

Thesis Submitted for the Degree of Doctor of Philosophy



The Design and Development of an HIV-1 vaccine to elicit a Broadly Neutralising antibody response

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To those who suffer from HIV/AIDS.

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Abbreviations

Ab	Antibody
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
ADCVI	Antibody-Dependent Cell-mediated Viral Inhibition
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
ANAb	Autologous Neutralising Antibody
APCs	Antigen Presenting Cells
APOBEC3G (A3G)	Apolipoprotein B mRNA-editing Enzyme Catalytic Polypeptide-like 3G
BCR	B cell Receptor
BN-PAGE	Blue-Native Polyacrylamide Gel Electrophoresis
BNAb	Broadly Neutralising Antibody
BP	Base-pairs (DNA)
BS³	Bis[sulfosuccinimidyl] suberate
BSA	Bovine Serum Albumin
bt	Biotin/Biotinylated
CA	Capsid protein (Gag)
CD4	Cluster of Differentiation 4
CD4bs	CD4 Binding Site
CD4i	CD4-induced
cDNA	Complementary DNA
CDR	Complementarity Determining Regions
CHR	C-terminus Heptad Repeat (gp41)
CRFs	Circulating Recombinant Forms
CT	HIV-1 Env Cytoplasmic Domain
CTL	Cytotoxic T-lymphocytes
Da	Dalton (Protein size)
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle's Medium Supplemented
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EC	Elite Controllers
ELISA	Enzyme-linked Immunosorbent Assays
Env	Envelope protein

Fab	Fragment Antibody-binding
Fc	Fragment Crystallisable
FACS	Flow Cytometry
FCS	Fetal Calf Serum
FP	Fusion Peptide (gp41)
GALT	Gut-associated Lymphoid Tissue
GP120	HIV-1 Glycoprotein 120kDa subunit
GP140	Env protein without transmembrane and cytoplasmic domain (Glycoprotein 140kDa)
GP160	Full Env protein (Glycoprotein 160kDa)
GP41	HIV-1 Glycoprotein 41kDa subunit
HA	Hemagglutinin (Influenza)
HAART	Highly Active Anti-Retroviral Therapy
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HV	Hypervariable region
I.M.	Intramuscularly
IAVI	International AIDS Vaccine Initiative
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMAC	Immobilised Metal ion Affinity Chromatography
IN	Integrase
KIRs	Killer immunoglobulin-like Receptor
LTNP	Long Term Non-Progressor
LTRs	Long tandem Repeats
MA	Matrix protein (Gag)
mAb	Monoclonal
MHC	Major Histocompatibility Complex
mITT	Modified Intention-to-treat
MPER	Membrane-Proximal External Region
MPL	Monophospholipid
mRNA	Messenger RNA
NAb	Neutralising Antibody
NC	Nucleocapsid
Nef	HIV-1 Negative Factor
NHR	N-terminus Heptad Repeat (gp41)
NK	Natural Killer

OD	Outer Domain (gp120)
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PIC	Pre-integration Complex
POC	Proof-of-Concept
PP	Per-Protocol
PR	Polar Region (gp41)
R5	Macrophage-tropic HIV-1
RNA	Ribonucleic Acid
RNAP	RNA Polymerase
RRE	Rev-response Element
RT	Reverse Transcriptase
S.C.	Subcutaneously
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SHIV	Chimeric SIV/HIV Virus
SIV	Simian Immunodeficiency Virus
SPR	Surface Plasmon Resonance
TBS	Tris-Buffered Saline
TCR	T cell Receptor
TLR	Toll-like Receptor
TM	Transmembrane Domain
TNF	Tumour Necrosis Factor
TRIM	Tripartite Motif
V1/V2 loop	Variable Loop 1/2
V3 loop	Variable Loop 3
VLPs	Virus-like Particles
Vpr	HIV-1 Viral Protein R
Vpu	HIV-1 Viral Protein U
X-Gal	β -Galactoside

Amino acid letter codes

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Nucleotide Letter codes

A	Adenosine
T	Thymidine
G	Guanidine
C	Cytidine
U	Uridine

Publication

Papers

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Design and Development of an HIV-1 vaccine to elicit a Broadly Neutralising antibody response

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Abstract

Despite 30 years of research, a prophylactic vaccine against HIV-1 is still lacking and is urgently needed in order to control the global AIDS pandemic. The discovery of broadly neutralising antibodies (BNABs) was an important step for HIV-1 research but no vaccine candidate tested so far has been able to reproduce responses containing such antibodies, and it remains unclear how this could be achieved via immunisation. In this thesis, I attempted to explore this gap of knowledge in two ways. First, certain features ('signatures') of the Env protein that were associated with a broadly neutralising response were identified through machine learning. Further characterisation of these signatures revealed several ways by which these naturally-occurring mutations might alter the immunogenicity of the Env protein that could result in the elicitation of a broadly neutralising response. The incorporation of such signatures in future vaccine design could be useful as the Env protein might adopt a conformation that encourages the elicitation of a broadly neutralising response. Second, 3 novel vaccination approaches were proposed aiming to induce a BNAB antibody response. The development of 2 approaches proved to be difficult and was not continued. For the third approach, non-neutralising immunogen-derived antibodies were used to mask immunodominant epitopes on the Env protein (i.e. 'antibody-shielding'), thus allowing the antibody response to be focused to the highly conserved CD4 binding site (CD4bs). Subsequent immunisation of the antibody-shielded gp120 proteins in mice and rabbits demonstrated that antibody-shielding was able to significantly dampen the V3-specific antibody response while retaining the CD4bs-specific response. However, the antibody response to the V1/V2 loop was enhanced upon V3-dampening which indicates that further optimisation of the antibody-shield is needed in order to eliminate any antibody response towards the immunodominant regions. In conclusion, these results are the first description of a number of novel vaccination ideas and provide valuable insights into how these approaches could be optimised to become effective HIV-1 vaccines that can lead to the elicitation of a broadly neutralising antibody response.

Chapter 1

Introduction

1.1 Combating the global AIDS pandemic

The discovery of vaccination in the 18th century is among the greatest achievements of modern medicine. With more than 70 vaccines licensed under FDA in the United States targeting over 20 diseases [sourced from FDA], vaccination has become the most effective and economical way of preventing infectious diseases such as Influenza, Hepatitis B, and Human Papillomavirus (HPV) etc. In particular, the eradication of Smallpox in the 20th century is the perfect example of how successful vaccination programmes can lead to the clearance of killer diseases in the human population (324). However, despite the success of these vaccination programmes, an effective vaccine against the **H**uman **I**mmunodeficiency **V**irus (HIV) – the causative agent of the **A**cquired **I**mmunodeficiency **S**yndrome (AIDS) – is still lacking.

Since its first description more than 30 years ago, AIDS has become one of history's worst pandemics with more than 25 million people dying of AIDS since 1981, and an annual toll of about 2 million deaths (UNAIDS global report 2010). Even though the numbers of newly infected (2.7 million) and AIDS-related deaths (1.8 million) per year have been declining since the late 90s, the number of infected individuals is still rising steadily. More than 33 million people are currently living with AIDS, of which 2.5 million are children less than 15 years of age (Fig. 1.1). To better contain the spread of HIV/AIDS, several strategies have been devised to reduce the rate of HIV infection in persons at risk of exposure. These methods include screening of blood donors, testing blood-derived products, counselling and safer sex campaigns etc. [reviewed in (140)]. In addition, apart from using traditional antiretroviral therapy as a post-exposure treatment, its potential to be used as a pre-exposure prophylaxis (PREP) was also explored in several studies, and have been correlated with a decrease in the risk of infection among men who have sex with

men (MSM), in heterosexual couples and in mother-to-child transmission (70, 107, 152, 390). Recently, male circumcision (17, 156, 429) and the use of tenofovir vaginal gel pre- and post-sexual intercourse has also shown to be able to reduce the rate of HIV-1 transmission (2).

Although the full implementation of these preventive measures has shown promise in controlling the spread of virus in a certain geographical setting (e.g. Uganda), the scaling-up of these measurements into a global setting is complicated by a number of logistical, economical and behavioural issues (UNAIDS global report 2010). Before these issues can be addressed fully, the development of a highly efficacious vaccine that would prevent HIV infection remains one of the major research focuses in modern medicine.

Global Prevalence of HIV, 2009

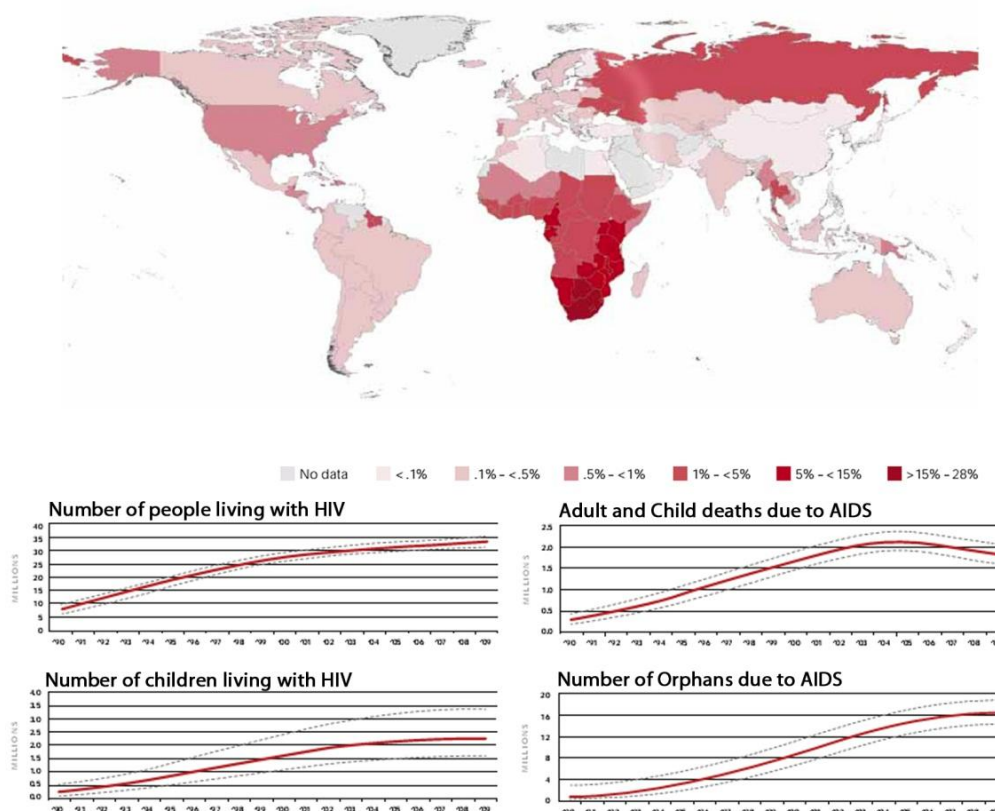


Figure 1.1 The Global AIDS Pandemic

(Top) Global Prevalence and (Bottom) Trends of the global AIDS pandemic. All figures were adapted from the UNAIDS AIDS Global Report in 2010.

1.2 The causative agent of AIDS – Human Immunodeficiency Virus

In 1983, two years after the description of the first case of AIDS, scientists from the Pasteur Institute isolated the virus that causes the disease (303), and this virus was officially named the Human Immunodeficiency virus (HIV) in 1986 (302). Two types of HIV have been described to date – HIV-1 and HIV-2 (140). Although both types of HIV-1 can cause AIDS, HIV-2 is considered less virulent and is geographically limited to West Africa, while HIV-1 is the most common and pathogenic virus that is responsible for the global AIDS pandemic and is therefore the major focus in HIV research.

1.2.1 The Structure of an HIV-1 virus

HIV belongs to the Lentivirus genus in the *Retroviridae* family that also includes the Simian Immunodeficiency Virus (SIV) from which HIV-1 is derived. Like all lentiviruses, HIV-1 is an enveloped RNA virus with each virus possessing 2 copies of single-stranded positive sense RNA (Fig. 1.2). A mature HIV-1 virion has a spherical morphology of 100-120nm in diameter, and each virion consists of an internal cone-shaped capsid surrounded by the viral lipid envelope that is wrapped on a scaffold of matrix proteins. The lipid shell of the HIV-1 virus is derived from the host cell membrane during the budding stage of its life cycle (see section 1.3 later), and the proteins that form the matrix scaffold and the inner cone-shaped capsid – p17 (MA) and p24 (CA) respectively – are cleaved products of the viral protein Gag. It is estimated that roughly 1,500 Gag molecules are required for the formation of an intact HIV-1 virus particle (60). On the viral surface, about 7-15 of the viral Envelope (Env) proteins are embedded in the viral membrane and are involved in the viral entry into the target cells (246, 456). The viral genomic RNA, which is essential for the replication of the virus, can be found inside the cone-shaped capsid together with the viral protease (PR), reverse transcriptase (RT), integrase (IN), Vpu, Vif, Vpr and Nef, and other cellular factors.

1.2.2 The HIV-1 genome

The HIV-1 viral genome is roughly 9.8kbp long, and consists of 9 opening reading frames that encode

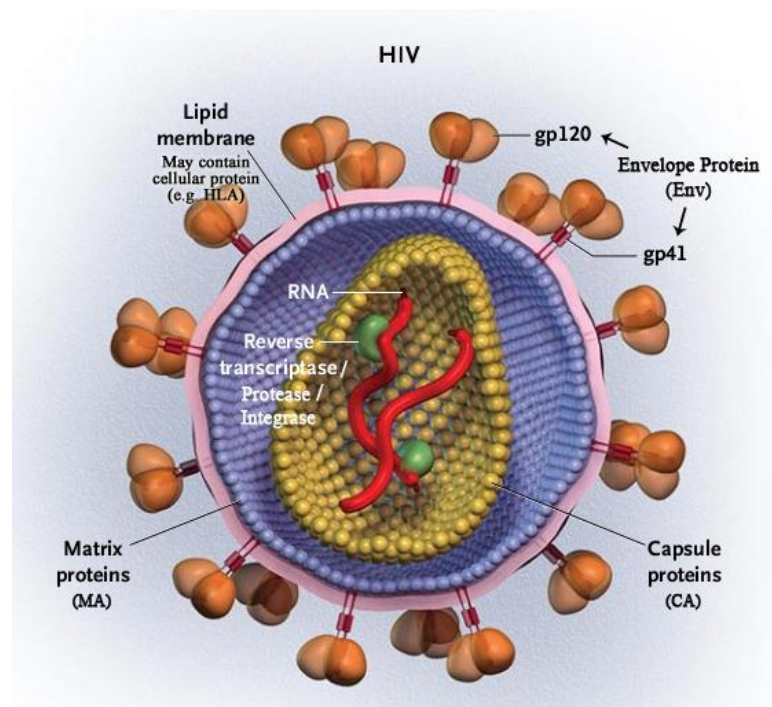


Figure 1.2 **Structure of a mature HIV-1 virion**

HIV virion with the structural and other virion proteins identified. The exact locations of Nef, Vpu and Vpr are not shown here. This figure was adapted and modified from (199).

all the proteins that are essential for the virus to replicate and survive (*gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vif*, *vpu* and *vpr*) (Fig. 1.3). The viral genome is flanked by Long Terminal Repeats (LTRs) at both ends, which contains the promoter and enhancer sequences for the regulation of gene expression. The viral proteins can be classified into 3 groups – **Structural** (Gag, Pol and Env), **Regulatory** (Tat and Rev) and **Accessory** (Vpu, Vpr, Vif and Nef) – and they are all derived from the same primary transcript (317). The timing of which these viral proteins are translated depends mainly on the level of splicing – transcripts that encode for the regulatory proteins are fully spliced and are expressed early, whereas transcripts that encode for the structural and accessory transcripts are either singly- or un-spliced and are expressed later in the replication cycle (see section 1.3.3). The function and importance of these viral proteins are summarised in Fig. 1.3.

MA, matrix (p17)

Undergoes myristylation that helps target Gag to lipid rafts;
Involved in nuclear import of HIV pre-integration complex (PIC)

CA, capsid (p24)

Binds cyclophilin A; Forms the viral inner core in the mature HIV virus

NC, nucleocapsid (p7)

RNA-binding protein; Helps in reverse transcription

p6

Interacts with cellular ESCRT machinery to promote to fission and release of virions from the cell surface

Gag

Overcome inhibitory effects of the cellular viral inhibitors APOBEC3G

Transcriptional Activator

Binds to the trans-activating response element (TAR) which are found at the 5' LTR of the nascent HIV transcripts, and increases the production of full-length viral RNA via the recruitment of p-TEFb

Tat

Interacts with newly synthesised CD4 in the ER and mediate proteasomal degradation of CD4; Counteracts Tetherin

Vif**Vpr****Vpu****Nef****Rev****Env****Negative effector**

Enhances uncoating of the viral core by inducing local pH change; Down-modulates CD4 and MHC class I from the cell surface to facilitate the release of fully infectious virions, and to protect the infected cells against CTL lysis; Elevate the transcription of the viral LTR promoter and viral replication via the induction of cellular transcription factors, e.g. NF-AT, NF- κ B;

Binds to RNA response element (RRE); Inhibits viral RNA splicing and promotes nuclear export of singly- and un-spliced RNA for the synthesis of later-stage viral proteins (e.g. Env)

Pol

Encodes for a number of viral proteins:

Reverse Transcriptase (RT)

Transcribes the viral RNA into dsDNA

Integrase (IN)

Integrates the viral DNA into host's genome

Protease (PR)

Cleaves newly synthesised polyproteins to create the mature protein components of an infectious HIV virion (e.g. Env, RT, IN)

Vpr

Part of the PIC and helps entry into the nucleus of cells; Promotes G2 cell cycle arrest; Enhancement of the fidelity of reverse transcription

Env

Contains RNA response element (RRE) that binds Rev; gp160 envelope protein cleaved in trans-Golgi to gp120 and gp41:

gp120 (SU) mediates CD4 receptor and chemokine (CCR5/CXCR4) receptor binding

gp41 (TM) mediates fusion

Figure 1.3 Genomic map of HIV-1 and summary of the function for the viral proteins

Shown here is the genomic map of HIV-1 showing the 9 opening reading frames (ORFs) that encode for the viral proteins. The central figure was adapted from the HIV-1 database (www.hiv.lan.gov) with the numbering referring to the codon position of the genome of the reference strain HxB2. Additional information regarding the key functions of the various viral proteins were summarised from (157, 214).

1.2.3 Diversity of HIV-1

The vast diversity observed in HIV-1 is mainly due to several factors: an error-prone RT (345), a high susceptibility to recombination (40), an extremely rapid turnover rate (345) and selection pressure exerted by the host immune response (272). While all of the viral genes are susceptible to mutation and protein variation, the greatest diversity is observed in the Env proteins as they are under constant selection pressure from the host immune response (267). HIV-1 can be subdivided into 4 groups based on the Env sequence, with the sequence diversity between groups differing by as much as 50%. The different HIV-1 groups include: the pandemic group M (Major) that is responsible for more than 90% of HIV/AIDS cases globally, the less prevalent groups O (Outlier) and N (Non-M, Non-O), and the recently identified group P ('Proposed') (140, 321). Virus strains in group M can be further divided into 9 phylogenetically-linked

subtypes/clades designated A-K (A-D, F-H, J and K), and sequences of distinct clades can vary by as much as 35% across clades, and 15% within clades (251, 304). Furthermore, there are also ‘circulating recombinant forms (CRFs)’ of HIV-1 which are generated through the recombination of viral genomes as a consequence of co- or super-infection of individuals with viruses belonging to different clades, e.g. CRF01_AE is the recombinant form of HIV-1 viruses from clade A and E (251). According to the Los Alamos HIV-1 Database, more than 40 CRF recombinants have been described to date. In the case of influenza vaccine, it was discovered that a < 2% amino acid difference can cause a failure in cross-reactivity of the polyclonal response to the vaccine (134). Based on such observation, it was therefore suggested that, unless a broadly neutralising response is to be elicited, an HIV-1 vaccine developed for one specific clade is highly unlikely to confer universal protection against heterologous HIV-1 challenges (32, 200, 327).

1.3 Different Stages of the HIV-1 Life Cycle

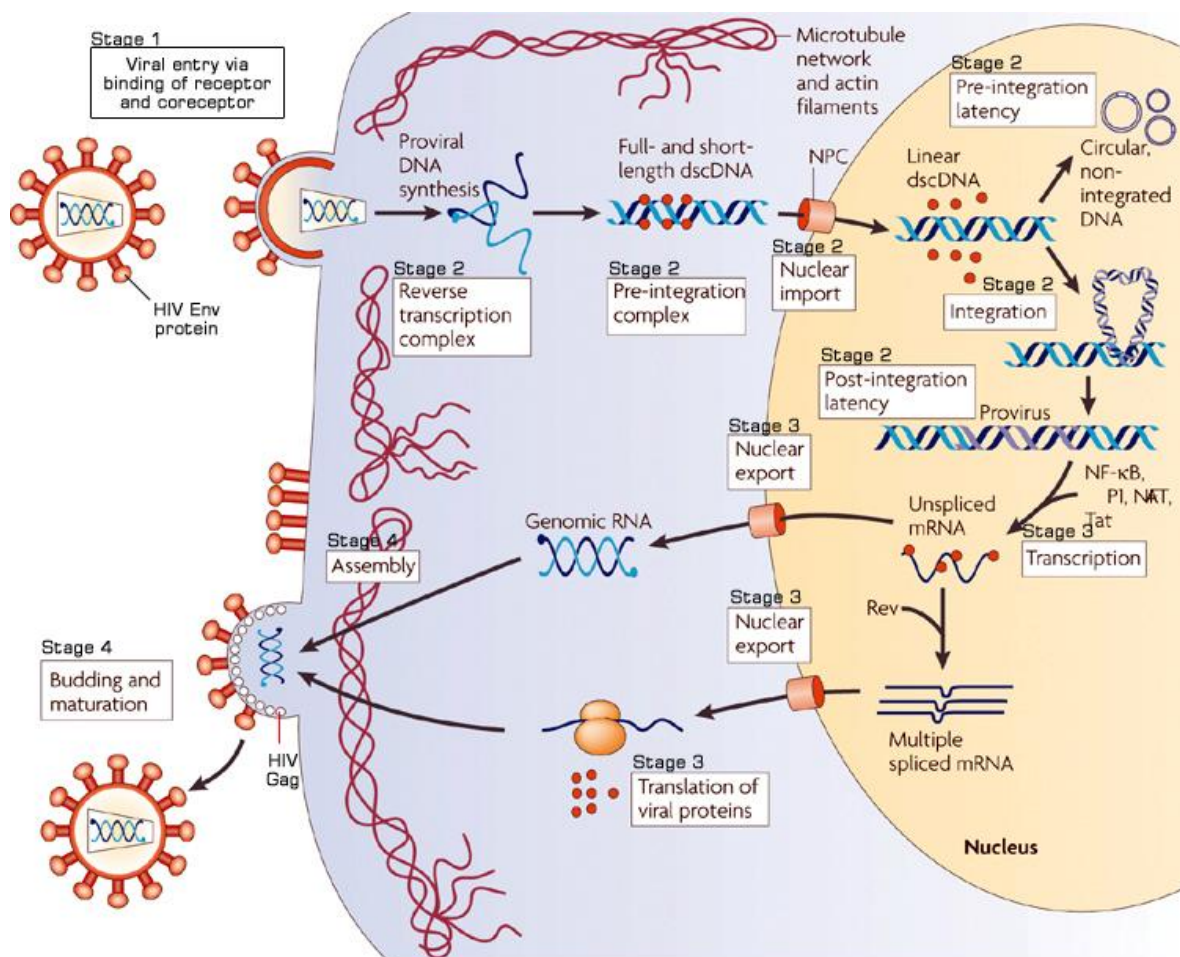
1.3.1 Stage 1 – Viral Entry into the target cell

An overview of the HIV-1 life cycle is shown in Fig. 1.4. The replication of HIV-1 begins with the attachment of the virion to the cell surface which is mediated through the binding of **Env** protein to the cellular CD4 receptor (Fig 1.4 & 1.5). The Env protein is a 160kDa glycoprotein that is cleaved into a gp120 external subunit and a gp41 transmembrane subunit, and assembles non-covalently to form a trimeric spike on the viral surface (see section 1.7.1 for more details on the Env protein). Upon CD4 binding, gp120 undergoes a conformational change that exposes the otherwise hidden binding site for the chemokine co-receptor, CCR5 or CXCR4 (233) (Fig. 1.5). The binding of CD4 and the co-receptor then leads to further conformational changes in gp41, which springs open to project 3 fusion peptides that ‘harpoon’ the lipid bilayer of the plasma membrane of the target cell (67). Further structural rearrangement of gp41 eventually draws the viral and cellular membrane together, initiating fusion and enabling viral entry into the cell (67).

Although the direct fusion of the viral and cell membrane has long been considered as the major pathway for HIV-1 internalisation (28, 253, 384), there is also increasing evidence that points to an endocytic route for HIV-1 entry (57, 116, 281, 312, 316). Nevertheless, the exact contribution and mechanism of this pathway remains to be fully understood.

1.3.2 Stage 2 – Reverse Transcription of viral genome and integration into host chromosome

Inside the cytoplasm, the uncoating of the viral core is initiated through a number of events, including the phosphorylation of the **MA** proteins (64) and the local pH change induced by the association between **Nef** and the V-ATPase in a way similar to the influenza M2 protein (346). Once the viral core is successfully uncoated, this is followed by the reverse transcription of the viral RNA to cDNA through the assembly of the reverse transcription complex, which comprises the viral RNA, tRNA^{Lys} primer, RT, IN, MA,



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Figure 1.4 Overview of the HIV-1 life cycle

Shown here is an overview of the HIV-1 life cycle. The classification of different stages (above each box) is as discussed in the text. Abbreviations used in the figure: ‘dscDNA’ – double-stranded cDNA; ‘NCS’ –nuclear pore complex. This figure was adapted and modified from (79).

nucleocapsid (NC), Vpr and various host proteins (206). However, during reverse transcription, the cellular antiretroviral protein APOBEC3G can hypermutate the viral cDNA that leads to many lethal changes and stop codons, and the viral protein **Vif** is crucial to counteract the action of this protein (205, 258, 259, 372). Once the reverse transcription is completed, this forms the pre-integration complex (PIC) which comprises the double-stranded viral cDNA, IN, MA, Vpr, RT and host transcription proteins (279). With the help of **Vpr** (453), the PIC then enters the nucleus through the nuclear pore, where the viral

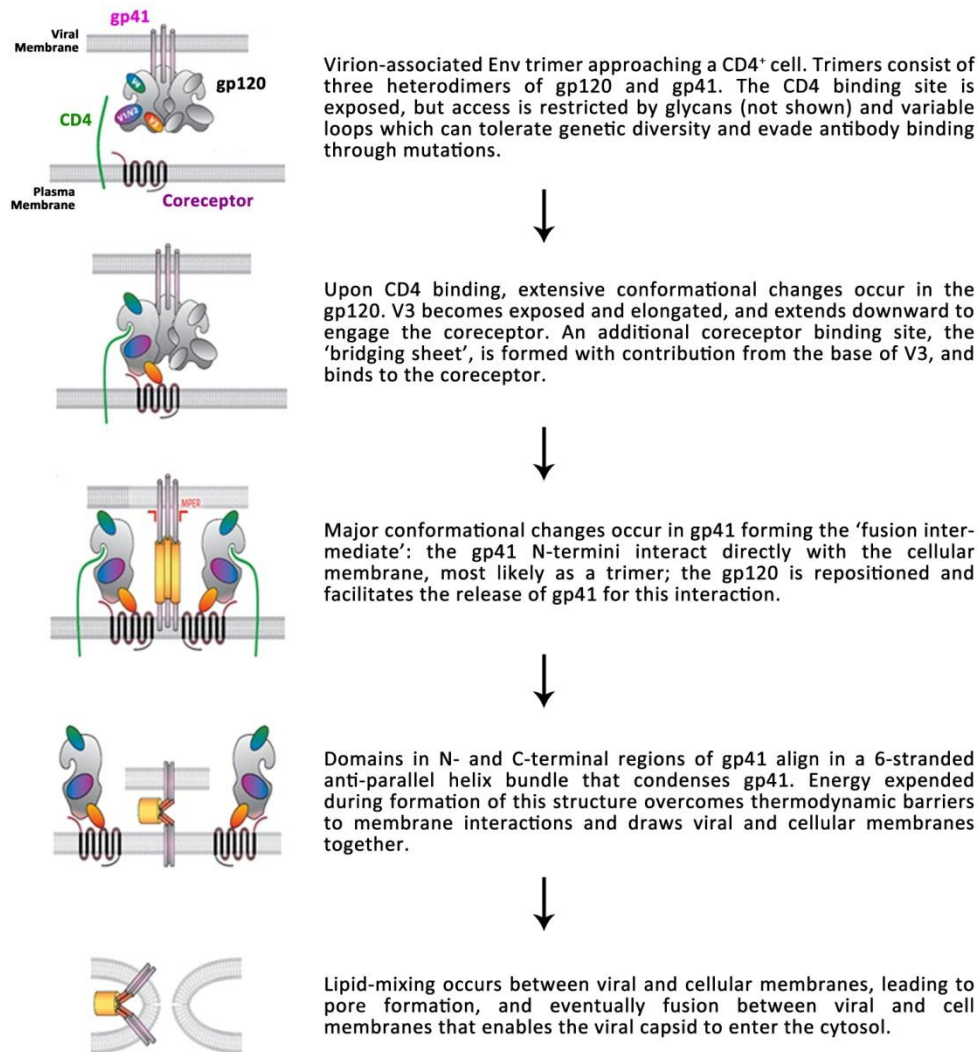


Figure 1.5 Stages of HIV-1 entry into host cells

Entry is associated with binding of a virion-associated Env trimer to a target cell expressing CD4 and a co-receptor, either CCR5 or CXCR4. The molecular interactions between viral and cellular membranes are outlined in each stage. This figure was adapted from (187).

cDNA translocates into the host nucleus and integrates into the host cell chromosomes via the action of **IN** (53, 367, 409). However, successful integration of the provirus DNA does not directly lead to the transcription of provirus DNA into RNA (i.e. transcriptionally active). In some cases, the infected cells remain in a transcriptionally latent state where there is no production of viral RNA and onward viral assembly, and a productive infection may not develop for some time (89).

1.3.3 Stage 3 – Replication and Expression of viral genome

In cells that are transcriptionally active, the assembly of the RNA polymerase at the LTR triggers the transcription of the viral genome (157). Using the provirus as a template, 3 kinds of viral mRNAs can be produced: (i) multiply-spliced mRNAs encoding Tat, Rev and Nef, (ii) singly-spliced mRNAs encoding Env, Vpu, Vif and Vpr, and (iii) unspliced mRNAs encoding Gag-Pol serving as genomic RNA. In the early phase of the replication cycle, only the multiply-spliced mRNAs are transported into the cytoplasm while the singly-spliced or unspliced mRNAs are retained in the nucleus (157). The translation of the multiply-spliced mRNA in the cytoplasm results in the production of Tat, Rev and Nef, in which both Tat and Rev play a significant role in regulating the expression of other viral proteins – **Tat** binds to the TAR element on the nascent HIV-1 transcript and promotes efficient elongation of the viral transcripts and the synthesis of proper genomic mRNA (164), while **Rev** binds to the Rev-response element (RRE) that is presented in singly and unspliced mRNAs and facilitates their export to the cytoplasm before further splicing, thus allowing expression of the ‘later-stage’ viral proteins that support further replication of the HIV-1 virus (329). Newly synthesised Env precursors are oligomerised in ER followed by cleavage in the Golgi by furin to form mature Env trimers. The Env trimers are then inserted in the plasma membrane so as to allow their later incorporation into any newly-budded viruses (71, 143)

1.3.4 Stage 4 – Assembly and Budding of new virions

All of the components of the HIV-1 virion that are synthesised in the previous stage eventually assemble at the plasma membrane where the budding of new virions occurs – a process which is mainly directed by **Gag** proteins (123). Viral assembly begins with the formation of the immature HIV-1 capsid by Gag proteins and the assembly of other components, e.g. 2 copies of the full-size genomic RNA, viral enzymes, the cellular tRNA^{Lys} primer etc. (157, 436). This capsid then buds through the plasma membrane and produces an immature virion. The maturation of the non-infectious HIV-1 particles is triggered by the **PR**-associated cleavage of Gag proteins into 3 independent proteins – matrix (MA), capsid (CA) and nucleocapsid (NC). These individual proteins then undergo further rearrangement – CA forms the conical

capsid core, MA remains bound to the viral membrane and NC remains associated with the viral RNA to form the nucleocapsid – which turns the immature virion into a mature, infectious HIV-1 virus (130, 265). To prevent any newly budded viruses from binding to the cell, **Nef** accelerates the endocytosis and degradation of surface CD4 and MHC molecules (90), while the presence of **Env** proteins in the ER traps any newly-synthesised CD4 molecules and eventually leads to a **Vpu**-induced degradation of the CD4 molecules (73, 83, 257).

1.4 Clinical Stages of HIV-1 infection

There are a number of ways by which an individual can be infected with HIV-1, e.g. unprotected sexual intercourse, blood-blood contact, or mother-to-child transmission (196). However, regardless of the transmission route – whether it be vaginal, oral, rectal, penile or intravenous (78) – the majority of the HIV-infected individuals (roughly 70-80%; considered as *typical progressors*) follow a similar infection process that can be characterised as 3 stages (Fig. 1.6) (305): (i) **Primary/Acute HIV-1 infection**, which is associated with a large increase in viral load followed by a decrease to a viral set-point; (ii) the **Asymptomatic/Chronic phase**, which is associated with a slow increase in viral load from the viral set-point and a gradual depletion of CD4⁺ T cell counts; (iii) the **Symptomatic phase**, where the host immune system becomes severely damaged and unable to control the viral replication, thus leading to the progression of AIDS (140, 208) .

1.4.1 Primary/Acute HIV-1 infection

In primary HIV-1 infection, the virus undergoes a rapid replication in the CD4⁺ T cells prior to the start of the HIV-1-specific immune response, resulting in an explosion of viraemia and spread of the virus to various organs and lymphoid tissues. In the first hours/days of infection, viruses (or infected cells) cross the mucosal barrier and become established at the point of entry. Although the inoculum (e.g. semen, blood) contains a mixture of viral quasi-species, roughly 80% of mucosally-transmitted HIV-1 infections are initiated by a single virus (4, 210, 354). Unlike other quasi-species that arise later in the infection, these early viruses have a greater efficiency at infecting the resting memory CD4⁺ CCR5⁺ T cells (278, 354), and the preferential selection of R5 HIV-1 strains during transmission and early infection is highlighted by the prevention of HIV-1 infection by a deletion of 32 base pairs in the CCR5 gene in both alleles (CCR5Δ32) (95, 248, 313). Roughly 10 days after transmission, HIV-1 viruses and infected cells start to disseminate to the draining lymph nodes and subsequently to other lymphoid tissue compartments, particularly the gut-associated lymphoid tissue (GALT), where they target the activated CD4⁺ CCR5⁺ T cells for further infection. The rapid replication of HIV-1 then leads to an exponential increase in the

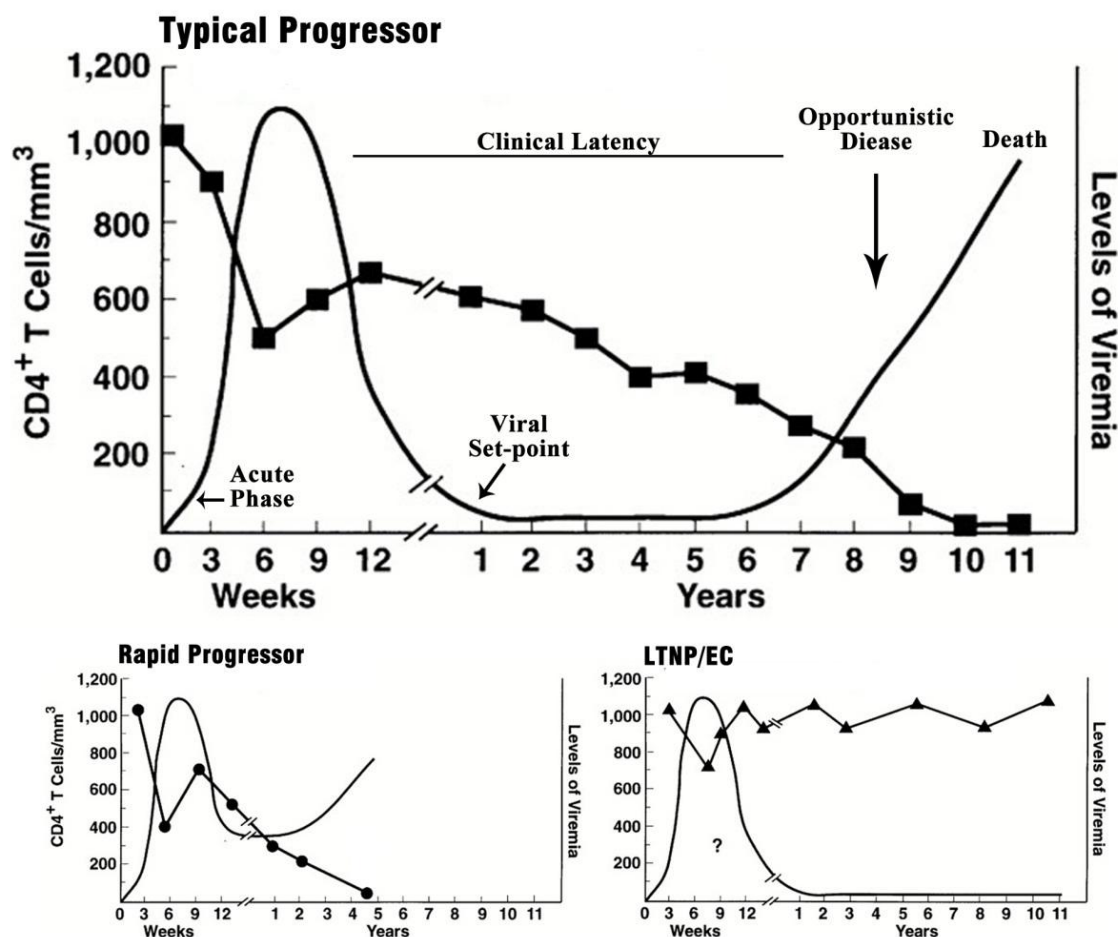


Figure 1.6 Clinical Course of HIV-1 infection in different rate of disease progression

Systematic representation of the different courses of HIV-1 infection on the basis of the changes of viraemia (—) and CD4⁺ T-cell count (■/●/▲) over time. Abbreviations used in the diagram: ‘LTNP’ – Long Term Non-Progressor and ‘EC’ – Elite Controller. EC is classified as a small group of individuals who control plasma viral RNA load of < 50 copies/ml on at least 3 occasions during a 1-year period. All figures were adapted and modified from (305).

plasma viraemia that can reach a peak level of $>1 \times 10^6$ RNA copies/ml of blood, and this is mirrored by a severe loss of CD4⁺ T cells in the GALT (over 80%) through apoptosis (272). However, within weeks after the peak viraemia is reached, the viral load starts to decline as a consequence of an effective immune response (see section 1.6) and due to the limitation of target cells (44, 333, 363). This allows the number of CD4⁺ T cells to return to a near-normal level in the blood, but not in the GALT (272). The decline of viraemia usually lasts over 12-20 weeks until a stable plateau (**viral set-point**) is reached, and at this stage, the infected individual enters the chronic/clinical latency stage of HIV-1 infection (272).

1.4.2 Chronic infection and Clinical latency

The viral set-point is a strong predictor of the time of onset of clinical symptoms, with a higher viral load being linked to a faster onset and *vice versa* (94, 274). During clinical latency, low number of viral particles can still be found in the bloodstream or in peripheral blood lymphocytes, and viruses continue to replicate in the lymphoid tissues which result in a progressive loss of CD4⁺ T cells. This continued loss of CD4⁺ T cells has a serious impact on disease progression – individuals with CD4⁺ T cell counts greater than 500/μl generally remain symptom-free but symptoms become more frequent when the cell counts drops below this level. Approximately 3 months after transmission, neutralising antibodies (NAbs) against autologous virus begin to develop slowly. Under constant immune pressure, the circulating viruses begin to diversify as multiple escape mutants are selected (44, 272, 333).

1.4.3 Progression to AIDS

Once the CD4⁺ T cell count drops below the critical level of 200/μl, the virus can no longer be effectively contained and this results in a rapid rise in plasma viraemia (94, 274). The patient will now show severe and persistent immunodeficiency symptoms, with an increased susceptibility to opportunistic infections and other AIDS-related diseases (305). Without proper treatment, the patient will eventually die from AIDS-related diseases.

1.4.4 Different rates of progression to AIDS

Described above is the infection process of **Typical Progressors** which normally takes about 7-11 years to progress to AIDS. However, this is not the only outcome of HIV-1 infection as the course of clinical infection is highly variable among individuals (305) (Fig. 1.6). Approximately 10-15% of infected individuals are **Rapid Progressors** as they experience a fast decline in CD4⁺ T cell counts and an unusually rapid progression to AIDS that occurs within 1-3 years of primary infection. For a smaller portion of infected-individuals (< 5%), they do not experience progression of disease for an extended period of time (> 10 years) despite not being treated with antiretroviral therapy, and are thus referred to as

Long Term Non-Progressors (LTNP). For an even smaller subset of infected-individuals (< 1%), referred to as **Elite Controllers (EC)**, they are able to control the plasma viral RNA load to < 50 copies/ml on at least 3 occasions during a 1-year period without antiretroviral therapy. The slower progression of disease observed in the LTNP and EC groups have been shown to associate with a number of host-related factors, e.g. the presence of an effective antibody and T cell response (44, 104, 277), a strong innate response (194), the CCR5 Δ 32 phenotype (95, 248, 313), and particular HLA alleles such as HLA-B57, HLA-B27 and HLA-B51 (62, 272). In addition, the observation of a lower HIV-1 genetic diversity in EC compared to infected individuals may be indicative of several viral-related factors that influence the replication and diversification of HIV-1 viruses in EC (237).

1.5 Innate immune response against HIV-1 infection

Innate immune response is the first line of defence against pathogens and is critical for the generation of an effective adaptive immune response. It is usually rapid and non-specific to the pathogen, and does not acquire memory from the infection that is central to all vaccination (61, 239, 425). Instead of utilising a 'specialised' group of cells (e.g. T and B lymphocytes as in the adaptive immune response; see section 1.6), innate response is constituted by a number of different cellular components that can be categorised into 3 groups – cellular, extracellular and intracellular. However, while innate immune response has been shown to contribute to the control of HIV-1 infection, several properties of the antiviral response may also have the potential of enhancing disease progression, thus making innate immune response a double-edged sword against HIV-1 infection.

1.5.1 Cellular components

A number of immune cells are involved in the cellular component of innate immune response and among these, strong interest in the role of Dendritic Cells (DCs) and Natural Killer (NK) cells against HIV-1 infection have been developed in recent years (239).

Dendritic cells are professional antigen-presenting cells (APC) as they possess both classes of Major Histocompatibility Complex (MHC) for antigen presentation (12). DCs detect viruses through the pattern recognition receptors (PRRs) on their surface, such as Toll-like Receptors (TLR) for the recognition of viral RNA, and C-type lectins (e.g. DC-SIGN) for the recognition of viral glycoproteins. Following activation and viral uptake, DCs migrate to the draining lymph nodes where they promote the activation of a number of immune cells (e.g. Natural Killer cells). In particular, DCs are considered to be the most effective cells for the initial activation of naïve T cells because they are able to process and present antigens on MHC class I and II, as well as provide the necessary co-stimulatory molecules (e.g. CD28, CD40) for the activation and further differentiation of T cells. Furthermore, DCs are also able to secret

various antiviral molecules, e.g. type-I interferons such as IFN- α and IFN- β . Broadly speaking, DCs can be divided into 2 major categories – **myeloid DCs (mDCs)** which secrete IL-12 to support naïve T cell activation, and **plasmacytoid DCs (pDCs)** which produce type I interferon (e.g. IFN α , IFN β) that have both immunostimulatory and antiviral activity (264). By comparing with patients who progress to AIDS, the significance of DCs in the control of HIV-1 is evident with a higher level of pDC being found in LTNPs, and the mDC in ECs also exhibited enhanced antigen-presenting properties (396). However, on the contrary, some properties exerted by the DCs can also be detrimental as they promote systemic HIV replication in two major ways (45): First, they mediate the trans-infection of T cells in draining lymph nodes, particularly to the antigen-specific CD4⁺ T cells with which they interact, thus simultaneously driving virus amplification and impede the HIV-specific CD4⁺ T cell response; Second, the immunostimulatory properties of the type-1 IFN secreted by DCs may attract T cells into local infected sites, thus indirectly enhancing the replication and establishment of viral infection at the early stage of infection.

In addition to DCs, increasing evidence has also pointed to a significant contribution from **Natural Killer cells** in the containment of viral replication. NK cells promote antiviral activity in a number of ways: production of inflammatory cytokines and chemokines, lysis of infected or transformed cells, and interaction with T cells and DCs to shape the magnitude and quality of adaptive immune responses (12). In particular, NK cells that express the killer-cell-immunoglobulin-like receptors (KIRs), especially KIR3DS1 and KIR3DL1 in conjunction with HLA-Bw480I, have been shown to expand rapidly during acute HIV-1 infection, and correlated with a lower viral set-point and a slower progression to AIDS (11, 260). In addition, the observation that HIV-1 has developed strategies to modulate the expression of surface ligands (e.g. MHC class I) of the infected cells in order to maximise the inhibition and to minimise the activation of NK cells also highlights the importance of NK cells in controlling HIV-1 infection (10, 42, 46, 426). However, similar to DC, the ability to promote immune activation by NK cells also means that they may enhance viral replication and dissemination.

1.5.2 Extracellular components

The extracellular components of innate immune response are cytokines and chemokines that are secreted in time with the increase in plasma viraemia, and the resulting '*cytokine storm*' observed in HIV-1 infection is much greater than that observed in hepatitis B or C infection (272). At this stage, the cellular sources of these molecules have not been fully elucidated, although it is most likely to include the activated DCs and NK cells as described above. The extracellular compartment of the innate immune response is comprised of many different cytokines and chemokines that exhibit different antiretroviral properties. For instance, *cytokines* such as IL-2 and IL-15 regulate the activation and proliferation of T and NK cells, whereas IL-4 promotes the proliferation and differentiation of B cells, and *chemokines* such as RANTES, MIP-1 α and MIP-1 β act by inhibiting HIV-1 infection by blocking the HIV-1 co-receptor CCR5 (425). However, as in the case of DCs and NK cells, such intense immunostimulatory responses in HIV-1 infection may instead fuel viral replication (45).

1.5.3 Intracellular components

Similar to the extracellular component, some of intracellular innate proteins are considered to play a key role in restricting HIV-1 infection and replication – e.g. APOBEC3G, tripartite motif (TRIM) 5 α and Tetherin – and their importance can be highlighted by the fact that they are counteracted by numerous viral proteins. The **APOBEC3G** – apolipoprotein B mRNA-editing, enzyme-catalytic polypeptide-like-3G – is an intracellular cytidine deaminase that is packaged into the viral particles and acts by deaminating cytidines (dC) to uridine (dU) in the cDNA, thus lead to the guanine (dG) to adenine (dA) substitution in the protein-coding DNA strand that might inactivate viral gene products or regulatory genetic elements (193, 239). In addition, the presence of dU in DNA strands also triggers the upregulation of ligands the activating NK cell receptor (NKG2D) that sensitise cells for NK recognition (87). For the virus to penetrate this host defence, it requires the action of the HIV-encoded Vif protein to counteract APOBEC3G by targeting it for ubiquitylation and subsequent degradation in the proteasome. **Tetherin** is another host restriction factor that has been under the spotlight recently. Tetherin is a transmembrane

protein that contains a C-terminal glycosyl-phosphatidylinositol (GPI) anchor that allows it to ‘tether’ the mature virions to the cell surface and prevents the release of progeny viruses (i.e. tethered viruses) (261, 388). Similar to APOBEC3G, the viral protein Vpu is present as a countermeasure that down-regulates the expression and degradation of tetherin from the plasma membrane. Another host restriction factor ***TRIM5α*** is present in the cytoplasm but little is known about this protein at this stage. TRIM5α have been shown to exert a protective role in primates against SIV infection, and it has been shown to be upregulated by interferon in HIV-1-infected individuals. It is believed that TRIM5α either leads to the direct uncoating of the virus through binding to the capsid protein, or that it blocks the reverse transcription by degrading of the TRIM5α-associated virus in the proteasome (193, 368, 389).

1.6 Adaptive Immune response against HIV-1 infection

In contrast to innate immune response, the adaptive immune response is a highly pathogen-specific and specialised system aiming to eliminate any pathogens that evade innate immune response. In addition, one important aspect of the adaptive immune response is the ability to generate memory of antigen-specific cells that allows a more rapid and intensive response in the future. The adaptive immune response is comprised of 2 major components – the cell-mediated (cellular) and humoral immune response. The outline/timeline of the HIV-specific adaptive immune response is shown in Fig. 1.7.

1.6.1 Cellular Immune response

Cellular immune response is an arm of the adaptive immune response that relies on the action of T lymphocytes (or T cells) for the control of pathogens. T cells are initially produced in the bone marrow and later migrate to the thymus for maturation. Broadly speaking, all T cells can be classified into 2 categories – the helper $CD4^+$ T cells and the cytotoxic $CD8^+$ T cells (CTLs) – and each has a distinctive role in the control of pathogens.

1.6.1.1 Helper $CD4^+$ T cell response

The helper $CD4^+$ T cells are crucial for the maintenance of an effective, long-lasting CTL and humoral response, and they also modulate the functions of APCs and other innate cells, e.g. NK cells, through the secretion of multiple cytokines (396). Although they are mainly considered to be helper cells of the immune system, there are also reports suggesting that they possess cytotoxic activity (280, 294, 300, 451). $CD4^+$ T cells are activated through the recognition of antigen, which is presented as a peptide by MHC class II molecules on the surface of APCs, through their surface T-cell receptor (TCR). $CD4^+$ T cells are central in controlling HIV-1 infection despite being the primary target for HIV-1. Specific insights into the HIV-specific $CD4^+$ T cell response can be obtained by studying such responses in EC and progressors. From these analyses, viral control and slow disease progression have been shown to correlate with HIV-specific $CD4^+$ T cells that exhibit: (i) strong proliferative potential (41, 348), (ii) IL-2 producing

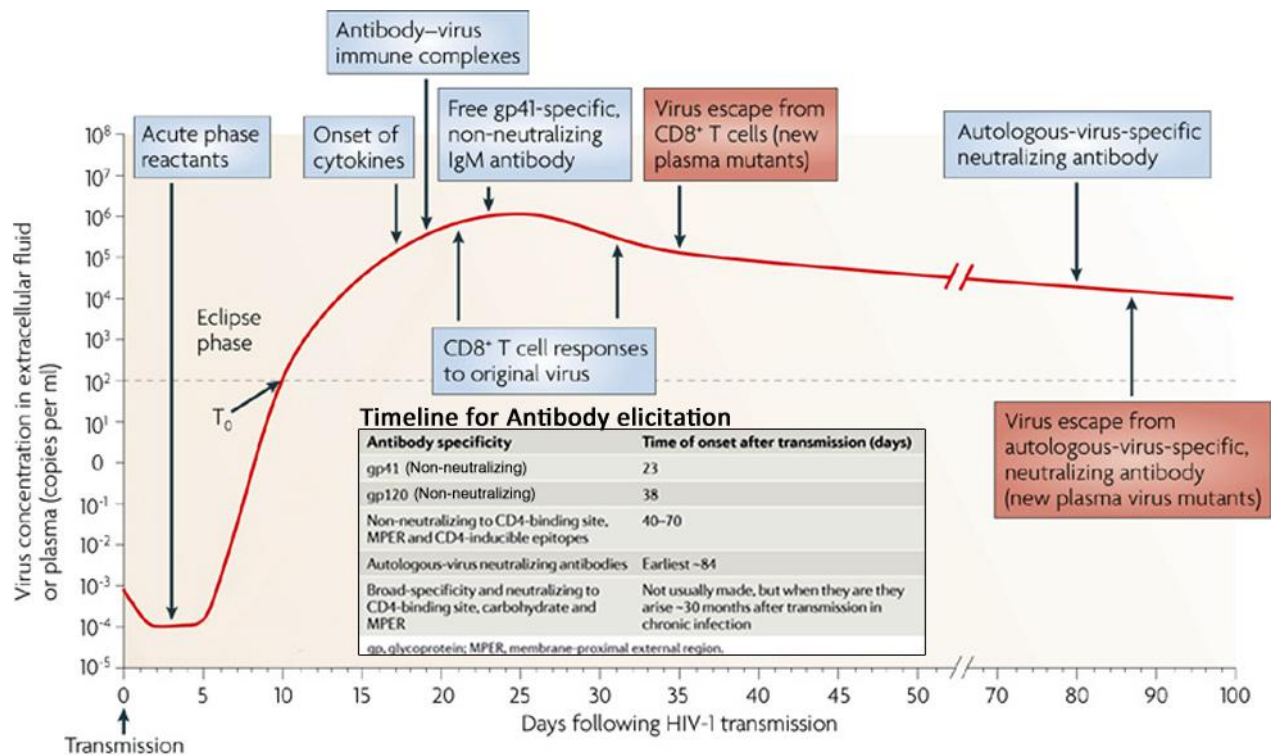


Figure 1.7 Adaptive immune response against HIV

Shown here is the onset of different immune response in HIV-1 infection. T_0 is referred at the point when virus is first detected in the plasma. The plasma viraemia increases exponentially within days and the antibody-virus immune complexes are first detected at this point. The onset of the CTL response is associated with the control of virus (1-2 weeks post- T_0), followed by the elicitation of non-neutralising antibodies. The earliest ANAbs are detected months following infection. An approximate timeline for antibody elicitation is listed in the table. The figure and table were adapted from (272).

capacity, an effector function that was impaired in progressors (396), (iii) poly-functionality, which refers to the ability to perform multiple effector functions, e.g. the ability to secrete multiple cytokines (e.g. IL-2, IFN- γ , TNF- α etc.) while cells from progressors secrete only IFN- γ (140, 255); (iv) higher functional avidity for the HLA class II-peptide complex, so that a higher magnitude of HIV-specific CD4⁺ T cell response can be triggered in response to minimal amounts of HIV-1 antigen (396).

1.6.1.2 Cytotoxic CD8⁺ T cell response

The cytotoxic CD8⁺ T cells (CTLs) are another group of T cells that possess both cytotoxic (i.e. lysis of infected cells) and non-cytotoxic activity (i.e. secretion cytokines and chemokines). Similar to CD4⁺ T cells, the activation of CTL is achieved through the recognition of antigens by TCR, in which the antigen

is presented as a peptide on the MHC class I complexes, rather than the on the MHC class II complexes (271).

Several lines of evidence have demonstrated the importance of the CTL response in the control of HIV-1 infection. During acute HIV-1 infection, a sharp decline in virus load is associated with the emergence of an HIV-specific CTL response (44, 222), and the role of CTL response in chronic HIV-1 infection was clearly demonstrated in several depletion experiments in SIV/macaque models (162, 365). In these experiments, the injection of anti-CD8 antibodies into ‘controller’ macaques led to a rapid and dramatic increase in viral load and a decline in the CD4⁺ T cell response, and the control of viraemia could only be re-established once the CD8⁺ T cell level was restored. In addition, specific HLA class I alleles in humans (e.g. HLA-B57, -B27 and -B58) are over-represented in ECs, and they have been shown to be associated with good control of the viraemia and a slow progression to AIDS (62, 272). Furthermore, the immunologic pressure mediated by CTL recognition has also been shown to be responsible for the outgrowth of escape mutants during early HIV-1 infection (144). These findings together provide a strong indication that CTLs are important for the containment of viral replication. To better elucidate the type of CTL response required for the control of viraemia, the CTL response of EC and progressors were compared. Similar to the CD4⁺ T cell response, poly-functional CTLs that possess strong proliferative potential and the ability to produce cytotoxic proteins (e.g. perforin) are significantly more frequent in ECs than in progressors (84, 114, 163, 276, 301, 351).

1.6.2 Humoral Immune response

Humoral immune response (HI) is the other arm of the adaptive immune response that relies on the antibody-producing cells called the B lymphocytes (B cells). Each B cell is equipped with a B-cell receptor (BCR), i.e. precursor of the soluble antibody, that is distinct from other B cells to ensure maximal recognition of antigens (229) (see section 1.6.2.2. for more detail).

1.6.2.1 *Structure of human antibodies*

Antibodies are Y-shaped glycoproteins that can be divided into 2 regions – the variable (V) region that defines the antigen-binding properties (Fab, fragment antigen binding), and the constant (C) region that interacts with effector cells and molecules (Fc, fragment crystallisable) (Fig. 1.8) (424). Based on the C region, antibodies can be classified into 5 classes – IgA, IgD, IgE, IgM and IgG – and they exist in different oligomeric states, e.g. dimeric (IgA) or pentameric (IgM). On the monomeric level, each antibody is composed of 2 identical heavy (H, 50kDa) and light (L, 25kDa) chains, with 4 inter-chain disulphide bonds holding them together. The Fab region of the antibody is formed by the V region of both the heavy and light chain, where they can be further divided into 3 hypervariable sequences (HV1-3; also referred to as complementarity determining regions (CDR)) sandwiched between four framework sequences (FR1-4). Among all the CDRs, the third CDR on the heavy chain (hCDR3) is particularly variable and often makes a crucial contribution to HIV-1 Env recognition (169). The Fc region is composed of the remaining sequences of the heavy chain and it binds to the Fc receptor on various cells (e.g. macrophages) or complement components to trigger the other arms of the immune system (120, 424).

1.6.2.2 *The Developmental pathway of B cells*

The development of B lineage cells from hematopoietic stem cells (HSCs) is a highly regulated pathway which initiates in the bone marrow and continues into periphery lymphoid organs, e.g. spleen (Fig. 1.9; *top figure*) (273). All HSCs share the same immunoglobulin (Ig) loci at the germline level, and antibody diversity is generated through a series of ordered V(D)J gene rearrangements in the heavy (IgH) and light chain (IgL) of the antibody (*‘somatic recombination’*) (9, 400). Once the gene rearrangements are completed, the heavy and light chain loci are silenced to ensure that only one type of antibody can be expressed in each B cell (*‘allelic exclusion’*) (273). The heavy and light chains of the antibody then assemble to form the B cell receptor (BCR) and localise onto the cell surface as immunoglobulin M (IgM). These B cells, which are now referred to as ‘immature B cells’, then undergo several ***negative selection***

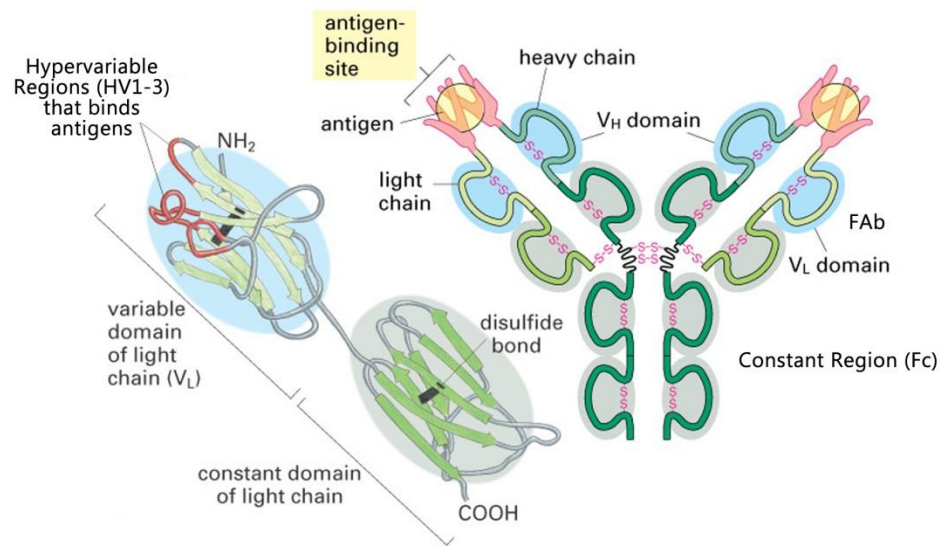


Figure 1.8 Schematic diagram of a typical antibody molecule

An antibody is composed of 4 polypeptide chains (2 heavy chains and 2 light chains) held together by disulphide bridges. The antigen-binding site (Fab) is formed by the variable domain of both a heavy-chain (V_H) and a light-chain (V_L). These are the domains that differ most in their sequence and structure in different antibodies. The ribbon drawing of a single light chain showing the parts of the V_L domain most closely involved in binding to the antigen in red (HV1-3). This figure was reproduced from Fig.4.32 in Essential Cell biology (@2004 Garland Science).

checkpoints to check for auto-reactivity of the BCR (337), and B cells that are not auto-reactive will proceed and develop into mature (naïve) B cells in the periphery lymphoid organs. In contrast, for B cells that are auto-reactive, their fates are largely dependent on the affinity to the antigen: if the binding is strong, the B cell will be eliminated from the population via apoptosis; if the binding is weak, the B cells will either be inactivated by **anergy**, or have their receptor replaced by a secondary light chain gene recombination (**‘receptor editing’**) (135, 336, 397). However, not all auto-reactive B cells are deleted during this process – roughly 20-25% of auto-reactive B cells can continue their development into mature (naïve) B cells alongside other B cells (427, 428).

1.6.2.3 B cell activation upon antigen recognition

Similar to T cells, B cells are activated upon antigen recognition and this can be achieved with or without T cell help (337). For the **T cell-independent** pathway, multimeric antigens directly stimulate the proliferation and differentiation of B cells by crosslinking multiple BCRs to generate a signal that is of

sufficient strength to activate the cell. However, the antibody response generated by this pathway is usually of low affinity and limited in effector function (97). In contrast, a much stronger B cell response could be elicited through the ***T cell-dependent*** pathway, which requires the activation signal from CD4⁺ T cells in addition to the crosslinking of BCRs. Protein antigens taken up by B cells through the BCR are processed into peptides inside the cell, and the resulting peptides are then presented by MHC class II and subsequently recognised by CD4⁺ T cells (Fig. 1.9; *lower figure*) in a phenomenon called ‘linked recognition’ – which indicates that a B cell can only be activated by a CD4⁺ T cell that responds to the same antigen as the B cell (322). Such interaction between B cell and T cell then initiates the formation of the germinal centre (GC) in the lymph nodes, and triggers the affinity maturation of antibodies in the B cells (128, 252, 454). ***Affinity maturation*** is a crucial step for the induction of an effective antigen-specific immune response as it allows the generation of a secondary antibody repertoire with improved antibody functionality and affinity towards the specific antigen (374, 385, 386). A series of key events take place during this process: first, ***somatic hypermutation*** occurs where multiple random mutations are introduced to the HV regions of the heavy and light chains of the antibody (337) (Fig. 1.8). Since such mutations may change the binding characteristics of the antibody, B cells carrying the mutated antibody will then undergo several ***positive selection*** checkpoints where the recognition of the specific antigen on DCs triggers the clonal expansion of that specific B cell. Hence, B cells that possess the antibody with the highest affinity towards the antigen are preferentially expanded. This is followed by the ***class-switching*** of surface IgM/IgD to other antibody classes (e.g. IgG, IgA or IgE) to ensure that a diverse function and distribution of the antibody within the host immune system can be achieved (337). These positively-selected B cells then differentiate into either long-lived plasma cells for high level antibody secretion, or into memory B cells where they remain in the resting state and respond quickly upon re-exposure of the same antigen in the future.

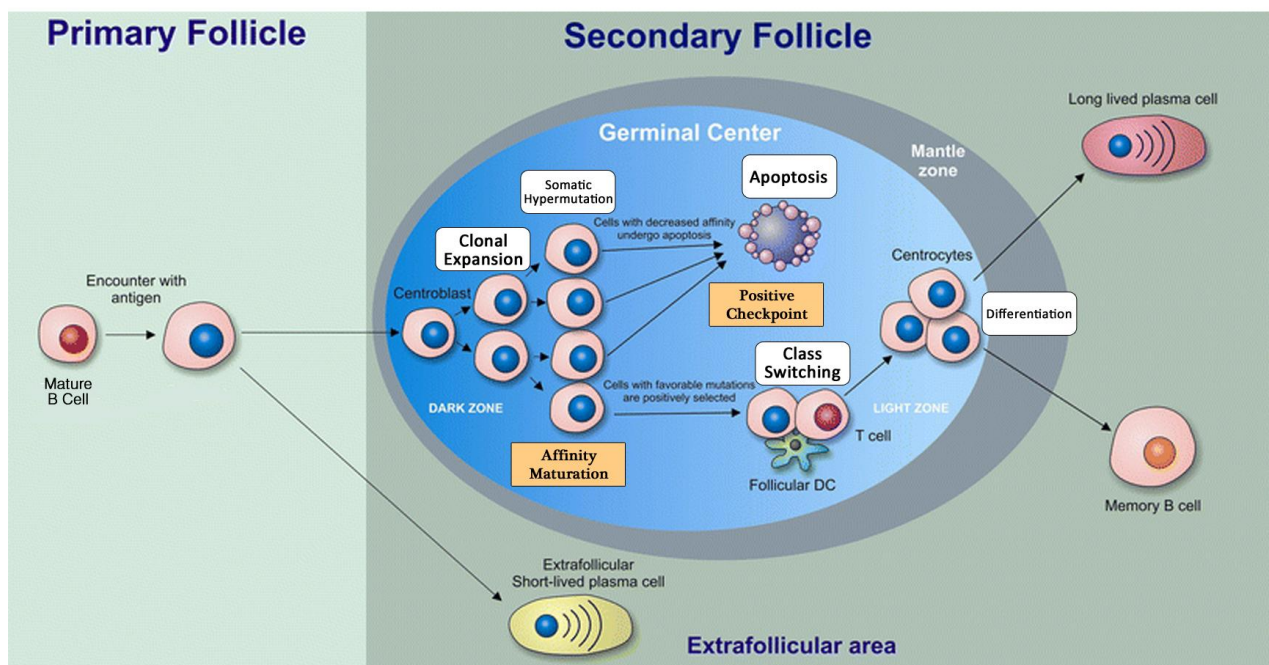
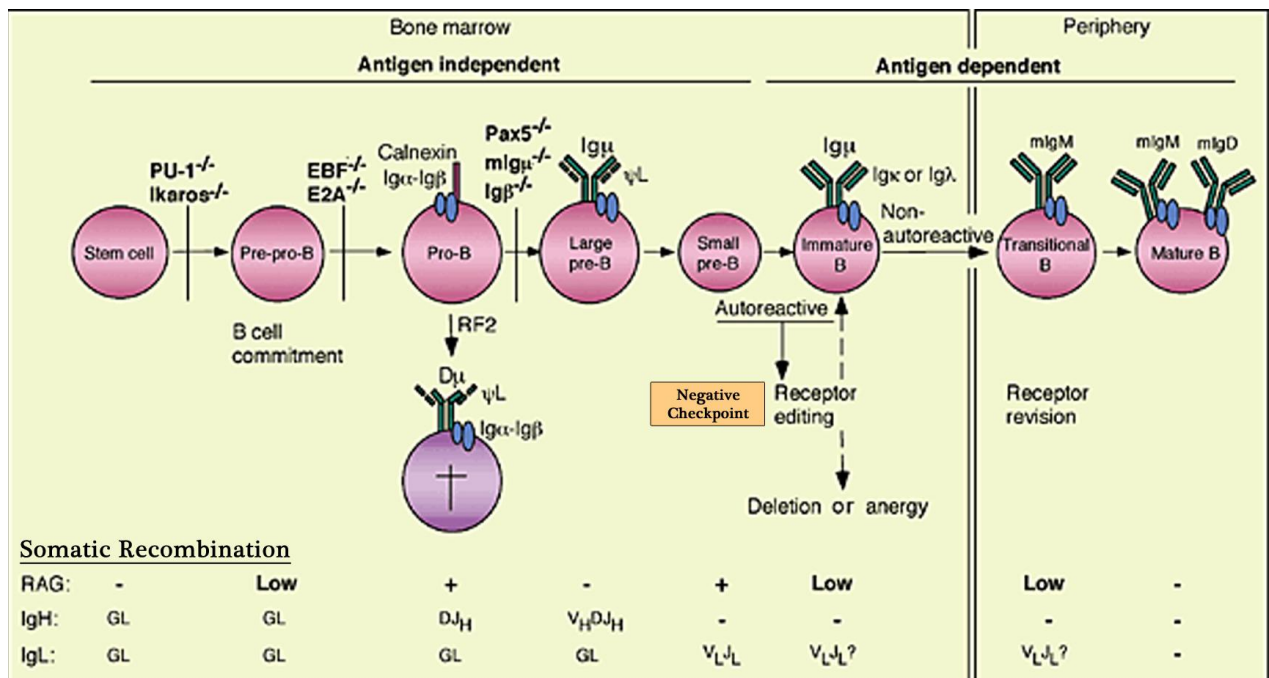


Figure 1.9 The maturation and activation of B cells

(Top) The maturation pathway of B cells. This figure was adapted from (273). (Bottom) The activation of mature naïve B cells upon encountering of antigens. This figure was adapted from (357).

1.6.2.4 *Humoral response against HIV*

For most viruses, such as influenza and hepatitis B virus, the immune correlate of protection is a strong neutralising antibody (NAb) response that inhibits viral infection through the binding of surface Env protein of the virus and prevents their entry into the target cell (323). However, it remains uncertain how significant humoral response is in controlling HIV-1 infection as a clear correlate of protection is still unknown at this stage (140). In light of the findings of the recent RV144 trial (see section 1.8.5), several studies have demonstrated a role for vaccine-induced V1/V2-specific antibodies to protect against HIV-1 infection (25, 168, 346).

Humoral immune response is activated soon after HIV-1 transmission (Fig. 1.7). Typically, Env-specific antibodies can be detected as early as 18 days after transmission in the form of antibody-virus complexes, and this is followed by the circulation of gp41-specific soluble antibodies that usually arise 5 days later (399). Antibodies that target gp120 do not normally occur until approximately 38 days after transmission (6, 7, 98, 153). Nevertheless, these antibodies are non-neutralising as they fail to neutralise the virus and, unlike the initial CTL response, they do not select for virus escape mutants (23, 399). It is not until 3 months post-infection that autologous neutralising antibodies (ANAbs) start to be detected (153, 342, 431). This early ANAbs response is most likely to be targeting the variable regions of the Env protein, although the exact target for the ANAbs remains unclear. Traditionally, the variable loop 3 (V3) of the Env protein is considered the principal neutralising domain (PND) as antibodies against this region are among the first to be elicited (91, 92, 288). However, the neutralising capacity of these antibodies has been brought into question as they can only neutralise most of the neutralisation-sensitive ‘Tier 1’ viruses, but not the more resistant ‘Tier 2’ viruses where the V3 loop is occluded in the native trimeric Env protein (91, 92, 166, 182, 225, 319). In contrast, there is increasing evidence suggesting a role for the V1/V2 loop as an early neutralising target: (i) a study by Sagar *et al* demonstrated that the substitution of an early V1/V2 loop into a heterologous viral backbone resulted in a transfer of neutralisation sensitivity to autologous plasma (352); (ii) the discovery of broadly neutralising antibodies that target the V2 loop (e.g. PG9/16, discussed

in section 1.7.2.3) provides a strong indication that neutralising epitopes are presented in this site (15, 419); (iii) the recent RV144 trial and a macaque challenge study also identified a possible correlation between V2-specific antibodies and a reduced risk of HIV-1 infection (26, 168, 346). Further investigations on the exact onset time of this V2-specific NAb response would be of particular interest. In addition to the V1/V2 and V3 loops, the C3/V4 domain has also been proposed to be potential neutralisation target in clade C viruses (288).

Although an ANAb response is elicited over the course of HIV-1 infection, such response is mostly strain-specific, and the fact that they recognise the immunodominant variable regions of the Env protein can be problematic because these regions can tolerate a high level of mutations (e.g. single point mutations, insertions, deletions and ‘evolving glycan shield’) without paying a significant fitness cost (188, 288, 377). As a result, the virus rapidly escapes from antibody recognition and the contemporaneous antibodies are only able to exert weak or no neutralisation of autologous viruses, thus fail to control the establishment of viral infections (126, 342, 431). As the disease progresses into the chronic phase, the continual replication of HIV-1 leads to the damage of the peripheral B cell pool that is reflected in the hyperactivation and hyporesponsiveness of B cells (55, 108, 125, 170). This includes the increased expression of activation molecules on circulating B cells, hyperglobulinemia and the increased production of auto-reactive antibodies, as well as increased levels of transitional B cells and exhausted B cells. Together, these eventually result in a diminished humoral response against, not only HIV-1, but also to other pathogens, and infected individuals thus become more prone to opportunistic infections.

1.6.2.5 *Broadly neutralising antibody response*

Although the early ANAbs response is highly strain-specific, around 20-30% of chronically infected subjects develop a broadly neutralising antibody response over time (typically 1-3 years after infection) that can neutralise roughly 70-80% of HIV-1 strains. Among all, about 1% of these individuals elicit serum monoclonal antibodies that can broadly and potently neutralise a diverse set of HIV-1 strains (361,

373, 379). Although the factors associated with the development of such a broadly neutralising response have not been fully understood, broad neutralisation appears to correlate with a greater time of infection, higher viral load, CD4⁺ T cell counts at the viral set point, and binding avidity to the Env protein. This therefore suggests that chronic antigen exposure and extensive affinity maturation of the B cell are most likely to be needed in order for these BNABs to be induced (51, 109, 361). A more detailed discussion of BNABs is included in section 1.7.2.

1.6.2.6 Other Roles of Antibodies in HIV-1 infection

Apart from the direct neutralisation of viruses, antibodies can also control viral infection through a number of Fc-dependent effector functions (176, 392), including: (i) **antibody-dependent cell-mediated cytotoxicity (ADCC)**, i.e. the Fc-mediated recruitment of innate immune cells, such as NK cells, that leads to killing of infected cells (142, 207); (ii) **antibody-dependent cell-mediated viral inhibition (ADCVI)**, i.e. the Fc-mediated recruitment of innate cells that leads to inhibition of viral replication (119, 399); (iii) phagocytosis of antibody-coated virions (266); (iv) activation of the complement system (191). The potential contributions of these non-neutralising antibodies have been demonstrated in SIV models and in humans: first, in a study by Hessel *et al*, Fc-mediated functions have been shown to be important for b12 protection against SHIV challenge in non-human primates (176); second, the appearance of ADCVI was found to be as early as the CTL response, i.e. much before the ANAb response, and therefore may contribute to early viral control (387); third, the ADCC activity was notably higher in EC compared to other viraemic individuals (235); finally, over 75% of the vaccinees in the RV144 clinical trial developed ADCC activity in their serum. Although the titres were unstable and waned over 20 weeks, this still suggests a role of these non-neutralising effector functions in the control of HIV-1 infection (170, 328).

1.7 The HIV-1 Envelope Protein and Broadly Neutralising Antibodies

1.7.1 The HIV-1 Envelope (Env) protein

The HIV-1 Env protein is a trimer of non-covalently associated heterodimers (175, 433), composed of the exterior gp120 glycoprotein and the transmembrane gp41 glycoprotein. Both of these proteins are generated from the precursor gp160 protein, which is cleaved in the trans-Golgi network by furin or equivalent endoproteases into gp120 and gp41 (240, 326).

1.7.1.1 The surface unit – gp120

GP120 is heavily glycosylated with almost half of the molecular mass composed of N-linked, and a few O-linked, glycans (8, 33). GP120 can be divided into variable (V1-V5 loops) and conserved (C1-C5) regions (282, 307, 437) with around 18 Cys residues (9 disulphide bridges) located throughout (131, 198). The conserved regions are mostly responsible for forming the Env protein ‘core’, which contains the highly conserved binding site for the CD4 receptor and the chemokine co-receptors. The variable regions are responsible for various functions and display different immunogenicity. The *V1/V2 loop* is one of the most variable regions on the Env protein in both sequence and length, and is immunogenic in ~20-45% of HIV-1 infected individuals (182). Functionally, apart from the well-recognised role of masking neutralisation determinants (188, 233), the V1/V2 loop also contributes to the Env trimer formation (72, 246, 456) and involves in co-receptor binding (459). In contrast, the *V3 loop* is relatively constant in length at 34-35 amino acids, although its sequence can vary considerably across and within strains (except in clade C where the variation is restricted to only ~20% of all sequences (134)). The V3 loop is highly immunogenic and anti-V3 antibodies are induced in almost all infected individuals and in the immunisation of gp120 (182, 369, 370). The V3 loop is functionally important for HIV-1 as the deletion of this loop has been shown to completely abrogate virus infectivity given its involvement in co-receptor binding (58). In contrast, the V4 and V5 loop (which is the only variable loop lacking a disulphide bridge) do not demonstrate any significant neutralisation capability and functional importance (182).

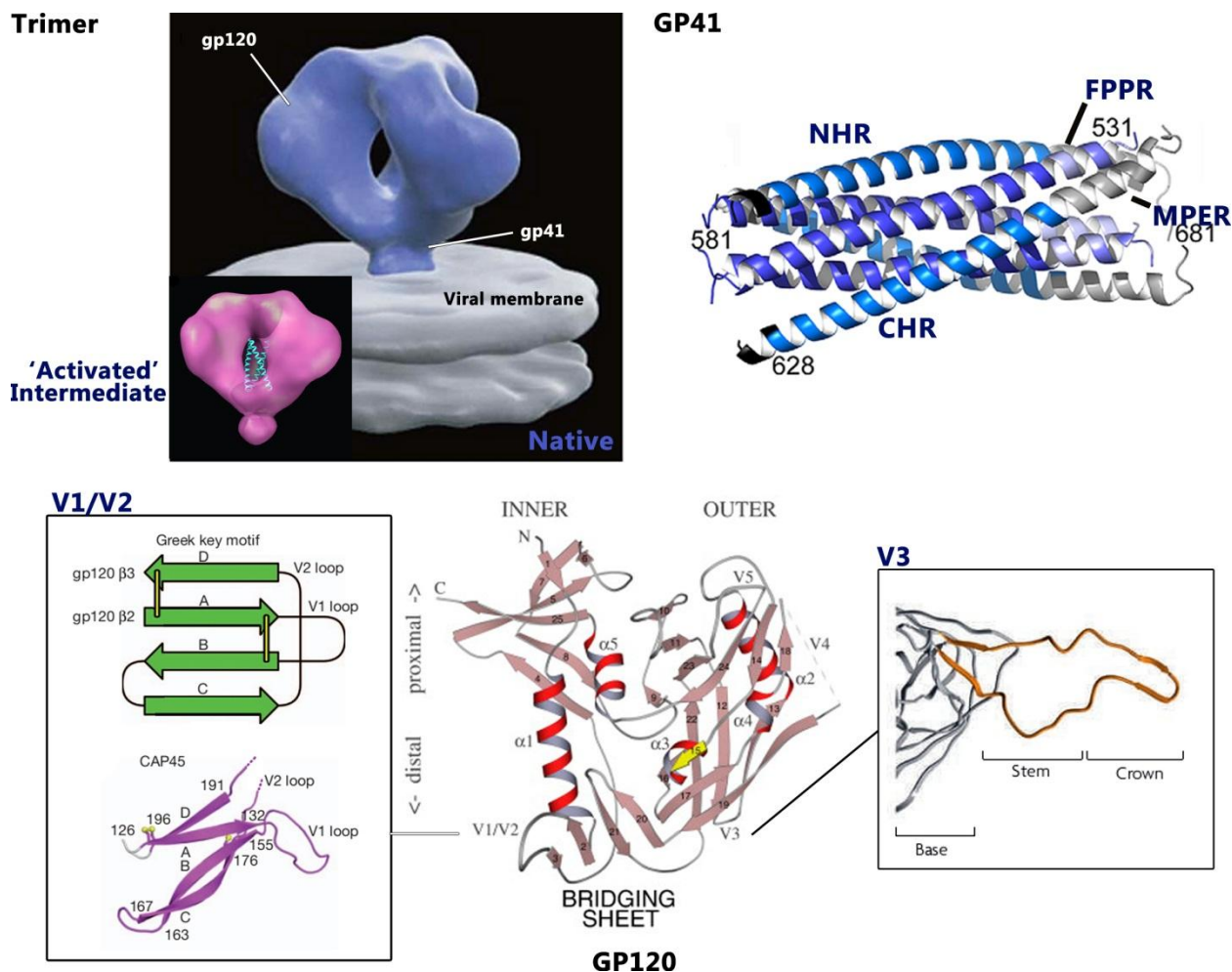


Figure 1.10 Structures of the HIV-1 Env protein

(Upper Left) Cryo-EM structure of the native Env trimer spike (unliganded 'closed' state) (246). Shown in the lower left corner inset (in pink) is the cryo-EM structure of a 17b-bound Env SOSIP trimer spike ('activated' intermediate state) (403). The 3 peptides in cyan indicated the proposed position of the 3 NHR in such state; (Upper Right) 2Å crystal structure of the complete gp41 lacking the fusion peptide and the cysteine-linked loop (394). The polar region (FPPR), NHR, CHR and MPER are labelled accordingly. (Bottom) 2.5Å crystal structure of gp120 core in the CD4-bound state (233). The structure of the V3 loop on the right was adapted from (182) and the structure of the V1/V2 loop on the left from (270). The V1/V2 in this structure was grafted onto a scaffold and in complex with the BNAb PG9.

A large collection of gp120 crystal structures has been published to date. Given the flexibility observed in the variable loops, gp120 used in these studies mostly consisted of the core protein (lacking variable loops and C-/N-terminus of the protein) only, *except* in one study by Huang *et al* where the V3 loop was successfully included in the structure (188) (Fig. 1.10). By comparing the CD4-bound and unliganded structures, valuable insights into the mechanism of gp120 interaction with CD4 can be obtained (233, 442):

In the unliganded state, residues that are in contact with CD4 were found to be widely dispersed in gp120, with C1, C3 and C4 being the principle determinants of the CD4 binding site (CD4bs) (224, 233, 236, 299). Upon CD4 binding, gp120 undergoes a dramatic conformational change which results in the formation of the co-receptor binding site (CoRbs) from residues that are otherwise spatially separated in the unliganded state (mainly on V1/V2, V3 and C4) (72, 233, 344). This mechanism ensures that the formation of the neutralisation-sensitive CD4bs and CoRbs is delayed until the Env protein is in close contact with the target cell, by which time steric hindrance may prevent NAb access to its respective epitope. In this CD4-bound state, gp120 forms a specific conformation that consists of an ‘inner’ domain and an ‘outer’ domain linked by a 4-stranded ‘bridging sheet’ (233). As for the variable loops on gp120, structural information of the V3 loop was determined by crystallography of the V3 peptide and revealed a rather conserved β -turn structure with a disulphide-bridge-stabilised base (188, 380-382) (Fig. 1.10). As for the V1/V2 loop, its structure was also determined as a separate unit to gp120 protein by McLellan *et al*, where the V1/V2 loop was fused to a scaffolding protein and complexed with the BNAb PG9 (270). In this structure, the V1/V2 loop adopted a 4-stranded β -sheet configuration, although it remains unclear whether this reflects the true conformation of the V1/V2 loop in the native Env protein (256) (Fig. 1.10).

1.7.1.2 Transmembrane domain – gp41

The gp41 protein can be divided into 3 major domains: the extracellular domain (ectodomain), the transmembrane (TM) domain and the intracellular cytoplasmic (CT) domain (71). The ectodomain can be further classified into several regions, including an N-terminal hydrophobic fusion peptide (FP) followed by a polar region (PR), 2 hydrophobic α -helical coiled structures (N- and C-terminus Heptad Repeat; NHR and CHR) that are connected by a disulphide-bridged loop, and a highly conserved tryptophan-rich region referred to as the membrane proximal external regions (MPER) (Fig. 1.10). Comparing to gp120, gp41 is less glycosylated with only 3-5 N-linked glycans (285, 338). Functionally, the FP is crucial for the fusion of viral and plasma membrane by inserting itself into the target membrane, the NHR and CHR are important for the assembly and stability of the Env trimer, and the MPER is required for the fusion and

infectivity of the virus (71, 286, 350). The TM domain, as its name suggests, consists of ~25 highly conserved amino acids that anchor the Env trimer in the phospholipid bilayer (71). The internal CT domain, although unlikely to be targeted by antibodies, is responsible for regulating Env incorporation, virus infectivity and also influencing the stability and presentation of the Env protein on the viral surface (115, 124, 129, 204, 290, 415, 449).

In contrast to gp120 protein, the structure of gp41 protein is less well-defined as gp41 proteins used for structural determination only consisted of the **core** of the ectodomain that is without the disulphide-bridged loop, the TM and the CT domains. In addition, most crystal structures are believed to represent the ‘post-fusion’ conformation of gp41 with the NHR and CHR forming a 6-helix coiled-coil bundle. At this stage, the conformation of gp41 at the ‘pre-fusion’ state remains unclear (54, 66, 247). To date, the most ‘complete’ crystal structure of gp41 protein is the one published by Buzon *et al*, where they successfully crystallised the NHR/CHR core of the ectodomain of gp41 together with the PR and MPER regions (Fig. 1.10). Consistent with other studies, the crystal structure revealed a ‘post-fusion’, NHR/CHR coiled-coil bundle for gp41 protein, and the proposed position of the FP and PR were found to be in contact with MPER. In addition, a section of the MPER that is juxtaposed to the TM domain was found to bend at 90°, thus positioning the aromatic side chains of 3 amino acids towards the membrane. It was proposed that this kink could induce membrane curvature and facilitate fusion of the viral and plasma membrane.

1.7.1.3 *Structure of the native Env trimer*

Unlike gp120 and gp41 proteins, a crystal structure for the full length Env trimer (spike) has not been determined at this stage. To date, most of the structural information obtained for the Env spike is obtained from cryoelectron microscopy (cryo-EM), e.g. cryo-electron tomography and single particle cryo-EM, of the HIV/SIV Env spike either unliganded or bound to CD4 and different antibodies (165, 246, 256, 434, 435, 439, 450, 457) (Fig. 1.10). However, despite recent advances in cryo-EM techniques, the correct

structure of the Env trimer is still unclear with the conformation of gp41 still under intense debate over the years. Even to this date, there is evidence supporting either the stalk-like model determined by Liu *et al* (165, 246, 256, 403, 434, 435), or a conflicting ‘tripod-like’ model determined by Zhu *et al* (439, 456, 458). In the trimer Env spike, the V1/V2 and V3 loops were situated at the top of gp120 and, as observed in gp120 crystal structures, cryo-EM structures also revealed a dramatic conformational change from the unliganded ‘closed’ state to the CD4-bound ‘opened’ state of the Env spike upon CD4 binding (165, 434, 435). Interestingly, while most structures only represent the Env spike in the ‘opened’ or ‘closed’ state, a recent ~9Å cryo-EM model determined by Tran *et al* revealed an in-between state (‘activated’ intermediates) where the 3 NHRs of gp41 protein are nestled at the centre of the complex and are not in the same compact structure as in the ‘post-fusion’ state (Fig. 1.10 inset) (403). This model may therefore provide an extra structural template for structure-based vaccine design other than the post-fusion 6-helix bundle.

1.7.2 Broadly neutralising antibodies

Understanding the interaction between broadly neutralising antibodies (BNABs) and the Env protein is crucial for vaccine design as it reveals regions on the Env protein that are vulnerable for antibody neutralisation, and provides valuable insights into how such a response can be elicited via vaccination. To date, a number of BNABs have been isolated and characterised (summarised in Fig. 1.11).

1.7.2.1 The 1st generation BNABs – b12, 2G12, 4E10 and 2F5

Until the late 2000s, only a small number of BNABs were isolated and characterised. These include: (i) b12, which targets the well-conserved CD4 binding site (CD4bs) of gp120 protein with an extended ‘finger-like’ hCDR3 region (241, 360); (ii) 2G12, which targets the glycans on gp120 protein by a unique V_H domain-swapped configuration of an IgG-dimer (56, 358, 362); (iii) 2F5 and 4E10, 2 antibodies that target adjacent epitopes on the highly conserved MPER on gp41 (291, 460). One unusual characteristics that is observed in 2F5 and 4E10 is their cross-reactivity with phospholipids of the plasma membrane,

Critical Antigen-Antibody Interaction

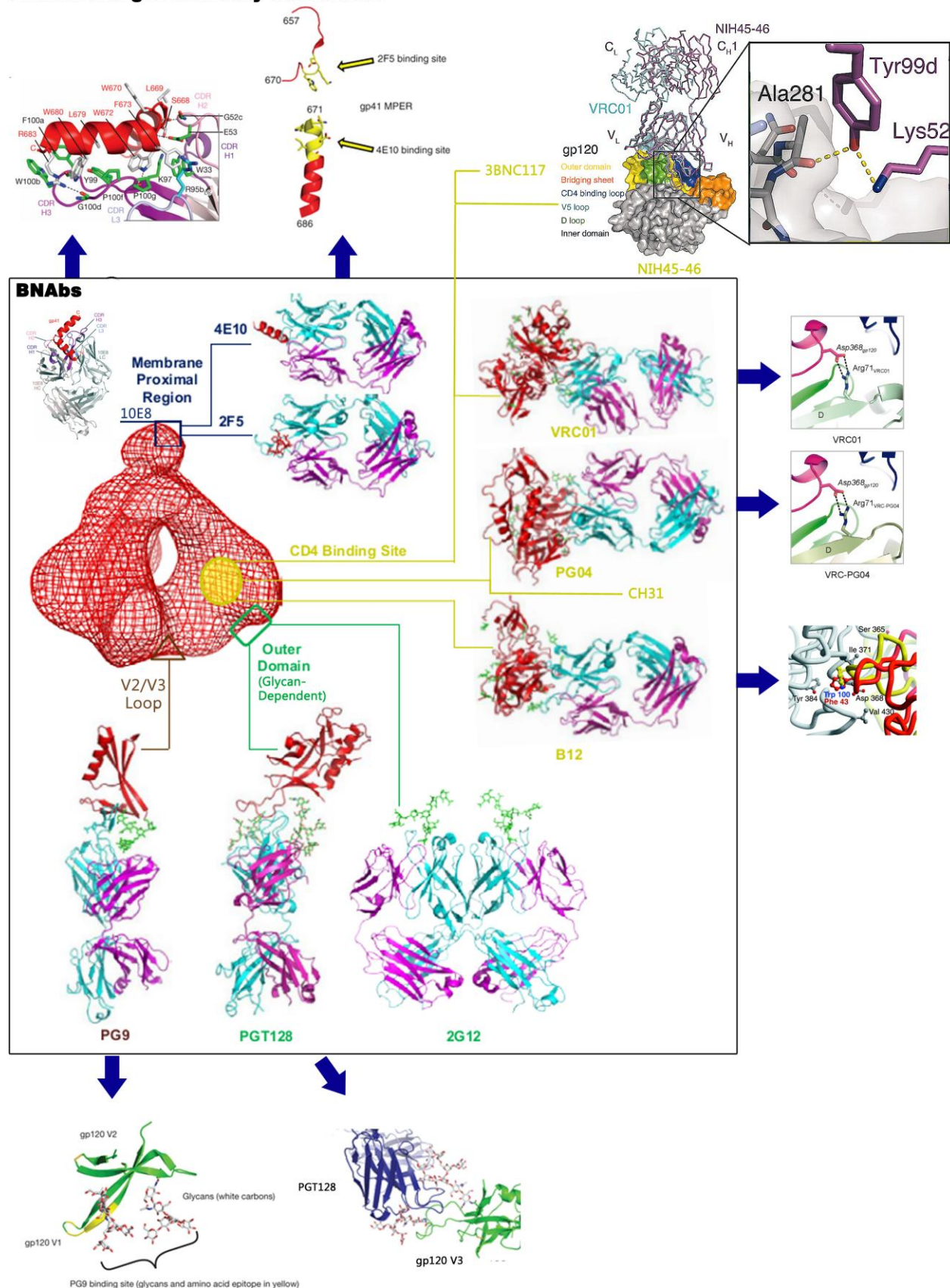


Figure 1.11 Broadly Neutralising antibodies against HIV

The central figure (labelled ‘BNAbs’) was adapted and modified from (218) except for the full structure of 10E8 which was obtained from (190). The binding sites for the known BNABs are shown in this diagram and a full antibody-antigen structure was included when available – the heavy and light chain of the antibody is shown in cyan and magenta, Env epitopes are shown in red and the glycans are shown in green. Structural information on the critical interaction between the antibody and antigen was shown in the periphery of the central figure (labelled ‘Critical Antigen-Antibody Interaction’) and figures were adapted from multiple sources: PG9, PGT128, 4E10 and 2F5 were obtained from (169); NIH45-46 from (103); VRC01 and PG04 from (441); b12 from (360); 10E8 from (190).

which is thought to be crucial for the binding of both antibodies towards the MPER (6, 167, 168). The breadth and potency of these first-generation BNABs have been clearly demonstrated in a study by Binley *et al* alongside other modestly-neutralising antibodies (39) (Table 1.1), and their ability to confer protection against SHIV challenges have been shown in a number of passive immunisations studies in macaques (177, 179, 181, 262). However, although the increased understanding of the BNAB-Env interaction has led to the design of many different immunogens, no vaccine candidate has been able to reproduce such response *in vivo*. It was therefore realised that the information obtained from the characterisations of these BNABs was insufficient, and considerable efforts were put back into the discovery of more BNABs in the late 2000s.

1.7.2.2 New generation of BNABs

By screening a much larger cohort of HIV-infected individuals and taking advantage of newly developed high throughput techniques [reviewed in (283)], more and more BNABs were successfully identified and characterised since 2009. The first study that marks the opening of the floodgate is the study by Walker *et al* in which **PG9** and the somatically-related **PG16** were isolated from a clade A-infected donor (419). Both PG9 and PG16 were able to neutralise 70-80% of primary HIV-1 isolates. Subsequent characterisations of these antibodies revealed that they are glycan-dependent and recognise a novel epitope on the conserved regions of the V2 and V3 loops, and that they preferentially bind to the functional Env trimer over the monomeric gp120 (270, 343, 418). Interestingly, structural information of the PG9 in

		b12		b6		2G12		447-52D		58.2		X5		2F5		4E10		5-mAb cocktail		clade B HIV+ plasma	
clade	#viruses	#neut	%	#neut	%	#neut	%	#neut	%	#neut	%	#neut	%	#neut	%	#neut	%	#neut	%	#neut	%
A	12	4	33	0	0	6	50	0	0	0	0	0	0	11	92	12	100	12	100	5	42
B	29	21	72	5	17	21	72	13	45	9	31	3	10	23	79	29	100	29	100	22	76
C	12	8	67	0	0	0	0	0	0	0	0	0	0	0	0	12	100	12	100	11	92
D	11	8	73	0	0	3	27	1	9	0	0	0	0	5	45	11	100	11	100	5	45
F	3	0	0	0	0	2	67	1	33	0	0	0	0	3	100	3	100	3	100	1	33
G	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	100	2	100	1	50
J	2	0	0	0	0	0	0	0	0	0	0	0	0	2	100	2	100	2	100	1	50
AC	2	0	0	0	0	2	100	0	0	0	0	0	0	1	50	2	100	2	100	2	100
AE	10	3	30	0	0	0	0	0	0	0	0	0	0	10	100	10	100	10	100	10	100
AG	2	0	0	0	0	1	50	0	0	0	0	0	0	1	50	2	100	2	100	1	50
BF	4	0	0	0	0	1	25	1	25	0	0	0	0	3	75	4	100	3	75	3	75
BG	1	1	100	0	0	1	100	1	100	1	100	0	0	1	100	1	100	1	100	1	100
Total	90	45	50	5	6	37	41	17	19	10	11	3	3	60	67	90	100	90	100	63	70

#viruses number of primary viruses included in that clade

#neut number of primary viruses neutralised by the antibody (neutralised with an $IC_{50} < 50\mu g/ml$)

% percentage of total primary viruses neutralised by the BNAbs

Table 1.1 Cross-clade neutralisation analysis of the 1st generation BNAbs

The breadth and potency of the 1st generation BNAbs (b12, 2G12, 4E10 and 2F5), and other anti-HIV mAbs, was measured against a panel of HIV-1 isolates from different clades. Shown in each box is the total number of viruses neutralised by the antibody with an IC_{50} below $50\mu g/ml$, regardless of potency. The data was colour-coded as follows: white box = no viruses were neutralised; green box = 1-30% of viruses neutralised; yellow box = 31-60%; orange box = 61-90%; red box = > 90%. Addition mAb included: b6 – a CD4 binding site-specific mAb; 447-52D – a V3-specific specifically targeting the GXGR motif, 58.2 – a mouse mAb directed towards V3 loop with HIGPGRAF motif; X5 – recognises a CD4-induced epitope on gp120; the 5-mAb cocktail consisted of b12, 2G12, 2F5, 4E10 and 447-52D. This table was adapted from (39).

complex with the V1/V2 loop revealed that the binding of PG9 to the Env protein involves 3 different interactions – glycan, electrostatic and sequence-independent (i.e. PG9 recognises the backbone of the protein instead of the variable side chains) (270) – and it is believed that the combined effects of this multi-component recognition mechanism are essential for the potent neutralising activity observed for PG9.

Many of the newly-identified BNAbs target the highly conserved CD4bs. These include: (i) **HJ16**, which was isolated by the immortalisation of memory B cells from a clade C-infected donor and exhibit a neutralisation breadth similar to b12 (81); (ii) **VRC01**, which was isolated from clade B-infected donors by screening B cells using a resurfaced stabilised core (RSC), where gp120 was engineered to alter the antigenic surface with the closely-related SIV gp120 while preserving the CD4bs of the HIV-1 Env through the introduction of numerous internal disulphide bridges (440). VRC01 was able to neutralise over 90% of circulating HIV-1 isolates with high potency; (iii) **PGV04** and **CH31**, isolated from a

recombinant clade A1/D and B donor, were also purified using a similar RSC approach and share the VRC01 germline V_H gene (IGHV1-2*02) (117, 441). Both antibodies demonstrated similar neutralisation properties to VRC01; (iv) **3BNC117** and **NIH45-46**, which were reported in the same study by Scheid *et al* (371). Respective B cells were isolated by sorting with a stabilised gp120 core protein, and specially-designed primers were used to target a more conserved upstream region of the immunoglobulin gene for amplification. Both antibodies demonstrated exceptionally broad neutralising capabilities that were highly comparable, if not better, than VRC01. In addition to these naturally-induced BNABs, an engineered variant of NIH45-46 (namely **NIH45-46W** which incorporates a G54W mutation) also demonstrated a greater potency than the original NIH45-46 and VRC01 (103).

In possibly the biggest boost in the search of novel BNABs, Walker *et al* unveiled a total of 17 antibodies in a single study that are at least 10-fold more potent than PG9, PG16 and VRC01 (418). These BNABs are referred to as the **PGT antibodies** (i.e. PGT121-123, 125-128, 130, 131, 135-137, 141-145) and they recognise novel epitopes on the Env protein that are previously uncharacterised. Interestingly, similar to PG9, the binding of PGT antibodies was found to be glycan-dependent and the deglycosylation of Env protein led to a loss of binding for all PGT antibodies (418). Subsequent structural analysis of PGT128 revealed that the antibody penetrates the glycan shield by binding to 2 relatively well-conserved glycans and a β -strand segment of the V3 loop (315). Recently, one of the PGT antibodies (PGT121) was also shown to confer protection against mucosal SHIV challenge in macaques (284). In addition to these PGT antibodies, a new MPER-targeting, 4E10/2F5-like antibody was also revealed in a publication by Huang *et al* (190). This antibody, namely **10E8**, binds to a region in proximal to 4E10 and demonstrated a neutralisation breadth and potency that is highly comparable to VRC01. Interestingly, unlike 4E10 and 2F5, this antibody is not self-reactive and does not bind to the phospholipids on the plasma membrane. With the elicitation of glycan- and MPER-specific antibodies proving to be difficult, the different properties observed in the PGT antibodies and 10E8 might provide additional insights into how glycan- and MPER-specific antibodies could be induced via vaccination.

	Env epitope specificity	V _H mutation (%)	hCDR3 length (aa)	Polyreactivity
B12	GP120 CD4bs	13.1	20	YES
HJ16	GP120 CD4bs	14.6	21	NA
VRC01	GP120 CD4bs	32.1	14	NO
CH31	GP120 CD4bs	24.0	15	NO
NIH45-46	GP120 CD4bs	35.7	18	YES
3BNC117	GP120 CD4bs	NA	10	YES
PG9, PG16	GP120 conformational (V1/V2-directed)	16.7-20.5	30	NO
2G12	GP120 carbohydrates	31.7	16	NO
PGT128	GP120 carbohydrates + V3 loop	19.0	19	NO
2F5	GP41 MPER	15.2	24	YES
4E10	GP41 MPER	15.6	20	YES
10E8	GP41 MPER	21.0	22	NO

aa – amino acids; NA – not available; hCDR3 – CDR3 region on heavy chain; V_H – Variable region on heavy chain

Table 1.2 Summary of characteristics of BNABs

Most data presented in this table was adapted from (169) *except* for (i) 3BNC117, in which data for this particular antibody was obtained from (371) and (ii) 10E8 from (190).

1.7.2.3 What makes BNABs so unusual and rare?

Despite the increasing number of BNABs being characterised, a number of unusual properties of these BNABs have posed serious barriers to the elicitation of similar antibodies in natural infection or through vaccination (refer to table 1.2). First, most of these BNABs have experienced a **high level of somatic mutation** during the affinity maturation stage (see section 1.6.2.3). Typically, antibodies accumulate only 5-15% changes in the variable region during the process, but the level of somatic mutation is markedly increased in these BNABs with over 30% changes observed for 2G12 and VRC01 (441). As a result, an extended period of time might be needed in order for the necessary mutations to accumulate and evolve to good effect. In addition, as exemplified by 2F5 and 4E10, **poly-reactivity** for host antigens is also considered as a key attribute for some of these BNABs (6, 167, 168). However, as discussed earlier in section 1.6.2.2, the majority of these poly-reactive B cells are eliminated during various selection checkpoints, meaning that the size of the B cell pool that is capable of maturing into BNABs is lower than expected (289). Finally, the unusually **long hCDR3 region** observed in some BNABs also led to a

proposed connection between hCDR3 length and broad neutralisation (59, 167, 297, 360, 419, 461). However, similar to poly-reactive B cells, any B cells that bear an elongated hCDR3 are also subject to elimination in various selection checkpoints (411). A better understanding in the biological requirement for the elicitation of BNAbs – particularly the role of B cell dysfunction, and the contribution of CD4⁺ follicular T (Tfh) cells that are specialised in B cell help – is urgently needed in order to accelerate the design of an HIV vaccine that could replicate such a response *in vivo* (51).

1.8 Development of a prophylactic HIV-1 vaccine

The main goal of vaccination is the induction of a protective immunological memory through the generation of memory T and B cells against a specific antigen so that a secondary response of higher magnitude can be induced rapidly upon the re-encountering of the same pathogen (65, 355). However, several major challenges have been hindering the development of an HIV-1 vaccine over the years, and different types of HIV-1 vaccines have been developed in an attempt to overcome these problems.

1.8.1 Major challenges in the development of an effective HIV-1 vaccine

The Env proteins have been a focal point in HIV-1 vaccine development as important T cell and NAb epitopes have been found to be located on the Env protein. However, a number of features of the Env protein prevents an effective immune response from being elicited via vaccination: first, the extensive sequence diversity observed in the Env proteins from different HIV-1 clades is likely to limit the vaccine-induced immune response to being relatively strain-specific (see section 1.2.3) (267); second, the immunodominant variable regions of the Env protein can tolerate extensive mutations with little fitness cost, thus allowing the rapid escape from NAb recognition (127). These regions are also externally facing in the trimeric conformation thus protecting the underlying conserved core domains from antibody binding (442); third, specific genetic changes in the Env protein result in the acquisition and rearrangement of glycan motifs, thus allowing the continuous evolution of the glycan shield that permits escape from antibody recognition ('evolving glycan shield') (335, 432); finally, the highly conserved CD4 and co-receptor binding sites are formed by discontinuous residues within gp120 core, and the formation of the highly conserved co-receptor binding site only occurs shortly after the engagement of CD4, meaning that the timeframe for antibody recognition is extremely short (306). Although it is widely believed that a vaccine that can lead to the elicitation of BNABs would be able to overcome the above problems, the requirement for a complex B cell maturation pathway means that the induction of such antibodies appears to be more difficult than initially expected (169), and such an issue is further complicated by the suggestion that the low density of Env spikes on the viral surface can only induce a rather poor activation

of B cells (51). Additional concerns regarding immunogen design and production – e.g. the co-expression of non-functional and native Env spikes on the surface of virus-like particles (86, 401), the inability for rationally-designed immunogens to mimic the native conformation of the Env (211, 238, 416, 420, 447, 452) etc. – are also problems that need to be addressed in order to facilitate the development of an effective HIV-1 vaccine.

1.8.2 Different types of HIV-1 vaccine

It is widely considered that the ideal HIV-1 vaccine should be able to generate ‘sterilising immune response’ in the form of BNABs to prevent the virus from establishing an infection, and at the same time, induce a T cell response that could control the scale of infection, which may affect the long-term mortality and morbidity of the host (51, 412). Over the years, the development of HIV-1 vaccines has followed 3 major overlapping paradigms – from the first wave of candidate vaccines focused on the induction of NABs, followed by a second wave focused on the stimulation of HIV-specific CTL response, and the current wave of optimising both humoral and cellular immune response by heterologous prime-boost strategies.

Traditionally, **live attenuated viruses** (e.g. measles, mumps, yellow fever etc.) have been used as a vaccine against many viral pathogens as they are able to elicit strong cellular and humoral immune response, and confer protection that is long-lasting even with a single immunisation (13, 51, 244). However, their uses against HIV-1 have been overshadowed by safety concerns that they may revert to become pathogenic viruses through mutations or recombination with wild-type viruses (19, 185). Another traditional vaccination approach was through the use of **whole inactivated viruses** (e.g. influenza, polio etc.). Using SIV as a model, inactivated SIV and SHIV vaccines were prepared by either mild oxidation with alditriol-2 to remove the Zn²⁺ ions which is essential for the function of Gag, or by UV inactivation of the viral RNA with the help of psoralen (140). However, the immunogenicity of such vaccine preparations was only modest due to the low number of Env proteins naturally presented on the viral surface. For this

reason, the focus of HIV-1 vaccine development has therefore switched to the use of alternative vaccination techniques.

One such technique is ***virus-like particles (VLPs)***-based vaccines that form the basis of the recently licenced HPV vaccines – Merck’s Gardasil® and GSK’s Cervarix® (51). HIV-VLPs, like all other VLPs, are non-pathogenic virus mimics that can be synthesised by the co-expression of HIV-1 Gag and Env proteins in mammalian cells or baculovirus systems, where the Gag proteins assemble to form the VLP thus allowing the Env proteins to be presented on the viral surface as in the actual virus (105). There are a number of advantages in using VLPs as a vaccine candidate: first, since the VLPs do not contain the HIV-1 genome, they are unable to replicate and the reversion to pathogenic strains is therefore avoided (49, 105, 250); second, similar to traditional vaccines, VLPs are capable of inducing both T cell and B cell responses as they can be taken up by DCs and drive the differentiation of T and B cells into effector and memory cells; third, different variations of the VLPs can be produced by using different combinations of expression vectors – e.g. chimeric SIV/HIV-VLPs (e.g. VLPs made of SIV Gag presenting HIV-1 Env) (445, 446), or HIV-VLP that expresses chimeric/engineered-Env proteins (e.g. uncleaved/internally-stabilised trimers or gp41 MPER fused to hepatitis B surface antigen) (85, 318). However, one particular concern regarding the use of VLP is the presence of non-functional Env spikes that leads to the elicitation of non-neutralising antibodies. Encouragingly, 2 studies have shown that these non-functional Env spikes could be selectively cleared from VLP surfaces by *in vitro* enzyme digests that involve Endo H, chymotrypsin, subtilisin and proteinase K. Subsequent antigenicity test of VLPs that bear ‘pure’ Env trimers demonstrated that only neutralising mAb could recognise these VLPs, meaning that the non-functional Env spikes have been selectively removed (86, 401).

Other than virus mimics, alternative vaccination approaches also include the use of soluble proteins or DNA antigens as they offer several advantages that make them favourable as a vaccine candidate, e.g. a high safety profile, high stability and relative low cost to synthesise. However, compared to traditional

vaccines, the immunogenicity of these vaccines is weak and often requires repeated high-dose immunisations and co-administration with adjuvants in order to boost the immune response. In light of the successful hepatitis B vaccine, initial development of **protein vaccines** was based on the immunisation of monomeric gp120 proteins (30, 106, 209). However, the NAb response generated with these proteins was strain-specific (151, 197, 263), and subsequent phase II and III trials (notably the Vaxgen trials) using gp120 vaccines also failed to display any significant reduction of HIV-1 infection in the vaccinees (118, 137-139, 150, 320). As a result, there has been a switch of emphasis to use soluble trimeric gp140 proteins (full Env trimer lacking the TM and CT domains) as they demonstrate better immunogenicity over monomeric gp120 (112, 223). However, one major challenge in the use of fully cleaved, trimeric gp140 is the dissociation of gp120 and gp41s (269, 287, 325). To stabilise the Env protein, different variations of the gp140 trimer have been developed, including: (i) uncleaved gp140 proteins where the furin-cleavage site is mutated (111), (ii) gp140 trimers that are internally stabilised by the addition of disulphide bridges between gp120 and gp41 (SOS gp140) (38) and with an additional mutation on gp41 (SOSIP gp140) (359), and (iii) gp140 proteins stabilised by the addition of heterologous trimerisation domains at the C-terminus of gp41 (443). Although the immunogenicity of these recombinant Env trimers was not significantly improved, the information obtained through immunisation with these proteins and the recent cryo-EM structures of the native trimer spike will no doubt aid the design of recombinant trimers in the future (see section 1.7.1.3).

The other types of subunit vaccines are DNA vaccines and live recombinant vaccines. **DNA vaccines** make use of plasmid DNA that encodes the antigen of interest under the control of a mammalian promoter. Once administrated, the transcription of the DNA plasmid leads to the expression of antigen in the cytoplasm of the cell, in which they are subsequently converted to peptide strings and presented to T cells on both MHC class I and II molecules (3, 230). Unfortunately, despite promising results shown in small animal models, DNA vaccines were poorly immunogenic in macaques and humans due to the low expression level of the antigen (3, 29, 77, 230). In contrast, **live recombinant vaccines** involve the use of

live viral or bacterial vectors engineered to carry the genes of the desired HIV/SIV antigens, and are attractive immunogens as they are able to elicit a strong CTL and humoral response (140). Nevertheless, one disadvantage of using recombinant vectors is the presence of pre-existing immune response (PEI) against the vector (393). Not only would this lead to a reduction of vaccine efficacy, the T cell response elicited against the vector and the mucosal homing of T cells may also indirectly enhance HIV-1 infection. The potentially deleterious role of PEI was highlighted in the STEP trial based on the adenovirus 5 (Ad5) vector, where an increased acquisition of HIV-1 infection was observed among Ad5-seropositive individuals in the vaccinated group (155). Safety concerns regarding the use of live recombinant vaccines was therefore raised, and a search for a genuinely safe vector with minimal PEI is still on-going at this stage. Recently, a study by Hansen *et al* described a cytomegalovirus (CMV)-based SIV vaccine that appears to function via CTL immune response and restricts the replication of SIV to a very low level in a significant fraction of vaccinated animals (161). Further investigation of this CMV-based vaccine in humans would be of particular interest.

1.8.3 Heterologous Prime-Boost Immunisations

While protein and DNA vaccines failed as stand-alone vaccines, improved immunogenicity could be achieved through the combined use of these vaccines in a ***heterologous prime-boost immunisation*** regime. This involves the sequential inoculations of different immunogens – DNA as a prime, follow by protein as the boost – that target related antigens so that a more complete immune response could be achieved. Indeed, an improvement in both breadth and potency of the antibody response against HIV-1 was observed in a number of prime-boost studies in animals as well as in humans (219, 405, 406, 421, 422). Most significantly, the beneficial role of the prime-boost approach was further highlighted in the RV144 Phase III clinical trial in Thailand, where the combined use of 2 previously unsuccessful vaccines, ALVAC (vector) and AIDSVAX (Clade B/E gp120 protein), were able to display a 31.2% efficacy in preventing HIV-1 infection (further discussion in section 1.8.5). In addition to the DNA prime-protein

boost regime, it is also possible to adopt a DNA-prime, live recombinant vector-boost as exemplified by the on-going Phase II HVTN 505 trial (31).

1.8.4 Adjuvants

Vaccine adjuvants are defined as substances that enhance the adaptive immune response to an antigen but have minimal toxicity or lasting immune effects of their own. There are 2 main groups of adjuvants though they are not entirely mutually-exclusive: **immunostimulants** (e.g. Toll-like receptor (TLR) agonists, cytokines, bacterial exotoxin etc.) that act directly on the immune system to increase response against the antigen, and/or **‘vehicles’** that present the antigen or deliver immunostimulants in the optimal manner (e.g. mineral salts, oil-water emulsions, liposomes etc.) (63). Among the different types of adjuvants, TLR agonists are currently under intensive investigation as they are a potent stimulator of both antibody and T cell responses, hence making them the ideal adjuvants for HIV-1 vaccines (63). To date, a large variety of adjuvants has been tested alongside HIV-1 antigens and some have shown to enhance the titre and/or breadth of the resulting antibody response. Notable examples include: Aluminium salts (63, 113); oil-in-water (O/W) formulations, e.g. MF59 (203, 292); TLR agonists, e.g. CpG oligodeoxynucleotide (CpG ODN; TLR9 ligand) (50, 217, 268, 398, 413); Freund’s Complete Adjuvant (inactivated and dried mycobacteria in O/W emulsion) (226); Ribi adjuvant system (refined mycobacterial products, e.g. cell wall skeleton, and Monophospholipid A (MPL) mixed with squalene oil and emulsified with saline containing the surfactant Tween 80) (21, 243, 375); Carbopol (polyanionic carbomer gel) (101, 226).

1.8.5 Past HIV-1 clinical trials and The RV144 study

Human clinical trials are an important phase in vaccine development since they allow the safety and efficacy of the vaccine to be measured in humans prior to license approval. Clinical trials can be categorised into 3 phases: **Phase I** trial is conducted in a small number ($n < 100$) of HIV-negative, healthy, low risk adult volunteers with the primary objective of determining the safety and immunogenicity of the

vaccine. Following Phase I, **Phase II** trial is carried out where the safety and immunogenicity of the vaccine candidates are further tested with a larger number of recipients ($n > 100$). It is at this stage that important information such as dosage, route of administrations and timing of vaccinations are determined for the optimisation of the vaccination protocol. Once the Phase II trial is completed and results analysed, the most promising vaccines and the delivery strategies are then examined further in the **Phase III** trial, where several hundreds to thousands of HIV-uninfected high risk volunteers from the same region as phase II participate in the study. The aim of the Phase III trial is to allow the safety and immunogenicity profile, the efficacy of the vaccine and the delivery strategy to be finalised for the licensing of the vaccine upon approval. It is noteworthy that, sometimes, clinical trials of different stages can be combined, e.g. Phase I/II is the combination of phase I and II by assessment of both toxicity and efficacy of the vaccine in one single study.

Since the early 80s, approximately 30 HIV-1 vaccine candidates have been tested in roughly 200 phase I/II clinical trials worldwide, with an extra 30 Phase I trials currently on-going. However, despite the vast array of vaccine candidates, only 5 trials have successfully reached the clinical efficacy stage (Phase IIb/III), among which only the RV144 trial demonstrated a significant result (339, 349, 404). The first 2 trials that have completed Phase III were 2 protein vaccines sponsored by **VaxGen**. The vaccines were based on 2 different yet related monomeric HIV-1 Env gp120 – the clade B gp120 (VAX004, AIDSVAX B/B) and the clade B/E gp120 proteins (VAX003, AIDSVAX B/E) (35, 201, 320). In spite of being safe and well-tolerated, these vaccines failed to protect against HIV-1 infection because it was believed that the antibodies elicited could not neutralise primary HIV-1 isolates. In light of these results, there was therefore a switch of focus towards the elicitation of a CTL response. Two ‘proof-of-concept’ Phase IIb trials – the **STEP** and its companion, the **Phambili** trial – were conducted in 2005 using a replication-incompetent recombinant adenovirus serotype 5 (Ad5) vector expressing HIV-1 Gag, Pol and Nef. The aim of both studies was to trigger a CTL response that could prevent HIV-1 infection or reduce viral loads

post-infection in adults at high risk of infection (48). Nevertheless, both trials were suspended in 2007 because of the disappointing results of the STEP trial.

The only clinical trial that has been able to display a significant result is the **RV144** trial (339, 349, 404). This Phase III trial is the largest HIV-1 vaccine study ever conducted in humans with more than 16,000 HIV-negative Thai men and women enrolled into the program. The vaccine used in this trial is a combination of 2 previously unsuccessful vaccines given in a ‘prime-boost’ approach – a canarypox virus-based vector (ALVAC-HIV) and the AIDSVAX B/E gp120 used in the VaxGen trial. The rate of infection in vaccine and placebo recipients was compared using 3 different analyses – intention-to-treat (ITT) (all enrolled, $n = 16,402$), per-protocol (PP) (all completed, $n = 12,542$) and modified ITT (mITT) in which 7 volunteers were removed from the ITT analyses as they were seropositive for HIV-1 at the time of the first vaccine administration ($n = 16,395$). Despite the difference in total numbers, a trend of lower infections in the vaccine recipients was consistently observed in all 3 analyses, of which a modest yet significant 31.2% vaccine efficacy ($p = 0.04$) with a 95% confidence level was observed in the mITT analysis. Two strong correlates of infection risk were identified in this trial: (i) the plasma concentration of V1/V2-specific IgG was inversely correlated with infection risk and supported by a recent immunisation study in macaques (26), and (ii) high plasma concentration of Env-specific IgA was directly correlated with acquisition of infection (168). Further analysis is now underway to examine the significance of these findings, and the results observed should accelerate research efforts towards a more effective HIV-1 vaccine.

1.9 Rational antibody-based HIV-1 vaccine design

One major focus in the field of HIV-1 vaccine research is the use of rationally-designed immunogens to elicit a broad and potent neutralising antibody response that resembles known BNABs. Generally speaking, strategies used in the development of rationally-designed vaccines fall into 2 categories: (i) ***Epitope mimicry***, which is a structural-assisted method that presents specific epitopes in the conformation that is recognised by BNABs. It is hoped that the antibodies elicited against such immunogens can exhibit similar binding properties that resemble the original BNAB; or (ii) ***Epitope focusing ('Immunofocusing')***, which relies on the focusing (or re-directing) of B cell responses from known immunodominant regions towards other immuno-recessive yet highly conserved regions of the Env protein. Different immunogens have been proposed and tested over the years for the elicitation of antibodies against the highly conserved CD4bs, MPER and glycan motifs on the Env protein.

1.9.1 Targeting the CD4bs on gp120

The CD4bs is an attractive target for vaccine design because it is targeted by numerous BNABs and because of its high degree of functional conservation among different HIV-1 clades. Different approaches have been used aiming to focus the antibody response towards the CD4bs: (i) alanine substitution, where selected amino acids around the CD4bs were mutated to eliminate the binding of non-neutralising CD4bs-specific antibodies (e.g. b6, b3 and F105) but retain the binding of the BNAB b12 (241, 309, 369); (ii) hyperglycosylation of Env proteins, where extra glycans were strategically introduced to the variable loop of the Env protein for the masking of non-neutralising epitopes on gp120 but not the b12 binding site (308, 309, 369); (iii) expression of CD4bs-containing outer domain (OD)-only Env proteins, where the unrelated inner domain of gp120 was excised (34, 74, 444). However, these immunogens have all failed to elicit a CD4bs-specific response that is comparable to known BNABs. Recently, 2 novel immunogens were designed with a similar aim and have attracted a lot of interest. The first immunogen is the RSC used in the isolation of VRC01, where amino acids of gp120 core protein outside the CD4bs were replaced with those from the structurally-related SIV molecule (440). As demonstrated in the *in vitro* study, the

preferential binding of CD4bs-specific BNABs makes this RSC protein an attractive candidate for the elicitation of CD4bs-specific antibodies, although the immunodominance of the ‘substituted SIV-shell’ is yet to be determined *in vivo*. For the second immunogen, the backbone and side chains that constitute the b12 epitopes were grafted onto an unrelated molecular scaffold (18) (Fig. 1.12). Characterisation of the final scaffolds showed that this molecule bound to b12 with high specificity and affinity, and crystallographic analysis revealed high structural mimicry of gp120-b12 complex structure. Immunogenicity studies of both constructs are currently underway and it would be interesting to see whether a BNAb-like response can be elicited.

1.9.2 Targeting the glycan-motifs on gp120

The HIV-1 Env protein is heavily glycosylated and the presence of a ‘glycan-shield’ enables the HIV-1 virus to escape antibody recognition. Even though the individual glycans are ‘self’ molecules, the high density of the glycans observed in the trimeric spike is naturally absent in the host and therefore may create epitopes for BNAb binding (e.g. 2G12, PG9 and the recently identified PGT antibodies) (270, 315, 358). Numerous immunogens have been designed, in the form of glycoconjugates, that mimic the oligomannose cluster recognised by 2G12, e.g. oligomannose dendrons (423), Man₄-containing neo-glycoconjugates (16) and cyclic glycopeptides (202). Although glycan-specific antibodies were induced in these studies, these antibodies failed to cross-react with gp120 or neutralise any HIV-1 isolates. Recently, glycan-specific antibodies that can cross-react with gp120 were successfully elicited by using yeast-derived glycan mutants (110, 249). However, the neutralisation capacity of these antibodies failed to mimic that of 2G12, therefore strongly indicating that the glycan epitopes recognised by these antibodies are different to that of the 2G12 epitope. Unless the elicited antibodies could replicate the domain-swapped configuration that is characteristic of 2G12 (56), it is still unclear how a glycan-specific neutralising response could be elicited at this stage. Based on the binding characteristics observed in PG9/16 and PGT antibodies, other antibody-antigen interactions are most likely to be required in order for these glycan-dependent, or glycan-specific, BNABs to be induced via vaccination (270, 315, 343).

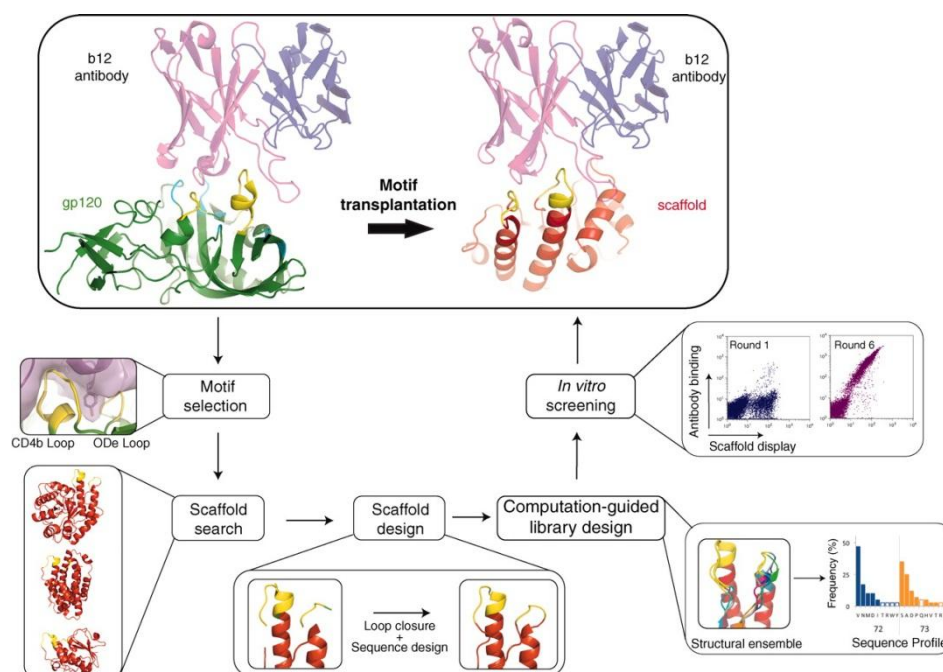


Figure 1.12 Grafting of key b12 epitope onto molecular scaffolds

Outline of a novel vaccine design utilising computation method and experimental selection for grafting the key b12 epitope onto an unrelated molecular scaffold. The process started with the selection of suitable scaffolds to accept the backbone segments comprising the motif. The resultant b12 mimic, which was grafted onto the scaffold, was iteratively modelled and mutated so that it binds to the b12 with an affinity similar to the native antigen. This figure was adapted from (18).

1.9.3 Targeting the MPER on gp41

Similar to the CD4bs, the MPER region is highly conserved in HIV-1, and the MPER-specific BNAb 10E8 is one of the most broad-acting and potent antibodies to date (39). However, the elicitation of MPER-specific antibodies has proved to be extremely difficult because: (i) antibody access to the MPER is restricted as the MPER is ‘sandwiched’ between gp120 and the viral membrane (232, 364), and (ii) poly-reactivity is thought to be critical for the binding of these antibodies to the MPER (6, 167, 171). To circumvent these problems, MPER-containing immunogens typically involve the use of heavily-engineered gp41 core proteins or chimeric proteins (e.g. gp41/HA protein) expressed on virus-like particles (211, 238, 322, 416, 420, 447, 452), or through the grafting of 4E10/2F5 epitopes onto unrelated scaffold proteins in a way similar to the grafting of b12 epitope mentioned above (80, 296). It was hoped that the elimination of gp120 and the presence of the viral membrane might encourage the elicitation of 2F5/4E10-like antibodies. However, the antibody responses elicited against these immunogens are mostly

non-neutralising as they failed to recognise the MPER in its native conformation and they also lack the poly-reactivity that is thought to be important for the binding of MPER-specific antibodies. Further characterisation of the recently discovered 10E8, which is more potent than 4E10 and is not poly-reactive, is therefore important and this may inspire a new wave of strategies for the elicitation of a MPER-specific neutralising antibody response that can be compared to 4E10, 2F5 and 10E8.

1.9.4 Other novel strategies for the elicitation of BNABs

In addition to the methods described above, there are also other novel strategies aiming to replicate a broadly neutralising response *in vivo*. In recent years, advances in our understanding in the maturation process of BNABs have led to the proposal of a B cell-lineage-based approach to vaccine design (Fig. 1.13) (169, 441). The goal of this approach is to derive antigens with an enhanced affinity for the unmutated and intermediate ancestor antibodies of a BNAB clonal lineage (e.g. IGHV1-2*02 as shared by several CD4bs-specific BNABs) compared to existing antigen. In this way, it has been hypothesised that the maturation of these BNAB lineage cells can then be ‘guided’ by sequential immunisation with different antigens, which would eventually lead to the induction of a broadly neutralising response. Furthermore, as an alternative approach to immunisation, vector-mediated gene transfer – namely vectored immunoprophylaxis (VIP) – was also employed with the aim of secreting known BNABs into the circulation (20). By using adeno-associated virus vectors as a carrier for the gene of the full-length BNAB 4E10, it was shown that a single muscular injection of this VIP vector into mice achieved life-long expression of 4E10 *in vivo*. Most importantly, mice receiving VIP appeared to be fully protected from HIV-1 infection even with a high dose of virus injected intravenously. Given such promising results in mice, the successful translation of this approach to humans might be an alternative mean to confer protection against HIV-1 infection.

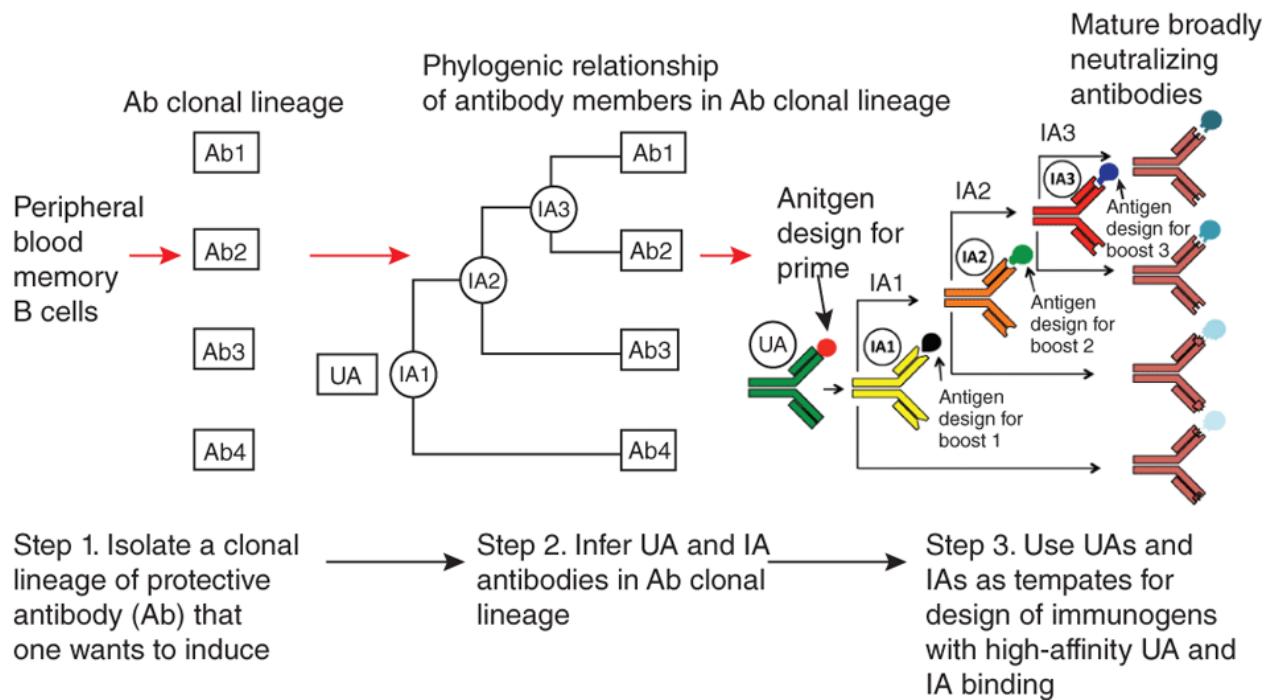


Figure 1.13 Illustration of the proposed B cell-lineage-based approach to vaccine design

Outline of the proposed B cell-lineage-based approach to vaccine design. The first step of the procedure involves the identification of a set of clonally-related B cells that contains BNABs, and this is followed by the inferring of the mutated ancestral (UA) BCR, along with probable intermediate ancestors (IA) BCRs at key clonal lineage branch points. Finally, this leads to the design and sequential immunisation of immunogens with an enhanced affinity for the UA and IA BCRs. This should therefore allow these B cells to be 'guided' through the maturation process, thus resulting in the elicitation of the BNABs. This figure was adapted from (169).

1.10 Scope of this thesis

The RV144 clinical trial is the first demonstration that protection against HIV-1 is achievable when the right strategy is used. Together with the discovery of an increasing number of BNABs, it is now believed that an HIV-1 vaccine that can lead to the elicitation of a BNAB-like response can be used to confer protection against the diverse strains of HIV-1 globally. However, the lack of know-how in eliciting such a broadly neutralising response remains one major bottleneck in HIV-1 vaccine development. Despite all the efforts, no immunogens have yet succeeded in achieving this with a number of challenging issues yet to be resolved (see section 1.8.1). In order to contribute to this area of research, I focused my doctorate on addressing 2 key questions:

- 1) *As the key determinant of humoral immune response, are there any features of the Env protein that are associated with a broadly neutralising response? If so, how can their presence in the Env protein encourage the elicitation of such a response and benefit future vaccine design?*

In **Chapter 3**, an *in silico* study was carried out to identify possible features of the Env protein that are associated with a broad neutralising antibody response (i.e. signatures). These signatures were subsequently characterised with existing structural and biochemical data to reveal their potential significance and contribution towards such a response, and how these findings could be used in future immunogen design.

- 2) *Immunodominant regions of the Env protein often 'distract' the host immune response from recognising the conserved regions that are the targets of BNABs. What vaccination approach can be used to focus the immune response back towards the conserved regions?*

Novel vaccination strategies were designed aiming to encourage the elicitation of BNABs that specifically target the CD4bs and MPER. In **Chapter 4**, I describe the development of 2 novel ideas which involve (i) the selective activation of CD4bs-specific B cells for the elicitation of CD4bs-

specific antibodies, and (ii) the use of VLPs bearing engineered gp41 proteins for the elicitation of MPER-specific antibodies. In **Chapter 5 and 6**, I report the development of an antibody-shielding technique where immunogen-derived antibodies were used for the masking of immunodominant regions of gp120. It was hoped that the use of such technique could eliminate the antibody response towards the immunodominant regions so that a more site-specific response that targets the highly conserved CD4bs could be induced.

Chapter 2

Materials and Methods

2.1 Antibodies

	Target	Concentration*	Source
Primary Antibodies			
<i>Penta-His</i>	His ₆ tag	100ng/ml (WB); 1µg/ml (E)	Qiagen
<i>HIVIG</i>	Multiple regions on HIV-1 Env	100ng/ml (WB); Varies (E)	Kindly provided by Dr. Chris Scanlan (Oxford)
<i>b12</i>	Env gp120 CD4bs	50ng/ml (WB); Varies (E)	NIBSC Centre for AIDS Reagents
<i>b6</i>		Varies (E)	
<i>VRC01</i>		Varies (E)	
<i>3074</i>	Env gp120 V3	Varies (E)	
<i>447-52D</i>		25ng/ml (WB); Varies (E)	
<i>2G12</i>	Env gp120 Glycan	Varies (E)	
<i>697-D</i>	Env gp120 V2	Varies (E)	
<i>2F5</i>	Env gp41 MPER	100ng/ml (WB); Varies (E)	
<i>4E10</i>			
<i>Mouse anti-p24 monoclonal</i>	Gag p24	100ng/ml (WB)	
<i>Rabbit anti-CD4 polyclonal</i>	Human CD4	500ng/ml (WB)	Thermo Scientific
Detection Antibodies			
<i>Goat anti-mouse HRP</i>	Mouse IgG	1/2000 dilution (WB); 1/10,000 dilution (E)	Sigma Aldrich
<i>Goat anti-human HRP</i>	Human IgG		
<i>Goat anti-rabbit HRP</i>	Rabbit IgG		
<i>Streptavidin-HRP (ExtrAvidin-HRP)</i>	Biotin	1/5000 dilution (WB); 1/10,000 dilution (E)	Invitrogen
<i>Goat anti-human (Fc-specific)-FITC</i>	Human IgG	1/1000 dilution (FACS)	Invitrogen

* Typical concentration used unless otherwise stated; WB – Western blot; E – ELISA; FACS – Flow Cytometry

2.2 General Methods

Listed in this section are methods and techniques that were commonly used throughout my study. Additional information or modifications made to any of these techniques will be described in the chapter-specific sections later in the chapter.

2.2.1 Polymerase Chain Reaction (PCR)

PCR-based techniques were extensively used for: (i) the reverse transcription of viral RNA to cDNA, (ii) the amplification of the *env* region from the whole viral cDNA or other *env* plasmids, and (iii) the introduction of various mutations to the Env protein by overlapping PCR – which relies on the joining of different DNA fragments that contains a complementary ‘linker’ to generate a single mutated DNA product (Fig. 2.1). The following enzymes/kits were used: Advantage 2 Polymerase mix (Clontech), KOD polymerase (Novagen), PrimeSTAR Max DNA polymerase (Clontech) and SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen). The typical reaction cycle used for each polymerase is listed below:

Step		Advantage 2	KOD	PrimeSTAR MAX	One-step RT-PCR
	cDNA synthesis	Not applicable			55°C 1min
1	Primary denaturing	94°C 2mins	95°C 2mins	98°C 1min	94°C 2mins
2	Denaturing	94°C 15s	95°C 25s	98°C 10s	94°C 15s
3	Primer annealing	60°C 30s	60°C 30s	55°C 5s	65°C 30s
4	Extension	68°C 1.5mins	72°C 1.5mins	72°C 10s	68°C 4mins
5	Cycle	Repeat step 2-4 for 20-30 cycles			
6	Final Extension	68°C 8mins	72°C 4mins	72°C 1min	68°C 8mins

2.2.2 Purification of DNA products

The purification of DNA products was achieved by gel purification using QIAquick Gel Extraction Kit (Qiagen). The DNA products were separated by 10% agarose gel (supplied with 2% ethidium bromide) and the desired band was identified and excised under UV. The DNA in the band was purified using the kit as per the manufacturer’s protocol.

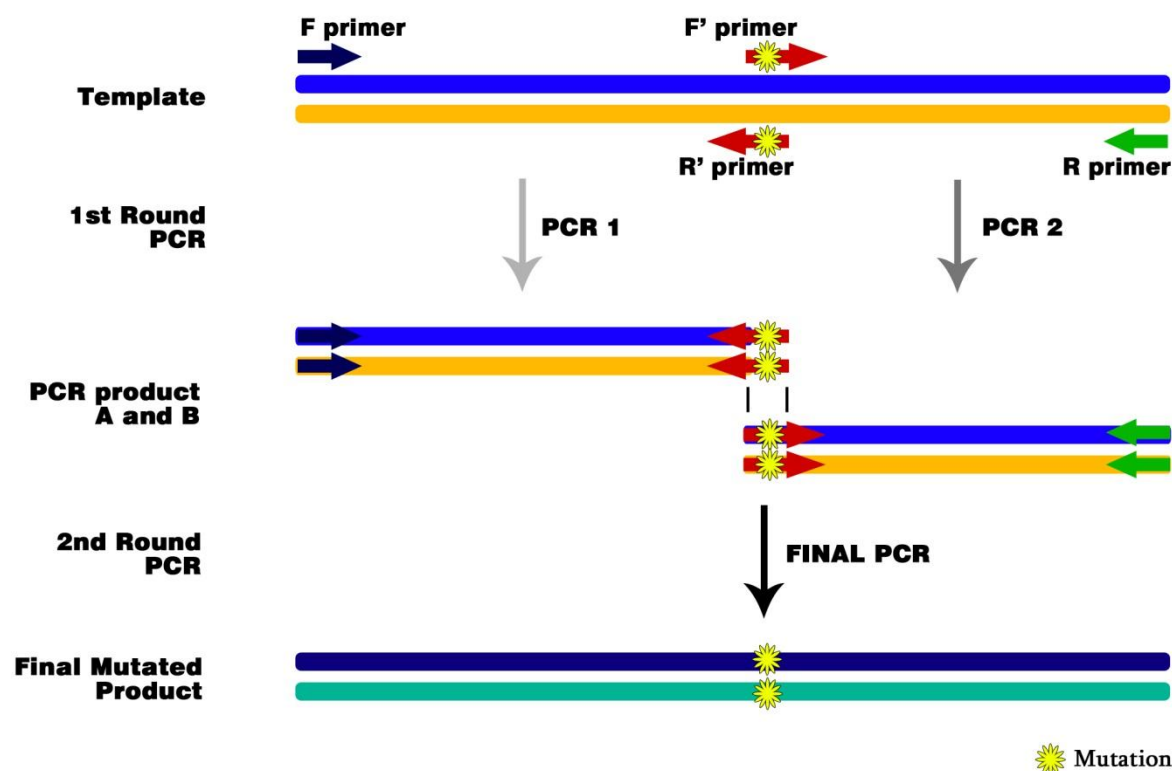


Figure 2.1 Introduction of mutation by overlapping PCR

Specific primers that hold the desired mutation (F' and R' complementary primers) are used for the amplification of individual segments from the template alongside with both 5'-end and 3'-end primers (F and R primer). The presence of the complementary sequences in both PCR products (A and B) allows the annealing of the 2 products in the subsequent PCR round(s) into one final chimeric DNA product.

2.2.3 Cloning and amplification of *env* plasmid

Both the *env* constructs and expression vectors were digested with EcoR1 and Xho1 (NEB) at 37°C for 1 hour and purified from the reaction mixture by gel purification as described above. Purified *env* constructs were ligated to the expression vector using the T4 DNA Ligase (NEB) at room temperature for 1 hour. The *env* plasmid was then transformed into Library Efficiency DH5α *E.coli* (Invitrogen), and colonies were selected on agar plates supplemented with carbenicillin (100µg/ml; Melford). For the amplification of the plasmids, depending on the required yield, either QIAprep spin Miniprep kit (routine ~1mg plasmid amplification) or EndoFree Plasmid Giga kit (>10mg for protein expression) (Qiagen) was used as per

the manufacturer's protocol. The amplified plasmids were then validated by using a combination of EcoR1 and Xho1 digestion, PCR or Sanger sequencing.

2.2.4 Protein Expression and purification

All proteins were expressed as a mixture of monomeric and oligomeric species by transient transfection of 293T cells. Cells were transfected with *env* plasmids using polyethylenimine (PEI) (Polysciences, Inc) as the transfection reagent (DNA:PEI ratio = 1:1.8) and cultured in DMEM supplemented with 2% FCS, 1% Penicillin-Streptomycin (PS; Sigma Aldrich). Cell culture supernatants were collected after 72 hours incubation (first round collection), and the cells were allowed to grow in fresh DMEM (2% FCS, 1% PS) for an additional 72 hours with supernatant collected (second round collection). All supernatant was combined, pre-cleared by centrifugation (7,000rpm, 3 hours), and filtered through Express PLUS® 0.22µm filter (Millipore). The pH of the filtered supernatants was then adjusted to approximately 8.0 by the addition of Tris-buffered saline (TBS) powder (pH 8.0) (Sigma Aldrich). With the presence of a C-terminus His₆Tag in the soluble Env proteins, purification of proteins from the supernatant was achieved through Immobilised Metal ion Affinity Chromatography (IMAC). All His₆tag-proteins were captured by cobalt-based IMAC purification using Talon Superflow Metal Affinity Resins (Clontech), washed by 3-column volumes of wash buffer (TBS pH 8.0) before elution with 2-column volumes of elution buffer (250mM Imidazole in wash buffer). The eluted proteins were further purified by size exclusion chromatography on HiLoad 16/60 Superdex-200 column (GE Healthcare) that was pre-equilibrated with endotoxin-free phosphate-buffered saline (PBS; Sigma Aldrich). Fractions containing the monomeric gp120 proteins were identified by SDS-PAGE analysis and combined (see section 2.2.7).

2.2.5 Virus-Like Particle (VLP) production

VLPs were produced by co-transfection of 293T cells with the corresponding *env* plasmids and pNL4-3.Luc.R-E-*, which encodes for HIV-1 Gag proteins, using polyethylenimine (PEI) (Polysciences, Inc) as described in section 2.2.4. After 72 hours of culturing, supernatants were collected and pre-cleared by low

speed centrifugation (4,000rpm for 30mins). The media were then filtered through Stericup-HV 0.45µm filter (Millipore), and VLPs were pelleted at 40,000xg for 2 hours. The VLP-pellet was washed with 1ml of endotoxin-free PBS and re-centrifuged in a bench top centrifuge at maximum speed for 1 hour. VLPs were then re-suspended in endotoxin-free PBS and stored at -80°C for future use.

** Kindly provided by Dr. James Binley, Torres Pines Institute for Molecular Studies*

2.2.6 Determination of protein concentrations

Protein concentrations were measured by UV spectroscopy using the BIO-RAD Protein Assay (BIO-RAD) as per the manufacturer's protocol.

2.2.7 SDS-PAGE

All samples were mixed with the 5x Lane Marker Sample Buffer (Thermo Scientific) prior to loading. For the reduction of protein samples, dithioereitol (DTT; Sigma Aldrich) was added to the samples at a final concentration of 100mM, and the samples were subsequently boiled at 100°C for 3mins. Afterwards, samples were loaded onto NuPAGE 4-12% Bis-Tris gel (Invitrogen) and ran in 1X NuPAGE MOPS SDS Running buffer (Invitrogen) at 150V until completion. Estimation of sample size was achieved by comparing the sample band with the Novex Sharp Pre-stained protein standard (Invitrogen). The completed gel was subjected to either Coomassie Blue staining or Western blotting (described below in section 2.2.9 and 2.2.10).

2.2.8 Blue-Native PAGE (BN-PAGE)

In SDS-PAGE, SDS is used as a charge-shift molecule that binds to the protein and confers a negative charge to the protein. However, the protein would also be denatured in this process. On the other hand, BN-PAGE relies on the use of Coomassie G-250 as a charge-shift agent and one advantage of BN-PAGE is that the protein will remain in its native conformation during separation (438). Samples were mixed with 2x Sample Buffer [100 mM MOPS, 100 mM Tris-HCl (pH 7.7), 40% glycerol, and 0.1% Coomassie

blue (Brilliant Blue G; BioRad)] before loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen), and separated at 4°C at 100V with (i) 50 mM MOPS/50 mM Tris (pH 7.7) as the anode buffer, and (ii) 50 mM MOPS/50 mM Tris (pH 7.7) with 0.02% Coomassie Blue as the cathode buffer. The completed gel was then subjected to either Coomassie Blue staining or Western blotting (described below in section 2.2.9 and 2.2.10).

2.2.9 Protein Staining

For the visualisation of the proteins on the gel, proteins were stained with Coomassie G-250 (Brilliant Blue G; BioRad) by incubating the gel in staining buffer (0.1% w/v Brilliant Blue G, 50% v/v Methanol, 10% v/v Glacial Acetic acid) for 1 hour at room temperature, before replacing with the destaining buffer (50% v/v Methanol, 10% v/v Glacial Acetic acid) for another hour. The destaining buffer was then discarded and the gel was left in distilled water overnight at room temperature for the removal of non-specific staining.

2.2.10 Western Blot

For the blotting of SDS-PAGE, the gel was incubated with 2x NuPAGE Transfer Buffer (Invitrogen) for 20-30mins before blotting onto Amersham Hybond-P PVDF membrane (GE Healthcare). The membrane was then transferred to blocking buffer (2% nonfat milk in PBS) for 1 hour at room temperature before probing with the primary antibody (diluted in blocking buffer) overnight at 4°C. The membrane was then washed twice by incubating in washing buffer (PBS with 0.05% Tween-20) for 15mins. This was followed by the detection with secondary antibody (diluted in blocking buffer) for an hour at room temperature. Again, the membrane was washed with 3 x 15mins wash-steps before development using Amersham ECLTM Western Blotting Detection Reagents and Films (GE Healthcare). **For the blotting of BN-PAGE**, the entire procedure was identical except the addition of a destaining step after blotting. Here, membranes were preliminarily destained and fixed in destaining buffer (50% v/v Methanol, 10% v/v

Glacial Acetic acid) for 10mins, followed by another destaining step with 100% methanol for 2-3 minutes. The completely destained membrane was then blocked and processed as described for the SDS-PAGE.

2.2.11 ELISA

Various forms of ELISA were performed in my thesis but all shared a similar protocol. Briefly, proteins (50ng/well in PBS) were coated onto High-Binding 96 well ELISA plates (Greiner) at 4°C overnight. Coated-plates were then washed 3 times with washing buffer (0.05% Tween-20 in PBS) before blocked with the blocking buffer (200µl/well; 2% nonfat milk in PBS). Plates were then washed for 3 times before the addition of the primary antibody/serum at 50µl per well, and was left at room temperature for 2 hours with gentle shaking and later washed 3 times. Subsequently, 50µl of the secondary detection antibody were added to each well for 1 hour at room temperature with gentle shaking. For detection, the plate was washed 3 times and 50µl of 1-step Ultra TMB-ELISA (Thermo Scientific) was added and left until the colour develops. The reaction was stopped by adding 50µl of 2M sulphuric acid to each well and the plate was measured at OD₄₅₀. All dilutions of antibodies/serum were made in sample buffer (1% BSA, 0.05% Tween-20 in PBS).

2.3 Chapter-specific methods

2.3.1 Chapter 3 – Associations between HIV-1 Env Traits and the generation of Broadly-Neutralising antibody response

Bioinformatics analysis in this study is a collaborative work with Dr. Joe Parker (Queen Mary, University of London). Methods performed by Dr. Joe Parker will be stated where applicable.

2.3.1.1 Cohort

Sample collection and neutralisation assays used to determine the neutralisation score (based on the neutralisation breadth and potency of the antibody) were undertaken and provided by the International AIDS Vaccine Initiative (IAVI) as part of their Neutralising Antibody Consortium (373). In summary, sera were collected from HIV-1-infected volunteers in Australia, the United Kingdom, Rwanda, Zambia,

Ivory Coast, Thailand, Kenya, Uganda, and the United States. All donors were age 18 years or older, were HIV-1 infected for at least 3 years prior to the day of screening, were clinically asymptomatic, without evidence of progression to AIDS based on WHO stage III or IV criteria or a CD4⁺ T cell count of < 200/μl, and were not on antiretroviral therapy (ART) for at least the previous 1 year. These samples were then screened for neutralising activities using various pseudovirus panels composed of clade A, B, C, D and CRFs viruses. For each sample, a neutralisation score (ranging from 0 – 2.83) was computed and samples with a score greater than or equal to 2.5 were classified as elite neutralisers.

2.3.1.2 Isolation, PCR amplification and sequencing of env from viral RNA

For the construction of *env* database, the viral RNAs were first isolated from plasma samples and the *env* region was subsequently amplified in a one-step RT-PCR reaction. The PCR products were subsequently cloned into the pCR4-TOPO vectors from the TOPO TA Cloning Kit for Sequencing (Invitrogen) and sequenced. The details of the RT-PCR, cloning and sequencing were as described previously (353).

2.3.1.3 Sequence assembly and initial phylogenetic analyses

The following work was performed by Dr. Joe Parker. Full-length *env* sequences were assembled automatically and the open reading frames (ORFs) from the contigs were extracted using the EMBOSS tool getorf, followed by the alignment of the ORFs (DNA and protein) using MUSCLE. Initial phylogenetic analyses were performed to validate the input data, and then sought to obtain a robust estimate of the best phylogeny, substitution model and parameters for later selection analyses. ModelTest (331, 332) (implemented in HYPHY(330)) was then used to identify the best-fitting model for the data, and the model of GTR + γ + I was used in all future analyses wherever possible. The phylogeny and model parameters for this refined data set were then iteratively optimised in GARLI (47) and PHYML respectively. Global (*env*) and site-wise dN/dS ratios were estimated from the best available topologies in HYPHY using the model GTR + γ + I as identified above. Furthermore, values for global parameters such as the length, net charge and number of glycosylation of the entire gp160, and of each variable loop, were

obtained through PERL scripting on the dataset (<http://www.perl.org>). Details for the above experiments were as described previously (353).

2.3.1.4 Preliminary analysis of obtained sequences in association with neutralisation score

For the preliminary analysis of the sequences, the values for the global parameter obtained for each *env* sequence were plotted against the respective neutralisation score in Graphpad PRISM (ver. 5.04). All statistical calculations/correlations were calculated in PRISM.

2.3.1.5 Decision Tree making

The generation of Decision Trees was performed by Dr. Joe Parker, while the identification of individual signatures was performed by myself. Open source machine learning software Weka 3.5 (Waikato Environment for Knowledge Analysis) was used to develop Decision Trees by the C45/J48 algorithm (160). The minimum number of objects per leaf node was tested and set to 25 objects in all classification experiments to confine the complexity of the models. To reduce overfitting and underfitting bias, iterative model testing was employed to improve model precision. The dataset was spilt randomly into a training set for model derivation (30% of all sequences), and a test set for testing and validation of the model (remaining 70%). Briefly, different parameters or prediction models were inputted into WEKA for model testing and the output (score %) for that particular run was recorded through PERL scripting. The score (%) is defined as how accurate the derived model can reflect the neutralisation profile of sequences in the test set. Another set of parameters/models was then selected for the next round of model testing, and the whole process was repeated until all possible combinations of parameters were exhausted. The final output was displayed as a table with the scores for each parameter/model that was involved. The average score (%) for each parameter/model was then used as an indication of its relative importance in predicting the neutralisation profile within the given dataset. In the end, the **5 parameters with the highest average score (%)**, together with **the identified positively selected sites**, were used in model derivation for associations determination. Similarly, with the derived model, the parameters were inputted into WEKA and were tested against all sequences in the dataset with self-validation. The score (%) of each model was recorded

at the end of the analysis and the combination(s) of parameters that led to the best neutralising group (i.e. group of highest neutralisation score) were inspected visually.

2.3.1.6 Generation of Consensus Sequence and structural mapping of signature positions

Consensus sequences for the clade B and C datasets were generated using the Advanced Consensus Maker on the Los Alamos HIV-1 database using the default settings (<http://www.hiv.lanl.gov/content/sequence/CONSENSUS/AdvCon.html>). For structural mapping of the signatures, the core structure of gp120 corresponding to the crystal structure of CD4- and X5-bound clade B JRFL gp120 was used (188). All signature positions were mapped onto this structure based on the alignment of the JRFL and HxB2 sequences, with all positional numberings based on the HxB2 sequence. Three-dimensional images were generated using the Visual Molecular Dynamics (VMD) software (192).

2.3.2 Chapter 4 – Development of immunofocusing techniques for the elicitation of a broadly neutralising antibody response

Approach [1] - Selective activation of CD4bs-specific B cells

2.3.2.1 Production of Env plasmids

Both the wildtype and the CD4bs-defective gp120 (D368R-gp120) proteins were derived from the subtype B JRFL strain. The sequence of the JRFL gp120 was codon-optimised using Gene Designer 2.0 (<https://www.dna20.com/tools/genedesigner.php>). A His₆Tag sequence was added to the C-terminus end of gp120 protein for later purification and detection. The introduction of D368R mutation was achieved by overlapping PCR using KOD DNA polymerase (Novagen) and the primers listed in the table below. All PCR products were gel purified and cloned into the highly-optimised mammalian expression vector *PLEC* (14) as described in the general methods (see section 2.2.3). All plasmids were checked with EcoRI and XhoI digest or PCR, and later verified by Sanger sequencing using the sequencing primers *PlecF* and *PlecR*.

Primers (5' → 3')

D368R mutation	
JRFL_D368R_F	CAC AGC TCT GGT GGT AGG CCT GAG ATT GTG ATG
JRFL_D368R_R	CAT CAC AAT CTC AGG CCT ACC ACC AGA GCT GTG
Sequencing Primer	
PlecF	CGT GCT GGT TAT TGT GCT
PlecR	GGT ATT TGT GAG CCA GGG

The D→R mutation is highlighted in **RED**

PCR PLAN	
Introduction of D → R mutation at amino acid 368 on gp120	
1 st round PCR	
CONSTRUCT A	JRFL_D368R_F + PlecR
CONSTRUCT B	JRFL_D368R_R + PlecF
2 nd round PCR	
Mixing Construct A and B with PlecF and PlecR as PCR primers	

2.3.2.2 Env Protein expression and purification

Expression and purification of the His₆Tag-Env proteins were as described in section 2.2.4.

2.3.2.3 SDS-PAGE and Western Blot Analysis of Env proteins

Detection of Env protein was achieved using SDS-PAGE and Western blot as described in the general methods (see section 2.2.7 & 2.2.10). Western blot was performed using a cocktail of anti-Env antibodies (mAbs; HIVIG & 447-52D), and detected with a goat anti-mouse HRP-conjugated antibody.

2.3.2.4 Biotinylation of D368R-gp120 proteins

D368R-gp120 Env proteins were biotinylated using the biotin ligase *BirA* (Avidity, LLC) as per the manufacturer's protocol, and all biotinylated proteins were purified using size exclusion chromatography as described in section 2.2.4. Purified biotinylated proteins were identified by Western blot using ExtrAvidin-HRP as described in 2.2.10.

2.3.2.5 Expression and Purification of Streptavidin

The streptavidin is modified to include a free cysteine at the C-terminus and the design was similar to the one described previously (340). A his₆tag was added immediate after the cysteine so to allow later purification by IMAC. The pETT22b+ plasmid (Novagen) containing the streptavidin insert was

transformed into DH5 α Library Efficient *E.coli* (Invitrogen), and expressed as inclusion bodies. Solubilised streptavidin was refolded in refolding buffer (1M Arginine, 10mM DTT, pH7.0). Refolded streptavidin was buffer-exchanged into endotoxin-free PBS before being purified by cobalt-based IMAC followed by size exclusion chromatography as described in section 2.2.4.

2.3.2.6 Auristatin cytotoxicity

72-hour drug exposure assay

The cytotoxicity of auristatin was evaluated in 2 human cell lines – 293T cells and Sup-B8 cells. 293T cells were grown in DMEM (Sigma Aldrich), whereas Sup-B8 cells were grown in RPMI-1640 (Sigma Aldrich). All media were supplemented with 10% FCS and 1% PS. 293T cells were seeded on 24-well plates at a density of 5×10^4 cells/well, and Sup-B8 cells were seeded on 24-well plates at a density of 1×10^5 cells/well. After 24 hours, the culture media were replaced by media (10% FCS, 1% PS) containing various concentrations of auristatin (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1 μ M and 10 μ M). After 72 hours of incubation, the number of viable and dead cells in each well was analysed by Trypan blue staining (Sigma Aldrich). Since the auristatin was dissolved in 20% DMSO (Sigma Aldrich), potential inhibitory effect of DMSO was also assessed separately by adding the equivalent amount of DMSO into each well. All experiments were conducted in duplicates. The half maximal inhibitory concentration (IC₅₀) was determined in Graphpad PRISM by fitting the data into a dose-response curve.

Auristatin Removal assay

The recovery from cell cycle arrest upon auristatin removal was also evaluated in 293T and Sup-B8 cells. Again, 293T cells were seeded on 24-well plates at a density of 5×10^4 cells/well, and Sup-B8 cells were seeded at a density of 1×10^5 cells/well. In the ‘**removal**’ group, cells were grown in media (10% FCS, 1% PS) that contained different concentrations of auristatin (1nM, 10nM, 100nM) for 8 hours, before replaced with normal media and cells were allowed to grow for another 72 hours. In the ‘**continuous**’ group, cells were continuously exposed to auristatin over the course of the experiment (total incubation of 80 hours).

The number of viable and dead cells was analysed at various time points (t = -8, 0, 24, 48, 72 hours, where auristatin is removed at t = 0) by Trypan blue staining. All experiments were performed in duplicates, and statistical analysis was performed using GraphPad Prism.

2.3.2.7 Conjugation of auristatin to the streptavidin

The cysteine-containing streptavidin (~1mg/ml; cys-strept) in endotoxin PBS was treated with 10mM DTT at 37°C for 30mins before buffer exchanged into endotoxin PBS by gel filtration. At 4°C, the purified cys-strept was added to maleimide-containing auristatin at a molar ratio of 1:10 (2.5 auristatin equivalent/SH group). After 1 hour, the reaction was quenched with excess cysteine (Sigma Aldrich) and gel purified as stated in section 2.2.4.

Approach [2] - Focusing the antibody response to the MPER by gp41-VLP

2.3.2.7 Production of Env plasmids

All gp41 proteins were derived from the wildtype subtype B JRFL strain (note: not codon-optimised version as used in 2.3.2.1). Overlapping PCR by KOD DNA polymerase (Novagen) was used to construct various gp41 constructs (see table on the next page). All gp41 PCR products were then gel purified and cloned into *PLEC* as described in section 2.2.3. All plasmids were checked with EcoRI and XhoI digest or PCR, and later verified by Sanger sequencing using the sequencing primers *PlecF* and *PlecR*.

Primers (5' → 3')	
Truncated gp41 proteins	
41FL_F	GGA CAG AAT TCC ATG GCA GTG GGA ATA GGA GCT GTG TTC CTT GGG
41FL_R	CCG CTC GAG CTA GTG ATG ATG GTG ATG GTG AAC TCT ATT CAC TAT
41_GSSGG_F	TGC GGT TCT TCT GGT GGT TGC ACC ACT GCT GTG CCT TGG AAT GCT AGT TGG AGT
41_GSSGG_R	GCA ACC ACC AGA AGA ACC GCA ACC CCA AAT CCC CAG GAG CTG TTG ATC CCC
41Q_F	GGT TCT TCT GGT GGT TGC ACC CAA CAA GAA AAG AAT GAA CAA GAA TTA TTG
41Q_R	GGT GCA ACC ACC AGA AGA ACC GCA ACC ATT GTT CTG CTG TTG CAC
41NQ_F	TGC GGT TCT TCT GGT GGT TGC ACC GAA TTA TTG GAA TTA GAT AAA TGG GCA
41NQ_R	ACC ACC AGA AGA ACC GCA ACC ACC AGA CAA TAA TAG TCT GGC CTG TAC CGT
41Δ2t_F	TGC GGA TCC TCT GGT GGC TGC ACC AGT TGG AGT AAT AAA TCT CTG
41Δ2t_R	GCA GCC ACC AGA GGA TCC GCA ACC ATC CCC TAG GTA TCT TTC CAC

41Δ4t_F	TGC GGA TCC TCT GGT GGC TGC ACC GAT AGG ATT TGG AAT AAC ATG
41Δ4t_R	GCA GCC ACC AGA GGA TCC GCA ACC AGC CAG GAC TCT TGC CTG GAG
41Δ6t_F	TGC GGA TCC TCT GGT GGC TGC ACC ACC TGG ATG GAG TGG GAA AGA
41Δ6t_R	GCA GCC ACC AGA GGA TCC GCA ACC ATG CTT GAT GCC CCA GAC TGT
41Δ8t_F	TGC GGA TCC TCT GGT GGC TGC ACC GAA ATT GAC AAT TAC ACA AGC
41Δ8t_R	GCA GCC ACC AGA GGA TCC GCA ACC GAG TTG CAA CAT ACG CTG TTG
Sequencing Primer	
PlecF	CGT GCT GGT TAT TGT GCT
PlecR	GGT ATT TGT GAG CCA GGG

PCR PLAN [1]	
Replacement of GSSGG loop for the immunodominant loop on gp41	
1st round PCR	
CONSTRUCT A	<i>41FL_F + 41_GSSGG_R</i>
CONSTRUCT B	<i>41FL_R + 41_GSSGG_F</i>
2nd round PCR	
Mixing Construct A and B with 41FL_F and 41FL_R as PCR primers	

PCR PLAN [2]	
Truncation of gp41 proteins (using the final product from PCR [1] as template)	
1st round PCR	
CONSTRUCT A	<i>Respective F primer + 41FL_R</i>
CONSTRUCT B	<i>Respective R primer + 41FL_F</i>
2nd round PCR	
Mixing Construct A and B with 41FL_F and 41FL_R as PCR primers	

2.3.2.8 Production of VLPs

VLPs were produced by co-transfection of 293T cells with the corresponding Env plasmids, and all VLPs were purified as described in section 2.2.5. A pCAGGS-based Env plasmid expressing the full length gp160 lacking the cytoplasmic domain (gp160ΔCT*) was used as the positive control.

* Kindly provided by Dr. James Binley, Torres Pines Institute for Molecular Studies

2.3.2.9 Western blot detection of gp41-VLPs

All VLP samples were lysed in solubilisation buffer (0.12% Triton X-100 in 1 mM EDTA, 1.5M aminocaproic acid in PBS with a protease inhibitor P-2714 (Sigma Aldrich)) overnight at 4°C, followed

by SDS-PAGE and Western blot analysis as described in section 2.2.7 & 2.2.10 respectively. For the detection of Env proteins, human MPER-specific BNABs (4E10 or 2F5 alone) were used and detected with goat anti-human HRP-conjugated antibody. For the confirmation of VLP production, anti-HIV p24 antibody was used followed by detection with goat anti-mouse HRP-conjugated antibody.

2.3.2.10 Flow Cytometry

293T cells transfected with respective gp41 constructs were detached from the flask and into suspension by agitation with PBS. 1×10^6 cells per sample were washed with FACS wash (PBS, 2% FCS) at 1,500rpm for 5mins. Afterwards, 250 μ l of primary antibodies (either 4E10 or 2F5 at 1 μ g/ml) were added to the sample and incubated at 4°C for 1 hour. Another 1ml of FACS wash was added to each sample before spinning at 1,500rpm for 5mins. This procedure was repeated once. Cells were then stained with goat anti-human IgG (Fc-specific)-FITC for 1 hour under foil at 4°C. Finally, cells were washed twice with FACS wash at 1,500rpm for 5mins, and were subsequently fixed in 250 μ l of Fixing solution (FACS wash with 1% Paraformaldehyde). All experiments were carried out in triplicates. Data analysis was performed using FlowJo (version 9.4.1; TreeStar): cells were gated on singlets and analysed based on their FITC expression. Statistical calculations were performed in Graphpad PRISM.

2.3.3 Chapter 5 - Dampening of immune response towards immunodominant regions of the HIV-1 Env protein by ‘Antibody-shielding’

2.3.3.1 Crosslinking of proteins

As described in previous studies (99, 121), crosslinking of proteins was achieved by the addition of 0.5mM of Bis(sulphosuccinimidyl) suberate (BS³; Pierce) for 1 hour at room temperature and at 4°C overnight. The reaction was quenched with the addition of Tris-HCL pH 8.0 to a final concentration of 50mM at room temperature for 30mins.

2.3.3.2 Production and purification of the CD4-gp120 complex

Crosslinked gp120-CD4 complexes were generated by incubating sCD4* and JRFL gp120 at a molar ratio of 2:1 at 37°C before BS³ crosslinking as described above. Crosslinked proteins were purified from the reaction mixture by size exclusion chromatography as described in section 2.2.4, and purified complexes were examined by SDS-PAGE under reducing condition to verify success crosslinking. Validation of the final product was achieved by Western blot probing with a cocktail of anti-HIV antibodies (447-52D and HIVIG) and rabbit anti-CD4 antibody.

* Kindly provided by Ms. Mai Vuong (Simon Davis' group, Oxford University).

2.3.3.3 Synthesis of mutated JRFL gp120 Env plasmids

Using the codon-optimised JRFL gp120 plasmid as a template, different versions of the mutated JRFL gp120 proteins were synthesised by overlapping PCR using PRIMESTAR Max DNA polymerase. These included: (i) the CD4bs-defective gp120 (D368R-gp120), (ii) the V1/V2-loop deleted gp120 (Δ V1/V2) and (iii) the V3-loop deleted gp120 (Δ V3). The synthesis of the D368R-gp120 was as described in Chapter 4 (see section 2.3.2.1), while the design of the variable loop-deleted gp120 proteins was based on previous studies in which the V1/V2 and V3 loops were substituted to the DAG and GAG motif respectively (43, 245, 359). The primers used were listed in the table on the next page, and the cloning, expression of purification of these proteins were as described in section 2.2.3 and 2.2.4.

Primers (5' → 3')	
Variable loops mutation	
J_ΔV1V2_DAG_F	CCT GAA GCC CTG TGT GAA GCT GAC CCC CCT GTG TGT GGA CGC CGG AAG CTG TGA CAC CTC TGT GA
J_ΔV1V2_DAG_R	GGG GCA GGC TTG GGT GAT CAC AGA GGT GTC ACA GCT TCC GGC GTC CAC ACA CAG GGG GGT CAG
J_ΔV3_GAG_F	TGC ACC GGC GCT GGA CAC TGC AAC ATC AGC AGG GCC AAG
J_ΔV3_GAG_R	GCA GTG TCC AGC GCC GGT GCA GTT GAT CTC CAC AGA CTC CTT
Sequencing Primer	
PlecF	CGT GCT GGT TAT TGT GCT
PlecR	GGT ATT TGT GAG CCA GGG

PCR PLAN	
Deletion of variable loops on gp120	
1 st round PCR	
CONSTRUCT A	Respective F primer + PlecR
CONSTRUCT B	Respective R primer + PlecF

2 nd round PCR
Mixing Construct A and B with <i>PlecF</i> and <i>PlecR</i> as PCR primers

2.3.3.4 *Measuring of antibody binding to the CD4-gp120 complex and Ab-gp120 proteins*

The binding of various anti-HIV antibodies to the CD4-gp120 complex and Ab-gp120 proteins was measured by an ELISA-based approach (see section 2.2.11). Plates were coated with either CD4-gp120 complex or Ab-gp120 proteins in PBS overnight and each mAb (primary antibody) was added at a saturating concentration for 2 hours at room temperature, followed by detection with goat anti-human HRP-conjugated antibody. All OD₄₅₀ readings were normalised against the reading of wildtype gp120.

2.3.3.5 *First-round and Second-round mouse immunisations*

(i) *First-round Immunisation (Generation of shielding antibodies)*

A group of 10 female balb/c mice (6–8 weeks of age) were intramuscularly primed with 100µg DNA at week 0, followed by 2 protein-boosts (10µg CD4-gp120 complex) injected subcutaneously at the neck region at week 3 and 6. All mice were pre-bled 1-week prior to DNA inoculation (week -1), and 7-10 days after each inoculation. Mice were sacrificed and terminally bled 10 days after the final immunisation. All immunogens were co-administrated with Ribi adjuvant (Corixa) for the boosting of antibody response.

(ii) *Second-round Immunisation (Assessing the immunogenicity of Ab-gp120 proteins)*

3 groups of female balb/c mice, each group consisting of 5 animals (6-8 weeks of age), were administrated with 10µg (Env-equivalent) protein immunogens subcutaneously at the neck region at week 0 and received 2 further boosts with the same immunogen at week 3 and 6 (see table below). All animals were pre-bled at week -1, and bled at 7-10 days after each inoculation. Mice were sacrificed and terminally bled 10 days after the final immunisation. All immunogens were co-administrated with Ribi adjuvant (Corixa).

Group	Immunogen
1	Native, unconjugated gp120 (control)
2	Uncrosslinked, antibody-conjugated gp120 (gp120 _{UNC-S})
3	Crosslinked, antibody-conjugated gp120 (gp120 _{C-S})

2.3.3.6 Serological Analysis of immune sera

ELISA plates were coated with either the CD4-gp120 complex, monomeric JRFL gp120 or sCD4 in PBS (see section 2.2.11). Serum samples were serially diluted in sample buffer and transferred to the coated plate for 2 hours with gentle shaking. Goat anti-mouse HRP-conjugated antibody was used for detection. All samples were performed in triplicates. Antigen-specific antibody responses were reported as reciprocal endpoint titres, i.e. the highest serum dilution giving an absorbance greater than the cut-off threshold (mean of background reading plus 2 standard deviation points). This was calculated by fitting a sigmoidal dose-response (variable loop) curve to the background-subtracted data using GraphPad PRISM.

2.3.3.7 Protein G purification of mouse IgG

For the purification of mouse IgG antibodies, sera samples were diluted at least 1:10 with endotoxin-free PBS before applying to the protein G column and allowed to flow by gravity. The column was then washed with 5-column volumes of endotoxin-free PBS. IgGs were eluted with 3-column volumes of elution buffer (Glycine, pH 2.0) and was immediately adjusted to pH 7.4 by the addition of an equal volume of 1M Tris buffer (pH 7.4). All eluted IgG antibodies were then purified by size exclusion chromatography on HiLoad 16/60 Superdex-200 column that has been pre-equilibrated with endotoxin-free PBS. Fractions containing the monomeric IgGs were identified by SDS-PAGE/Western blot analysis and combined.

2.3.3.8 Antibody-blocking ELISA

Antibody-blocking ELISA was used to characterise the shielding antibodies obtained from the first round immunisation. This ELISA-based assay (see section 2.2.11) was similar to the one described previously (369). Plates were coated overnight at 4°C with monomeric JRFL gp120. Purified IgG antibodies were added at an Env:Ab ratio of 1:16 for 1 hour, washed 3 times before the addition of anti-HIV mAbs. Goat anti-human HRP-conjugated antibody was used for detection. In principle, the anti-human detection antibody should not react with the mouse IgG antibodies, but as a precaution, potential cross-reactivity between mouse IgG antibodies and the anti-human detection antibody was also measured by detecting the

OD₄₅₀ in the absence of any competing proteins/antibodies. Any cross-reactivity was calculated by subtracting the OD₄₅₀ value with the background and under the criteria that all values must be greater than 0 (negative values may be obtained when no cross-reactivity was detected). The resulting OD₄₅₀ value was designated as *xreactOD*₄₅₀. The final OD₄₅₀ was obtained by subtracting the sample OD₄₅₀ with the background and *xreactOD*₄₅₀. This was then normalised with the OD₄₅₀ of the unshielded gp120 control. All experiments were performed in triplicates.

2.3.3.9 Surface Plasmon Resonance

Surface plasmon resonance (SPR) was used to assess the binding of sCD4 and b12 after the coating of gp120 with the purified IgG. Biosensor data were collected on a BIACORE 3000 optical biosensor (Biacore AB). 1000 response units (RU) of biotinylated monomeric JRFL gp120 was immobilised onto separate flow cells within the same streptavidin-coated sensor chip (GE Healthcare). For the coating of gp120, the IgG purified from the mice sera and anti-JRFL gp120 IgG were injected into separate flow cells (Fc 1 and 2 respectively) at a concentration of 1mg/ml, and at a flow rate of 1 µl/min. Once the coating of gp120 with both types of IgG plateaued, sCD4 and b12 were injected to saturation at a flow rate of 10µl/min. The binding of both sCD4 and b12 was compared based on the difference in RU between groups.

2.3.3.10 Preparation of Ab-gp120 proteins

For the preparation of Ab-gp120 proteins, purified IgG antibodies were incubated with monomeric JRFL gp120 proteins overnight at 4°C before crosslinking with BS³ as described above (see section 2.3.3.1). The Ab-gp120 proteins were purified from the reaction mixture by size exclusion chromatography on HiLoad 16/60 Superdex-200 column that was pre-equilibrated with endotoxin-free PBS. Final purified products were examined by SDS-PAGE and Western blot probing with human anti-Env cocktail (HIVIG, 447-52D, b12) and anti-mouse HRP antibody.

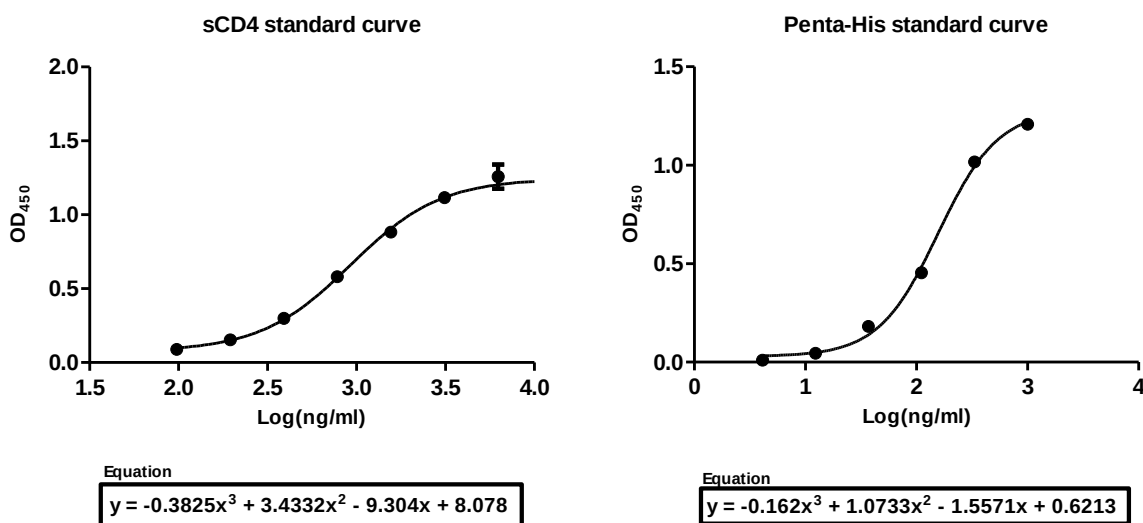
2.3.3.11 Determination of Env-content of CD4-gp120 complex and Ab-gp120

(i) CD4-gp120 complex

The Env content of the CD4-gp120 complex was determined by a 'Penta-His binding' standard curve which measures the binding of Penta-His antibody to the His₆tag on gp120, and compared to the binding of the same antibody towards a known amount of gp120 (on next page). The OD₄₅₀ readings were background-subtracted and a standard curve was generated by fitting a best-fit polynomial curve through all data points based on the sigmoidal dose-response (variable slope) model in Graphpad PRISM. The Env content of the Ab-gp120 was then determined by measuring the OD₄₅₀ of the sample and interpolated from the standard curve. All experiments were performed in triplicates.

(ii) Ab-gp120

In the case of shielded immunogens, the standard curve was based on the binding of sCD4 to the Env protein because the ELISA and SPR data demonstrated that the binding of sCD4 was not affected by the Ab-shield (see Chapter 5, section 5.2.5 & 5.2.6). Similar to the methods described above, a standard curve was created by measuring the binding of sCD4 towards a known amount of gp120.



2.3.3.12 Normalisation of molar concentration of various mutant gp120 for ELISA plate coating

Since the molar mass/molar concentration for each mutant gp120 protein was different, the molarity of all mutant gp120 proteins needed to be normalised prior to plate-coating so as to ensure that the final results of each assays were comparable. This was achieved by measuring the binding of Penta-His antibody towards these proteins based on the rationale that each gp120 protein can only bind to a single Penta-His antibody. Using an ELISA-based system (see section 2.2.11), plates were coated with either wildtype or mutant gp120 proteins and Penta-His antibody was added at 1µg/ml as the primary antibody. Goat anti-mouse HRP-conjugated antibody was used for detection, and the final OD₄₅₀ value for each mutant gp120 protein was normalised against the wildtype gp120. A ‘normalised’ stock for each mutant gp120 protein was prepared and the whole process was repeated until no significant difference between the OD₄₅₀ ($p < 0.01$) could be observed. The final ‘normalised’ stocks were then used for the future coating of ELISA plates. All measurements were performed in triplicates.

2.3.3.13 Epitope Mapping by ELISA-based assays

(i) Cross-competition ELISA

Serially-diluted mouse sera were pre-mixed with inhibiting human mAbs or biotinylated sCD4 (at a pre-determined K_D concentration) before they were added onto gp120-coated ELISA plates (see section 2.2.11). Anti-human HRP-conjugated antibody was used for the detection of human mAbs, whereas the ExtrAvidin-HRP was used for the detection of sCD4. The OD₄₅₀ obtained for these data were designated as *compOD*₄₅₀. Again, the cross-reactivity between mouse IgG antibodies and the detection antibodies was also measured as a precaution (see section 2.3.3.9) and the final cross-reactivity OD₄₅₀ was presented as *xreactOD*₄₅₀. For the processing of cross-competition data, the *compOD*₄₅₀ were subtracted with the background and *xreactOD*₄₅₀. Half-maximal inhibition titres (IC₅₀) were determined by fitting a three-parameter dose response curve to the data in GraphPad Prism under the restriction of limiting the ‘Top’ value to 1.0 and ‘Bottom’ value greater than 0. For statistical analysis, a One-Way Anova including a

Bonferoni post-test was performed on the logarithm of the IC₅₀. All experiments were performed in triplicates.

(ii) *Serum mapping by measuring the binding of mice sera towards different mutant gp120*

The binding of mice sera towards various mutant gp120 proteins was measured to determine the site-specific responses of the immune sera. The procedure of this ELISA was identical to the serological analysis described in 2.3.3.13 above *except* using the ‘normalised’ mutant gp120 proteins for coating. The measurement of EC₅₀, i.e. the effective serum dilution that leads to 50% binding, was determined by first transforming the serum dilution into log₁₀ scale, before fitting a sigmoidal dose-response (variable slope) curve through all data points in GraphPad PRISM. All experiments were performed in triplicates. The % of site-specific response was determined by comparing the EC₅₀ of wildtype and mutated gp120 proteins using the following equation: $(1 - \text{EC}_{50} \text{ of mutated gp120} / \text{EC}_{50} \text{ of wildtype gp120}) \times 100\%$.

2.3.3.14 Bandshifting BN-PAGE

Bandshifting BN-PAGE was used to analyse the binding of mouse IgG antibodies to gp120 proteins as previously described (85, 401). IgG antibodies were incubated with gp120 proteins overnight with gentle mixing. The crosslinking of gp120 and antibodies was as described in section 3.3.1.1, and the separation of proteins by BN-PAGE was as described in the general methods (see section 2.2.8).

2.3.4 Chapter 6 – Further optimisation of the antibody-shielding technique for the elicitation of a CD4bs-specific antibody response

2.3.4.1 Cloning, Expression and purification of P7A-gp120 proteins

The original P7A gp120 construct (with a C-terminus His₆Tag) was incorporated in a PTT-based vector (pOPINTTG). To sub-clone into *PLEC*, the P7A gp120 sequence was amplified by PCR so that the EcoRI and XhoI digestion sites could be introduced to the 5’ and 3’ end of gp120 *env* sequence respectively. The primers used for the amplification of P7A were listed in the table below. The subsequent cloning, expression and purification were as described in the general methods (see section 2.2.3 & 2.2.4) *except*

that the addition of an extra purification step using Concanavalina A Agarose (ConA; Vector Laboratories) prior to size exclusion chromatography. ConA acts by binding to the glycan shield of the Env protein. Briefly, the IMAC-purified Env proteins (in PBS) were passed through the ConA column via gravity. Afterwards, the column was washed with 3-column volumes of endotoxin-free PBS, followed by elution using 2-column volumes of 1M α -methyl mannoside (pH 4.0) (Sigma). The pH of the eluted fractions was immediately adjusted to roughly pH 7.4 by the addition of an equal volume of 1M Tris buffer (pH 7.4).

Primers (5' → 3')	
Cloning of P7A gp120 from pOPINTTG to PLEC	
TTG2Plec_F	GGC GAA TTC CTA GCG CCA CCA TGC CTG CGC TGC TCT CCC TCG TGA GCC
TTG2Plec_R	CGG CCA ACT CGA GTC AGT GGT GGT GGT GGT GGT GTT TGC CTT CGT GCC ATT CGA TTT TCT GAG CCT CGA AGA TGT CG

2.3.4.2 Synthesis of various mutant P7A gp120 proteins

The cloning of these mutant P7A gp120 proteins was achieved by overlapping PCR using PrimeSTAR Max DNA polymerase as described in Chapter 5 (see section 2.3.3.3) *except* using a different set of primers and the P7A gp120 as the template:

Primers (5' → 3')	
Variable loop deletion	
P7A_D368R_F	TTT GCT AAC GCC TCA GGA GGG CGA TTA GAA ATC ACA ACA CAT AGT
P7A_D368R_R	ACT ATG TGT TGT GAT TTC TAA TCG CCC TCC TGA GGC GTT AGC AAA
P7A_DV1V2_DAG_F	TGT AAT GGC GCT GGA AAT TGT AAT ACC TCA GCC ATT ACA CAG GCT TGT CCA AAG GTA
P7A_DV1V2_DAG_R	TGA GGT ATT ACA ATT TCC AGC GCC ATT ACA ATC TAA AGT AAC GCA AAG AGG GGT TAA CTT TAC ACA
P7A_DV3_GAG_F	TGT CTC GGC GCT GGA CAT TGT AAT ATT AGT AAA ACA GAA TGG AAT AAC ACT TTG AAA
P7A_DV3_GAG_R	ACT AAT ATT ACA ATG TCC AGC GCC GAG ACA AGT AAT GCT TAC AGG CTT GGC AAG TTG TAC TAG TAT

2.3.4.3 Antigenicity and Immunogenicity of the wildtype P7A gp120

The antigenicity of the P7A gp120 was examined by antibody-binding ELISA. Plates were coated with P7A gp120 and various human anti-HIV mAbs (primary antibody) were serially-diluted and added to P7A gp120-coated plates for 2 hours at room temperature, followed by detection with goat anti-human HRP-conjugated antibody. As a control, the binding of these antibodies towards clade B JRFL gp120 was also determined using the same protocol *except* that JRFL gp120 was used for coating. The immunogenicity of

the P7A gp120 was determined by measuring serum binding to various P7A gp120 mutants as described in section 2.3.3.13.

2.3.4.4 Normalisation of mutant gp120 proteins for ELISA plate coating

This method was performed as described in section 2.3.3.12.

2.3.4.5 Production of CD4-gp120 complex

This method was performed as described in section 2.3.3.2 except that the P7A gp120 was used rather than JRFL gp120.

2.3.4.6 Purification of IgG by Protein A agarose

IgG antibodies were purified from rabbit immune-sera using Protein A Agarose (Thermo Scientific) as described in section 2.3.3.7 except that Protein A binding buffer (Thermo Scientific) was used in place of the endotoxin-free PBS for the dilution of rabbit sera, and for the subsequent wash-steps. All purified IgG antibodies were buffer-exchanged to TBS (pH 7.4) using size exclusion chromatography that was pre-equilibrated the column with TBS.

2.3.4.7 Coupling of Env proteins to paramagnetic beads

Paramagnetic bead-immobilised CD4-gp120 complexes were used for the isolation of shielding antibodies. These were prepared by covalently coupling the CD4-gp120 complex (**biotinylated through sCD4**) to the Dynabead M-280 Streptavidin magnetic beads (Invitrogen) as per the manufacturer's protocol. Briefly, the beads (40mg/ml) and the biotinylated gp120 proteins (10mg/ml), both in endotoxin-free PBS, were mixed and incubated for 1 hour at room temperature with gentle rotation. Generally, 10mg of beads were used for every 1mg of biotinylated complexes to ensure maximum protein absorption. The beads were then separated by magnet and centrifugation, and were washed 3 times with PBS containing 0.1% BSA, followed by 3 more washes with TBS (pH 7.4) to ensure that all phosphate-based buffers were completely removed as the presence of phosphates will interfere with the downstream purification of shielding antibodies using the Gentle Ag/Ab elution buffer (Pierce). The integrity of the bead-coupled Env protein

was confirmed by using for the absorption of anti-CD4, 3074 and HIVIG antibodies, and the bound-antibodies were eluted in acidic condition as previously described (295, 314) and tested in ELISA.

2.3.4.8 Immunoprecipitation of shielding antibodies

Purified IgG antibodies in TBS (10mg/ml) were incubated with the paramagnetic bead-immobilised CD4-gp120 complex at room temperature for 2 hours with gentle rotation, and were then separated by magnets and centrifugation. The supernatant, i.e. unbound pool of IgG antibodies, was removed to another falcon tube for further adsorption. The beads were then washed 3 times with TBS before elution with Gentle Ag/Ab Elution Buffer (Pierce), which is a high-salt solution optimised for the elution of antibodies in near-neutral (pH 6.55) condition. The elution was carried out 3 times to ensure that as many antibodies were eluted as possible. All 3 fractions were combined, buffer-exchanged to TBS, and the protein concentration was determined by OD₂₈₀. The beads were then washed 5 times with TBS and this whole procedure was repeated with the beads for further adsorption of any antigen-specific antibodies that were unbound in the previous round. In situations where no antibodies were eluted from the beads (background OD₂₈₀), an extra round of elution was carried out by exposing the beads to increasingly acidic conditions as previously described (295, 314). This was to ensure the full recovery of any antibodies that could not be eluted by the high salt solution. Briefly, the beads were first incubated in 100mM glycine-HCl elution buffer (pH 2.7) for 30s with vortexing, and separated by magnets and centrifugation. The eluted IgG antibodies were removed and placed in another tube where the pH was adjusted to roughly 7.4 with 1M Tris buffer (pH 8.0). The beads were then exposed to glycine-HCl buffer at pH 2.0, followed by pH 1.7 subsequently, and all 3 elutions were buffer-exchanged to TBS and combined with other eluted IgG antibodies. All beads were discarded and freshly-prepared CD4-gp120-immobilised beads were used for the new rounds of purification. The antibody pool was defined as exhaustively absorbed once antigen-specific antibodies were no longer eluted from the fresh beads. All eluted IgG antibodies were further purified by size exclusion chromatography with HiLoad 16/60 superdex-200 column that was pre-equilibrated with endotoxin-free PBS.

2.3.4.9 Digestion of IgG antibodies to Fab fragments by papain

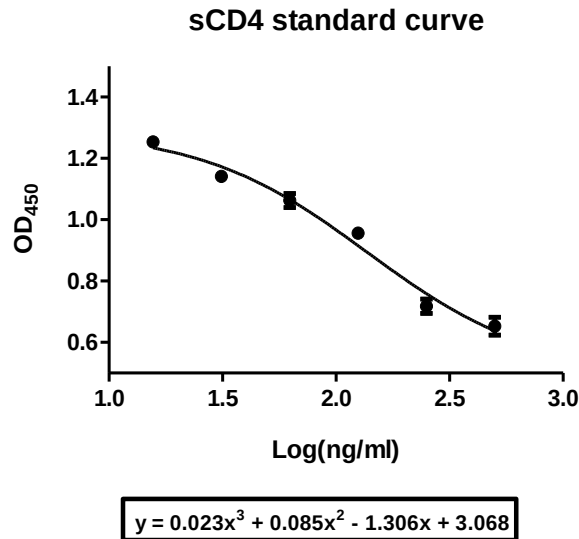
Concentration of purified IgG antibodies in sample buffer were adjusted to roughly 20mg/ml, and incubated overnight with Immobilised Papain (Thermo Scientific) following the manufacturer's protocol. For the purification of the Fab fragments (Fabs), the reaction mixture was passed through a Protein A column as described above since Protein A only binds to the Fc domain but not to the Fabs, therefore leaving the Fabs in the flowthroughs (see section 2.3.4.6). All the fractions (flowthroughs, washes, and elutions) were analysed using reducing and non-reducing SDS-PAGE, and were stained with Coomassie Blue as described in section 2.2.7 and 2.2.9. Fractions containing the Fabs were combined and purified by size exclusion chromatography on HiLoad 16/60 Superdex-200 column that was pre-equilibrated with endotoxin-free PBS.

2.3.4.10 Preparation of Ab-gp120

Preparation of Ab-gp120 was similar to the methods described in section 2.3.3.10 *except* using Fabs instead of IgG antibodies, and P7A gp120 instead of JRFL gp120.

2.3.4.11 Determination of Env-content in Ab-shielded immunogen

To ensure that all comparisons between wildtype gp120 and Ab-gp120 were valid, whether it be an *in vitro* experiment or the actual rabbit immunisation, the Env-content of the Ab-gp120 was determined based on their ability to bind to sCD4 as it was shown that the binding of sCD4 was not affected by the 'Ab-shield' (see Chapter 6, section 6.2.4). The determination of Env-content was achieved by plotting a 'sCD4-binding' standard curve as described in section 2.3.3.11.



2.3.4.12 Biotinylation of Rabbit Fab

For the biotinylation of the purified rabbit Fabs, the EZ-link NHS-Biotin kit (Pierce) was used as per the manufacturer's protocol. Briefly, the concentration of Fabs was adjusted to 10mg/ml and biotin was added at 10-fold molar excess based on the calculation provided by the manufacturer. The reaction was incubated at 4°C overnight and the biotinylated-Fabs were purified from unliganded biotin by size exclusion chromatography using the HiLoad 16/60 Superdex-200 column that was pre-equilibrated in endotoxin-free PBS. Successful biotinylation was confirmed by Western blot probing with ExtrAvidin-HRP antibody as described in section 2.2.10.

2.3.4.13 Characterisation of the Ab-shielded gp120 by antibody-binding ELISA

To assess the antigenicity of the Ab-gp120, antibody-binding ELISA was performed where the binding of mAbs to the Ab-gp120 was measured as described in section 2.3.4.3 *except* that the ELISA plates were either coated with the wildtype P7A gp120 (positive control), mutated P7A gp120 or BSA (negative controls). As a precaution, potential cross-reactivity between rabbit Fabs and the anti-human detection antibody was also accounted for as described in section 2.3.3.8.

2.3.4.14 Rabbit Immunisation

Two groups of 4 female New Zealand White rabbits (1.0-1.5kg) were administrated with 30µg (Env-equivalent) of either wildtype P7A gp120 (*control; group 1*) or the Ab-gp120 (*shielded; group 2*) with Ribi Adjuvants (Corixa). The following immunisation routes and dosage were used: 0.2ml intramuscularly into each hind leg and 1.0ml subcutaneously around the neck region. All animals were pre-bled and received the first inoculation at week 0, and 2 more protein inoculations were given 4 weeks apart. All bleeds were collected 7-10 days after each injection and the terminal bleed was collected 10 days after the final immunisation.

2.3.4.15 Serological analysis of immune sera

The end-point titre of the antibody response elicited in each rabbit was determined as described in section 2.3.3.6 *except* that the ELISA Plates were coated overnight with P7A gp120 and detection was achieved by using goat anti-rabbit HRP-conjugated antibody.

2.3.4.16 Epitope Mapping of the serum antibody response

(i) Cross-competition ELISA and measuring the binding of rabbit sera towards mutant gp120

Both methods were performed as described in section 2.3.3.13 *except* that serially-diluted rabbit sera were used in place of mouse sera, and the use of mutant P7A gp120 proteins for plate coating.

(ii) Peptide ELISA

A set of 15 overlapping linear peptide corresponding to the V1/V2 and V3 loops of the P7A gp120 was used. These peptides were designed by using the PeptGen peptide generator available on the Los Alamos HIV-1 Database with the length restricted to 18-mers with an overlapping region of 10 amino acids (<http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html>). These peptides were kindly synthesised by Dr. Zhanru Yu (HIU facility) and were dissolved in DMSO (Sigma Aldrich). The protocol of this peptide ELISA is as previously described (369, 370). Briefly, ELISA plates were coated with 200µg/well of individual peptides in PBS overnight at 4°C. Plates were then washed before the addition of

rabbit sera (diluted 1/100 in dilution buffer) and left for incubation at room temperature for 2 hours. Detection was achieved by using goat anti-rabbit HRP-conjugated antibody and plates were read at OD₄₅₀. All readings were reported as background-subtracted OD₄₅₀, and presented as the average titre obtained from triplicate analyses.

Chapter 3

Associations between HIV-1 Env Traits and the generation of a Broadly-Neutralising antibody response

In this chapter, the bioinformatics analysis was the work of Dr. Joe Parker (Queen Mary, University of London). This includes the initial phylogenetic analysis (Fig. 3.3 and Table 3.1), the identification of positively-selected sites, the iterative model-testing for the construction of the final association model (Fig. 3.5 and Table 3.2). He also generated the raw data for the identification of signatures using Decision Tree making. The construction of env database, preliminary correlation study as well as the analysis of raw Decision Tree data were performed by myself.

3.1. Introduction

The elicitation of a broadly neutralising antibody response is not as rare as previously thought – it has been discovered that about 20% of HIV-infected individuals developed a broadly neutralising antibody response over time, and about 1% of these individuals elicited serum antibodies that can broadly and potently neutralise a diverse set of HIV-1 strains (361, 373, 379). However, no vaccine candidates have thus far been able to replicate such a response *in vivo* and it remains unclear how this could be elicited via vaccination. Since the sequence and conformation of the Env protein are both highly critical in shaping the antibody response, it was therefore hypothesised that there are common features of the Env proteins that are associated with the elicitation of a broadly neutralising response, and the design of vaccines that incorporate such naturally-occurring features – i.e. ‘signatures’ – may be beneficial as they may improve the immunogenicity of the Env protein.

In recent years, there has been a growing interest in employing computational analysis to look for such ‘signatures’ on the Env protein (141, 213, 227, 347). Although none of these studies have presented any

follow-up *in vitro* characterisations of these signatures, a study by Gnanakaren *et al* showed that the signatures predicted *in silico* did agree with the findings of other structural or mutational studies, thus validating the use of computation strategy for the prediction of neutralisation phenotypes (141). From these studies, different signatures in gp120 and gp41 were identified – some might be in direct interaction with antibodies, some might change the conformation of the Env protein and modulate antibody recognition through quaternary and allosteric effects, and others might affect glycan motifs that change the glycosylation status of the Env protein (141, 213, 227, 347). Comparing to common *in vitro* studies (e.g. crystallography and alanine scanning) which focus mainly on the direct antibody-Env interface, computational analysis could be a useful alternative as it also allows the identification of naturally-occurring features/mutations distant to the antibody binding site that may contribute to a broad neutralising activity. These findings may subsequently provide interesting signature patterns on the Env protein that can be considered for the design of an HIV-1 vaccine.

In this study, the identification of signatures was achieved by using Decision Tree, which was previously employed for the prediction of neutralisation phenotypes (141). Decision Tree is a form of machine learning which allows the identification of ‘features’ (i.e. neutralisation phenotype in the case of this study) in large datasets by passing the dataset through a series of ‘decision nodes’ until they can be clearly classified (136, 140). Figure 3.1 demonstrates a simple example of Decision Tree: the dataset is being passed through a series of ‘decision nodes’ where a specific question is asked, i.e. ‘decision-making’. By passing through each node, the dataset starts to divide into smaller and smaller subsets and this process is continued until a clear classification is reached. As a result, Decision Trees not only allow the classification of a large dataset, they also enable the identification of a series of events or conditions that lead up to the specific outcome. For the determination of neutralisation phenotypes, while the underlying principle of Decision Tree remains the same, the actual Decision Tree is more complex as *env* sequences are more diverse than the given example, meaning that more parameters or ‘decision nodes’ are required

for a clear classification of data. An example of a Decision Tree generated from the determination of neutralisation phenotypes of Env proteins is shown in Fig. 3.1.

Through collaboration with the International AIDS Vaccine Initiative (IAVI), I obtained 185 patient serum samples (93 clade B, 92 clade C) that were previously collected by Simek *et al* (373) where the PGT antibodies were isolated. As described in their publication, these patients were HIV-1 infected for at least 3 years, clinically asymptomatic and without evidence of progression to AIDS, and were not on ART for at least the previous 1 year. The breadth and potency of the serum antibody response was determined by a series of neutralisation assays and represented as a neutralisation score (0 – 2.83), where a score of 0 represents limited neutralising activity, and a score of 2.83 represents the most broad and potent neutralisation. Serum samples with a score higher than 2.5 were classified as elite neutralisers. It must be noted that only samples with a score of 0, 0.33, 0.5, 0.83, 1, 1.33, 1.5, 1.83, 2, 2.33, 2.5 and 2.83 were provided by IAVI for this study.

The primary objectives of this study include:

- 1) Construction of an *env* sequence database based on the IAVI samples by cloning and sequencing of the entire *env* region of the serum-isolated virus;
- 2) Identification of clade-specific signatures that are associated with broad and potent neutralisation phenotype by Decision Tree;
- 3) Characterisation of the identified signatures using existing structural and biochemical data to reveal their potential significance and contribution towards the broad neutralising phenotype.

3.2. Results

3.2.1 Generation and validation of the gp160 env sequence

* *Dr. Joe Parker was responsible for aligning all env sequences with HxB2 and obtaining the values of various global parameters of the Env protein*

A total of 1,168 clonal full length *env* nucleotide sequences were obtained from the plasma of 185 HIV-infected patients, among which 605 are classified as clade B and 563 as clade C. Based on the phylogenetic analysis of the full-length gp160 *env* sequences, 2 levels of clustering were observed: monophyletic clusters of sequences isolated from the same patient, and clustering of sequences of the same clade (Fig 3.2). All *env* sequences were subsequently translated into amino acid sequences and codon-aligned with the Los Alamos (LANL) reference strain HxB2, thereby allowing the identification of the conserved and variable domains of the Env protein. As expected from chronic HIV-1 infection, considerable variations were observed in the sequence and length of each variable loop, while limited variations were found in the conserved regions (Table 3.1). These sequences were also compared to all HIV-1 Env sequences in the LANL database for further validation of the obtained sequences (Fig. 3.3), and overall, no significant discrepancies between the 2 datasets were observed. Since the intrinsic diversity of Env protein in the 2 clades may potentially affect the identification of signatures, all analyses were performed in a clade-specific manner.

3.2.2 Preliminary study on the correlation of Env features with broad neutralisation

A preliminary study was carried out to identify any possible correlations of the Env features with broad and potent neutralisation. Several global parameters of the Env protein were selected for the analysis, including the length, net charge and number of PNLG sites of the entire Env gp160 protein, and of each variable loop. The final results were analysed with respect to the neutralisation score of each individual sequence (Fig 3.4). For the clade B dataset, a broad and potent neutralisation was found to correlate with a longer overall gp160 sequence as well as a longer V2 loop ($p < 0.001$). A similar phenomenon was also

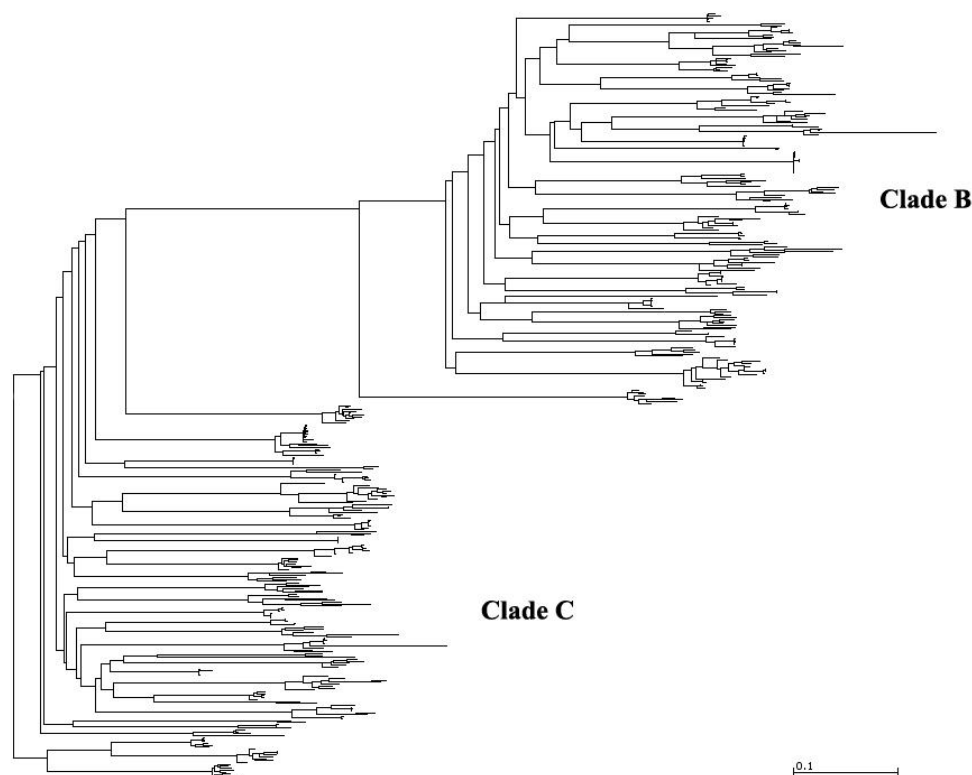


Figure 3.2 Maximum likelihood tree indicating the phylogenetic relationships of the gp160 sequences in the IAVI env database

Phylogenetic tree of the sequences obtained in this study revealed 2 levels of clustering - monophyletic clusters of sequences isolated from individual patients, and clustering of sequences of the same clade.

observed in the clade C dataset with a longer Env sequence ($p < 0.01$) and V1 loop length ($p < 0.05$) found to be correlated with a high neutralisation score.

3.2.3 The use of Decision Tree for signature identification

* This work was performed by Dr. Joe Parker

To identify signatures using Decision Tree, all sequences were grouped into 4 distinct categories based on their neutralisation score, and a total of 11 global and site-specific parameters were selected for the initial model building (Table 3.2). However, the number of parameters to be introduced into the prediction model must be controlled as over- or under-fitting of the model could influence the accuracy of the final prediction. If an excess of parameters were included, i.e. over-fitting bias, the prediction would become overly-strict and this could lead to the omission of relevant meaningful signatures. In contrast, if not

Overall		
Total number of Samples	185	
Total number of Sequences	1,168	
Neutralisation Score	0 (Limited breadth) – 2.83 (Broad neutralisation)	
Clade B	Clade C	
Distribution		
93	Total Samples	92
605	Number of Sequences	563
0 – 2.33	Neut. Score range	0 – 2.83
Characteristics		
857.83 ± 9.04	Overall Length	858.91 ± 13.76
30.35 ± 2.61	Total number of PNLG sites	30.15 ± 2.72
27.26 ± 3.62	Overall net charge	26.35 ± 4.11
Variable Loop 1		
29.64 ± 6.16	Length	28.58 ± 5.70
3.82 ± 1.27	PNLG sites	4.07 ± 1.08
-1.04 ± 1.43	Net Charge	-0.32 ± 1.24
Variable Loop 2		
43.53 ± 3.13	Length	41.51 ± 4.43
3.15 ± 0.75	PNLG sites	3.27 ± 0.92
0.85 ± 1.38	Net Charge	0.96 ± 1.66
Variable Loop 3		
34.72 ± 1.35	Length	34.84 ± 1.73
1.79 ± 0.46	PNLG sites	1.72 ± 0.48
4.94 ± 1.10	Net Charge	3.97 ± 0.90
Variable Loop 4		
31.94 ± 2.63	Length	28.32 ± 4.99
4.38 ± 0.84	PNLG sites	4.07 ± 1.06
-0.53±1.55	Net Charge	0.36 ± 1.60
Variable Loop 5		
15.23 ± 1.81	Length	15.26 ± 2.37
1.64 ± 0.66	PNLG sites	1.47 ± 0.74
-0.59 ± 0.87	Net Charge	-0.67 ± 1.00

Table 3.1 Summary of the IAVI Env sequence database

All the data were determined based on the protein sequence of the Env protein.

enough constraints were used, i.e. under-fitting bias, the prediction model would not be accurate enough in determining the actual neutralisation phenotype. To improve the precision of the model, the significance of each parameter was therefore tested iteratively. Sequences in each dataset were randomly divided into a training set (30% of all sequences) for the derivation of prediction model, and a test set (70% of all sequences) for the validation of the prediction model. A score (%) was generated for each parameter that represents its significance in predicting the neutralisation phenotype of the test set (Fig. 3.5).

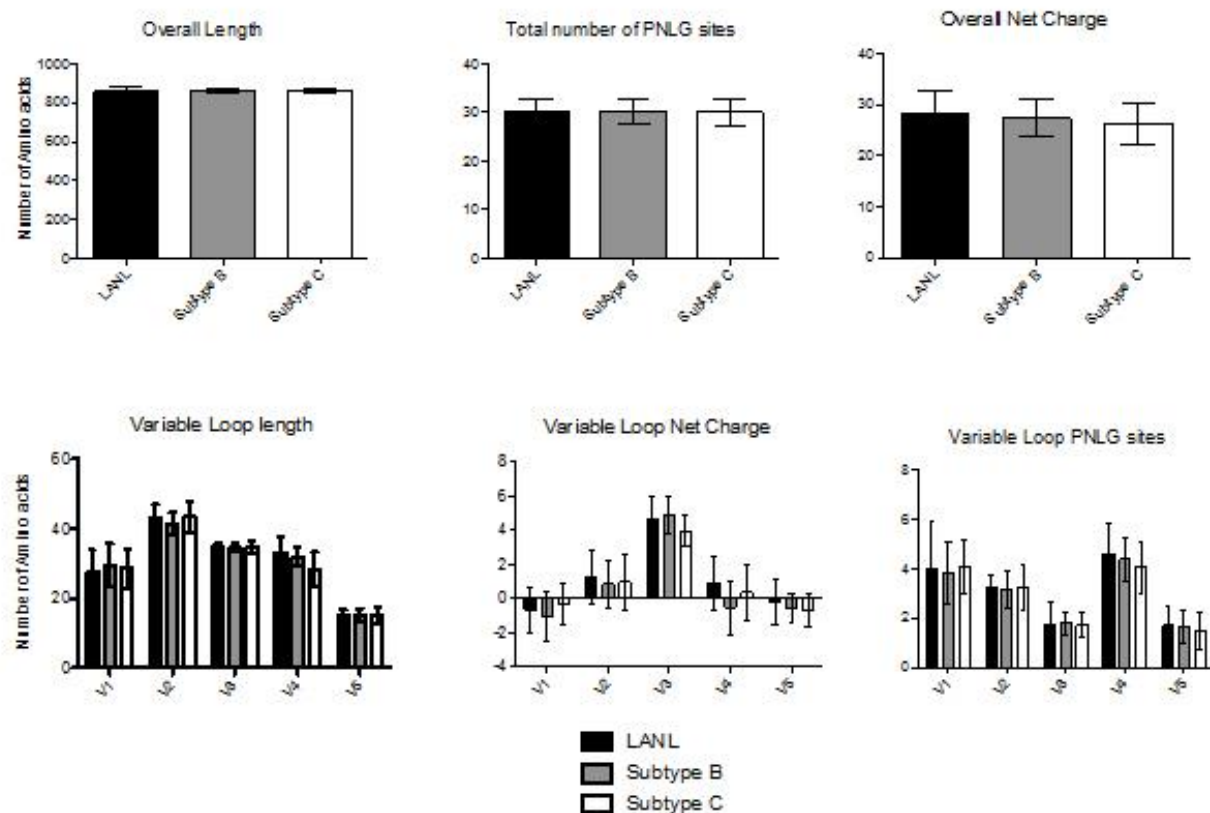


Figure 3.3 Validation of the Env protein sequence of the IAVI dataset

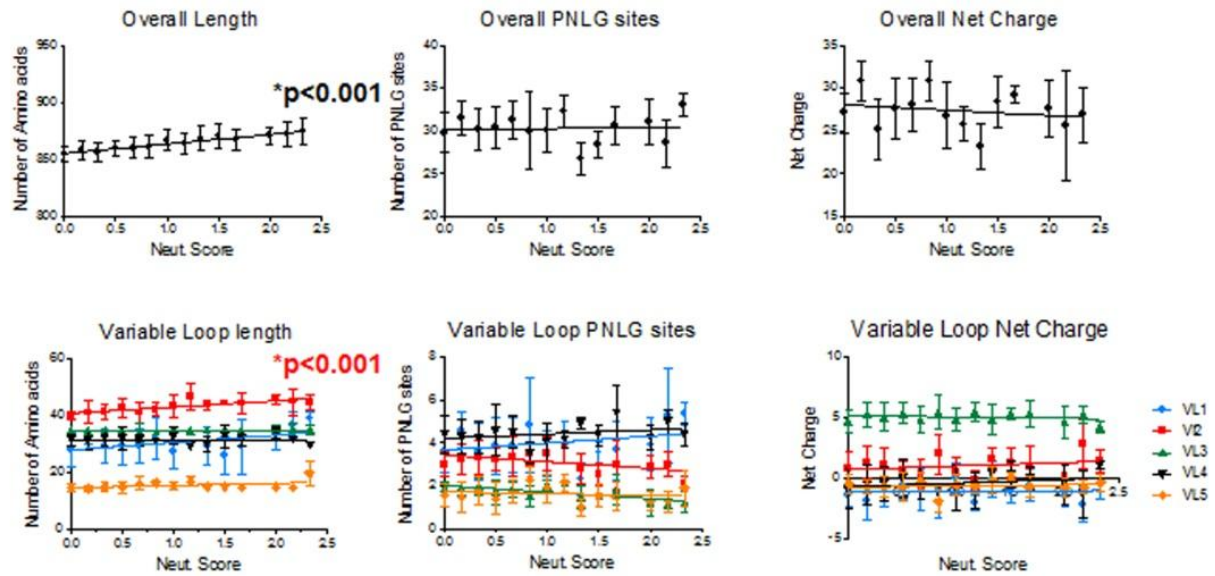
The Env sequences obtained in this study were validated by comparing to all the Env sequence available from the Los Alamos (LANL) database. Top panel displays attributes of the entire Env protein, including (left) overall length, (middle) total number of potential N-linked glycosylation (PNLG) sites and (right) overall net charge. Lower panel displays the attributes of each variable loops: (left) length, (middle) net charge and (right) number of PNLG sites. In all diagrams, the error bars indicate the standard deviation.

3.2.4 Determination of the final prediction model for Decision Tree

* This work was performed by Dr. Joe Parker

Based on the respective score, all parameters (except the positive-selected amino acid sites) was ranked based on their score. Interestingly, the top 4 parameters identified in both clades were the same, which included: the immunodominant region on gp41, the CD4 binding site, the length and net charge of each variable loop (Fig 3.5). This highlights the potential importance of these features in determining the broad neutralising phenotype. All parameters were then combined with the set of positively-selected sites for the construction of different prediction models. Each prediction model was tested iteratively based on the same algorithm as described above, and the predictive power (i.e. accuracy) of each particular model

Clade B



Clade C

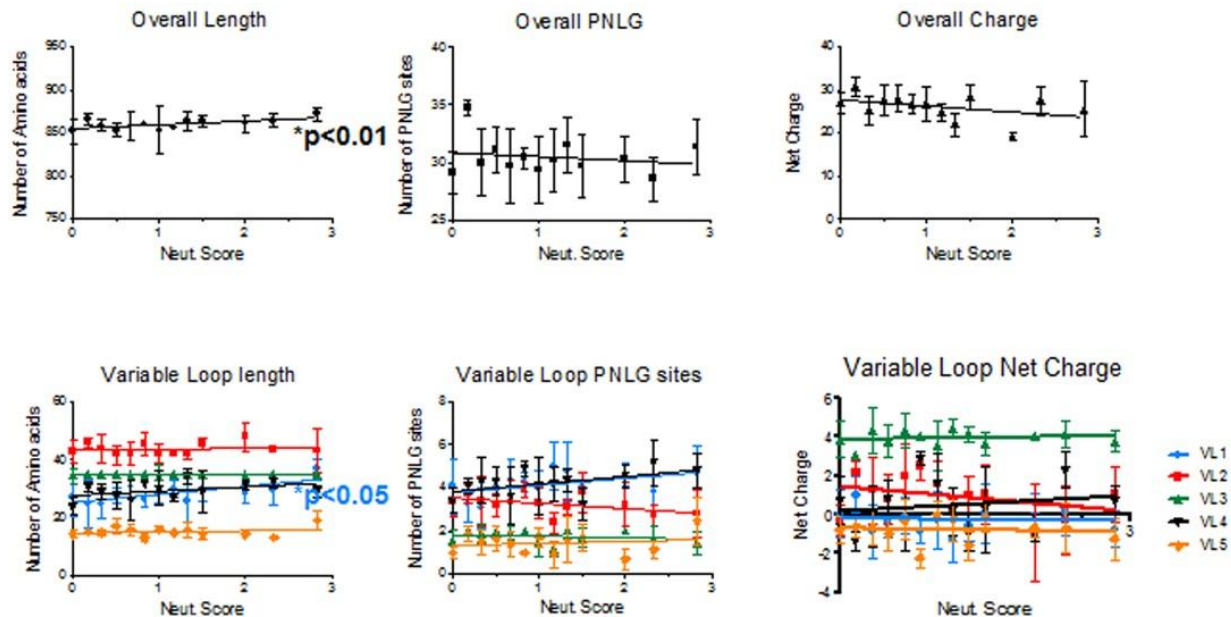


Figure 3.4 Preliminary analysis of the Env sequence

Shown here are the analyses of various characteristics of sequences with different neutralisation scores for each clade aiming to determine, if any, possible correlations with broad neutralisation. Attributes showing significant correlation were highlighted with an asterisk* [clade B – overall length & V2 length; clade C – overall length & V1 length]. All error bars indicate the standard deviation. Significant correlation was calculated by PRISM.

<u>Neutralisation scores</u> 0, 0.33, 0.5, 0.83, 1, 1.33, 1.5, 1.83, 2, 2.33, 2.5* and 2.83*			
Group	Neut. Score (S)	Group	Neut. Score (S)
1	S ≤ 0.83	3	1.5 < S ≤ 2.33
2	0.83 < S ≤ 1.5	4*	S ≥ 2.5

*** Samples with score greater than or equal to 2.5 are considered as Elite neutralisers (373)**

Global Parameters	Site-specific Parameters
Overall Length of gp160	CD4 binding site [HxB2 368, 380, 427, 430]
Length of each variable loops	Immunodominant region (IDR) [HxB2 588-607]
Overall Net charge of gp160	Tropism (CXCR4/CCR5)
Net Charge of each variable loops	Positively selected site (p<0.1)*
Total Number of PNLG sites	2F5 epitope [HxB2 position 662-667]
	4E10 epitope [HxB2 position 671-676]

**Not included in the parameter selection and iterative model testing*

Table 3.2 Grouping of sequences and parameters selected for Decision Tree analysis

(Top) All sequences were grouped in 4 distinct groups based on the neutralisation score provided by IAVI. (Bottom) The parameters that was included in the iterative model testing where the relative importance of each parameter was being evaluated.

was judged based on the final score. For both datasets, the same prediction model – **top 5 scoring parameters** combined with **the set of positively-selected sites** – was found to provide the best predictive power of the neutralising phenotypes (Fig. 3.5). This model was subsequently used for the identification of signatures that are associated with a broadly neutralising phenotype.

3.2.5 Terminology used for the interpretation of results in Decision Tree

Apart from the basic **score** (%), which represents the accuracy of the prediction model, several outputs can also be obtained from Decision Tree and are defined as follows:

- Individual signatures** – This consists of 2 elements: a *position numbering* indicating the location of the signature (HxB2 numbering), as well as an *amino acid code* which represents the amino acid that was found to be enriched in the Env sequence that display broad neutralising phenotype, e.g. the signature of L835 represents leucine at site 835;
- Combinations** – this refers to the number of different ‘routes’ that determine the same predicted outcome (i.e. broad neutralising phenotype). Consider the scenario where broad neutralising

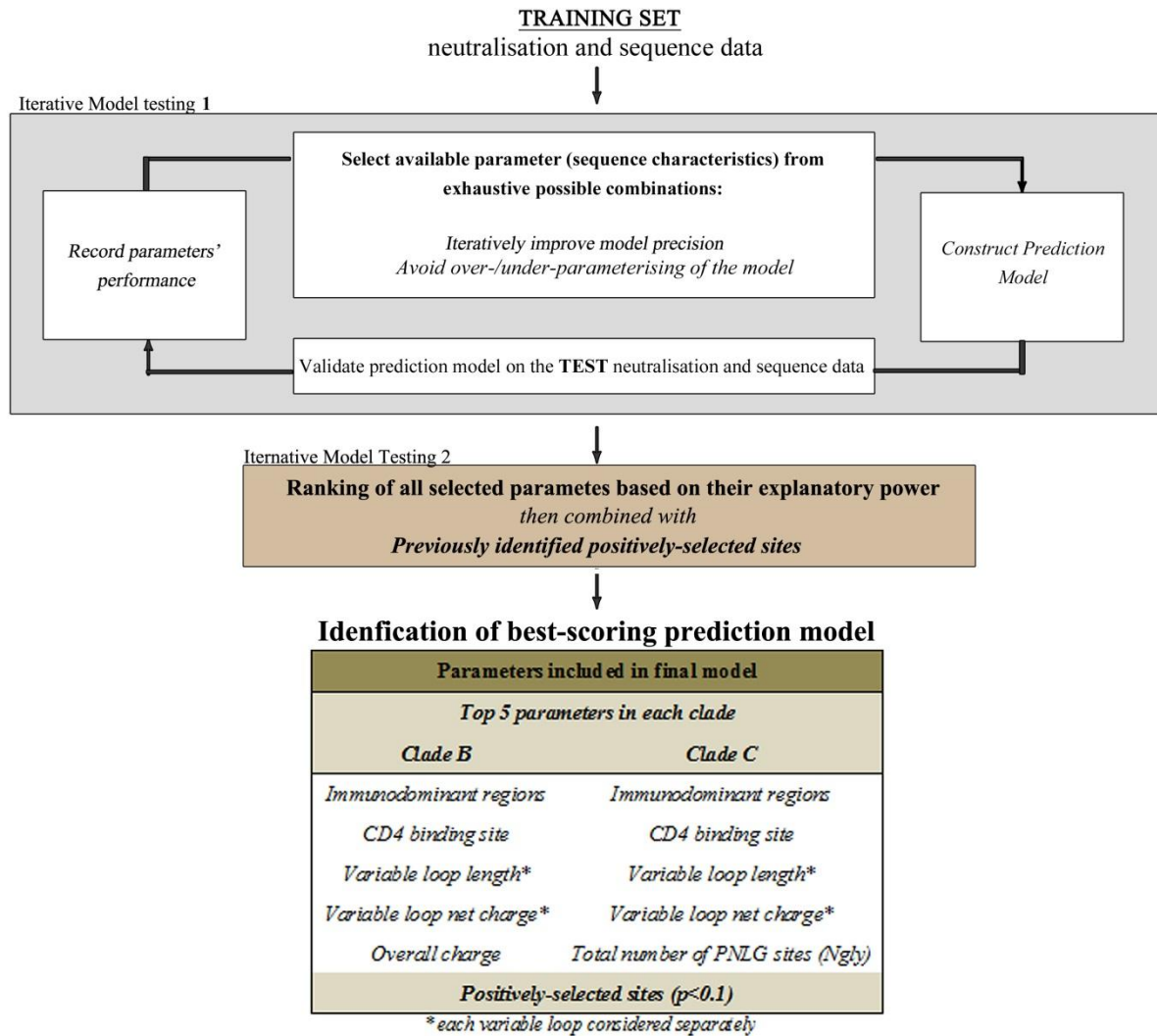


Figure 3.5 Systemic parameter selection, iterative model testing and the determination of the final prediction model

The relative importance of each parameter was tested iteratively and a score (%) was used to indicate the accuracy of each parameter in prediction of the neutralisation score. The final model used for the signature identification included the top 5 parameters plus the positively-selected sites ($p < 0.1$) for each clade.

phenotype can be determined by 2 sets of signatures – A101 + C102 or D201 + E202 – this will be described as 2 combinations and 4 signatures;

- (iii) **Contribution** – this is the % of sequences that are determined by a specific combination. For instance, if the combination of A101 and C102 is found in 30 out of the 50 possible broadly neutralising sequences, the contribution of this A101 + C102 combination is described as 60%.

3.2.6 Signatures identification by Decision Tree

* *The raw data (i.e. Decision Tree) of this section was obtained from Dr. Joe Parker.*

Using the prediction model described above, a final score of 88.9% and 92.1% was obtained for the clade B and C dataset respectively (Table 3.3; summarised in Table 3.4). For the clade B dataset, a total of 11 signatures (10 site-specific and 1 global feature) were identified over 3 combinations – including *T209*, *A275*, *M427*, *A430* and *E501* on gp120 and *A583*, *T589*, *T613*, *L815* and *L835* on gp41, together with a global feature that indicates the V2 loop length must be longer than 43 amino acids long. For the clade C dataset, 10 site-specific signatures were identified over 3 combinations – including *S275*, *P299*, *F317*, *I435* and *S443* in gp120 and *A596*, *L754*, *G798*, *A812* and *L835* in gp41. No global features were identified as signatures for this dataset. In both datasets, each of the 3 combinations contributed differently to the overall result, with one particular combination of signatures accounting for approximately 50% of all broad neutralising sequences (combination 2 in table 3.3). In addition, certain signature patterns were determined in more than 1 combination within each dataset (**bolded signatures**; table 3.3), and despite the vast diversity observed between clade B and C viruses, the signature L835 and 275 (serine or alanine) were identified in both datasets (**labelled in red**; table 3.3). Although the significance of these findings is yet to be determined, this still strongly suggests that these signatures may synergistically promote an Env conformation that facilitates the elicitation of a broadly neutralising response. The position of each signature was highlighted on the respective consensus sequence that was generated from all sequences in each clade (Clade B – Fig. 3.6; Clade C – Fig. 3.7).

3.2.7 Characterisation of signatures identified in each dataset

To obtain a better understanding of their biological significance and contribution towards a broad and potent neutralising phenotype, each signature was mapped onto: (i) the crystal structure of a V3-containing clade B JRFL gp120 core protein that was bound by CD4 and X5 (targeting CD4i epitopes) (PDB: 2B4C) (188), and (ii) a gp41 model due to a lack of representative crystal structures for gp41. Although it was possible to map the clade C signatures onto the recently determined structure of the clade

Clade B [score = 88.9%]		
Combinations	Signatures	Contribution*
1	A275 + E501 + L815 + L835	20.8%
2	Length V2 > 43 + T209 + A275 + T613 + L815 + L835	45.8%
3	T209 + M427 + A430 + A583 + T589	33.3%

Clade C [score = 92.1%]		
Combinations	Signatures	Contribution*
1	S275 + L754 + L835	12.5%
2	P299 + F317 + I435 + S443 + L754 + G798 + A812 + L835	66.7%
3	A596 + L754 + A812 + L835	20.8%

* Contribution = % number of sequences in the broad neut. group defined by this combination

Table 3.3 Combinations and signatures for each clade identified by Decision Tree

Signatures that appeared in more than one combination are shown in bold for clarification. Signatures that appeared in both clades are highlighted in red. The amino acid position identified for each signature is based on HxB2 numbering.

C gp120 core (CAP210), the location of the signatures did not show any significant differences as the root mean square deviation (RMSD) of the CD4-bound JRFL and CAP210 gp120 proteins showed that both structures are highly similar (102, 213). For this reason, the JRFL structure was used as it allows the mapping of signatures that are located within the V3 loop.

For the clade B dataset, the signature at position E501 could not be shown on the crystal structure due to a lack of structural information (188). All signatures in gp120 are located within the constant core of Env (Fig. 3.6 & 3.8), with several of these signatures situated in key functional areas of Env: (i) M427 and A430 are located within the CD4 binding site and are involved in the binding of CD4 and the broadly neutralising antibodies b12 and VRC01 (37, 241), (ii) M427 and E501 are located in gp120-gp41 interface and a mutation at site 501 has been associated with a complete dissociation of the Env protein (174). Although no clear role was suggested for the signatures of T209 and A275, their outward-facing position on gp120 reveals that they could be solvent-exposed and could therefore be involved in direct antibody binding. Of the 5 signatures within gp41, signatures A583, T589 and T613 are located in

the central region of the ectodomain (Fig. 3.6 & 3.8) – an area flanking and including the disulphide-bonded loop that was found to be crucial for gp120-gp41 interaction (195, 254, 448). Two additional signatures, L815 and L835, were situated in the intracellular cytoplasmic domain of gp41 which is inaccessible to antibodies.

Similarly for the clade C dataset, 3 out of 5 signatures (S275, I435 and S443) identified were located in the constant core region (Fig. 3.7 & 3.8). However, no specific information was available for these signatures: the externally-facing S275 and I435 could be involved in direct antibody binding, whereas the signature S443, which is in proximity to gp120-gp41 interface, may interfere with the conformation or stability of the Env protein (220). The remaining 2 gp120 signatures – P299 and F317 – were situated in the V3 loop, a region that is generally conserved in clade C viruses (Fig. 3.8) (134). In particular, the signature of F317 is located at the crown of the V3 loop and it forms the epitope for a number of anti-V3 broadly-reactive monoclonal antibodies (mAbs), including 3074 and HGN194 (22, 81, 391, 430). Furthermore, it is also directly involved in the binding site for the CXCR4 co-receptor (27). Again, the locations of gp41 signatures in this dataset are highly similar to the clade B dataset (Fig. 3.7 & 3.8): A596 is located at the central ectodomain of gp41, whereas 4 of the 5 signatures (L754, G798, A812 and L835) are located in the intracellular cytoplasmic domain of the protein.

Figure 3.6 – 3.8 on the following pages

	Env Signal Peptide <=> gp120 start						
B_CONSENSUS	MRVKGIRKNC	QHLWRWGIML	LGMLMICSAA	EKLWVTVYYG	VPVWKEATTT	LFCASDAKAY	
Signatures							
B_CONSENSUS	DTEVHNWAT	HACVPTDPNP	QEVVLENVTE	NFNMWKNNMV	EQMHEDIISL	WDQSLKPCVK	
Signatures							
	=> V1----- => V2-----						
B_CONSENSUS	LTPLCVTLNC	TDLNNNTNT	NIYSSRNKME	KGEIKNCSEFN	ITTSIRDQM	KEYALFYKLD	
Signatures							
	----- 209						
B_CONSENSUS	VVPIDNNNTS	YRLISCNTSV	ITQACPVSF	EPIPIHYCAP	AGFAILKCND	KKFNGTGPCK	
Signatures			T.....				
	275 => V3--						
B_CONSENSUS	NVSTVQCTHG	IRPVVSTQLL	LNGSLAEDEV	VIRSENFNTN	AKTIIVQLNE	SVEINCTRPN	
Signatures			A.....				

B_CONSENSUS	NNTRKSIHIG	PGRAFYATGE	IIGDIRQAH	CNISRTKWNNT	LKQIVKKLRE	QFGNKTIVFN	
Signatures							
	=> V4-----						
B_CONSENSUS	QSSGGDPEIV	MHSFNCGGEF	FYCNTTQLFN	STWNSNNIIT	NNNDENDTIT	LPCRIKQIIN	
Signatures							
	427 430 => V5-----						
B_CONSENSUS	MWQEVGKAMY	APPIRGQIRC	SSNITGLLLT	RDGGNNNNNT	ETFRPGGGDM	RDNWRSELYK	
Signatures	M...A.....						
	501 => gp41 start						
B_CONSENSUS	YKVVKIEPLG	VAPTKAKRRV	VQREKRAVGI	GAMFLGFLGA	AGSTMGAASM	TLTVQARQLL	
Signatures		E.....					
583							
B_CONSENSUS	SGIVQQQNNL	LRAIEAQOHL	LQLTVWGIKQ	LQARVLAVR	YKDDQQLLGI	WGCSGKLICT	
Signatures				A.....	T.....		
	613						
B_CONSENSUS	TAVPWLASWS	NKSLDEIWDN	MTWMQWEREI	DNYTGIIYTL	IEESQNQQEK	NEQELLELDK	
Signatures	T.....						
	=> TM domain => CT domain						
B_CONSENSUS	WASLWNWFDI	TNWLWYIKIF	IMIVGGLIGL	RIVFAVLSIV	NRVRQGYSP	SFQTRLPPR	
Signatures							
B_CONSENSUS	GPDRPEGIEE	EGGERDRDRS	GRLVNGFLAL	IWDDLRLSLCL	FSYHRLRDL	LIVARIVELL	
Signatures							
	815 835						
B_CONSENSUS	GRRGWEALKY	WWNLLQYWSQ	ELKNSAVSL	NATAIAVAEG	TDRIIEVVQR	ICRAILHIPR	
Signatures			L.....		L.....		
B_CONSENSUS	RIRQGLERAL	L					
Signatures							

Figure 3.6 Site-specific signatures of the clade B dataset

The consensus sequence was generated from all clade B sequences involved in the analysis.

	Env Signal peptide < => gp120 start						
C_CONSENSUS	MRVRGILRNC	QQWWIIWGILG	FWMLMICNVV	GNLWVTVYYG	VPVWKEAKTT	LFCASDAKAY	
Signatures							
C_CONSENSUS	EKEVHNWVAT	HACVPTDPNP	QEMVLENVTE	NFNMWKNDMV	DQMHEIDIISL	WDQSLKPCVK	
Signatures							
	V1 ----- V2 -----						
C_CONSENSUS	LTPLCVTLNC	TNVTINNTTN	NYNTNNDNIT	EEMKNCSFNT	TTEIRDKXXX	EYALFYRLDI	
Signatures							

C_CONSENSUS	VPLMSSNYSE	YRLINCNTST	ITQACPKVSF	DPIPIHYCAP	AGYAILKCNN	KTENGTPCNC	
Signatures							
C_CONSENSUS	NVSTVQCTHG	IKPVVSTQLL	LNGSLAEEEI	IIRSENLTND	AKTIIVHLNE	SVEIKCTRPS	
Signatures				S.....		P.	
	-----317-----						
C_CONSENSUS	NNTRKSIRIG	PGQTFYATGD	IIGDIRQAHG	NISKDKWNKT	LQKVREKLKE	HFPNKTIFK	
Signatures		F.....					
				V4 -----			
C_CONSENSUS	PSSGGDLEIT	THSFNCRGEF	FYCNTSRLFN	STYNINNNNT	NETITLPCRI	KQIINMWQEV	
Signatures							
	435 443			V5 -----			
C_CONSENSUS	GRAMYAPPIA	GNITCKSNIT	GLLLTRDGGN	KNNKTETFRP	GGGDMRDNR	SELYKYKVVE	
Signatures	A.....	..S.....					
				=> gp41 start			
C_CONSENSUS	IKPLGVAPTK	AKRRVVEREK	RAVGIGAVFL	GFLGAAGSTM	GAASITLTVQ	ARQLLSGIVQ	
Signatures							
				596			
C_CONSENSUS	QQSNLLRAIE	AQQHMLQLTV	WGIKQLQARV	LAIERYLKDQ	QLLGIWGCSG	KLICTTTVPW	
Signatures					A.....		
C_CONSENSUS	NSSWSNKSQK	DIWDNMTWMQ	WDREINNYTD	TIYRLLEDSD	NQQEKNEKDL	LALDSWKNLW	
Signatures							
	=> TM domain			=> CT domain			
C_CONSENSUS	NWFDITNWLW	YIKIFIMIVG	GLIGLRIIFA	VLSIVNRVRQ	GYSPLSFQTL	TPNPRGPDRL	
Signatures							
				754			
C_CONSENSUS	GRIEEEGGEQ	DRDRSIRLVN	GFLALAWDDL	RSLCLFSYHR	LRDFILIAAR	AVELLGRSSL	
Signatures			L.....				
				798 812 835			
C_CONSENSUS	RGLQRGWEAL	KYLGSLVQYW	GLELKKS AIS	LLDTIAIAVA	EGTDRIIELI	QRICRAIRNI	
Signatures		G.....	A.....			Q.....	
C_CONSENSUS	PRRIRQGFEA	ALL					
Signatures							

Figure 3.7 Site-specific signatures of the clade C dataset

The consensus sequence was generated from all clade C sequences involved in the analysis.

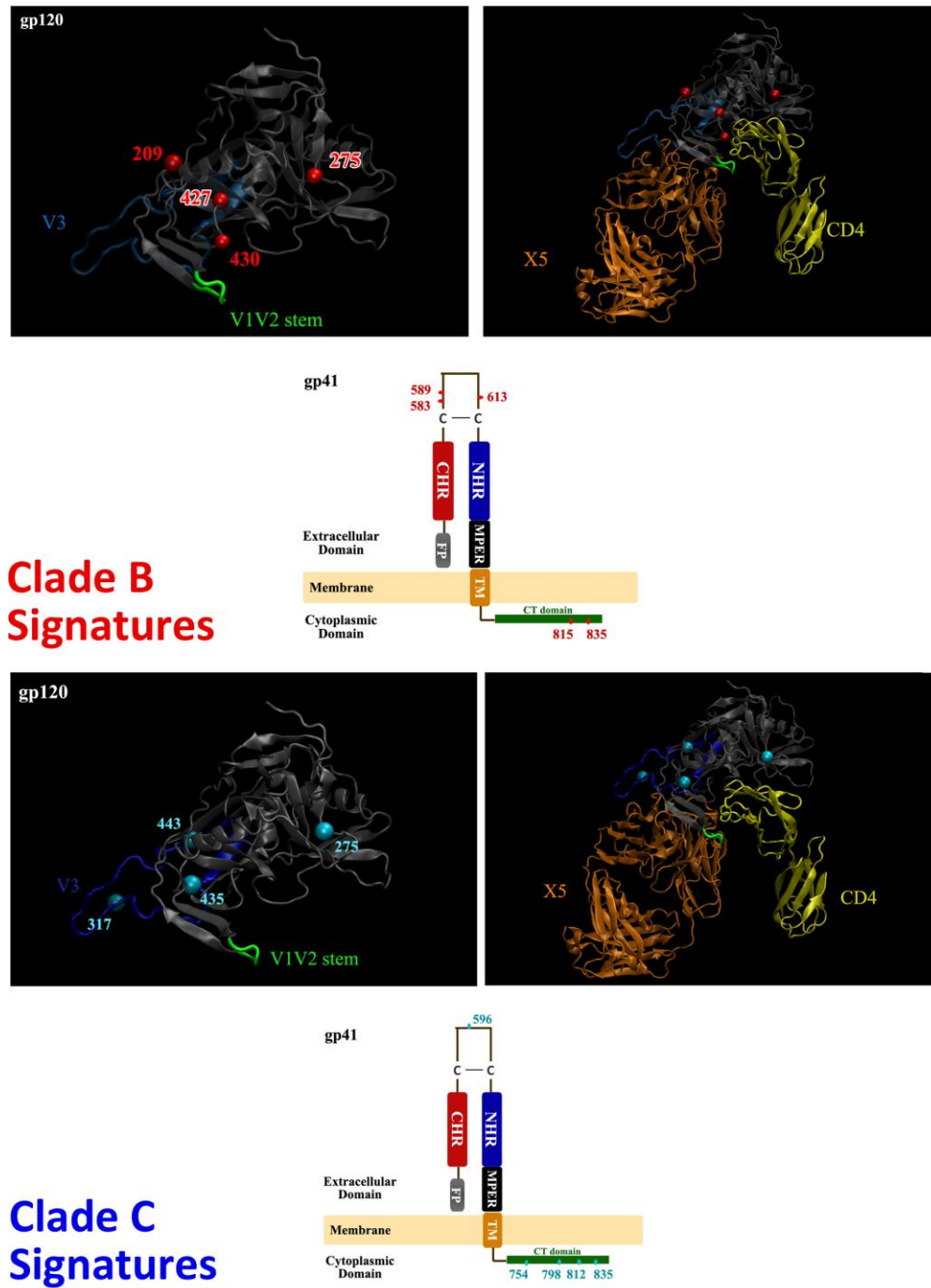


Figure 3.8 Structural Mapping of the identified signatures on the Env protein

All gp120 signatures of both clade B (red dots; *top figure*) and C dataset (blue dots; *bottom figure*) were mapped onto the crystal structure of the CD4- and X5-bound clade B JRFL gp120 core with V3 loop, which lacks the V1/V2 loop and portion of the N- and C-terminus (188). All gp41 signatures of both clade B (red dots; *top figure*) and C dataset (blue dots; *bottom figure*) were mapped onto a cartoon model of gp41 protein due to a lack of representative crystal structure for this region.

3.3. Discussion

In this study, I set out to identify signatures on the Env protein that are associated with the elicitation of a broadly neutralising response, and assess how these naturally-occurring features/mutations may contribute to the elicitation of such a response. Through collaboration with the International AIDS Vaccine Initiative (IAVI) (373), a database consisting of 1,168 clonal *env* sequences with varying neutralisation phenotype was generated from a total of 185 sera samples. After validating the sequence with those in the LANL database (Fig. 3.3), a preliminary study aiming to identify possible correlations between the global parameters of the Env protein and a broad neutralising phenotype was performed. It showed that, for both clades, sequences with a broader neutralisation profile tend to have a longer gp160 and V1/V2 loop (V2 for clade B and V1 for clade C) (Fig. 3.4). While a similar correlation was also observed in other studies (352, 407, 408), a longer V1/V2 loop was thought to be the consequence of broad neutralisation rather than the cause.

Decision Tree was subsequently used for the identification of signatures. A total of 11 and 10 signatures were obtained in both clade B and C datasets respectively. It appeared that the global features of the Env protein (e.g. variable loop length, net charge etc.) did not contribute significantly to a broad neutralising phenotype, as almost all of the signatures identified were site-specific associations (Table 3.3 & 3.4). The only global parameter that was identified as a signature was in the clade B dataset, and it specifies that the length of the V2 loop has to be longer than 43 amino acids. Although this finding agrees with the preliminary analysis, the biological significance of this feature is unclear since the average length of the V2 loop in clade B is also 43 amino acids long (Table 3.1), thereby suggesting that a long V2 loop might co-evolve with the induction of a broad neutralising response. Given that the V2 loop contains a conserved region that is recognised by the BNABs PG9 and PG16 (419), it is possible that a change in the length of the V2 loop may affect the binding of V2-specific antibodies towards this region. Indeed, such a view is in agreement with a recent study which demonstrated that alterations in the length of the V2 loop could

affect the binding of PG9 and PG16 (343). In addition, considering that the V2 loop was also found to be important for Env trimer formation, a longer V2 loop might also alter the conformation, or presentation, of the Env protein on the viral surface and indirectly affect antibody recognition towards the Env protein (72, 182). Further analysis, e.g. generation of Env proteins with varying V2 loop length, is therefore required to analyse the significance of this feature in determining the neutralisation phenotype of the Env protein.

For the site-specific associations, the position and potential significance of each individual signature for both clades are summarised in table 3.4. Although the signatures identified in both clades were different, they are all located within the conserved regions of gp120, as well as in the ectodomain and cytoplasmic domain of gp41. Such observation was in line with a similar study by Kulkarni *et al* and indicates that amino acids within both gp120 and gp41s could contribute to a broadly neutralising response (227). In both datasets, certain sets of signatures (Clade B – A275/L815/L835; Clade C – L754/A812/L835) were repeatedly determined by different combinations, thereby suggesting that the synergistic effect of these signatures might form the foundation towards broad neutralisation. Interestingly, signatures at 275 (alanine or serine) and L835 were determined in both clade B and C. Although a different amino acid was identified for the signature site at 275, the substitution from the rather bulky side chain of glutamate (E) to the smaller side chain of serine (S) or alanine (A) implies that a smaller amino acid is favoured at this position for the elicitation of a broadly neutralising response. Further investigation would be required to confirm this viewpoint. In any case, no specific information was available at this stage to pinpoint the relative importance of both signatures, but the fact that they are identified in 2 diverse clades strongly indicates that they may have an important role in the elicitation of a broad neutralising response.

Subsequent characterisations of individual signatures revealed a number of common observations that are shared in both datasets. First, numerous signatures were found to be located in the conserved regions of gp120 and are directly involved in the binding of numerous broadly neutralising antibodies. In the clade B

Clade B (10 site-specific associations)				
Signature	Location*	Com**	AA***	Possible Contribution****
209	gp120 core	2	S→T	CD4 binding; recognised by CD4, b12 and VRC01
275	gp120 core	1,2	E→A	
427	gp120 core	3	W→M	
430	gp120 core	3	V→A	
501	gp120 core	1	A→E	gp120-gp41 interface; different mutations at this site shown to affect the stability of gp120-gp41 interaction
583	gp41 ecto.	3	V→A	gp120-gp41 interface
589	gp41 ecto.	3	D→T	
613	gp41ecto.	2	S→T	
815	gp41 CT	1,2	L→L	Env protein stability and incorporation onto the viral membrane. Mutation at this region have shown to alter the conformation of the Env protein that result in antibody escape
835	gp41 CT	1,2	R→L	

Clade C (10 site-specific associations)				
Signature	Location*	Com**	AA***	Possible Contribution****
275	gp120 core	1	E→S	Co-receptor (CXCR4) binding; recognised by 3074 and HGN194
299	gp120 V3	2	P→P	
317	gp120 V3	2	F→F	
435	gp120 core	2	Y→I	
443	gp120 core	2	I→S	gp120-gp41 interface
596	gp41 ecto	3	W→A	gp120-gp41interface; the exact substitution to alanine at this site has previously shown to cause extensive gp120 shedding
754	gp41 CT	1,2,3	A→L	Env protein stability and incorporation onto the viral membrane. Mutation at this region have shown to alter the conformation of the Env protein that result in antibody escape
798	gp41 CT	3	S→G	
812	gp41 CT	2,3	I→A	
835	gp41 CT	1,2,3	R→L	

* Location of the signature. Ecto - ectodomain; CT – intracellular cytoplasmic domain

** The combination that the signature was determined in

*** Consensus amino acid → **particular amino acid association with the signature**. In the case where both amino acids are the same, it represents a conservation of the amino acids

**** Known information of this site gathered from other studies as discussed in the results and discussion.

Table 3.4 Summary of all site-specific signatures identified in each dataset

This table summarises the information of all the signatures identified for each clade. Within each clade, signatures that were repeatedly determined in more than 1 combination were shaded in **grey**. Signatures that were identified in both clades were highlighted in **red**.

dataset, the signatures of M427 and A430 are located in the conserved CD4 binding site – a region that has been shown to be a major target for broadly neutralising antibodies (BNABs) with a number of the characterised BNABs found to be directed towards this site (81, 103, 117, 314, 371, 403, 440, 455). Both signatures are found to be involved in the binding of CD4 and the BNABs b12 and VRC01 (37, 241). As a result, the identification of these signatures not only reiterates the importance of the CD4bs in the

generation of a broadly neutralising response, this also suggests a potential role for these signatures to be in direct contact with the CD4bs-specific antibodies. Likewise, the same could also be applied to the clade C signature F317 which is located in the generally conserved V3 loop (134). Similar to the clade B signatures mentioned above, the signature site of 317 is recognised by numerous V3-specific broadly-reactive antibodies (HGN194 and 3074) (22, 27, 81), and is also involved in the co-receptor binding site (CoRbs) of gp120 – a domain which is also highly conserved with several relatively broadly-reactive antibodies found to be targeting this particular region (e.g. 17b, X5) (96, 154, 242). The identification of a signature within the CoRbs is rather fascinating as such a domain is only briefly exposed upon CD4 binding (344, 356, 394), and the binding of CoRbs-specific antibodies is usually associated with a large conformational change in gp120 (231). As a result, while it is possible that the signature of F317 could directly involve in the binding of V3-specific or CoRbs-specific antibodies, it might also play an indirect role that modulates the conformation of adjacent regions, e.g. the CD4bs, in a way that is similar to the conformational changes induced by gp120-CD4 binding.

Apart from residing in the conserved regions on gp120, a large collection of signatures in both clades is also found to be located within gp120-gp41 interface [Clade B – M427, E501, A583, T589 and T613; Clade C – I435, S443 and A596]. Given that gp120 and gp41s are non-covalently associated, gp120-gp41 interface is a particularly important region in the Env protein as any changes in its amino acid composition might affect the stability and presentation of the Env protein. Indeed, several of the identified signatures have been previously reported to affect gp120-gp41 association – the introduction of a disulphide-bridge between site 501 (clade B) and 605 on gp41 has led to the generation of a stabilised and properly folded gp120-gp41 complex (SOS), while alanine substitution at sites 501 (clade B) or 596 (clade C) has led to the destabilising of the Env protein that resulted in the extensive shedding of gp120 (174, 195). Considering that the highly conserved membrane proximal external region (MPER) is ‘sandwiched’ between gp120 and the viral membrane (232, 296, 364), the shedding of gp120 caused by the A596 signature might therefore improve the accessibility of the MPER for antibody neutralisation, and lead to

the elicitation of MPER-specific antibodies that resemble the BNABs 2F5, 4E10 and 10E8 (190, 291, 460). Additional signatures were also identified in the cytoplasmic domain of gp41 [Clade B – L815 and L835; Clade C – L754, G798, A812 and L835]. Although the cytoplasmic domain is internal and rarely targeted by antibodies, mutations within this region have been shown to affect the neutralisation phenotype of the Env protein by mediating allosteric changes that affect epitopes in the outward-facing gp41 ectodomain and gp120 (115, 204, 415).

Concluding from these results, the identification and characterisation of different signatures suggest that these naturally-occurring mutations could impact the neutralisation phenotype of the Env protein by either locating in conserved regions on the Env protein and serve directly as targets for neutralising antibodies, or they could involve in an indirect manner by inducing specific conformational changes that affect epitopes in distant regions of the Env protein. Similar to other computational studies, the results generated here were preliminary and further analysis would be needed to validate the signatures identified here as, at this stage, it is difficult to clearly distinguish whether the signatures obtained here are the true cause of the broadly neutralising response, or merely escape mutations as a consequence of the immune pressure. Future work of this study should therefore focus on the validation of signatures. A logical first step would be to incorporate these signatures into Env proteins either individually or in combinations, followed by the immunization of these Env mutants into mice to assess if the signatures were able to improve the immunogenicity of the Env protein compared to the wildtype. In the case where the incorporated signatures were able to elicit a broader and more potent antibody response, the signatures involved would be studied *in vitro* so as to determine its true contribution. In addition, the Decision Tree here only analysed the epitopes recognised by the 1st generation BNABs (e.g. b12, 2F5 and 4E10). Given the vast amount of BNABs discovered in recent years, further analysis that includes the epitopes recognised by the new generation of BNABs (e.g. PG9, PG16 and PGT antibodies) would be worthwhile.

Chapter 4

Development of immunofocusing techniques for the elicitation of a broadly neutralising antibody response

4.1. Introduction

The isolation of broadly neutralising antibodies (BNAbs) from HIV-infected individuals is a pivotal point in HIV-1 research as it demonstrates that the host immune system is capable of producing antibodies that can neutralise HIV-1 strains from different clades. Subsequent characterisation of these antibodies allows the identification of numerous regions on the Env protein that are vulnerable to antibody neutralisation – e.g. the CD4 binding site (CD4bs) on gp120 and the membrane proximal external region (MPER) on gp41. The highly conserved nature of these sites among HIV-1 viruses makes them attractive targets for HIV-1 vaccine design. Unfortunately, the elicitation of a broadly neutralising response is complicated by the presence of several immunodominant non-neutralising domains on the Env protein which distracts the host immune response from recognising these conserved regions. Hence, antibody responses towards these immunodominant sites must be reduced or eliminated in order for the host immune response to be focused back onto the conserved epitopes of the Env protein ('immunofocusing').

In this chapter, I present 2 novel immunofocusing strategies aiming to encourage the elicitation of a broadly neutralising response that specifically targets either the CD4bs or the MPER. I would first describe the hypothesis and principles behind both techniques, followed by the production and *in vitro* characterisation of all the immunogens.

Approach [1] - Principle

Selective activation of CD4bs-specific B cells

The synthesis and conjugation of Auristatin is a collaborative work with Prof. Ben Davis' Group, Department of Chemistry, Oxford University

The B cell repertoire contains a pool of B cells and each B cell contains a unique B-cell receptor (BCR) that recognises a specific epitope on the antigen. Upon BCR binding of the antigen, the activation and proliferation of the respective B cell triggers a series of events which ultimately lead to the elicitation of the epitope-specific antibodies (273, 337). With the discovery of CD4bs-specific antibodies, this indicates that CD4bs-specific B cells are naturally present in the B cell pool and it was therefore hypothesised that a CD4bs-specific response can be elicited if B cells that recognise the CD4bs (CD4bs-B cells) are selectively activated, whilst B cells that recognise the immunodominant regions (ID-B cells) are inhibited and remain inactivated.

To test this hypothesis, an immunisation regime that involves the selective activation of CD4bs-B cells was devised (Fig. 4.1). A toxin-conjugated gp120 (toxin-gp120) protein was specially designed for the inhibition of ID-B cells, and this was composed of 2 key components – a gp120 protein for the targeting of HIV-specific B cells, and a toxin for the inhibition of cell growth. gp120 used here contains a D → R mutation at position 368 (D368R-gp120) that has previously been shown to abolish the binding of CD4 and numerous CD4bs-specific antibodies (237, 314, 395). As a result, the use of D368R-gp120 would deliver the toxin to the ID-B cells, but not to the CD4bs-B cells. For the toxic component of the toxin-gp120, the anti-mitotic agent auristatin – which acts by inducing apoptosis via the inhibition of tubulin polymerisation – was used because it has demonstrated potent cytotoxic activities in numerous clinical trials for cancer therapy (215, 341, 366). For the production of the toxin-gp120, auristatin was first conjugated to streptavidin followed by the binding of biotinylated D368R-gp120 onto the streptavidin. The immunisation of the toxin-gp120 should therefore result in the inhibition of ID-B cells. By co-immunising toxin-gp120 proteins with wildtype gp120 proteins, this should allow the preferential

activation and proliferation of the CD4bs-B cells and lead to the elicitation of a CD4bs-specific antibody response that resembles b12 and other CD4bs-specific BNABs.

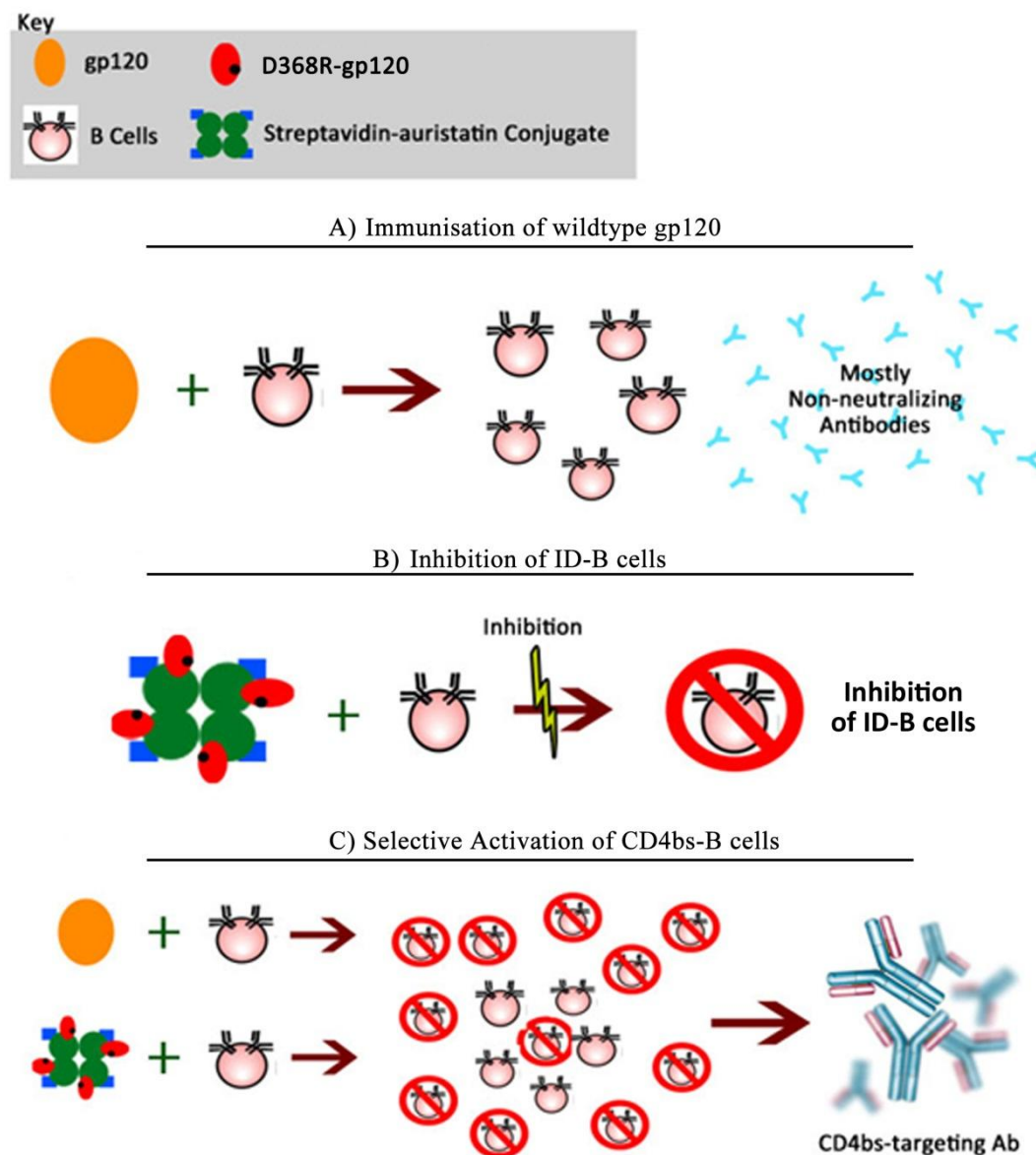


Figure 4.1 Illustration of approach [1] - Selective activation of B cells

(A) Recognition of epitopes on gp120 leads to the activation and proliferation of B cells, which eventually result in the production of soluble antibodies. However, these antibodies are mostly non-neutralising as they recognise epitopes on the highly variable immunodominant regions. (B) The toxin-gp120 would preferentially inhibit the activation and proliferation of B cells that recognise the immunodominant regions (ID-B cells), thus preventing the elicitation of antibodies from these cells. B cells targeting the CD4bs (CD4bs-B cells) will remain unaffected. (C) Co-immunisation of the toxin-gp120 and the wildtype gp120 should therefore lead to the selective activation of CD4bs-B cells, and the resultant antibody response should now be focused towards the CD4bs.

Approach [2] - Principle

Focusing the antibody response to the MPER by gp41-VLP

The MPER is an attractive target for HIV-1 vaccine development as it is highly conserved among HIV-1 (286), and the MPER-specific antibody 10E8 is among the most broad and potent BNABs known to date (39, 190). However, the elicitation of MPER-specific antibodies is extremely rare in natural infection and vaccination because: first, anti-gp41 antibodies generally target the immunodominant disulphide-bridged loop and other core regions of gp41 rather than towards the immuno-recessive MPER (37, 82, 241, 286, 318, 420, 447), and second, the steric effect of having the MPER region ‘sandwiched’ between gp120 and the viral membrane means that the accessibility of the antibody towards the MPER is most likely to be hindered (232, 296, 364). To overcome these problems, here I explored whether the use of heavily-truncated gp41 proteins could facilitate the elicitation of MPER-specific response. It was hypothesised that the occlusion of gp120 would improve the antibody access to the MPER, and the deletion of most immunodominant epitopes of gp41 protein would allow the focusing of antibody response back towards the immuno-recessive MPER region.

Based on the crystal structure of gp41 protein published by Buzon *et al* (PDB: 2X7R) (394), 2 designs of gp41 immunogens were proposed (Fig. 4.2): one contained a complete deletion of both the N- and C-terminus Heptad Repeat (NHR and CHR) regions, while the other retained a glutamine ring (Q-ring) at the base of gp41 core. To date, this Q-ring at the base of the NHR/CHR bundle has not yet been characterised and it is intriguing to examine if it is important for the structural integrity or immunogenicity of gp41 protein. In addition, further modifications were also introduced to these immunogens to stabilise gp41 constructs: firstly, the disulphide-looped bridge that connects the NHR and CHR was retained as it was found to be crucial for the conformational integrity of gp41 protein (195, 314), and its sequence was mutated to GSSGG that has previously been shown to reduce the immunogenicity of this region (211); secondly, both the fusion peptide (FP) and polar region (PR) on the N-terminus of gp41 were also retained as they appeared to be in contact with the MPER and increase the stability of gp41 protein (394); thirdly,

these heavily-truncated gp41 proteins were expressed on the surface of virus-like particles (gp41-VLP) as the presentation of these proteins in a membrane context may enhance their stability (86), and the plasma membrane is also thought to be necessary for the optimal neutralisation by MPER-specific antibodies (6, 167, 171). Taken together, it was hoped that the use of these heavily-truncated gp41 immunogens could facilitate the elicitation of a MPER-specific response that is comparable to 4E10 and 10E8.

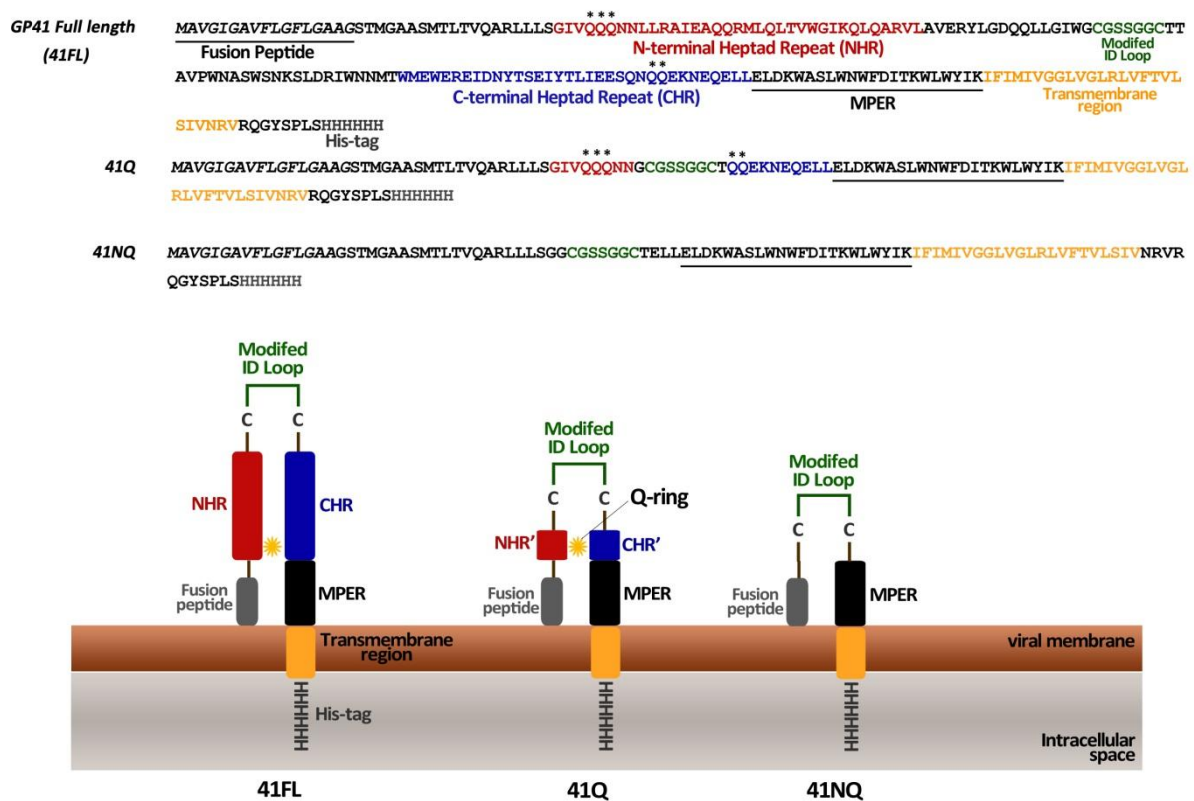


Figure 4.2 Illustration of approach [2] - Focusing the antibody response to the MPER by gp41-VLP

Two different gp41 immunogens, 41Q and 41NQ, were designed based on gp41 structure aiming to refocus the immune response towards the MPER on gp41. The NHR and CHR regions of gp41 were partially, or completely, removed in 41Q and 41NQ respectively, leaving only the fusion peptide (FP), polar region (PR), the MPER region and the disulphide-bridged, immunodominant (ID) loop to maintain the structural integrity of the protein. The only difference between the 2 immunogens is the inclusion of a glutamine (Q) cluster in both NHR and CHR that forms a Q-rich ring that can be seen in the six-helix bundle of gp41 and may be potentially immunogenic (highlighted by asterisks *). The ID loop was replaced with a potentially less immunogenic GSSGG motif (211).

4.2. Results

Approach [1] - Selective activation of CD4bs-specific B cells

4.2.1 Production of the toxin-gp120 components

The toxin-gp120 consists of 3 major parts: (i) a D368R-gp120 protein, (ii) a modified streptavidin with 4 additional cysteines for the conjugation of auristatin, and (iii) auristatin with the addition of a maleimide group for the conjugation to the cysteines on the streptavidin, and a valine-citrulline cathepsin B labile linker for protease-mediated endosomal release of the toxin. The monomeric D368R-gp120 was purified and validated by Western blot using a cocktail of anti-Env antibodies (HIVIG and 447-52D), and the protein was biotinylated to enable the coupling of the protein to the auristatin-conjugated streptavidin (Fig. 4.3). The cysteine-containing streptavidin used for the conjugation of toxin was previously synthesised and purified in the lab (unpublished data), and the auristatin was produced by Prof. Ben Davis' group at Oxford University (the experimental procedure is shown in supplementary materials).

4.2.2 Determination of the cytotoxicity of the synthetic auristatin

Once the auristatin was produced, an *in vitro* cytotoxicity assay was carried out to validate the toxin by assessing the functionality of the synthetic product. A series of toxin concentrations were tested here – from a relatively low concentration of 1pM to a high concentration of 10µM – and the cytotoxicity of the synthetic auristatin was evaluated in 2 different human cell lines: (i) 293T cells, which are widely-used in cell biology research, and (ii) the Sup-B8 cell line, which is a human B cell line that has been previously described (242). The use of 2 different human cell lines would allow a better understanding of the cytotoxic effect of the auristatin. The cytotoxicity of the auristatin was assessed in terms of the number of viable cells after 72 hours of continuous exposure to the toxin, and cells that were not toxin-treated were used as a control. In addition, since the auristatin was dissolved in 20% DMSO due to its hydrophobicity, the potential inhibitory effect of DMSO was also examined in a separate setting by adding the equivalent amount of DMSO to the cells.

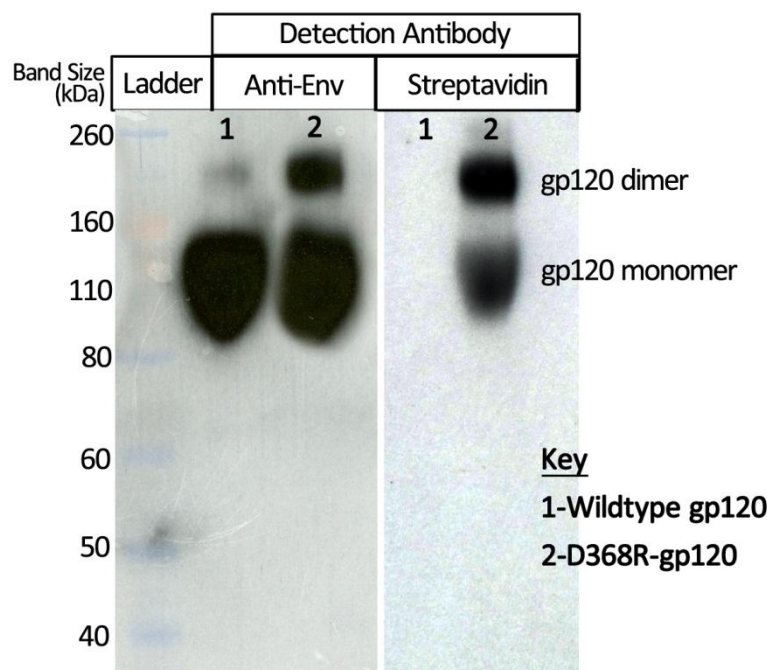


Figure 4.3 Expression and biotinylated of the wildtype and D368R-gp120

Both the synthesis of wildtype and D368R-gp120, and the biotinylation of D368R-gp120, were analysed by non-reducing SDS-PAGE and Western Blot using a cocktail of anti-Env antibodies (HIVIG, 447-52D and b12) and ExtrAvidin (streptavidin-HRP conjugate).

In both the 293T and Sup-B8 cells, a similar correlation between viable cells and toxin concentration was observed. Overall, the cytotoxicity of the auristatin was most effectively observed in the nM range, with minimal cytotoxicity in the lower pM concentration and maximal cytotoxicity in the μ M range (Fig.4.4). No cytotoxic effect was observed with DMSO at all concentrations used, thus confirming that all cytotoxicity detected was due to auristatin. From these data, an IC_{50} at 2.79 nM and 4.82nM was determined for the 293T cells and Sup-B8 cells respectively (Fig. 4.4). With a similar IC_{50} in the lower nM range previously reported for auristatin (215), these results therefore agree well with other published findings and validate the auristatin synthesised for this study.

4.2.3 Recovery of cells upon auristatin removal

Although a potent inhibition in cell growth was observed upon continuous exposure of the auristatin, this may not best reflect the situation *in vivo* since target ID-B cells are only transiently exposed to the toxin as

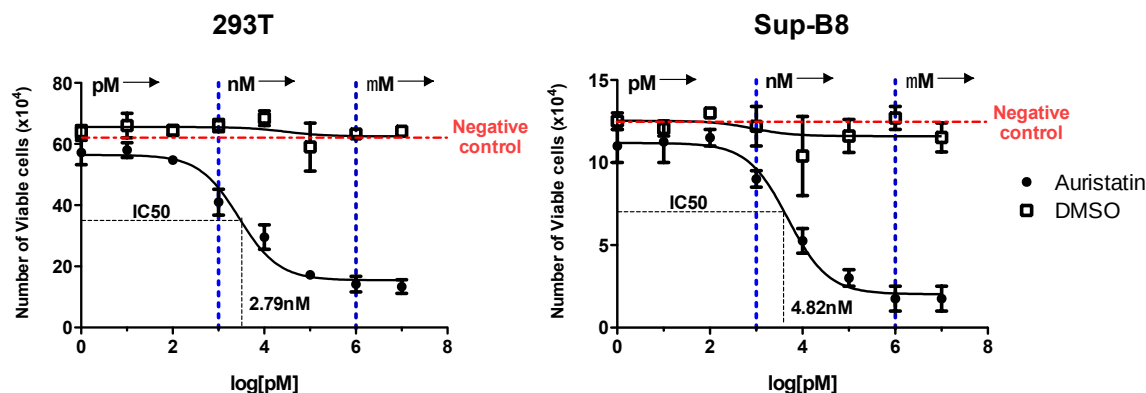


Figure 4.4 *In vitro* cytotoxicity assay and the determination of IC₅₀ of auristatin

The number of viable cells (●) in (left) 293T and (right) Sup-B8 cell-line was measured at the end of the 72-hour incubation trypan blue staining and were plotted against the concentration of the auristatin. Potential inhibitory effect of DMSO (□) was also assessed by using the equivalent amount of DMSO in each toxin concentration. All error bars represent the standard deviation of duplicate samples.

the toxin-gp120 would eventually be cleared from the system. In these situations, the previously-arrested cells might recover from cell-cycle arrest, and the subsequent activation of ID-B cells would not be ideal for this approach. To investigate whether cell recovery is possible, cells were exposed to various concentrations of auristatin (1nM, 10nM and 100nM) and divided into 2 groups: in one group, designated the ‘*removal*’ group, cells were exposed to auristatin for 8 hours before the toxin was removed, and the cells were grown for a further 72 hours. For the other group, designated the ‘*continuous*’ group, cells were continuously exposed to the auristatin over the course of the experiment. Measurements of cell growth were based on the number of viable cells at 24-, 48- and 72-hour post-removal.

Overall, the results obtained from 293T and Sup-B8 cells were highly similar and are therefore discussed together. To examine whether the cells are able to recover from cell cycle arrest, the number of viable cells in the ‘removal’ and ‘continuous’ groups at the final time-point of the experiment (t = 72 hours) was compared. It was assumed that the viable cell count would be similar in both ‘removal’ and ‘continuous’ group if the cells are permanently inhibited, whilst the viable cell count would be higher in the ‘removal’ group than in the ‘continuous’ group if the cells are able to recover from growth inhibition. From the data

obtained from both cell lines, it was clear that the number of viable cells of the ‘removal’ group was higher than the ‘continuous’ group across all toxin concentration (Fig. 4.5), and the difference was more profound when a concentration above the calculated IC_{50} was used (i.e. 10nM and 100nM). By looking at the rate of cell growth at each time-point (Fig. 4.6), cells that were exposed to a higher toxin concentration appeared to be growing at a significantly slower rate than cells that were exposed to a lower toxin concentration (i.e. 100nM vs. 10nM, and 10nM vs. 1nM). Taken together, these results therefore indicate that: one, upon drug removal, cells were able to recover from cell cycle arrest meaning that the inhibitory effect of auristatin is likely to be reversible rather than permanent; two, the rate of recovery was largely dependent on the concentration that the cells were pre-exposed to.

4.2.4 Conjugation of auristatin to streptavidin

It was hoped that, with a successful conjugation reaction, the number of auristatin molecules conjugated to the streptavidin could be modulated. The conjugation of auristatin to streptavidin was performed in 20% DMSO due to the hydrophobicity of the auristatin, but unfortunately, no protein peak could be observed when the product was purified on a size exclusion chromatography column, and it was suspected that the loss of proteins was due to the insolubility of the streptavidin-auristatin conjugate. This therefore led to a re-designing of the toxin-gp120 protein by substituting the auristatin with another toxin called calicheamicin, which is more hydrophilic and is used as a toxin conjugate in therapeutic antibodies in humans (e.g. Mylotarg aka Gemtuzumab ozogamicin) (25, 383). In addition, the use of a hydrazine-derivative of the calicheamicin (hydrazine-calicheamicin) would allow the direct conjugation of toxin to the glycans of the Env protein (183), thus avoiding the multiple conjugate steps as postulated with the use of auristatin. Disappointingly, at the time of submission of this thesis, an IP agreement with Pfizer (who holds the right for the use of this molecule) has yet to be reached and it remains to be seen whether this approach could lead to the elicitation of a CD4bs-specific response.

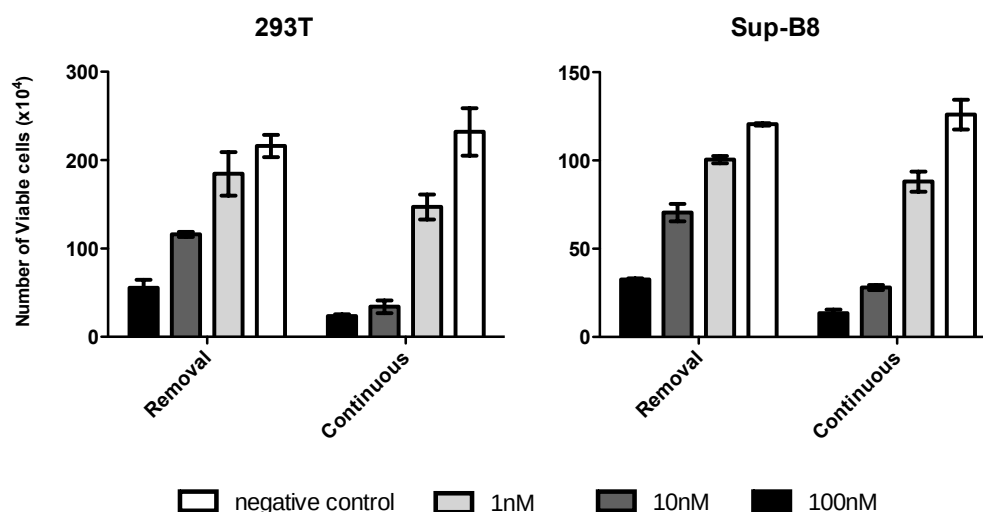


Figure 4.5 Investigation of cell recovery upon auristatin removal after 8 hours of incubation

For the ‘removal’ group, both (*left*) 293T and (*right*) Sup-B8 cells were exposed to various concentration of auristatin for 8 hours before the auristatin-containing media were replaced with normal growth media, and cell growth was monitored for the next 72 hours. For the ‘continuous’ group, the same procedure was used without the removal of auristatin, i.e. the cells were continuously exposed to the drug for a total of 80 hours. The error bars represent the standard deviation of the duplicate samples.

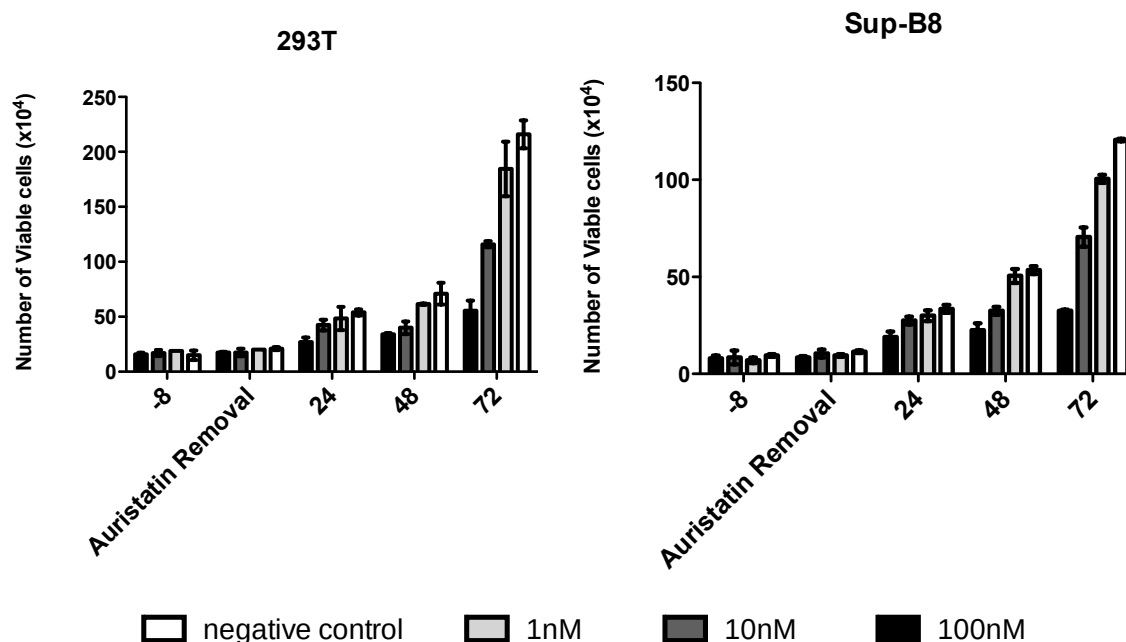


Figure 4.6 Cell recovery upon auristatin removal

(*left*) 293T and (*right*) Sup-B8 cells were incubated in various concentrations of auristatin. After 8 hours of incubation ($t = 0$), media containing the auristatin was replaced with normal growth media, and cell growth were monitored at $t = 24, 48$ and 72 hours after the removal of the toxin. Error bars represent the standard deviation of the duplicate samples.

Approach [2] - Focusing the antibody response to the MPER by gp41-VLP

4.2.5 Production of gp41 plasmids

Various forms of gp41 immunogens were used in this study, including (i) a wildtype, full length gp41 (41FL) protein, (ii) a ‘partially-truncated’ gp41 protein with the Q-ring (41Q), and (iii) a fully-truncated gp41 protein, i.e. 41No-Q (41NQ) (Fig. 4.2). The genetic products of these gp41 immunogens were synthesised by overlapping PCR based on a JRFL gp160 protein that lacks the cytoplasmic domain (gp160 Δ CT). The final product was size-checked by electrophoresis (Fig. 4.7) and validated by sequencing before they were cloned into the *PLEC* vector.

4.2.6 Expression of gp41-VLPs containing truncated gp41 proteins

Plasmids of various gp41 constructs were co-transfected with the gag-expressing pNL4-3.Luc.R-E-* plasmids for the production of gp41-VLPs. In this experiment, VLPs bearing the gp160 Δ CT (gp160 lacking the cytoplasmic domain) were included as a positive control, whereas VLPs bearing no Env proteins (naked-VLPs) were used as a negative control. The expression of gp41-VLPs was confirmed by Western blot analysis using anti-p24 antibodies under reducing condition (Fig. 4.8). The incorporation of different Env proteins into VLPs was also analysed by Western blot under reducing condition, probing with either 4E10 or 2F5 (Fig. 4.8). As expected for the gp160 Δ CT, 2 proteins bands were found in both analyses with the upper band representing the full-length Env protein (gp120 plus gp41 Δ CT), while the lower band represents gp41 of the dissociated Env protein (gp41 Δ CT only). For the 41FL, a single gp41 band was observed in both 4E10 and 2F5 Western blots, thus confirming the incorporation of 41FL in gp41-VLPs. However, the expression for both 41Q and 41NQ was not detected in both Western blots, thus strongly suggests that both proteins were not incorporation into gp41-VLPs.

4.2.7 Detecting cell surface expression of 41 immunogens by flow cytometry

The failure for both 41Q and 41NQ to be incorporated into VLPs suggests that both proteins were not expressed on the cell surface of the 293T cells. To further examine the expression of these gp41 immunogens on the cell surface, the same gp41 plasmids were used for the transfection of 293T cells

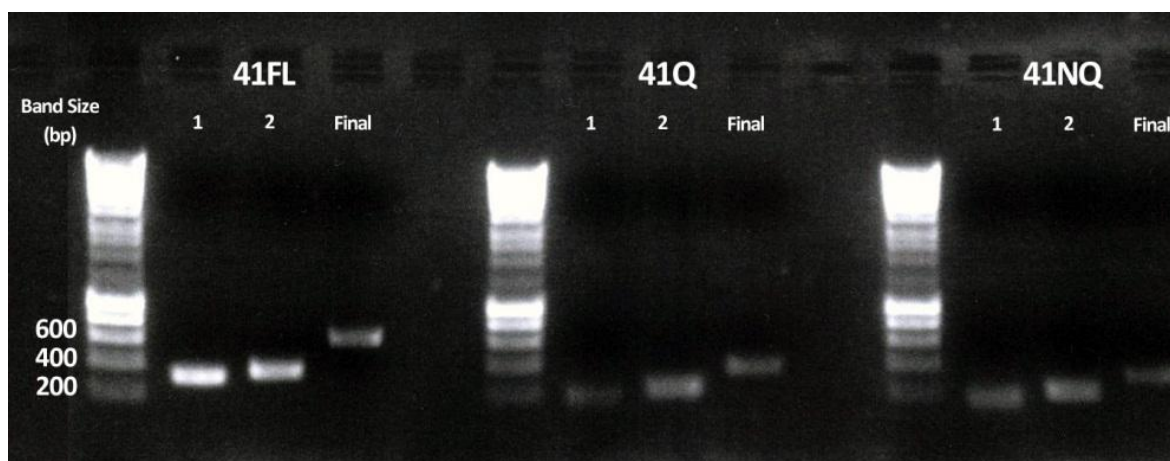


Figure 4.7 Construction of gp41 plasmids

Overlapping PCR results for the construction of gp41 immunogens. The final PCR product (marked as final) was amplified by mixing PCR product 1 and 2 (labelled respectively). The final product was validated by sequencing. [Predicted size of each gp41 constructs: 41FL – 623bp; 41Q – 414bp; 41NQ – 371bp]

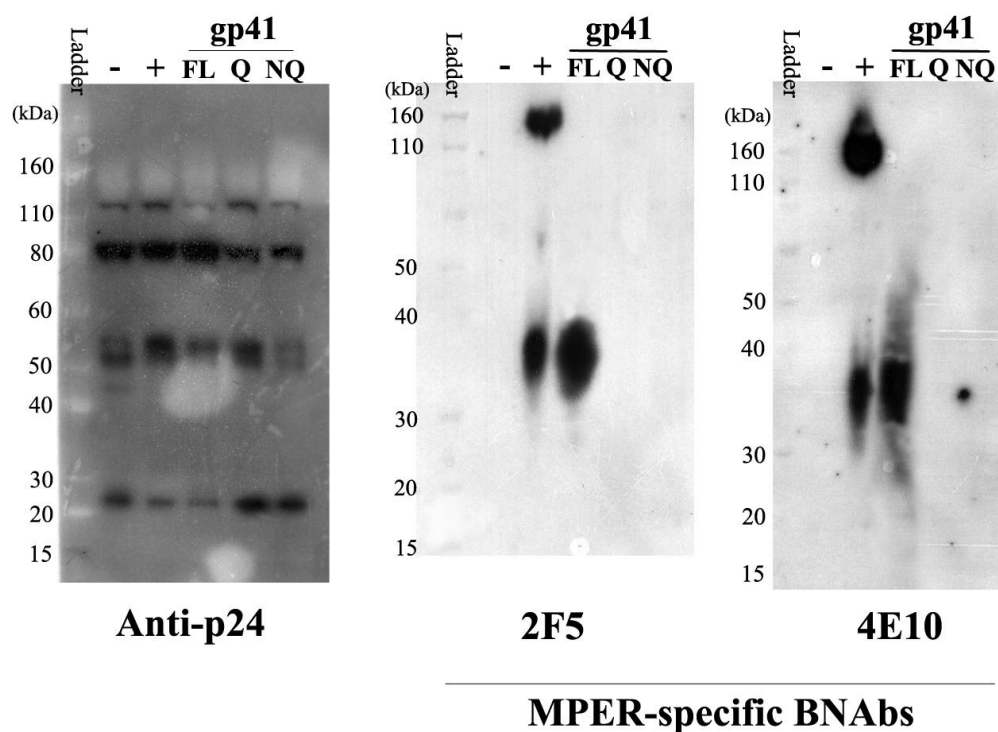


Figure 4.8 Detection of the expression of 41FL, 41Q and 41NQ in gp41-VLPs

Western blot analysis of the purified VLPs under reducing conditions probing p24-antibodies and the MPER-specific broadly neutralising antibodies (2F5 and 4E10) respectively. Negative control (-) refers to naked VLP which have the Env plasmid omitted in the transfection. Positive control (+) refers to the wildtype JRFL gp160ΔCT.

without the pNL4-3.Luc.R-E-* plasmid, and the expression of these proteins on the surface of the 293T cells was analysed by flow cytometry staining with 4E10 and 2F5. In this experiment, gp160 Δ CT-transfected cells were used as the positive control, while untransfected cells were used as the negative control. As a precaution, gp41-transfected cells were also stained with the secondary FITC-antibody alone in case the antibodies cross-react with gp41 proteins or the plasma membrane of the 293T cells and therefore gives a false-positive signal.

The results obtained here were in good agreement with the Western blot analysis described above (Fig. 4.9). Compared to the negative control, quantification of the 4E10⁺ or 2F5⁺ population confirmed a substantially higher expression of gp160 Δ CT and 41FL ($p < 0.01$), whilst the expression of both 41Q and 41NQ was insignificant. In all cases, staining with FITC-antibody (without 4E10 or 2F5) alone did not yield any positive signal, meaning that all positive signals were strictly resulted from the binding of gp41 proteins by 4E10 or 2F5 (not shown). As a result, these results confirmed the expression of 41FL on the cell surface, and the lack of surface expression of both 41Q and 41NQ therefore explains the failure for these proteins to be incorporated into respective gp41-VLPs.

4.2.8 Production and expression of gp41 proteins with stepwise deletion

Since the interaction between NHR and CHR is involved in maintaining the structural integrity of gp41 (66, 172, 173, 394), extensive mutation within these sites in 41Q and 41NQ may therefore have an adverse effect on their expression and subsequent incorporation into the VLP. To examine the effect of NHR and CHR deletion on the surface expression of gp41 immunogens, 4 additional truncated-gp41 proteins were designed where a stepwise deletion of 7 amino acids in both the NHR and CHR regions was introduced to each protein (Fig. 4.10). The deletion of 7 amino acids, which corresponds to 2 α -helical turns, was necessary in order to retain the helical periodicity of both NHR and CHR. The introduction of these proteins would bridge the gap between 41FL and 41Q, thereby providing a better understanding on the consequence of the NHR/CHR deletion. These stepwise mutants were referred to as 41 Δ 2t, 41 Δ 4t,

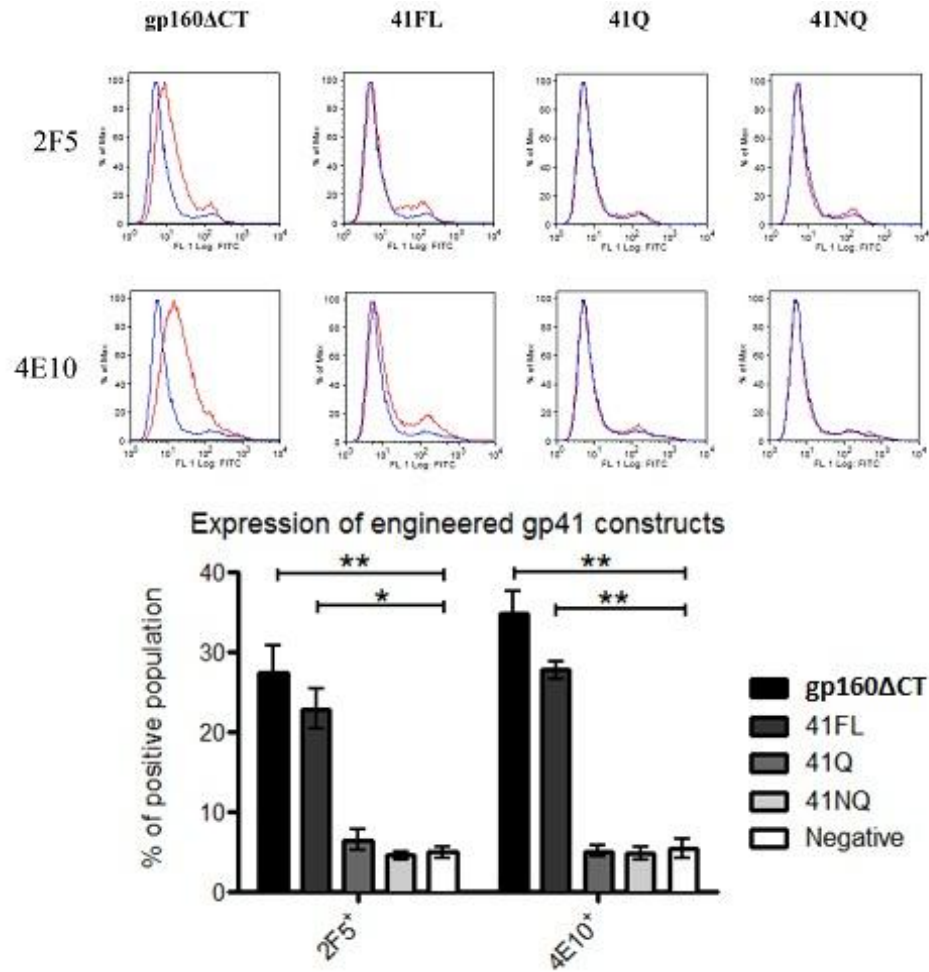


Figure 4.9 Flow cytometry analysis of cell surface expression of 41FL, 41Q and 41NQ

Cells were stained with either MPER-specific 2F5 or 4E10 followed by detection with anti-human FITC antibody. (Top) Representative histogram showing the expression of various proteins, as indicated by the intensity of FITC, on the membrane surface of the 293T cells. The blue line in each plot represents the negative control while the red line represent the respective sample. (Bottom) Quantification of 4E10⁺ and 2F5⁺ FITC signal. The error bars represent the standard deviation of the triplicates. [*p<0.01; **p<0.001]. Statistics were calculated using One way ANOVA with a Bonferroni post-hoc test.

41Δ6t and 41Δ8t respectively, where Δxt refers to the number of turns deleted. Again, the production of these constructs was achieved through overlapping PCR, and were validated by electrophoresis and sequencing (Fig 4.10).

Together with 41FL, which was used as the positive control, these constructs were transfected into 293T cells and the surface expression of all proteins was examined by flow cytometry as in the previous assay.

As previously observed, 41FL proteins were found to be expressed on the cell surface (Fig. 4.11). However, the negative impact of NHR and CHR deletion started to become detectable in the truncated gp41 proteins. Although the expression of 41 Δ 2t was still detectable on the cell surface, the expression level dropped by almost 50% compared to the 41FL ($p < 0.05$), and for the rest of gp41 constructs (i.e. 41 Δ 4t, 41 Δ 6t and 41 Δ 8t), further deletion into the NHR and CHR completely eradicated the expression of these proteins on the cell surface. Hence, these results confirm that that deletion of even a small portion of the NHR and CHR (e.g. 2 α -helical turns) is intolerable for gp41 protein. With a lack of expression for the proposed 41Q and 41NQ immunogens, this vaccination approach was therefore not continued.

Figure 4.10 and 4.11 on the following pages

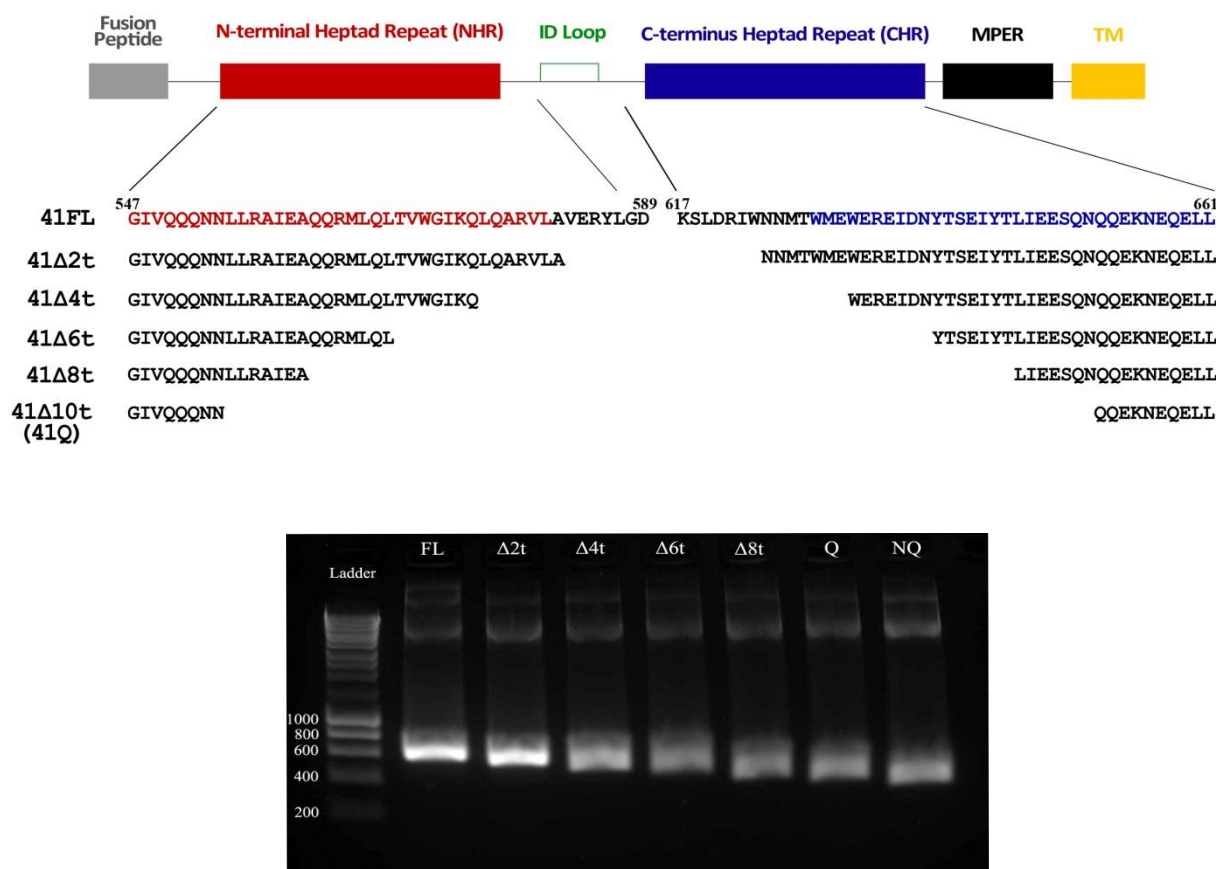


Figure 4.10 Amino acid sequences and the plasmid construction of the NHR/CHR step-deletion mutants

(Top) Amino acid sequences of each stepwise mutants given in the one-letter code. The numbering indicates the HxB2 position of the amino acids. (Bottom) All stepwise mutants were synthesised by overlapping PCR and shown here are the final products of each mutant. [Predicted size of each gp41 constructs: 41FL – 623bp; 41Δ2t – 581bp; 41Δ4t – 539; 41Δ6t – 497; 41Δ8t – 455bp; 41Q – 413bp; 41NQ – 371bp]

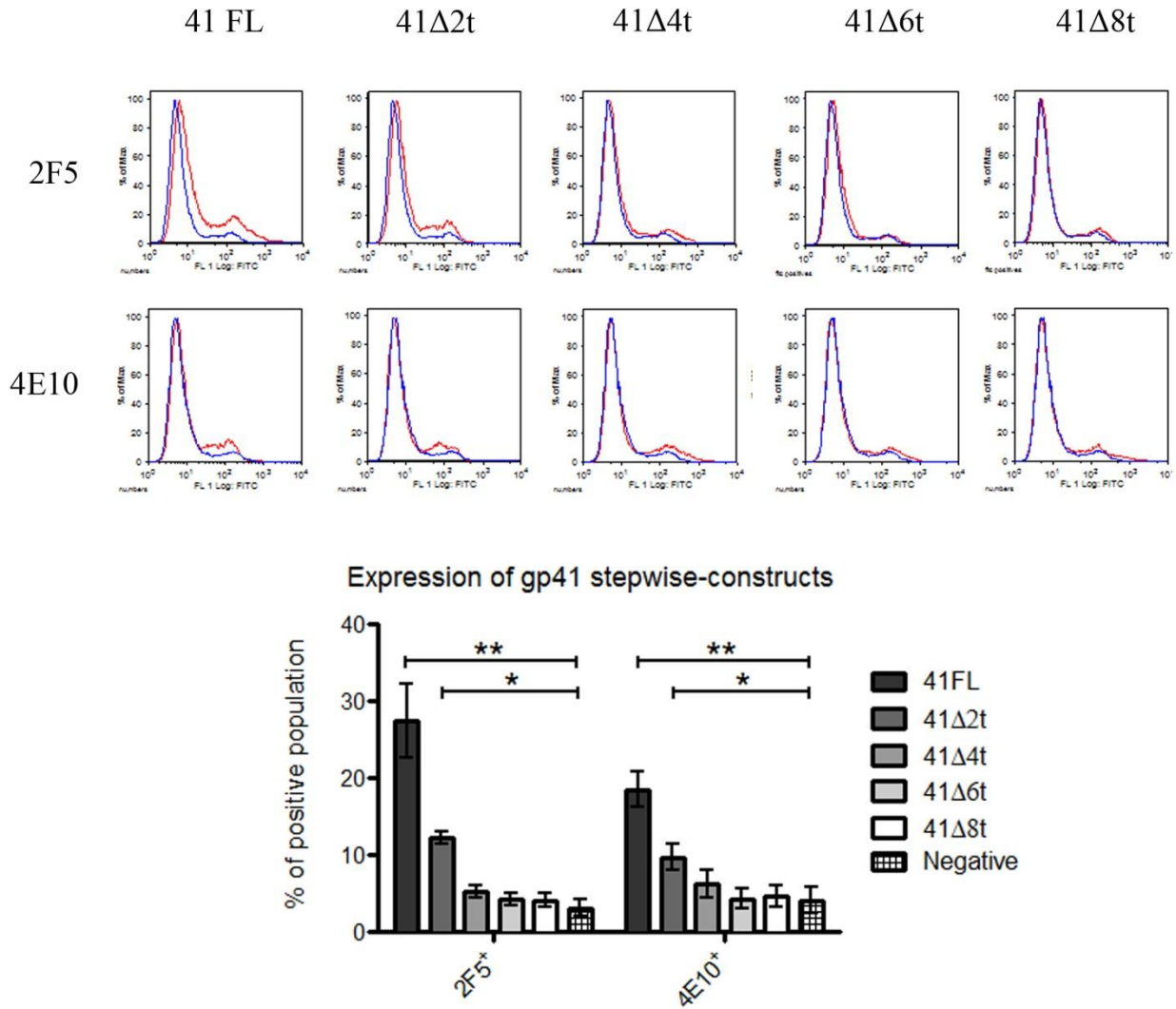


Figure 4.11 Flow Cytometry analysis of the stepwise mutants

All cells were stained with either MPER-specific 2F5 or 4E10 followed by detection using anti-human FITC antibody. (Top) Representative histogram showing the expression of the stepwise mutants on the membrane surface of the 293T cells, as indicated by the intensity of FITC. The blue line in each plot represents the negative control while the red line represent the respective sample labeled in the title. (Bottom) Quantification of 4E10⁺ and 2F5⁺ FITC signal. The error bars represent the standard deviation of the triplicate samples. [*p<0.05; **p<0.001]. Statistics were calculated using One way ANOVA with a Bonferroni post-hoc test.

4.3. Discussion

In this chapter, I describe 2 novel ‘immunofocusing’ approaches with the specific aim of eliciting CD4bs- and MPER-specific antibodies. However, for different reasons, the development of both techniques was hindered or discontinued.

Approach [1] - Selective activation of CD4bs-specific B cells

The objective of the first approach was to elicit CD4bs-specific antibodies through the selective activation of CD4bs-targeting B cells (CD4bs-B cells). The development of this approach started with the preparation of the toxin-conjugated gp120 complex (toxin-gp120), which was composed of a CD4bs-defective gp120 (D368R-gp120) for the targeting of non-CD4bs-targeting B (ID-B) cells, and the highly potent antimetabolic auristatin (i.e. the toxin) for the inhibition of the ID-B cells. To validate the functionality of synthetic auristatin, its potency was assessed in an *in vitro* cytotoxicity assay by exposing both 293T and Sup-B8 cells to various concentrations of auristatin for 72 hours (Fig. 4.4). The synthetic auristatin demonstrated a potent cytotoxic effect with an IC_{50} within the lower nM range (293T: 2.79nM; B8: 4.82nM) that was comparable to other studies (93, 158, 186, 215, 298, 311) and therefore validates the functionality of the auristatin. Although a potent cell growth inhibition was observed upon continuous exposure of the auristatin, this may not best reflect the situation *in vivo* since the target cells are more likely to be transiently exposed to the auristatin as the toxin-gp120 would eventually be cleared from the host system. For this reason, the consequence of toxin clearance was assessed by monitoring the growth of both 293T and Sup-B8 cells after they were incubated for 8 hours in various auristatin concentrations. The results demonstrated that, upon auristatin removal, the cells were able to recover from the inhibitory effect of the toxin (Fig. 4.5, and the rate of recovery was also found to be dependent on the toxin concentration, i.e. the higher the auristatin concentration, the slower the rate of recovery was. Whether the use of an even higher concentration would induce permanent cell arrest was not explored in this study. In addition, while a potent inhibitory effect was observed with the use of 2 different cell lines, further assessment of the

toxin component in primary B cells would be worthwhile as the effect imposed on the cell lines by the toxin might be different in primary B cells.

Unfortunately, despite the promising start of the project, the conjugation of auristatin and streptavidin was unsuccessful and therefore led to the designing of a completely different toxin-gp120 immunogen. In the new design, a more hydrophilic toxin – calicheamicin – is used as the substitute to auristatin (25, 383), and this toxin will be conjugated directly onto the glycan of gp120 based on a previously described method by Hinman *et al* (183). However, the development of this newly-designed toxin-gp120 was hindered by unresolved IP agreement with Pfizer, and it remains to be seen how the use of calicheamicin would differ to that of the auristatin.

Approach [2] - Focusing the antibody response to the MPER by gp41-VLP

The second approach of this study focuses on the elicitation of MPER-specific antibodies by immunising with virus-like particles bearing heavily truncated gp41 proteins (gp41-VLPs) – 41Q and 41NQ – in which the majority of gp41 protein outside the MPER were partially or completely removed (Fig. 4.2). Only the disulphide-bridged loop, the fusion peptide, the polar region of gp41 protein were retained as their presence has shown to increase the stability of gp41 conformation. The production of gp41-VLPs was confirmed by anti-p24 Western blot (Fig. 4.8), although not all gp41 constructs were found to be incorporated into the VLPs. Based on the results of the 4E10/2F5 Western blots and flow cytometry (Fig. 4.8), only the gp160 Δ CT and full length gp41 (41FL) could be detected on the VLP, while the lack of expression of 41Q and 41NQ suggests that both gp41 immunogens were not expressed on the cell surface (Fig. 4.9).

With the interactions between NHR and CHR – especially the **LLSGIV** motif (residues 544-549) and the **LQLTVWGI** motif (residues 567-574) on the NHR, and the **RIWNNMT** (residues 621-627) on the CHR – found to be crucial for the correct folding and stability of gp41 proteins (66, 68, 172, 173, 394), it was

suspected that the extensive mutation within both regions might have an adverse effect on the expression and presentation of the 41NQ and 41Q on the cell surface. To examine this, another set of mutant gp41 proteins (41Δ2t, Δ4t, Δ6t and Δ8t) were synthesised, with each consisted of a stepwise-deletion of 2 α -helical turns in the interacting regions of the NHR and CHR (Fig. 4.10). Among these constructs, only the 41Δ2t protein was expressed on the cell surface, but the partial deletion of the RIWNNMT motif on CHR has caused the expression level of this protein to drop by almost 50% (Fig. 4.11). This finding therefore agrees well with previous studies by He *et al* in which they demonstrated that this RIWNNMT motif is the key determinant for the stabilisation of gp41 6-helix bundle (172, 173). Further eliminations of the LQLTVWGI and RIWNNMT motifs in the NHR and CHR respectively were not tolerated as none of the 41Δ4t, 41Δ6t and 41Δ8t proteins could be detected on the cell surface by flow cytometry. Although the LLSGIV motif in the NHR was still retained in the 41Q and 41NQ, the results here suggest that this motif alone is not sufficient to stabilise gp41 protein in the right conformation, and the expression of MPER-only proteins might not be possible without structural support from gp41 NHR/CHR core, heterogeneous oligomerisation domains or other structural scaffolds (e.g. HA-gp41 chimeric protein) as demonstrated in other studies (211, 238, 416, 420, 447, 452).

In light of these results, a logical way to move this study forward was to express the MPER region together with a heterologous oligomerisation domain to stabilise the protein, and such approach has already been tested by Toukam *et al*. Although these MPER-containing proteins were successfully expressed onto the VLPs via the introduction of a heterologous leader peptide and an isoleucine zipper on either ends of the MPER (322), MPER-specific antibodies were only elicited in 3 out of 5 of the immunised mice and no neutralising activities could be detected from these antibodies. Like most gp41 immunogens studies to date, it was believed that the lack of neutralising activities in the elicited antibodies was mostly due to their inability to recognise the native conformation of the MPER of the Env spike (211, 238, 322, 416, 420, 447, 452). This therefore indicates that even the incorporation of an oligomerisation domain in gp41 immunogens is not enough for the MPER to retain its native conformation. Furthermore,

with self-reactivity (lipid-binding) considered essential for the optimal binding and neutralisation of MPER-specific antibodies 4E10 and 2F5 (167, 169, 171), it is still unsure how such a unique feature of these antibodies could be induced *in vivo*. A complex B cell maturation pathway was likely to be involved for the elicitation of these antibodies, and the incorporation of MPER-containing immunogens into VLPs is unlikely to provide the solution to this problem. For these reasons, the recent discovery of another MPER-specific BNAbs 10E8 could be important as this antibody is not self-reactive and demonstrates a neutralisation breadth and potency that is superior to 4E10 and 2F5 (190). This finding therefore indicates that self-reactivity, after all, might not be necessary for the antibody binding to the MPER and further characterisation of 10E8 might inspire a new wave of vaccination strategies that could finally lead to the elicitation of a MPER-specific neutralising response.

Chapter 5

A Pilot Study – Dampening of immune response to the immunodominant regions of the HIV-1 Env protein by ‘Antibody-shielding’

5.1 Introduction

As mentioned in the previous chapter, one major hurdle in HIV-1 vaccine design is the presence of immunodominant regions on the Env protein as they ‘distract’ the immune system from recognising the conserved regions that are targeted by known BNABs. To circumvent this problem, different strategies have been devised aiming to dampen the antibody response towards the immunodominant regions, e.g. the deletion of such regions on gp120 and gp41 protein (43, 76, 212, 322, 376, 378), or the masking of immunodominant epitopes by hyperglycosylation (133, 308, 309, 369, 370). Here, I describe the development of a novel immunofocusing technique that hypothesises the use of immunogen-derived antibodies for the shielding of immunodominant regions of the Env protein. This approach was designed so that the CD4 binding site (CD4bs) would remain available for antibody recognition after antibody-shielding. It was hoped that greater focus towards the CD4bs could be achieved through this approach, so that a CD4bs-specific response that resembles b12 and other CD4bs-specific BNABs could be elicited.

The principle of antibody-shielding is rather similar to the use of hyperglycosylated Env protein, where extra glycans were added to the immunodominant V1/V2 and V3 loops in an attempt to focus the antibody response towards the CD4bs (308, 309, 369, 370). However, while the CD4bs-specific response in these studies was retained, the antibody response towards all immunodominant variable loops was not completely dampened by the added glycans. In light of these results, I therefore proposed the use of immunogen-derived antibodies for epitope-masking as it was believed that an ‘antibody-shield’ could be a useful alternative to a ‘glycan-shield’: Firstly, the hyperglycosylation of the Env protein could in principle

provide additional epitopes for the elicitation of glycan-specific antibodies, e.g. 2G12 (110, 358, 362), or glycan-dependent antibodies, e.g. PG9 and PGT antibodies (270, 315, 343). In contrast, host-derived antibodies are true ‘self’ molecules and an antibody response against the Ab-shield is highly unlikely; Secondly, this approach prevents any reduction in Env folding efficiency that is caused by the additional glycan (218) and the addition of antibody-shield after protein folding means that the Env protein would be natively folded; Thirdly, the presence of the Fc domain of IgG antibodies in the Ab-shield may also enhance the antibody response against the antigen due to an increased transport to the lymph nodes and processing of complexed immunogen by antigen-presenting cells (APC) via the Fc/FcγR interaction (1, 184, 228, 414). For these reasons, the use of antibodies as a shielding device is an attractive option as the several benefits of the Ab-shield may encourage the elicitation of a more robust site-specific antibody response.

In this study, a 2-stage mouse immunisation regime was devised as a ‘proof-of-concept’ experiment (Fig. 5.1): the first-round immunisation was for the generation of shielding antibodies, while the second-round immunisation was for the assessment of the Ab-shielded immunogens *in vivo*. To ensure that the access to the CD4bs was not hindered by the Ab-shield, CD4-gp120 complexes were used as the first-round immunogen where the CD4bs was blocked by chemically crosslinking a soluble CD4 (sCD4) to gp120. Such CD4-gp120 complexes have previously been used in immunisation studies and the immune sera against this complex have demonstrated strong cross-reactivity towards the wildtype gp120 (99, 100, 121, 122). With the CD4bs blocked by sCD4, antibodies that bind directly or in close proximity to the CD4bs would not be elicited, and none of these antibodies would therefore be used in the construction of the Ab-shield. In addition, the use of CD4-gp120 complexes is also a better option than the CD4bs-defective (D368R mutation) gp120 because not all CD4bs-specific antibodies are sensitive to the D → R mutation, e.g. HJ16 (81, 241). Following the first-round immunisation, all IgG antibodies from the sera were purified and used for the construction of the Ab-shield. The shielded Env protein (Ab-gp120) was then

used in the second immunisation and the final antibody response was assessed to see if the elicitation of CD4bs-specific antibodies could be enhanced by this method.

The primary objectives of this study were:

- 1) To examine whether the antibody response towards the immunodominant regions of gp120 could be dampened by the Ab-shield;
- 2) To examine whether the presence of the Ab-shield could focus the antibody response towards the CD4bs so that a broadly neutralising, CD4bs-specific response could be elicited;
- 3) To identify any shortcomings associated with this technique in light of the immunisation results to assist further development and improvement of the technique.

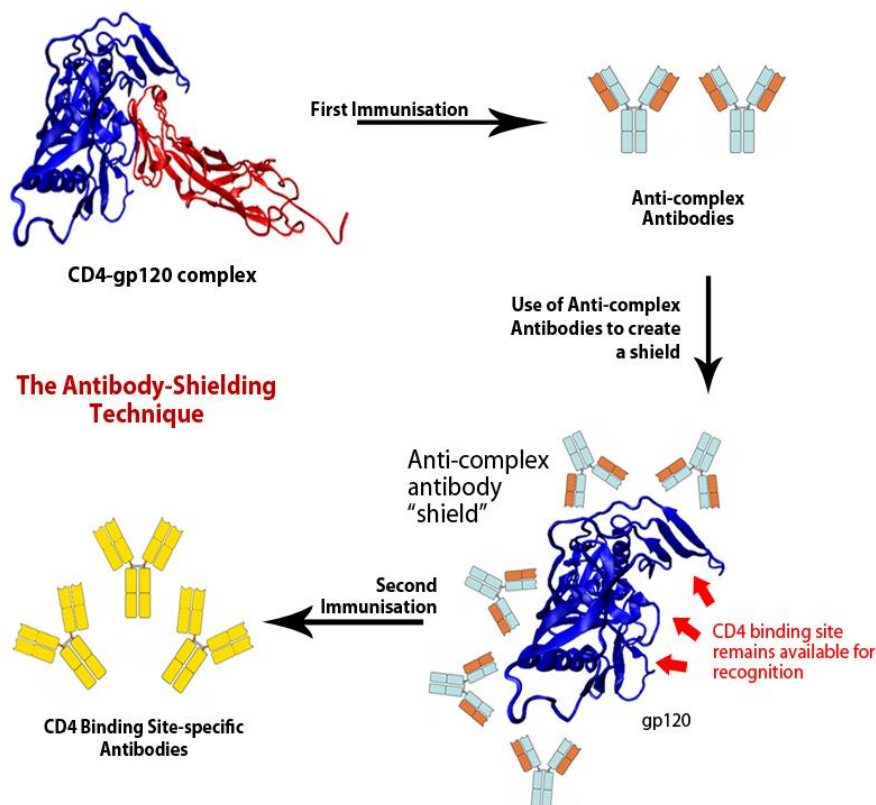


Figure 5.1 Illustration of the antibody-shielding technique

Both the crystal structures of the CD4-gp120 complex and gp120 protein was adapted from (189).

5.2 Results

The development of this antibody-shielding technique is described in 3 sections:

- Stage I** Preparation of the first-round immunogen and the first-round immunisation;
- Stage II** Production and Characterisation of the shielded gp120 (Ab-gp120) and the second-round immunisation;
- Stage III** Assessment of the final serum antibody response.

Preparation of first-round immunogen and the first-round immunisation

5.2.1 Production of the CD4-gp120 complex

Gp120 used in this study was based on the subtype B JRFL strain. The CD4-gp120 complex was synthesised by chemically crosslinking sCD4 and gp120 with BS³, and the final product was analysed with SDS-PAGE under reducing condition (Fig. 5.2). Protein staining with Coomassie blue revealed a single protein band at a size of roughly 160kDa, and Western blot probing with either anti-CD4 or anti-gp120 antibodies also showed a band of the same size. From these results, it can be concluded that the complex was composed of a 1:1 ratio of sCD4 (40 kDa) and gp120 (120 kDa), and that the 2 molecules were successfully crosslinked to each other since the complex did not disassemble under reducing condition (Fig. 5.2).

5.2.2 Characterisation of the CD4-gp120 complex

To ensure that the CD4bs was properly blocked in the CD4-gp120 complex prior to the first-round immunisation, the binding of a number of human anti-Env monoclonal antibodies (mAbs) to the CD4-gp120 complex was measured in an antibody-binding ELISA (Fig. 5.3). The following antibodies were included in this experiment: b12, b6 and F105, directed to the CD4bs (24, 52, 237); X5 and 17b, directed to the CD4-induced (CD4i) epitopes (234); 447-52D, directed to the V3 loop (147, 149). All mAbs were added at a saturating concentration, and the binding of these mAbs to CD4-gp120 complexes and wildtype

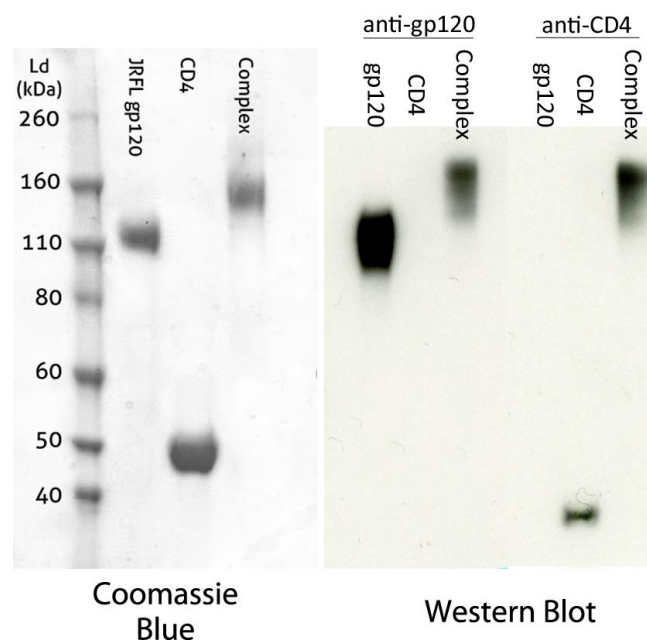


Figure 5.2 Validation of the CD4-gp120 complex by SDS-PAGE under reducing condition

Purified CD4-gp120 complex was analysed by SDS-PAGE. *(Left)* Coomassie blue staining of the SDS-PAGE. *(Right)* Western blot analysis with anti-gp120 cocktail (HIVIG and 447-52D) and anti-CD4 antibody as shown on top of each blot.

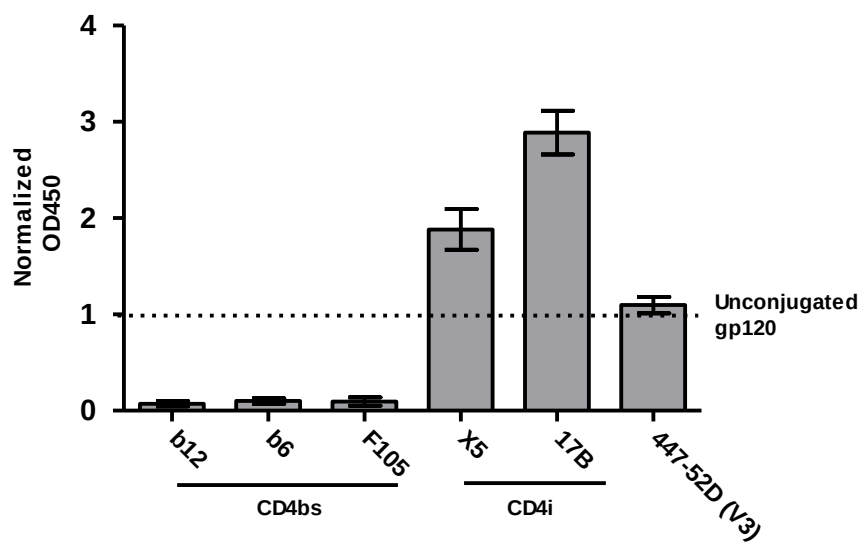


Figure 5.3 Characterisation of the CD4-gp120 complex

ELISA was performed to characterise the purified CD4-gp120 complexes by measuring the binding of various HIV-specific mAbs. All the OD₄₅₀ results were normalised with unconjugated gp120. Error bars represent the standard deviation obtained from triplicate samples.

gp120 proteins was measured. Subsequently, the result of wildtype gp120 proteins was used for the normalisation of the measurement of the CD4-gp120 complex. For the CD4-gp120 complex, the binding of sCD4 and all 3 CD4bs-specific antibodies was successfully abolished as the CD4bs was blocked in this complex. In contrast, the binding of 447-52D to the CD4-gp120 complex was no different to that of the wildtype gp120, thus indicating that these epitopes were not affected in the CD4-gp120 complex. Since gp120 of the complex was locked in the CD4-bound conformation, antibody binding to the otherwise-hidden CD4i epitopes was significantly enhanced in the CD4-gp120 complex.

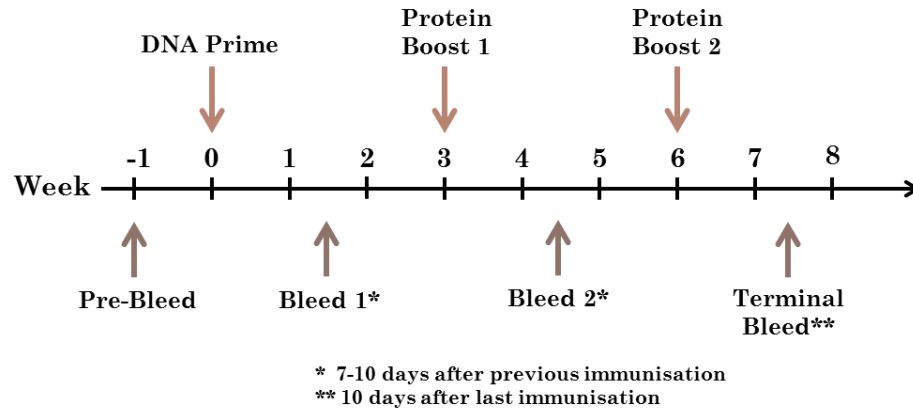
5.2.3 First-round mice immunisation

A group of 10 balb/c mice were immunised with the CD4-gp120 complex in order to generate the antibodies for shielding. A DNA-prime protein-boost regime was devised (Fig. 5.4): mice were DNA-primed with JRFL gp120 DNA at week 0, followed by 2 boosts with the CD4-gp120 complex 3 weeks apart. Bleeds were collected 1 week before the DNA-prime (week -1), and then 7-10 days after each inoculation. All mice were sacrificed 10 days after the final immunisation and terminal bleeds were collected. For the boosting of antibody response, the CD4-gp120 complex was co-administrated with Ribi adjuvant, which has previously been used in several immunisation studies with Env proteins (243, 293, 375).

Production and Characterisation of the Ab-gp120 and the second-round immunisation

5.2.4 Antibody binding titres following the first-round immunisation

All mouse sera were pooled together and an ELISA assay was used to measure the serum antibody titre against the CD4-gp120 complex over the course of the immunisations. Typical of a prime-boost regime (Fig. 5.5; black line), the priming of DNA did not elicit a detectable antigen-specific antibody response in the pooled mice sera. An antigen-specific response only started to become detectable after the first protein boost and it continued to rise over the course of the immunisation. At the end of the immunisation, a total IgG endpoint titre of $\sim 10^8$ (reciprocal serum dilution) was obtained. In addition, the antibody



	Number of Mice	DNA Prime	Protein Boost	Adjuvant
CD4-gp120 complex	10 female (6-8 weeks old)	100µg JRFL gp120 DNA (IM)	10µg CD4-gp120 complex (SC)	Ribi adjuvant

IM – Intramuscular; SC – Subcutaneous

Figure 5.4 First-round mouse immunisation

A group of 10 mice were immunised with the purified CD4-gp120 complexes. (Top) The timeline of the immunisation displaying the time points of each immunisations and bleeds. All mouse sera from the terminal bleed were pooled together and mouse IgG antibodies were subsequently purified. (Bottom) Table summarising the details of the immunisation, including the dosage, immunisation route and the adjuvant used.

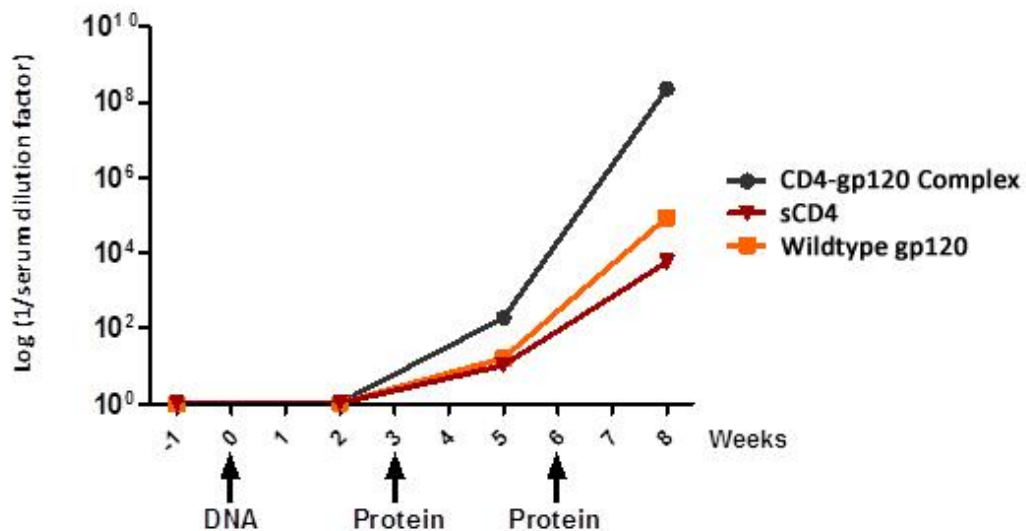


Figure 5.5 Serological analysis of the first-round immunisation

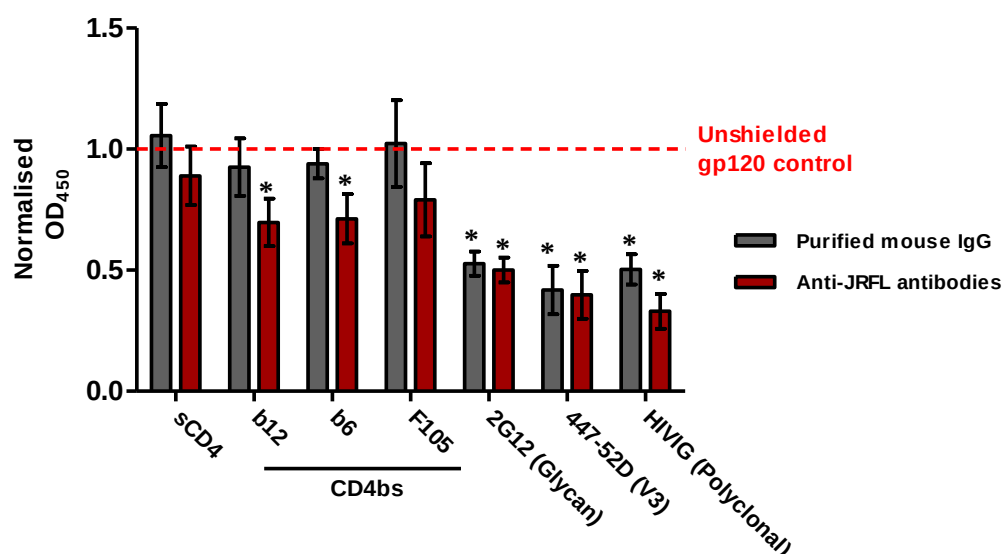
The antigen-specific response of the pooled sera sample (●) at each time point was analysed and reported as end-point titre (reciprocal serum dilution). The antibody response against sCD4 (▼) and gp120 (■), the 2 constituents of the CD4-gp120 complex, was also measured. The time-point of each bleed is labelled in the x-axis.

responses against the 2 constituents of the antigen – sCD4 and gp120 – were also measured separately and an endpoint titre of $\sim 10^4$ and $\sim 10^5$ were obtained for the respective protein (Fig. 5.5; red and orange line respectively). The lower antibody response towards the wildtype gp120 therefore indicates that a considerable amount of the antibodies were specific to the CD4-bound conformation of the gp120 and do not recognise the wildtype gp120.

5.2.5 Characterisation of the IgG Ab-shield by antibody-blocking ELISA

For the *in vitro* characterisation of the Ab-shield, mouse IgG antibodies were purified from the pooled sera and used for the coating of gp120 in an antibody-blocking ELISA. The purpose of this assay was to assess whether the CD4bs would remain exposed upon antibody-shielding. Here, purified mouse IgG antibodies were added to gp120-coated ELISA so that an Ab-shield could be formed around the Env protein prior to the addition of sCD4 and human anti-Env mAbs. If the binding site for sCD4/mAb was masked by the Ab-shield, their binding towards gp120 would be affected and this would be reflected in the reduction of sCD4 or mAb binding in the final results. In a separate setting, polyclonal anti-JRFL antibodies were also used for the construction of the Ab-shield, and the Ab-shield created by both sets of antibodies was compared. The human mAb used in this assay included: b6, b12 and F105 (CD4bs), 447-52D (V3) and 2G12 (glycan). The inclusion of sCD4 and 3 CD4bs-specific antibodies should provide a good assessment of the accessibility of the CD4bs as each of these molecules binds differently to the CD4bs (75). In addition, the polyclonal HIVIG antibodies, which recognise multiple regions on the Env protein (88), were also included in the ELISA to provide a more general view of the level of shielding on gp120. In this assay, unshielded gp120 proteins were used as a positive control and for the normalisation of the final results.

For the Ab-shield created using the purified IgG antibodies, the binding of sCD4 and CD4bs-specific mAbs was not affected by the Ab-shield, meaning that the CD4bs on the shielded gp120 was well-exposed for antibody recognition (Fig. 5.6). Outside the CD4bs, the epitope-masking potential of this Ab-shield



	% Reduction*						
	sCD4	b12	b6	F105	2G12	447-52D	HIVIG
Purified mouse IgG	-	7.6	6.0	-	47.3	58.2	49.7
Anti-JRFL antibodies	11.0	30.3	28.8	20.9	49.9	60.2	67.0

*Compare to unshielded control

Figure 5.6 Characterisation of the shielding antibodies generated in the first-round immunisation

Purified mouse IgG antibodies (Grey) and anti-JRFL antibodies (Red) were used for the coating of gp120 proteins. The binding of sCD4 and different anti-HIV antibodies was then assessed by ELISA. Uncoated gp120 proteins were used as a control and standard for the normalisation of all OD₄₅₀ results. The % reduction in binding of sCD4 and antibodies was listed in the table below. Error bars represent the standard deviation obtained from triplicate samples. Asterisks (*) were used to indicate statistical significance ($p < 0.05$). This was calculated using one-way ANOVA with Bonferroni post-hoc test.

was demonstrated with the binding of 447-52D and the polyclonal HIVIG reduced by about 50% in the shielded gp120, thus indicating that the binding site for these mAbs were likely to be masked by the Ab-shield. The reduction of 2G12 binding was rather interesting since glycan-specific antibodies are rarely elicited following Env protein immunisation (369, 370). Instead of directly masking the epitopes, this was more likely to be due to the steric hindrance caused by the nearby shielding antibodies, and therefore demonstrates a secondary shielding effect exerted by the Ab-shield. For the Ab-shield constructed by anti-JRFL antibodies, a similar reduction of 447-52D, 2G12 and HIVIG binding was detected in this group, but a considerable reduction of b12, b6 and F105 binding was also observed, thereby indicating that the access

to the CD4bs was blocked by the Ab-shield. Hence, the use of the CD4-gp120 complex for the generation of shielding antibodies was necessary to ensure that the CD4bs remain accessible after antibody-shielding. Moreover, the reduced binding of a number of mAbs also serves as a first demonstration that the use of Ab-shield could indeed be used for the masking of epitopes on gp120 protein.

5.2.6 Further evaluation of the accessibility of CD4bs by Surface Plasmon Resonance

To provide further evidence that the accessibility of the CD4bs was not affected by the Ab-shield, surface plasmon resonance (SPR) was performed to support the antibody-blocking ELISA above as this allows the binding kinetics of sCD4 and b12 to be measured at greater depth (Fig. 5.7). Similar to the previous assay, both the purified mouse IgG antibodies and anti-JRFL antibodies were used for the coating of gp120 separately (shaded grey in Fig. 5.7), and the unshielded gp120 was used as the positive control. After the coating of gp120 has plateaued, either sCD4 or b12 was injected onto the sensor chip and the binding of both proteins to the shielded gp120 was monitored (shaded green in Fig. 5.7).

The first observation that could be made from this SPR study was that the level of gp120 coating with mouse IgG antibodies was less than that of the anti-JRFL antibodies (Fig. 5.7). Such discrepancy was not surprising as a portion of the Env protein was blocked in the CD4-gp120 complex used to generate the shielding antibodies, meaning that there is less exposed surface on gp120 for antibody binding. However, the consequence of this was rather significant. When mouse IgG antibodies were used, the binding of sCD4 and b12 did not appear to be affected by the presence of the Ab-shield as they demonstrate a binding kinetics that resembled the unshielded control. However, the binding of both b12 and sCD4 was significantly reduced when the anti-JRFL antibodies were used. These results were therefore in line with the antibody-blocking ELISA and confirmed that the Ab-shield created by the purified mouse IgG antibodies does not affect the accessibility of the CD4bs of gp120.

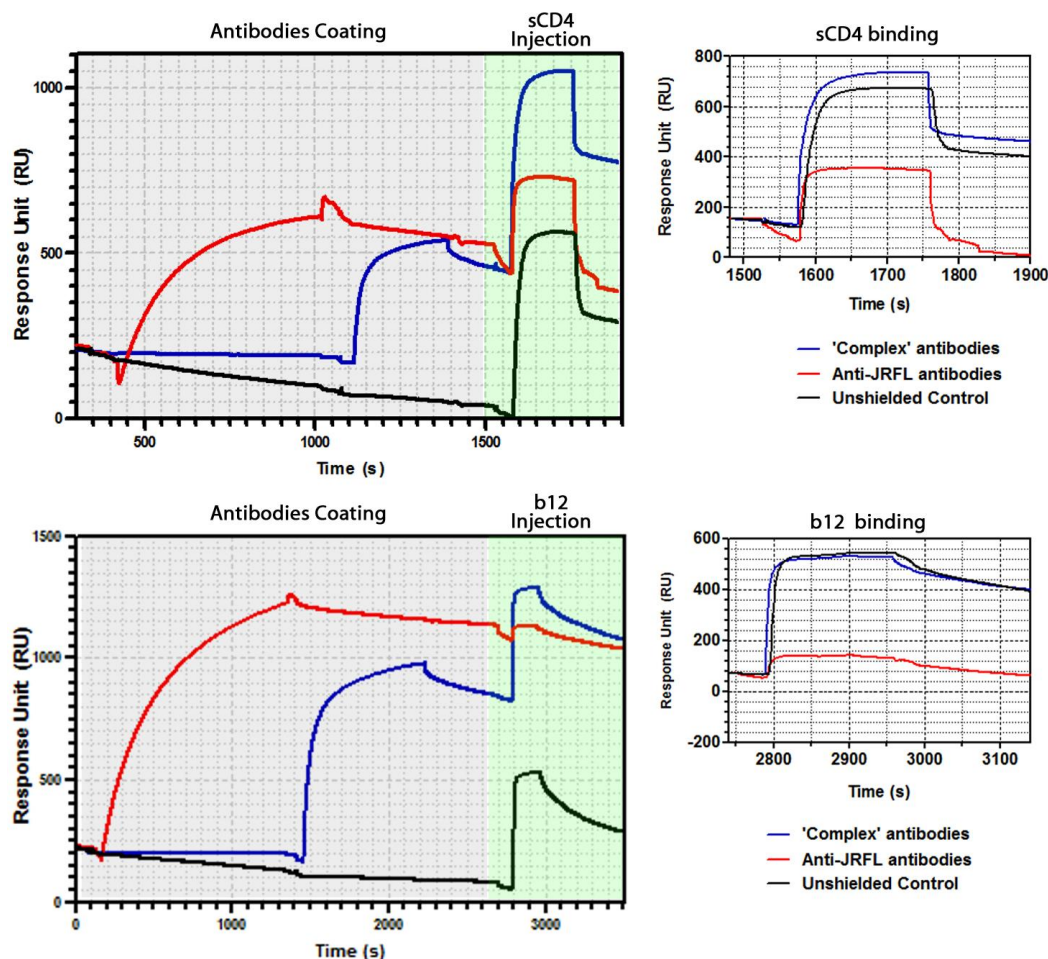


Figure 5.7 Further evaluation of the accessibility of the CD4bs after antibody-shielding by surface plasmon resonance (SPR)

Purified mouse IgG antibodies ('complex') and polyclonal anti-JRFL antibodies (anti-JRFL) were used to coat pre-immobilised gp120 proteins on the SPR chip (*shaded grey*), and uncoated gp120 proteins were used as a control. Either sCD4 or b12 was then injected onto the flow cells. The binding of sCD4 and b12 to the coated/uncoated proteins were measured and compared based on the increase in response unit (*shaded green*).

5.2.7 Stability of the Ab-shield on the coated gp120

Although the potential of antibody-shielding was demonstrated in the ELISA and SPR, the stability of the Ab-shield on gp120 was a concern prior to the second-round immunisation since any dissociation of the shielding antibodies would allow the re-exposure of undesired epitopes for antibody recognition. For this reason, it was therefore important to examine whether the shielding antibodies should be crosslinked to gp120 in order to maximise the effect of the Ab-shield. To test this, JRFL gp120 proteins were mixed with the purified antibodies in different Env:Ab ratios (ranging from 1:1-1:16), with and without

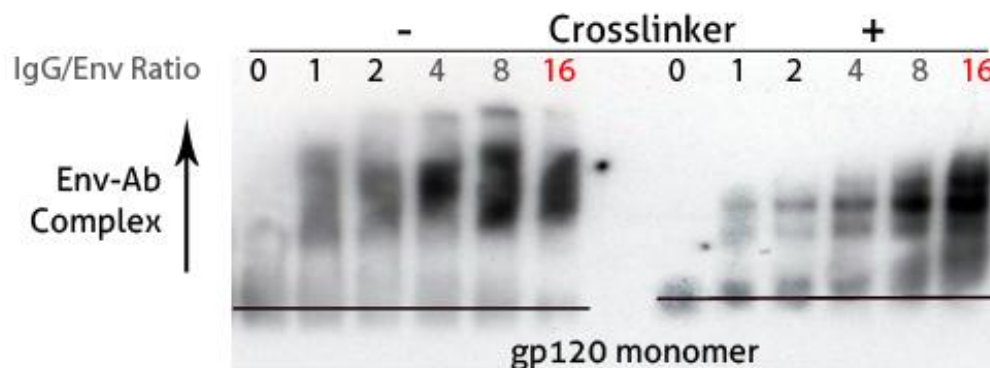


Figure 5.8 Evaluating the stability of the Ab-gp120 by Bandshift BN-PAGE

Bandshifting BN-PAGE was carried out to investigate the effect of crosslinking on the stabilisation of the shielding antibodies on gp120 protein. Shielding antibodies were mixed at various IgG:Env ratios, as indicated by the marking above each lane. The 1:16 ratio was marked in red since it was the ratio used for the final preparation of shielded-gp120. BS³ crosslinker was added to one group (+) but not the other (-). Any bands that are above gp120 control in the BN-PAGE are Ab-gp120 complexes.

BS³ crosslinker. Samples were then analysed by bandshifting Blue Native-PAGE (BN-PAGE) and Western blot using a cocktail of anti-HIV antibodies (b12, 447-52D and HIVIG) (Fig. 5.8).

Based on the premise that antibody-gp120 complexes (Ab-gp120) migrate more slowly than unliganded gp120 proteins, any protein bands that are above gp120 control were defined as Ab-gp120 (Fig. 5.8). Overall, the formation of Ab-gp120 was notable and more Ab-gp120 could be found whenever a higher Env:Ab ratio was used. However, without BS³, the monomeric gp120 band was consistently visible in all Env:Ab ratios meaning that the Ab-shield was not particularly stable, and unliganded gp120 proteins were constantly present in these mixtures. In contrast, when BS³ was added, a complete depletion of the gp120 proteins was observed at the highest Env:Ab ratio (1:16), thus suggest that the Ab-shield was stabilised by the crosslinking. Furthermore, the Ab-gp120 bands appeared to be more discrete when compared to the uncrosslinked samples. As a result, the crosslinking of the Ab-shield seems to be necessary in order to avoid any dissociation of shielding antibodies *in vivo*, and therefore required further investigation in the second-round immunisation by the use of both crosslinked and uncrosslinked Ab-gp120.

5.2.8 Production of Antibody-shielded immunogens

In light of the BN-PAGE results, 2 Ab-gp120 immunogens – with and without crosslinking – were prepared for the second-round immunisation. The production of Ab-gp120 began with the mixing of gp120 and purified antibodies at a highest-possible ratio of 1:16. For the crosslinked samples, Ab-gp120 complexes were purified from unliganded gp120 proteins and antibodies by size exclusion chromatography. The eluted fractions were analysed with Western blot using either anti-mouse antibodies for the detection of Ab-shield, or a cocktail of anti-gp120 antibodies (b12, 447-52D and HIVIG) for the detection of gp120 protein (Fig. 5.9). Both Western blots displayed a similar profile thus confirming that the higher-order complexes were Ab-gp120 rather than gp120-gp120 or IgG-IgG complexes. Further purification was carried out until all unliganded gp120 or IgG antibodies were removed from the final product. The final purified product was then analysed by SDS-PAGE and a mixture of unevenly-shielded Ab-gp120 complexes were observed (Fig. 5.10). The stoichiometry of gp120 (120kDa) and IgG antibodies (150kDa) of the Ab-gp120 complexes could be estimated based on the size of each protein band, e.g. the bottom band represents the binding of 1 gp120 protein with 1 IgG antibody, while the next band represents the binding of 1 gp120 protein with 2 IgG antibodies and so on. Furthermore, the smearing of bands observed under reducing condition indicates that the Ab-shield was stabilised by various crosslinking reactions between gp120 protein and the IgG antibodies. For the preparation of the uncrosslinked Ab-gp120, it was observed in a preliminary study that the Ab-gp120 complex would dissociate during the size exclusion purification step. For this reason, the uncrosslinked Ab-gp120 complexes were immunised as an antibody and gp120 mixture.

5.2.9 The second-round mouse immunisation

In the second-round immunisation (Fig. 5.11), 3 groups of 5 balb/c mice each were immunised with wildtype gp120 (control; group 1), uncrosslinked shielded-gp120 (gp120_{UNC-S}; group 2) and crosslinked shielded-gp120 (gp120_{C-S}; group 3). All mice received 3 protein inoculations at week 0, 3 and 6. To ensure that the equivalent amount of Env proteins was immunised across all groups, the Env content of the

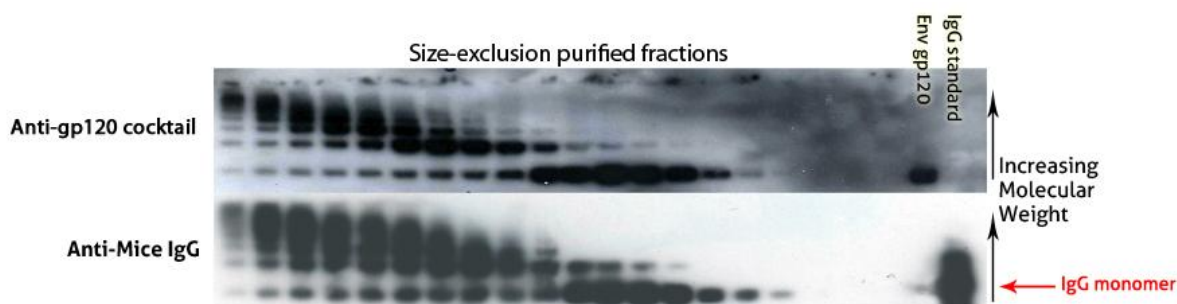


Figure 5.9 Western blot analysis of the eluted fractions from the size exclusion chromatograph

Eluted fractions from the size exclusion chromatograph was analysed by Western blot probing with a cocktail of anti-Env antibodies (b12, 447-52D & HIVIG) and anti-mouse antibodies. Env gp120 and mouse IgG standard was used as a control and as a size reference.

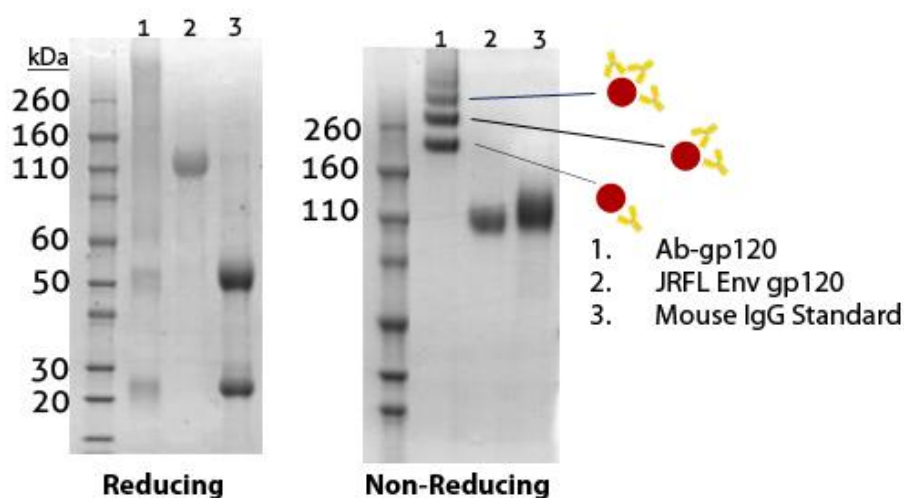


Figure 5.10 SDS-PAGE analysis of the final purified Ab-gp120

The final purified Ab-gp120 was analysed by SDS-PAGE under (*left*) reducing and (*right*) non-reducing condition. JRFL Env gp120 and mouse IgG standard were used as a control. Shown also is possible Env:IgG stoichiometry of the final Ab-gp120 (*red closed circle* – Env gp120; *yellow IgG* – shielding antibodies).

gp120_{UNC-S} and gp120_{C-S} was standardised based on sCD4 binding by ELISA (see Chapter 2, section 2.3.3.12). Bleeds were collected 1 week before the first protein injection and then 7-10 days after each immunisation. All mice were sacrificed 10 days after the final immunisation and terminal bleeds were collected. As with the first-round immunisation, all proteins were co-administrated with Ribi adjuvant for the boosting of the antibody response.

Assessment of the final serum antibody response.

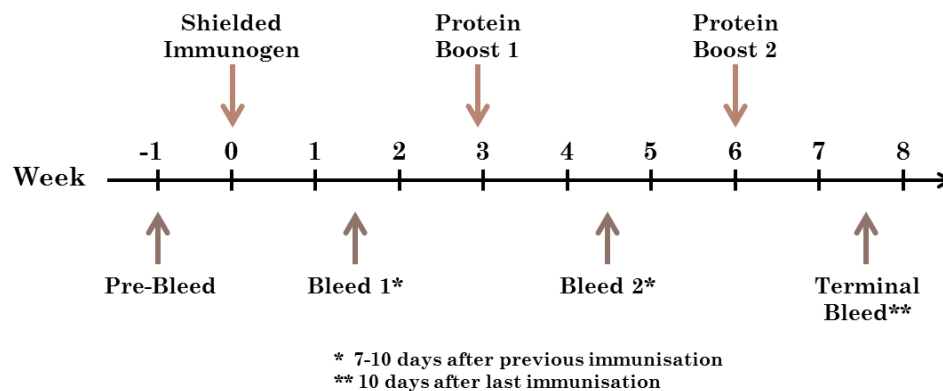
5.2.10 Antibody titres in the second-round immunisation

At the end of the second-round immunisation, all sera from the same group were pooled together due to limited sera obtained from each mouse. The antibody response against the wildtype gp120 was measured for each group (Fig. 5.12). All immunised groups responded to the immunisation with the anti-gp120 antibody response became detectable after the first protein immunisation and continued to rise in subsequent injections. At the end of the immunisation, serum end-point titres in the range of 10^4 - 10^5 were obtained for the 3 immunised groups.

5.2.11 Mapping of serum antibody response by cross-competition ELISA

To explore whether the elicitation of site-specific antibodies was affected by the Ab-shield, cross-competition ELISA was used for the mapping of serum antibody response where sCD4 and human mAbs were used to compete with the mouse sera for the binding of specific sites on the Env protein. The results were reported as IC_{50} , which is defined as the reciprocal dilution of the mice sera that caused 50% inhibition of the mAb binding, i.e. the higher the IC_{50} , the stronger the site-specific antibody response. In this assay, the mAbs used included b12, b6 and F105 (CD4bs), 447-52D (V3), 697-D (V2) and 2G12 (glycan). The pre-bleed sera of all groups were used as the baseline as no antigen-specific antibodies could be present at that time. In all cases *except* 2G12, a significantly greater competition with sCD4 and mAbs was observed in the terminal bleed sera as compared to the pre-bleed sera, meaning that CD4bs-, V1/V2- and V3-specific antibodies were elicited in all groups over the course of immunisation (Fig.5.13).

Overall, there is no significant difference in the results obtained from gp120_{UNC-S} group and the control group as the immune sera of both groups competed equivalently with sCD4 and other mAbs (Fig. 5.13). This therefore strongly indicates that, as postulated earlier in this study, the Ab-shield of gp120_{UNC-S} groups might have dissociated from gp120, and crosslinking is therefore required for the Ab-shield to remain attached to the Env protein *in vivo*. In contrast, with a stabilised Ab-shield, the serum



Group	Immunogen	Number of Mice	Dosage	Route	Adjuvant
1	Unshielded wildtype gp120 (Control)	5 female (6-8 weeks old)	10µg Env- equivalent	Subcutaneous (Neck region)	Ribi Adjuvant
2	Uncrosslinked Ab-gp120 (gp120 _{UNC-S})				
3	Crosslinked Ab-gp120 (gp120 _{C-S})				

Figure 5.11 Second-round mouse immunisation

3 groups of 5 mice were immunised with unshielded wildtype gp120, uncrosslinked Ab-gp120 (gp120_{UNC-S}) and crosslinked Ab-gp120 (gp120_{C-S}) respectively. (Top) The timeline of the immunisation displaying the time points of each immunisations and bleeds. (Bottom) Table summarising the details of the immunisation, including the dosage, immunisation route and the adjuvant used.

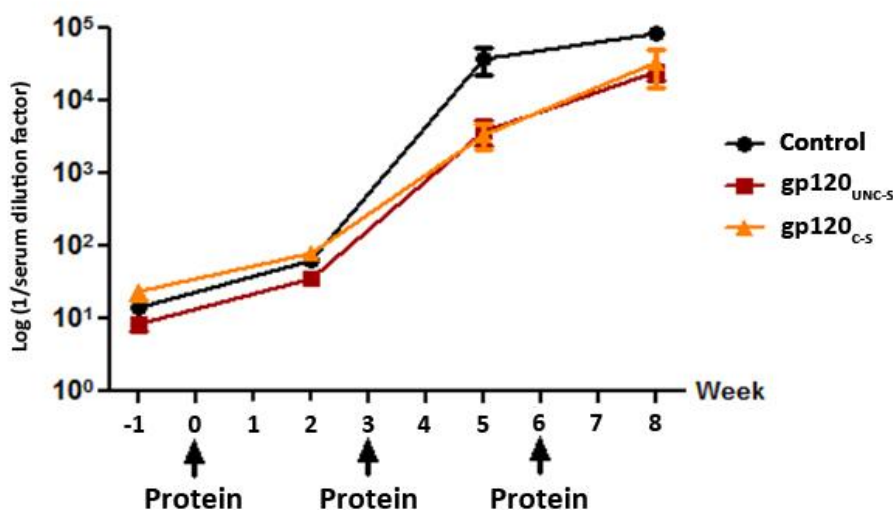


Figure 5.12 Serological analysis of the mouse sera in the second-round immunisation

For all 3 groups, the antigen-specific (anti-gp120) response of the pooled sera sample at each time point was analysed and recorded as end-point titre (reciprocal serum dilution). The time-point of each bleed is labelled in the x-axis.

antibody response towards the variable loops in gp120_{C-S} group was significantly different to the control and gp120_{UNC-S} groups. Serum competition with the V2-specific antibody 697-D was considerably stronger in gp120_{C-S} group than in the control group, while the competition with the V3-specific antibody 447-52D was weaker in gp120_{C-S} group. Hence, the V2-response was boosted while the V3-response was dampened by the Ab-shield. In addition, a trend of a slightly stronger CD4bs-specific response was also observed in gp120_{C-S} group, although statistical significance was not obtained. This low level of CD4bs refocusing therefore highlights the sub-dominance of this site in comparison to the V1/V2 loops, despite the shielding of key immunodominant sites (e.g. V3 loop) of the Env protein.

5.2.12 Further mapping and quantitative analysis of site-specific response

The site-specific responses were also mapped by measuring the serum reactivity towards several mutated JRFL gp120 proteins, including the CD4bs-defective gp120 (D368R-gp120), the V1/V2 loop-deleted (Δ V1/V2) gp120 and the V3-loop deleted (Δ V3) gp120 (Fig 5.14). One advantage of this assay is that it allows the *entire* region to be examined, rather than focusing on a single epitope as in the cross-competition ELISA. By comparing the EC₅₀ (i.e. the reciprocal serum dilution that accounts for 50% of the maximum binding) of the serum against different gp120 proteins, the relative proportion (%) of the site-specific response can be estimated using the following equation: $(1 - \text{mutant-EC}_{50}/\text{wildtype-EC}_{50}) \times 100\%$. Using the data in the control group as an example (Fig. 5.15) – with an EC₅₀ of 300.7 and 277.8 obtained for the binding of the wildtype and D368R-gp120 respectively, it can be estimated that 7.6% of the serum antibody response in the control group was specifically targeting the CD4bs $[1-(277.8/300.7) \times 100\%]$.

Again, the serum antibody response was highly similar in the control and gp120_{UNC-S} group with the V3-response dominated at over 40% and V1/V2-response at approximately 20% (Fig. 5.15). Only a limited amount of the CD4bs-response was detected in both groups – 7.6% for the control group and 6.4% for gp120_{UNC-S} group. For gp120_{C-S} group, as seen in the cross-competition ELISA, a significant

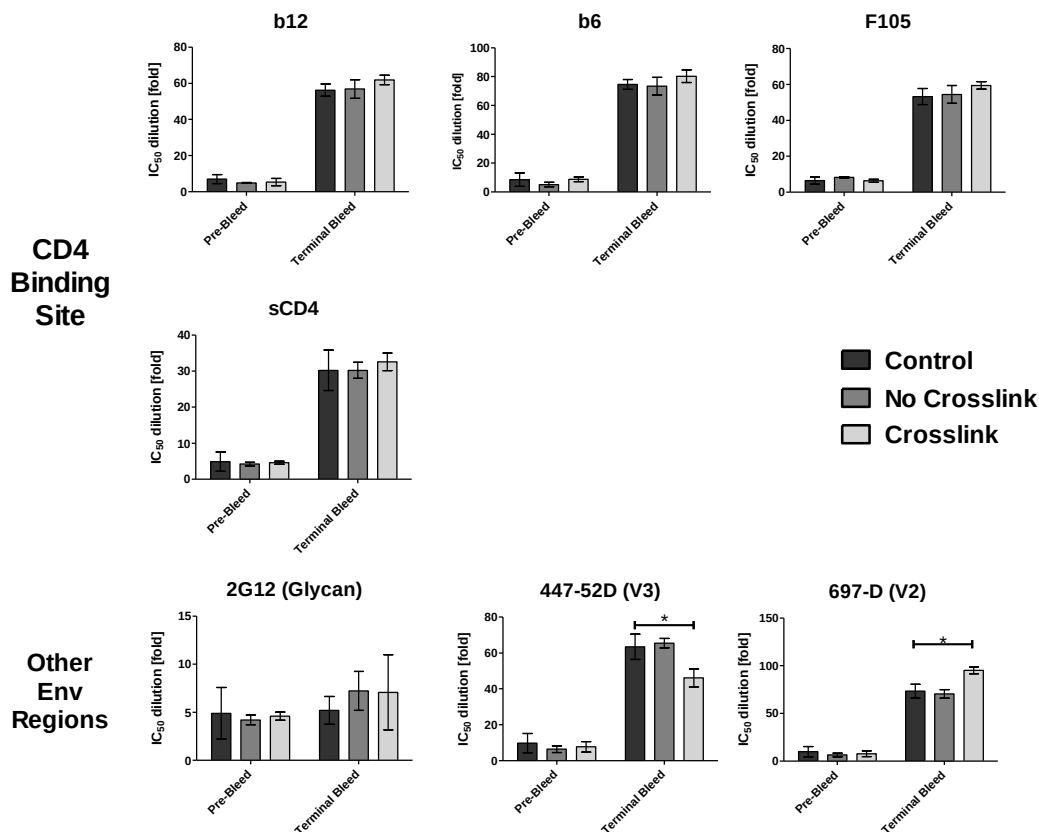


Figure 5.13 Mapping of serum antibody response by cross-competition ELISA

Serum competition with sCD4 and various human anti-HIV mAbs were measured for the mapping of antibody response towards specific region of the Env protein. Asterisks (*) show cases where statistical significance was achieved ($p < 0.05$). All error bars represent standard deviations obtained from triplicate samples. Statistics were calculated using One way ANOVA with a Bonferroni post-hoc test.

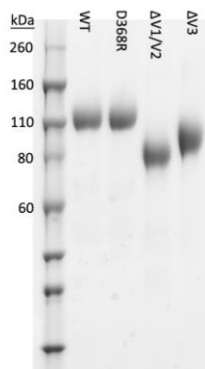
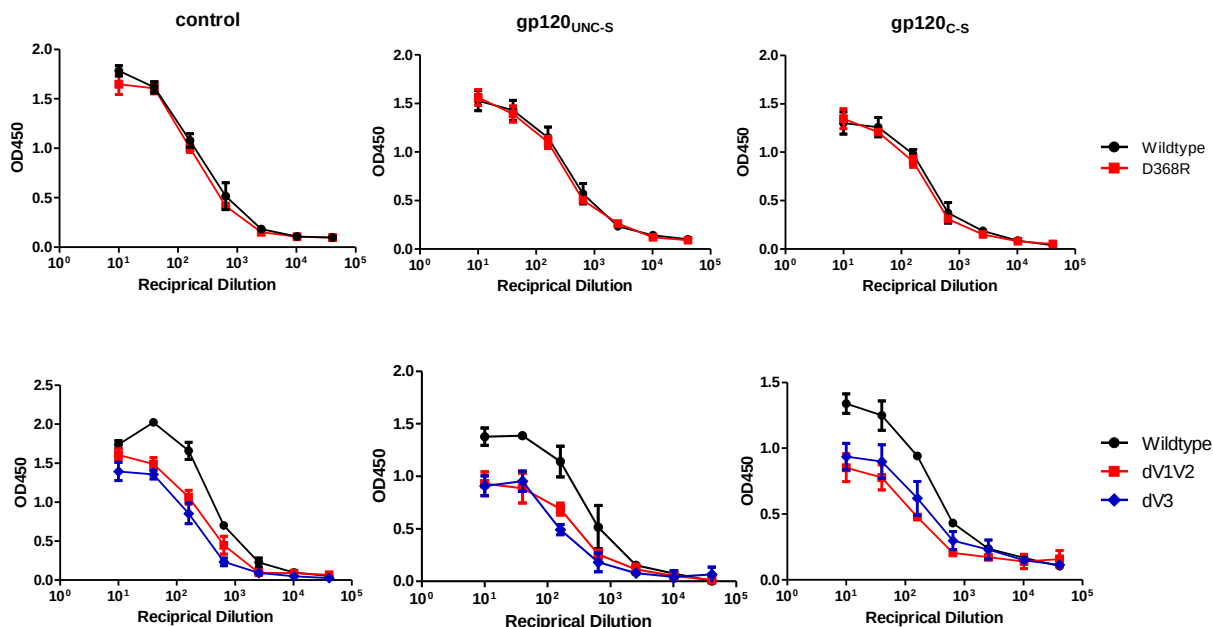


Figure 5.14 SDS-PAGE of various JRFL mutant gp120 proteins

Purified JRFL mutant gp120 proteins were analysed by SDS-PAGE under non-reducing condition and proteins were made visible by Coomassie blue staining. The wildtype gp120 is abbreviated as 'WT', CD4bs-defective gp120 as 'D368R', V1/V2 loop-deleted gp120 as 'ΔV1/V2' and V3 loop-deleted gp120 as 'ΔV3'.

reduction in the V3-response was observed as only 27.9% of the serum antibody response was targeting this region. Such dampening of the V3-specific response was mirrored with an increased V1/V2-specific response at 41.3%, thus making it the primary target for the serum antibodies in the presence of the Ab-shield. Once again, the increase in the CD4bs-specific antibodies was insignificant (7.6% → 8.9%). In any case, these results were in good agreement with the cross-competition ELISA by clearly demonstrating a shift of immunodominance from V3 to V1/V2 in the presence of a functional Ab-shield, and therefore highlighted the epitope-masking potential of the antibody-shielding technique.

Figure 5.15 on next page



	Wildtype		Mutant		
Site	Protein	EC ₅₀ ^A	Protein	EC ₅₀ ^B	% site-specific response
Control					
CD4 binding site	Wildtype gp120	300.7	D368R	277.8	7.6
V1/V2 loop		526.2	ΔV1/V2	415.7	20.9
V3 loop			ΔV3	299.9	43.0
Gp120 _{UNC-S} (Uncrosslinked)					
CD4 binding site	Wildtype gp120	361.5	D368R	338.4	6.4
V1/V2 loop		441.1	ΔV1/V2	334.3	24.2
V3 loop			ΔV3	266.9	39.5
Gp120 _{C-S} (Crosslinked)					
CD4 binding site	Wildtype gp120	251.2	D368R	228.8	8.9
V1/V2 loop		450.3	ΔV1/V2	264.3	41.3
V3 loop			ΔV3	324.7	27.9

The calculation of the final % site-specific response: $1 - (B/A) \times 100\%$.

Figure 5.15 Further mapping and quantitative analysis of serum antibody response

For further mapping of the serum antibody response, serum binding to the wildtype gp120 (●) and various mutant gp120 (D368R ■; ΔV1/V2 ■ and ΔV3 ◆) was measured. (Top) Raw data obtained for the sera binding to various mutant gp120. The error bars represent the standard deviation obtained from triplicate samples. (Bottom) Table summarising the raw data displaying the EC₅₀ determined in each case. % site-specific response was calculated using the formula listed at the bottom of the table.

5.3 Discussion

In this chapter, I describe the development of a novel immunofocusing technique with the hypothesis that immunogen-derived antibodies could be used for the masking of immunodominant epitopes of the Env protein. Through this process, it was hoped that a greater focus of host immune response to the immunorecessive CD4 binding site (CD4bs). The assessment of this technique was focused mainly on addressing 3 key questions on: (i) whether crosslinking of the Ab-shield is required to maximise the effect of the Ab-shield, (ii) whether the Ab-shield can reduce the antibody response towards undesired epitopes on the Env protein, especially the V1/V2 and V3 loops, and (iii) whether an improved targeting to the CD4bs could be obtained in presence of antibody-shielding.

The development of this technique started with the generation of shielding antibodies from the immunisation of a CD4-gp120 complex (*stage I*), and the epitope-masking capability of the purified mouse IgG antibodies was subsequently examined *in vitro* by ELISA and SPR (*stage II*) (Fig. 5.6 & 5.7). By coating gp120 proteins with the purified mouse IgG antibodies, the binding of non-CD4bs-specific monoclonal antibodies (mAbs) (e.g. 2G12, 447-52D and HIVIG) was impeded by the Ab-shield, while the binding of sCD4 and CD4bs-specific mAbs (e.g. b12, b6 and F105) remained unaffected. Hence, the shield constructed using the purified mouse IgG antibodies selectively masked regions outside the CD4bs but not to the CD4bs itself. Although this observation was encouraging, one particular concern regarding the immunisation of antibody-shielded gp120 (Ab-gp120) proteins was that the Ab-shield might dissociate from gp120 protein *in vivo*, thus allowing the re-exposure of previously-masked epitopes for antibody recognition. Indeed, notable dissociation of Ab-shield was observed in the bandshifting BN-PAGE analysis (Fig. 5.8) and the crosslinking of Ab-shield onto gp120 appeared to be necessary to prevent the Ab-shield from dissociating. Hence, the benefit of the crosslinking of the Ab-shield was assessed in the subsequent immunisation by immunising with 2 different Ab-gp120 proteins – one with an uncrosslinked Ab-shield (gp120_{UNC-S}) and the other with a crosslinked Ab-shield (gp120_{C-S}) (*stage III*).

The serum antibody response of all groups was subsequently mapped by cross-competition ELISA and by measuring serum binding to various mutant gp120 proteins (Fig. 5.13 & 5.15) and the results of both assays were in good agreement with each other. For gp120_{UNC-S}, the antibody response elicited against this protein was no different to the unshielded gp120 control, with the V3 loop being the most immunodominant region of gp120, the V1/V2 loop being the secondary antibody target and only a limited amount of antibodies were targeting the CD4bs. Such dominance of the V3-response is typically seen in Env immunisation (182, 369, 370) and with a wildtype-like immunogenicity demonstrated by gp120_{UNC-S} protein, this strongly indicates that the Ab-shield might have dissociated from gp120 *in vivo*. Indeed, the importance of crosslinking the Ab-shield to the Env protein was reflected in the immunisation of gp120_{C-S} as a rather striking change in the serum antibody response was observed. In the presence of a stabilised Ab-shield, the V1/V2-response was enhanced and became the primary antibody target (20.9% → 41.3%), while the supposedly dominant V3-response was reduced by almost 50% (43.0% → 27.9%). In addition, a slight increase in the CD4bs-specific response was also seen (7.6% → 8.9%) even though statistical significance could not be obtained. Although a significant improvement of the CD4bs-response was not achieved in this study, the reduction of the V3-specific response and the increase in the V1/V2-specific response are clear indications that the dampening of responses to undesired epitopes on gp120 is achievable with this novel ‘antibody-shielding’ technique.

Although the dampening of the V3-specific response was a promising start to the development of this novel technique, the increase of antibodies to the V1/V2 loop rather than to the CD4bs was rather disappointing. In light of the serum-mapping results in this study, the inability to completely dampen the V1/V2- and V3-specific response is a major setback of the current approach, and further development of this technique must therefore focus on improving the masking ability of the Ab-shield. For this reason, the current approach of antibody-shielding was evaluated. Firstly, one major improvement that could be implemented in the future is to use only the maximally-shielded Ab-gp120 protein for immunisation. As shown in Fig. 5.10, a mixture of unevenly-shielded Ab-gp120 complexes were included in this study, and

although it was not determined how this has affected the final result, the inadequate shielding in the lightly-shielded Ab-gp120 complexes would no doubt diminish the effect of this technique. It is believed that the amount of shielding antibodies obtained from 10 mice were insufficient for the construction of a proper Ab-shield, and the use of rabbits as the animal model in the future would be beneficial as more shielding antibodies could be obtained from each animal. By using a larger quantity of shielding antibodies, it is hoped that a higher Env:Ab ratio could be used in the construction of Ab-gp120 immunogens, thereby allowing the formation of a larger proportion of the highly-shielded immunogens. Secondly, another change that might improve the current approach is the use of smaller antibody fragments (e.g. Fabs) for shielding instead of the full IgG antibody. With the Fabs being smaller and more compact than the rigid ‘Y-shape’ IgG antibodies, this means that more shielding antibodies could be accommodated around the Env molecule and allows the formation of a more densely-packed Ab-shield. Additionally, the reduced size of the overall Ab-shield might exert less steric hindrance at the B cell surface which allows better access to the CD4bs on the Env protein.

In conclusion, this pilot study was a useful first step in the development of this novel immunofocusing approach as it highlights the potential of this technique in dampening the immune response towards undesired epitopes on the Env protein. Although the ideal result was not achieved, this study allows the identification of a number of shortcomings in the current protocol that may aid further development of this technique. Further work was focused on improving the Ab-shield so that all responses towards the immunodominant variable regions could be eliminated. The results of the follow-up study are presented in ***Chapter 6*** of this thesis.

Chapter 6

Further optimisation of the antibody-shielding technique for the elicitation of a CD4bs-specific antibody response

6.1 Introduction

In the previous chapter, I reported the results of a ‘proof-of-concept’ (‘POC’) immunisation study with an antibody-shielded gp120, where it was hypothesised that immunogen-derived antibodies could be used for the masking of undesired epitopes on gp120 protein (e.g. V1/V2 and V3 loops), and that the host immune response could then be focused towards the immuno-recessive CD4 binding site (CD4bs) (see Chapter 5, Fig. 5.1). It was hoped that this would lead to greater focus towards the CD4bs thus leading to an improved CD4bs-specific response. Results of the POC study demonstrated that the masking of epitopes could be successfully achieved by the use of an antibody-shield (Ab-shield). However, an increased response towards the V1/V2 loop indicates that not all immunodominant epitopes were sufficiently masked. Further improvements to this approach are therefore necessary to significantly improve the performance of the Ab-shield.

To optimise the level of shielding, 2 major changes to the previous approach were implemented in this study with the aim of improving the overall quantity and quality of the shielding antibodies. First, rabbits were used for the immunisation instead of mice so that a greater amount of immune sera (i.e. purified shielding antibodies) could be obtained per animal, and used for the construction of a proper Ab-shield. Second, instead of using whole IgG antibodies for shielding, only the antigen-binding fragments (Fabs) of the antibody were used. The rationale behind this change was two-fold: one, the rigid ‘Y-shape’ IgG antibody might cause steric hindrance at the B cell surface that restricts the accessibility of epitopes (e.g. CD4bs) and this could be avoided when the smaller Fabs are used; two, the compact size of Fabs might

allow the construction of a more densely-packed Ab-shield on gp120 protein and contribute to a better epitope-shielding effect.

In this study, the immune sera used for the generation of shielding antibodies were obtained from a previously unpublished immunisation study in collaboration with the International AIDS Vaccine Initiative (IAVI-study). Rabbits in this IAVI-study were immunised with a wildtype clade A gp120 (designated P7A; originally obtained from Dr. A. Iversen group) and a robust antigen-specific antibody response was detected in these sera. As in the ‘POC’ study, CD4-gp120 complexes were used for the purification of gp120-specific antibodies from wildtype-gp120-immune sera to ensure that any antibodies that bind directly or proximal to the CD4bs would not be isolated and used for the construction of the Ab-shield. The purified antibodies were then digested into Fabs and used for the construction of an Ab-shield on gp120 protein. The antigenicity and immunogenicity of the shielded-gp120 (Ab-gp120) were subsequently assessed *in vitro* and in an *in vivo* immunisation study in rabbits.

The primary aims of this study were similar to the ‘POC’ study in Chapter 5:

- 1) To investigate whether the antibody response against regions other than the CD4bs, especially the immunodominant V1/V2 and V3 loops, can be reduced or abolished;
- 2) To examine whether a greater focus to the CD4bs could be achieved that lead to an improved CD4bs-specific response;
- 3) In the case where an enhanced CD4bs-specific response was demonstrated, the neutralising breadth and potency of the antibody response will be determined against a panel of HIV-1 pseudoviruses.

6.2 Results

6.2.1 Characterisation of the Clade A P7A gp120

The clade A P7A gp120 used here was previously used in an unpublished immunisation study (IAVI-study). In the IAVI-study, the P7A gp120 was immunised into rabbits and demonstrated a robust antibody titre even though neutralisation capacity could not be detected. For this reason, it was believed that the antibodies obtained from these rabbit sera would be suitable for this approach, and the P7A gp120 protein was therefore selected for this study. To obtain a better understanding of this clade A Env protein, several preliminary studies were carried out to obtain a basic understanding of the antigenicity and immunogenicity of the P7A gp120 prior to its use in subsequent experiments.

(i) Sequence

The protein sequence of the P7A gp120 was aligned with: (i) the clade A consensus, which was obtained from the Los Alamos (LANL) HIV-1 Database, and (ii) the LANL reference strain HxB2 (clade B) so that the conserved and variable regions could be identified (Fig 6.1). As expected from strains of different HIV-1 clades, a considerable variation in the length and sequence was observed between the P7A and HxB2, while differences between the P7A and consensus A were restricted mainly to the sequence of the variable regions. The C-terminus His₆Tag was introduced to allow purification and identification of the protein.

(ii) Antigenicity

The wildtype P7A gp120 protein was synthesised (Fig 6.2), and using antibody-binding ELISA, the conformational integrity and antigenicity of the P7A gp120 was determined by measuring the binding of sCD4 and various anti-HIV monoclonal antibodies (mAbs) (Fig 6.3; black line). The subtype B JRFL gp120 was used as a positive control and reference in this experiment (red line). As expected, the clade A P7A gp120 exhibited a very different antigenicity profile to the clade B JRFL gp120: First, the V2-

	1				50
CONSENSUS A	MRVMGIQRNC	QHL.LRWGTM	ILGMIICSA	AENLWVTVYY	GVPVWKDAET
P7A	M	PAL.LSLVSL	LSVLLMGCVA	ETGLWVTVYY	GVPVWKDAET
HXB2	MRVKEKYQHL	WRWGWRWGTM	LLGMLMICS	TEKLWVTVYY	GVPVWKEATT
	51				100
CONSENSUS A	TLFCASDAKA	YETEMHNVWA	THACVPTDPN	PQEIHLNVT	EEFNMWKNNM
P7A	TLFCASDAKA	YETERHNVWA	THACVPTDPN	PQEIQLNVT	EEFNMWKNSM
HXB2	TLFCASDAKA	YDTEVHNVWA	THACVPTDPN	PQEVVLNVVT	ENFNMWKNDM
	101			V1	150
CONSENSUS A	VEQMHTDIIS	LWDQSLKPCV	KLTPLCVTLN	CSNVNVTNNT	TNTHE.....
P7A	VEQMHTDIIS	LWDQSLKPCV	KLTPLCVTLD	CNNVT...NI	TTEMG.....
HXB2	VEQMHTDIIS	LWDQSLKPCV	KLTPLCVSLK	CTDLKNDTNT	NSSSGRMIME
	151	V2			200
CONSENSUS A	.EEIKNCFSN	MTTELDRKKQ	KVYSLFYRLD	VVQINENNSN	SS..YRLINC
P7A	.GEIKNCFSN	MTTELDRDRKQ	KVYSLFYRLD	VVQINGNQGN	NSNQYRLINC
HXB2	KGEIKNCFSN	ISTSIRGKVQ	KEYAFFYKLD	IIPID....N	DTTSYKLTSC
	201				250
CONSENSUS A	NTSAITQACP	KVSFEPIPIH	YCAPAGFAIL	KCKDKEFNGT	GPCKNVSTVQ
P7A	NTSAITQACP	KVSFEPIPIH	YCAPAGFAIL	KCKDKEFNGT	GPCKNISTVQ
HXB2	NTSVITQACP	KVSFEPIPIH	YCAPAGFAIL	KCNNKTFNGT	GPCTNVSTVQ
	251				V3
CONSENSUS A	CTHGIKPVVS	TQLLLNGSLA	EEVIIRSEN	ITNNAKTIIV	QLTKPVKINC
P7A	CTHGIKPVVS	TQLLLNGSLA	EREVKIRSEN	ITNNAKNILV	QLAKPVSITC
HXB2	CTHGIRPVVS	TQLLLNGSLA	EEVVIRSVN	FTDNAKTIIV	QLNTSVEINC
	301				350
CONSENSUS A	TRPNNNTRKS	IRI..GPGQA	FYATGDIIGD	IRQAHCNVSR	SEWNKTLQKV
P7A	LRPNNNTRKG	IHI..GPGQA	FYARDNIIGD	IRQAHCNISK	TEWNNTLKRV
HXB2	TRPNNNTRKR	IRIQRGPGRA	FVTIGKI.GN	MRQAHCNISR	AKWNNTLKQI
	351			V4	400
CONSENSUS A	AKQLRKYF.K	NKTIIFTNSS	GGDLEITTHS	FNCGGEFFYC	NTSGLFNSTW
P7A	VTQLRITYF.K	NKTIIFANAS	GGDLEITTHS	FNCGGEFFYC	NTSGLFNSTW
HXB2	ASKLREQFGN	NKTIIFKQSS	GGDPEIVTHS	FNCGGEFFYC	NSTQLFNSTW
	401				450
CONSENSUS A	NNG	TM...KNTIT	LPCRIKQIIN	MWQRAGQAMY	APPIQGVIRC
P7A	IHNANLTESN	ST...NDTIT	LQCRMKQIIN	MWQRTGQAMY	APPIEGVIRC
HXB2	FNSTWSTEGS	NNTEGSDTIT	LPCRIKQIIN	MWQKVGKAMY	APPISGQIRC
	451	V5			500
CONSENSUS A	ESNITGLLLT	RDGGN...NN	TNETFRPGGG	DMRDNRSEL	YKYKVVKIEP
P7A	ESNITGLILT	RDGGDGECSN	TTETFRPGGG	DMRDNRSEL	YKYKVVKIEP
HXB2	SSNITGLLLT	RDGGS...NN	ESEIFRPGGG	DMRDNRSEL	YKYKVVKIEP
	501		517		
CONSENSUS A	LGVAPTRAKR				
P7A	LGVAPTKAKR	KHHHHHH			
HXB2	LGVAPTKAKR				

Figure 6.1 Alignment of gp120 protein sequences of the clade A consensus, P7A and the clade B reference strain HxB2 (grey).

specific antibody 697-D failed to recognise the P7A gp120 as the 697-D epitope – ISTSIRGKVQKEYAFFYKLD – was not present in the clade A P7A (145). The glycan-specific 2G12 also did not recognise the P7A gp120; Second, the CD4bs-specific antibodies b12 and F105 only bound weakly to the P7A; Third, the presence of a GPGQ epitope in the crown of V3 (Fig. 6.1), instead of the

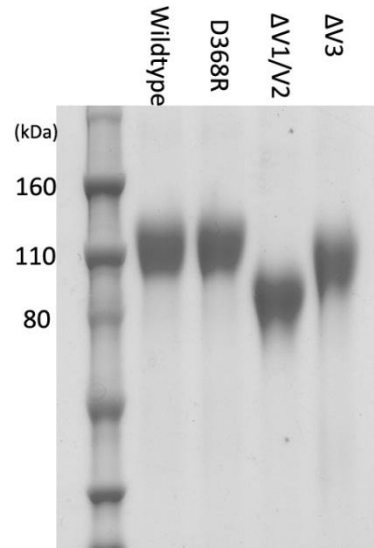


Figure 6.2 Synthesis of wildtype and mutated versions of the P7A gp120

Wildtype and mutated P7A gp120 proteins were synthesised and analysed by SDS-PAGE under non-reducing conditions. The protein bands were made visible through Coomassie blue staining. The CD4bs-defective gp120 was referred to as 'D368R', V1/V2-deleted gp120 as 'ΔV1/V2' and V3-deleted gp120 as 'ΔV3'.

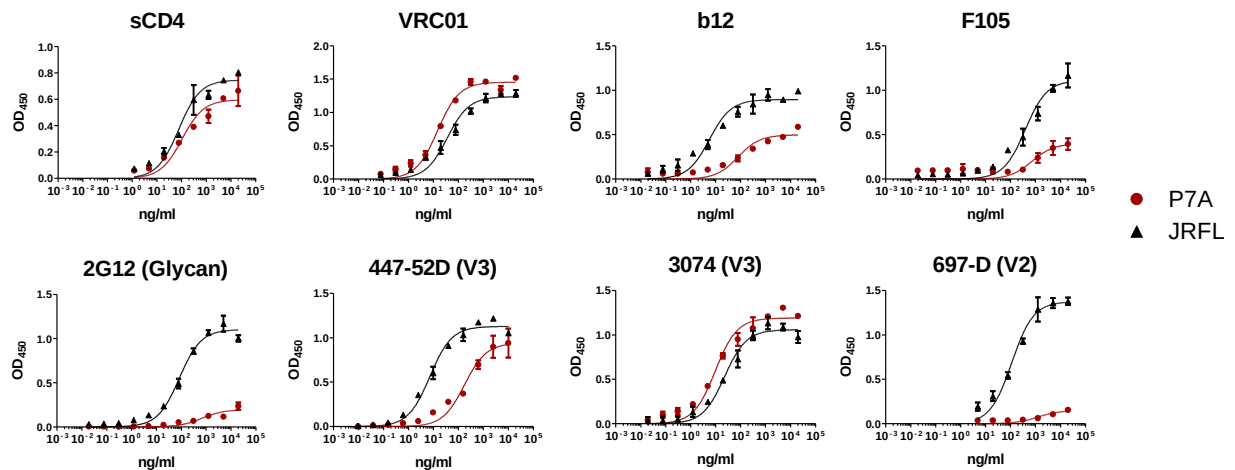


Figure 6.3 Antigenicity of the P7A gp120

The binding of sCD4 and a number of anti-HIV mAb were measured against the P7A gp120 in order to measure the antigenicity of the P7A gp120 protein (●). The clade B JRFL gp120 was used as a positive control (▲). The top row shows results from sCD4 and other CD4bs-targeting mAb, while the bottom row contains mAbs that target various regions of the Env protein (The sites these mAbs recognise were stated in the headings) All error bars represent standard deviations obtained from triplicate repeats.

GPGR motif common among clade B strains, also led to a weakened recognition by 447-52D that was in line with previous findings (275, 410); Lastly, the binding of sCD4, the CD4bs-specific antibody VRC01 and the V3-specific antibody 3074 were comparable for both proteins. These results therefore confirmed the conformational integrity of this newly reported P7A gp120.

(iii) Immunogenicity

In order to gain an understanding of the immunogenicity of the P7A gp120 protein, the immune-sera obtained from the unpublished IAVI-study were also analysed and mapped to different Env regions by measuring its binding to different mutant gp120 proteins, including the CD4bs-defective gp120 (D368R-gp120), the V1/V2-deleted gp120 (Δ V1/V2) and V3-loop-deleted gp120 (Δ V3) protein (Fig 6.2 & 6.4). The basis of this assay was explained in detail in the previous chapter (see Chapter 5, section 5.2.12). Overall, the immunogenicity of the P7A gp120 is highly similar to other gp120 proteins (133, 212, 369, 370), with the V3 and V1/V2 loops of the P7A gp120 being the primary and secondary antibody target respectively, accounting for roughly 40% and 25% of the total gp120-specific antibodies. In contrast, the antibody response towards the CD4bs is limited to only 5% of the total response, thus highlighting the sub-dominance of the CD4bs in comparison to the variable loops.

6.2.2 Purification of shielding antibodies

To obtain shielding antibodies for the construction of the Ab-shield, rabbit IgG antibodies were first isolated from the rabbit sera by Protein A agarose, and all antigen-specific antibodies were then purified from the total IgG pool by immunoprecipitation using paramagnetic bead-immobilised CD4-gp120 complexes (Fig. 6.5). Antibodies were eluted under high salt conditions instead of the acidic condition that is commonly used in other established methods (295, 314). The rationale behind this is that the extremely low pH of the elution buffer would likely denature the CD4-gp120 complex that is coupled to the beads. With the loss of conformational integrity, the use of these denatured CD4-gp120 complex in further

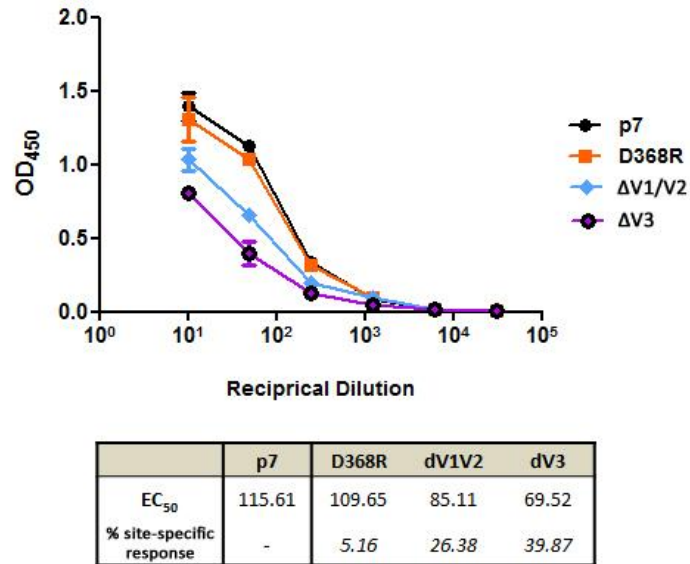


Figure 6.4 Immunogenicity of the P7A gp120

The P7A gp120-immunised sera obtained from the IAVI-study were analysed by measuring the serum binding to various mutated P7A gp120 so that the site-specific response can be determined. The % reduction (shown in the table) was calculated by using the equation: $(1 - EC_{50} \text{ of mutant gp120} / EC_{50} \text{ of wildtype gp120}) \times 100\%$. All error bars represent standard deviations obtained from triplicate repeats.

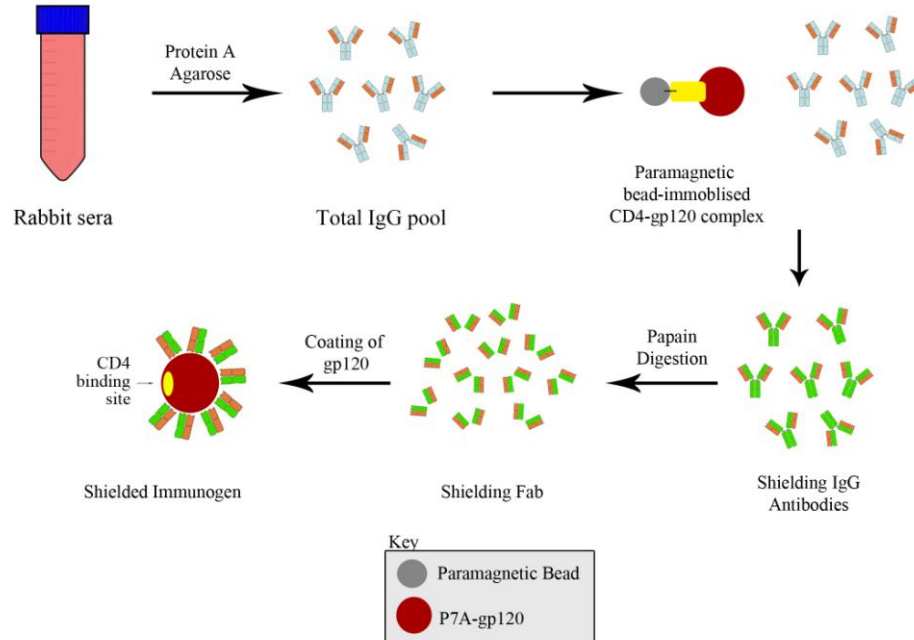


Figure 6.5 Systemic diagram of the preparation of Ab-shielded Env protein for immunisation

Rabbit IgG antibodies were isolated from the rabbit sera by Protein A agarose, and gp120-specific antibodies were purified by affinity-chromatography using paramagnetic-beads-immobilised CD4-gp120 complexes. Once the shielding antibodies were obtained, they were subsequently digested into their Fab fragments (Fabs) and the purified Fabs were then used for the construction of the Ab-shield onto the P7A gp120.

purification steps might fail to purify any conformation-specific antibodies that could be used in the subsequent shielding of gp120. All purified IgG antibodies were subsequently digested to their Fab fragments (Fabs) by papain, and purified from the reaction mixture by Protein A agarose as Protein A only binds the Fc fragments but not Fabs. The purified fractions were then analysed by SDS-PAGE (Fig. 6.6) – since the Fabs are the only antibody fragments that contain the light chain of the antibody, in oppose to the Fc which is composed of 2 identical heavy chains, the Fabs were identified in the flow-through of the Protein A purification (Fig 6.6; Lane 2). These Fabs were further purified by size exclusion chromatography.

6.2.3 Preparation of antibody-shielded immunogen

For the construction of the Ab-shield, all the purified Fabs were mixed with the P7A gp120 proteins at an Env:Fab ratio of 1:8 before they were crosslinked onto the Env protein by BS³ to prevent the dissociation of the Ab-shield *in vivo*. The reaction mixture was then purified by size exclusion chromatography, and this was repeated until all the unliganded and lightly-shielded gp120 species (i.e. gp120 with only 1-2 Fab attached) were removed. The final purified product (Ab-gp120) was analysed by SDS-PAGE under non-reducing and reducing conditions (Fig. 6.7), and the results showed that the final purified product contained 2 Ab-gp120 species. Further purification of these 2 species by size exclusion chromatography was not possible due to the small difference in their sizes. In addition, the smearing of the bands under reducing condition provides a strong indication that the Ab-shield was stably crosslinked to gp120 as the Ab-gp120 did not disassemble into their individual components (i.e. gp120 and the heavy and light chains of an antibody).

Based on the size of each protein bands, the stoichiometry of Fabs bound to gp120 could be estimated with the following ‘size’ assumptions – the size of the P7A gp120 proteins was assumed to be **120kDa**, and the molecular weight of the Fab was assumed to be **37.5kDa**. This was estimated by combining the

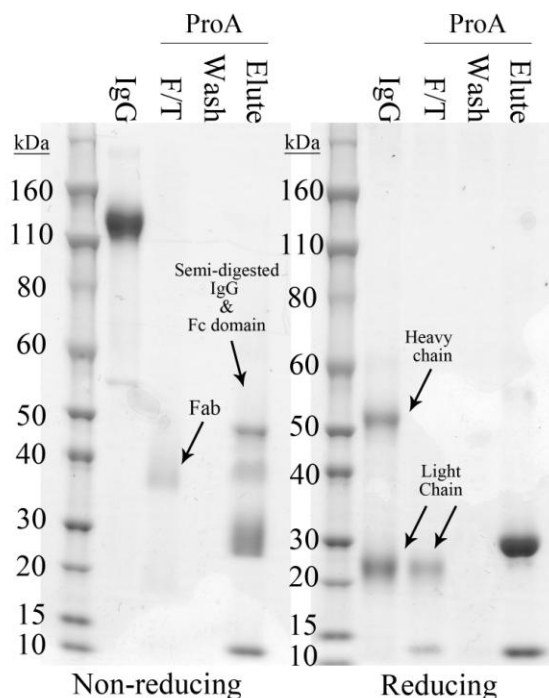


Figure 6.6 Generation of shielding Fabs from purified IgG antibodies

The IgG antibodies purified from the immunoprecipitation was digested by papain and the Fabs were purified from other IgG digests by Protein A agarose. The flow-through (F/T), wash and elute fractions from the Protein A purification were analysed by SDS-PAGE under (*left*) non-reducing and (*right*) reducing conditions. The protein bands were made visible by Coomassie Blue staining.

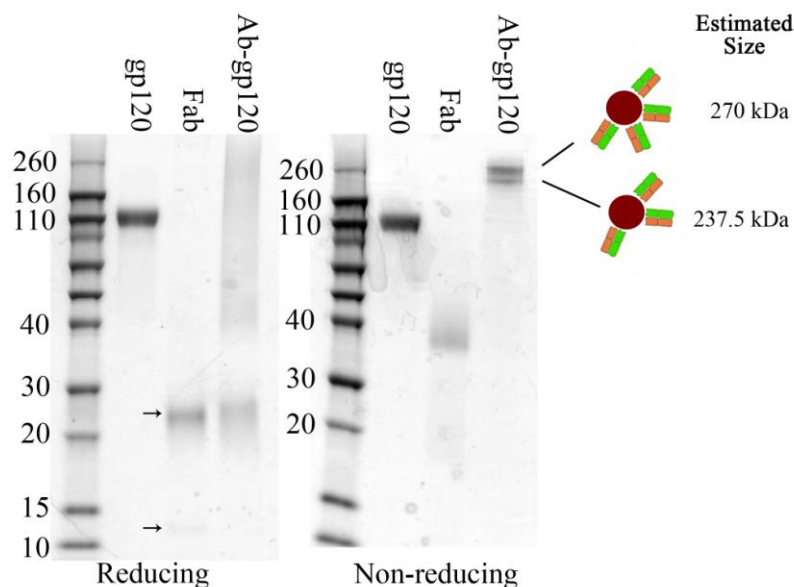


Figure 6.7 SDS-PAGE analysis of the final purified Ab-gp120 product

The *final* purified Ab-gp120 was analysed by SDS-PAGE under (*left*) reducing and (*right*) non-reducing conditions, visualised by Coomassie Blue staining. The monomeric P7A gp120 (gp120) and Fabs were used as a size control. In the reducing SDS-PAGE, the heavy and light chains of the Fab were highlighted. For the calculation of the size of the Ab-gp120, the size of gp120 proteins and Fabs are estimated as 120kDa and 37.5kDa respectively.

size of the heavy and light chain bands (25 and 12.5kDa) as observed in the reducing SDS-PAGE (Fig. 6.6). Under these assumptions, Ab-gp120 with a single Fab would therefore have a size of 157.5 kDa and Ab-gp120 with 2 Fabs would be 195 kDa and so on. As a result, based on the size of the 2 Ab-gp120 bands, it was estimated that the Ab-shield of the upper/larger Ab-gp120 band might contain 4 Fabs to each gp120 (Fab₄-gp120; combined size of 270 kDa), whereas the lower/smaller Ab-gp120 band might have 3 Fabs attached to gp120 protein (Fab₃-gp120; combined size of 237.5 kDa).

6.2.4 *In Vitro* Characterisation of the Ab-gp120

Prior to the immunisation study, the purified Ab-gp120 was characterised by measuring the binding of sCD4 and a number of different HIV-specific mAbs (pink line; Fig. 6.8), including the CD4bs-specific antibodies *b12* and *VRC01*, and the V3-specific antibodies *3074* and *447-52D*. The polyclonal *HIVIG*, which recognise multiple epitopes on the Env protein, was also used to inspect the level of shielding of the whole gp120 protein. Furthermore, the rabbit Fab that were used for the construction of the Ab-shield were also **biotinylated** and used to examine the integrity of the Ab-shield (Fab-bt). The binding of sCD4 and antibodies was also measured against the wildtype P7A gp120 as the positive control (blue line), and the appropriate negative control (grey line): the D368R-gp120 for sCD4, *b12* and *VRC01*; the ΔV3 gp120 for the *3074* and *447-52D*; BSA for the polyclonal *HIVIG* and Fab-bt (Fig. 6.8).

First, the rabbit Fabs used for the construction of the Ab-shield was biotinylated and used to assess whether all the possible sites that these Fabs recognised have already been occupied by the Ab-shield – in other words, is the Ab-shield obtained for the Fab₄-gp120 the maximum level of shielding that can be achieved in this study? If this is true, no ‘re-binding’ of the Fab-bt should be observed given the lack of exposed epitopes. By comparing the binding of the Ab-gp120 to the wildtype gp120 and the negative control (BSA), the results here demonstrated that very minimal ‘re-binding’ of the Fab-bt (Fig. 6.8). The slight ‘re-binding’ measured could be due to the presence of the ‘lighter-shielded’ Fab₃-gp120 species in the final purified product, although this was not further investigated here. In any case, this strongly

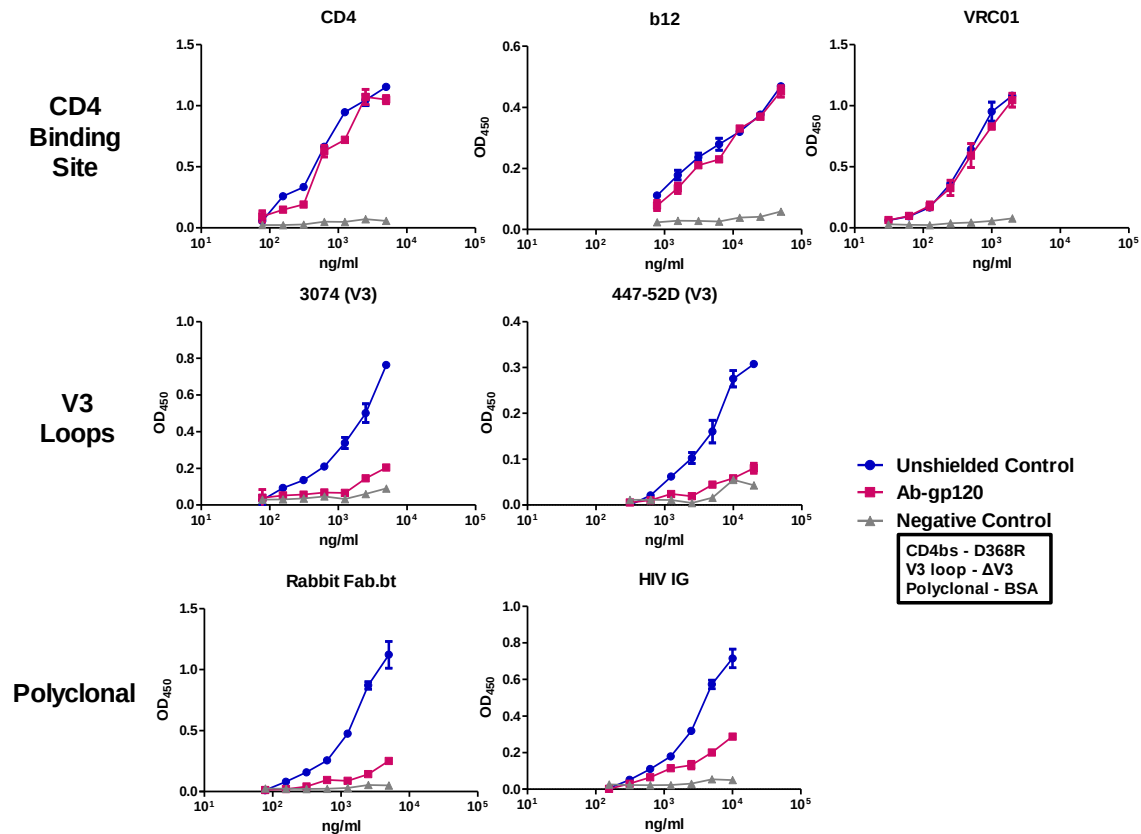


Figure 6.8 Antigenicity of the purified Ab-gp120

The antigenicity of the final purified Ab-gp120 (■) was assessed by measuring the binding of sCD4 and anti-HIV mAbs. The wildtype P7A gp120 (unshielded control; ●) was used as a positive control and reference, whereas various mutant gp120 proteins and BSA were used as negative controls (▲; as listed in the legend). All error bars represent standard deviations obtained from triplicate repeats.

indicates that the level of shielding was close to its maximum level and the Fab₄-gp120 was most likely to be the most thoroughly-shielded gp120 that can be achieved using these Fabs.

For the antigenicity of the Ab-gp120, the binding of sCD4, b12 and VRC01 was largely unaffected as they recognise both the wildtype gp120 and Ab-gp120 almost equally (Fig. 6.8). This therefore demonstrates that the accessibility of the CD4bs was not blocked, or hindered, by this Ab-shield. In contrast, the binding of both V3-specific antibodies (447-52D and 3074) was significantly reduced for the Ab-gp120 and resembled more the ΔV3 gp120 (negative control) than the wildtype gp120. Similarly, a significant

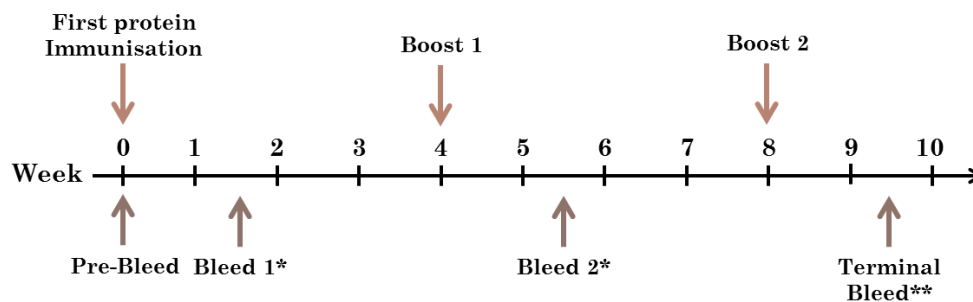
reduction in the binding of the polyclonal HIVIG antibodies to the Ab-gp120 was also observed, meaning that the majority of the HIVIG epitopes were masked by the Ab-shield. The residual binding observed here could be resulted from the binding of CD4bs-specific antibodies that are naturally present in the HIVIG. With the confirmation that the CD4bs of the Ab-gp120 was available for antibody recognition, and the Ab-shield was able to prevent the binding of numerous anti-HIV antibodies, this study then proceeded to the next immunisation stage where the immunogenicity the Ab-gp120 was assessed *in vivo*.

6.2.5 Rabbit Immunisation

For the immunisation, 2 groups of 4 rabbits each were immunised with the wildtype gp120 (unshielded control; group 1) and the Ab-gp120 (shielded; group 2) (Fig. 6.9). All rabbits received 3 protein inoculations at week 0, 4 and 8 through the subcutaneous and intramuscular routes, and Ribi adjuvant was co-administrated for the boosting of the antibody response (243, 293, 375). To ensure that the equivalent amount of Env proteins were immunised in both groups, the Env content in the Ab-gp120 immunogens was determined by a standard curve based on the binding of sCD4 (see Chapter 2, section 2.3.4.9). Pre-bleeds were collected at week 0 and all serum samples were collected 7-10 days after each inoculation. All rabbits were sacrificed and terminal bled 10 days after the final immunisation.

6.2.6 Serological Analysis of the immune sera

The serum antibody responses against the wildtype P7A gp120 were determined for both immunised groups (Fig. 6.10). In both groups, a gp120-specific response was detected after the first protein inoculation, and continued to increase over the next 2 protein inoculations. Interestingly, the kinetics of the antibody response of the 2 groups appeared to be different. In the control group, the immunisation of wildtype gp120 saw a sharp rise of the antibody response after the second immunisation (between bleed 1 and 2), while the response appeared to be reaching a plateau after the final immunisation (bleed 2 onwards). In contrast, the increase of the antibody response in the Ab-gp120 group appeared to be rising more consistently over these time-points, thus suggesting a different immunogenicity of the unshielded gp120 and Ab-gp120.



* 7-10 days after previous immunisation

** 10 days after final immunisation

Group	Immunogen	No. of Mice	Route	Adjuvant
1	30µg Unshielded wildtype gp120 (Control)	4 female (1.0-1.5kg)	Subcutaneous (neck region) and	Ribi
2	30µg Ab-gp120		Intramuscular (each hind leg)	

Figure 6.9 Rabbit Immunisation

2 groups of 4 rabbits each were immunised with unshielded wildtype gp120 and Ab-gp120 respectively. (Top) The timeline of the immunisation displaying the time points of each immunisations and bleeds. (Bottom) Table summarising the details of the immunisation, including the dosage, immunisation route and the adjuvant used.

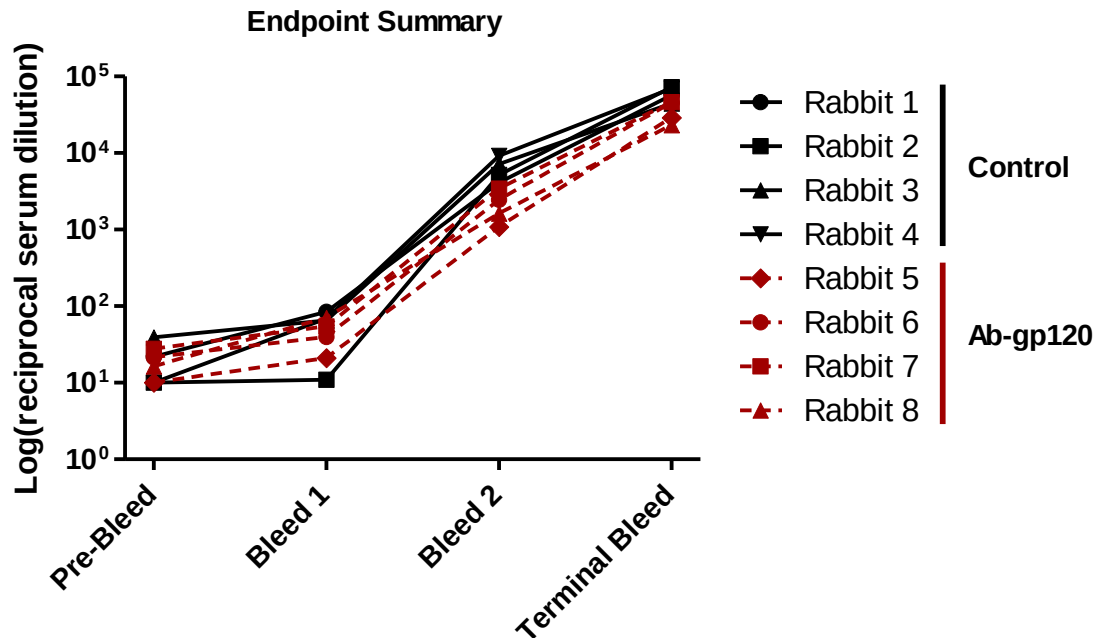


Figure 6.10 Serological Analysis of the immune sera

The antigen-specific (anti-gp120) response of the rabbit sera in each group was analysed at each time point and reported as end-point titres, i.e. the reciprocal serum dilution that gives an absorbance greater than the cut-off threshold (mean of background reading plus 2 standard deviation points).

6.2.7 Mapping of the serum antibody response

Several mapping analyses were performed with individual rabbit serum in order to explore the site-specific response towards the V1/V2, V3 and the CD4bs:

(i) Cross-competition ELISA

Cross-competition ELISA maps the site-specific response by measuring the competition between mAb/sCD4 and serum antibodies for the binding of specific sites on the Env protein, e.g. the CD4bs (sCD4, b12 and VRC01) and the V3 loop (3074 and 447-52D). Unfortunately, due to a lack of antibodies that bind to the V2 loop, the V2-specific response could not be determined in this assay. Pre-bleed serum of each rabbit was used as a negative control because Env-specific antibodies could not be present in the pre-bleeds. All results were reported as IC_{50} , which is defined as the reciprocal dilution of the rabbit sera that caused 50% inhibition of the mAb binding, i.e. the higher the IC_{50} , the stronger the site-specific antibody response and *vice versa*.

By comparing the sera from the pre-bleeds (PB; grey symbols in Fig. 6.11) and terminal-bleeds (TB; black symbols) within each immunisation group, a significantly stronger inhibition of sCD4 and mAbs was measured in the TB than in the PB, thus indicates that both CD4bs- and V3-specific antibodies were elicited over the course of the immunisation. The effect of the Ab-shield was then assessed by comparing the TB between both groups. The CD4bs-specific response appeared to be retained as both groups competed equally with sCD4, VRC01 and b12. On the other hand, serum competition with both V3-specific antibodies (3074 and 447-52D) was significantly weaker for the shielded group than for the control group, thus indicating a lower level of V3-specific antibodies in the shielded group. This result was therefore in line with the ‘proof-of-concept’ study by demonstrating that the Ab-shield has successfully masked the V3 loop from antibody recognition.

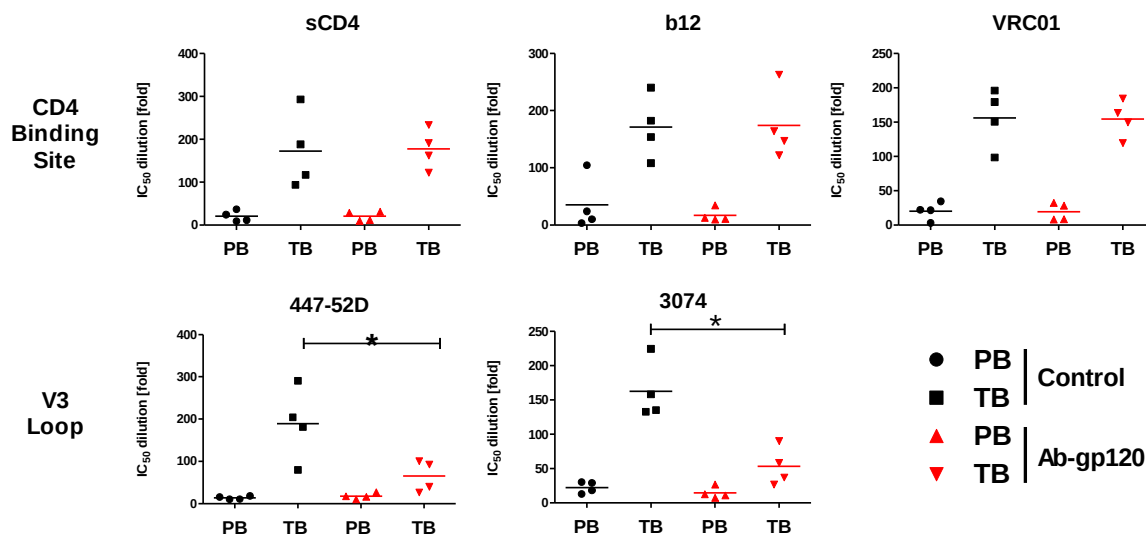


Figure 6.11 Serum Mapping analysis by cross-competition ELISA

Serum competition with sCD4 and various human anti-HIV mAbs were used for the mapping of antibody responses towards specific regions of the Env protein (CD4bs and V3 loop). In all cases, the pre-bleed and the terminal bleed were used for the analysis. The mean of the data within each analysis is shown as a line. Asterisks (*) were shown where statistical significance was achieved ($p < 0.01$). All error bars represent standard deviations obtained from triplicate repeats. Statistics were calculated using One way ANOVA with a Bonferroni post-hoc test.

(ii) Serum binding to mutant P7A gp120

The serum antibody response was also mapped by measuring the serum binding to different mutant gp120 proteins as, in contrast to cross-competition ELISA, this allows the mapping of the entire region rather than a single epitope. Again, the D368R-, Δ V1/V2- and Δ V3 mutant gp120 proteins were used and the site-specific responses was quantified by measuring the reduction of the EC₅₀ to each of these particular proteins (for more details, see Chapter 5, section 5.2.12). For each immunised group, the pre-bleed sera from the 4 rabbits were pooled together and used as the negative control.

Overall, the results of this assay were in good agreement with the cross-competition ELISA (Fig. 6.12). The CD4bs-specific response elicited in both immunised groups was largely similar with roughly 8% of the serum antibodies targeting this particular region, and the V3-specific response observed in the control group (~50%) was significantly stronger than the shielded group (~12%), thus providing further evidence

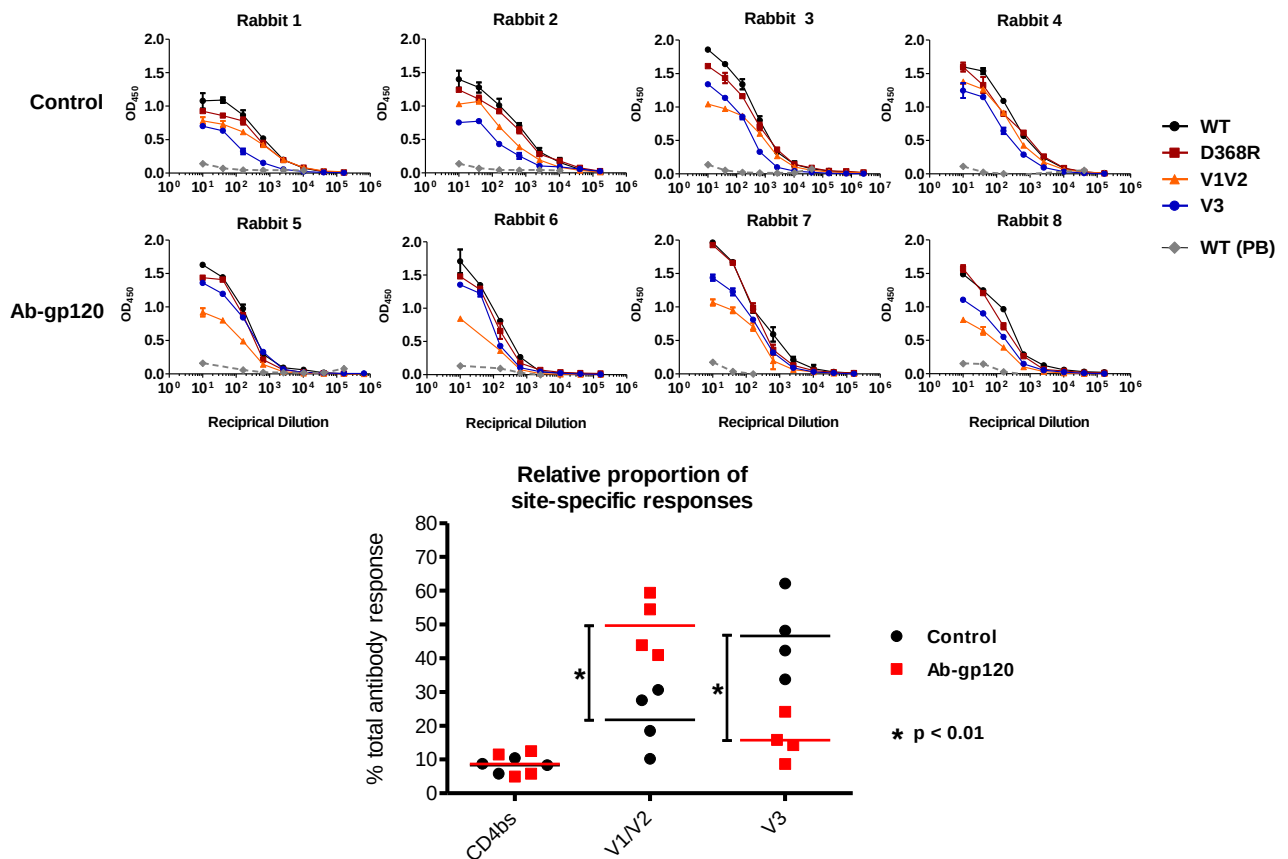


Figure 6.12 Serum Mapping by measuring serum binding to mutant gp120

(Top) Raw data obtained for the sera binding towards various mutant gp120. The binding of wildtype gp120 by the pooled pre-bleed (PB) serum of the 4 rabbits was shown as a negative control. (Bottom) Figure summarising the estimated relative proportion (%) of the site-specific response by comparing the EC₅₀ of the wildtype and respective mutant gp120. The equation used for the calculation = $(1 - EC_{50} \text{ of mutant gp120} / EC_{50} \text{ of wildtype gp120})$. All error bars represent standard deviations obtained from triplicate repeats. Statistics were calculated using One way ANOVA with a Bonferroni post-hoc test.

that the V3-specific response was dampened by the Ab-shield. As for the V1/V2 loop, which was not assessed by the cross-competition ELISA, the presence of the Ab-shield led to a 2-fold increase in the serum V1/V2-specific antibody response (from ~20% in the control group to ~50% in the shielded group). Such an increase in the V1/V2 response in the presence of V3 dampening was in line with the previous ‘proof-of-concept’ study, and once again suggested the insufficient shielding of all immunodominant loops.

(iii) Peptide ELISA

To obtain a detailed view on the shift of immunodominance between the variable regions, the serum antibody response was assessed by peptide ELISA using a set of overlapping peptides that correspond to the V1/V2 and V3 loops (Fig. 6.13). In comparison to the above assays, the peptide ELISA allows a much more precise mapping of epitopes by dissecting these immunodominant regions into smaller fragments (18-mers peptides). However, this assay is not without its limitations: first, the epitopes identified represent only those that are linear or conformations that can be folded by the 18 amino acid long peptides. Antibodies that bind to discontinuous epitopes, or conformations that are derived from longer peptides, will not be detected; second, since the peptides are overlapping (by 10 amino acids), in the situation where consecutive peptides are found to be reactive, it cannot be determined whether such reactivity was against multiple independent epitopes on each peptide, or the same epitope that are presented in all peptides; finally, antibodies that are glycan-dependent, e.g. PG9 and the PGT antibodies, will not be detected given the lack of glycosylation for these peptides.

For the immunodominant V3 loop, the serum antibodies of the control group were found to be reactive against a number of peptides that correspond to the crown of the V3 loop (V3 peptides 2-4). In particular, the strongest response was directed towards peptide 3 – TRKGIHIGPGQAFYARDN – which contains the immunodominant GPGQ motif right in the centre of the peptide (148, 453). While the adjacent peptides (2 and 4) also contain part of the GPGQ motif, the relatively weaker response towards both peptides suggests that the majority of the V3-specific antibodies were targeting the full GPGQ motif, or that the GPGQ motif needs to be in the middle of the peptide so that it can exhibit a proper conformation in order for it to be antigenic. On the other hand, such strong response towards the crown of the V3 loop (especially towards the V3 peptide 3) was significantly reduced in the shielded group. Hence, this provides strong indication that the presence of the Ab-shield has successfully dampened the antibody response towards this particular region of gp120.

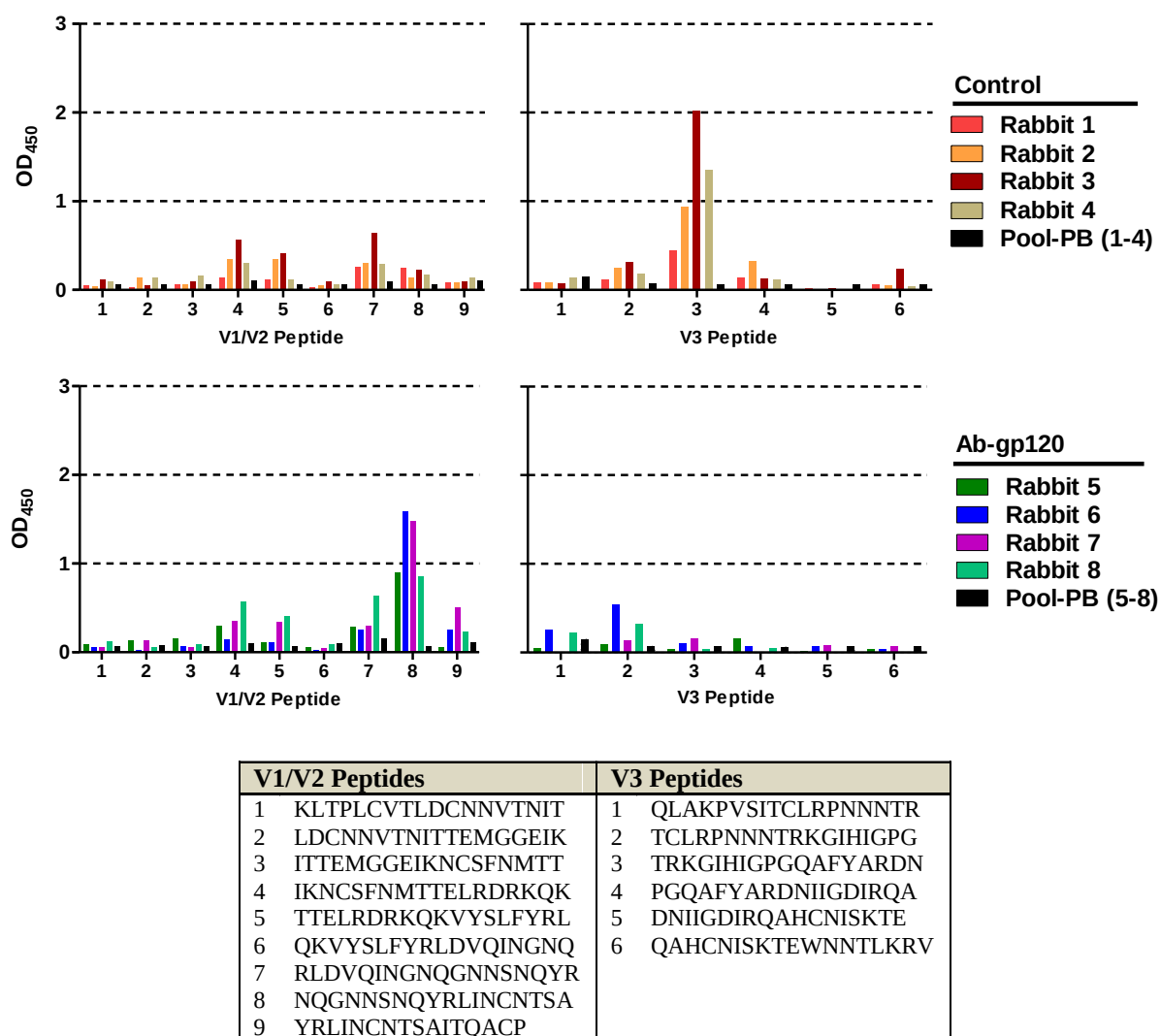


Figure 6.13 ELISA of serum binding to linear overlapping V1/V2 and V3 peptides

(Top) The upper panel represents the immune sera from the wildtype-gp120-immunised rabbits, while the lower panel represents the sera from the Ab-gp120-immunised rabbits. In both cases, the pre-bleed sera of 4 rabbits were pooled together and used as a negative control. The results here are averages of triplicate repeats. (Bottom) The sequences of the overlapping 18-mers peptides used in this assay.

For the V1/V2-specific response, which was found to be the secondary antibody target in previously described assays, rabbit sera of the control group did not recognise any of the V1 peptides (V1/V2 peptides 1-3) but they did demonstrate a moderate response towards a number of the V2 peptides (V1/V2 peptides 4-9). This therefore indicates that the majority of the V1/V2-specific response was directed

towards the V2 loop but not the V1 loop. Similarly, for the shielded group, the V1/V2-specific response was directed towards the V2 but not the V1 loop. As observed in the above assays, the loss of V3-specific response was mirrored by an increase in the V1/V2-specific response, especially to peptides that correspond to the C-terminus end of the V2 loop (V1/V2 peptides 8-9). The results here therefore suggested an intra-domain immunogenicity-hierarchy within the V2 loop by showing that the C-terminus of the V2 loop was more immunogenic than the rest of the V1/V2 loop.

Taken together, the findings of these 3 mapping studies are in good agreement with each other by demonstrating a shift of antibody response from the V3 loop to the V2 loop. Despite all the changes implemented to this approach, results here did not suggest that the implemented changes did not improve the masking performance of the Ab-shield.

6.3 Discussion

Experiments described in this chapter were a follow-up from the ‘proof-of-concept’ (‘POC’) study in Chapter 5. The aim of this study was to investigate whether the changes implemented to the approach could improve the overall performance of the antibody-shielding technique – i.e. a complete dampening of antibody responses to the immunodominant V1/V2 and V3 loops that could lead to an increased elicitation of CD4bs-specific response that resembles b12 and other CD4bs-specific BNABs.

The preparation of the Ab-shielded immunogen began with the purification of gp120-specific Fab antibodies from the rabbit sera (Fig. 6.5 & 6.6), and these Fabs were then used for the construction of an Ab-shield on gp120. The final purified product (Ab-gp120) was analysed by SDS-PAGE and an antibody-binding ELISA (Fig. 6.7 & 6.8). It was revealed that the final purified products contained a mixture of different levels of shielding – one species with 3 Fabs attached (Fab₃-gp120) and the other with 4 Fabs (Fab₄-gp120) attached. The Fab₄-gp120 is believed to be the highest possible shielding that is going to be achieved in this study as very limited re-binding of the rabbit Fabs was observed (Fig. 6.8). Next, the epitope-masking potential of the Ab-shield was assessed: one, the binding of the V3-specific antibodies (3074 and 447-52D) and the polyclonal HIVIG antibodies was significantly reduced by the Ab-shield, meaning that multiple epitopes of gp120 were masked by the Ab-shield; two, the binding of sCD4 and other CD4bs-targeting antibodies was not affected by the Ab-shield, meaning that the CD4bs of the Ab-gp120 was not masked by the Ab-shield. Following these results, the Ab-gp120 proteins were immunised into rabbits so that the immunogenicity of the Ab-gp120 could be assessed *in vivo* (Fig. 6.9 & 6.10).

The serum antibody responses of the control and shielded groups were mapped by 3 different assays – cross-competition ELISA, peptide ELISA and by measuring the serum binding to mutant gp120 proteins. Overall, the results of these assays were in good agreement with each other (Fig. 6.11 – 6.13). In the presence of the Ab-shield, the antibody response against the V3 loop was significantly reduced in the

shielded group as demonstrated through a weaker serum competition with the V3-specific antibodies 447-52D and 3074, a reduced reactivity towards the V3 peptides and a stronger recognition of Δ V3-gp120 than the wildtype sera. In particular, the most significant dampening of the antibody response was found to be at the crown of the V3 – which contains the immunodominant motif GPGQ and is the prime target in wildtype gp120 immunisations (133, 182, 212, 369, 370). In line with the ‘proof-of-concept’ study, such dampening of the V3-specific response was mirrored by an increased response towards the V2 loop with the serum antibodies of the shielded group demonstrated greater reactivity towards the V2 peptides and a weaker binding of the Δ V1/V2-gp120 proteins than the control group. Interestingly, a preferential increase of the V2- but not the V1-specific response was observed here, although the nature of the peptide ELISA means that conformation-specific antibodies towards the V1 loop could still be present in the immune sera.

As for the antibody response to the CD4bs, the sera from both immunised groups competed equivalently with b12, VRC01 and sCD4, and they also bound the D368R-gp120 to an equal extent. Similar to the ‘POC’, the result here means that the CD4bs-specific response was retained and not affected by the Ab-shield. In conclusion, as in the ‘proof-of-concept’ study, these results clearly demonstrate that antibody-shielding can be used for the dampening of the antibody response towards undesired epitopes. Comparing to the ‘POC’ study, the implemented changes did improve the overall quality of the Ab-shield by demonstrating a much-improved masking of the V3 loop (from ~50% $\rightarrow$ ~12%). However, an increase in the V1/V2-specific antibodies means that the improved Ab-shield still failed to mask all the immunodominant epitopes of the Env protein completely. Further assessment of the current approach is required in order for this technique to evolve into a true HIV-1 candidate vaccine.

The inadequate shielding of the entire V1/V2 loop, and possibly other regions of the Env protein that were not explored in this study, remains a major bottleneck in the development of this technique. In light of the results here, it is clear that the use of a larger quantity of shielding antibodies was unlikely to be the solution to this problem. Several adjustments to the approach are worth considering in the future in order

to obtain a more thorough shielding of undesired epitopes: first, instead of using the monomeric gp120, the trimeric gp140 could be used as the compact nature of the trimer means that there is less exposed surface on each monomeric unit; second, while it is possible to repeat the immunisation step until all antibody targets outside the CD4bs are masked, this is not ideal as it is difficult to pinpoint how many animals and immunisations are required. One possible alternative is to use a mixture of well-characterised human mAbs and this would also allow more control over which regions are to be shielded. For instance, 447-52D and 39F could be used for the shielding of the crown and N-terminus of the V3 respectively (275, 310, 391, 453), while 697-D could be used for the shielding of V2 (145), and so on; finally, the purification strategy could be optimised from the current use of size exclusion chromatography as this failed to resolve high-order Ab-gp120 complexes due to the small size difference. Further purification could be achieved by using the shielding antibodies in affinity chromatography for the capturing of any ***under-shielded*** or ***unliganded*** Env proteins. All ***maximally-shielded*** proteins should remain in solution as all epitopes have already been shielded. With these changes, it would be interesting to see if a significant improvement of this antibody-shielding technique could finally be achieved.

Chapter 7

General Discussion

With the demonstration that broadly neutralising sera can be developed over time in some HIV-infected individuals, there has been a renewed optimism in the development of HIV-1 vaccine as this proves that the human immune system is capable of inducing a response with enough potency and breadth to neutralise HIV-1 isolates of different clades (361, 373, 379). A number of broadly neutralising antibodies (BNAbs) have been isolated from these individuals (36, 417), and the potential for these BNAbs to confer protection against HIV-1 has been demonstrated in a number of passive immunisation studies in non-human primate models (178, 180, 181). Further characterisations of these BNAbs have led to the identification of numerous sites on the Env protein that are vulnerable to antibody neutralisation, including the CD4 binding site (CD4bs), unique patterns of glycosylation, quaternary epitopes on the V2 and V3 loops of gp120 protein, and the membrane proximal external region (MPER) of gp41 protein (169, 216). These findings have since served as the template for the design of many different HIV-1 vaccines. However, despite the vast array of HIV-1 vaccines being tested, none of these candidates were able to elicit an antibody response that resembles these BNAbs and it remains unclear how a broadly neutralising antibody response can be induced via vaccination. With over 30 million people currently living with HIV-1 and million cases of new infection every year, there is an urgent need for novel immunisation technologies to be developed. In my doctorate, I therefore attempted to address this gap of knowledge in 2 directions.

In the first part of this thesis (**Chapter 3**), in collaboration with IAVI and Dr. Joe Parker, more than 1,000 Env sequences (clade B and C) were obtained from patient plasma that displayed a neutralisation profile of varying breadth and potency. The primary objective of this study was to identify naturally-occurring features/mutations of the Env protein (referred as ‘signatures’) that may contribute to a broadly

neutralising response seen in some HIV-infected individuals. These signatures would be useful in future vaccine design as their presence in the Env protein may trigger a specific conformation that encourages the elicitation of a broad neutralising response. Using Decision Tree, a total of 21 signatures were identified in the cohort (11 for clade B and 10 for clade C), and suggested a number of ways these signatures might contribute to a broad neutralising phenotype – they could either locate in the conserved regions (e.g. the CD4 binding site, V3 loop and the co-receptor binding site) and directly involve in antibody-recognition, or situate in key functional areas of the Env protein (e.g. gp120-gp41 interface and the cytoplasmic domain of gp41) that could affect the overall conformation and stability of the Env protein, thus indirectly altering the epitopes in distant regions of the Env protein. In addition, the length of the V2 loop was also found to be associated with the development of a broadly neutralising response, although the exact mechanism of its contribution remains to be determined. In the context of immunogen design, the identification of these signatures represents the very first step in vaccine development and a number of changes that might improve the immunogenicity of the Env were highlighted here. Further characterisation of these signatures *in vitro* would be a useful first step in order to identify any ‘beneficial’ mutations that could improve the immunogenicity of the Env protein.

One major obstacle in the development of an HIV-1 vaccine is that the immune system is often ‘distracted’ towards the immunodominant regions (e.g. V1/V2 and V3 loops on gp120) instead of focusing on the conserved regions of the Env protein. In the second part of my thesis (**Chapter 4 – 6**), 3 vaccination approaches were presented with the primary aim of eliciting a site-specific antibody response towards the highly conserved sites on the Env protein, e.g. CD4 binding site (CD4bs) and MPER. These techniques are referred as ‘immunofocusing’. In **Chapter 4**, two of these immunofocusing techniques were described. One approach proposed that an MPER-specific response could be elicited through the immunisation of virus-like particles (VLPs) bearing heavily-truncated gp41 proteins. Unfortunately, the extensive deletion of gp41 appeared to be intolerable for gp41 proteins as they were not expressed on the cell surface and were therefore not incorporated into the VLPs. For this reason, this approach was not carried forward. The

other approach hypothesised that the CD4bs-specific response could be elicited through the selective activation of B cells that possess a CD4bs-specific B-cell Receptor (BCR). However, the development of the toxin-conjugated immunogen (toxin-gp120), which was designed for the inhibition of non-CD4bs-specific B cells, was unsuccessful and a ‘proof-of-concept’ immunisation study has yet to be carried out. For this reason, several key questions remain to be answered for this technique. One key concern is that, assuming the toxin could kill the ID-B cells, how frequent are these ID-B cells being replenished by the host? How can the ID-B cell populations be continually suppressed in order for this technique to succeed? Different immunisation procedures (e.g. the use of adjuvant, more frequent inoculation of immunogen etc.) should be considered in order to find out the optimal strategy to retain the level of B cell suppression *in vivo*. Furthermore, an additional concern regarding this approach is that dendritic cells (DCs) may also recognise the toxin-gp120 via the PRR (see section 1.5.1). The intake of toxin-gp120 into the DCs may trigger the release of toxin and results in the inhibition of DCs, which could have an adverse effect on the T-cell activation and prevent an effective antibody response from being elicited. The design of the current immunogen might therefore need to be revised in the future so that it could specifically target B cells only.

In **Chapter 5 and 6** of this thesis, I presented the development of a novel ‘antibody-shielding’ technique that hypothesised that immunogen-derived antibodies could be used for the shielding of immunodominant epitopes on the Env protein. Through this, it was hoped that a greater focus of the antibody response towards the highly conserved CD4bs could be achieved, thus leading to the elicitation of a CD4bs-specific response. This antibody-shielding technique was assessed in 2 animal models – a ‘proof-of-concept’ study in mice (Chapter 5) and a follow-up study in rabbits (Chapter 6). The results of the 2 immunisation studies were highly similar, where the V3-specific response was significantly dampened by the Ab-shield and that the CD4bs-specific response was retained to a level comparable to the unshielded control. However, in both studies, an increase in the antibody response towards the V1/V2 loop was observed, thereby indicating that V1/V2 loop was not completely masked by the Ab-shield. Although the primary aim of this approach was not met, the significant reduction of the V3-response proves that antibody-shielding could

be a useful tool for the masking of immunodominant epitopes on the Env protein. The next phase in the development of this antibody-shielding technique should therefore concentrate on improving the overall level of shielding of the Ab-shield so that the antibody response can be focused towards the CD4bs on the Env protein (see discussion in Chapter 6).

In addition to the original idea, the development of this antibody-shielding technique can also evolve in a number of different directions. For instance, there has been a growing interest in the V2-specific response in recent years: first, the discovery of a number of BNABs that recognise conserved epitopes on the V2 loop – PG9/16 and 2909 – indicates that antibodies against the V2 loop could be potentially broadly neutralising (69, 146, 270, 343, 419); second, in light of the human RV144 trial and a recent SHIV-challenge study by Barouch *et al* (26, 168), vaccine-induced V2-specific antibodies have demonstrated a correlation in reducing the risk of HIV/SIV acquisition *in vivo*. Together, these findings strongly suggest that the V2 loop could be a useful target for future vaccine design. Can this antibody-shielding technique be used for the elicitation of a PG9-like broadly neutralising response? Does the ‘exposed’ nature of the V2 loop make it a better target than the typically ‘recessed’ CD4bs? Instead of using the full Env protein, can the V1/V2-scaffold protein designed by McLellan *et al* be used as a potential immunogen for this approach (270)? These are all interesting questions that are worthy of further investigations.

The antibody-shielding technique was designed with the primary aim of focusing the antibody response onto the highly conserved epitopes on the Env protein. However, it should be taken into caution that the focusing of antibody response on a highly conserved site did not necessarily guarantee the elicitation of a broadly neutralising response. As demonstrated in the immunisation of hyperglycosylated proteins and a number of MPER-only immunogens, the resulting site-specific responses elicited against these immunogens were mostly non-neutralising (80, 296, 369, 371, 402). As discussed in these studies, the failure for these immunogens to present the desired epitopes in their most native conformation could be one reason why a neutralising antibody response was not elicited. Another potential reason is that these

immunogens were intended as B cell immunogens, where the design and development of these immunogens were predominantly focused on the interactions between the immunogen and the B cells, yet they failed to account for the fact that the elicitation of an effective antibody response also require the ‘immunological help’ from dendritic cells and T cells (see section 1.6.2.3 for more details). As a result, for the antibody-shielding approach to elicit a broadly neutralising response, these factors must be taken into consideration in the future.

There are a number of ways in which a better ‘immunological help’ could be induced. Traditionally, adjuvants have been used to boost the antibody response elicited against an immunogen. In my mice and rabbit studies, Ribi was inoculated with the antibody-shielded immunogens as it has been extensively used in HIV-1 immunogen research and has shown to improve the antibody response (21, 243). However, other adjuvants should also be considered and compared in the future as 2 studies have demonstrated that an improved CD4bs-specific neutralising response could indeed be elicited by using other adjuvants (e.g. QuilA and Carbopol) ((5); Bowles *et al*, article in press). In addition, another way to improve DCs and T-cell help would be to adopt heterologous prime-boosting (see section 1.8.3 for more detail), as 2 recent studies shown that such an approach could help to focus antibodies to the target epitope (159, 296). Recently, Garcia *et al* described a DC-based HIV-1 vaccine that was able to induce a strong T-cell response that could control HIV-1 infection in humans (132). Based on this observation, it would be interesting to see if such DC-based HIV-1 vaccines could be used in the context of prime-boosting and confer ‘immunological help’ that could result in an enhanced antibody response. Moreover, Guenaga *et al* also showed that the additional of a T-helper epitope to the immunogen improved titres against the target epitope, thus demonstrating how weak immunogenicity of a target epitope could be improved with this approach (159). All these approaches should be tested with the shielded immunogen in the future, although at this moment, the construction of an antibody-shield that could mask all undesired epitopes of the Env protein remain the utmost priority in the development of the antibody-shielding technique.

Taken together, the work of this thesis describes the early phases of the development of a number of novel vaccination ideas, and point to a number of directions in which these approaches could be optimised and progressed into effective candidate HIV-1 vaccines. However, it must be noted that the development of an immunogen that could lead to the elicitation of a broadly neutralising response is merely the very first step in HIV-1 vaccine development with many questions still to be addressed – such as the actual correlate(s) of protection, the efficacy of the vaccine-induced effector mechanisms and the capacity of the induced response to maintain its activity over time. In this respect, further human clinical trials similar to the RV144 and the on-going HVTN 505 would be particularly useful as they provide the ideal opportunity to generate hypotheses in relation to these knowledge-gaps, and the results would no doubt assist the design of vaccine candidates with higher efficacy. In addition, systems-based studies of other successful human vaccines, such as the yellow fever virus vaccine (YF-17D), could also be beneficial as this may reveal previously unexpected genes or pathways that may contribute to a highly protective B cell, or even T cell, vaccine-derived response (334).

In recent years, advances in our understanding in the maturation process of BNABs have led to the proposal of a B cell-lineage-based approach to vaccine design, where it was proposed that BNAB-lineage cells can be ‘guided’ towards maturation that led to the elicitation of BNABs (169, 441). Although the development of this approach is still in its infancy, the underlying principle of this method is the perfect example of how improvements in our knowledge can lead to the continual evolution of different vaccine designs. With the discovery of more BNABs, a growing understanding of the host anti-HIV immune response and the development of exciting new technologies, there is certainly a renewed enthusiasm in the field of HIV-1 vaccine research and a successful vaccine might just be around the corner in the next human trial.

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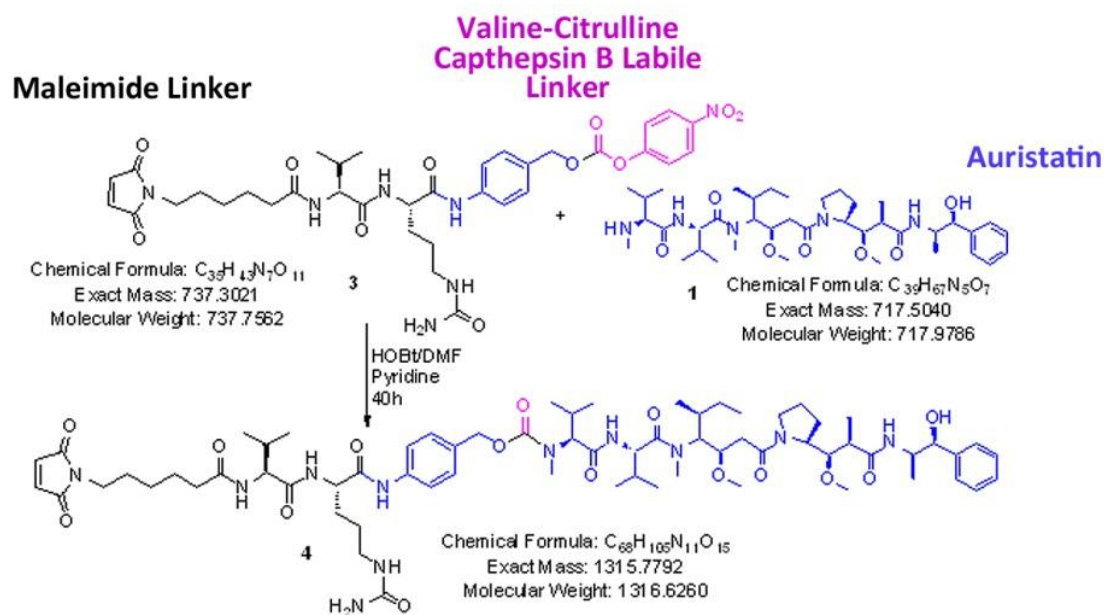
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Supplementary Materials

Functional Sites	
CD4 Binding Site	D368 E370 W427 V430 D457 I371 N425 M426 G473
Co-receptor binding site	
<i>Outside V3</i>	
<i>Inside V3</i>	I420 K421 Q422 I423 P438 R440 G441 K121 T123 T202 K207 E381 F383 K117 N377 R419 P438 Q442
Co-receptor specific (R5/X4) sites	R298 N301 T303 R306 R308 G312 P313 R315 F317 Y318 I323 D325
(outside V3)	I326 R327 H330
<i>Outside V3</i>	
<i>Inside V3</i>	R166 195 N197 T198 V200 A204 A221 I424 M434 R440 N301 302 T303 R306 I309 A316 F317 K322 I323 M326
gp120 interface with gp41	V65 V85 N92 V36 Y40 R252 F382 W427 A433 G495 T232 N234 P238
gp41 interface with gp120	W596 G597 S618 S528 M530 L555 Q562 V608 L593 K601 W610
Antibody Binding sites	
2G12	N295–297 N332–334 N392–394 N386–388 N448–450
b12	T257 N280 A281 S365 D368 P369 E370 I371 Y384 N386 P417 R419 V430 D457 D474 M475
2F5	E662 L663 D664 K665 W666 A667
4E10	N671 W672 F673 N/D674 I675 T/S676 L679 W680
Z13	N671 W672 F673 N/D674 I675 T/S676

Supp. Table 1 Critical positions in the HIV-1 Envelope

List of amino acids that take part in key functional and structural interactions of HIV-1 Env. All amino acids are obtained from either the HIV-1 database, or from (221), and all position numberings are based on the HxB2 reference strain.



Supp. Figure 1 Synthesis of Auristatin for the toxin-gp120

For the production of toxin-gp120, the auristatin was synthesised with the addition of a maleimide linker and a valine-citrulline cathepsin B labile linker for the conjugation onto streptavidin.