar level as they did NP2/CD4/CCR5 cells. HIV-1 92UG037.A9, but not HIV-1 SF162, was also able to infect CCC/CCR5 cells, the target cell line lacking CD4, although very inefficiently, to a titre that was approximately 4 logs lower than the titre on CCC/CD4/CCR5 cells (Fig. 5.2.3.1). This observation indicates that the inability of sCD4 (and perhaps anti-CD4bs antibodies such as the VHH) to inhibit 92UG037.A9 infection is not due to ability of the 92UG037.A9 virus to infect target cells in a CD4-independent manner. Further studies, possibly involving fluorescent activated cell sorter (FACS) analysis of infected cells, are needed to shed light on why sCD4 and the VHH are unable to neutralise 92UG037.A9 virus despite being able to bind to 92UG037 gp140.

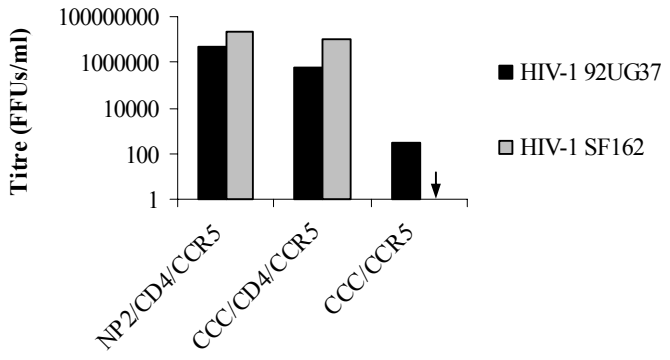


Figure 5.2.3.1. HIV-1 92UG037.A9 titre on cells lacking CD4. HIV-1 92UG037.A9 and SF162 were titrated onto NP2/CD4/CCR5, CCC/CD4/CCR5 and CCC/CCR5 cells. At 72 hours post-infection, cells were fixed and stained for HIV-1 p24 protein, as described in chapter 2. Titre was determined as number of focus-forming units (FFU) per ml.

Overall, the observation that the VHH could bind to recombinant gp120 and gp140 derived from HIV-1 92UG037 in ELISA, despite not being able to neutralise 92UG037.A9 virus, may suggest that these recombinant envelope constructs are not good representatives of the functional spike. Such antigens may not be suitable as an immunogens and for panning of VHH libraries.

Interestingly, VHH binding to recombinant 92BR025.C1 gp120 seemed to correlate with ability to neutralise 92BR025.C1 virus. A12 and C8 were able to bind well to recombinant 92BR025.C1 gp120 in ELISA, whereas D7 bound with weaker signal (Fig. 5.2.2.2). This observation correlates with the corresponding neutralisation data, where A12 and C8 neutralise HIV-1 92BR025.C1 with IC_{50} titres of 0.2 and 2 μg/ml, respectively, whereas the IC_{50} for D7 were found to be >50 μg/ml (Table 4.2.4.3.1). This finding may indicate that recombinant 92BR025.C1 gp120 represents a good mimic of the 92BR025.C1 gp120 structure in the functional spike. This notion is, however, speculative and further studies are needed to evaluate the antigenic properties of this gp120.

5.3 Discussion

In this chapter, it has been shown that the VHH bind to recombinant envelope proteins in two different biochemical assay settings, and that they do so with affinities that are close to the suggested affinity ceiling for *in vivo* maturated antibodies. Furthermore, it has been shown that they compete with sCD4 for this binding, indicating that they recognise epitopes that overlap or are in proximity to the CD4bs. Envelope binding competition analysis, using a set of anti-gp120 mAbs with defined epitopes, further confirmed that the VHH somehow target the CD4bs. Hence, it is likely that the VHH neutralise HIV-1 by interacting with the envelope spike prior to its interaction with CD4.

For VHH A12 and D7, the K_D values obtained in surface plasmon resonance experiments differ from the concentrations yielding half-maximal binding to IIIB gp120 in the direct binding ELISA described in Fig. 5.2.1.2.1, whereas for VHH C8 the values are in agreement. Such discrepancies have been described previously (1240).

The results described in this chapter further highlight the cross-subtype reactivity of the selected VHH. VHH A12, D7 and C8 were found to bind to envelope proteins of subtype A, B and C, indicating that they bind to a motif that is relatively conserved across HIV-1 subtypes. The lack of ability of the VHH to neutralise some of the corresponding viruses does, however, indicate that their epitopes are either not accessible or not present in the context of the functional envelope spike, despite being present on the soluble recombinant envelopes.

Thus, the observations described in this chapter confirm previous findings that antibody binding to recombinant envelope proteins not necessarily correlates with ability to neutralise virus. Such a result is expected, since the structure of soluble, recombinant envelope glycoproteins is likely to be different from the structure of the envelope glycoproteins in the context of the functional spike (138, 140, 259). This has been previously observed in, for example, the case of mAbs b12 and b6, where both b12 and b6 compete with sCD4 for binding to envelope proteins, whereas b12 is the only of the two that is able to neutralise the corresponding virus (1009). The non-neutralising human mAb b6 can capture virus particles and inhibit capture by mAb b12, but has no effect on the neutralisation activity of b12, suggesting it can bind to non-functional envelope spikes present on the virus surface (142, 897, 1018, 1194). Whether the VHH can bind to non-functional spikes present on the surfaces of viruses that they are unable to neutralise, as in the case of mAb b6, has yet to be determined.

In conclusion, the results reported in this chapter have shown that the VHH behave as anti-CD4bs antibodies. More work, such as alanine scanning of gp120 as well as structural studies, would shed further light on their epitopes. The crystal structure of the unliganded VHH D7 has been solved to a resolution of 2.4 Å by Winfried Weissenhorn at the European Molecular Biology Laboratory (EMBL) in Grenoble, France. This structure, in combination with molecular modelling experiments and mutagenesis studies, may provide insight into the way in which VHH D7 and the D7-like VHH interact with gp120. Atomic structures of the VHH in complex with recombinant gp120 would define their precise epitopes. Once their epitopes are known, they may prove useful in characterising recombinant envelope proteins.

Chapter 6

Final discussion

Our study shows that HIV-1 neutralising VHH can be isolated from llamas immunised with recombinant gp120 (1241). To increase the chances of isolating neutralising VHH, we employed a functional selection approach which enabled the identification of VHH that compete with CD4. By panning of phage libraries displaying the VHH repertoires of the immunised animals on recombinant gp120, and by introducing a competitive elution step using sCD4, we could select for VHH targeting the CD4bs of gp120. Immunising with gp120 derived from an HIV-1 CRF07_BC primary isolate, followed by panning on gp120 from either a subtype B virus or sequentially on gp120 from subtypes A, B and C, allowed for selection of VHH with cross-subtype neutralising activity. Three VHH (A12, D7 and C8) able to neutralise HIV-1 primary isolates of subtype B and C were identified. These were shown to bind to recombinant envelope proteins with affinities that are close to the suggested affinity ceiling for *in vivo* maturated antibodies, and to compete with sCD4 and anti-CD4bs mAbs for this binding, indicating that they somehow target the CD4bs of gp120. In light of this observation, it is likely that they are able to inhibit HIV-1 entry into target cells by interacting with the functional envelope spike prior to its interaction with the main cellular CD4.

Given the unique properties attributed to VHH, in terms of solubility, thermal and chemical stability, and high expression levels leading to a low production cost (1109, 1119, 1121, 1122), neutralising VHH might prove useful in a number of applications, for example as candidate HIV-1 microbicides as well as anti-retroviral drugs or prophylactics. Topical application of mAb b12 has been shown to protect macaques from infection after vaginal challenge with SHIV, which supports the potential use of antibodies for topical prevention of HIV-1 transmission (668). Further studies are needed to establish whether the identified VHH would have preventive effects *in vivo*. Anti-envelope VHH may also be of use in characterising epitopes on HIV-1 envelope proteins. Attempts to design an

HIV-1 immunogen that can elicit a broadly neutralising humoral immune response have so far failed (259, 754, 1229), although a recent study reports that extensively cross-reactive antibodies (albeit at low titre) have been induced by immunisation of rabbits with a recombinant gp140 (1046). It is possible that a set of neutralising and non-neutralising VHH may become useful in the design of such an immunogen.

The stability and solubility of VHH allows for engineering of multivalent and bispecific molecules (1123). Engineered multivalent VHH to other antigens have previously been shown to exhibit better binding and neutralising properties than their monovalent counterparts (1124). It is possible that introduction of multivalency will increase the potency of the identified anti-gp120 VHH. Construction and evaluation of such multivalent VHH, using the VHH identified in this study, is ongoing, but has so far not led to increased neutralisation potency or breadth (data not shown). Selecting for VHH recognising other epitopes and linking them to the CD4bs-targeted VHH described in this study, thus producing a bispecific VHH, might also result in more potent binding and neutralisation properties.

The neutralising activities of the VHH were evaluated in the TZM-bl neutralisation assay against a panel of 65 HIV-1 isolates from subtype A, B, C, D, CRF02_AG and CRF07_BC. The most potent and broadly reactive VHH was A12. VHH A12 was able to neutralise 59% of the subtype B viruses and 48% of the subtype C viruses included in the study on an IC_{50} level, compared to 77% and 56% for the broadly neutralising anti-CD4bs mAb b12. The difference in neutralisation breadth between VHH A12 and mAb b12 was even more pronounced at an IC_{90} level. While mAb b12 neutralised 64% of subtype B viruses and 36% of subtype C viruses on an IC_{90} level, VHH A12 neutralised only 36% of subtype B viruses and 22% of subtype C viruses, indicating that VHH A12 is less potent and less broad in its cross-subtype neutralisation ability than mAb b12. Moreover, the VHH were less potent at neutralising HIV-1 in primary PBMC. Furthermore, it should be noted that the VHH are approximately ten times smaller than mAb b12, making mAb b12 more potent on a molar level. On the other hand, mAb b12 has two antigen-binding sites. To be able to make a

direct comparison of neutralisation ability, the VHH would need to be expressed in the context of a full immunoglobulin.

Interestingly, the VHH were found to neutralise a different spectrum of viruses compared to mAb b12. These differential neutralisation reactivities may indicate that the VHH and mAb b12 recognise different epitopes on gp120, despite competing with each other for binding to gp120 in ELISA. VHH A12 and C8 were also observed to neutralise different but overlapping sets of viruses, indicating that they may be directed at different CD4bs-related regions of gp120. VHH D7, on the other hand, was observed to neutralise a subset of the viruses neutralised by VHH A12. This finding together with the notion that VHH A12 and D7 showed a high degree of sequence similarity indicated that they may recognise similar epitopes. To further characterise the VHH epitopes and to determine whether they overlap with the mAb b12 epitope, additional studies are needed, including structural studies of VHH in complex with gp120, mutagenesis studies, and neutralisation escape studies. Determining the epitopes of these VHH may provide additional information about vulnerable sites in proximity to the CD4bs of gp120.

The CD4bs is thought to be a good target for HIV-1 vaccine design (754), as it is functionally conserved, and furthermore targeted by the extensively studied, broadly neutralising mAb b12, which binds to an epitope overlapping the CD4bs (1008). In addition, Li *et al*. (985) have recently mapped the specificities of the neutralising activity in some broadly neutralising sera from HIV-1-seropositive individuals to the CD4bs. These anti-CD4bs antibody fractions were able to neutralise viruses not neutralised by b12, indicating that they contain antibodies directed at epitopes that may be different to the b12 epitope. Moreover, in a recent study, Binley *et al*. (1242) mapped the neutralising activities in plasma from 24 individuals with broad neutralising responses, and found that a large proportion of the neutralising activity (50-99%) was directed to CD4bs epitopes in 9 out of the 24 plasmas, further highlighting the notion that the CD4bs is a good target for vaccine design. In contrast, antibodies to the V3 region or to 2G12-like epitopes did not seem to contribute to the neutralisation activity. However, the specificities of around two-thirds of the neutralising activity could

not be determined, indicating that the sera contained neutralising antibodies directed against epitopes that have not yet been characterised. The characterisation of such novel neutralising epitopes may aid vaccine development. It is possible that a panel of anti-envelope VHH, which can be selected from immunised llamas using the versatile phage display technology, may help to define such epitopes. More specifically, a panel of neutralising VHH recognising CD4bs-related epitopes may help to further characterise the functionally important CD4bs, and may possibly help to define additional neutralising epitopes in the vicinity of it.

The immunogen, CN54 gp120, showed poor binding to sCD4 in biochemical assays and did not mediate infection when expressed on viruses. It was chosen as immunogen due to its availability before these properties were known. Nonetheless, despite immunising with an immunogen that appeared to be non-functional, broadly neutralising VHH could be isolated. It is possible that immunising with an immunogen that better exposes the CD4bs would have resulted in more potent and broadly reactive VHH. For example, a gp120 construct engineered to be locked in a CD4-bound conformation by the introduction of two point mutations in the Phe43 cavity has been shown, by Dey *et al*. (1066), to be better at eliciting neutralising antibodies compared to the corresponding native gp120. Immunising with such an antigen, alternatively including it in the panning step, may result in the isolation of more potent and broadly neutralising VHH.

Overall, immunising llamas and selecting for anti-CD4bs VHH using an antigen that is a better representation of the functional envelope spike might result in VHH with broader cross-subtype neutralising activities and lower inhibitory doses. Again, identification of such an envelope antigen is one of the major challenges when screening for agents that can act as HIV-1 entry inhibitors as well as in HIV-1 vaccine design (259). Previous studies have shown that purified trimeric envelope proteins are somewhat better at eliciting cross-subtype-neutralising antibodies than monomeric recombinant gp120 (1036, 1038-1041, 1243). Immunising llamas with recombinant CN54 gp140 in this study did not, however, result in the isolation of neutralising VHH.

Zhang *et al*. (1046) immunised rabbits with a recombinant gp140 construct from an HIV-1-seropositive individual whose serum exhibited broad neutralising activity, and found that it elicited broad, although not so potent, neutralising serum responses. It is possible that immunising llamas with recombinant envelope glycoproteins from individuals with broadly neutralising antibody responses, or including such an antigen in the panning protocol, may result in increased chances of isolating broadly neutralising VHH. Additional VHH selection strategies may include panning on virus-like particles or infected cells to expose them to native spike, followed by elution with sCD4.

In this study, the phage libraries were panned on gp120 derived from the TCLA isolate IIIB, or on gp120 derived from HIV-1 primary isolates of subtype A or C. Panning was carried out on each antigen alone, or on the same antigens alternated in various combinations in two subsequent rounds of panning, always using the competitive elution with sCD4. Interestingly, using this method of selection, we could select for VHH that could bind to envelope proteins of subtype A, B, and C, regardless of which of the above antigens that were used in the panning. However, when selecting using HIV-1 IIIB gp120 only, a range of VHH were isolated that could be grouped into two families, whereas when the subtype A and C envelopes were included in the panning, only the most potent and broadly reactive VHH in the set were selected. This finding suggests that alternating the antigen in the panning procedure enables selection of VHH with better cross-reactive properties. Sequential alternation of the antigen in the panning step has previously been shown to result in the isolation of antibody fragments with more broad and potent neutralising activity (1020, 1205, 1206).

In conclusion, this study provides proof-of-concept that the camelid immune system can be used to generate anti-envelope VHH with relatively broad neutralising activity across HIV-1 subtypes. Three cross-reactive VHH able to neutralise several HIV-1 primary isolates of subtype B and C were characterised. These VHH competed with sCD4 and anti-CD4bs mAbs for binding to recombinant gp120 and gp140. To our knowledge, this is the first description of broadly neutralising mAbs to HIV-1 envelope, which are derived from an immunised animal, as all previously reported broadly neutralising anti-HIV-1

mAbs have been a result of natural infection rather than immunisation (259, 754, 1229). The results indicate that heavy-chain antibody fragments have a possible use as potent HIV-1 entry inhibitors. Since VHH are stable and can be produced at a relatively low cost, they may be considered for applications such as HIV-1 microbicide development. Combined with structural studies, such VHH could be used to characterise conserved neutralising epitopes on the HIV-1 envelope spike, which may be of value for HIV-1 immunogen design.

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