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Functional Studies on the Interaction of Imunoglobulins with HIV-2 Envelope

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Abstract

Infection with HIV-2 has a much more limited geographic distribution than HIV-1, being less readily transmitted and is generally less pathogenic. The role of immunoglobulins in defence against pathogens in different body compartments is known, where IgG is the principal isotype in the blood and extracellular fluid while IgA predominates in the mucosal compartment. The aim of this thesis was to study the interaction of IgA and IgG with the HIV-2 envelope in an attempt to understand the role of the humoral immune response in controlling HIV-2 infection.

Since HIV is a sexually transmitted disease, IgA may play a role in controlling transmission of HIV-2 across mucosal surfaces. IgA was purified from the sera of HIV-2 infected individuals. The antigenic sites of HIV-2 envelope important for binding IgA were studied using recombinant gp105 and gp36, as well as peptides spanning the whole envelope. Prominent IgA binding was defined to the central region of gp36, corresponding to residues 644-658. The purified IgA demonstrated neutralizing activity against HIV-2 in 59% of the sera tested, suggesting a role for IgA in preventing HIV-2 transmission.

The V3 region expressed on the HIV envelope has been identified as a main neutralization target for both HIV-1 and HIV-2. However, studies have indicated that this site is not exposed in HIV-1 primary isolates. To study the exposure of the V3 region, both functionally and structurally, the envelope gene from a primary CD4-independent HIV-2 isolate was cloned, and two constructs of HIV-2 gp125 were designed as previously described for HIV-1 gp120, where in the gp125$\Delta v_1 v_2$ construct the V1/V2 region was excluded. These gp125 constructs were expressed in CHOlec cells as glycosylated proteins with minimal glycosylation heterogeneity, and were characterized using circular dichroism and mass spectrometry analyses.

Two V3-specific murine monoclonal antibodies (7C8 & 3C4) were used to study the exposure of the V3 region on gp125, 7C8 being linear site-specific, while 3C4 is conformationally sensitive. Using different lectin affinity chromatography techniques, both monomeric and oligomeric forms of the gp125 proteins were purified. These monomeric and oligomeric gp125 proteins were recognized by 7C8, while only gp125 oligomers were recognized by 3C4. Previous studies have demonstrated that HIV-1 binding to CD4 causes conformational changes in the envelope, where the V1 and V2 loops reorient and expose the V3 region. Surface plasmon resonance analysis of CD4 binding to gp125 and gp120 glycoproteins demonstrated that CD4 binds to gp125 at a lower affinity and with different kinetics compared to gp120. Molecular modeling of gp125 revealed a residue that fills the hydrophobic cavity implicated in CD4 binding which may be responsible for the CD4-induced conformation of gp125. Furthermore, the accessibility of the V3 region in polymeric gp125 suggests that gp125 does not require CD4 to reorient the position of the V1 and V2 loops in order to expose the V3 region.

To understand the role of the gp125 V3 region in neutralization of HIV-2, the murine antibody responses induced by immunization with recombinant gp125 proteins, and the neutralization capacity of V3-specific murine mAbs were studied. Antibodies at titres of 8000 to 128000 recognizing gp125 or gp125$\Delta v_1 v_2$ were induced in the sera of the immunized mice with either recombinant gp125 protein. However, none of the sera neutralized HIV-2 or bound a peptide spanning the center and C-terminus of the V3 region. While neither 7C8 nor 3C4 mAbs neutralized HIV-2, their respective Fab fragments blocked infection.

In conclusion, the results from this thesis suggest a role for IgA and V3-specific IgG in controlling infection by HIV-2. The analysis of the conformation of gp125 and the exposure of the V3 region presented in this work could provide an explanation underlying the CD4-independence observed for most HIV-2 isolates. However, while the V3 region may be exposed on HIV-2, smaller Fab fragments are required to neutralize HIV-2. The specific conformation of gp125, exposing the V3 region, and V3-specific small molecules may provide clues for the design of vaccines against HIV.

Keywords: HIV, Glycoprotein, Antibodies IgA/IgG, V3, CD4, Neutralization, Immunization

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To My Parents

"One becomes entitled to ask questions and seek illumination only if one has observed control of the senses and always followed *Truth*, and only then will one's questions deserve to be answered."

The Bhagavad Gita; Chapter 2

According to *Mahatma Ghandi.*

List of Papers

This thesis is based on the following papers and manuscripts:

I. Qin Lizeng, Pia Skott, **Samer Sourial**, Charlotta Nilsson, Sören Andersson, Mariethe Ehnlund, Nuno Taveira and Ewa Björling.
Serum immunoglobulin A (IgA)-mediated immunity in human imunodeficiency virus type 2 (HIV-2) infection.
Virology 2003, 308;225-232.

II. **Samer Sourial**, Anette Wärnmark, Charlotta Nilsson, Ewa Björling, Adnane Achour and Robert Harris.
Cloning, expression and purification of HIV-2 gp125: A target for HIV vaccination.
Molecular Biotechnology 2005, 31:155-162.

III. **Samer Sourial**, Charlotta Nilsson, Anette Wärnmark, Adnane Achour and Robert Harris.
Characterization of the V1 and V2 loop deletion on the accessibility of the V3 loop in HIV-2 gp125.
Manuscript submitted.

IV. **Samer Sourial**, Qin Lizeng, Adnane Achour, Robert Harris and Charlotta Nilsson.
HIV-2 V3-specific Fab fragments neutralize HIV-2 and SIV.
Manuscript.

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Abbreviations

AA	amino acid
Ab	antibody
ADCC	antibody-dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
C1-5	constant region 1-5
CA	capsid protein
CAF	cellular antiviral factor
CDC	complement-dependent cytotoxicity
ConA	concanavalinA
CTL	cytotoxic T lymphocyte
DB	dot blot
DC	dendritic cell
dIgA	dimeric IgA
ELISA	enzyme linked immunosorbent assay
ENV	envelope protein
ESN	exposed seronegative
Fv	variable fragment of antibody
GN	galanthus nivalis
gp125, gp36	glycoprotein125, glycoprotein36
GS	glutamine synthetase
HA	hemagglutinin-A
HIV	human immunodeficiency virus
IFN	interferon
Ig(A/G/M/E)	immunoglobulin(A/G/M/E)
IgA_{sec}	secretory IgA
IL	interleukin
IN	integrase enzyme
IP	immunoprecipitation
IPC	IFN producing cell
k_a	association constant
k_d	disassociation constnat
LTR	long terminal repeats

Amino acid

A	alanine
C	cysteine
D	Apartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
N	aspargine
P	proline
Q	glutamate
R	arginine
S	serine
T	threonine
V	valine
W	tryptophane
Y	tyrosine

MA	matrix protein
mAb	monoclonal antibody
MBL	mannose binding lectin
MSX	methionine sulphoxamine
МΦ	macrophage
nAb	neutralizing Ab
NC	nucleocapsid protein
NK	natural killer cell
PBMC	peripheral blood mononuclear cell
PR	protease enzyme
rgp125	recombinant gp125
RT	reverse transcriptase
SC	secretory component
SDF	stromal-derived factor
sIgA	serum IgA
SIV	simian immunodeficiency virus
SLPI	secretory leukocyte protease inhibitor
SP	signal peptide
SPR	surface plasmon resonance
SU	surface unit
TC	thrombin cleavage site
TCLA	T cell line adapted
T_H *(cell)*	T helper *(cell)*
TLR	toll like receptor
TM	transmembrane
TNF	tumor necrosis factor
V1-5	variable region 1-5
V_L	light chain variable region
V_H	heavy chain variable region
WB	western blot

1. Introduction

More than two decades have passed since the isolation of the human immunodeficiency virus (HIV; *Barre-Sinoussi, 1983*), which is associated with the clinical syndrome named Acquired Immune Deficiency Syndrome (AIDS; *Gallo, 1984; Levy, 1984*). Despite extensive HIV research, AIDS remains a global threat, with more than 40 million persons being estimated to be living with HIV (*UNAIDS, 2004*).

While HIV-1 is the main causative agent for AIDS cases worldwide, infection with HIV-2 is geographically restricted (*Schim van der Loeff, 1999*). Moreover, HIV-2 is less readily transmitted and has a lower pathogenicity than HIV-1 (*Jaffar, 2004*). Other characteristics that distinguish HIV-2 from HIV-1 are summarized in Table 1. Differences between HIV-1 and HIV-2 could be attributed to the phylogenetic relationship between HIV-1, HIV-2 and the simian immunodeficiency virus (SIV), where HIV-2 is closer to SIV_{SM} (isolated from the sooty-mangabey monkeys), than to HIV-1 (Figure 1).

	HIV-1	**HIV-2**	
Geographic distribution	Global	West Africa	*Reeves, 2002*
Sexual spread	Higher	Lower	*Kanki, 1994*
Mother-to-child transmission	20-25%	<5%	*Schim van der Loff, 1999.*
Time to AIDS	10-12 years	>20 years	*Marlink, 1994*
Genetic similarity*	40-60%		*Guyander, 1987*

Table 1 *Comparison of HIV-1 and HIV-2 features.*

*Genetic variation is dependent on gene analyzed.

The origin of HIV is disputed, yet it has been suggested that these viruses have entered the human population through zoonotic, or cross-species transmission from non-human primates (*Hahn et al., 2000; Marx et al., 2004*). HIV-1 shares an identical genomic organization as that of SIV infecting chimpanzees (SIV_{CPZ}), whereas the genomic organization of HIV-2 and SIV_{SM} are identical (*Hrisch, 1989; Huet, 1990*). While SIV_{CPZ} and SIV_{SM} do not cause disease in their natural hosts (*Hrisch, 1989; Gao, 1999*), there are reports on the viral

infection of new (unnatural) simian hosts by SIV_{sm}, which resulted in pathology (*Murphey-Corb, 1986; Chakrabarti, 1986*).

HIV-2 appears to be less virulent in humans than HIV-1, with only a smaller proportion of HIV-2-infected individuals developing AIDS (*Poulsen, 1997*). Since HIV-1 is responsible for most AIDS cases reported, most HIV research has been focused on this virus. However, identification of the factors responsible for the lower pathogenicity of HIV-2 may assist in learning more about the virus and host factors involved in HIV pathogenesis, which may in turn assist in vaccine development.

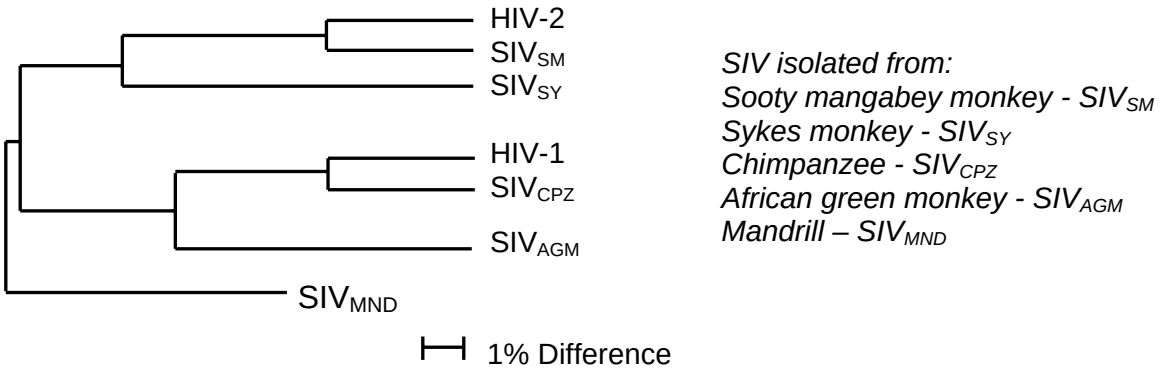


Figure 1 *Phylogenetic relationship among primate lentiviruses.*

Representative lentiviruses compared by using *pol* gene nucleotide sequences. Adapted from *Flint, 2000.*

1.1. HIV

HIV and SIV are members of the lentivirus subfamily of retroviruses which infect human and non-human primates. Lentiviruses are enveloped RNA viruses that have a slow onset of disease, causing neurological disorders and immunosuppression (*Murray, 2002*).

1.1.1. The virion

Both HIV-1 and HIV-2 have similar genetic organizations encoded in a single positive-stranded RNA (Figure 2). The genome is flanked by long terminal repeats (LTR) and consists of structural *(gag, env)*, enzymatic *(pol)*, regulatory *(tat, rev)*, and accessory genes *(nef, vif, vpr/vpx)*. The genome of HIV-2 (9670 nucleotides) is slightly longer than the genome of HIV-1 (9200 nucleotides), which is partly due to larger LTRs at the ends of the HIV-2 genome (*Guyander, 1987*). The accessory gene *vpu* is unique for HIV-1 and SIV_{CPZ} (Huet, 1990), and

is replaced with *vpx* in HIV-2 and SIV_{SM} (*Hirsch, 1989*). The functions of HIV gene products are summarized in Table 2.

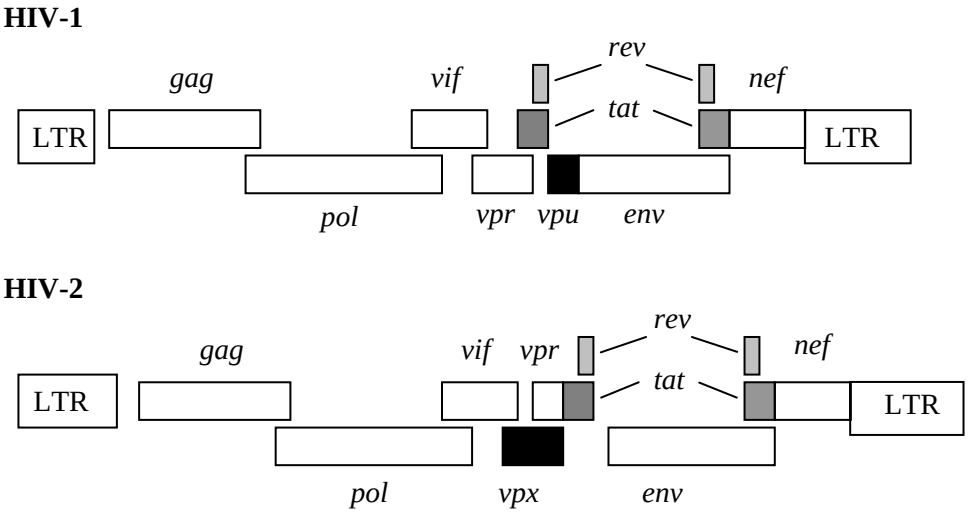


Figure 2 *Genomes of HIV-1 and HIV-2.*

Gene		**Gene product/function**
gag	Group-specific antigen	Matrix, capsid, and nucleocapsid proteins
pol	Polymerase	Reverse transcriptase, protease and integrase enzymes
env	Envelope	Surface unit and transmembrane glycoproteins
tat	Transactivator	Stimulation of transcription
rev	Regulator of viral expression	Regulation of viral mRNA production and transport of unspliced transcripts from nucleus
vif	Viral infectivity	Increases viral infectivity and affects virion assembly
vpr	Viral protein R	Transports DNA to nucleus, increases virion production and causes cell arrest
vpu	Viral protein U	Affects virus release and downregulates CD4
vpx	Viral protein X	Nuclear entry of preintegration complex
nef	Negative factor	Augments viral replication and downregulates CD4

Table 2 *Function of HIV gene products.*

A mature HIV virion comprises two identical RNA copies stabilized by nucleocapsid proteins (NC). Together with the viral protease (PR), integrase (IN) and the reverse transcriptase (RT), the RNA strands are engulfed in a cone-shaped core composed of capsid (CA) proteins. A shell composed of matrix (MA) proteins surrounds the core and is associated with a lipid bilayer, to which the transmembrane (TM) glycoprotein and other host cell-derived molecules are anchored. The surface unit (SU) glycoprotein is non-covalently associated with the TM glycoprotein, forming a heterodimer. These in turn form trimers on the surface of the virion.

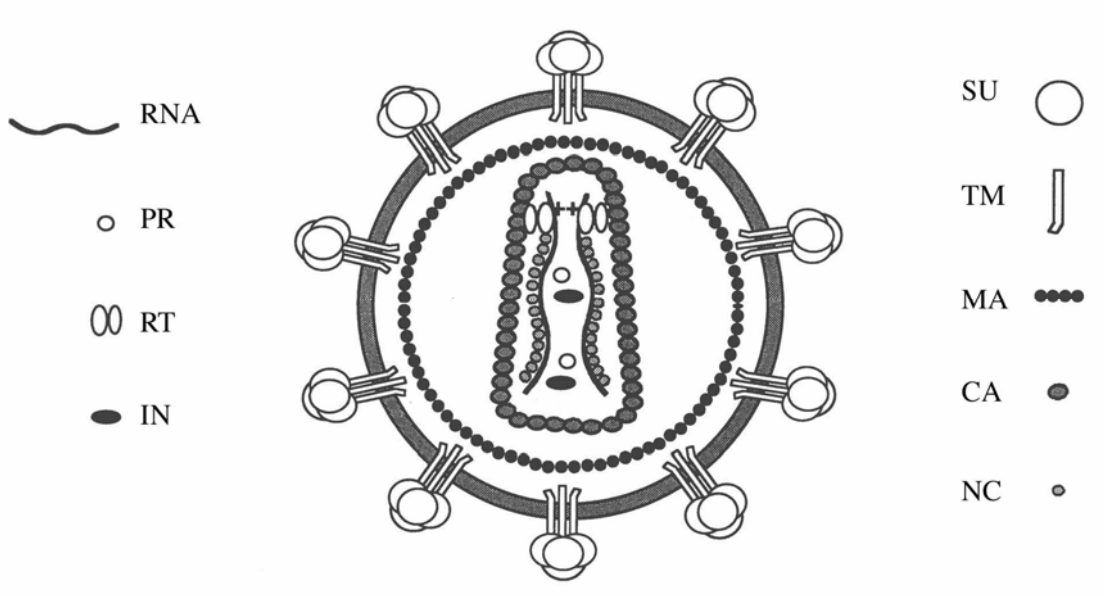


Figure 3 *The molecular organization of HIV virion.*
Modified from *Luciw, 1996*. (Courtesy of Mörner A).

The SU of HIV consists of five conserved domains (C1-C5) and five variable regions (V1-V5), with several N-linked glycosylation sites and conserved disulfide bonds (*Modrow, 1987; Starcich, 1986; Willey, 1986*). The SU and TM glycoproteins of HIV-1 are called gp120 and gp41, respectively, while gp125 and gp36 are the respective SU and TM glycoproteins of HIV-2.

Structural studies of the core of gp120 have indicated that this glycoprotein folds into two domains with a four-stranded "bridging" β-sheet (*Kwong, 1998*). Figure 4 depicts the domains of gp120, where computational-modelling suggested the exposure of the "outer" domain on trimeric spikes on HIV virions, while the "inner" domain is predominantly concealed (*Wyatt, 1998a*).

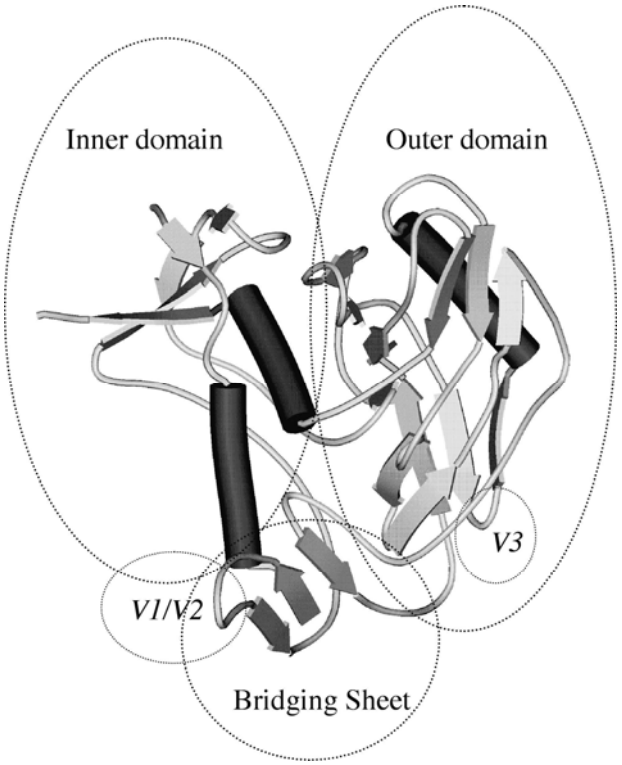


Figure 4 *Structure of the core of gp120 (Kwong, 1998).*

The inner and outer domains, β-strands "bridging sheet" and the V1/V2 as well as the V3 domains are encircled. α-helices and β-strands are depicted as barrels and arrows, respectively.

1.1.2. Transmission & propagation

HIV is most commonly transmitted through virus contact with mucosal surfaces during sexual contact (*Mestecky, 1994*). Other main routes include vertical transmission from mother to infant, needle stick (in drug-use or accidents), blood transfusion and organ transplantation (*Mastro, 1998*).

While heterosexual transmission accounts for >80% of all HIV infections in Africa (*Piot, 1994*), the sexual transmission of HIV-2 is less efficient than for HIV-1 (*Kanki, 1994*). This is illustrated in the prevalence rates of HIV-1 and HIV-2, where a substantial portion of HIV-1-infected individuals are at age of 15-20 years, and the peak prevalence is at age 20-30 years (*Anderson, 1991*), whereas only few HIV-2-infected individuals are at age 15-20 years, and the rise in prevalence is up to 50-55 years (*Poulsen, 1989*). Furthermore, a recent study suggested that sexual transmission might not be sufficient to establish an HIV-2 epidemic and that parenteral exposure (use of injections, vaccination campaigns, blood transfusion and traditional practices) might be the principal route for HIV-2 spread (*Gomes, 2003*). The mother-to-child transmission is not a principal route for HIV-2 spread, as it only occurs in <5% of the cases studied (*Andreasson, 1993; Adjorlolo-Johnson, 1994*).

There are a number of mechanisms for the transmission and propagation of HIV after contact with the mucosal surfaces (Figure 5):

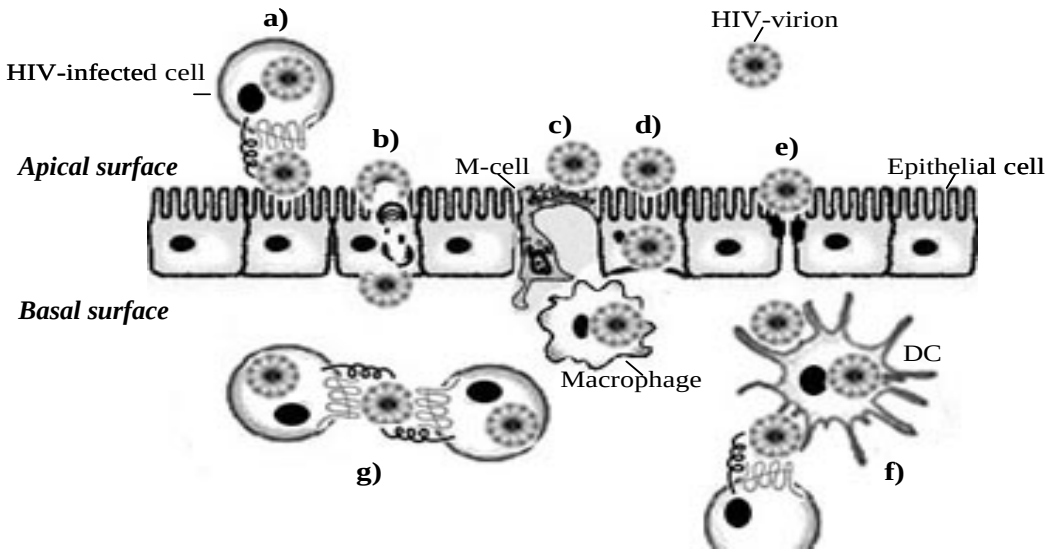


Figure 5 *Possible mechanisms for HIV transmission and propagation at mucosal surface.*

a) HIV-infected T cell or macrophage causes cell-to-cell infection of epithelial cells via receptors such as galactosyl ceramide (*Shattock, 2003; Delezay, 1997*).

b) Cell-free virions interact with various potential receptors on epithelial cells (*Pierson, 2003*).

c) HIV is trancytosed across mucosal microfold cells (M cells), which are specialized cells that bind macromolecules or microorganisms at their apical surface and transport to the basal surface without degradation (*Amerongen, 1991; Neutra, 1996*). Once past the epithelial barrier, immunocompetent cells can be infected (*Kraehenbuhl, 2000*).

d) HIV-infected T cell engages an epithelial cell, and the virus is transcytosed across the cell into the underlying tissue, infecting immunocompetent cells (*Bomsel, 1997; Spira, 1996*).

e) Disruption of epithelial cell layer of mucosal surface allows direct access of HIV virions to immunocompetent cells (*Pope, 2003*).

f) Dendritic cells (DC) at mucosal surface capture and internalize cell-free virions through interaction of DC-*SIGN* receptors and present them to T cells which can get infected (*Turville, 2002*).

g) Infected T cells can infect another T cell through cell-to-cell transmission without the release of free virions (*Piguet, 2004*).

1.2. HIV Infection

Most of what is known about HIV infection is based on studies of HIV-1. The course of HIV infection evolves into three phases (*Murray, 2002*); an acute infection followed by a chronic latent infection and the final AIDS phase. The acute phase lasts approximately two weeks and is characterized by rapid viral replication with a burst in plasma viremia (*Clark, 1991; Daar, 1991*). After the acute phase the plasma viremia drops to a relatively low set point due to effective host immune responses (reviewed in *Fauci, 1996*) and the chronic phase commences. HIV-1 and HIV-2 infections are in principle the same, except the drop in plasma viremia is more significant in HIV-2 than in HIV-1 infections (*Shanmugam, 2000*), and the clinical latency period is substantially longer in HIV-2 infections (*Marlink, 1994*). During the course of the HIV infection, the $CD4^+$ T cell-count gradually decreases (*Lang, 1989*), the plasma viremia increases (*Mellors, 1997*) and the patients enter the final phase of AIDS, where opportunistic infections predominate. Yet with HIV-2 infections, the majority of infected individuals are regarded as long-term non-progressors (*Lisse, 1996; Whittle, 1994*), who do not develop AIDS.

1.2.1. Cellular tropism

Both HIV-1 and HIV-2 infect the same $CD4^+$ target cells, including T cells (*Klatzmann, 1984*) and macrophages (*Gartner, 1986; Koenig, 1986*). HIV uses CD4 as a main receptor to interact with these target cells, and requires the use of a seven transmembrane G protein-coupled co-receptor to infect the cell. The CCR5 and CXCR4 chemokine receptors are the main co-receptors used by HIV-1 (*Deng, 1996; Feng, 1996*), and the use of each reflects the biological phenotype of HIV (*Björndal, 1997*). CCR5-using strains (R5), described as viruses that infect macrophages and primary T cells (M-tropic), replicate slowly and yield low virus production, while X4-viruses use CXCR4, infect predominantly T cells (T-tropic), replicate fast and produce high virion titers (*Berger, 1998*). Furthermore, R5 HIV-1 strains are generally the transmitted viruses, whereas X4-strains emerge later during infection and are associated with a rapid progression to AIDS (*Schitemaker, 1992*). A similar R5-to-X4 transition is not obvious in HIV-2-infected individuals, as many primary strains isolated from infected individuals use a range of coreceptors (including CXCR4 and CCR5) and a limited number of X4 viruses have been isolated from symptomatic HIV-2-infected individuals (*Guillon, 1998; Mörner, 1999*).

1.2.2. Cell entry & replication cycle

The replication cycle of HIV is initiated by the attachment of the viral SU glycoprotein to the N-terminal domain of the CD4 receptor on the target cell (*Habeshaw, 1989*). The binding of HIV-1 gp120 to CD4 has been reported to induce conformational changes, which in turn enable gp120 to interact with a chemokine coreceptor (*Trkola, 1996; Wu, 1996*). These conformational changes alter the position of the V1/V2 and V3 regions (*Wyatt, 1995; Sullivan, 1998*) and expose a highly conserved region in gp120 (*Rizzuto, 1998*), which is also present in the equivalent HIV-2 and SIV SU glycoproteins (*Reeves, 2002*). While infection of all primary HIV-1 isolates is dependent on CD4 binding, most primary HIV-2 isolates need only the chemokine receptor in order to infect cells (*Liu, 2000; Reeves, 1999*). Some of the T cell line-adapted (TCLA) HIV-1 isolates are capable of infecting co-receptor^{+} cell in the absence of CD4. While as many as seven mutations were required in HIV-1 *env* to induce CD4-independence in one of the HIV-1 isolates (*Dumoncequx, 1998*), only three mutations in HIV-2 gp125 induced CD4-independence in HIV-2 (*Reeves, 1997*). The factors that induce CD4-independence in HIV-1 have not yet been compared to HIV-2, nor has the conformation of HIV-2 gp125 yet been studied.

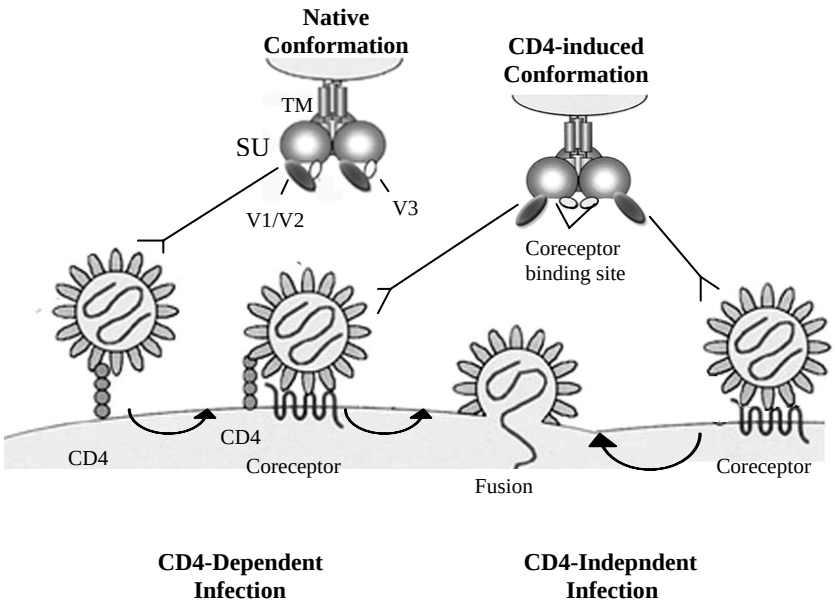


Figure 6 *CD4-dependent and CD4-independent routes of cell entry by HIV.*

After the interaction of HIV with the coreceptor, the TM glycoprotein alters its conformation to a fusogenic state and forces the viral membrane to fuse with the cell membrane (Caffrey, 1998). The remaining stages of the HIV replication cycle are summarized in Figure 7.

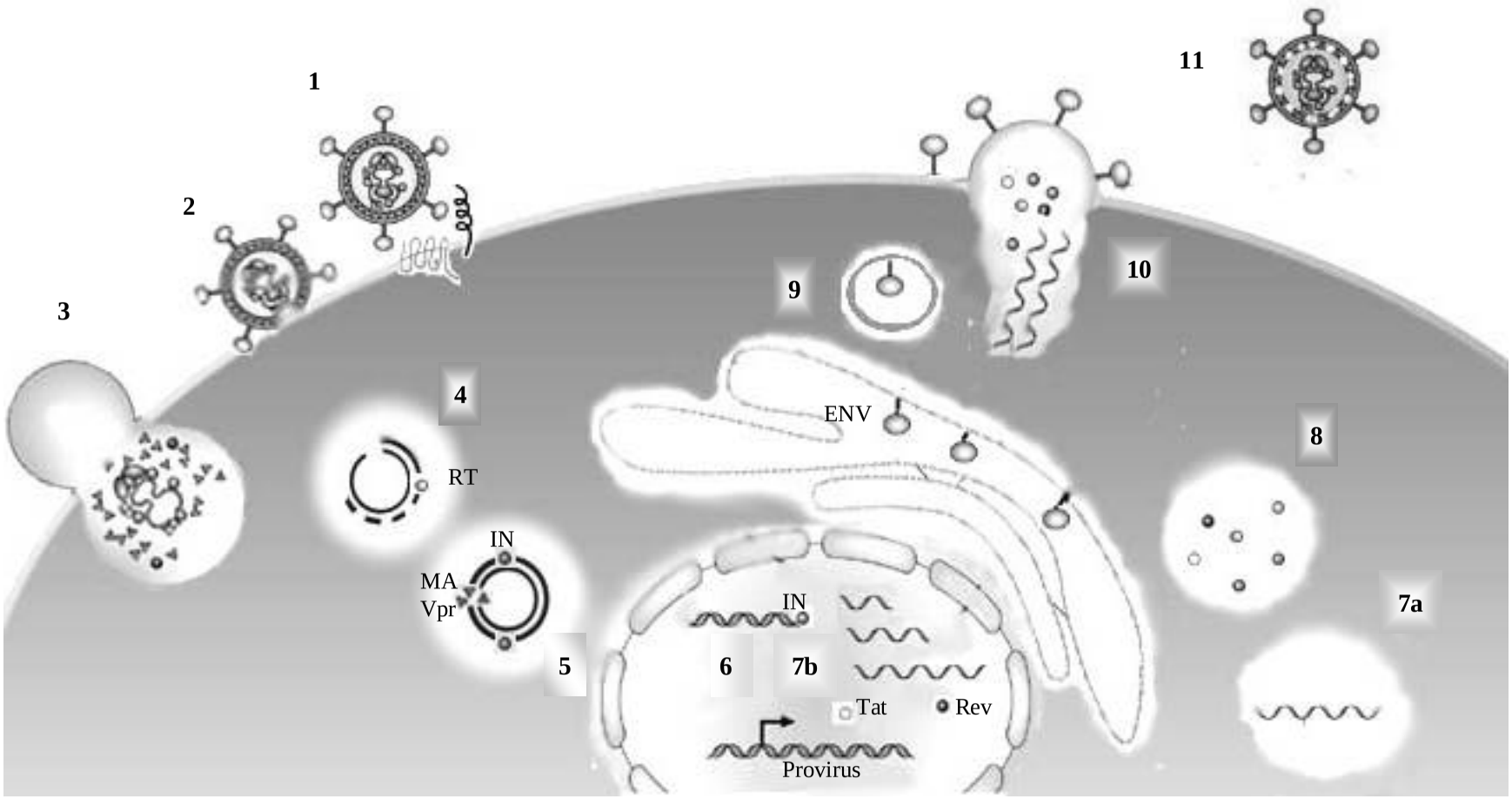


Figure 7 *Replication cycle of HIV.*

1) The SU glycoprotein of HIV interacts with CD4 on target cell.

2) Conformational changes in HIV SU and the interaction of HIV TM glycoprotein with co-receptor on target cells induces further conformational changes in the TM glycoprotein, which fuses the viral membrane with the membrane of the target cell.

3) The viral core is deposited into the cytoplasm and the nucleocapsid is uncoated, releasing viral RNA into cell.

4) The viral RNA is reverse transcribed by the virally encoded RT, forming cDNA and degrading the viral RNA.

5) The double-stranded DNA forms a pre-integration complex with Vpr, MA, integrase (IN) enzyme and other cellular proteins, and is transported to the nucleus.

6) In the nucleus, the viral DNA is covalently integrated into the host genome through IN, forming the provirus.

7) Provirus remains latent until host cell (T cell) is activated.

    a) T cell activation induces low-level transcription of the provirus.

    b) Cellular enzyme, RNA polymerase II, transcribes full-length viral transcripts from promoter located in the 5´LTR. RNA transcripts are multiply spliced, allowing the translation of the early genes *tat* and *rev*. Tat amplifies transcription, while Rev increases transport of singly spliced or unspliced RNA to the cytoplasm.

8) The unspliced and singly spliced transcripts are translated to produce the structural components of the viral core and envelope, together with RT, IN and PR.

9) The ENV precursor polyprotein is synthesized in endoplasmatic reticulum. After folding glycosylation and oligomerization in the golgi apparatus, the ENV is cleaved into SU and TM and transported to the host membrane.

10) At the host membrane, the Gag and Gag-Pol precursor polyproteins are incorporated in the budding immature virions together with the viral RNA genome.

11) The produced virion then matures, where the Pol portion of the Gag-Pol polyprotein is cleaved into PR, IN and RT. The Gag polyprotein is cleaved by PR into MA, CA and NC.

1.3. Host Defense

Mucosal surfaces are epithelial-lined surfaces that are continuously exposed to a wide variety, and often large quantities, of foreign antigens in the environment. The challenge of differentiating large quantities of benign antigenic substances, such as ingested food, from potential pathogens, has led to the development of a highly specialized immune system (*Veazey, 2003*). Foreign antigens are initially encountered by components of the "natural" or innate host defence. The innate immune system responds rapidly to pathogens, initiating antimicrobial activity, and provides time for the subsequent development of adaptive immune responses (*Janeway, 1999*). The innate immune system recognizes pathogens by the pattern of their microbial surface components, while the adaptive immune system responds to specific antigenic sequences (*Medzhitov, 2000*), and is divided into cellular and humoral immune systems. Figure 8 depicts the different cellular and soluble components of the innate and adaptive system which combat HIV.

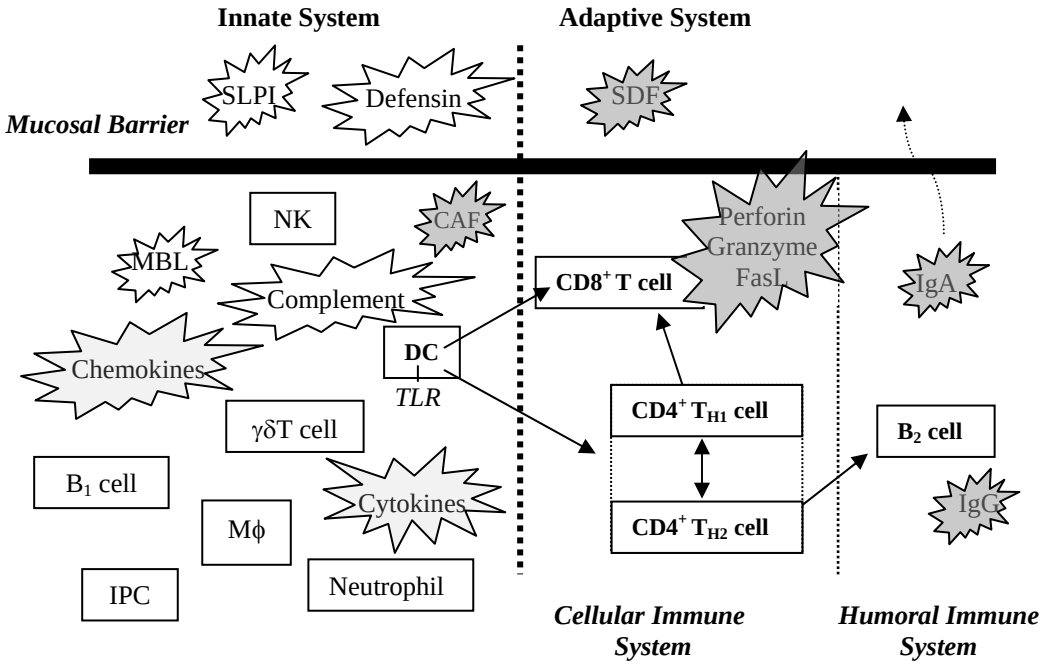


Figure 8 *Components of the innate and adaptive immune systems.*

Soluble components are indicated in 'stars'; dark grey and white-shading indicate soluble components produced by cells (in boxes) of the adaptive and innate systems, respectively. Soluble components produced by both systems are shaded in light grey. Interactions between key immune cells (in bold) in HIV infection are indicated by arrows. IgA crossing mucosal epithelial cells is indicated by a dashed arrow.

Innate immune responses may play a critical role in the early stages of HIV. Secretory leukocyte protease inhibitor (SLPI) found in saliva, has an anti-HIV activity (*McKneely, 1997*) and may contribute to the infrequent oral transmission of HIV (*McKneely, 1995*). High concentrations of the ligand of the CXCR4 receptor, stromal-derived factor (SDF), is expressed in human cervico-vaginal and rectal epithelial cells (*Agace, 2000*), and may prevent the transmission of X4-viruses across mucosal surfaces (*Lehner, 2003*). Among the soluble components of the innate immune system with anti-HIV activity are mannose-binding lectins (MBL) and complement (Figure 8). These soluble products bind to HIV and either lyse the virus directly or induce macrophages (Mϕ) to phagocytose the virus (*Levy, 2001; Sullivan, 1996*). HIV induces the production of defensin, which is produced by mucosal epithelial cells and other immune cells (*Alfano, 2005*). The anti-microbial mechanism of defensins is not fully understood, but it is generally believed that it disrupts microbial membranes (*Yang, 2002*). The cellular antiviral factor (CAF) is a soluble factor that is produced by $CD8^+$ T cells of the adaptive system, and has anti-HIV activity (*Levy, 1996*). The release of CAF does not appear to be induced by particular HIV proteins, where the release of this factor displays the characteristics of an innate immune response (*Levy, 2001*). Release of chemokines and cytokines is induced by the interactions of pathogens with different cells of the innate system. Chemokines are part of the homeostatic immune cell-trafficking (*Rossi, 2000*), whereas the binding of β-chemokines (RANTES, MIP-1α and MIP-1β) is capable of blocking HIV access to the CCR5 coreceptors (*Cocchi, 1995*). Innate immune cytokines such as interleukin-12 (IL-12) and IL-2 can modulate the adaptive immune response (Figure 9), while others such as tumor necrosis factor α (TNF-α) and interferons (IFNs), can affect the extent of HIV replication (*Graziosi, 1996*). IFNs primarily produced by IFN-producing cells (IPC; *Siegal, 1999*), can have direct inhibitory effects on HIV and can block HIV replication (*Pomerantz, 1987*).

Dendritic cells (DCs) trap HIV at mucosal sites and elicit protection from viral infection through the production of chemokines and IFNs (*Levy, 2001*). Toll-like receptors (TLR) expressed on the surface of DCs (*Moll, 2003*) are receptors that recognize certain patterns on microbial agents and induce production of IFN (*Akira, 2003*). Furthermore, DCs are mediators of antigen recognition and bridge the innate and adaptive immune responses through secretion of cytokines.

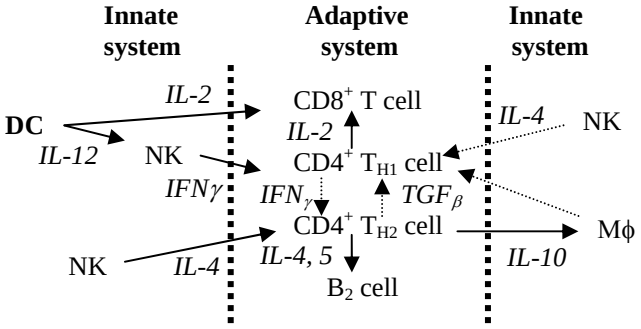


Figure 9 *Interplay between innate & adaptive immune systems through cytokines.*
Activating and inhibitory factors are indicated by solid and dashed arrows, respectively.

Other cellular components of the innate system include γδ Tcells, which are involved in mucosal protection and can lyse HIV-infected target cells (*Wallace, 1996*). Neutrophils are the most abundant innate immune cells responding early to infections, and possess viricidal effects on HIV (*Klebanoff, 1992*), whereas natural killer (NK)-cells eliminate HIV-infected cells directly or through antibody-directed cellular cytotoxicity (*Biron, 1999*). While B_2 cells produce epitope-specific antibodies, B_1 cells make little contribution to the adaptive immune response and have a rapid antibody response to polysaccharide antigens (*Murakami, 1995*). Antibodies produced by these cells are polyreactive, binding to numerous different ligands at low affinity, and have been demonstrated to react with HIV Tat protein (*Rodman, 1999*).

1.3.1. Cellular immune response

The cellular immune system consists of $CD8^+$ T cells, involved in the killing of infected cells, and $CD4^+$ T cells that activate different cells of the immune system. $CD4^+$ T cells are described as T-helper (T_H) cells and are divided into T_{H1} cells that promote the activity of $CD8^+$ and activate infected macrophages, and T_{H2} cells that activate specific B_2 cells to make antibody (*Janeway, 1999*).

$CD8^+$ T cells are also known as cytotoxic lymphocytes (CTLs) which have the ability to kill HIV-infected cells through the production of perforin and granzymes (*Shankar, 1999*). Indeed, CTL responses are responsible for the fall of viremia during primary HIV infection (*Schmitz, 1999; Wilson, 2000*). As previously mentioned, $CD8^+$ T cells express CAF in a manner resembling an innate immune response (*Levy, 2001*), and this response has been described to have non-cytotoxic, HIV inhibiting effects.

It is not clear which of the functions of $CD8^+$ T cells are most important for controlling HIV (*McMicheal, 2001*). Box 1 summarizes the inhibitory effects of $CD8^+$ T cells.

Box 1 - $CD8^+$ T cell Effectors	
Cytotoxic effect	
Perforin/ Granzyme	*Shankar, 1999*
FasL	*Hadida, 1999*
Non-Cytotoxic effect	
IFN_γ	*Meylan, 1993; Emilie, 1992*
CC-chemokine	*Wagner, 1998; Price, 1998*
CAF	*Levy, 2001*

$CD8^+$ T cells are capable of killing HIV-infected cells within five hours after initial infection, before new virions are generated (*Yang, 1996*). Despite this effective response, HIV is not completely eliminated, where cells infected with viruses with mutations in critical amino acids of dominant epitopes are selected for (*McMicheal, 2001*). Furthermore, impaired CTL function allows virus level to increase which therefore causes more mutations with the increased virus replication (*Appay, 2000*). As the virus levels rise, the CTL effect progressively becomes less efficient (*McMichael, 2001*).

Several studies have indicated that high production of chemokines and $CD8^+$ T cells non-cytotoxic antiviral responses may delay disease progression in HIV & SIV infections (*Ahmed, 1999; Zagury, 1998; Ullum, 1998*). Indeed, strong $CD8^+$ T cell non-cytotoxic responses are reported in HIV-exposed seronegative individuals (*Stranford, 1999; Ahmed, 2001*). A recent study has described the production of β-chemokines by most HIV-2-infected individuals (*Ahmed, 2005*), and suggested that the production of T cell non-cytotoxic antiviral factors may contribute to the low transmission of HIV-2 and disease progression in HIV-2-infected patients compared to patients infected with HIV-1. A higher proportion of the $CD8^+$ T cells from HIV-2-infected patients produce IL-2 compared to HIV-1-infected patients, and this cytokine allows CTLs to proliferate and survive (*Sousa, 2001*).

A strong $CD8^+$ CTL response is dependent on the presence of functional $CD4^+$ T_{H1} cells (Box 2). Early studies of HIV-1-infected patients demonstrated a weak proliferative response of HIV-1-specific lymphocytes (*Wahren,1987; Schrier, 1989*). This was later explained by the weak T_H response in people with chronic progressive HIV-1 infection, where this response is not sufficient to allow full maturation of CTLs for the control of infection (*Rosenberg, 1998*).

Box 2 – Functions of $CD4^+$ T_{H1} cells	
Priming $CD8^+$ T cells	*Ridge, 1998*
Maintaining $CD8^+$ T cells memory	*Walter, 1995*
Regulating $CD8^+$ T cells functions	*Zajac, 1995*

Strong proliferative T cell response demonstrated in HIV-1-exposed uninfected individuals, indicates the role of $CD4^+$ T_H activity in controlling infection (*Rosenberg, 1997*). Recent report on the more prevalent $CD4^+$ T_H responses in HIV-2-infected individuals (48%) compared to HIV-1 (8%), further explains the stronger immune response and slower disease progression observed in HIV-2-infected patients (*Zheng, 2004*).

1.3.2. Humoral immune response

The production of antibodies (Abs) by B cells (*the humoral immune response*) has been described to be necessary and sufficient for protection against many viruses (reviewed by *Robbins, 1995*). The abrogation of virus infectivity *in vitro*, by the binding of Abs to the virus, is defined as neutralization (*Klasse, 2002*). B cells produce different *isotypes* of immunoglobulins (Igs), where isotype-switching is induced by different cytokines (*Stavnezer, 1996*). T_{H2} cells stimulate the proliferation and differentiation of antigen-binding B cells through secretion of IL-4, IL-5 and IL-6.

The different isotypes are directed to different compartments of the body, in which their distinct effector functions are appropriate (*Janeway, 1999*). Immunoglobulin M (IgM) is the first isotype produced during the humoral immune response, usually has a low affinity for the antigen, and is confined to the blood. IgG is the principal isotype in the blood and extracellular fluid. While IgA predominates in secretions across epithelia, including breast milk, it is the second major isotype in extracellular fluid. There are different subtypes of IgG and IgA and their respective concentrations in serum are indicated in Table 3. IgE is mainly found as mast-cell associated antibody just beneath epithelial surfaces.

	IgA_1	**IgA_2**	**IgG_1**	**IgG_2**	**IgG_3**	**IgG_4**
Serum Conc. *mg/ml*	3.0	0.5	9.0	3.0	1.0	0.5

Table 3 *Comparison of serum IgA and IgG.*

(*Lizeng, 2005*)

Igs are composed of two polypeptide chains; a heavy chain of 50kDa and light chain of 25kDa, which in turn are divided into variable and constant regions (*Edelman, 1991*). Two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond (Figure 10). An antibody is organized into two Fab fragments and one Fc fragment. These three globular portions are joined by a flexible stretch of polypeptide chain known as the hinge region.

The variable domains of the Fab fragments contain the antigen-binding site. At the tip of the variable domain of the heavy (V_H) and light (V_L) chains are three variable loops denoted CDR1, CDR2 and CDR3 (*Chitarra, 1993*). The contribution of the CDRs from both V_H and V_L domains determines the final antigen specificity. Furthermore, the CDR3 loop of the heavy chain (CDRH3) of antibodies plays a distinctive role in determining antibody specificity (*Wu, 1993*).

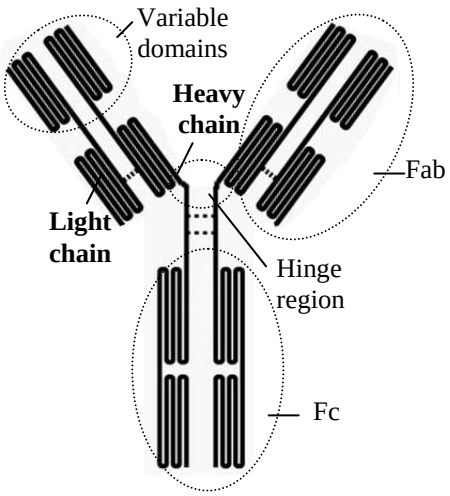


Figure 10 *Molecular organization of an immunoglobulin.*

The Fc portion of an antibody confers other effector functions that are distinct for each of the various isotypes. Abs are capable of coating the surface of a pathogen, and the Fc portion of IgG_1 and IgG_3 allows antibodies to be phagocytosed through the Fc-receptors of macrophages (*Jefferis, 1995*). The Fc portion of antigen:antibody complexes can activate the *complement* system, which is characteristic for IgM, IgG_1 and IgG_3 (*Sensel, 1997*). Abs are also capable of binding antigens on the surface of infected cells. Antibody-coated infected cells can be killed by NK cells in a process known as antibody-dependent cell-mediated cytotoxicity (ADCC), where CD16 receptors on NK cells recognizes the Fc domain of the bound Ab (*Lanier, 1988*). Finally, the Fc portion allows the transport of IgA across epithelial cells at mucosal surfaces (*Mostov, 1994*). B cells in the mucosal compartment express dimeric forms of IgA (dIgA), composed of two monomeric IgA (mIgA) molecules connected with a 15kDa polypeptide (J-chain). The mIgA and the J-chain are connected to each other by disulfide bonds (*McCune, 1981*). Figure 11 describes the pIgR transport of dIgA (*transcytosis*) from the basal surface of epithelial cells to the apical surface, releasing secretory IgA (IgA_{sec}) into the lumen of mucosal surfaces.

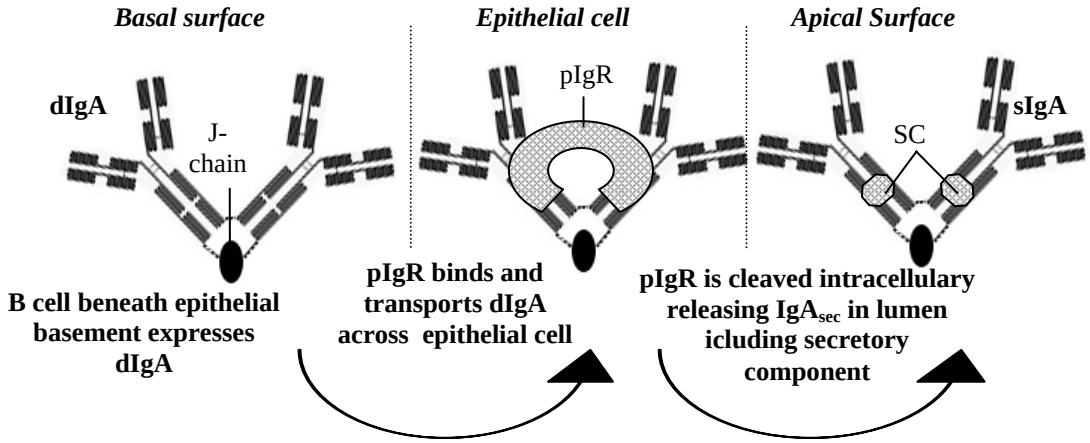


Figure 11 *Transcytosis of dIgA and production of sIgA.*

The majority of IgA_{sec} produced in humans is derived from mucosal surfaces (Jonard, 1984). The secretory component (SC) of IgA_{sec} protects IgA from the protease-rich environment in mucosal compartments (*Lindh, 1975*). Furthermore, the polymeric structure of IgA_{sec} enhances its avidity to antigens, making this antibody particularly well-suited to protecting mucosal surfaces (*Kerr, 1990*).

mIgA makes up to 80-99% of serum IgA (sIgA), with the majority (85%) of sIgA being of subtype IgA_1 (*Russell, 1992*). Differences between IgA_1 and IgA_2 are attributed to a 13-amino acid hinge region containing five carbohydrate moieties that is lacking in IgA_2. The absence of this region renders IgA_2 less flexible than IgA_1, but makes IgA_2 less susceptible to bacterial proteases at mucosal surfaces (Batten, 2003; Kilian, 1981). The functional difference between the two subtypes of IgA is not fully understood, but it has been suggested that protein antigens elicit IgA_1, while IgA_2 reacts to polysaccharides (*Russell, 1992*).

1.4. HIV Neutralization

The view regarding the role of antibodies (Abs) in preventing infection with HIV-1 has varied markedly over the past 20 years, ranging from optimistic views in the late 1980s, to predictions of the mid 1990s that HIV-1 isolated from patients (*primary isolates*) were resistant to antibody-mediated neutralization (*Zolla-Pazner, 2004a*). Weak neutralizing activity of primary HIV-1 isolates has been reported for Abs isolated from HIV-1-infected patients (*Montefiori, 1996; Moog, 1997*), and non-neutralizing Abs were often detected (*Poignard, 1999*). Nevertheless, detailed analysis of recently HIV-1-infected individuals has indicated the involvement of neutralizing Abs (nAbs) in controlling viral replication during the first month after infection, exerting significant pressure on HIV-1 (*Richman, 2003*).

Up to the mid 1990s, HIV neutralization has been assessed using HIV isolates cultured in $CD4^+$ T cell lines (*Poignard, 1996*). High neutralization titers were detected in sera of infected individuals and immunized animals, using T cell line adapted (TCLA) viruses (*Nara, 1991; D´Souza, 1991*). Primary HIV-1 isolates are more difficult to neutralize than TCLA strains (*Moore, 1995*). While TCLA strains are more adapted to infecting $CD4^+$ cells, nAbs selective pressure on primary HIV isolates led to the development of different mechanisms to evade neutralization (*Poignard, 1996*). The proofreading activity lacking in HIV RT generates several mutants that can escape neutralization (*Telesnitsky, 1997*). Other mechanisms adopted by primary HIV isolates in escaping nAbs are summarized below.

Mechanism of evading neutralization	***Effect***	
Trimerization	Conceals "*neutralizing face*" of HIV ENV. Non-covalent interaction allows shedding and exposure of "non-neutralizing face"	*Wyatt, 1998b; Chan, 1997*
Glycosylation	Glycan shield covers HIV ENV, exposing "*silent face*", and covering neutralization sites.	*Johnson, 2002*
CD4-dependence	Conceals major neutralizing sites that are only exposed upon interaction with CD4.	*Hoffman, 1999*
Variable loops	Elicits strain-specific Abs, while conserved neutralizing sites are poorly immunogenic	*Haigwood, 2003; Moore, 1994*

Table 4 *Characteristics of primary HIV envelope (ENV) involved in evasion of nAbs.*

The trimerization of the HIV envelope (ENV) through non-covalent interaction has a consequence on HIV-1 susceptibility to neutralization. HIV-1 ENV has been described to have three faces; *neutralizing*, *non-neutralizing* and *silent* faces (*Wyatt, 1998b*). The "outer domain" of HIV-1 gp120, suggested to be exposed on gp120 trimers, is heavily glycosylated and therefore is regarded as a "silent" face. The *neutralizing* face of gp120 includes sites that are not exposed in trimeric ENV, and are only exposed upon binding to CD4 (*Sullivan, 1998*). Non-covalent association between gp120 and gp41 allows the shedding of monomeric gp120 through interaction with CD4 (*Sattentau, 1993*), which in turn exposes the *non-neutralizing* face of the gp120 inner domain (*Wyatt, 1998b*), limiting the efficiency of the humoral immune response *(Parren, 1999*).

The structural organization of HIV-2 ENV has not yet been studied, but previous research indicates that HIV-2 is more readily neutralized than HIV-1 (*Björling, 1993; Fenyö, 1996*).

Furthermore, CD4 does not cause disassociation between gp125 and gp36 as readily as is observed for HIV-1 ENV components (*Sattentau, 1993*). The fact that most HIV-2 primary isolates are CD4-independent (*Liu, 2000; Reeves, 2002*) may explain the higher neutralization susceptibility of primary HIV-2 isolates compared to primary HIV-1 isolates.

1.4.1. Mechanism of viral inhibition

In spite of the dispute regarding the role of nAbs in preventing HIV infection, passive immunization experiments have repeatedly established that antibodies can provide sterilizing immunity against HIV-1 (*Shibata, 1999; Mascola, 2000*). The different mechanisms adopted by Abs in inhibition of viral infection are summarized in Box 3, and the mechanisms characterized for HIV-1 inhibition of infection are indicated with an asterisk. As previously discussed, HIV is capable of crossing mucosal surfaces through trancystosis. The role of IgA in preventing HIV-1 infection has been described in the genital tracts of HIV-1 exposed but persistently seronegative (uninfected) individuals (*Belec, 2001; Kaul, 1999*). Furthermore, IgA has been reported to neutralize HIV-1 intracellulary, by inhibiting HIV-1 transfer across epithelial cells (*Devito, 2000; Bomsel, 1998*).

Box 3 Viral Inhibition Mechanisms

Prevention of adhesion to target cell molecules:*	
Lectins	*Scanlan, 2002*
Adhesion molecule	*Hioe, 1998*
CD4	*Ugolini, 1997*
Co-receptor	*Thali, 1993*
Inhibition of fusion*	*Golding, 2002*
Inhibition of viral budding	
Inhibition of viral core uncoating	
Inhibition of endocytosis*	*Bomsel, 1998*; *Devito, 2000*
Inhibition of cell-to-cell transmission*	*Pantaleo, 1995*
Complement activation*	*Spear, 1994*
ADCC*	*Tyler, 1990*
Inhibition of viral enzymatic reaction	
Aggregation of virions*	*Parren, 1999*

While nAbs are described as Abs that are capable of inhibiting infections, non-neutralizing antibodies have the capacity of mediating antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) as well as complement-dependent virolysis or phagocytosis, through Fc-mediated effector systems (*Burton, 2002*). Higher concentrations of Abs are required in association with ADCC and CDC than for the neutralization of cell-free virions (*Hezareh, 2001*). Non-neutralizing antibodies can also interact synergistically with poorly neutralizing Abs, increasing the neutralization susceptibility of HIV-1 (*Cavicini, 2002*).

While the Fc portion of Abs is capable of inducing HIV inhibitory effects, the Fc region can also be responsible for the enhancement of viral infection (*Takada, 2003*). The interaction of the Ab Fc portion with Fc-receptors on target cells may be responsible for antibody-mediated enhancement of infection (*Takeda, 1990*), where the interactions of CD4 and co-receptor may also be involved (*Schutten, 1995, Guillon, 2002*). Moreover, it has also been suggested that low Ab concentrations may be responsible for enhancing HIV infection (*Nottet, 1992*).

The Fc region of some Abs reduces its capacity to neutralize HIV-1 (*Roben, 1994; Labrijn, 2003*). Steric hindrance can prevent Abs from neutralizing HIV-1, where even single fragments of an Ab variable region (Fv) have been described to neutralize HIV-1 (*Labrijn, 2003; Dey, 2003*). Different mechanisms for neutralization have been described for Abs compared to Fabs, where an Ab has been described to neutralize HIV-1 through inhibition of viral fusion with the host membrane, while its Fab fragment inhibited the virus at a later stage after cell entry (*McInerney, 1997*).

Epitope	**mAb**	**Mechanism of Neutralization**	
Transmembranal region in gp41	2F5	Inhibits viral fusion with host membrane.	*Trkola, 1995*
gp120 CD4-binding domain.	b12	Inhibits interaction with CD4.	*Ho, 1991*
α mannose residue	2G12	Inhibits interaction with lectin receptor?	*Scanlan, 2002*
CD4-induced epitope	17b	Prevents interaction with co-receptor by binding CD4-induced epitope.	*Xiang, 2002*
V3 region	447D	Interferes with interaction with co-receptor?	*Gorny, 2002*

Table 5 *Neutralizing epitopes utilized by exemplary HIV-1 monoclonal antibodies (mAbs).*

A variety of different epitopes on ENV have been described to be recognized by Abs, enabling HIV neutralization through different mechanisms. The neutralizing epitopes for HIV-1 have been well-characterized and are summarized in table 5 above. Most of the neutralizing epitopes characterized to date are recognized by the IgG isotype. HIV-1 neutralizing sites recognized by IgA have been mapped to the extramembranal region of the TM glycoprotein, gp41 (*Muster, 1994; Pastori, 2000*). However, the HIV-2 neutralizing site recognized by IgA has not been identified as yet. Neutralizing sites on HIV-2 ENV have generally not been studied as extensively as for HIV-1 ENV, where few HIV-2-specific mAbs have been generated and characterized. One of the neutralizing sites that has been studied for HIV-1 and HIV-2 is the SU glycoprotein V3 region.

1.4.2. V3 region & nAbs

The V3 regions of both gp120 and gp125 have been described as the principal neutralizing determinants for both HIV types (*Norrby, 1991*). The GPGR/Q motif at the crown (residues 319-322) of the gp120 V3 region is conserved and elicits Abs that can neutralize TCLA HIV-1 strains (*Javaherian, 1989*). Similarly, the gp125 V3 region has been described as being immunogenic (*Björling, 1991, Matsushita 1995, McKnight 1996*), with residues FHSQ (amino acids 315-318) at the crown of the V3 region (*Björling, 1994*).

Other than the conformation-sensitive, V3-specific mAb 447D (*Gorny, 2002*), most Abs recognizing the linear site at the crown of gp120 V3 region neutralize TCLA strains and are not capable of neutralizing primary HIV-1 isolates (*York, 2001*). Indeed, the capacity of this linear site to elicit Abs that can neutralize primary HIV-1 isolates is disputed, and it is generally believed that this site is inaccessible in primary isolates (*Burton, 2004; Spenlehauer, 1998*). The V3 regions may be accessible on the monomeric form of gp120, but it is enclosed in the oligomeric state (*Bou-Habib, 1994; Satamatatos, 1995*).

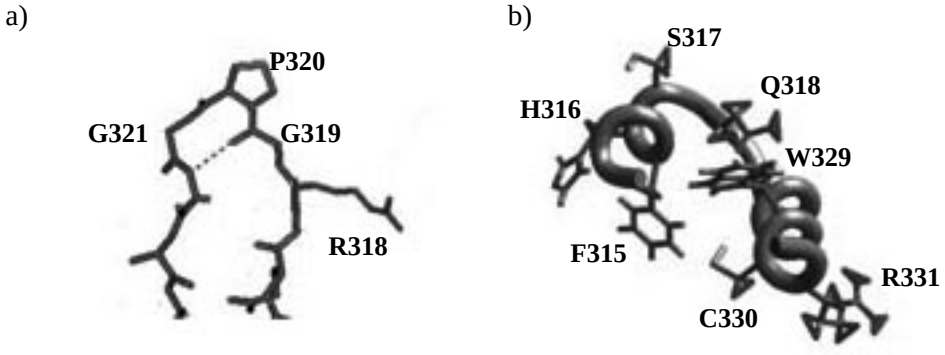


Figure 12 *Molecular organization of HIV-1 and HIV-2 V3 crown.*
a) Adapted from *Rosen, 2005*. b) Adapted from *Mörner, 1999*.

The V1/V2 region defines the accessibility of the V3 region in primary HIV-1 isolates and renders HIV-1 more sensitive to neutralization when deleted (*Cao, 1997; Kang, 2005; Wyatt, 1995*). Furthermore, glycans on the V1/V2 region occlude the tip of the V3 region (*Losman, 2001*). As previously mentioned, primary HIV-1 isolates require CD4 binding to reorient the position of the V1/V2 region, exposing the V3 region as well as other neutralization sites (*Sullivan, 1998*). The few HIV-1 CD4-independent isolates characterized to date, display similar sensitivities to neutralization as described for HIV-2 (*Edwards, 2001; Hoffman, 1999; Kolchinsky, 2001*).

While exposure of the V3 region on primary HIV-2 isolates has not yet been studied, mAbs recognizing the linear (FHSQ) site on gp125 could not neutralize HIV-2 isolates (*Björling, 1994*). A conformational epitope (Figure 12b) composed of FHSQ and WCR (residues 329-331) has been described in the V3 region of gp125 (*Björling, 1994; Mörner, 1999*), and mAbs recognizing conformational eitopes in the gp125 V3 region could neutralize HIV-2 isolates (*Björling, 1994; McKnight, 1998*). Conformational epitopes in gp120 have generally been described to elicit Abs with potent neutralizing capacity (*Gorny, 2002; Spenlehauer, 1998*).

1.5. Protective Immunization

The eradication of smallpox in 1978 is an example of the success of a vaccine against viral infections. Several different strategies have been employed in the design of vaccines that can elicit specific immune responses (Box 4). These responses are a combination of Abs that neutralize virus (nAbs), cytotoxic killing of infected cells (CTL), or activating the $CD4^+$ cells which in turn induce humoral and cellular immune responses (CD4).

Box 4 Vaccine strategies

Type of vaccine	Immune response
Attenuated virus	nAbs, CTL
Killed virus	nAbs, CD4
Viral vectors	CTL, CD4
DNA vaccine	CTL, CD4
Protein/peptides	nAbs, CD4

Type of adjuvant	Immune response
Alum	T_{H2}
MF59	T_{H2}
IL-2	CD4
TGF_β	IgA producion
Cholera toxin	IgA production

First generation vaccines were exclusively live attenuated viruses, which included polio, measles and mumps (*Pantaleo, 2004*). Vaccination with attenuated SIV, in which the *nef* gene has been deleted, induced sterilizing immunity against homologous and heterologous viral challenges in non-human primates. However, the vaccine strain eventually reverted to a pathogenic virus encoding full-length *nef* (*Ruprecht, 1999*). Safety concerns led to the development of another generation of vaccines which included the use of viral protein units and viral peptides. Advances in molecular biology and genetic engineering enabled the design of recombinant proteins and synthetic peptides, which are either purified or expressed as DNA- or virus vector-based vaccines. Moreover, DNA vaccines are combined with recombinant protein immunization, in an attempt to induce both cellular and humoral immune responses.

The induction of V3-specific nAbs is one of the strategies investigated in inhibiting HIV infection. In a recent study, Rhesus primates were immunized with peptides of the V3 region and then challenged with SIV/HIV chimeric virus (*Letvin, 2001*). While nAbs were induced,

V3 peptide immunization did not provide protection against pathogenic strains of the virus. Furthermore, HIV-1 V3-specific nAbs have been demonstrated to be strain-specific (*Someya, 2005; Haigwood, 2003*). The use of a cocktail of peptides known as hypervariable epitope constructs has been suggested to induce antibodies against a broader range of HIV-1 isolates (*Anderson, 1994; Meyer, 1998*), whereas the use of conjugated peptides such as multiple antigen peptides improved the presentation of conformational epitopes (*Hewer, 2002; Iglesias, 2005*).

While the use of peptides has been generally associated with the induction of linear-specific Abs, the use of proteins such as gp120 subunits can induce conformation-sensitive Abs (*Burton, 2002; Gorny, 2004*). Nonetheless, immunization with monomeric gp120 has been reported to induce nAbs primarily neutralizing laboratory-adapted HIV-1 strains (*Beddows, 1999; Belshe, 1998*), whereas immunization with the oligomeric form of gp120 (gp140) elicits nAbs against primary HIV-1 isolated as well as laboratory-adapted strains (*Earl, 1994*). Nevertheless, the role of the V3 region of oligomeric gp120 in eliciting nAbs appears to be insignificant, since less than 7% of these antibodies recognize the V3 region (*Earl, 1994; Grunder., 2005*). Conversely, studies in macaques have demonstrated an alteration in the immunogenicity of the V3 region after deletion of the V2 region in gp140, which increased the induction of nAbs (*Barnett, 2001; Srivastava, 2003*).

Other attempts to induce V3-specific nAbs includes expression or the conjugation of the V3 region with foreign antigens (*Someya, 2005; Eller, 2004; Hirio, 2001; Rubinstein, 1999*). Coupling of the V3 region to carrier proteins such as *Brucella abortus* promoted V3-specific nAb secretion at mucosal sites (*Eller, 2004*). Routes of immunization also affect the immune response, where a study has demonstrated that mucosal immunization of mice with the V3 region integrated into a Pseudomonas exotoxin (ntPE), elicited vaccine-specific immune responses in both systemic and mucosal compartments (*Mrsny, 1999*).

Furthermore, parenteral administration of immunogens in combination with an appropriate adjuvant formulation can elicit different immune responses (*Egan, 2004*). Adjuvants can be divided into *carriers* which physically associate with the immunogen and affect immunogen structure, release and targeting, and into *immunostimulants* that influence T_{H1}/T_{H2} responses. The only vaccine adjuvants approved for use in humans are aluminium salts, known as "*alum*", and in a oil in water emulsion called *MF59* (*Singh, 1999*). Vaccines combined with either adjuvant predominantly induce a T_{H2}-type immune response (*Goebel, 1999; Sandstrom, 1999*).

2. AIMS OF THESIS

The general aim of this thesis was to identify the characteristics of HIV-2 envelope that may be involved in neutralization. The specific aims were to address the following points:

- **Role of IgA in HIV-2 neutralization.**
  - Which epitopes are recognized by IgA isolated from HIV-2-infected individuals?
  - Does HIV-2-specific IgA neutralize HIV-2 *in vitro*?

- **Production of recombinant gp125.**
  - Construct gp125 proteins similar to previously crystallized gp120 (*Kwong, 1998*).
  - Express gp125 proteins suitable for structural and functional studies.

- **Accessibility of the V3 region on HIV-2 recombinant gp125 proteins.**
  - What is the difference between the non-neutralizing 7C8 and the neutralizing 3C4 V3-specific mAbs (*Björling, 1994*)?
  - Does the V1/V2 region affect accessibility of the V3 region on gp125?
  - Is there a correlation between CD4-independence and accessibility of V3 region?

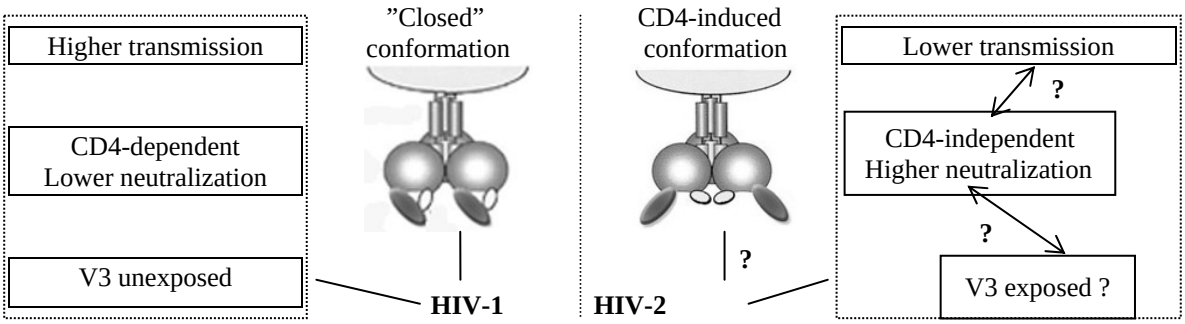


Figure 13 *Possible hypotheses that may explain differences between HIV-1 & HIV-2.*

- **Neutralization capacity of V3-specific antibodies and Fab fragments.**
  - Does the deletion of the V1/V2 region enhance exposure of neutralization sites?
  - Can HIV-2-neutralizing antibodies be induced by immunizing mice with recombinant gp125 proteins?
  - Is there a correlation between the size of a V3-specific antibody and its neutralization capacity?

3. MATERIALS & METHODS

The different materials and methods that have been used are described in detail in the individual papers of this thesis. Below is a brief description of the principles of the techniques employed and the reasoning behind the design of the different experiments.

3.1. Protein Production

Construction and expression of recombinant gp125 proteins

Peripheral blood mononuclear cells (PBMCs) were infected with a primary HIV-$2_{SBL6669}$, nuclear DNA was extracted from the infected cells and the *env* gene was cloned through nested PCR. Figure 14 describes the desig and cloning of both gp125 and gp125Δv_1v_2, including the base pairs (bp) that are included in the different constructs (numbering according to the ISY clone of HIV-$2_{SBL6669}$). XbaI and BamHI restriction sites were introduced in the 5' and 3' primers, respectively, which were used in cloning into the expression vector, *pBSKs*. The first 25 codons at the 5' end and 10 codons at the 3' end were excluded in both gp125 constructs, and the V1/V2 region was eliminated in gp125Δv_1v_2, being replaced with Gly-Ala-Gly residues. Cloning into *pBSKs*, introduced an Ig κ-chain signal peptide (SP), a hemagglutinin-A (HA) tag, and a thrombin cleavage (TC) site at the 5'end of both gp125 constructs. The gp125 constructs, including SP, HA and TC sites, were then subcloned into the expression vector, *pBJ5/GS*, which encodes the selection marker gene *glutamine synthetase* (GS). Chinese hamster ovarian (CHO*lec*) cells were transfected with

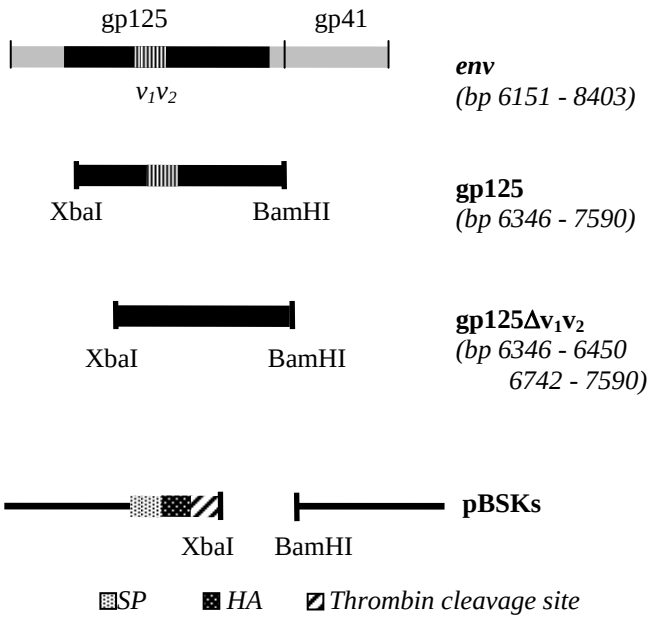


Figure 14 *Cloning and design of gp125 constructs.*

pBJ5/GS vector expressing gp125 or gp125Δv_1v_2. Transfected CHO*lec* cells were then cultured in glutamine-free medium including the selection marker toxin *L-methionine sulphoxamine* (MSX), where only cells expressing GS would survive. CHO*lec* clones expressing gp125 or gp125Δv_1v_2 were screened through ELISA and immunoprecipitation using an anti-HA monoclonal antibody. Selected clones were cultured in roller flasks, and gp125 proteins were purified from harvested supernatant.

Production of HIV-2 V3-specific mAbs and Fabs

Forty hybridoma cell lines expressing V3-specific monoclonal antibodies (mAbs) have been previously isolated from mice immunized with two overlapping peptides of the V3-region (Björling, 1994). Box 5 describes two of these V3-specific mAbs and the peptides they recognize. The supernatant of 7C8 or 3C4 expressing hyridoma cell lines was harvested from roller flasks, and mAbs were purified using *Protein A*-sepharose beads and eluted using glycine buffer.

Fab fragments were produced through papain digestion of purified mAbs. Papain digestion was performed at 37ºC, where the digestions of 7C8 and 3C4 were terminated after 1.5 and 3 hours, respectively. Fab fragments were separated from papain digestion products through size-exclusion chromatography.

Box 5 V3-specific mAbs

mAb	Peptide recognized*
7C8	^{311}SGRRFHSQKIINKKPR^{326}
3C4	^{311}SGRRFHSQKIINKKPR^{326} ^{322}NKKPRQAWCRFKGEWR^{337}

* Used in mice immunization (Börling, 1994). Numbering according to the ISY molecular clone of HIV2$_{SBL6669}$.

3.2. Protein Purification

Purification of recombinant gp125 proteins

CHO*lec* cells express glycosylated proteins with high-mannose sugar moieties, which enabled the use of lectin affinity chromatography in purifying the expressed proteins. Immobilized lectin from *Galanthus nivalis* (GN) was used to trap monomers of gp125 or gp125Δv_1v_2, and the bound glycoproteins were eluted using α-methyl-D-glycoside. *Concanavalin A* (ConA) lectin sepharose was used to trap different forms of glycosylated proteins, which were also eluted using the same substrate as used for GN-purification. Gp125 and gp125Δv_1v_2 were further separated from other glycosylated proteins through size-exclusion chromatography (using Sepharose 12) and the use of different ion-exchange chromatography (as described in

Box 6). The pH of gp125-containing eluted proteins was adjusted to 7.5, the gp125 was purified through *MonoQ* ion-exchange chromatography and eluted by increasing salt concentration. Gp125$\Delta v_1 v_2$ was purified by adjusting the pH to 6.0, followed by using *SP* ion-exchange and eluting by increasing both salt concentration and pH.

Box 6 Ion-exchange chromatography

Column	Function
MonoQ	Traps (-) charged proteins *gp125 pI = 6.0*
SP	Traps (+) charged proteins *gp125$\Delta v_1 v_2$ p I= 8.7*

Purification of IgA, IgG and Fab fragments

IgA and IgG were purified from sera using *Jacalin*- and *Protein G*-affinity chromatography, respectively. *Jacalin-agarose* beads were added to diluted sera (1:10) and the captured IgA was eluted using methyl-α-D-galactopyranoside. Residual IgG in the eluted IgA fractions was removed using *Protein G*-spharose beads. Similarly, IgG was purified from sera using *Protein G*-sepharose beads and was eluted using glycine-buffer. The 7C8 and 3C4 mAbs were purified from cell culture supernatant using *Protein A*-sepharose beads and eluted using glycine-buffer, while Fab fragments were purified through size-exclusion chromatography.

3.3. Protein Analysis

Sequence analysis

The amino acid (aa) sequences of the proteins analyzed, were generated through the translation of their respective genomic sequence. Gp125 was sequenced after cloning into the *pBSKs* vector, while the variable regions of 7C8 and 3C4 were sequenced using cytoplasmic RNA extracted from their respective hybridoma cell lines. The primers used in amplifying the variable regions of 7C8 and 3C4 heavy and light chains were also used in sequencing. DNA translations and alignments were performed using bioinformatic tools (www.expasy.com), while CDR predictions were made through http://imgt.cines.fr/home.html, and protein modelling was performed using the CPH models 2.0 homology modelling programme.

Mass spectrometry and circular dichroism spectroscopy

GN-purified gp125 or gp125$\Delta v_1 v_2$ were used in mass spectrometry analysis to determine their respective molecular weight. To ensure conserved structures of the gp125 proteins and that the deletion of the V1/V2 region did not alter the structure of gp125$\Delta v_1 v_2$, the secondary structure of both recombinant proteins was analyzed using circular dichroism spectroscopy.

Box 7 Peptides analyzed		
Peptide	Region	Residues
S1-2	V2	160-186
S1-9	V2/C2	189-205
S11-11	V3	283-297
A43-29	V3	311-330
A43-36	V3	318-337
S1-18	V4	399-419
S12-37	gp36	582-603
A43-9	gp36	615-634
A43-10	gp36	634-648
A43-12	gp36	644-658

Blotting analysis

The interactions between different mAbs or serum Abs with the recombinant proteins or peptide were analyzed in dot blot and western blot (WB) analyses. WB analysis identified protein components recognized by different antibodies, while the dot blot analysis gave an indication of the strength of Ab binding to different proteins or peptide. In dot blot analysis, a titration series of purified gp125 proteins or a peptide of the V3 region was applied directly onto nitrocellulose membranes and incubated with mAbs or sera. Bound Abs were detected using HRP-labelled anti-mouse or anti-human Abs, and the intensity of the signal produced was measured after scanning. Proteins recognized by Abs in WB analysis were similarly identified using HRP-labelled anti-mouse or anti-human Abs.

ELISA

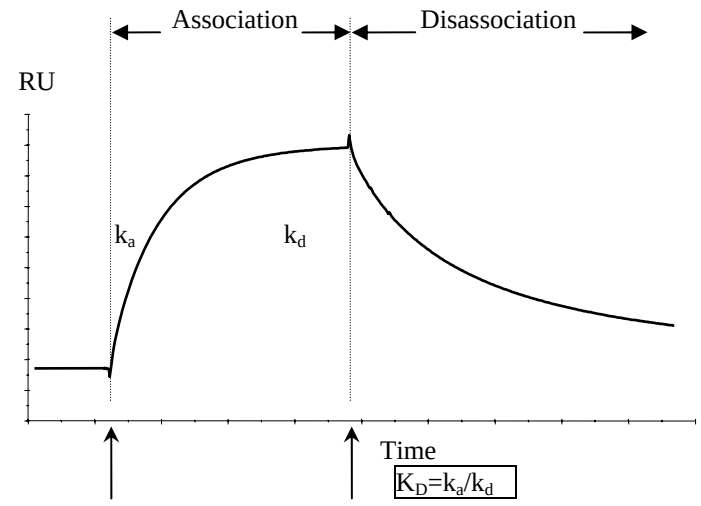
Screening of CHO*lec* clones expressing gp125 or gp125$\Delta v_1 v_2$ was performed through the coating of 96-well plates with cell culture supernatant diluted in sodium carbonate buffer. Expressed proteins were detected using biotinylated-HA and streptavidin. Ab binding to HIV-2 proteins or peptides was analyzed in ELISA through coating plates with recombinant glycoprotein or peptide (Box 7) and the antibody binding was studied by adding a titration series of the purified mAbs or sera tested. Bound antibodies were detected using HRP-labelled anti-mouse or anti-human Abs.

Box 8 Source of Abs analyzed	
3C4	V3-peptide immunized mice
7C8	V3-peptide immunized mice
$gp125^+$ sera	gp125-immunized mice
HIV-2^+ sera	Subjects from Guinea Bissau and Portugal
HIV-1^+ serum	Subject from Sweden
Negative controls	Subjects from Sweden Uninfected mice.

Surface plasmon resonance (SPR)

The kinetics of Ab binding to gp125 proteins (or peptide of the V3 region), and the interaction of soluble CD4 (*sCD4*) to HIV surface glycoproteins, was studied through surface plasmon resonance analyses. The interaction between two proteins was studied, by immobilizing one of the proteins onto a microchip (*CM5 chip*) and injecting the other over the surface of the chip. Interactions of the injected protein with the immobilized protein change the angle of the polarized light reflection, which is detected within the *Biacore system*. As described in Box 9, SPR analysis gave indication of strength of protein binding (*affinity*) and information on the real-time rates of protein interaction (*association (k_a) & disassociation (k_d)*).

Box 9 Principle of surface plasmon resonance analysis



Biacore sensogram

In Biacore systems, polarized light is projected onto a glass ***surface*** that contains an electrically conducting gold layer. This light causes free electron clouds of the gold to generate waves called ***plasmons*** which reduces the intensity of the reflected light. Interaction between two molecules (proteins) is analyzed by immobilizing one of the molecules on a surface and then injecting a buffer containing the other molecule, over this surface. The difference between the *refractive index* of the sensor surface and the buffer flowing over the gold layer affects the ***resonance*** angle at which the light is reflected, which is detected by the Biacore sytsem. As the buffer is injected, the molecules interact (associate) at a certain constant (k_a), and then start disassociating (k_d) after the injection is stopped. The strength of this interaction (affinity) is expressed by the K_D value.

3.4. Immunization and Neutralization Analyses

Mice were immunized twice with gp125 or gp125Δv_1v_2 absorbed to aluminium hydroxide (*alum*), with a three week interval. A dilution series was made of immunized mice sera, mAbs or Fabs, and was tested for HIV-2 neutralization capacity by incubating with HIV-2 for 1 hr at 37ºC, which were then mixed with PBMCs and incubated overnight at 37ºC. The medium of the PBMCs was changed on days one and four after infection, and on day seven the supernatant was collected and analyzed for HIV-2 antigen using an in-house capture ELISA (*Thorstensson, 1991*).

4. Results & Discussion

The lower transmission rate of HIV-2 compared to HIV-1, and the slower disease progression in HIV-2-infected individuals compared to HIV-1-infected patients, may be due to differences in the humoral immune response in HIV-2- and HIV-1-infected individuals, respectively. In the work presented herein we analyzed the interactions of immunoglobulins (IgA and IgG) with different regions within the HIV-2 ENV in an attempt to identify the different regions and characteristics of HIV-2 gp160 which may be responsible for HIV-2 neutralization.

4.1. Role of IgA in HIV-2 neutralization

The role of IgA in blocking viral infections has been described for many viruses such as poliovirus, cytomegalovirus and influenza virus (*van Ginkel, 2000*). The association of IgA with mucosal surfaces suggests a role for this immunoglobulin subtype in controlling sexual transmission of HIV (*Burrer, 2001*; *Devito, 2000*). In *paper I* we attempted to analyze the regions in HIV-2 ENV that are recognized by HIV-2-specific IgA and we studied the HIV-2 neutralization capacity of serum IgA (sIgA) purified from HIV-2-infected individuals.

Serum IgA (sIgA) purified from the HIV-2-infected individuals reacted with HIV-2 antigens from whole viral lysate (*Paper I*- Table 1). Of the purified sIgA 90%reacted with recombinant gp125 (rgp105), whereas all the sIgA displayed highest reactivity to recombinant gp36 (*Paper I* Fig. 2). To identify the regions withing HIV-2 ENV that are recognized by HIV-2-specific IgA, the binding of sIgA to peptides mimicking the V2, V3, and V4 regions of HIV-2 gp125, and peptides of the central region of gp36 (residues 582-658), was assessed in ELISA. The equivalent regions in HIV-1 gp120 have been suggested to be important for the binding of HIV-1-specific sIgA (*Skott, 1999*), where the C-terminal region of HIV-1 gp41 has been reported to include sites recognized by HIV-1 neutralizing antibodies (*Muster, 1994*; *Vanini, 1993*). Our results indicated that the recognition of the V3 region by HIV-2-specific sIgA ranged between 10% and 28%, while the V2 and V4 regions were recognized by 14% and 7%, respectively, of the purified sIgA. However, the peptide spanning the C-terminal region of gp36 (residues 644-658) was recognized by 72% of the purified sIgA analyzed (*Paper I* Table 1).

The C-terminal region of HIV TM includes the ELDKWA epitope recognized by the nAb 2F5 (*Trkola, 1995*), where immunization with immunogen including this epitope elicited both IgG and IgA capable of neutralizing HIV-1_{MN} and HIV-1_{RF} (*Muster, 1994*). Nevertheless, other neutralizing sites within HIV and SIV TM glycoproteins have been described to be

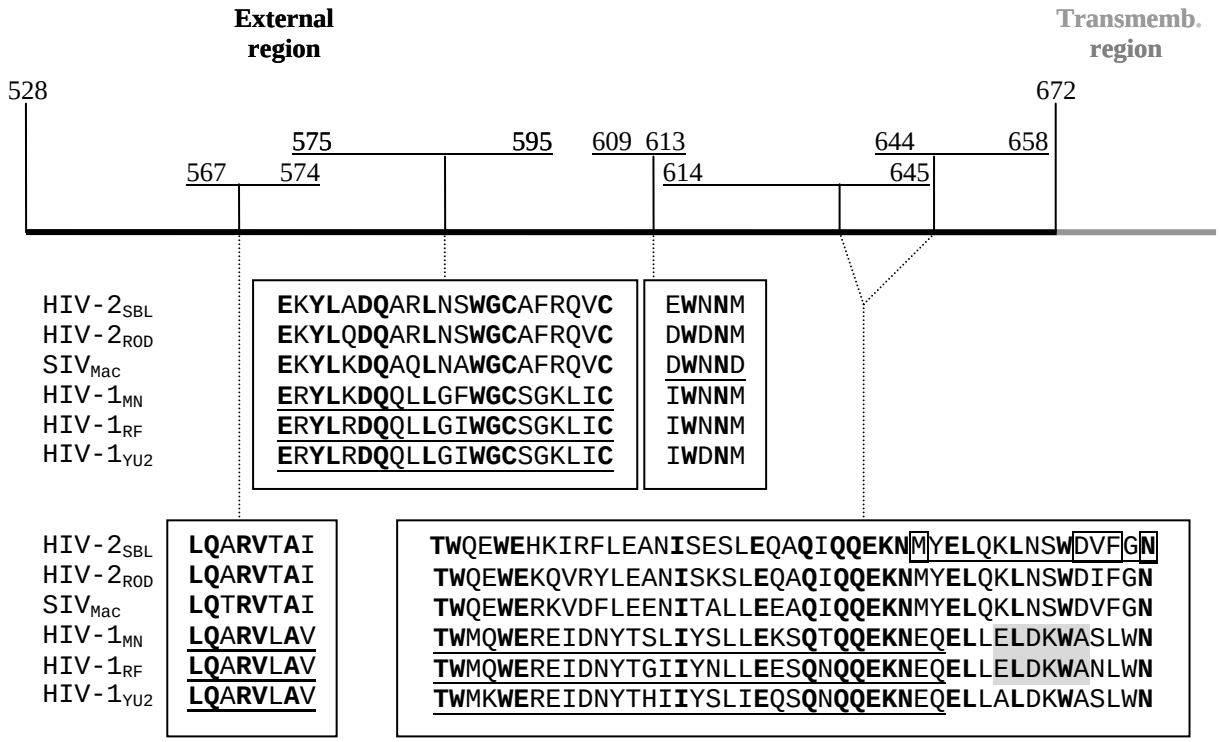


Figure 15 *Alignment of the neutralizing sites within the external regions of HIV and SIV TM glycoproteins.*

Amino acid numbering is according to ISY clone of HIV-$2_{SBL6669}$. Identical residues are in bold, IgA neutralizing sites (regions) in SIV (Kodama, 1991), HIV-1 (*Pastori, 2000*; *Clerici, 2002*) and HIV-2 (*Lizeng, 2003*) are underlined, respectively. The epitope recognized by the neutralizing mAb 2F5 (*Trkola, 1995*), is shaded. Residues within HIV-$2_{SBL6669}$ gp36 that are critical for antibody binding (*Skott, 2002*) are contained within rectangles.

recognized by IgA (summarized in Figure 15). Sequence analyses of these regions indicate that the ELDKWA-epitope is not found in HIV-2 and SIV and that most of the neutralizing sites are more conserved between HIV-2 and SIV. Almost all residues identified as being critical for the binding of HIV-2 antibodies (*Skott, 2002*) are conserved between HIV-2 and SIV isolates (Figure 15, residues in rectangles).

The recognition of gp125 and gp36 by HIV-2 sIgA has similarly been reported for HIV-1, where both gp120 and gp41 were recognized by IgA from HIV-1^{+} sera (*Pastori, 2000*). Conversely, the sera of HIV-1 exposed seronegative (ESN) individuals do not recognize gp120, while recognizing the N-terminal region (equivalent to residues 567-574 in Figure 15) of HIV-1 gp41 (*Pastori, 2000*; *Clerici, 2002*). Furthermore, another group could not confirm the recognition of the ELDKWA epitope or the V3 region by neutralizing IgA isolated from

the mucosal secretion of HIV-1-infected patients (*Moja, 2000*), which could suggest a difference in epitope recognition by serum and mucosal IgA, respectively.

IgA in humans is distributed in a secretory/mucosal and a plasma/systemic compartment. While these two compartments display a certain degree of independence, cross-talk over the mucosal and systemic barriers does occur (*Ogra, 1999*). Serum IgA may give an indication as to the IgA response in the mucosal compartment, as been described for HIV-1 ESN individuals (*Mazzoli, 1999*) where the same IgA activity was observed for sIgA and IgA from vaginal wash samples (*Mazzoli, 1997*). On the contrary, this correlation between serum and mucosal IgA appears to be lacking in HIV-1-infected individuals (*Schneider 1998*). Comparative analysis of healthy and HIV-1^+ individuals has revealed a significantly elevated level of immunoglobulins (Igs) in the intestinal mucosa compared to serum Igs of HIV-1^+ individuals (*Eriksson, 1995*), while both serum IgG and IgA are elevated in HIV-1^+ patients compared to in healthy individuals (*Schneider 1998*).

Our results indicated no significant difference in serum IgA- and IgG-level between HIV-2^+ and healthy individuals, whereas only sIgA and sIgG from HIV-2^+ individuals were capable of neutralizing HIV-2. The results from *paper I* thus suggest a functional role for HIV-2^+ IgA in controlling HIV-2 infection. While only sIgA was analyzed in our study, both serum and mucosal IgA have been described to be functionally similar, where IgA from both systemic and mucosal compartments were capable of inhibiting HIV transcytosis across human epithelial cells (*Devito, 2000*).

4.2.Production & purification of recombinant gp125 proteins

Investigating the humoral immune response in HIV-2 infection requires not only studying the interaction of immunoglobulin with HIV-2 ENV, but also the characterization of HIV-2 envelope subunits. Resolving the crystal structure of HIV-1 gp120 identified many of the regions involved in the interaction with immunoglobulins and cellular receptors (*Kwong, 1998*; *1999*; *Wyatt, 1998*).

In *paper II* we described the construction, expression and purification of two recombinant gp125 glycoproteinns. Both recombinant proteins were constructed in a similar manner as been described for gp120 (*Kwong, 1998*), with deletions at N- and C-termini (Figure 14), and in gp125Δv_1v_2 the V1/V2 region was eliminated and replaced with amino acids Gly-Ala-Gly. Chinese hamster ovarian (CHO*lec*) cells were used to express the gp125 glycoproteins with high mannose sugars, which enabled the purification of these glycoproteins through lectin

affinity chromatography, as well as the characterization the role of sugar moieties on the surfaces of these proteins. Furthermore, these mammalian cells express glycoproteins with minimal glycosylation heterogeneity, rendering the expressed gp125 proteins suitable for crystallization and structural analysis.

The exclusive α-D-mannosyl specificity of *Galanthus nivalis* (GN) allowed the selective binding of the high-mannose glycsoylated gp125 proteins. This lectin has previously been used to purify envelope glycoproteins of HIV-1, HIV-2, and SIV (*Gilljam, 1993*), trapping monomeric forms of their respective SU glycoprotein. Similarly, monomeric gp125 was trapped by the GN-column, while oligomers were detected in the eluent of GN-purified gp125$\Delta v_1 v_2$.

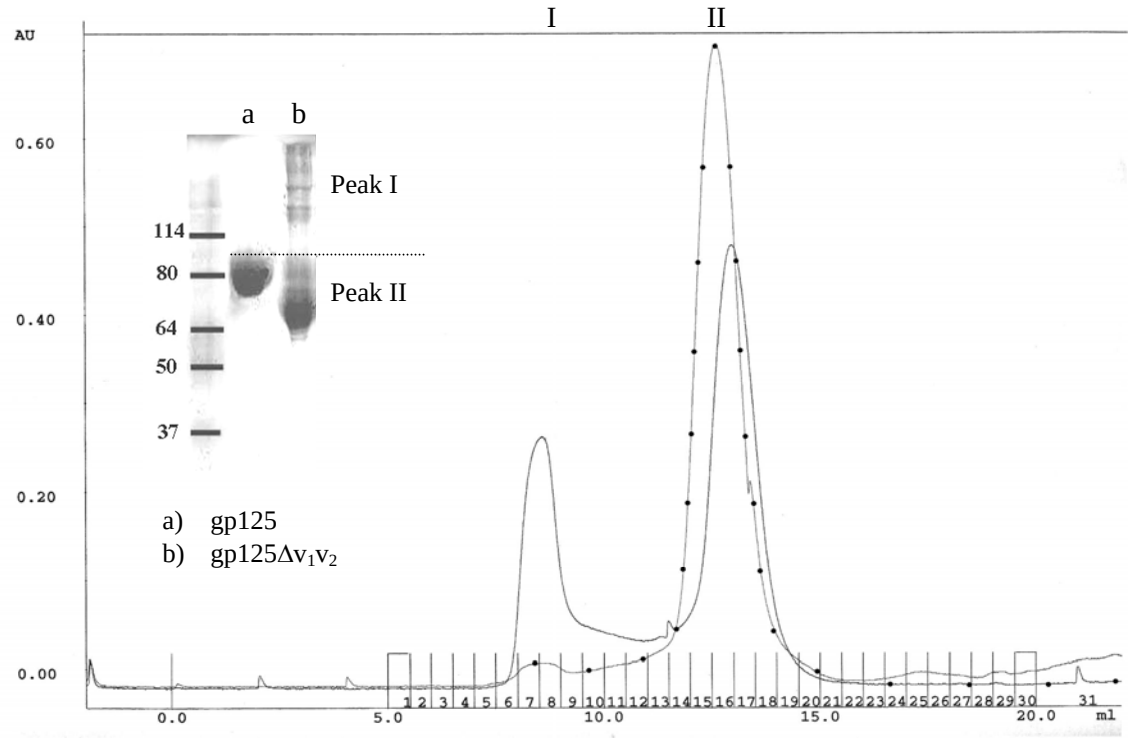


Figure 16 *Superimposition of the the size-exclusion separation chromatograms of GN-purified gp125* (•) *and gp125Δv₁v₂* (-).

Seperation products of the two peaks (I, II) are indicated on the SDS gel.

Figure 16 illustrates the separation of monomeric and oligomeric forms of the GN-purified glycoproteins, where a large portion of the GN-purified gp125$\Delta v_1 v_2$ is oligomeric, and a low proportion of GN-eluted gp125 is oligomeric. The oligomeric proteins were eluted in the first peak, whereas the monomeric proteins were eluted in the second peak. Western blot (WB) analysis of the gp125 proteins in non-reducing conditions confirmed the observation made in Figure 16, and demonstrated that gp125$\Delta v_1 v_2$ forms dimers and trimers (*Paper II* Figure 2C).

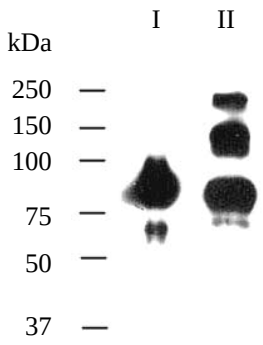


Figure 17 *WB analysis of ConA-purified gp125 in reducing (I) and non-reducing (II) conditions.*

However, mass spectrometry analysis of the GN-purified gp125 proteins indicated that both gp125 and gp125Δv_1v_2 form dimers and no trimers could be detected for gp125Δv_1v_2.

An alternative lectin with different sugar specificity was tested in an attempt to trap a broader variety of glycosylated proteins. Since *Concanavalin A* (ConA) lectin recognizes both α-D-mannosyl and α-D-glucosyl, more glycoproteins were trapped from the cell culture supernatant. To test if ConA was similarly able to trap oligomers of gp125 as those observed in GN-purified gp125Δv_1v_2 fractions, ConA-purified gp125 was analyzed in WB analysis using anti-HA antibody. As indicated in Figure 17, ConA lectin was capable of trapping both oligomeric and monomeric forms of gp125, similar to what was observed for GN-purified gp125Δv_1v_2 (*Paper II* Fig. 2C). ConA also trapped oligomers as well as monomers of gp125Δv_1v_2. However size-exclusion separation of the oligomers and the monomers indicated that more oligomers are produced by gp125Δv_1v_2 than by gp125 (first peak in Figure 18a & b). Moreover, freezing of either recombinant gp125 proteins destabilized the oligomers produced, where less oligomers were detected on the SDS gel after storing the proteins at -20ºC. While ConA trapped oligomers of both recombinant gp125 proteins, several other proteins were also captured from the cell culture supernatant. This consequently required further purification of both gp125 proteins using ion-exchange and size-exclusion chromatographies. The negative charge of gp125 at neutral pH allowed the purification of this protein using the anion exchanger (*MonoQ*) column, while the positive charge of gp125Δv_1v_2 was trapped by the cation exchanger (*SP*) column.

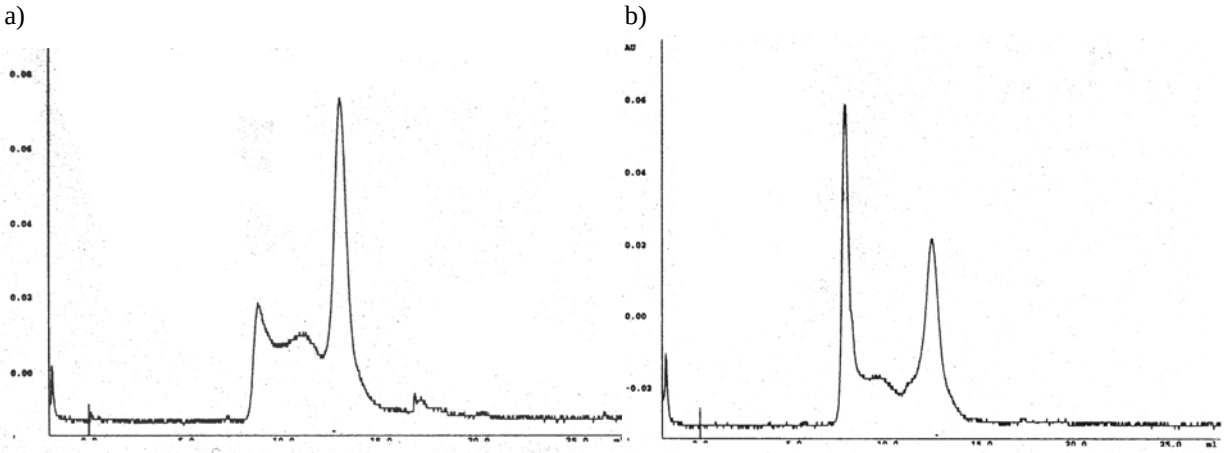


Figure 18 *Chromatograms of the size-exclusion separation of ConA- purified gp125 (a) and gp125Δv_1v_2 (b).*

4.3. Analysis of V3-specific mAbs

Forty murine hybridoma cell lines expressing V3-specific monoclonal antibodies (mAbs), have been previously produced (*Björling, 1994*). These have been generated through the immunization of mice with overlapping peptides of the V3 region (residues 311-326 and 322-337). The 3C4 mAb bound both peptides in ELISA and the ascetic fluid of the 3C4 hybridoma cell line neutralized HIV-2 isolates. Conversely, the 7C8 mAb recognized the peptide spanning the center of the V3 region (residues 311-326), and could not neutralize HIV-2. Since the 3C4 mAb could bind both peptides of the V3 region and was capable of neutralizing HIV-2, it has been suggested that this mAb may be conformationally sensitive. In our analyses we used a single peptide (A43-29) spanning the center and C-terminus of the V3 region (residues 311-330), and we tested the binding of the 3C4 and 7C8 mAbs to this peptide. While the 7C8 mAb bound the V3 peptide (A43-29) in dot blot and SPR analyses (*Paper III* Figure 4) as well as in ELISA (*Paper IV* Figure 1c), weak binding of the 3C4 mAb to this peptide could only be detected in ELISA (*Paper IV*- Figure 1c). Furthermore, the binding of the 3C4 and 7C8 mAbs to the recombinant gp125 and gp125Δv_1v_2 proteins was assessed in WB, dot blot and SPR analysis. All the results indicated that 7C8 bound both monomers and oligomers of the recombinant proteins, in reducing and non-reducing conditions, respectively. Conversely, the 3C4 mAb only bound oligomers of the recombinant proteins, and was sensitive to the conformation of the proteins. Furthermore, comparison of the binding capacity of the 3C4 mAb to the oligomers and monomers of the recombinant protein indicated that 3C4 mAb is oligomeric-specific (Figure 19), which further suggests that this mAb is conformation-sensitive.

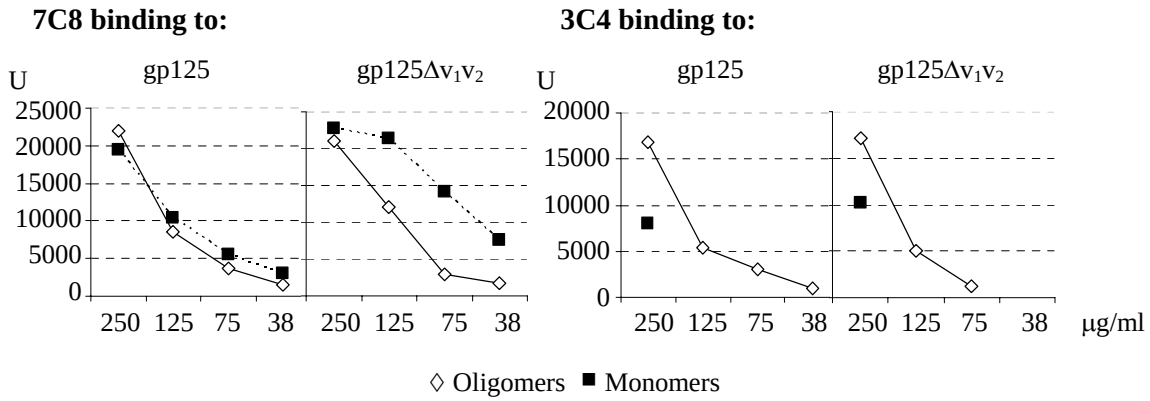


Figure 19 *Dot blot analysis of the 7C8 & 3C4mAbs binding to gp125 proteins.*

Oliogomers of ConA-purified gp125 (1st peak of Figure 18a) or gp125Δv_1v_2 (1st peak of Figure 18b), and monomers of ConA-purified gp125 (3rd peak of Figure 18a) or gp125Δv_1v_2 (3rd peak of Figure 18b) were titrated onto nitrocellulose membranes and 7C8 & 3C4 binding was assessed.

4.4. Accessibility of the V3 region & CD4-independence

The use of the 7C8 and 3C4 mAbs facilitated our analysis of the accessibility of the V3 region in recombinant gp125 proteins. While the linear (GPGR/Q) site at the crown of HIV-1 is believed to be inaccessible on gp120 (*Burton, 2004*; *Splenlehauer, 1998*), the binding of the 7C8 and 3C4 mAbs to monomeric and oligomeric gp125 in blotting analyses (Figure 19; *Paper III* Figure 5) suggests that the V3 region is exposed in HIV-2. Furthermore, SPR analysis of the 7C8 mAb-binding to the recombinant gp125 proteins demonstrated that the deletion of the V1/V2 region did not increase the exposure of the V3 region (*Paper III* Figure 6a). Conversely, removal of glycans on the V1/V2 region of HIV-1 increased the accessibility of the V3 region on gp120 (*Losman, 2001*; *Ly, 2000*).

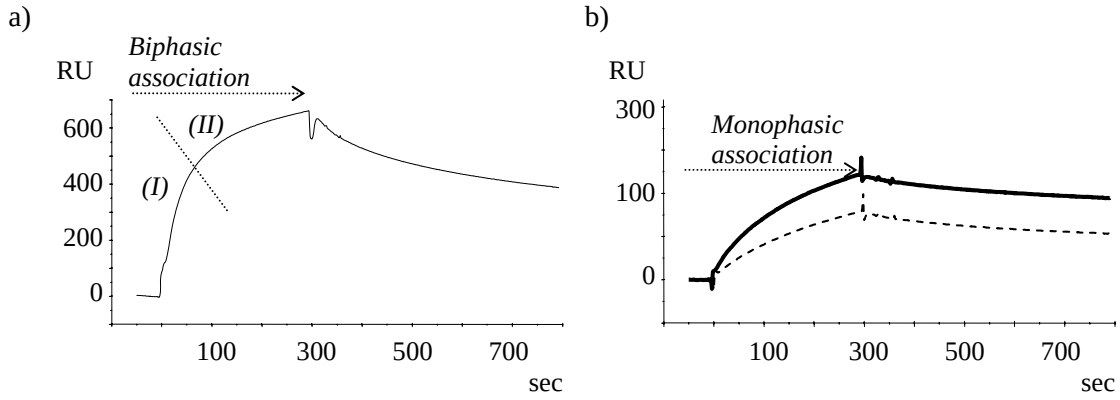


Figure 20 *SPR analysis of sCD4 binding to gp120 and gp125 recombinant proteins.*
a) Biphasic association of sCD4 with gp120, with an initial rapid association (I) followed by conformational changes in gp120 (II). b) CD4 binding to gp125 (bold line) and gp125$\Delta v_1 v_2$ (dashed line).

The exposure of the V3 region and the interaction with a chemokine co-receptor in HIV-1 infections are associated with interactions with CD4 (*Trkola, 1996*; *Wu, 1996*). In *paper III*, we analyzed the binding of soluble CD4 (sCD4) to gp120 and gp125 proteins. HIV-1 gp120 bound sCD4 at a higher affinity than to both gp125 proteins, and displayed a different association pattern to sCD4 than that observed for the gp125 proteins (Figure 20). The binding pattern of sCD4 to gp120 displayed a biphasic association, with an initial rapid association of sCD4 with gp120 followed by a conformational adaptation curve, whereas the binding of sCD4 with gp125 followed the pattern of a monophasic association. The biphasic pattern of sCD4 binding to gp120 confirms the conformational changes induced by sCD4, where sCD4 binding alters the position of the V1/V2 region, exposing the V3 region as well as other neutralizing sites (*Sattentau, 1991*; *Sullivan, 1998*; *Wyatt, 1995*).

Our results, demonstrating a relatively low affinity of sCD4 for gp125, confirms previous reports of the differential affinity of CD4 to gp125 compared to gp120 at levels ranging from two-to eight-fold (*Sattentau, 1993*) to 280-fold lower affinity (*Ivey-Hoyle, 1991*). An increase in CD4 affinity has been described for laboratory-adapted compared to primary HIV-1 isolates (*Moore, 1992*; *Ivey-Hoyle, 1991*). Furthermore, laboratory-adapted HIV-1 isolates have been reported to be more sensitive to neutralization than are primary isolates (*Moore, 1995*), and many of them have been reported to be CD4-independent (*Edwards, 2001*; *Dumonceaux, 1998*; *Kolchinsky, 1999*). While primary HIV-1 isolates are CD4-dependent, a few CD4-independent HIV-1 strains have been characterized. Box 10 summarizes the features described for HIV-1 CD4-independence.

Box 10 CD4-independent HIV-1 characteristics & mutations

Co-receptor use	Region	Mutation	
CCR5	V1/V2 stem	Loss of glycosylation sites	*Kolchinsky, 1999*
CCR5	Hyrophobic cavity	S-to-W mutation	*Xiang, 2002*
CXCR4	C2-V3-C3	Seven point mutations	*Dumonceaux, 1998*
CXCR4	V3, V4/C4	Single point mutation	*Edwards, 2001*

Like most primary HIV-2 isolates, HIV-$2_{SBL6669}$ is a CD4-independent virus (*Liu, 2000*; *Reeves 2002*). Our results, suggesting the accessibility of the V3 region in HIV-$2_{SBL6669}$, may explain the higher neutralization susceptibility of CD4-independent HIV-2 compared to CD4-dependent HIV-1. While HIV-2 displays similar sensitivity to neutralization as described for laboratory-adapted HIV-1 strains, it does not have a higher affinity to CD4 compared to primary HIV-1 isolates, as previously reported for laboratory-adapted or CD4-independent HIV-1 (*Moore, 1992*; *Xiang, 2002*). We analyzed the amino acid (aa) sequence of the HIV-$2_{SBL6669}$ gp125 that we have expressed, and compared the aa sequence with the factors associated with HIV-1 gp120. In *paper III* we give an example of such a comparison, and we demonstrate that none of the aa that are required for HIV-1_{NDK} (*Dumonceaux, 1998*) CD4-independence are present in HIV-2 gp125, and that these residues are conserved between our CD4-independent HIV-2 and the CD4-dependent HIV-2_{ROD}. Analysis of the mutations causing loss of glycoslytion in the V1/V2 and V3 regions that are associated with HIV-1 CD4-independence (*Kolchinsky, 1999; Edwards, 2001*), were not present in our sequence, Furthermore, these residues were also conserved between CD4-independent and CD4–

dependent HIV-2. The only mutation described for CD4-induced conformation of HIV-1 that was also present in our sequence, is the substitution of a serine (S) in the gp120 hydrophobic cavity involved in CD4 binding to a tryptophan (W). We suggested that this mutation may be responsible for the CD4-induced conformation of gp125, allowing the accessibility of the V3 region in HIV-2. The filling of the hydrophobic cavity induces a CD4-induced conformation (*Xiang, 2002*), and this is also evident in HIV-1_{NDK} where this S residue is mutated into a larger hydrophobic residue, methionine (M). However, this single mutation could not be responsible for CD4-independence in HIV-1, since this mutation is not present in the two other characterized CD4-independent HIV-1 isolates (*Kolchinsky, 1999*; *Edwards, 2001*). Furthermore, while this residue may explain a CD4-induced conformation of HIV-2 gp125, it may not be responsible for HIV-2 CD4-independence, since this residue is also present in CD4-independent HIV-$2_{SBL6669}$ and CD4-dependent HIV-2_{ROD}. Interestingly, SIV_{SM} which is closely related to HIV-2, possesses the same W residue, whereas SIV_{CPZ} which is more related to HIV-1, possesses the same M residue present in HIV-1_{NDK}. While CD4-independence is not characterized for SIV, the difference between SIV_{CPZ} and SIV_{SM} is another indication for the difference in the evolution of the CD4-induced conformations of HIV-1 and HIV-2.

4.5. V3-specific antibodies and HIV-2 neutralization

The neutralization capacity described for the 3C4-containing ascitic fluid has been previously suggested to be due to the recognition of a conformational epitope in the V3 region of HIV-2 gp125 (*Björling, 1994*; *Mörner, 1999*). Conformation-sensitive antibodies have been reported to be more potent in neutralizing HIV-1 (*Binley, 2004*; *Conley, 1994*; *Zolla-Pazner, 2004b*), whereas antibodies recognizing the linear (GPGR/Q) site on the crown of gp120 are not capable of neutralizing primary HIV-1 isolates (*Javaherian, 1989*; *York, 2001*). Similarly, mAbs recognizing peptide including the equivalent linear (FHSQ) site on the crown of gp125 were not capable of neutralizing HIV-2 (*Björling, 1994*). While our results confirmed the conformation sensitivity of the 3C4 mAb (*PaperIII*), protein A-purified 3C4 mAb was not capable of neutralizing any of the HIV-2 isolates tested (*Paper IV*).

Since immunization with peptides has been previously reported to induce linear site-specific antibodies (*Burton, 2002*; *Gorny, 2004*), we tested the immunogenicity of the recombinant gp125 proteins. The immunogenicity of gp125 was compared to that of gp125$\Delta v_1 v_2$, where deletion of the V1/V2 region in gp125$\Delta v_1 v_2$ did not increase the immunogenicity of gp125, and both gp125 and gp125$\Delta v_1 v_2$ elicited equivalent antibody titers. The fact that sera from the

gp125Δv_1v_2-immunized mice could cross-react equally well with both gp125 and gp125Δv_1v_2 (*paper IV* Figure 2), could suggest that no new epitopes have been exposed after the deletion of the V1/V2 region. Conversely, it has been demonstrated that the V1/V2 region shields the V3 region as well as other neutralizing targets on HIV-1 gp120 (*Wyatt, 1995*; *Cao 1997*; *Kang, 2005*). Furthermore, immunization trials with gp120 having large or partial deletions in the V1/V2 region demonstrated that these deletions alter the immunogenicity of gp120 and direct the immune responses to the V3 region as well as to other neutralizing sites (*Barnett , 2001*; *Srivastava, 2003*; *Kim, 2003*).

While a high immunogen-specific antibody titer was induced in mice by both gp125 proteins, none of the mouse sera recognized the V3 peptide, A43-39 (residues 311-330), in ELISA and blotting analyses, nor did they neutralized HIV-2. The lack of neutralizing capacity of the immunized mouse sera could be due to the relatively short CDR3 loop of their heavy chain (CDRH3), as the average length of mouse CDRH3 is between 8 and 9 aa (*Wu, 1993*). The third CDR of the heavy chain (CDRH3) of an antibody plays a distinctive role in determining antibody specificity (*Wu, 1993*), and human anti-gp120 neutralizing antibodies have a CDRH3 of >19 aa (*Kwong, 1998*; *Saphire, 2001*; *Zwick, 2004*).

While none of the V3-specific mAbs were capable of neutralizing HIV-2, their respective Fab fragments neutralized both primary and TCLA HIV-2 isolates. However, only intact Fab fragments were capable of neutralization, while over-digested fragments did not neutralize HIV-2. Both 3C4 and 7C8 mAbs had a slightly longer CDRH3 loop than average mouse antibodies. Nevertheless, we propose that the smaller size of Fab fragments compared to whole antibodies enables these full access to the V3 region on the virion surface (Figure 21).

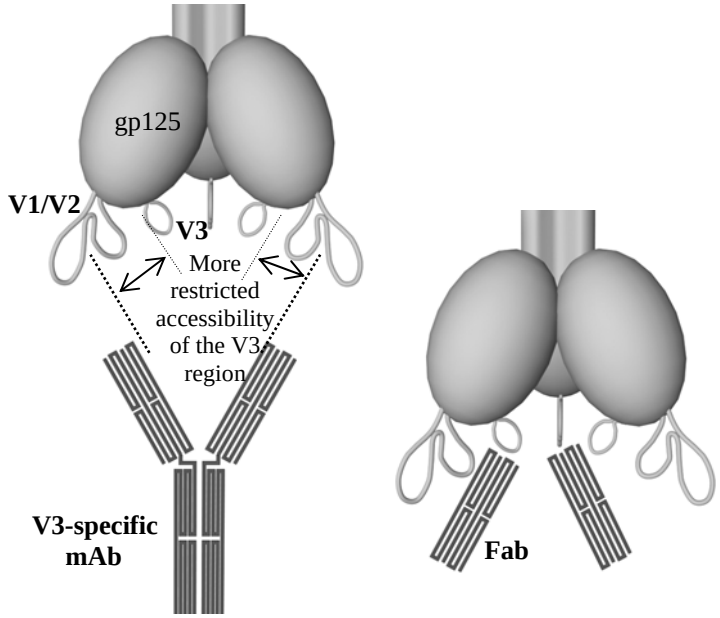


Figure 21 *Schematic presentation of the accessibility of the V3 region on HIV-2 surface.*

Left: A model of the HIV-2 ENV with the V3 region accessible on the surface of the virion. The Fc region of a mAb prevents full access of the Fab arms to the V3 region. *Right:* Removal of the Fc region allows full access of V3-specific Fabs to the V3 region.

5. GENERAL ASPECTS

The lower sexual transmission and stronger humoral immune response of HIV-2 infections compared to infections with HIV-1 suggest that HIV-2 may posses characteristics useful as a model for the design of vaccines against HIV. In this thesis the interaction of HIV-2-specific IgA with HIV-2 ENV was studied to assess the role of IgA in neutralizing HIV-2, which may be responsible for the low transmission of HIV-2. Furthermore, the accessibility of the V3 region was studied in correlation to the conformation of gp125 and the neutralization sensitivity of HIV-2.

The results presented in this study indicate that HIV-2-specific IgA primarily recognizes the C-terminal region of extracellular gp36, whereas the neutralization capacity of the HIV-2-specific IgA suggests a role for IgA in preventing transmission of HIV-2. Moreover, the lack of hyper-production of sIgA and IgG in HIV-2-infected individuals compared to healthy individuals suggests that HIV-2 infections do not disregulate B cell activity as been described for HIV-1 infections. While IgA production in HIV-2 infections may resemble the IgA response of HIV-1 ESN individuals, HIV-2-specific IgA binding to the N-terminal region of extracellular gp36 remains to be analyzed, to investigate whether HIV-2-specific IgA recognizes similar regions as been described for the IgA isolated from HIV-1 ESN. Other potential experiments are summarized in Table 6.

Our analyses suggest that the conformation of gp125 allows the accessibility of one of the main neutralizing site (V3 region), which may in turn explain the neutralization sensitivity of HIV-2. Structural studies of gp125 will define the conformation of HIV-2 SU and may provide further explanation for the neutralization sensitivity of HIV-2. The recombinant gp125 proteins described in this thesis are suitable for crystallization, and their glycosylation homogeneity may also enable the analysis of glycan moieties on HIV-2 surface.

While our results suggest an accessible V3 region on the surface of HIV-2, only intact HIV-2-specific Fab fragments were capable of neutralizing HIV-2. Further analyses of the papain digestion products, such as N-terminal sequencing, are required to verify the role of the Fv region in neutralization. Furthermore, we have crystallized both V3-specific Fab fragments, and resolving their structure may explain the significance of the CRH3 loop of these murine antibodies.

Topic	Paper	Conclusions	Future Studies
HIV-2-specific serum IgA (sIgA)	I	• HIV-2-specific sIgA recognizes C-terminal region of gp36. • HIV-2-specific sIgA neutralizes HIV-2 *in vitro*. • No difference in IgA level between HIV-2-infected and healthy individuals.	• Test if sIgA recognizes peptides of gp36 N-terminal region. • Test if IgA from mucosal secretions (dIgA) also neutralizes HIV-2. • Test if IgA recognizes conformational epitopes in V2 region, by comparing IgA-binding to gp125 versus gp125$\Delta v_1 v_2$.
Conformation of gp125	II, III	• Deletion of the V1/V2 region increases oligomerization of gp125. • V3 region is accessible on gp125. • Features responsible for CD4-independence are not common for HIV-1 & HIV-2. • Filling of the hydrophobic cavity with W may be responsible of the CD4-induced conformation of gp125. • CD4 binds to gp125 at lower affinity and with different kinetics compared to gp120.	• Crystallize gp125 proteins described in paper II, resolve the structure of these glycoproteins and define conformation of HIV-2 SU. • Compare CD4 binding to recombinant gp125 to native GN-purified gp125.
V3-specific mAbs	IV	• 7C8 and 3C4 mAbs recognize a linear site and conformational epitope, respectively. • Only intact V3-specific mAbs neutralize HIV-2 and SIV.	• N-terminal sequencing of Fab and over-digested Fab, to verify role of Fv region. • Test neutralization capacity Fabs from other mAbs. • Test HIV-1 cross-neutralization capacity of 3C4 and 7C8 Fabs.
gp125 immunization	IV	• Deletion of the V1/V2 does not increase the immunogenicity of gp125. • Sera of gp125-immunized mice do not neutralize HIV-2.	• Immunize rabbits with recombinant gp125 proteins, and test if the long CDRH3 loop of their antibodies can neutralize HIV-2.

Table 6 *Summary of the conclusions made from the different studies of this thesis and proposal of future experiments.*

Investigations of HIV infection indicate that the differential evolution of HIV-2 and HIV-1 has led to the development of one virus type (HIV-2) sensitive to neutralization by human antibodies, while the other type (HIV-1) is more resistant to neutralization. The outcome of viral infection is also determined by the type of species infected (Figure 22), where sooty mangabey monkeys are the natural host for SIV_{SM}, whereas this virus causes AIDS in rhesus macaques. However, while most individuals infected with HIV-1 progress to AIDS, there are also reports of HIV-1 exposed uninfected (HIV-1 ESN) individuals. These facts suggest a role for genetic factors in determining the outcome of an infection. Moreover, sexual exposure to certain HIV strains at the "correct" dose and at appropriate frequencies may also allow certain individuals to be naturally vaccinated against HIV.

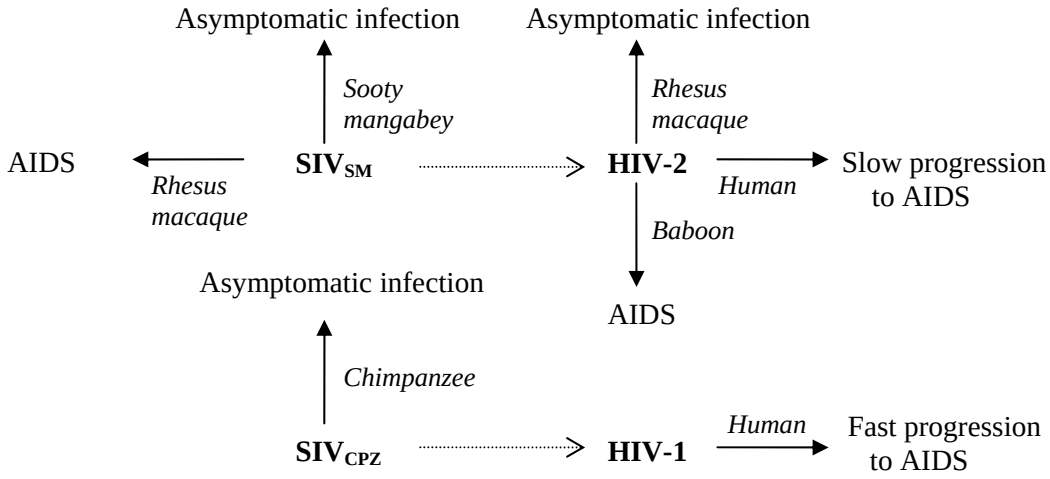


Figure 22 *Examples of natural and cross-species infections of SIV and HIV.*

The exposure of neutralizing sites on HIV-2 gp125, and the early reports of the cross-reactivity of HIV-2 ENV-specific antibodies with HIV-1 ENV, supports the concept of using HIV-2 gp125 as an immunogen in the formulation of a vaccine against both HIV-1 and HIV-2. While there is no clear evidence of HIV-2-infections causing protection against HIV-1 infections in human, *in vitro* studies indicate that HIV-2 infection down-regulates infection with HIV-1. Box 11 gives examples of potential use of recombinant gp125 proteins in HIV vaccination trials, where the possibility of generating gp125-specific antibodies that can neutralize HIV-1 needs to be tested. Alternatively, gp125/gp120 hybrids could be constructed, making use of the CD4-induced conformation of gp125 exposing neutralizing sites, while replacing gp125 variable (V1-V3) regions with gp120 variable regions. While HIV-1 is CD4-dependent and several of HIV-1 neutralizing sites are accessible only after CD4 binding, pre-

immunization with recombinant gp125 proteins may generate antibodies that can act during this narrow window of opportunity.

Box 11- Potential HIV prophylactic concepts		
Recombinant gp125 proteins	*Active immunization* →	Induce cross-reactive HIV-1 nAbs.
Combine gp125 core with gp120 variable loops	*Active immunization* →	Induce nAbs specific for HIV-1 unexposed sites.
Protein engineered stabilized Fab	*Passive immunization* →	Inhibit acute viral infection
DNA vector encoding Fab fragments	*Active immunization* →	Inhibit viral infection at local infection sites

Another concept that arose in this thesis is the use of V3-specific Fabs in HIV neutralization. While Fab fragments are not naturally produced in humans, passive immunization of these molecules may be useful during critical physiological conditions, such as at child birth. However, Fab fragments are less stable and may have a shorter half-life than whole antibodies. Nevertheless, the smaller size of Fab fragments compared to whole antibodies may allow these chains to be more easily stabilized through protein engineering and/or expressed through DNA vectors. Furthermore, expression through DNA vectors will enable the manipulation of generating Fab molecules under certain promoters, which may direct the production of these components to specific regions, such as mucosal surfaces.

Finally, whereas the results from this thesis may provide clues to HIV prophylactic concepts, the evaluation of HIV inhibition is equally important for the development of anti-HIV agents. Moreover, functional analyses need to be combined with structural studies to design more specific agents. While HIV-2 infections appear to induce more potent immune responses and is better tolerated in humans than HIV-1 infections, further analyses of alternative lentiviruses in other species may lead to the discovery or development of viruses or viral components that are tolerated in humans, providing cross-protection against HIV strains.

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