ANTIGENIC AND IMMUNOMODULATORY PROPERTIES OF HIV-1 GP120 N-LINKED GLYCOSYLATION

By

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Antigenic and Immunomodulatory Properties of HIV-1 gp120 N-linked Glycosylation

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Abstract

The HIV-1 surface glycoprotein, gp120, is made of a rapidly mutating protein core and an extensive carbohydrate shield which are, respectively, encoded by the viral genome and synthesised by the host cell. In contrast to host cell glycoproteins however, gp120 contains a population of unprocessed oligomannose-type glycans that interact with host lectins, promote HIV infection, and alter cell signalling. They also form the basis of the epitopes of several broadly neutralising antibodies isolated against HIV, making them a key feature for immunogen design. The mechanistic basis of how HIV glycans are differentially processed by the host cell was demonstrated on a recombinant gp120 model, suggesting that steric occlusion within the patch of densely packed glycans lead to lack of processing by ER and Golgi α-mannosidases. Furthermore, an elevated level of oligomannose-type glycans was evidenced on gp120 isolated from HIV-1_{JRCSF} virions produced in PBMCs, compared to recombinant material (respectively ~79% and ~29% of total N-linked glycans), along with a subset of highly processed and sialylated, bi-, tri- and tetra-antennary complex-type glycans, which could be involved in direct interaction with key host cell immune receptors and strongly suppress both antibody and T-cell immune responses. The effect of variation in viral production systems was analysed, with envelope glycoprotein derived from pseudoviral particles produced in HEK 293T cells exhibiting predominantly an oligomannose population (98%), compared to gp120 isolated from a single-plasmid infectious molecular clone (56%). Finally, mutation of one or several glycosylation site(s), known to be required for oligomannose-restricted neutralizing antibodies, was shown to induce a subtle redistribution within the oligomannose series whilst maintaining overall oligomannose levels. The gp120 glycan profile is therefore robust to mutations and also remarkably similar across primary viral isolates from Africa, Asia and Europe and consequently represents an attractive target for vaccine development.

List of publications

The work described in this thesis and related studies has formed the basis of published manuscripts which are listed below.

1. Dunlop D.C., Bonomelli C., Mansab F., Vasiljevic S., Doores K.J., Wormald M.R., Palma A.S., Feizi T., Harvey D.J., Dwek R.a., Crispin M. and Scanlan C.N. **2010**. Polysaccharide mimicry of the epitope of the broadly neutralizing anti-HIV antibody, 2G12, induces enhanced antibody responses to self oligomannose glycans. *Glycobiology* 20: 812-23
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3. Kong L., Sheppard N.C., Stewart-Jones G.B., Robson C.L., Chen H., Xu X., Krashias G., Bonomelli C., Scanlan C.N., Kwong P.D., Jeffs S.A., Jones I.M. and Sattentau Q.J. **2010**. Expression-system-dependent modulation of HIV-1 envelope glycoprotein antigenicity and immunogenicity. *J Mol Biol* 403: 131-47
4. Bonomelli C.*, Doores K.J.*, Dunlop D.C., Thaney V., Dwek R.a., Burton D.R., Crispin M. and Scanlan C.N. **2011**. The Glycan Shield of HIV Is Predominantly Oligomannose Independently of Production System or Viral Clade. *PloS one* 6: e23521
5. Harvey D.J., Sobott F., Crispin M., Wrobel A., Bonomelli C., Vasiljevic S., Scanlan C.N., Scarff C.A., Thalassinos K. and Scrivens J.H. **2011**. Ion mobility mass spectrometry for extracting spectra of N-glycans directly from incubation mixtures following glycan release: application to glycans from engineered glycoforms of intact, folded HIV gp120. *J Am Soc Mass Spectrom* 22: 568-81
6. Goodfellow J.J., Baruah K., Yamamoto K., Bonomelli C., Krishna B., Harvey D.J., Crispin M., Scanlan C.N. and Davis B.G. **2012**. An endoglycosidase with alternative glycan specificity allows broadened glycoprotein remodelling. *J Am Chem Soc* 134: 8030-3
7. Harvey D.J., Scarff C.A., Crispin M., Scanlan C.N., Bonomelli C. and Scrivens J.H. **2012**. MALDI-MS/MS with Traveling Wave Ion Mobility for the Structural Analysis of N-Linked Glycans. *J Am Soc Mass Spectrom* 23: 1955-66

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Chapter 1

Introduction

1.1. The human immunodeficiency virus

1.1.1. The HIV/AIDS pandemic

The HIV/AIDS epidemic has spread rapidly, and has global long-lasting effects on demography, communities, firms, agriculture, education, and economic development (United Nations 2004). According to the WHO/UNAIDS report, 2.6 million people became infected with HIV-1 in 2009, with more than 95% of infections occurring in developing countries. This brought the total number of people living with the virus close to 33.3 million, of which over 22.5 million are estimated to be living in sub-Saharan Africa. Around 22 million people are estimated to have died of AIDS since the beginning of the epidemic, with 1.8 million deaths recorded in 2009.

There were few reports of HIV/AIDS-related cases before the 1980s. However, the first infection and transmission events are thought to have taken place in the 1930s (Korber *et al.* 2000; Salemi *et al.* 2001), with the earliest sample being identified as being present in the Democratic Republic of Congo in 1959 (Zhu *et al.* 1998). The first public report of AIDS was made on the 5th June 1981 by the Centre for Disease Control and Prevention in Atlanta, after observation of a rare lung infection (*Pneumocystis carinii pneumonia)* in five young men. One year later, the name 'Acquired Immune Deficiency Syndrome' was introduced.

In 1983, a virus believed to be the causative agent of AIDS was isolated in the Institut Pasteur in France (Barre-Sinoussi *et al.* 1983), and later named lymphadenopathy-associated virus (LAV). In 1984, the National Cancer Institute in the US isolated a virus named human T lymphocytropic virus type 3 (HTLV-III) (Gallo *et al.* 1984). It was subsequently demonstrated that LAV and HTLV-III were the same virus responsible for AIDS, and the name Human Immunodeficiency Virus (HIV) was confirmed by the International Committee on Taxonomy of Viruses in 1987.

The transmission routes for HIV were determined, and preventive measures, such as blood screening and needle exchange schemes, were introduced in an effort to reduce transmission. Thirteen years after the discovery of the virus, the announcement of the anti-retroviral therapy (ART) was made at the International AIDS Conference in Vancouver in 1996. Highly active anti-retroviral therapy (HAART) is composed of three antiretroviral drugs targeting different viral proteins, such as the viral reverse transcriptase and protease (Greene 2004). HAART has decreased mortality rates by delaying the progression to AIDS (Palella *et al.* 1998), and it has also significantly reduced mother-to-child transmission (Cooper *et al.* 2002). However, HAART has significant side effects, can lead to drug-resistant strains, and does not eradicate the virus (Finzi *et al.* 1997). Nearly 30 years after the discovery of HIV there is neither a cure for AIDS, nor is a preventive vaccine available. The development of a prophylactic vaccine is perhaps especially important for populations that cannot readily access HAART treatments, and will be crucial to control the HIV/AIDS epidemic. Several vaccine candidates have entered clinical trials in the last twenty years, and the moderate positive results obtained recently with the RV144 trial indicate that HIV prevention might be achievable (section 1.5.) (Rerks-Ngarm *et al.* 2009).

1.1.2. The antigenic diversity of HIV-1

Two distinct types of human viruses, HIV-1 and HIV-2, both originating from zoonotic infections, can lead to AIDS. The closest relative of HIV-1 is the simian immunodeficiency virus SIV_{cpz}, whose natural host is the chimpanzee *Pan troglodytes troglodytes*. HIV-1 is likely to have originated from a cross-species infection, through cuts, butchery, or close contacts between chimpanzees and humans in central parts of sub-Saharan Africa (Gao *et al.* 1999; Hahn *et al.* 2000; Korber *et al.* 2000); HIV-2 originated from the sooty mangabey monkey *Cercocebus atys* and is mainly restricted to West Africa (Lemey *et al.* 2003). SIV infection has been reported for 36 African primate species (Klatt *et al.* 2012). There are five main phylogenetic SIV lineages, and SIV shows long-term host-dependent co-evolution. Unlike HIV-1, SIV has low virulence and simian lentiviruses do not typically cause significant pathology in their natural hosts, except for SIV_{cpz} (Sharp *et al.* 2011). However, cross-species transmission of primate lentiviruses to new hosts can cause epidemic spread providing they adapt to their new hosts, as was observed for example with SIV_{smm} infection of macaques (Letvin *et al.* 1985; Murphey-Corb *et al.* 1986).

HIV-1 isolates are classified into four evolutionary linked groups on the basis of genetic similarities and phylogenetic analysis (Korber *et al.* 2001). The groups M, O, N, and P, account for all of the different HIV-1 lineages that have been isolated and characterised to date. The ‘major’ group M isolates, which cause more than 90% of total HIV-1 infections, have evolved from a single transmission of SIV_{cpz} from chimpanzees (Gao *et al.* 1999). The group is further subdivided based on sequence similarity into nine subtypes or clades, A, B, C, D, F, G, H, J, and K. The sequences of the structural protein Gag and the envelope glycoprotein Env vary by at least 15% and 20% respectively between isolates from two different clades. The different clades can be related to different geographical areas, with clade C dominant in sub-Saharan Africa and India, and clade B predominant in America, Australia,

Japan and Western Europe. Recombination between HIV-1 isolates present in the same infected cell gives rise to circulating recombinant forms (CRFs) (Hu *et al.* 1990; Robertson *et al.* 1995). Notably, CRF01, which originated in central Africa, is the predominant subtype in Southeast Asia. The much less prevalent 'outlier' group O arose from a cross-species infection with a chimpanzee or gorilla lentivirus, and is mainly distributed across west central Africa (Gurtler *et al.* 1996; Van Heuverswyn *et al.* 2006). The group N ('non-M, non-O') is composed of viruses isolated in a few individuals in Cameroon and can be traced to a cross-infection with SIV_{cpz} (Simon *et al.* 1998; Keele *et al.* 2006). Lastly, an isolate closely related to SIV_{gor} has been characterized and attributed to a new group P (Plantier *et al.* 2009).

In the majority of cases, a single transmitted virus is responsible for infection (Keele *et al.* 2008; Abrahams *et al.* 2009). However, mutations alter around 10% of the envelope gene sequence over the course of infection, giving rise to a set of viruses in each infected individuals, commonly referred to as viral quasi-species (Goodenow *et al.* 1989; Meyerhans *et al.* 1989). The diversity and frequency of forms present in the viral population arise from several factors. High mutation rates introduce on average one mutation in every three genomes (Mansky *et al.* 1995), mainly due to the lack of proof-reading mechanisms in the viral RNA reverse transcriptase (RT) (Ji *et al.* 1992), with additional changes in the viral genome generated by host RNA polymerase II and DNA polymerases. Viral quasi-species can also arise by recombination. Finally, whilst a rapid viral turnover gives rise to a large viral population (Ho *et al.* 1995; Wei *et al.* 1995; Perelson *et al.* 1996), selective pressure by therapy or immune system (Richman *et al.* 2003; Frost *et al.* 2005b) are responsible for the frequency of the various viral forms observed. The presence of such a varied viral population within an infected individual, which is comparable to the global genetic variation of an influenza virus (Korber *et al.* 2001), is a major obstacle for successful anti-retroviral therapy and vaccine development.

1.1.3. HIV-1 viral structure

HIV-1 is a lentivirus belonging to the retrovirus family. A virion particle contains two identical molecules of positive sense single-stranded RNA (Figure 1.1). The viral genome encodes in total 15 proteins, distributed between structural proteins (Gag, Env), enzymes (Pol), regulatory proteins (Tat and Rev), and accessory proteins (Vif, Vpu, Vpr and Nef), that are further processed and assembled into a mature infectious virion (Figure 1.2).

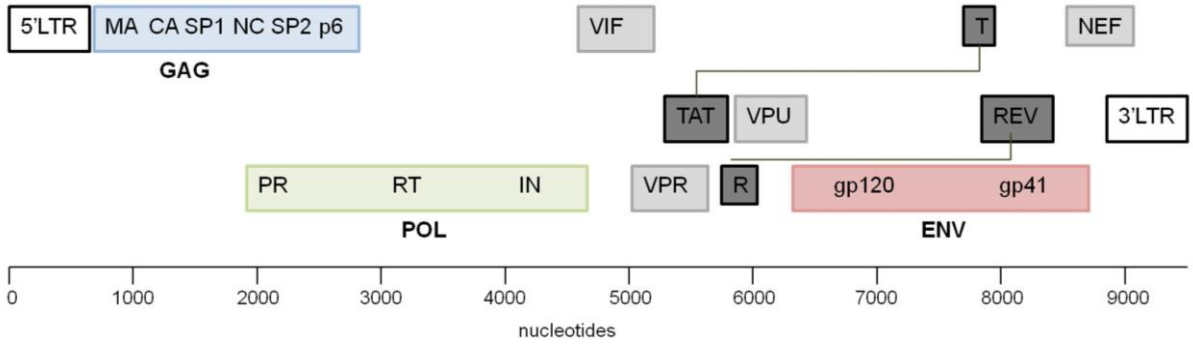


Figure 1.1. The HIV-1 genome organisation
Open-reading frames of the HIV-1 genome (approximately 9.8kb). Structural proteins (encoded by GAG) are highlighted in blue, viral enzymes (encoded by POL) in green, envelope glycoproteins (encoded by ENV) in red, regulatory proteins (Tat and Rev) in dark grey, and accessory proteins (Vpu, Vif, Vpr and Nef) in light grey. The following abbreviations are used: matrix (MA), capsid (CA), spacer peptide 1 and 2 (SP1 and SP2), nucleocapsid (NC), protease (PR), reverse transcriptase (RT), integrase (IN), and long terminal repeat motif (LTR).

The Gag polyprotein precursor p55 assembles at the plasma membrane, and is responsible for the internal structural properties of the virus. The assembly of Gag polyproteins results in an intracellular immature, non-infectious, virus-like particle. During viral assembly, the precursor is cleaved by viral aspartyl protease (PR), encoded by the POL gene, and the conformational changes induced lead to the formation of mature and infectious virions (Figure 1.2) (Gottlinger *et al.* 1989). The matrix (MA or p17) binds to the plasma membrane through insertion of its myristoyl group into the lipid bilayer. The conical capsid (CA or p24) confines the NC, RT, and IN. The nucleocapsid (NC or p7) coats the viral RNA

genome. The C-terminal domain of Gag (p6) interacts with the Endosomal Sorting Complex Requiered for Transport (ESCRT) pathway leading to budding.

The envelope glycoprotein (Env) precursor gp160 is cleaved into gp120 and gp41 by furin in the *trans*-Golgi network at the C-terminus of the consensus sequence Arg-Glu-Lys-Arg (Thomas 2002). The two glycoproteins then further assemble via non-covalent interactions into a trimer of heterodimers on the viral surface (Figure 1.2). Attachment to the host cell is mediated by gp120 binding first to the CD4 receptor and then the CXCR4/CCR5 co-receptor following induced conformational changes (section 1.2). Meanwhile, gp41 is responsible for the fusion of viral and cell membranes.

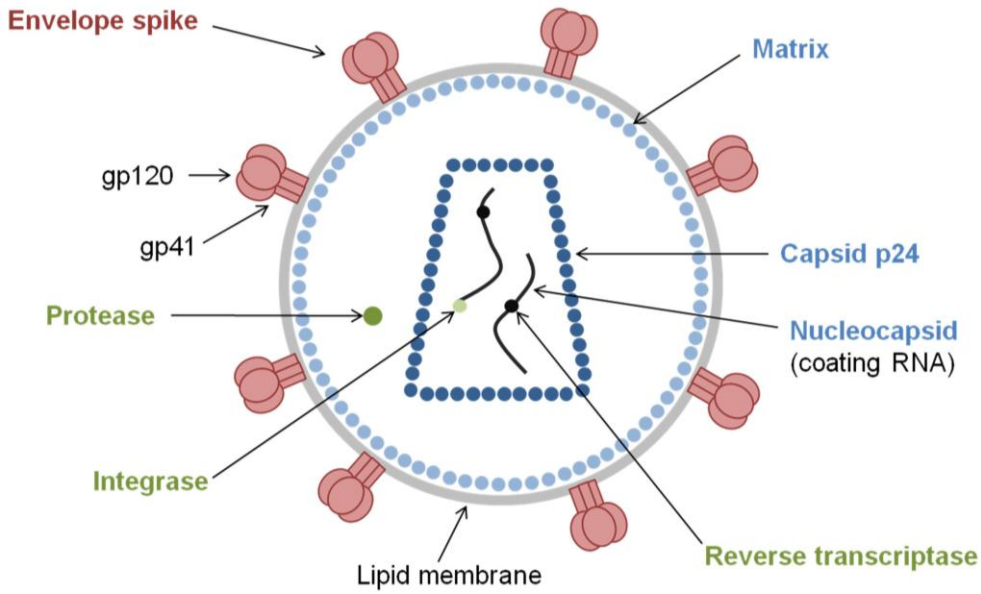


Figure 1.2. Schematic model of a mature HIV-1 virion
A spherical virion particle has a diameter of approximately 120 nm (Gelderblom *et al.* 1987). Mature virions exhibit a capsid of conical shape that encloses two copies of positive single-stranded RNA associated with viral cofactors. As budding occurs from the host plasma membrane, virions acquire a lipid envelope. There are between 9 to 14 envelope spikes displayed on the viral surface (Zhu *et al.* 2003).

The viral enzymes protease (PR or p10), reverse transcriptase (RT or p50), RNase H (or p15), and integrase (IN or p31) are cleaved from the Pol precursor p160 by PR (Frankel *et al.* 1998). The protease also cleaves Gag at several sites to produce the MA, CA, NC, and p6

proteins, inducing the conformational changes required to form infectious mature virions. Reverse transcription of the viral genome is performed by RT, which catalyses RNA- and DNA-dependent DNA polymerisation, with RNase H digesting the RNA portion of the RNA-DNA hybrids generated. The $tRNA_3^{Lys}$ primer used to initiate the reaction is incorporated in virions during assembly and budding. Finally, IN is responsible for the integration of the viral genome into the host chromosome, by first removing two 3′ nucleotides on each DNA strand, and then annealing them to the 5′ ends of target DNA. Following integration, the DNA is repaired by cellular enzymes.

The HIV-1 genome also comprises additional regulatory genes, such as the RNA-binding proteins Tat and Rev. Tat activates and greatly enhances transcription initiation and elongation from the LTR promoter by binding to TAR (trans-activating response element), an RNA hairpin located downstream of the transcription start site (Feng *et al.* 1988; Roy *et al.* 1990). Rev binds to the Rev response element (RRE), an RNA sequence that is present only on non-spliced or singly-spliced mRNAs, promoting their nuclear export (Malim *et al.* 1989). This increases the cytoplasmic concentration of mRNAs that code for Gag, Pol, Vif, Vpu, and Vpr, instead of multiply-spliced RNAs that are produced in the early phases of cellular infection.

Finally, the HIV-1 genome also comprises accessory proteins that whilst dispensable *in vitro*, are nevertheless crucial for infectivity *in vivo* (Malim *et al.* 2008). The viral infectivity factor (Vif) is a cytoplasmic protein that is essential to the formation of infectious virions (Fisher *et al.* 1987; Strebel *et al.* 1987), by counteracting the antiviral effects of members of the APOBEC (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) family (Sheehy *et al.* 2002; Marin *et al.* 2003). Amongst the APOBEC family of cellular cytidine deaminases, APOBEC3G exhibits the highest antiviral properties. In the absence of Vif, it becomes encapsulated in the budding virion, and induces guanosine to adenosine

mutations on the HIV-1 genome by deaminating cytidine to uridine in the first-strand reverse transcript (Harris *et al.* 2003; Mangeat *et al.* 2003; Marin *et al.* 2003; Zhang *et al.* 2003). Vif binding to APOBEC3G is followed by the formation of a functional ubiquitin ligase complex that leads to the degradation of APOBEC3G and to the inhibition of its DNA mutator activity (Marin *et al.* 2003).

The viral protein U, Vpu, is likewise an antagonist of another host restriction factor, tetherin, a type II membrane protein that limits budding and release of virions from infected cells (Neil *et al.* 2008). Additionally, Vpu is involved in the degradation of CD4 in the ER, which subsequently leads to enhanced release of Env molecules and down-regulation of CD4 on the cell surface (Willey *et al.* 1992).

Similar to Vpu, Nef down-regulates host proteins expression on the cell surface, such as CD4 (Garcia *et al.* 1991; Chaudhuri *et al.* 2007), and the major histocompatibility complex (MHC) class I (Schwartz *et al.* 1996), promoting immune evasion by disrupting antigen presentation. Moreover, the selective down-regulation of HLA-A and HLA-B, but not HLA-C and HLA-E, protect the infected cells from NK cells killing (Cohen *et al.* 1999). Nef helps maintain high viral load, enhance viral infectivity, and is a critical determinant of disease progression (Kirchhoff *et al.* 2008). It is one of the first viral proteins produced, and is anchored in the plasma membrane through its myristolic residue.

Finally, the viral protein R, Vpr, is a multifunctional protein that has been shown to assist nuclear import of pre-integration complexes, being therefore crucial for productive infection of macrophages, as well as to induce G_2 arrest, apoptosis, transactivate viral and cellular genes, and facilitate immune evasion (Jowett *et al.* 1995; Ayyavoo *et al.* 1997; Vodicka *et al.* 1998; Romani *et al.* 2009).

1.1.4. HIV-1 life cycle

Binding of gp120 to the CD4 receptor defines HIV's primary cellular tropism (Figure 1.3). CD4 is expressed on the surface of T helper cells, dendritic cells, monocytes and macrophages. Upon CD4 binding, conformational changes within gp120 allow for binding to chemokine co-receptors (Feng *et al.* 1996; Sattentau 1998; Berger *et al.* 1999; Doranz *et al.* 1999), where the choice between CCR5 or CXCR4 has consequences for pathogenesis, disease progression, and transmission (Moore *et al.* 2004). CCR5 is the main co-receptor used both during viral transmission, early stage and acute infections. A shift to the CXCR4 co-receptor is sometimes observed several years after infection, and the use of this co-receptor is associated with rapid disease progression. However, a change in co-receptor is not always observed and R5-viruses from late stages of HIV-1 infection exhibit enhanced macrophage-tropism, increased fitness, more efficient usage of CCR5, increased ability to cause apoptosis of CD4+ T cells, and enhanced kinetics of HIV-1 entry (Gorry *et al.* 2011).

Following binding, conformational changes within the Env spike lead to fusion of the virus and the host cell membrane, which permits core entry. The viral RT produces a dsDNA from the uncoated viral RNA template. A pre-integration complex formed of dsDNA, RT, IN, MA, Vpr and other cellular co-factors is formed and imported into the nucleus. IN then integrates viral DNA into the host genome, and transcription and translation subsequently occur as described in section 1.1.3.

A significant body of evidence supports that the plasma membrane is the main location for HIV-1 assembly and budding (Bieniasz 2009). However, some studies have shown the formation of virion particles from late endosomal membranes, especially in macrophages (Orenstein *et al.* 1988; Raposo *et al.* 2002; Pelchen-Matthews *et al.* 2003; Pelchen-Matthews *et al.* 2004; Kramer *et al.* 2005). HIV and SIV Env have been shown to have highly conserved trafficking sequences, including at least two endocytosis signals that

interact with clathrin adaptor complexes (Wyss *et al.* 2001), and prevent Env accumulation on the plasma membrane (LaBranche *et al.* 1995). Moreover, recent findings have shown that the first step of assembly occurs on endosomal membranes. The virion then buds as a non-infectious immature particle, with infectious virions formed following cleavage of Gag by the activated viral protease, and rearrangement of its subunits.

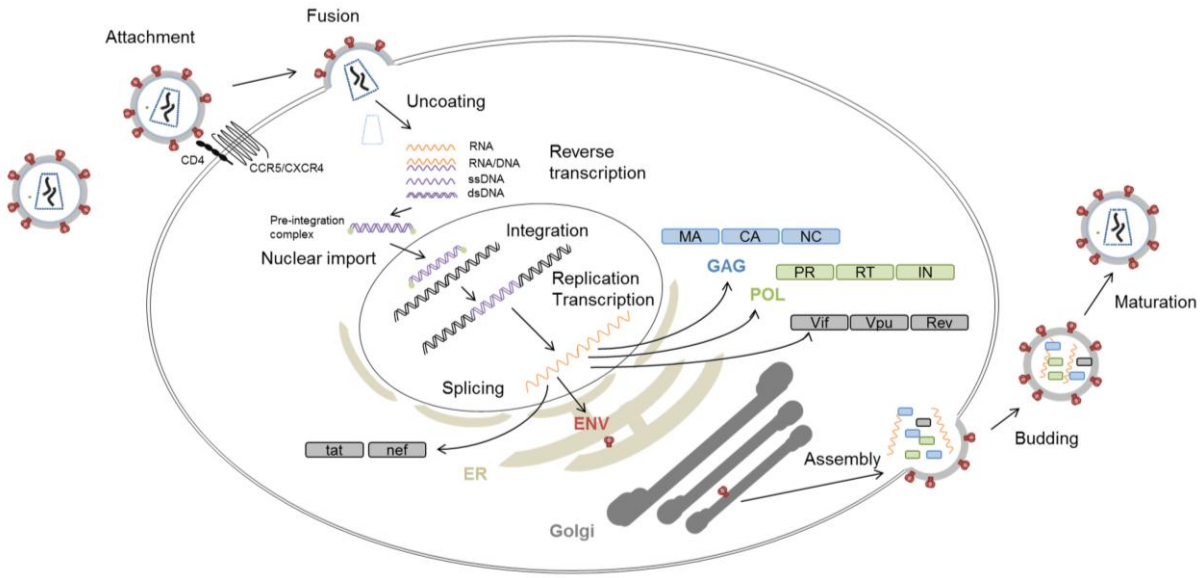


Figure 1.3. HIV life cycle
HIV-1 infection of host cells starts with the binding of gp120 to the CD4 receptor, followed by interaction with the chemokine co-receptor (CCR5 or CXCR4). Conformational changes within the envelope spike and gp41 lead to virus-cell membrane fusion, followed by entry of the core into the cell. Genomic RNA is reverse-transcripted into dsDNA by RT, and a pre-integration complex is formed and imported in the nucleus (the viral and host proteins participating in the pre-integration complex are omitted for clarity). IN catalyses the integration of the viral DNA into a host chromosome, and viral transcripts are further expressed from the promoter in the 5'LTR region – the transcription being enhanced by Tat. Spliced and genomic-length RNAs are regulated by Rev. Translation of Gag and Pol occurs in the cytoplasm, and Env is secreted in the ER/Golgi. The core particle then assembles at the plasma membrane, buds, and further matures upon Gag cleavage by Pol.

1.2. HIV-1 envelope glycoproteins structure and glycosylation

1.2.1. Envelope glycoprotein mediated viral attachment and entry

The viral envelope spikes are composed of a trimer of heterodimers of the two envelope glycoproteins, gp120 and gp41. The two glycoproteins are associated by non-covalent interactions, with gp41 anchoring the spike in the membrane. The attachment glycoprotein gp120 is responsible for cell tropism. Analysis of sequence variations between different isolates has shown that gp120 is composed of five conserved domains (C1-C5) interspaced with five hypervariable domains (V1-V5 loops) as highlighted in Figure 1.4.A (Willey *et al.* 1986; Modrow *et al.* 1987; Holmes *et al.* 1992). Virus entry into the host cell is mediated by binding of gp120 to the CD4 receptor (Figure 1.4.B) (Dalgleish *et al.* 1984; Klatzmann *et al.* 1984; Maddon *et al.* 1986; McDougal *et al.* 1986).

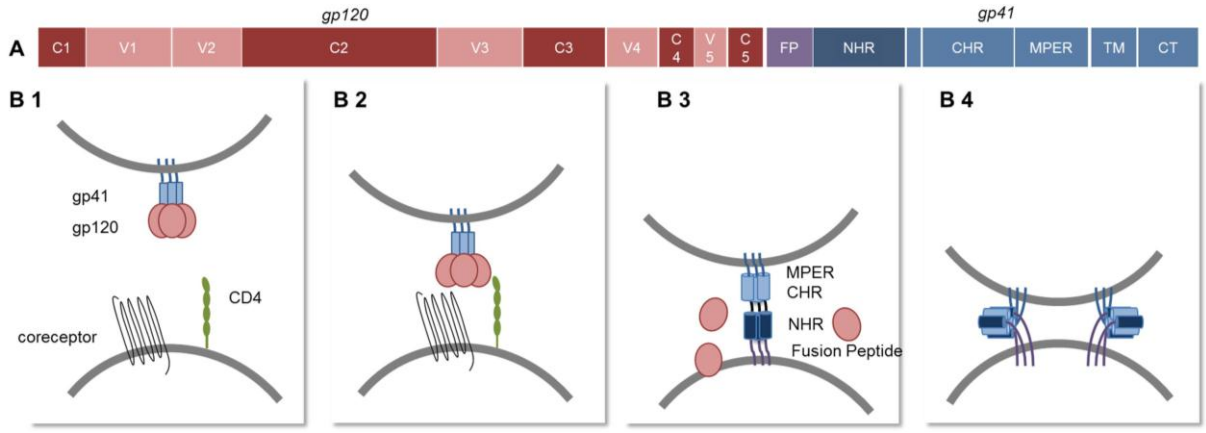


Figure 1.4. Virus attachment and fusion into host cells
(A) Schematic sequences of the envelope glycoproteins gp120 and gp41, highlighting gp120 constant (C1-C5) and variable (V1-V5) regions in red, and gp41 domains in blue, where FP is the fusion peptide, NHR the N-terminal heptad repeat helix, CHR the C-terminal heptad repeat helix, MPER the membrane proximal external region, TM the transmembrane domain, and CT the cytoplasmic tail. **(B) (1-2)** Virus attachment to the host cell is mediated by gp120 binding to the host receptor CD4 and co-receptor CCR5/CXCR4; **(3-4)** gp41 fusion peptide is inserted into the host membrane, leading to the formation of a six-helix bundle that brings host and viral membrane together and results in the formation of a fusion pore.

The binding of gp120 to CD4, shown in Figure 1.5.A, is mediated by the interaction of highly conserved gp120 residues (Asp368, Glu370, Trp427) with predominantly two CD4 residues, Phe43 and Arg59, as previously demonstrated by mutagenesis studies (Kowalski *et al.* 1987; Cordonnier *et al.* 1989; Olshevsky *et al.* 1990; Pollard *et al.* 1991; Choe *et al.* 1992; Ryu *et al.* 1994).

Two key X-ray crystal structures were solved and allowed further study on gp120 conformational changes upon CD4 binding, a key mechanism of HIV entry into cells. The crystal structure of an unliganded glycosylated SIV gp120 core (without the V1/V2, V3 loops) (Chen *et al.* 2005) was compared to the crystal structure of a deglycosylated gp120 core (Kwong *et al.* 1998) in complex with the two N-terminal domains of CD4 and the Fab region of the 17b antibody, which targets a binding site present upon CD4 binding (CD4i) (Figure 1.5.B and C). Two major folding domains were identified in the latter, referred to as the 'inner domain' and 'outer domain', with the former proximal to the C and N termini.

The co-receptor binding site overlaps with the 'bridging sheet', a structure that is present between the inner and outer domains in the CD4-bound state. Upon CD4 binding, the inner domain undergoes several conformational rearrangements, ultimately leading to the formation of the bridging sheet that is in close proximity to the target cell membrane. The outer domain also rotates with respect to the inner domain, resulting in the exposure of the newly-formed co-receptor binding site, which is positioned away from gp41 and close to the host cell membrane. The V3 loop, which is also involved in CD4 interaction, has its base adjacent to the bridging sheet involved in co-receptor binding (Hwang *et al.* 1991; Shioda *et al.* 1991; Carrillo *et al.* 1996; Malenbaum *et al.* 2000). Consequently, the V3 loop is mainly responsible for the chemokine co-receptor specificity, and it was shown that a few amino acid changes or alterations in N-linked glycosylation within the V3 loop led to a switch in co-receptor usage from CCR5 to CXCR4 (Yang *et al.* 2004; Ogert *et al.* 2001).

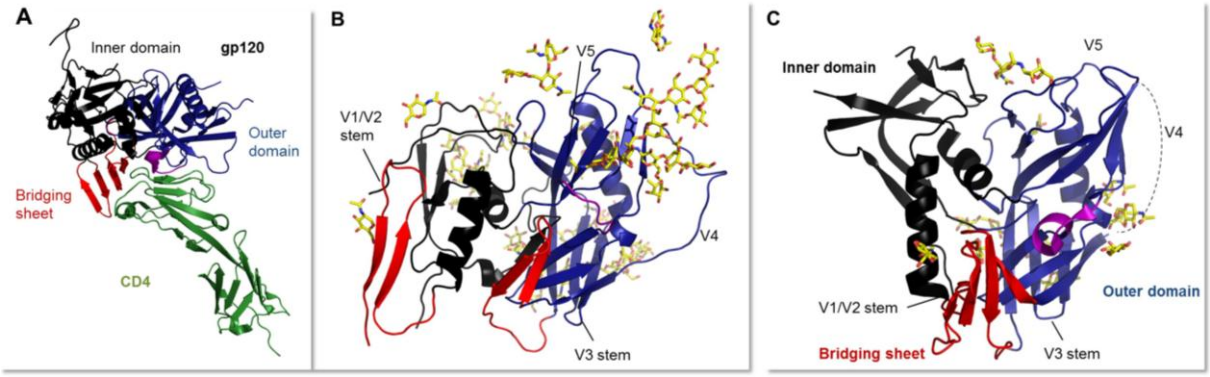


Figure 1.5. Molecular modelling of gp120 interaction with CD4
Molecular models of **(A)** gp120 bound to CD4; **(B)** unliganded SIV gp120 core; **(C)** deglycosylated CD4-bound HIV-1 gp120 core highlighting the conformational changes induced upon CD4 binding. The gp120 outer domain is shown in blue, gp120 inner domain in black, gp120 bridging sheet in red, the CD4-binding loop in purple, and the two CD4 N-terminal domains in green. The figure was constructed using pymol with the pdb 3JWD, 2BF1, and 1GC1 respectively.

Upon gp120 binding to CD4 and co-receptor CXCR4/CCR5, gp41 undergoes a series of conformational changes (Figure 1.4.B). Each gp41 ectodomain is composed of a fusion peptide, two α-helical regions (N-terminal and C-terminal helices) separated by a small disulfide loop, and a membrane-proximal external region (MPER) (Figure 1.4.A). Following the insertion of the hydrophobic fusion peptide in the target cell membrane, a central α-helical coiled coil is formed by three adjacent N-terminal helices. Three C-terminal helices subsequently pack into the hydrophobic grooves of the N-terminal helices, in an anti-parallel manner, resulting in the formation of a rod-like structure of three hairpins (Chan *et al.* 1997; Weissenhorn *et al.* 1997) that brings both cell and viral membranes together.

The long cytoplasmic domain of gp41, whilst dispensable for viral replication, is implicated in regulating the rate of endocytosis, Env cell surface expression, fusogenicity and association with MA during assembly (Gabuzda *et al.* 1992; Lambele *et al.* 2007). It was recently shown to induce the NF-κB transcription factor that further binds to HIV LTR and enhances transcription (Alcami *et al.* 1995; Postler *et al.* 2012).

1.2.2. Trimeric envelope spike structure

There are currently no X-ray models of the fully glycosylated Env trimer. Use of the cryoelectron microscopy (cryo-EM) single particle technique, which collects a large number of protein images and then reconstructs the 3D structure, has led to the determination of the structure of an unliganded, HIV-1_{JR-FL} Env trimer, with a truncation of gp41 cytoplasmic domain, at around 11 Å resolution (Figure 1.6.A) (Mao *et al.* 2012). The three gp41 subunits associate around the trimer axis at the plasma membrane. Each gp120 monomer occupies around 30 Å around that axis, and is further interacting with the two other gp120 subunit at the apex of the trimer. The gp120 inner and outer domains, obtained from previous crystal structures of gp120 bound to CD4, could not be fitted together into the cryo-EM density map, consistent with a conformational change induced by CD4 binding within the Env trimer. It was also noted that the unbound conformation was different for the SIV unliganded core and the HIV-1 Env trimers. The CD4 binding site (CD4BS) is located inside the cavity formed by each gp120 monomer, reducing its accessibility to neutralising antibodies.

The unbound trimer conformation is of a significant, metastable higher state of energy relative to the fusion one (Kwon *et al.* 2012). The native unliganded state is kept together by key contacts between gp41 subunits in the transmembrane domain and MPER, and also by interactions at the apex of the trimer between residues in the V1/V2 and V3 loops of gp120 'trimer association domain' (Mao *et al.* 2012). More specifically, unliganded gp120 conformation varies upon gp41 interaction with gp120 and the length and composition of the V1/V2, and V3 loops (Liu *et al.* 2008).

The structures of the unliganded Env, the Env bound to the anti-CD4BS antibody b12, and the Env bound to both CD4 and the antibody 17b were determined at around 20 Å resolution, and analysed using the X-ray crystal structures of the monomeric gp120 core obtained previously as a guide (Liu *et al.* 2008). The V1/V2 loops were found to be present at

the apex of the trimer, consistent with the cryo-EM map. The CD4BS is located 20 Å from the top of the spike, indicating that CD4 N-terminal D1 and D2 domains must reach into the spike, consequently driving further conformational changes within the spikes. Furthermore, CD4's own conformational changes bring the spike closer to the host cell membrane. Upon CD4 binding, conformational changes within each gp120 monomer as well as a change in each of their relative orientation together result in an opening-up of the spike (Figure 1.6) (Liu *et al.* 2008). This phenomenon is driven by the displacement the V1/V2 loops of gp120 and the lifting up the V3 loop, and was confirmed by cryoelectron tomography analysis of two unliganded SIV envelopes (White *et al.* 2010) and by single particle cryoelectron microscopy analysis of unliganded and liganded HIV-1 spikes (Wu *et al.* 2010a).

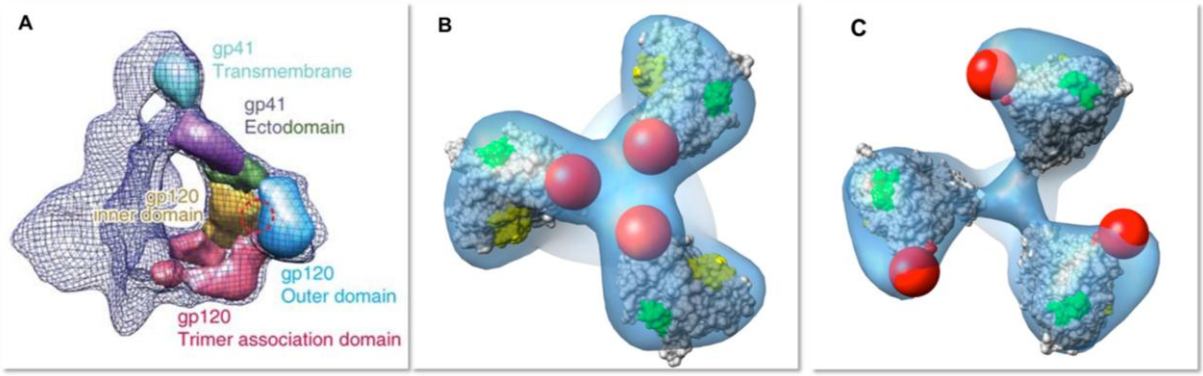


Figure 1.6. Env trimeric spike structure
(A) Cryo-EM density map of an unliganded HIV-1_{JR-FL} trimer (figure reprinted by permission from Macmillan Publishers Ltd: Nature Structural and Molecular Biology (Mao *et al.* 2012), copyright 2012). The Env trimer is represented as a solid-surface meshwork, highlighting monomer segments corresponding to gp41 TM domain (cyan), gp41 ectodomain (purple), gp120 outer domain (blue), gp120 inner domain (yellow), gp120 trimer association domain (pink) and the CD4BS (dashed red circle). The opening-up of the spikes upon CD4 binding can be visualised on the cryo-electron tomography density maps fitted with gp120 coordinates of **(B)** unliganded and **(C)** CD4-bound state of SIV Env spikes (top view). The V1/V2 loops are modelled by red spheres, and are found on the top of the spike. CD4 binding site residues are shown in yellow, and residues in the stem of the V3 loop in green. Figure adapted from White *et al.* 2010.

Cryo-electron tomographic studies of both HIV and SIV unliganded Env have shown that the gp41 region is either compacted (Zanetti *et al.* 2006; Liu *et al.* 2008; White *et al.*

2010) or formed of tripods with legs 70 Å apart (Zhu *et al.* 2006; Wu *et al.* 2010a). Furthermore, the putative structure of soluble trimers bound to 17b at a 9 Å resolution was obtained with cryo-EM (Tran *et al.* 2012). The conformation of an activated intermediate, precursor of the pre-hairpin intermediate in the fusion process is described. The gp41 fusion peptides face the host cell membrane, whilst the N-terminal helices, arranged around the three-fold axis of the trimer, are less tightly packed than in the fusion conformation.

1.2.3. N-linked glycosylation and processing of viral Env

HIV envelope glycoproteins are extensively modified with covalently attached, host-derived N- and O-linked oligosaccharides (Allan *et al.* 1985; Montagnier *et al.* 1985; Ratner *et al.* 1985; Lasky *et al.* 1986; Leonard *et al.* 1990; Bernstein *et al.* 1994; Zhu *et al.* 2000). Protein N-linked glycosylation is one of the main co-translational modifications, and is involved in protein folding, solubility and stability, as well as interactions with other proteins and further signalling (Varki *et al.* 2009).

The N-glycosylation of the viral envelope gp160 (later cleaved by furin in the Golgi apparatus into gp120 and gp41) begins in the endoplasmic reticulum (ER) with cotranslational addition of a dolichol-pyrophosphate(PP)-linked glucosylated oligomannose precursor, $Glc_3Man_9GlcNAc_2$, by the oligosaccharyltransferase to the free amide of asparagines residues within the nascent polypeptide (Figure 1.7) (Stanley *et al.* 2009). Transfer is directed to asparagines within glycosylation sequons Asn-Xaa-Ser/Thr-X (where Xaa is not Pro) (Kornfeld *et al.* 1985). This glycan precursor is trimmed by ER α-glucosidase I and II, to yield an intermediate monoglucosylated species that interacts with the molecular chaperones calnexin and calreticulin. The N-linked glycans present on the correctly folded protein are further trimmed by α-glucosidase II and ER α-mannosidase I to yield $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$. The latter is then processed by Golgi α-mannosidases IA–C in the

intermediate compartment (IC)/*cis*-Golgi compartments to form the $Man_5GlcNAc_2$ intermediate.

The first key step towards the synthesis of complex-type glycans is performed by the medial-Golgi-resident enzyme *N*-acetylglucosaminyltransferase I (GnT I) which catalyses the addition of a single β1→2-linked N-acetylglucosamine residue (GlcNAc) to form GlcNAc β1→2$Man_5GlcNAc_2$. This biosynthetic intermediate then undergoes further Golgi-resident glycosidase and glycosyltransferase processing, which catalyse the assembly of a wide array of mature, complex-type glycans. The differential expression of Golgi transferases between cell types can lead to significant differences in N-linked glycosylation: the major determinant in the chemical nature of complex-type glycans is generally the producer cell-type, not protein structure. A particular example is the tissue-specific expression of sialyltransferases, which catalyse the addition of a terminal sialic acid residue in a α2-3, α2-6 or α2-8 linkage (Harduin-Lepers *et al.* 2001). Sialic acids are negatively charged moieties that are involved in recognition mechanisms as 'self' elements, through receptors highly specific of particular linkages (Varki *et al.* 2012). The Siaα2-6Gal linkage, catalysed by the β-galactoside α2,6-sialyltransferase, a glycosylation enzyme expressed notably in B-lymphocytes, is illustrated in Figure 1.7.C. N-acetylneuraminic acid (Neu5Ac) is the dominant form of sialic acids found in humans.

Finally, in contrast to this cell-directed glycosylation model, whereby the structure of complex-type glycans is directly related to the cell-specific expression of glycosyltransferases, several proteins have been reported to exhibit a restricted set of glycans, dependant of glycan accessibility, protein structure and/or trafficking, referred to as protein-directed glycosylation. Human IgG bears a conserved glycosylation site at residue Asn297 in the Fc region, which has been shown to be a complex-type bi-antennary glycan. However, alternative glycosylation is observed for this glycan (Heil *et al.* 2004), and has high

implication for IgG Fc-mediated effector functions. It was indeed shown that core fucosylation of the heavy chain glycan reduces binding to the activatory FcγRIIIa receptor (Duh *et al.* 1989), whilst sialylation confers anti-inflammatory properties (Folks *et al.* 1989). Lack of terminal galactose residues on IgG Fc N-linked glycan is observed in autoimmune disease such as rheumatoid arthritis (Suzuki *et al.* 2009) and is associated with low enzyme activity of the β4-galactosyltransferase in B cells of rheumatoid arthritis patients (Suzuki *et al.* 2003). Agaloctasyl IgG has been shown to bind to mannose binding lectin and activate the complement cascade. Likewise, the three-dimensional shape of the protein and limited glycan accessibility can also lead to protein-directed glycosylation, as reported for HIV envelope glycoproteins.

Host glycoproteins have on average two thirds of the potential *N*-linked glycosylation sites occupied, with the occupancy rate of potential glycosylation sites increased by the presence of a threonine instead of a serine in the glycosylation sequon, and by the presence of neighbouring hydrophobic residues at the Asn-2 position (Petrescu *et al.* 2004).

The experimentally determined mass of gp120 closely matches that theoretically predicted for a complete occupancy of all the N-linked glycosylation sites present in the envelope gene, which has been confirmed by site analysis glycosylation (Leonard *et al.* 1990; Zhu *et al.* 2000). The number of sequons varies from different viral isolates, with a median at 25 N-glycosylation sites, within a range of 18–33 N-linked glycans per polypeptide chain, with 75% of isolates having between 23 and 27 sequons (Korber *et al.* 2001). The outer domain of gp120 contains a higher NXT sequon density and is more glycosylated than the inner domain which is involved in gp41 and trimer interface (Rao *et al.* 2010).

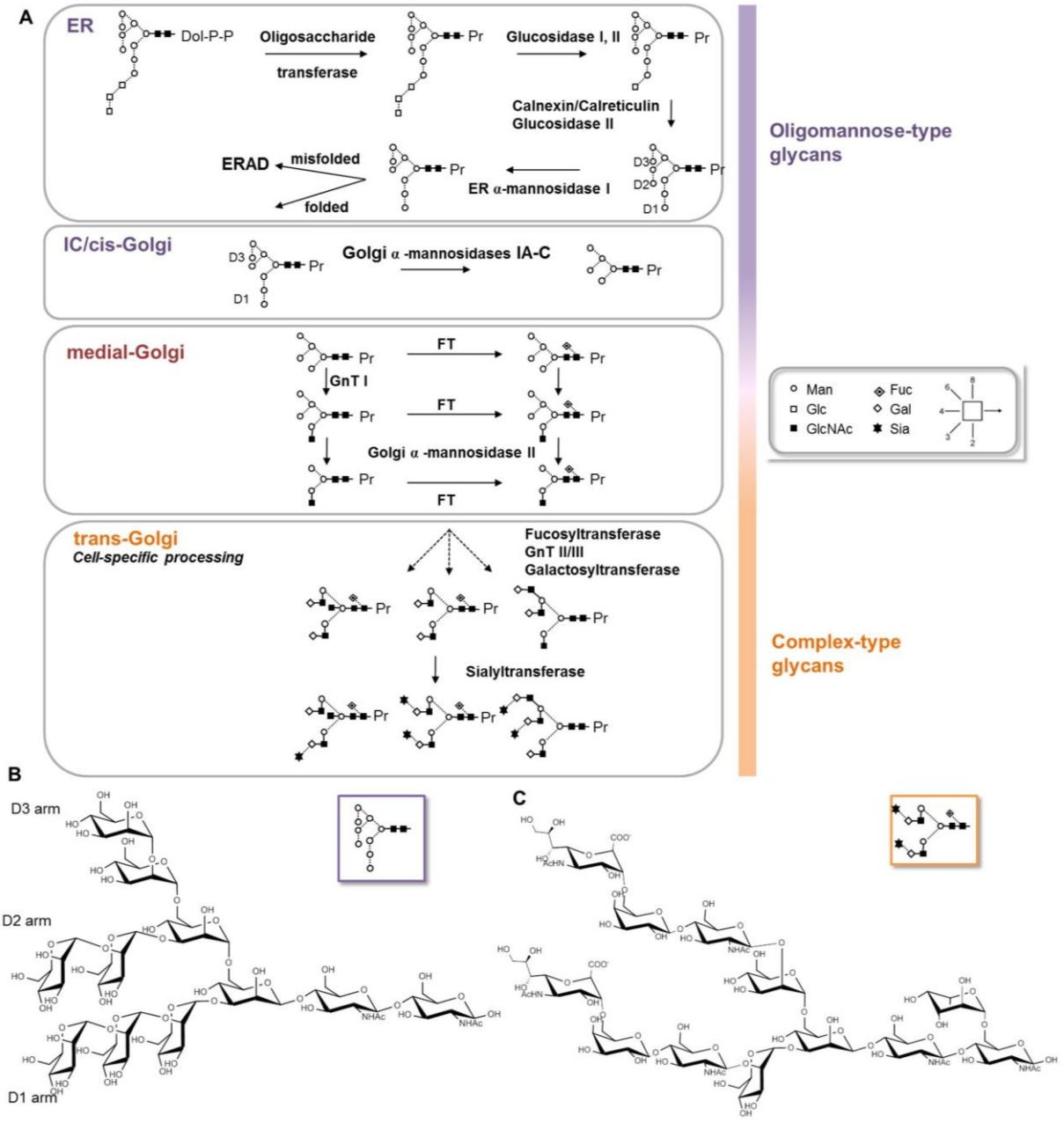


Figure 1.7. N-linked glycosylation pathway

(A) N-glycosylation of a glycoprotein (Pr) results in co-translational attachment of glycans to the amide group of asparagine in the ER lumen. Folding of the nascent chain starts in the ER, accompanied by the formation of disulfide bonds in this oxidizing milieu (10 in total for gp160, including 9 for gp120), and helped by the protein chaperones calnexin and calreticulin. Unprocessed, immature oligomannose-type glycans arise through processing by ER and Golgi α-mannosidases. Upon GnT I transfer of a N-acetylglucosamine residue in the medial Golgi, diversification of glycans occurs, leading to the presence of branched, galactosylated and sialylated complex-type glycans in the *trans*-Golgi network. Fucosylation of the reducing-terminal GlcNAc in the α1→6 position is performed by the Golgi-resident α1→6-fucosyltransferase (FT), and can occur in a GnT I-independent manner (Crispin *et al.* 2006). Glycan representation was done according to the guidelines from *Harvey et al. 2008*. Chemical structures of **(B)** the $Man_9GlcNAc_2$ glycan highlighting the D1, D2 and D3 arm, and **(C)** an α2-6 sialylated, biantennary and fucosylated complex-type glycan.

Envelope glycoproteins isolated from clade A and C infected individuals, but not clade B, show an increase in the number of potential glycosylation sites as the infection progresses (Sagar *et al.* 2006) – suggesting that virions with lower glycosylation sites are selected from the donor during transmission to a new recipient (Derdeyn *et al.* 2004; Chohan *et al.* 2005; Frost *et al.* 2005a; Li *et al.* 2006). There appears to be a balance between extensive glycosylation – which provides protection from the immune system recognition, but induces a slower rate of production plus difficulties in receptors and co-receptors binding and infection of new cells – and lesser glycosylation, which enhances binding to receptors whilst decreasing viral fitness due to increased sensitivity to neutralising antibodies (Zhang *et al.* 2004). Furthermore, whilst the number of gp120 glycosylation sequons has overall remained constant since the beginning of the epidemic, the NXT/NXS ratio, as well as the distribution of sequons, have changed to increase viral fitness.

1.2.4. Analysis of monomeric gp120 glycosylation

A variety of techniques have been employed in the analysis of the glycans of HIV. Antibody or lectin binding can be used to detect specific mono- or oligosaccharides present on a glycoprotein, as well as probe for linkage specificity, with the glycan chemical structure being elucidated by a combination of mass spectrometry (MS) and chromatography on protein-released glycans. The analysis of the glycome is somewhat complicated by the complex branched structure of carbohydrates compared to DNA and protein molecules, the microheterogeneity observed at a PNGS, i.e. different glycan structures can be present at the same site on different protein molecules, and finally by the presence of isomeric structures that result from different stereochemistry of individual monosaccharides and linkages between them.

Several glycosylation site analyses have been conducted on recombinant monomeric gp120 (Figure 1.8). The first study was performed by Leonard *et al.* on recombinant monomeric HIV_{IIIB} gp120 expressed in Chinese hamster ovary (CHO) cells Leonard *et al.* 1990. Oligomannose and complex-type glycans were released from tryptic peptides by using the peptide-N-glycosidase F (PNGase F), a routinely used endoglycosidase that cleaves the bond between the GlcNAc moiety and the Asn residue, converting it to Asp. Oligomannose-type glycans were specifically cleaved using the endoglycosidase H (Endo H), that cleaves oligomannose- and most hybrid-type glycans between the two core GlcNAc residues, but not complex-type glycans. Tryptic peptides were analysed by RP-HPLC and identified by amino acid analysis. 14 glycopeptides were identified and their sensitivity to Endo H treatment was then used to determine the nature of the glycan structure i.e. oligomannose or complex-type glycans. Overall, the 24 PNGS on $gp120_{IIIB}$ were found to be fully occupied, with 13 bearing complex-type glycans. The 11 oligomannose-type glycan occupied PNGS were amongst the 13 most highly conserved glycosylation sites found on $gp120_{IIIB}$. As this analysis was based on relative retention time shifting upon PNGase F and Endo H digests only, no define chemical structure of the glycan at each PNGS was determined.

Progress in mass spectrometry allowed for more precise characterization of gp120 glycans, including microheterogeneity at individual PNGS. The analysis of monomeric HIV_{SF2} gp120 expressed in CHO cells was conducted with MALDI and nanoelectrospray MS and MS/MS techniques on RP-HPLC fractionated glycopeptides Zhu *et al.* 2000. Of HIV_{SF2} gp120's 26 potential glycosylation sites, the glycans structures at 25 of these sites were characterized further. The relative abundance between different glycan species was also determined at the majority of PNGS. As observed on the previously characterized $gp120_{IIIB}$, $gp120_{SF2}$ exhibits a population of unprocessed oligomannose-type glycans, together with bi- and tri-antennary complex-type glycans, that can be fucosylated and sialylated. A follow-up

study using nanocapillary HPLC coupled with ESI and MALDI-MS aimed to further characterize the sialylation, and eight sialylated glycans on monomeric $gp120_{SF2}$ were observed Cutalo *et al.* 2004.

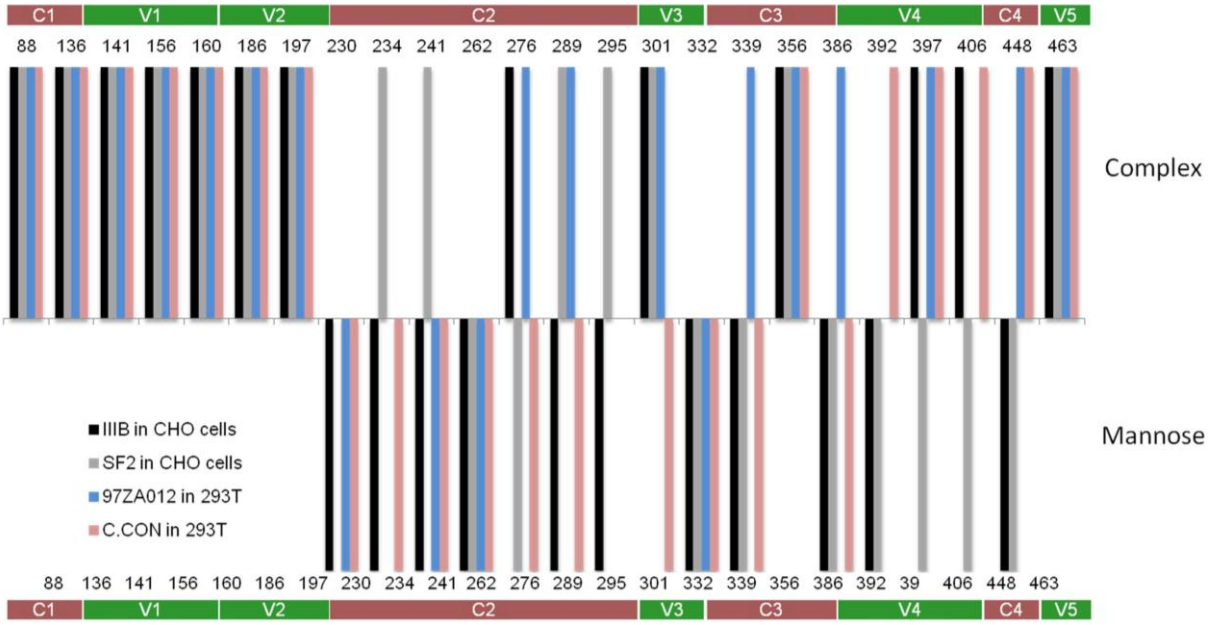


Figure 1.8. Site analysis glycosylation of monomeric gp120
Diagram showing the presence of complex- or oligomannose-type glycans at a specific position within the amino acid sequence of $gp120_{IIIB}$ in black, $gp120_{SF2}$ in grey, $gp120_{97ZA012}$ in blue and $gp120_{C.CON}$ in red. This figure was derived from the results of Go *et al.* 2009; Zhu *et al.* 2000; Leonard *et al.* 1990. The numbering is based on the HIV_{HxB2} isolate Env sequence. The position of the constant and variable regions is indicated with red and green boxes respectively (not to scale).

Analyses were also conducted on consensus sequences for the group M (CON-S) (Go *et al.* 2008), the clade C (C.CON) (Go *et al.* 2009), and a clade C primary isolate 97ZA012 by Go *et al.* Based on the number of different glycan structures found at a given PNGS, a ratio between oligomannose- and complex-type glycans was determined, which might not reflect entirely the relative abundance between these structures. It was found that $gp120_{C.CON}$ (29 potential N-glycosylation sites, 22 occupied) exhibited a higher proportion of oligomannose-

type glycans than $gp120_{97ZA012}$ (24 potential N-glycosylation sites, 22 occupied), both predominantly distributed within the C2 and C3 regions.

The following conclusions can be drawn for Figure 1.8 which displays the results from these different analyses: (i) N-glycosylation sites are fully, or almost fully occupied on gp120; (ii) glycans in the C1 region, V1/V2 and V5 loops are complex-type glycans; (iii) glycans within the C2, C3 regions and the V3 loop are mostly oligomannose glycans, with the exception of N301 and N356; (iv) glycans in the V4 loop, C4 and C5 regions are mostly complex-type glycans.

Whilst the structure of the complex-type glycans will depend on the cells in which the glycoprotein is expressed, all analyses share the presence of a 'patch' of unprocessed oligomannose-type glycans around the immunodominant V3 loop. On the folded protein, these oligomannose glycans arrange on what has been named the 'immunologically silent face', as the underlying peptide epitopes are covered by carbohydrates which prevent antibody recognition (Wyatt *et al.* 1998a).

The previous analyses have been performed on recombinant material expressed in CHO cells that are routinely used for protein expression. However, the glycan structures at each specific site might differ on viral Env, as gp120 trimerisation could conceivably affect glycan processing, and the glycosylation machinery of human HIV-infected cells is different to the one of CHO cells (Lee *et al.* 1989). Analysis of gp120 directly isolated from virions produced in PBMCs is described in Chapter 4.

Several O-linked glycans are also observed on viral Env (Bernstein *et al.* 1994; Corbeau *et al.* 1995). In contrary to N-linked glycosylation, there are no consensus sequences for O-linked glycosylation sites, as O-GalNac glycosylation mainly depends on surface accessibility and three-dimensional, structural context. O-linked glycosylation begins with the addition of a core GalNAc residue to a serine or threonine residues, generating the Tn-

antigen, and is followed by addition of various GlcNAc, Fuc and sialic acid residues (Hart 1992). O-linked glycans are involved in protein stability, and also provide additional shielding from the humoral immune response. HIV gp120 contains between 4 and 8 O-linked glycans, with high strain-variation observed (Hansen *et al.* 1998). Interestingly, cross-neutralising antibodies were elicited against a Tn-epitope present on gp120 (Hansen *et al.* 1991).

1.2.5. HIV-1 Env glycans implications for infectivity and immune evasion

Three main roles can be attributed to HIV-1 Env N-linked glycans: (i) they assist protein folding and ensure protein stability, (ii) their presence and location provide a shield for underlying peptide epitopes from immune system recognition, (iii) their specific binding to receptors can modulate immune response and help infectivity.

Protein folding and stability

The first role of Env N-linked glycans is to facilitate protein folding, by stabilising folding domains, thus preventing aggregation, as well as interacting with molecular chaperones (Trombetta *et al.* 1998). It has been shown that nonglycosylated gp120 was unable to bind to CD4, albeit no glycans are involved in the binding interaction, indicative of the importance of glycans in reaching a proper folded state (Li *et al.* 1993).

The N301 glycan within the V3 loop has been shown to be important for infectivity via viral entry into host cell for some HIV isolates, by disrupting binding to the co-receptor while retaining CD4 binding (McCaffrey *et al.* 2004). The V3 loop partially shields the CD4BS, and some of its residues contribute, together with residues from the C4 region, to the co-receptor binding site.

Altering Env folding is the mode of action of *N*-butyldeoxynojirimycin (*N*B-DNJ) (Chapel *et al.* 2007). This compound is an inhibitor of α–glucosidase I and II, and has been

shown to inhibit viral and host cell membrane fusion, whilst retaining CD4 binding (Fischer *et al.* 1995). Liposome-encapsulated *N*B-DNJ was shown to inhibit viral infectivity against a cross-clade panel of 25 HIV-1 isolates, and infectivity was abolished after two rounds of *N*B-DNJ treatment (Pollock *et al.* 2008).

An evolving glycan shield providing neutralisation escape

The occlusion of potentially antigenic protein epitopes by large, closely-spaced, N-linked glycans plays a central role in protection against the host humoral response (Wei *et al.* 2003). The N-linked glycans of gp120 are synthesised by the host cell, and would not be expected to be immunogenic, as a result of tolerance to 'self' glycan structures. The intensive glycosylation of Env provides protection against antibody neutralisation to epitopes such as the CD4 binding site, the V1/V2 and V3 loops. Glycans partially overlap the epitope, yet still allow receptor binding.

Moreover, the addition and loss of glycosylation site(s) can dramatically affect antibody binding. A study on rhesus monkeys infected with SIV and V1 glycosylation deleted mutants demonstrated that glycosylation offers protection of some structural determinants, and decreases neutralisation by antibodies (Reitter *et al.* 1998b). Sensitivity to anti-V3 loop antibodies is augmented upon removal of glycans within the V3 loop (N301) (Back *et al.* 1994; Malenbaum *et al.* 2000; McCaffrey *et al.* 2004; Koch *et al.* 2003), the V2 loop (Ly *et al.* 2000), and the 'immunologically silent face' (V4 loop, C4 and C5 regions). Increased sensitivity to anti-CD4BS antibodies (McCaffrey *et al.* 2004) is observed upon removal of glycans within and around the V3 loop, including the C2 glycan N295 at the N-terminal base of the V3 loop, the C4 glycan N332 at the C-terminal base of the V3 loop, and the V3 loop glycan N301 (Back *et al.* 1994; Koch *et al.* 2003), glycans within the V2 loop (Ly *et al.* 2000) and glycans on the immunologically silent face. The V4 region of gp120 is involved in CD4

binding, and is surrounded by glycans from the immunological silent face that mask the CD4BS and the V3 loop from antibody recognition. It has been shown that removal of glycan N440 in the C4 region and glycan N456 in the V5 loop leads to increased susceptibility by CD4-binding site antibodies and several anti-V3 loop antibodies (McCaffrey *et al.* 2004). Sensitivity to anti-co-receptor antibodies increases upon removal of the V3 loop N301 glycan, as well as the C2 glycan N276 and the V4 glycan N386 (Koch *et al.* 2003). Upon removal of glycans within the C2 region (N295) (Li *et al.* 2006), C4, and V5 glycans (McCaffrey *et al.* 2004), neutralisation by anti-gp41 antibodies (2F5) decreases. This could be due to direct shielding of the gp41 epitope, conformational changes in the V3 loop, or even changes in the gp120/gp41 interaction.

Both gp120 N-linked glycans and variable loops provide evasion from the host immune responses. Over the course of infection, humoral immune escape is associated with a change in the length of the viral Env sequence, in particular the V1/V2 loops (Curlin *et al.* 2010), and a change in the location or an increase in the number of potential glycosylation sites, as shown by studies performed on macaques infected with SIV (Overbaugh *et al.* 1992; Reitter *et al.* 1998a; Rudensey *et al.* 1998; Cheng-Mayer *et al.* 1999) and SHIV (Narayan *et al.* 1999) – a simian immunodeficiency virus encoding several HIV-1 genes including ENV, TAT, and REV –, and on infected patients with HIV-1 clade B and clade A isolates (Sagar *et al.* 2006; van Gils *et al.* 2010). Changes in sequons select for mutants that escape immune system recognition whilst retaining Env function and host cell infectivity. The major changes occur in the C3 and V1/V2 region of gp120, resulting in evasion of antibody recognition against the V1/V2 loop.

Taken together, these results lead to the concept of an ‘evolving glycan shield’ (Wei *et al.* 2003), whereby glycans are shifted to protect neighbouring neutralisation-sensitive epitopes, while still allowing gp120 to interact with receptor and co-receptor of the host cell.

Interaction with cellular receptors

The composition of gp120/gp41 carbohydrates, as well as their relative positions, are both equally critical. Proper N-linked glycosylation is essential for HIV-1 pathogenesis (Montefiori *et al.* 1988), with for instance deglucosylated glycans essential for syncytia formation. The interaction of different types of glycans with immune receptors, leading to increased infectivity or immune regulation, will be described in further detail in section 1.3. and is briefly summarised below.

The population of unprocessed oligomannose-type glycans have been shown to increase viral uptake by dendritic cells and virus dissemination in the lymph nodes, to increase infection of dendritic cells, and to induce immunosuppressive responses (section 1.3.1.).

A residual population of complex glycans has been shown to modulate viral infectivity and binding of several antibodies. However, the nature and abundance of these complex-type carbohydrates varies considerably between cell-types and complete inhibition of complex glycan processing does not abrogate infectivity, indicative of their dispensability to replication and cell entry (Montefiori *et al.* 1988; Binley *et al.* 2010; Eggink *et al.* 2010).

A significant subset of sialylated, complex-type glycans is present on viral gp120. Infectivity was shown to be greatly enhanced when negatively charged sialic acid residues were removed by sialidase (Hu *et al.* 1996). As sialic acid seems to render virions less infectious, it has been suggested that these residues play a role in immunoregulation of immune cells response. Siglecs (sialic acid binding, immunoglobulin-like lectins) are expressed on immune system cells, and their binding to sialic acid modulates intracellular signalling pathways (Crocker *et al.* 2001; Crocker 2005; Crocker *et al.* 2007). Members of the human siglec family show cell-specific expression and different ligand specificity. Most siglecs have an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic

domain that negatively regulates the cells that express them, and point towards a role of siglecs in inhibiting intracellular signalling pathways. Indeed, it was shown that siRNA downregulation of several B cell inhibitory receptors, including siglec-6 and siglec-2, led to an increase of BCR-mediated proliferation and effector function for tissue-like memory B cells (Kardava *et al.* 2011). A recent study has shown that HIV gp120 glycans binding to siglecs on macrophages increased macrophages infection (Zou *et al.* 2011).

1.3. The interaction of cellular immunity with HIV-1 glycans

Although the primary target of HIV is $CD4^+$ T-cell, and depletion of such cells eventually leads to AIDS, nevertheless during HIV infection a broad range of immune system cells either become infected with HIV, carriers of HIV virions, or suffer bystander effects due to chronic immune activation. Moreover, HIV-1 glycans have been shown to be involved in infectivity and immune regulation.

1.3.1. Infection and destruction of T-cells by HIV

During acute infection, the first cells infected by the transmitted R5-tropic virus are $CD4^+$ $CCR5^+$ T-cells, such as memory T-cells which are abundant in gut-associated lymphoid tissues (GALT) (Poles *et al.* 2001; Douek *et al.* 2009), as well as proliferating naïve and resting T-cells present in lymphoid tissues (Zhang *et al.* 1999; Eckstein *et al.* 2001). Peripheral naïve $CD4^+$ T-cells are also moderately subject to infection at that stage. A latent pool of infected $CD4^+$ T-cells is thought to become established during the first two to three weeks of infection (Chun *et al.* 1998). The asymptomatic phase of infection is characterized by a low viral load set point, and normal $CD4^+$ T-cell count (around 1,000 cells/mm^3 of whole blood), that plateau as lymphocyte homeostasis reconstitute naïve T-cells stocks (Douek *et al.* 2003). However there is a persistent expression of viral particles, and thus high turnover of

$CD4^+$ T-cells. As HIV infection progresses, $CD4^+$ T-cell counts decrease due to three main factors: (i) death of $CD4^+$ infected and uninfected cells by apoptosis, (ii) altered homeostatic regeneration of peripheral $CD4^+$ cells, (iii) impaired production of T-cell progenitor cells in the thymus (Clark *et al.* 2000) as the thymic output decreases during HIV infection (Gaulton *et al.* 1997; Douek *et al.* 1998; Haynes *et al.* 2000; Douek *et al.* 2001). Progression to AIDS is associated with the destruction of $CD4^+$ T-cell regenerative ability (Douek *et al.* 2003; McCune 2001) and consequently low $CD4^+$ count (typically defined as <200 cells/mm^3 of whole blood). Rapid disease progression is sometimes associated with a change of co-receptor, the X4-virus being highly pathogenic, as CXCR4 is a receptor expressed on naïve T-cells and around 70% of progenitor cells thymocytes. Moreover, infection with X4-virus induces the formation of syncytia that have a short lifespan, and thus contribute to T cell depletion and disease progression (Lifson *et al.* 1986; Richman *et al.* 1994; Sylwester *et al.* 1997).

The average half-life of HIV-infected $CD4^+$ T-cells is 12-36 hours (Ho *et al.* 1995; Wei *et al.* 1995; Perelson *et al.* 1996). The depletion of $CD4^+$ HIV-infected T-cells following HIV infection is mainly due to apoptosis (Gougeon *et al.* 1996; Alimonti *et al.* 2003; Gougeon 2003). Env components increase the intrinsic mitochondrial apoptotic pathway by inducing cytochrome C production (Badley *et al.* 2000). Envelope glycoprotein gp120 also activates the extrinsic pathway (activation-induced cell death, AICD) by increasing the death receptors CD95 and FasL levels (Oyaizu *et al.* 1994; Tateyama *et al.* 2000), inducing effector caspases, such as caspase 3 (Cicala *et al.* 2000). Tat also increases levels in CD95, Fas ligand (FasL), up-regulates the apoptosis initiator caspase 8 (Ensoli *et al.* 1990; Westendorp *et al.* 1995; Bartz *et al.* 1999), induces pro-apoptotic Bax (Sastry *et al.* 1996) and Bim (Chen *et al.* 2002), and down-regulates the anti-apoptotic Bcl-2 levels (Sastry *et al.* 1996). Bcl-2 is also downregulated by HIV protease (Strack *et al.* 1996). Finally, Nef also contributes to inducing

FasL expression by binding to the zeta chain of the TCR complex (Xu *et al.* 1999; Zauli *et al.* 1999), as well as caspase 3 (Rasola *et al.* 2001).

$CD4^+$ T-cell depletion can also be attributed to other factors. Expression of $CD4^+$ T-cell co-stimulatory molecules is altered during HIV infection. The lack of a second contact between either CD28 on T-cells and CD80/CD86 on antigen presenting cells (APC), or CD40L on T-cells and CD40 on APC, results in apoptosis (Kammerer *et al.* 1996; Chougnet *et al.* 1998; Sousa *et al.* 1999). Syncytia formation observed with X4-tropic virus, continuous budding from the plasma membrane (Fauci 1988) and increased permeability due to Vpu (Gonzalez *et al.* 2001) both decrease cell viability, and cellular cytotoxicity has been linked to linear viral DNA (Levy 1993).

Whilst direct apoptosis of $CD4^+$ HIV-infected cells occur, 'bystander' or indirect apoptosis of uninfected cells remains the major cause of decline in $CD4^+$ and $CD8^+$ T-cells counts. Indeed, the death rate for $CD4^+$ and $CD8^+$ T-cells is similar during HIV infection (Mohri *et al.* 1998; Hellerstein *et al.* 1999). Whilst SIV infection is characterised by higher viral load than the one reported in HIV-infected individuals, elevated levels of pro-inflammatory cytokines and consequent induced apoptosis of $CD4^+$ T-cell might explain the disease progression to AIDS observed with HIV compared to SIV (Silvestri *et al.* 2003). Apoptosis of helper and cytotoxic cells is a key aspect of immune evasion, and whilst it can be delayed by Nef to ensure at least one round of replication, it can also be induced by HIV proteins released in the extracellular environment.

During HIV-1 infection, CD95 and CD95L have been shown to be up-regulated on an increasing number of T-cells from PBMCs isolated from HIV-infected individuals (Aries *et al.* 1995; Baumler *et al.* 1996; Estaquier *et al.* 1996; Silvestris *et al.* 1996). Binding of CD95L on infected $CD4^+$ T-cells to CD95 on $CD8^+$ T-cell leads to antigen-independent cell death of the $CD8^+$ T-cell (Piazza *et al.* 1997). Apoptosis of uninfected $CD4^+$ and $CD8^+$ T-cells can be

mediated by AICD following chronic immune activation, or can be induced by viral proteins (Holm *et al.* 2005). Activation of $CD4^+$ T-cells via non-infectious virion binding to chemokine co-receptors leads to release of soluble cytotoxic factors, including TNF-α, resulting in activation and apoptosis of $CD8^+$ T-cells. HIV-1 proteins Tat, Vpr, Nef, and most significantly Env, have cytotoxic effects (Azad 2000). Cross-linking of CD4 by gp120 in uninfected cells induce apoptosis (Banda *et al.* 1992; Algeciras *et al.* 1998; Arthos *et al.* 2002; Ahr *et al.* 2004) notably by macrophages (Badley *et al.* 1996; Badley *et al.* 1997) by inducing FasL expression. Binding of gp120 to CD4 also increases effector caspase 3, 6 and 8 activity (Cicala *et al.* 1999; Cicala *et al.* 2000; Algeciras-Schimnich *et al.* 2002). Tat secreted from infected cells can be endocytosed, subsequently upregulating caspase 8 and FasL, inducing apoptosis of T helper cells as well as neurons. Nef also induces neuronal and blood cell death via a Fas-dependent mechanism.

Finally, the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is also involved in $CD4^+$ T-cell death in HIV-infected individuals (Gougeon *et al.* 2012). TRAIL expression on pDCs (type I interferon producing cells) is induced by IFN-α, and is also up-regulated by Tat on monocytes (Yang *et al.* 2003; Kim *et al.* 2007). HIV-1 infection up-regulates the death receptor 5 (DR5, TRAIL's ligand) both on infected and uninfected $CD4^+$ T-cells, rendering these cells susceptible to TRAIL-mediated apoptosis.

Despite being the main target of HIV, $CD4^+$ T helper lymphocytes play a central role in modulating and regulating the immune system response to HIV-1 during acute (Copeland *et al.* 1996; Rosenberg *et al.* 1998) and chronic infection (Rosenberg *et al.* 1997), and can also help prevent infection (Fowke *et al.* 2000). They are crucial to initiate and control both humoral and cellular immune responses. Upon stimulation by $CD4^+$ T-cells, effector $CD8^+$ T-cells carry out cytotoxic T lymphocyte (CTL) responses and induce apoptosis of HIV infected cells (Kalams *et al.* 1999). CTLs together with anti-HIV antibodies control viremia during

acute infection (Clark *et al.* 1991; Kuroda *et al.* 1999), with around 10% of $CD8^+$ T-cells being HIV-specific at that stage (Wilson *et al.* 2000). A strong CTL response is observed in non-progressors, and individuals carrying human leukocyte antigen (HLA) alleles HLA-B27, HLA-B51 and HLA-B57 maintain high $CD4^+$ counts (Stephens 2005). Other alleles, such as HLA-B35, or HLA homozygosity, correlate with rapid progression to AIDS (Carrington *et al.* 1999; Tang *et al.* 1999). However, the CTL response eventually fails to control viremia, resulting in disease progression to AIDS. Firstly, HIV uses several mechanisms to delay apoptosis of infected cells to generate more virions. Nef (Schwartz *et al.* 1996; Collins *et al.* 1998) and Tat (Howcroft *et al.* 1993) down regulate expression of MHC-I, which prevents presentation of viral peptides to the T-cell TCR and thus recognition of the infected cells. Down-regulation of CD4 expression by Env (Crise *et al.* 1990), Nef (Salghetti *et al.* 1995) and Vpu (Willey *et al.* 1992) negates both HIV super infection and CD4-gp120 induced apoptosis (Tanaka *et al.* 2003). Secondly, $CD4^+$ helper T-cell depletion means that CTL do not mature properly (Champagne *et al.* 2001). Thirdly, HIV-specific $CD8^+$ T-cells are more susceptible to CD95/Fas-mediated apoptosis (Mueller *et al.* 2001). Finally, CTLs constantly select for escape mutants during both acute and chronic infection (Goulder *et al.* 1997).

Upon TCR activation, and appropriate cytokine stimulation, T helper cells are differentiated into four distinct lineages, T_H1, T_H2, T_H17 and iT_{reg}. Cytokine expression levels are altered in HIV-1 infected individuals. A decrease in the T_H1 response, characterised by IL-2, IL-12, TFNα and IFNγ production, that provide a bias towards cellular immunity is observed, compared to T_H2 cytokines (typically IL-4, IL-5, IL-6) (Clerici *et al.* 1994; Kedzierska *et al.* 2001). This shift in cytokine expression also leads to increased susceptibility to apoptosis, as for instance IL-2 was shown to decrease apoptosis (Adachi *et al.* 1996).

1.3.2. Dendritic cells as a means for viral dissemination

Dendritic cells (DCs) are antigen-presenting cells that are part of the first line of defence against infection, and by capturing pathogens and presenting pathogen-derived peptides to thymic T-cells, initiate an antigen-specific immune response. Upon virion capture and endocytosis, DCs mature (Mellman *et al.* 2001) and migrate to secondary lymphoid organs, where they display antigenic peptides on their MHC II to helper T-cells (Banchereau *et al.* 1998; Lanzavecchia *et al.* 2001). Interestingly, a small proportion of virions internalised by DCs are not degraded, but localise in tetraspanins CD81- and CD9-enriched, midly acidic, protective environment (Garcia *et al.* 2005), in a similar fashion to DCs exosome endocytosis and transmission to T-cells (Garcia *et al.* 2005; Wiley *et al.* 2006; Izquierdo-Useros *et al.* 2009). Virions within these vesicular compartments remain infectious for up to five days. Upon contact with $CD4^+$ T-cells, virions redistribute to form an infectious synapse, with a high concentration of HIV receptors on T-cells, allowing efficient *trans*-infection of T-cells (Cameron *et al.* 1992; Pope *et al.* 1994; McDonald *et al.* 2003; Arrighi *et al.* 2004; Turville *et al.* 2004). The first DCs to interact with HIV-1 during sexual transmission are Langerhan cells (LCs), present in the epithelial layer of mucosal areas, and immature DC (iDCs) that scan the mucosal environment for pathogens. Blood DCs have also been shown to mediate *trans*-HIV-1 transmission to T-cell during blood transmission (Engering *et al.* 2002b).

Finally, HIV-1 can also directly infect $CCR5^+$ DCs leading to *cis*-infection of $CD4^+$-T cells upon release of infectious particles produced by DCs (Bhoopat *et al.* 2001; Turville *et al.* 2004). This mode of transmission is more efficient for the CCR5-tropic virus, which is the predominant virus at transmission and during acute infection, and takes advantage of the expression of the chemokine receptor CCR5 on DCs.

HIV-1 Env oligomannose-type glycans have been shown to interact with the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), a

mannose-specific C-type (Ca^{+}-dependent) lectin that mediates HIV-1 uptake by DCs, and further facilitates *trans*-infection of T lymphocytes (Figure 1.9.A) (Geijtenbeek *et al.* 2000; Kwon *et al.* 2002; Geijtenbeek *et al.* 2003). DC-SIGN expression on DCs is induced upon Interleukin 4 (IL-4) stimulation, and down-regulated by Interferon α (IFN-α), IFN-γ, and Tumor growth factor β (TGF-β) (Relloso *et al.* 2002). A similar protein DC-SIGNR, which is not expressed on DCs, is expressed on endothelial cells in the liver, lymph nodes and placental capillary. DC-SIGN and DC-SIGNR are tetrameric type II transmembrane proteins, with each subunit carrying a C-terminal carbohydrate-recognition domain (CRD) (Mitchell *et al.* 2001). DC-SIGN acts as a recycling endocytotic receptor, being localised to endocytic vesicles (Engering *et al.* 2002a) and releasing its ligand at low pH values found in this particular microenvironment (Guo *et al.* 2004). Both DC-SIGN and DC-SIGNR bind to oligomannose-type glycans, with the highest affinity observed for $Man_{6\text{-}9}GlcNAc_2$ structures (Feinberg *et al.* 2001; Guo *et al.* 2004). As DC-SIGN is present as a tetramer on the cell surface, clustering of oligomannose glycans increases binding affinity (Mitchell *et al.* 2001). DC-SIGN also binds to a range of non-sialylated fucosylated glycans, including blood group antigens and Lewis antigens Le^x and Le^a (Guo *et al.* 2004; van Liempt *et al.* 2006). *Trans*-infection of T-cells can also be mediated by binding of immune complexes of HIV-1 virions to follicular DC receptors (Heath *et al.* 1995). Simultaneous binding of gp120 to DC-SIGN and of the Fc region of Abs bound to the virion to Fcγ receptors (FcγRs) on DCs leads to endocytocis of the immune complex (van Montfort *et al.* 2007). Upon T-cell stimultation infectious HIV-1 virions are released, whilst antibodies dissociate from the virions in the acidic endosomal compartments.

Along with DC-SIGN, other C-type lectin receptors (CLR) that are expressed on different of DC subsets are involved in HIV-1 capture and transmission to T lymphocytes (Figure 1.9.A) (Turville *et al.* 2001; Turville *et al.* 2002). The DC Inhibitory Receptor (DCIR)

has been shown to bind to HIV glycans and induce a negative regulatory effect through its ITIM cytoplasmic domain (Lambert *et al.* 2011). Between 30 and 50% of captured HIV-1 virions are bound either to DC-SIGN or to DCIR. DCIR has been shown to bind to oligomannose-type glycans, as well as to glycans bearing fucose, galactose, N-acetylgalactosamine, glucose and N-acetylglucosamine residues (Lee *et al.* 2011). The mannose receptor (MR) and DEC-205 are expressed on iDCs as well as macrophages, and are also involved in HIV-1 virion uptake (Bozzacco *et al.* 2007). This receptor, which contains eight CRDs, recognises terminal mannose, fucose and GlcNAc residues, usually found on pathogens but not self-antigens (Shepherd *et al.* 1981; Largent *et al.* 1984). Finally, on LCs, which are cells that do not express DC-SIGN, the Langerin receptor is crucial to virion uptake (Turville *et al.* 2002).

Changes in viral Env glycosylation can influence gp120 interaction with DCs' CLRs. Addition of a glycan at the base of the V2 loop of SHIV increased binding to DC-SIGN and subsequent *trans*-infection of T cells (Lue *et al.* 2002). A recent study has shown that virus uptake is increased for Env bearing predominantly oligomannose-type glycans (van Montfort *et al.* 2011). However, virions were directed to the endosomal pathway, and thus further degraded for presentation to T-cells instead of *trans*-infection of T-cells. This is consistent with C-type lectin mannose receptors (DC-SIGN, DCIR and the mannose receptor) targeting their ligands to endosomic mediated Ag-presentation.

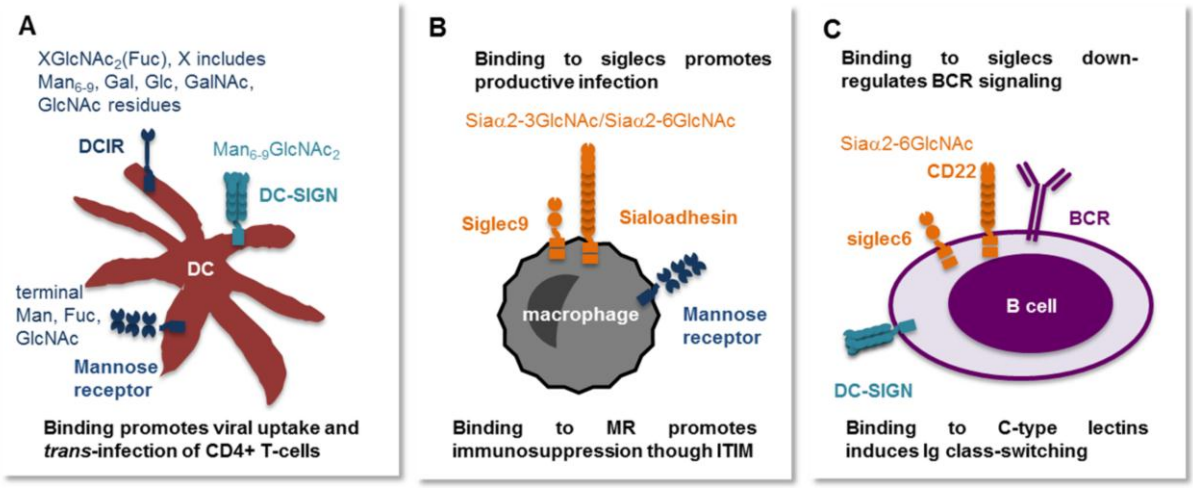


Figure 1.9. HIV-1 Env glycan interaction with host lectins on immune system cells
Schematic representation of selected lectins, highlighting their specificity, expressed on **(A)** dendritic cells, which includes DC-SIGN, DCIR and the mannose receptor, **(B)** macrophages, with siglec 9 and sialoadhesin, and **(C)** B-cells, with siglec 6, CD22, and DC-SIGN present on a subset of B-cells. Lectins specific to oligomannose-type glycans are shown in light blue, to oligomannose and specific complex-type glycans in dark blue, and to sialylated glycans in orange.

1.3.3. Macrophage infection and establishment of a latent reservoir

Like DCs, macrophages, as part of the innate immune system, constitute a first line of defence against foreign organisms. They are phagocytic, and secrete inflammatory cytokines. If stimulated by lymphocytes T and B, they can act as effector cells as part of the adaptive immune system response. Macrophages display both MHC I and MHC II, and can also act as antigen-presenting cells.

Macrophages express the CCR5 chemokine co-receptor, which is used during the first phase of HIV infection. Some macrophages are present in mucosal subepithelial tissues (together with DC and T cells). Macrophages can be directly infected by R5-tropic HIV-1 viruses, resulting in the establishment of a latent viral reservoir (Montaner *et al.* 2006; Shen *et al.* 2008).

A recent study demonstrated that siglecs expressed on macrophages can act as an additional receptor for HIV entry, consistent with low level expression of CD4 on the macrophage surface (Figure 1.9.B) (Zou *et al.* 2011). HIV glycans were shown to interact

with a particular subset of siglecs present on macrophages (siglecs 1, 3, 5, 7 and 9), both in solution, on transfected cells and on macrophages. Sialoadhesin (siglec-1) has the longest extracellular domain and as such its sialic acid binding pocket is not masked by neighbouring sialylated glycans present on the cell surface. As sialoadhesin exhibit the highest affinity for viral attachment, and is up-regulated in inflammation and infection, it was proposed that it might participate in enhancing HIV replication and viral dissemination.

Finally, as observed with DCs, mannose-binding lectins on macrophages (such as the macrophage mannose receptor and DEC-205) can capture HIV, resulting in viral internalisation for antigen presentation or *trans*-infection of T-cells (Nguyen *et al.* 2003; Hatsukari *et al.* 2007; Trujillo *et al.* 2007).

1.3.4. B-cells hyperactivation and dysfunction

B cells interact directly with HIV (Schnittman *et al.* 1986). Complement protein bound to the virus *in vivo* bind to the complement receptor CD21 (CR2) expressed on mature B cells (Moir *et al.* 2000). HIV binding to B-cells promotes infection of $CD4^+$ T-cells, as observed with follicular DCs that also express CD21 (Malaspina *et al.* 2002). Other receptors can mediate HIV binding to B-cells, such C-type lectins (He *et al.* 2006) including DC-SIGN (Rappocciolo *et al.* 2006), and surface immunoglobulins of the variable heavy chain 3 (V_H3) family (Berberian *et al.* 1993).

However, as with T-cells, direct HIV interaction is not sufficient to account for the dysfunction of B cells during infection – HIV viremia also has indirect effects on B-cells, such as B cell hyperactivation and dysfunction (Schnittman *et al.* 1986; Lane *et al.* 1983; Jacobson *et al.* 2002; Moir *et al.* 2009), which often leads to hypergammaglobulinemia (Moir *et al.* 2001; De Milito *et al.* 2004) and increased production of autoantibodies (Haynes *et al.* 2005). During the acute phase of infection, B-cell apoptosis occurs in germinal centres,

creating early damage to the generative B-cell population (Zhang *et al.* 2007). During chronic infection, the B-cell generative environment is also disrupted. Furthermore, the B-cell population in peripheral blood of HIV infected individuals is altered, with some additional sub-populations present compared to healthy individuals – immature transitional $B10^+$ B-cells (Malaspina *et al.* 2006), exhausted $CD20^{hi}CD27^-CD21^{lo}$ tissue-like memory B-cells (Moir *et al.* 2008), short-lived plasmablasts, and activated mature B-cells bearing for instance CD70, CD71, CD80 and CD86 – and a decrease in the usual B-cell sub-populations of resting antigen-specific memory B-cells. An increase in B-cell turnover, as with $CD4^+$ and $CD8^+$ T-cells, is observed during chronic immune-cell activation (De Boer *et al.* 2003). CD95 is upregulated on B cells from HIV-viraemic individuals which show increased levels of apoptosis (Titanji *et al.* 2005). Finally, several B-cell inhibitory receptors, markers of B-cell exhaustion, including FcRL4, CD22, and siglec-6, are up-regulated on tissue-like memory B cells (Kardava *et al.* 2011).

B cell hyperactivation by HIV viremia is not fully understood, however certain cytokines, such as IFNα, TNF, IL-6, IL-10, CD40L and the B-cell activating factor (BAFF) are thought to be involved (Rieckmann *et al.* 1991; Muller *et al.* 1998; Weimer *et al.* 1998; He *et al.* 2006). HIV proteins Nef and gp120 may also play a part in B cell hyperactivation. Nef, which inhibits CD40L-dependent Ig class-switching (Qiao *et al.* 2006), could also be involved in polyclonal B-cells activation, and induce macrophages to release pro-inflammatory cytokines and ferritin, leading to enhanced $CD4^+$ T-cell infection (Swingler *et al.* 2003; Swingler *et al.* 2008). HIV gp120 binding to B-cells C-type lectins induces Ig class-switching via up-regulation of the activation-induced cytidine deaminase (AID) (Schnittman *et al.* 1986; He *et al.* 2006) (Figure 1.9.C). The subsequent production of IgG and IgA via this CD40L-independent pathway impairs T-cell depend antibody responses. The serum levels of

IgG observed in HIV-1 infected individuals are elevated, characteristic of hypergammaglobulinemia, yet the Ag-specific antibodies titers are decreased.

The interaction between B-cells and HIV is accompanied by the production of soluble immunoglobulins. The first antibodies – IgM and IgG bound to virions – are detected 8 days after plasma viremia appears, followed at around 13 days post-infection by anti-gp41 antibodies (Tomaras *et al.* 2008). In the mucosal areas, the IgA and IgG response is similarly directed towards gp41, although the low IgA response facilitates viral dissemination (Mestecky *et al.* 2004). The initial B-cell response is, however, unable to control plasma viremia (Tomaras *et al.* 2008). The autologous neutralising antibody (nAb) response arises around three months after infection, and can select for escape mutants (Arendrup *et al.* 1992; Richman *et al.* 2003; Wei *et al.* 2003). As a result, antibodies present in a serum are able to neutralise autologous viruses from earlier in infection, but not current circulating viruses. The binding of nAbs to the Env spike prevents viral entry into host cells (Moore *et al.* 2006; Yang *et al.* 2006). Due to poor access to the conserved and immunogenic Env regions, the nAb response is not broad in the majority of cases – broadly neutralising antibody (bnAbs) responses are discussed in detail in section 1.4. Shedding of gp120 from the viral surface leads to exposure of non-neutralising epitopes, such as gp120 trimer interface, which are not present on the functional spike (Wyatt *et al.* 1998a). Finally, nAbs responses are dampened in the presence of effective anti-retroviral therapy, as a reduction in plasma viremia titre results in the loss of antigenic drive (Morris *et al.* 1998).

Whilst the Ab variable domain (Fab region) is responsible for antigen recognition and virus neutralisation, additional anti-viral responses are mediated by the constant crystallisable fragment (Fc) of Ab molecules. Binding to Fcγ receptors on host immune cells such as natural killer (NK) cells, macrophages, monocytes, and to complement proteins leads to infected cell and viral lysis (Holl *et al.* 2006; Ackerman *et al.* 2012). Upon IgG1 or IgG3 binding to their

FcγR-IIIa receptor, NK cells mediate antibody-dependent cellular cytotoxicity (ADCC) of the HIV-1 infected cell (Forthal *et al.* 2005). Antibody-dependent cell phagocytosis (ADCP) is induced by the interaction between mainly IgG2 sublass Fc and FcγRIIa on phagocytic cells. Fc binding to complement molecules induces antibody-dependent complement-dependent cytotoxicity (ADCDC). Antibody-dependent cell-mediated viral inhibition (ADCVI) refers to inhibition of viral growth, which can be obtained with a combination of ADCC and ADCP for example. There is a correlation between strong ADCC response and slower disease progression (Baum *et al.* 1996). Furthermore, it is thought that Fc-mediated effector functions could also assist in providing protection against infection. It was indeed shown that FcγR, but not complement binding, is crucial to mAb b12 protection against viral challenge (Hessell *et al.* 2007). Cross-reactive ADCC antibodies are first detected during the first three months of acute infection (Koup *et al.* 1991), however like the CTL and nAb responses, ADCC Abs can also select for escape mutants (Chung *et al.* 2011).

1.3.5. Viral Env glycans and immune evasion

Whilst HIV-1 Env N-linked glycans provide a mean of immunological escape from neutralising antibodies via the 'evolving glycan shield' (Wyatt *et al.* 1998b; Wei *et al.* 2003) (section 1.2.5), another interesting effect of N-linked glycosylation is immunosuppression, achieved when carbohydrates interact with specific immune receptors.

In addition to promoting *trans*-infection of $CD4^+$-T cells, binding of gp120 mannose moieties to DC-SIGN induces dendritic cell-mediated immunosuppression, via activation of the ERK1/2 signalling pathway that results in the release of the anti-inflammatory cytokine IL-10 (Shan *et al.* 2007). This is thought to dampen the humoral immune response and bias the quality of helper T-cell activity (with a shift from T_H1 to T_H2 type cytokines). IL-10 production is influenced by the recombinant gp120 concentration and strain, probably due to

differential glycosylation patterns. Binding of mannose moieties to mannose C-type lectin receptors on dendritic cells, including but not limited to DC-SIGN, was also shown to impair proliferation and IL-12 expression of co-cultured T-cells. Overcoming the immunosuppressive effect of mannose on immune system cells may be a requirement of a vaccine designed to elicit high-titre, long-lasting IgG responses (Banerjee *et al.* 2012). The persistence of the mannose patch on diverse isolates is therefore of central importance to immune evasion and will form the basis of the analysis conducted in Chapter 3 and 4.

Similarly, sialylated glycans present on gp120 could induce immunosuppressive response. Indeed, several pathogens, such as *Neisseria gonorrhoeae, Haemophilus influenzae*, group B *Streptococcus, Campylobacter jejuni, Trypanosoma cruzi*, present sialylated glycans on their surface, thus mimicking host cells and elude the immune response by interacting with regulatory molecules such as complement factor H (Gulati *et al.* 2005), and immunosuppressive lectins such as siglecs (Vimr *et al.* 2002; Avril *et al.* 2006). This effect has yet to be demonstrated for HIV, and is investigated in Chapter 6.

1.4. The broadly neutralising antibody response to HIV-1

Most HIV-1 infected individuals develop only strain-specific neutralising antibodies. Several years after infection, around 10–25% of HIV-1 infected individuals develop some degree of neutralisation breadth (Doria-Rose *et al.* 2009; Gray *et al.* 2011), and 1-2% of chronically infected patients – designated as ‘elite neutralisers’ – develop bnAbs that broadly and potently neutralise a majority of HIV strains tested (Simek *et al.* 2009). Heterologous nAb response is developed over time, and correlates with higher level of plasma viremia generated by chronic antigen exposure. The neutralisation breath is likely to arise through gradual maturation and refocusing of the B cell response on a conserved epitope (Stamatatos *et al.* 2009), and is discussed in further detail in section 1.4.5.

A broad panel of bnAbs have now been isolated and characterised (Figure 1.10.A), and are discussed in further detail in the next sections. The first generation of bnAbs, which includes mAbs b12, 2G12 and 2F5, helped understand the main targets of the neutralising antibody response on viral Env. Advances in memory B-cells screening has allowed the identification of new bnAbs from elite neutralisers from an IAVI cohort (protocol G) (Scheid *et al.* 2009; Walker *et al.* 2009). A second generation of bnAbs, namely PG9 and PG16, was found to target a previously uncharacterised quaternary epitope. Finally, over the last two years, a third generation of bnAbs targeting the V3 region, the PGTs antibodies, has been extensively studied, and PGT121 and PGT128 are now the most potent and broad anti-HIV antibody, with a 10-fold increase in potency compared to PG9/PG16, and a 100-fold increase compared to first-generation bnAbs (Figure 1.10.B).

1.4.1. The CD4 binding site as a bnAb epitope

The CD4BS is a conserved feature amongst HIV-1 isolates, and has been shown to be one of the main targets of the bnAb response (Li *et al.* 2007; Li *et al.* 2009; Lynch *et al.* 2012). The first anti-CD4BS bnAb was isolated by phage display (Burton *et al.* 1994). IgG1 b12 was shown to protect macaques against vaginal challenge with SHIV (Parren *et al.* 2001). The crystal structure of mAb b12 in complex with gp120 was determined at 2.3 Å resolution, showing that b12 binds through its heavy chain only to a conformationally conserved surface in gp120 outer domain that overlaps partially the CD4BS (Figure 1.11.A) (Zhou *et al.* 2007). Another anti-CD4BS bnAb, mAb HJ16, was isolated from immortalised B cells and exhibit the same breadth of neutralisation as b12 (around 40% of a panel of pseudoviruses) (Corti *et al.* 2010).

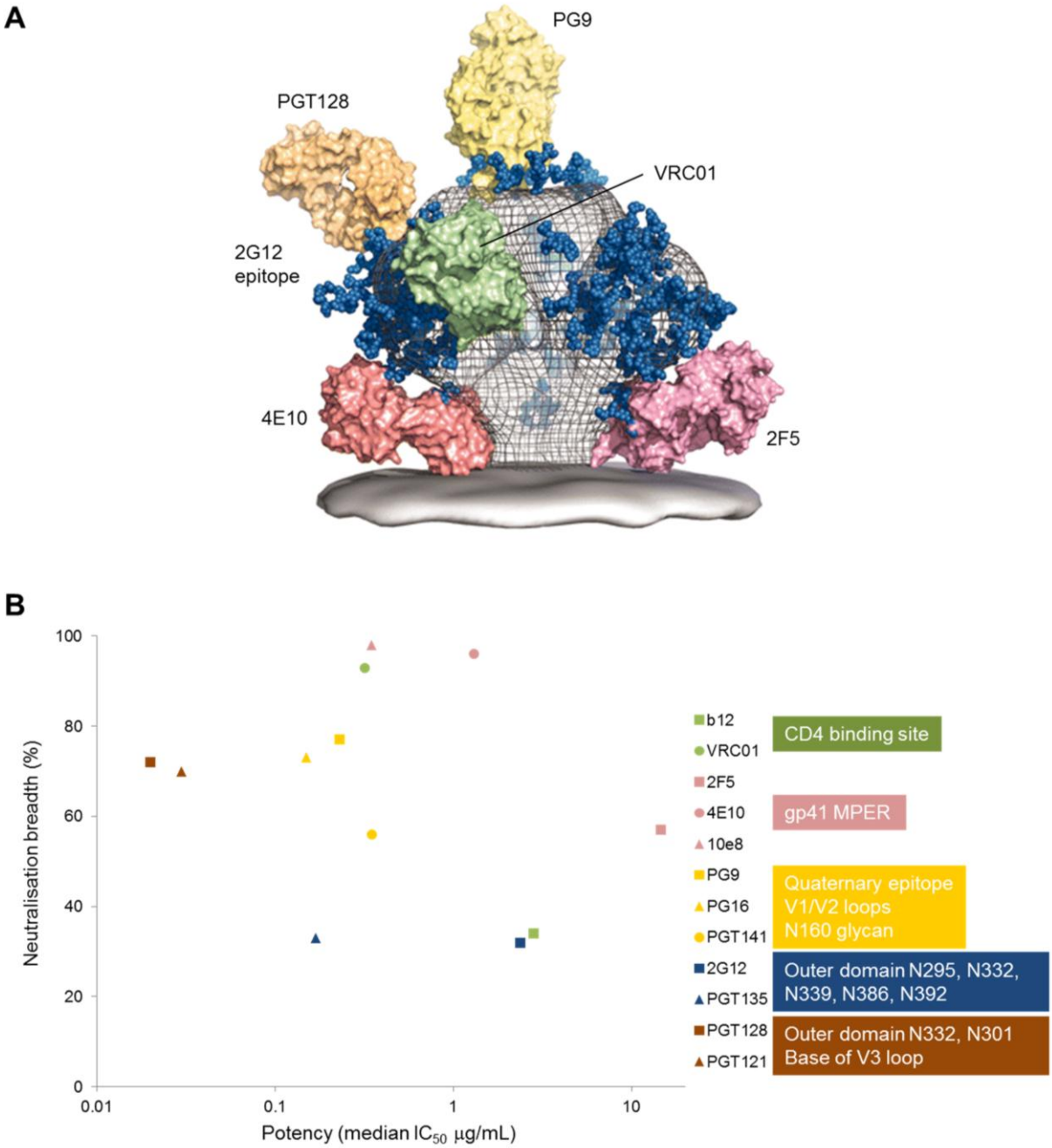


Figure 1.10. The broadly neutralising antibody response against HIV-1
(A) Sites of vulnerability on the envelope spike. Figure constructed using the cryoelectron tomography model of HIV-1 Env spike (Liu *et al.* 2008) and the Fabs of PG9 (McLellan *et al.* 2011) (yellow), PGT128 (Pejchal *et al.* 2011) (orange), VRC01 (Zhou *et al.* 2010) (green), 4E10 (Cardoso *et al.* 2007) (red), and 2F5 (Bryson *et al.* 2009) (pink). The $Man_9GlcNAc_2$ glycans are modelled as blue spheres. The approximate position of the 2G12 epitope is indicated. Reproduced from Burton *et al.* 2012 reprinted with permission from the AAAS **(B) Neutralisation breadth and potency of isolated anti-HIV bnAbs.** The neutralisation breadth and potency was assessed for b12, VRC01, PG9, PGT141, 2G12, PGT135, PGT128, and PGT141 against 162-pseudovirus panel (Walker *et al.* 2011), and for 2F5, 4E10, 10e8, and PG16 against a 181-pseudovirus panel (Huang *et al.* 2012). The neutralisation breadth is plotted against the median neutralisation potency obtained for pseudoviruses neutralised at a $IC_{50} < 50$ µg/mL.

A recent study using Env probes against individual B cells expressing anti-CD4BS antibodies resulted in the isolation of mAbs VRC01, VRC02 and VRC03 (Wu *et al.* 2010b). VRC01 and VRC02 were found to be somatic variants, and displayed a high level of mutation compared to the putative germline sequence. They also exhibit a long heavy chain CDR3 (complementarity-determining regions) of 14 amino acids. VRC01 was able to neutralise 91% of pseudoviruses with an IC_{50} < 50 μg/mL, in contrast to b12, which neutralised 41% of the panel.

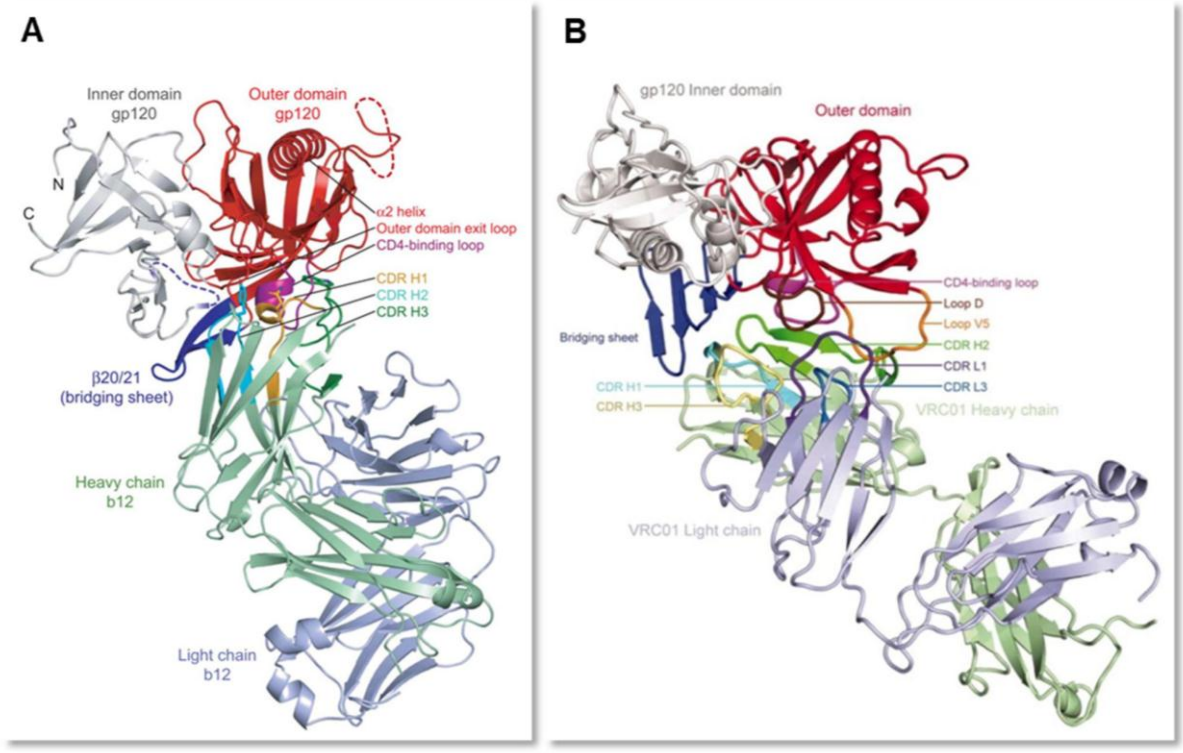


Figure 1.11. Structures of bnAbs b12 and VRC01 in complex with an HIV-1 gp120 core (A) Interaction between gp120 and mAb b12 (reprinted by permission from Macmillan Publishers Ltd: Nature (Zhou *et al.* 2007), copyright 2007). Polypeptide chains are depicted in ribbon. The gp120 outer domain is shown in red, the inner domain in grey, and the bridging sheet in blue. The CD4-binding loop (purple) is highlighted. The b12 light chain is shown in blue and b12 heavy chain in green, with CDR H1 in orange, CDR H2 in cyan and CDR H3 in dark green. (**B) VRC01 mimics CD4 binding to gp120** (reproduced from Zhou *et al.* 2010, reprinted with permission from the AAAS). Some additional features of gp120, such as the D loop (brown) and V5 loop (orange) are highlighted. VRC01 Fab light chain is shown in light blue, with CDR L1 in dark blue and CDR L3 in marine blue. VRC01 Fab heavy chain is shown in green, with CDR H1 in cyan, CDR H2 in green, and CDR H3 in yellow. Structure was determined at 2.9 Å resolution. HIV-1 $gp120_{97TH057}$ used for this study was composed of the inner and outer domain core, with truncations in the V1/V2 and V3 loops, and the C- and N-termini.

The crystal structure of VRC01 in complex with gp120 was subsequently determined (Figure 1.11.B) (Zhou *et al.* 2010). Both light and heavy chains of VRC01 Fab interact with gp120, with the CDR H2 making most contacts. Additional interactions are made with the CDR L1 and L3 and the CDR H1 and H3. VRC01 was shown to adopt a mode of binding that mimics CD4 interaction with gp120. However, VRC01 mainly targets a conformationally invariant part of the gp120 outer domain, with only a small proportion of contacts with the bridging sheet being dispensable. In contrast, CD4 contacts with the bridging sheet make up 33% of the total interactions, which might explain the extreme breadth and potency observed with VRC01, which requires only minimal conformational changes to bind to its epitope. Furthermore, VRC01 light chain interaction with the core GlcNAc residue of the N276 glycan turns this CD4BS occluding glycan into a binding site for the antibody.

However, poor accessibility and conformational/glycan masking of the conserved CD4BS makes it difficult for antibodies elicited during HIV infection to recognise the functional spike and subsequently neutralise the virus *in vivo* (Chen *et al.* 2009; Julien *et al.* 2012).

1.4.2. bnAbs targeting the fusion-exposed gp41 MPER

Another highly conserved Env region is the MPER domain on gp41 that is involved in fusion. Some anti-MPER mAbs also show cross-reactivity with cell membrane lipids – suggesting anti-MPER B-cells are potentially self-reactive and eliminated during B-cell differentiation (Haynes *et al.* 2005). However, several bnAbs have been isolated that target this domain – IgG3 2F5 and 4E10 (Zwick *et al.* 2001a), and more recently 10E8 (Huang *et al.* 2012).

4E10, which is amongst the broadest nAbs (Binley *et al.* 2004b), binds to a peptide epitope at the insertion of gp41 stalk into the virion lipid membrane, whilst 2F5 binds to a

peptide epitope in gp41 HR-2 region. Both bind to gp41 in its pre-hairpin intermediate conformational state (Frey *et al.* 2008). 4E10 epitope's helical structure (Cardoso *et al.* 2005) has been suggested to be more suited to bnAbs elicitation than 2F5 epitope's extended conformation (Ofek *et al.* 2004).

Recently, another anti-MPER bnAb, 10E8, has been isolated and characterised (Huang *et al.* 2012). The 10E8 epitope overlaps with that of 4E10. It adopts the same approach as 4E10 to gp41, however it does not have the same membrane contact. It is ten-fold more potent and exhibits a wider breadth of neutralisation – it was shown to neutralise 98% of the primary isolates tested with an $IC_{50} < 50$ μg/mL.

1.4.3. A quaternary epitope involving gp120 glycans and V1/V2 loops

Screening of antibody-containing memory B-cell supernatant derived from an African clade A-infected donor resulted in the isolation of the bnAbs PG9 and PG16 (Walker *et al.* 2009). These antibodies target a quaternary epitope involving gp120's V1/V2 loops, which are usually a critical feature for HIV immune evasion. They exhibit a high breadth of neutralisation, with respectively 73 and 79% of HIV-1 primary isolates neutralised (Walker *et al.* 2009; Pancera *et al.* 2010). Both exhibit preferential binding for the envelope spike, with PG9 only binding weakly to gp120 and recombinant trimers.

The crystal structure of PG9 Fab was determined in complex with the V1/V2 gp120 domains, and it was shown that PG9 recognises an epitope comprised of two glycans (present on the strand B at position N160 and N156/N173) and a peptide part within the strand C (Figure 1.12.A) (McLellan *et al.* 2011). PG9 long CDR H3 is able to penetrate through the glycans and make contacts with the underlying peptide, as well as the N160 glycan, which is a small $Man_5GlcNAc_2$ structure due to the expression of the V1/V2 construct in GnT I-deficient cells, that stall glycan processing at that intermediate. Additional interaction between the

N156/173 glycan and PG9 heavy chain occurs, however this glycan is not always necessary for PG9 binding, suggesting that it might be involved in providing a tunnelling structure for antibody access. The crystal structure of its somatic variant, PG16, was also determined (Figure 1.12.B) (Pancera *et al.* 2010). The long negatively charged CDR H3 region characteristic of anti-V1/V2 loops antibodies can be visualised.

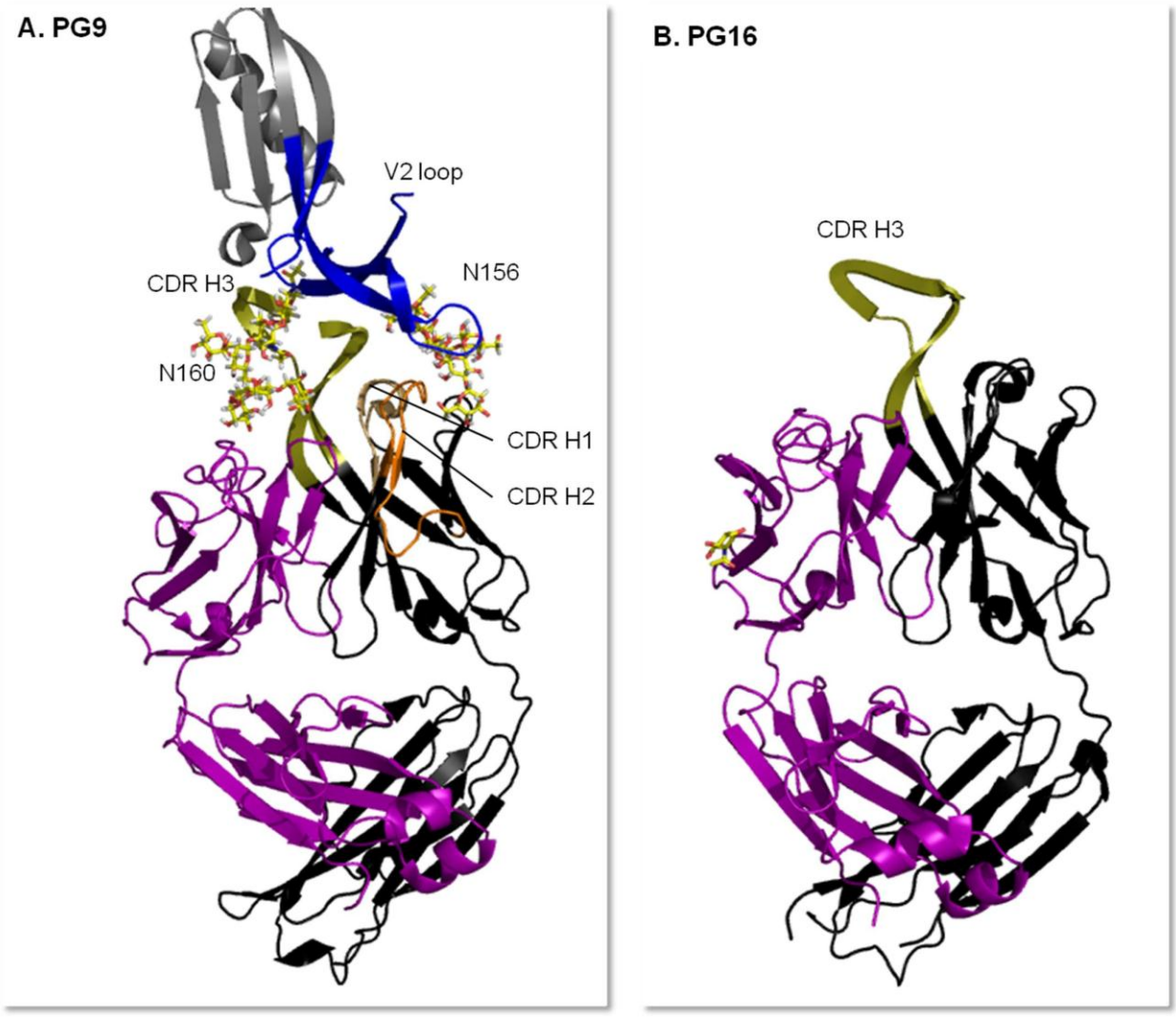


Figure 1.12. Structure of anti-V1/V2 bnAbs PG9 and PG16
(A) Structure of the complex between PG9 Fab and gp120$_{CAP45}$ V1/V2 domains, using ribbon representation. PG9 heavy chain is shown in black, and light chain in purple, with the CDR H1 in light yellow, CDR H2 in orange and CDR H3 in dark yellow. The 1FD6 scaffold used to achieve crystallisation is shown in grey, and the V1/V2 loops in blue. The N160 and N156 glycans are shown using the ball-and-sticks representation. The structure was constructed using pymol (pdb 34UK). (**B) Crystal structure of PG16 Fab**. The same colour-scheme as for PG9 is adopted. The long CDR H3 region is highlighted in dark yellow. The structure was constructed using pymol (pdb 3MME).

Recent results are indicative of the previously unsuspected presence of bnAbs directed against gp120 V1/V2 loops, domains dispensable for viral entry, but thought to be essential for neutralisation resistance. Screening of antibody-containing memory B-cells supernatant using a high-throughput method (Simek *et al.* 2009) from top four elite neutralisers amongst 1,800 HIV infected donors belonging to the IAVI Protocol G (Walker *et al.* 2009), resulted in the isolation of 17 new 'PGT' antibodies (Walker *et al.* 2011). Of these, the bnAbs PGT141-145 were found to be directed against a PG9/PG16-like quaternary epitope involving the V1/V2 loops. Furthermore, a recent screening of antibodies from a subtype C HIV-1 superinfected individual has shown that breadth of neutralisation was due to an antibody directed towards this epitope (Moore *et al.* 2011).

1.4.4. Anti-carbohydrate bnAbs directed towards gp120 outer domain

1.4.4.1. Recognition of the oligomannose cluster on gp120 outer domain by 2G12 and PGT135

IgG 2G12 is effective against a somewhat broad range of antigenically unrelated HIV-1 isolates (Binley *et al.* 2004b) – with the notable exception of the epidemiologically crucial Clade C, and has been shown to protect against mucosal SHIV challenge (Hessell *et al.* 2009b).

The structure of the 2G12 Fab in complex with $Man_9GlcNAc_2$ explains the basis for both its affinity and its selectivity (Figure 1.13.A) (Calarese *et al.* 2003). A unique domain-exchanged configuration provides an extended binding site, which is capable of multivalent interaction with two Manα1→2Man terminating oligomannose glycans. The accommodation of a third N-glycan at the V_H:V_H interface is suggested based on molecular packing within the crystallographic lattice oligomannose glycans. It was indeed shown that a non-domain exchanged mutant of 2G12 could recognise gp120 but not neutralise primary isolates, and it

was hypothesised that the domain exchange structure allowed 2G12 to recognise the closely packed HIV-1 Env glycans (Doores *et al.* 2010b).

The carbohydrate-binding pocket of 2G12 which is formed largely by CDR H3 loop shows extensive somatic mutations away from the germline D-segment. This relatively deep pocket accommodates Manα1→2Man but not Manα1→3Man or Manα1→6Man disaccharides explaining 2G12's selectivity for larger Manα1→2Man terminating oligomannose glycans. In addition to linkage specificity, the orientation of the carbohydrate binding pocket relative to the Fab surface permits binding of only one of the three Manα1→2Man termini of oligomannose glycans: the D1 but not D2 or D3 arms are accommodated. For example, glycan array analysis confirms 2G12 binding to Man_7D1 but not to Man_7D3. Consistent with this, the addition of a single D-glucose residue to the D1-arm prevents 2G12 binding, explaining the ability of glucosidase inhibitors to completely abolish the 2G12-gp120 interaction (Scanlan *et al.* 2002a). The recognition of the Manα1→2Man D1 motif relates to the ability of 2G12 to recognise its epitope despite the phenomenon of glycan microheterogeneity: the D1-arm is present on most of the biosynthetically common $Man_{7-9}GlcNAc_2$ oligomannose isomers formed by limited α1→2-mannosidase trimming.

The fine carbohydrate selectivity of 2G12 is dependent on strict geometric requirement of Manα1→2Man reducing termini determined by the spacing of gp120 Asn residues. The 2G12 epitope is dependent on a small number of oligomannose glycans – notably those attached to N295, N332, N339, N386 and N392 (Sanders *et al.* 2002; Scanlan *et al.* 2002a). Glycans at position N339 and N386 are believed to be important in preventing mannosidase processing of the glycans required in the 2G12 epitope (Scanlan *et al.* 2002a; Calarese *et al.* 2003). The ability of 2G12 to recognise a precise arrangement of conserved glycans also explains the sensitivity of this antibody to alterations in glycan geometry. Indeed, single point mutations resulting in the loss of a single oligomannose glycan can have dramatic

effects on antigenicity (Scanlan *et al.* 2002b; Lee *et al.* 2004b; Dunlop *et al.* 2010a). This sensitivity to glycan geometry is observed *in vivo*, as 2G12 is unable to neutralise most of the circulating viruses found in Sub-Saharan Africa (a major concern for vaccine development). This epidemiological pattern can be explained at a structural level: most clade C isolates lack one of the required glycans at N295 or N339 (Zhang *et al.* 2004). Similarly, passive transfer of 2G12 to infected patients rapidly selects for viral escape variants with mutations at key glycosylation sites (Manrique *et al.* 2007).

Mutants of 2G12 which interrupt domain exchange maintain the structure of the individual Fab units and bind to monomeric gp120, but do not neutralise HIV-1 pseudoviruses (Doores *et al.* 2010b). This requirement for domain exchange may be seen a contributing factor to the poor immunogenicity of the 2G12 epitope: antibodies with this unique structure would be expected to arise with extremely low frequency during an immune response. On the other hand however, it has been reported that domain exchange can occur with a surprisingly few number of germline V(D)J segments mutations (Huber *et al.* 2010). Furthermore, the observation that 2G12 requires domain exchange for binding does not necessarily imply that any antibody to gp120 glycans must adopt this configuration – as seen with the recently reported, broadly neutralising PGT antibodies.

The bnAbs PGT131-135 were shown to bind to a similar epitope as 2G12. Alanine scanning and crystallisation trials of PGT135 showed that glycans at position N295, N332, N339, N386 and N292 were involved in the epitope, together with amino acid residue H330. PGT135 was shown to bind to the D2, D3 arm of the glycan at position N332, the D1, D2 arm of N386 and the D2 arm of N392. Inability to neutralise viruses bearing mainly the $Man_9GlcNAc_2$ glycan is consistent with $Man_{7\text{-}8}GlcNAc_2$ and D2 arm specificity.

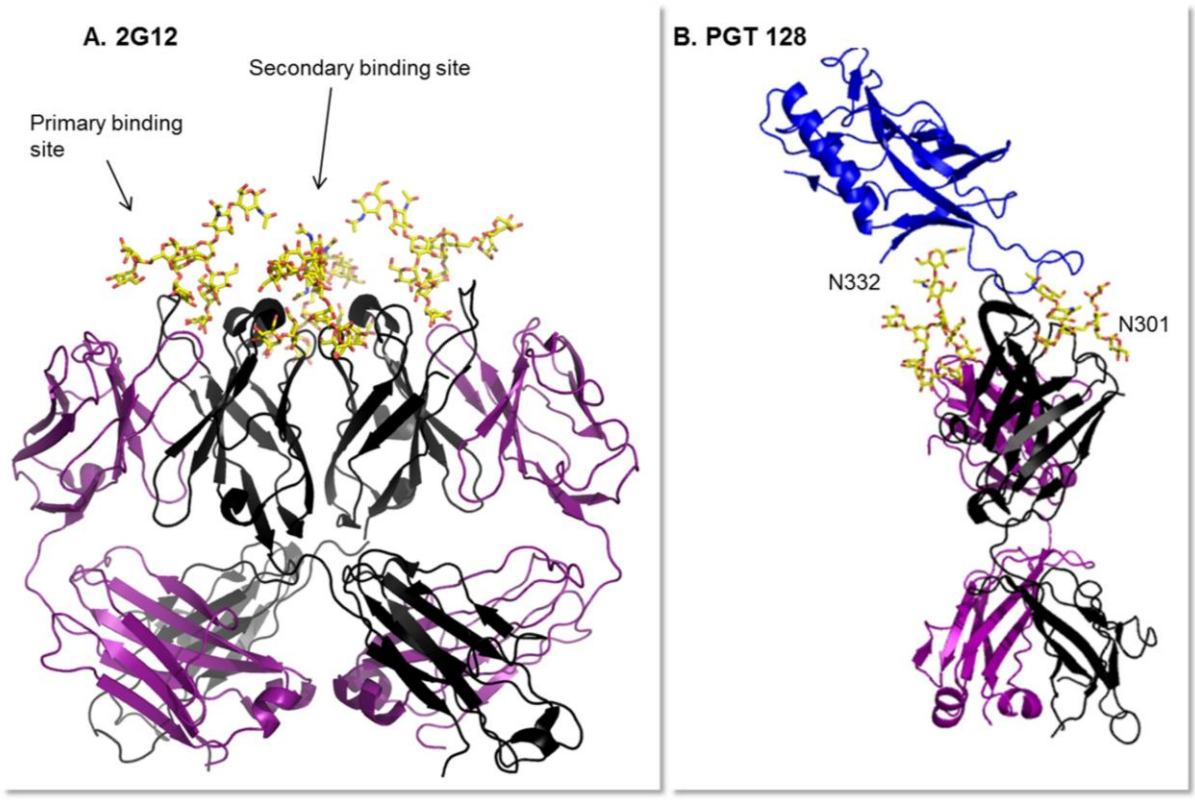


Figure 1.13. Anti-carbohydrates bnAbs 2G12 and PGT128 structure
(A) Crystal structure of 2G12 Fab dimer in complex with $Man_9GlcNAc_2$, highlighting 2G12's particular domain-exchanged conformation. 2G12 Fab heavy chain is shown in black, and light chain in purple. The glycans that take part in the 2G12 epitope are shown using the ball-and-sticks model. The primary and the secondary binding sites are highlighted. The structure was constructed using pymol (pdb 1OP5). (**B) Crystal structure of PGT128 Fab in complex with gp120 outer domain**. PGT128 heavy chain is shown in black, light chain in purple. The engineered glycosylated outer domain is depicted in blue ribbons, and its N-linked glycans as yellow (C), red (O), and blue (N) ball-and-sticks model. The primary binding site accommodates terminal mannose residues of the D1 and D3 arm of the N332 glycan, and the secondary binding site recognizes the N301 glycan. The structure was constructed using pymol (pdb 3TYG).

1.4.4.2. Specificity for the N332 glycan: PGT128 and PGT121

Two particular PGT antibodies (PGT121 and PGT128) proved to be almost ten-fold more potent than the previously described PG9, PG16, and VRC01 antibodies, and a 100-fold more than b12 and 2G12. At a low IC_{50} < 0.1 μg/mL, PGT128 still neutralised half of the 162-pseudovirus panel, and PGT121 44% (Walker *et al.* 2011). PGT121 was shown to provide sterilising immunity against vaginal challenge with $SHIV_{SF162P3}$ at the low dose of 1

mg/kg, which correspond to a serum concentration of 15 μg/mL, indicative of the potential of this antibody in future vaccine design (Moldt *et al.* 2012).

Interestingly, PGT 121-123, 125-128, 130 and 131, isolated from two elite neutralisers, belong to the same competition group as 2G12 (Walker *et al.* 2011). They were found to compete with 2G12 for binding to monomeric gp120, as well as with a V3-loop specific monoclonal antibody. It was shown that N-linked glycans at position N332 (72% conserved) and N301 (93% conserved) were crucial for neutralisation of HIV_{JRCSF} pseudovirus variants by PGT monoclonal antibodies 125-128, 130, 131 (alanine substitution). The carbohydrate nature of the epitope was highlighted by the lack of binding of PGTs 125-128 and 130 to Endo-H treated gp120, and glycan array analyses, which showed binding to $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$, but not Man_4 mimic of the D1 arm. These antibodies appear to be even more specific than 2G12 to an idiosyncratic arrangement of the whole $Man_9GlcNAc_2$.

Whilst PGT121 was shown to be sensitive to the N332A mutation (Mouquet *et al.* 2012), no binding to protein-free oligomannose-type glycans was detected on glycan array analyses, yet affinity towards complex-type glycans, such as mono- or bi-antennary galactosylated and α2-6sialylated complex-type glycans was observed (Walker *et al.* 2011; Mouquet *et al.* 2012). However, PGT121 was shown to retain binding to a gp120 glycoform bearing predominantly oligomannose glycans, suggesting that both oligomannose and complex-type glycans are involved in PGT121 recognition.

Structurally, PGT127 and PGT128 do not exhibit a domain-exchange structure (Figure 1.13.B) (Pejchal *et al.* 2011). Their crystal structures in complex with $Man_9GlcNAc_2$ showed exquisite interaction between the terminal mannose residue of the D1 and D3 arm. Residues within the CDR L3 region bind to the D3 arm. The D1 arm of $Man_9GlcNAc_2$ binds to the V_H FR2 antibody region, but also to six amino acid within the CDR H2 regions, that have been

inserted for PGT125 and PGT128 as a result of somatic mutation, and thus enhance Ab binding to gp120 Man_9D1 arm. The crystal structure of the bnAb PGT128 in complex with an engineered glycosylated gp120 outer domain demonstrates the existence of several binding sites (Pejchal *et al.* 2011). The first of these sites bind the D1 and D3 arms of the $Man_{8/9}GlcNAc_2$ oligomannose glycan linked to Asn332; the secondary site binds to the N301 glycan. PGT128 also interacts with the base of gp120 V3 loop via van der Waals and hydrogen bonds with PGT128 CDRH3.

The crystal structure of bnAb PGT121 was recently solved, and explains the basis of the binding of this antibody to a bi-antennary sialylated complex-type glycan (Mouquet *et al.* 2012). The glycan interact with PGT121 V_H domain and makes extensive contacts with CDR H1, H2 and H3, including direct contact with the sialic acid residue.

This epitope specificity brings up the concept of glycan promiscuity, which is further explored in Chapter 5, whereby the interaction with one glycan promotes binding to an additional, neighbouring glycan. In contrast to 2G12 which binds the tip of the $Man_9GlcNAc_2$ structure, PGT128 binds to residues closer to the core of the glycan, and could thus potentially accommodate complex-type glycans that share that pentasaccharide core, as seen with PGT121. It is interesting to note that while the glycan at position N332 is part of the epitopes of both 2G12, PGT135, PGT128 and PGT121, these antibodies all adopt a different angle of approach and mode of binding towards this glycan on the envelope spike.

1.4.5. Broadly neutralising antibodies: (un)common features

Broadly neutralising antibodies are not elicited until several years after infection (Mikell *et al.* 2011), a delay that is still not well understood. Three main steps are involved in B-cell maturation: recombination of germline antibody genes to produce unique antibody light and heavy chains; deletion of auto-reactive B-cells through clonal deletion (a major

problem for bnAbs such as 2G12, 2F5 and 4e10, but not VRC01); antigen-affinity maturation through hypermutation of the variable domains. Indeed, it was shown that all HIV-1 bnAbs have in common a long heavy chain CDR H3, used to gain exquisite affinity towards their specific epitopes. For instance, b12 long CDR H3 loop allows it to gain access to the recessed CD4BS. Furthermore, shorter light chain CDR loops correlate with increased neutralisation potency, as shown with the two amino acid deletion in VRC01 CDR L1 loop (Zhou *et al.* 2010), as well as commonly observed short CDR L3 for the VRC01-like bnAbs (West *et al.* 2012).

Moreover, a high level of affinity maturation is involved for HIV-reactive antibodies and neutralising antibodies (Scheid *et al.* 2009). For instance, VRC01 exhibits a high degree of affinity maturation, together with other uncommon features such as the presence of an extra disulfide bond, a site for N-linked glycosylation, and a two amino acid deletion in the antibody light chain. Within the extensively maturated interface between VRC01 and gp120, several amino acid reversions are crucial to VRC01 activity (Zhou *et al.* 2010). The deep sequencing technology has made it possible to study the precursor and maturation process of antibodies from HIV-1 infected individuals (Wu *et al.* 2011). It was shown that extensive somatic maturation (70-90 changes) within the precursor heavy chain gene allele IGHV1-2*02 is involved in the development of VRC01-like antibodies. Other CD4BS antibodies have been similarly characterised (Scheid *et al.* 2011). A comparable extensive maturation was observed for PG9 and PG16, which exhibit 20.4% of V_H and 15.1/21.2% of V_I genes respectively genetic divergence relative to the putative germline (Pancera *et al.* 2010).

B-cells expressing antibodies with either long CDR H3 and/or polyreactivity usually undergo negative selection, directly in the bone marrow or by B-cell tolerance mechanisms (Chen *et al.* 1994; Chen *et al.* 1995; Verkoczy *et al.* 2010). This may explain the relatively low occurrence of a cross-reactive neutralising antibody response amongst HIV-1 infected

individuals. The use HIV-1 bnAbs affinity maturation knowledge to develop immunisation schemes is detailed in section 1.5.5.

1.5. Towards a carbohydrate-based HIV vaccine

Current AIDS preventive strategy encompasses use of pre-exposure prophylaxis (antiretroviral chemoprophylaxis), use of topical microbicides, male circumcision, and mother-to-child prevention. Whilst advances in HAART have decreased HIV transmission and mortality rates from AIDS, a prophylactic vaccine remains an elusive component to the anti-HIV arsenal.

Given that HIV can integrate itself within the host genome, establishing latent viral reservoir, an efficient HIV-1 vaccine will need to entirely prevent infection, providing sterilizing immunity (Pope *et al.* 2003; Johnston *et al.* 2007; Haynes *et al.* 2008). This renders the development of a vaccine for HIV even more difficult than for other viral pathogens, which usually become eradicated at an early stage of dissemination, before disease onset.

1.5.1. Humoral vs cellular immunity as primary component of an anti-HIV vaccine

Previous vaccines were successful against diseases such as hepatitis B, measles, *Haemophilus influenzae* viruses, papillomavirus, and smallpox due to their ability to elicit an antibody response similar to that which arises during natural infection (Amanna *et al.* 2008). Memory-driven immunity is obtained through the adaptive immune response that induces rapid response against antigenically similar pathogens. The induction of neutralising antibodies is efficient at protecting against pathogens that circulate through the bloodstream to reach their target organs, or even pathogens that replicate at the mucosal surface where they can be neutralised by locally produced IgA, IgM, or IgG diffused from serum. Several vaccines, including polio, measles, rubella, human influenza type b, pneumococcus, have

been shown to provide sterilising immunity through the action of nAbs. Thus, eliciting broadly neutralising antibodies by vaccination constitute the main approach used for an HIV prophylactic vaccine development (Mascola *et al.* 2010), which is supported by several studies.

Broadly neutralising antibodies as a pivotal component of a prophylactic vaccine

Key experiments of passive transfer of bnAbs demonstrated that bnAbs could offer protection against intravenous, vaginal, rectal, and oral SHIV challenge in non-human primates (Mascola *et al.* 1999; Baba *et al.* 2000; Mascola *et al.* 2000; Parren *et al.* 2001; Nishimura *et al.* 2002; Veazey *et al.* 2003; Hessell *et al.* 2009a; Hessell *et al.* 2009b; Hessell *et al.* 2010). It was observed that high titers of bnAbs were required to protect against high-dose intraveneous (Mascola 2002), but much lower doses were required for mucosal challenge (Hessell *et al.* 2009a).

The presence of neutralising antibodies exhibiting some cross-reactivity in around 25% of HIV-1 infected patients show that these antibodies can be generated as part of the natural B-cell response (Simek *et al.* 2009). Moreover, studies have shown that nAbs elicited by vaccination of rhesus macaques offered protection against homologous SHIV challenge (Barnett *et al.* 2008; Bogers *et al.* 2008).

To offer protection, elicited antibodies must neutralise the virus very early after exposure, and exhibit a good breadth and magnitude of neutralisation. The response must also offer some form of long-term protection (Evans *et al.* 2004; Goepfert *et al.* 2007). However, the elicited nAbs in previous clinical trials have achieved only short half-lifes (McElrath *et al.* 2000; Goepfert *et al.* 2007) – in stark contrast to live attenuated vaccines such as smallpox, measles, mumps, rubella, varicella-zoster which have a serum half life of at least 50 years

(Amanna *et al.* 2007). Another limit to nAbs is the fact that they have been shown not to offer protection against super-infection (Blish *et al.* 2007).

Could non-neutralising Ab responses also protect against HIV infection?

Non-neutralising antibodies elicited by a vaccine could have potentially protective effects. Dimeric IgA and pentameric IgM can aggregate virions, subsequently preventing them from crossing the mucosal barrier and epithelial layers (Hladik *et al.* 2009).

More importantly, Fcγ receptors-dependent effector functions such as endocytosis, phagocytosis, and cell lysis, are important in targeting both free virions and HIV-1 infected cells. It was shown that a subset of elite controllers has high ADCC (Lambotte *et al.* 2009), and control of viremia correlates with high ADCC and ADCVI in non-human primate trials (Xiao *et al.* 2010; Florese *et al.* 2009; Gomez-Roman *et al.* 2005) and efficacy clinical trials, such as the Vax004 trial (Forthal *et al.* 2007) and the recent RV144 trial (discussed in more detail in the following section). No neutralising antibodies were elicited with the RV144 trial, however it was shown that elicited antibodies exhibit strong cross-clade ADCC and ADCVI activity (Bonsignori *et al.* 2012). Finally, binding to Fc receptors on effector cells lead to the release of antiviral chemokine and cytokines (DeVico *et al.* 2004).

The use of non-neutralising antibodies as a sole agent for protection against HIV-1 infection is still controversial. Before the RV144 trial, there was little evidence that non-neutralising antibodies would provide a correlate of protection. Moreover, non-neutralising antibodies showed little or no efficacy in protecting against SHIV challenge in non-human primates (Florese *et al.* 2009; Burton *et al.* 2011).

An ideal scenario would be to elicit neutralising antibodies that also induce strong ADCC, ADCVI or phagocytosis, thereby destroying infected cells as well as preventing

infection. ADCC and ADCVI have been detected on b12 (Hessell *et al.* 2007), 2F5/4E10 (Hessell *et al.* 2010; Tudor *et al.* 2011), and 2G12 (Klein *et al.* 2010).

Involvement of the cellular response in immunity

Even if antibodies prove to be the main correlate of protection, cellular immune responses will be needed, for instance in the $CD8^+$ T-cells control of viral replication, and $CD4^+$ T-cells mediated B-cells development and CTL responses. The establishment of a latent pool very early in infection has led to a great deal of interest for T-cell based vaccine approach, which would limit the destruction of the immune cells by the virus and allow for better control of the infection and disease development. A good example of this is the BCG (bacille Calmette-Guérin) vaccine against tuberculosis, where a strong cellular response is required to prevent disease, including $CD4^+$ T-cells IFN-γ production (Fletcher 2007), as well as $CD8^+$ T-cells that maintain the tubercle bacilli in a latent state (McShane 2004). Cellular immune response thus provide correlate of protection against the disease, whilst humoral immune response provides correlate of protection against infection.

$CD8^+$ T-cells are crucial in controlling early viremia, slowing disease progression (Schmitz *et al.* 1999) and selecting for mutant that have decreased viral fitness (Goulder *et al.* 2008). T-cell based vaccines target all viral antigens, including Gag epitopes that are critical for viral fitness (Troyer *et al.* 2009). However, the T-cell responses obtained during the clinical STEP trial, and SHIV mucosal challenge (Lakhashe *et al.* 2011) were unable to protect against infection or ineffective in lowering viral load. It has since then been proposed that a protective T-cell response should include characteristics such as a specific cytokine production profile (IFN-γ, TNF-α, IL-2, MIP-1β), T-cell proliferation, cytolytic potential, inhibition of HIV-1 replication within infected cells, long lived central memory cells and effector memory cells present at the mucosal barrier (McElrath *et al.* 2010).

In addition to CTL responses, stimulation of $CD4^+$ T helper cells also play a role in protection, and are crucial in generating long-lived effective $CD8^+$ T-cell and B-cell memory responses, as well as directing T-cells to the site of infection. The RV144 trial induced a robust $CD4^+$ T cell response (section 1.5.2).

Should the response be limited to T-cell however, the latent viral reservoir will remain. Therefore T-cell based vaccine, by reducing viral load, could impact upon both disease progression, and reduced transmission rates, despite offering little protection against infection. Moreover, the selection of escape mutants and CTL escape remains a major challenge for a cellular-based vaccine.

1.5.2. Efficacy trials to date

Several large-scale efficacy trials have been conducted since 1998 (Mascola *et al.* 2010; McElrath *et al.* 2010). As HIV is an integrative virus, live attenuated or killed viruses are not safe for vaccine use. However, a subunit vaccine has been used effectively against viruses such as Hepatitis B and papilloma virus (Villa 2011), and is a proof-of-concept that this kind of approach can elicit neutralising antibodies.

The first efficacy trial was a phase III double-blind, placebo-controlled, clinical trial, VAX004, composed of two subtype B strains derived recombinant gp120 (300 μg formulated with the adjuvant alum). The same trial was conducted in Asia, VAX003, with recombinant gp120 derived from subtype B and subtype E used. Both trials showed no efficacy either towards HIV protection or lowering of the viral load (Flynn *et al.* 2005; Pitisuttithum *et al.* 2006), despite eliciting nAbs against the vaccine strain.

The next efficacy trial was geared towards stimulating the cellular immune response in addition to providing cell-mediated immunity as well as reducing early plasma HIV-1 levels. The Merck STEP trial was a multi-centre, double blind, randomised and placebo-controlled

phase II trial. It used a Merck replication-incompetent Adenovirus type 5 vector encoding HIV-1 clade B Gag/Pol/Nef. It proved to be ineffective at protection and may even have increased infection rates (Buchbinder *et al.* 2008; Gray *et al.* 2010). More specifically, phase II studies have shown elicitation of CD4BS Abs that mediate ADCC (not broadly reactive) and CD4 lymphoproliferation (McElrath *et al.* 2000).

Recently, the RV144 trial has been the first efficacy trial showing HIV protection – a 31% of reduction in HIV-1 infection was reported (Rerks-Ngarm *et al.* 2009). The prime (months 0, 1, 3, 6) consisted of the live recombinant canarypox ALVAC-HIV vCP1521 encoding Gag, Pol, Nef, ALVAC-HIV-1, and membrane-anchored gp120 derived from the $HIV_{92TH023}$ strain. The boost (months 3 and 6) was a protein-based vaccine VaxGen gp120 from subtype B/E in alum. Whilst 99% of vaccinated subjects elicit Abs against the B/E Env, the duration of the protection was however limited, as titres decreased after 15-20 weeks. ADCC was observed in 75% of subjects, disappearing after 20 weeks, and there was no significant reduction in early viral load or $CD4^+$ T cell counts.

Finally, the efficacy trial HVTN 205 is currently under way. It is composed of a multiclade DNA plasmid prime encoding Gag, Pol, Nef, and clade A, B and C gp140s, and a boost by rAd5 encoding the same proteins except Nef. The vaccine is expected to elicit both cellular and humoral immune responses, and subsequently reduce the viral load, and perhaps offer prevention of HIV-1 infection.

To develop further vaccine strategies, analysis of the correlates of immunity of previous immunisation and clinical trials is crucial. The correlates of immune protection of the RV144 are currently studied in order to gain insight into the mechanisms associated with protection and further develop them in future immunisation (Karnasuta *et al.* 2005; Fuchs *et al.* 2010; Kim *et al.* 2010; Montefiori *et al.* 2012). The phases I and II of the identical vaccine regime led to a strong humoral and cellular response (Nitayaphan *et al.* 2004). The RV144

trial did not induce broad neutralising antibodies, and it is thought that protection was achieved through ADCC and cellular mechanisms. The whole cellular response was studied 6 months after the end of immunisation, and demonstrated a recognition pattern centred on epitopes within the Env V2 region (de Souza *et al.* 2012). Whilst the $CD8^+$ T-cell response was relatively weak, nevertheless a robust $CD4^+$ T-cell response induced production of IL-2 and IFN-γ. Such effector memory $CD4^+$ T-cells are crucial in developing IgG-secreting plasma B cells and maintaining a long-lasting B cell response.

1.5.3. The epitope-based vaccine design strategy

The impossibility of using a live attenuated or killed virus as a vaccine, and the failure of precedent efficacy trials described previously administrating Env as a subunit, have led to employ an alternative strategy to elicit bnAbs, which focus the immune response on specific conserved region of HIV Env spikes. The approach of the epitope-based vaccine design, or 'reverse vaccinology', frequently makes use of the analogy of a template where the neutralising antibody serves to imprint the structure required of an immunogen (Burton 2002; Walker *et al.* 2010a). The construction of the immunogen is thus directly based on the bnAb crystal structure, yet it is presented in a different immunological context, aiming at eliciting similary cross-reactive nAbs. It was indeed shown that V3 loop epitope mimics could elicit anti-HIV nAbs (Zolla-Pazner *et al.* 2011), albeit the breadth of neutralisation of such antibodies is limited.

The design of an epitope-based vaccine requires the solving of two distinct problems (Burton *et al.* 2004; Haynes *et al.* 2006). The first is to elucidate, at the structural level, the epitope to which a neutralising antibody binds. The second is to determine how this epitope, if used as an immunogen, might elicit similar neutralising antibodies. These two phenomena exemplify the immunological concepts of antigenicity and immunogenicity, respectively.

Whilst the antigenicity accounts for physical parameters such as valency and glycan chain length, immunogenicity refers to the ability of an antigen to induce a B-cell response. The characteristics of the latter include self-tolerance, cross-reactivity, affinity maturation, and B-cell germline affinity, and are imperfectly understood and less amenable to direct manipulation. The difficulty in translating antigenic, structural information into a successful immunogen arises partly from an intrinsic property of HIV: namely the poor immunogenicity of conserved regions of the viral Env, and the correlated immunodominance of variable regions, such as the V3 loop. This feature is an inevitable consequence of viral adaptation in the presence of the host antibody response. A lack of immunogenicity is, almost by definition, an inherent property of any epitope recognised by a broadly neutralising antibody, which is unfortunately the template for immunogen design. The improvement of the immunogenicity remains thus a major challenge in this epitope-based vaccine strategy.

The isolation of broadly neutralising HIV antibodies has led to the subsequent characterisation of their epitopes. The elucidation of their crystal structures has paved the way towards immunogen design via mimicking of their natural epitopes. There is currently a wide range of approaches towards immunogen design (reviewed in Hoxie 2010; Mascola *et al.* 2010), including but not limited to removal of immunodominant regions of Env, multivalent displays of Env, Env stabilisation, mimics of the native viral spike, and mimicking bnAbs epitopes. The next section will focus on the latter approach, in the particular case of 2G12/PGT128-like antibodies targeting the gp120 glycan shield.

1.5.4. Design of a carbohydrate-based immunogen

The oligomannose glycans of HIV are part of an extensive array of carbohydrates that prevents humoral recognition of the viral Env surface. Although the glycan shield of HIV is poorly immunogenic, it is nonetheless antigenically distinct from the glycosylation of the host

cell – as evidenced by the selective recognition by 2G12 and PGT128, both of which bind gp120 but not self glycans. Yet HIV is glycosylated entirely by the infected cell biosynthetic machinery. Critically, the processing of gp120 diverges from cellular glycan processing, giving rise to a large array of oligomannose glycans, including the epitope recognised by 2G12. Thus, the glycan shield of gp120 is simultaneously both a primary viral adaptation against antibody neutralisation and conversely a site of recognition by broadly neutralising antibodies, with key glycans, including N322, involved in epitopes that account for up to half of serum specificity (Walker *et al.* 2010b). The elucidation of the interaction between 2G12 and gp120, combined with an understanding of the biogenesis of HIV Env glycosylation provides a detailed, molecular-level blueprint for vaccine design.

The first carbohydrate-based vaccine was commercialised in 1983 against *Streptococcus pneumoniae*, and since similar vaccines targeting carbohydratres of several pathogens, including *Neisseria meningitides*, *Haemophilus influenza B*, *Staphylococcus aureus*, and *Salmonella typhi*, have been developed (Astronomo *et al.* 2010a). In contrast to protein antigens, carbohydrates usually induce T-cell independent B-cell responses, which are less robust, shorter lived, and lack antibody class-switching. As a result, T-cell epitopes are usually conjugated to the carbohydrate-based immunogen to obtain the crucial $CD4^+$ T helper cell response. Multivalent display, as detailed in section 1.4.4, can counteract the usually low affinity between carbohydrate and antibody. Finally, glycan microheterogeneity, which dilutes the antibody response, can be overcome, at least in the case of gp120, by targeting specific oligomannose-type structures.

A diverse set of chemical and biological approaches have yielded glycoconjugate antigens engineered to mimic the carbohydrates found on HIV recognised by 2G12. The first approach was to chemically synthesise carbohydrate mimics of the 2G12 epitope – mainly the D1 arm $Man_4GlcNAc_2$, and $Man_9GlcNAc_2$ – and conjugate several of these glycans onto

scaffold, protein carriers, or viral particles, in an arrangement mimicking the 2G12 epitope (Table 1.1). Alternatively to chemical synthesis, $Man_9GlcNAc_2$ glycans can be obtained by interfering with the glycosylation pathway. Several approaches have been used, including the use of enzyme inhibitors, such as kifunensine, an ER α-mannosidase I inhibitor that stalls glycan processing at the $Man_9GlcNAc_2$ intermediate, (Scanlan *et al.* 2007), or glycosylation enzyme knockouts in genetically engineered *Saccharomyces cerevisiae* strains (Table 1.2). A final approach is the use of bacterial or yeast proteins that exhibit polysaccharides which share antigenic similarities with the 2G12 epitope (Luallen et al. 2009), as summarized in Table 1.3.

Table 1.1. Chemical approaches to anti-carbohydrates HIV-1 immunogens design †

Immunogen	Results	Ref
The glycoconjugates were obtained by linking either $Man_9GlcNAc_2$ or a synthetic cluster of $Man_9GlcNAc_2$ onto a galactose ring, and then onto a protein carrier (either KLH or a T-helper epitope derived from tetanus toxoid).	Immunisation of rabbits resulted in antibodies mainly directed at the linker. Some level of gp120 binding was observed, however sera showed no neutralisation activity.	Wang *et al.* 2004; Ni *et al.* 2006
Two $Man_9GlcNAc_2$ moities conjugated onto a peptide scaffold were incorporated to an immunostimulatory protein carrier derived from *Neisseria meningitidis*.	Anti-carbohydrate Abs were elicited in guinea pigs and macaques, that did not recognise gp120, and were unable to neutralise viral isolates.	Joyce *et al.* 2008
Oligomannose dendrons: covalent binding of up to 10 D1-$Man_4GlcNAc_2$ units to BSA.	Anti-carbohydrate Abs were elicited in rabbits, but failed to bind gp120, and neutralise viral isolates.	Astronomo *et al.* 2008
Polyamidoamine scaffolds were used to generate oligomannose dendrons that were covalently coupled to the protein carrier CRM_{197}.	Immunisation in rabbits and mice resulted in Abs that recognised oligomannose glycans, but failed to recognise monomeric gp120.	Kabanova *et al.* 2010
Glycocojugates with multivalent Man_4 and Man_9 display on icosahedral capsids of the macrophage Qβ and cowpea mosaic virus (CPMV).	Qβ glycoconjugates elicited anti-mannose antibodies in rabbits that failed to recognise gp120 and neutralise HIV-1 isolates.	Astronomo *et al.* 2010b
A terminal non-self D-fructose residue was introduced on the D1 arm to break immune tolerance, on a bacteriophage Qβ icosahedral capsid conjugate.	Abs titers were higher than with the nonself immunogen in rabbits, and shown to cross-react with the self construct, but not with monomeric gp120.	Doores *et al.* 2010a

† immunogens tested in animal models

Table 1.2 Exploiting the cell glycosylation machinery to obtain anti-carbohydrates HIV-1 immunogens †

Immunogen	Results	Ref
Recombinant gp120 glycoforms were obtained by expressing gp120 in insect or mammalian cells, and with kifunensine.	Non-sialylated material resulted in increased immunogenicity. Antibodies obtained after immunisation of Balb/c mice with high-mannose gp120 glycoforms bind to wt-gp120, albeit with lower affinity.	Kong *et al.* 2010
ΔMmn1 S. cerevisiae -/- mutant displays predominantly Manα1-2Man terminating glycans, in a more immunogenic context than relative to gp120.	Immunisation of whole yeasts mutant in rabbits yielded sera that did bind to gp120 carbohydrates, and modest neutralisation was observed.	Dunlop *et al.* 2008 Dunlop *et al.* 2010b
The triple mutant (TM) *ΔOchp1 ΔMmn1 ΔMnn4 S. cerevisiae* exhibit mainly $Man_8GlcNAc_2$ glycans.	Rabbits immunised with whole TM yeasts elicited Abs that bind to gp120 carbohydrates, but did not have any neutralising activity.	Luallen *et al.* 2008; Luallen *et al.* 2010; Agrawal-Gamse *et al.* 2011

† immunogens tested in animal models

Table 1.3. Antigenic mimicry†

Immunogen	Results	Ref
Bacterial lipooligosaccharide (LOS) from the soil bacterium Rhizobium radiobacter Rv3 naturally exhibits carbohydrate structures analogous to the $Man_9GlcNAc_2$ D1 arm	Immunisation with heat-killed whole bacteria in mice elicited Abs that recognise monomeric gp120 but fail to neutralise viral isolates.	Clark *et al.* 2012

† immunogens tested in animal models

Surprisingly, given the variety of carbohydrate antigens and vaccine scaffolds employed, the antibody responses to these experimental antigens are similar. In no case has immunisation with a glycoconjugate yielded responses with serum antibody titres that would be capable of neutralising HIV. Nevertheless, in a number of cases antibodies with carbohydrates specificities similar to 2G12 have been elicited by glycoconjugates based on the 2G12 epitope. However, the differences in carbohydrates spacing and flexibility on these immunogens, compared to their presentation on viral Env spikes, might explain the lack of success obtained so far with carbohydrate-based immunogens. A deeper understanding of the

nature of the HIV-1 glycan shield and its cross-clade variability is necessary for the future developments of this field.

1.5.5. Future immunisation strategies

An ideal preventive vaccine would induce memory $CD4^+$ and $CD8^+$ T cells at mucosal sites, and long lasting protective broadly neutralising antibodies. Two different approaches could be used to obtain broadly neutralising responses. The first consists of using multiple immunogens corresponding to different epitopes (Zwick *et al.* 2001b). The second approach would be to direct the immune system into developing a response against a broadly neutralising epitope. Exploration of B-cell lineages provides insight into antibody maturation and nAbs response evolution, which can then be applied in vaccine development. The first step of such an immunisation strategy would be to elicit germline precursors to bnAbs previously identified (Ma *et al.* 2011), and subsequently boost with mimics of viral Env allowing induction of broadly neutralising antibodies (Malherbe *et al.* 2011).

Finally, with respect to the development of an anti-carbohydrate vaccine, it is crucial to determine the exact composition of Env N-linked glycosylation, as found on the virus. This will allow a proper understanding of antibody recognition, and aid further immunogen design. The HIV glycan shield can also interact with immune receptors, and such interactions might either be avoided or exploited in future immunogen design. The main aim of this thesis is therefore the extensive characterisation of the HIV-1 glycan shield, as detailed in Chapters 3, 4 and 5. The interaction of HIV gp120 glycans with several cellular receptors is discussed in Chapter 6, together with the results of an immunisation study based on a glycan-modified gp120.

Chapter 2

Materials and Methods

2.1. Materials

The plasmid construct encoding for $gp120_{BaL}$ inserted in the pHLsec vector (Aricescu *et al.* 2006) was a gift from Dr Guillaume Stewart-Jones (University of Oxford). The N332A-$gp120_{BaL}$ and N339A-$gp120_{BaL}$ mutants inserted in the pHLsec vector were obtained in collaboration with Cameron Dunlop (University of Oxford). The pSVIII vector encoding $gp120_{JRCSF}$ (accession number AAB03749) was a gift from Prof Dennis Burton (TSRI, USA). The pOTB7 vector containing a cDNA clone of the human ER α-mannosidase I gene (accession number BC002953) was purchased from OpenBiosystems (Huntsville, AL, USA). The pUNO1 vector containing a cDNA clone of the human CD22 gene was obtained from Invivogen (San Diego, CA, USA).

Enzymes were purchased from New England Biolabs (Ipswich, MA, USA), and chemicals from Sigma-Aldrich, unless otherwise specified. Primers for cloning, mutagenesis and sequencing were obtained from Thermofisher (Waltham, MA, USA) and are listed in section 2.2.3. The AmpliTaq Gold PCR Master Mix was obtained from Applied Biosystems (Carlsbad, CA, USA). GelRed, PE-anti human CD22, PE-anti human CD33, PE-anti human sg5/CD170, PE-anti human sg7/CD328, and PE-anti human siglec 9 antibodies were obtained from Cambridge Biosciences (Cambridge, UK). EndoFree Plasmid Maxi and Mega kits were purchased from Qiagen (Crawley, UK). NucleoSpin Plasmid and NucleoSpin Gel and PCR clean up kits were purchased from Macherey-Nagel (Düren, Germany). Transformation

competent cells were purchased from Stratagene (La Jolla, CA, USA). Sequencing was performed by Source Bioscience (Department of Biochemistry, Oxford, UK). Pre-cast NuPAGE 4-12% Bis-Tris polyacrylamide gels, NuPAGE LDS Sample Buffer, 3-morpholinoethane-sulfonic acid (MES) and Transfer buffers, AP (alkaline phosphatase) and HRP (horse radish peroxidase) conjugated anti-HIS C-terminus antibodies, BenchMark His-tagged protein standard, penicillin, streptomycin, fetal calf serum, Dulbecco's modified Eagle Medium (DMEM) and minimum essential medium (MEM) nonessential amino acids were obtained from Invitrogen (Paisley, UK). Kifunensine was obtained from Cayman Europe (Estonia). Precision Plus Protein Kaleidoscope Standard was obtained from BioRad (Hemel Hempstead, UK). Immobilon-P western polyvinylidene fluoride **(**PVDF) membrane was obtained from Millipore Ltd (Watford, UK). Vivaspin 20 centrifugation tubes were obtained from Sartorius (Goettingen, Germany). HisTrap FF crude purification columns were obtained from GE Healthcare (Waukesha, WI, USA). ELISA microtiter plates Costar type 3690 were obtained from Corning (New York, USA). The HRP developing solution 1-Step™ Ultra TMB-ELISA was obtained from Thermo Scientific (Rockford, IL, USA). D7324 antibody was obtained from Aalto Bio Reagents (Dublin, Ireland). Purified monoclonal antibodies IgG 2G12 and IgG b12 were a gift from Prof. Dennis Burton (TSRI, USA). Amide-2 SPE cartridges were obtained from Applied Separations (Allentown, PA, USA). CMP-sialic acid and UDP-galactose were obtained from Calbiochem. The Proteome Profiler Human Cytokine Array kit was purchased from R&D Systems.

2.2. Methods

2.2.1. Cloning into pHLsec

The residues 32–503 of $gp120_{JRCSF}$, and 19–686 of CD22 were cloned into the pHLsec vector for expression of soluble proteins in mammalian cells. Amplification of the insert of interest

was done with polymerase chain reaction (PCR) with primers introducing the AgeI and KpnI restriction site at the 3′ and 5′ end respectively. For inserts already containing KpnI restriction site in their sequence, the BsiWI restriction site was introduced at the 5′ end. The PCR product was analysed by electrophoresis with 1% agarose gel in Tris-acetate-EDTA (TAE) buffer, the bands corresponding to the correct size inserts were excised and purified using a gel extraction kit. The purified PCR product was then digested with restriction enzymes according to the manufacturer's directions, and purified with the PCR/gel extraction kit. The pHLsec vector was similarly digested with restriction enzymes, purified by gel electrophoresis, and treated with Antartic Phosphatase. For inserts with AgeI and KpnI restriction sites, the vector was similarly digested with AgeI and KpnI. For inserts with AgeI and BsiWI restriction sites, the vector was digested with AgeI and Acc65I. Ligation was performed by incubating for 20 min at room temperature 1 μL of T4 DNA ligase, 1 μL of T4 DNA ligase buffer, 1 μL (50 ng) of the purified vector and 7 μL (100 ng) of the purified PCR product. The final construct was transformed into XL-1 blue cells according to the manufacturer's instructions, and plated overnight on LB agar plates with carbenicillin (50 μg/mL) at 37°C. Individual colonies were picked and grown overnight at 37°C in a rotary shaker, in 10 mL of LB broth with carbenicillin (50 μg/mL). The plasmid was purified using NucleoSpin Plasmid kit and the sequence was confirmed by DNA sequencing.

2.2.2. Mutagenesis

Mutagenesis was carried out using a two-step overlapping PCR approach. In a first step, two separate PCRs were carried out, one amplifying from the 3′ end of the gene to the mutation primer, and the other one amplifying from the mutation primer to the 5′ end of the gene. Overlapping primers were used (see list below). The PCR products were analysed by agarose gel electrophoresis and purified using a gel extraction kit. In a second step, the PCR inserts

were mixed with the 3′ and 5′ vector primers. The final PCR product was digested with the appropriate restriction enzymes, and ligated into the pHLsec vector. DNA was purified and its sequence confirmed.

2.2.3. List of primers

Gene	Primer name	Forward sequence 5′ to 3′
$gp120_{JRCSF}$	AgeI_fwd	*CGAC*ACCGGTGCTGTAGAAAAGTTGTGGG
	BsiWI_rev	*CAG*CGTACGCCTTTTTTCTCTTTGCACC
	N295A_fwd	GAATGAATCTGTAAAAATTGCTTGTACAAGGCCCAGCAAC
	N295A_rev	GTTGCTGGGCCTTGTACAAGCAATTTTTACAGATTCATTC
	N332A_fwd	GACAAGCACATTGTGCCATTAGTAGAGCACAATGG
	N332A_rev	CCATTGTGCTCTACTAATGGCACAATGTGCTTGTC
	N339Q_fwd	GTAGAGCACAATGGCAGAACACTTTAAAACAG
	N339Q_rev	CTGTTTTAAAGTGTTCTGCCATTGTGCTCTAC
	N392Q_fwd	CAACACAACTGTTTCAGAGTACTTGGAATG
	N392Q_rev	CATTCCAAGTACTCTGAAACAGTTGTGTTG
$gp120_{BaL}$	AgeI_fwd	*CGAC*ACCGGTATGGACGCCATGAAGCG
	KpnI_rev	*CAG*CGTACGCACCACGCTGCTGATG
	N295A_fwd	GAATCTGTAGAAATTGCTTGTACAAGACCCAACAACAATACACGC
	N295A_rev	GCGTGTATTGTTGTTGGGTCTTGTACAAGCAATTTCTACAGATTC
CD22	AgeI_fwd	*CCGC*ACCGGTGACTCAAGTAAATGGGTTTTTG
	BsiWI_rev	*ATAA*CGTACGTCGCCTGCCGATGGTCTC
pHLsec	fwd	TGCTCTCCCTCGTGAGCCTTCTC
	rev	CCTGAGGAGTGAATTATCGCGATACTAGTCTCG

Cloning primers have a flanking region of 3-4 nucleotides (in italic in the table), a restriction site (underlined), and 15-20bp of the gene sequence. Mutagenesis primers were design to overlap the mutation site by a minimum of 10 nucleotides, and have a higher melting temperature. The mutation is underlined. pHLsec sequencing primers are also indicated.

2.2.4. Protein expression

Mammalian human embryonic kidney (HEK) 293T cells (ATCC number CRL-1573), and GnTI-deficient HEK 293S cells (Reeves *et al.* 2002) were cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin. When growing GnTI-deficient 293S cells, media was supplemented with MEM nonessential amino acids. Transient transfection was done as follow: for each T175 flask, 90 µg of polyethyleneimine (PEI-MAX) and 50 µg of DNA were incubated for 10 min in 5 mL of serum free media; then added to 80-

90% confluent cells cultured in 25 mL of serum free media. Transfection with kifunensine included 20 μM of kifunensine.

2.2.5. Protein purification

Culture supernatant was collected at 5 days post-transfection. Protease inhibitor was added to the collected supernatant, which was subsequently centrifuged, sterile filtered through a 0.22 μM pore sterile filter and then concentrated by centrifugal filtration using Vivaspin 20 devices. The concentrated supernatant was purified using a HisTrap column. Binding and washing was done with a 20 mM imidazole solution in a phosphate buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7.5). The protein was eluted with a 250 mM imidazole solution in phosphate buffer. Proteins were then purified by size exclusion chromatography using a Superdex 200 Prep Grade (GE Health Care) equilibrated in phosphate buffer saline (PBS). Protein concentration was determined using Nanodrop 1000 spectrophotometer (ThermoScientific).

2.2.6. Protein electrophoresis and western analysis

Samples were first incubated at 70°C for 10 min with the SDS loading sample buffer and then run on a NuPAGE gel in MES buffer for 60 min at 190 V. Gels were stained with Brilliant Blue R and destained using a water, methanol and acetic acid (ratio 5/4/1) solution. Unstained gels were blotted onto a conditioned nitrocellulose membrane by wet transfer, for 75 min at 30 V. Membrane was then blocked with 3% milk in PBS for 30 min at room temperature. The membrane was then incubated with HRP conjugated anti-HIS antibody (1/1000 dilution in PBS/1% milk) for 60 min at room temperature. The membrane was then washed twice with PBS, and incubated for 5 min with 1 mL of the detection substrate. The membrane was then visualised by chemiluminescence using a Fujifilm Las-1000 Intelligent DarkBoxII.

2.2.7. Enzymatic release of N-linked glycans

Oligosaccharides were released from glycoproteins with peptide-*N*-Glycosidase (PNGase) F from Coomassie blue-stained Nu-PAGE gels (Küster *et al.* 1997). Excised bands were washed six times alternatively with acetonitrile and deionised water, and rehydrated with a 3000 Units/mL of PNGase F water solution. After incubation overnight at 37 ˚C, the enzymatically released *N*-linked glycans were eluted with water, and dried using a Concentrator Plus (Eppendorf, Hamburg, Germany).

2.2.8. MALDI-TOF MS spectrometry

Glycan samples were cleaned on a Nafion 117 membrane for an hour (Bornsen *et al.* 1995), and mixed on a plate with the 2,5-dihydroxybenzoic acid (DHB) matrix (0.3 μL of a solution of 2,5-dihydroxybenzoic acid in acetonitrile:water (1:1, v:v)) and sodium chloride, then allowed to dry at room temperature. The sample/matrix mixture was then recrystallized with ethanol. Desialylation was performed by heating samples at 80°C for 1 hr with 1% acetic acid. Dried samples were redissolved in water. Mass spectra were recording using a Shimazu AXIMA TOF2 MALDI TOF/TOF mass spectrometer (Kratos Analytical, Manchester, UK) as previously described in Harvey *et al.* 2008.

2.2.9. ER α-mannosidase I time course assay

Recombinant $gp120_{BaL}$ (20 μg), and ER α-mannosidase I (1 or 5 μg) were separately pre-warmed at 37°C for 5 min in a reaction buffer (80 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (PIPES), 1 μg/μL BSA, 4 mM $CaCl_2$, 0.0016% NaN_3, pH 6.5). Samples were then mixed to a final volume of 250 μL and aliquots taken at different time point, and gp120 glycans were prepared and analysed by MALDI-TOF MS.

2.2.10. Production of hypergalactosylated gp120

Recombinant gp120 was first treated with neuraminidase according to the manufacturer's instructions, and purified by size-exclusion chromatography. The mouse B4GalT I was expressed in HEK 293T cells and purified by size-exclusion chromatography. The PBS buffer was then exchanged to a 150 mM sodium chloride buffer with protein concentrator devices, and finally to 25 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$ buffer, 10 mM $MnCl_2$, pH 7.5 buffer. Gp120 (1 mg) was then incubated with the B4GalT I enzyme (100 μg) and UDP-Galactose (50 μg) in a total reaction volume of 1 mL, for 1 hour at 37°C. After incubation, proteins were analysed by SDS-PAGE and glycans prepared for glycan analysis. Cloning of the mouse B4GalT I catalytic domain into the pHLsec vector was done by Ben Yu (University of Oxford).

2.2.11. Production of hypersialylated gp120

Recombinant gp120 was first treated with neuraminidase according to the manufacturer's instructions, and purified by size-exclusion chromatography. The rat ST6Gal I was expressed in HEK 293T cells and purified by size-exclusion chromatography. The PBS buffer was then exchanged to a 150 mM sodium chloride buffer with protein concentrator devices, and finally to a sodium cacodylate trihydrate buffer. Proteins (400 μg) were then incubated with the ST6 enzyme (200 μg) and (CMP) sialic acid (1.5 μg) in a total reaction volume of 2 mL, for 48 hours at 37°C. After incubations, proteins were analysed by SDS-PAGE and glycans prepared for HPLC analysis. Cloning of the rat ST6 catalytic domain into the pHLsec vector was done by Ben Yu.

2.2.12. Serum ELISA

Microtitre plates were coated with 5 μg/mL of protein solution in PBS, 50 μL/well, overnight at 4°C. The following steps were then performed at room temperature. The plates were washed twice with a PBS/0.05% Tween20 solution, and blocked for 1 hour with 100 μL/well of 3% bovine serum albumin (BSA) in PBS. 50 μL/well of the serum was then incubated for 2 hours. After incubation, the plates were washed five times with PBS/0.05% Tween20. Detection of bound antibodies was performed by incubating an AP-conjugated anti-rabbit antibody (1/10000 dilution in PBS) for 1 hour, washing the plate, and adding 50 μL/well of p-Nitrophenyl Phosphate (PNPP) substrate, and reading absorbance at 405 nm on a Spectramax M5 (Molecular Devices, California, USA) plate reader.

2.2.13. Flow cytometry

Cells were detached using a cell scraper, counted, and adjusted to $5x10^6$ cells/mL in separate aliquots. Cells were washed twice with PBS. PE-conjugated antibody was added according to the manufacturer's instructions, for 30 min on ice. Cells were washed once with PBS, once with FACS Buffer, and the cell pellet was resuspended in 400 μL FACS buffer and analysed using FACSCalibur (Becton Dickinson). Forward scatter (FSC), side scatter (SSC), green (FL1) and red (FL2) fluorescence were analysed on a logarithmic scale. A minimum of 10000 events were recorded for each sample.

2.2.14. PBMCs virus preparation

Human PBMCs were isolated from buffy coats obtained healthy blood donors, by $CD8^+$ T-cell depletion followed by Ficoll-Hypaque centrifugation, and stimulated with phytohemagglutinin for 72 hours (Mann *et al.* 2009). HIV-1_{JRCSF} virus stocks were grown and titered on these cells (Rusert *et al.* 2004), and virus production was monitored by p24 ELISA

(Aalto Bioreagents, Dublin, Eire). Production of virus in PBMCs was performed by Katie Doores (The Scripps Research Institute).

2.2.15. Pseudovirus preparation

HEK 293T cells were transfected with plasmids carrying the reporter gene expressing the virus backbone (pSG-3ΔEnv or pNL4-3ΔEnv) and the functional envelope clone at a ratio of 2:1 using Fugene (Roche) according to the manufacturer's instructions (Li *et al.* 2005).. Pseudovirus supernatants were harvested after 3 days. Pseudovirus preparation was performed by Katie Doores (TSRI).

2.2.16. Infectious molecular clone preparation

HEK 293T cells were transfected with the pLAI-JRCSF plasmid using Fugene (Roche) as described previously (Leaman *et al.* 2010). The pLAI-JRCSF plasmid carries the JRCSF ectodomain (aa 24-692), and the leading sequence and cytoplasmic tail of HIV_{LAI-2}. Supernatants were harvested after 3 days. Infectious molecular clone preparation was performed by Katie Doores (TSRI).

2.2.17. Envelope glycoproteins isolation

Virus particles were pelleted by ultracentrifugation (20000 rpm, 1 hour). Virus pellets were lysed with NP-40 (1% in PBS with protease inhibitors, 20min at 4°C). The debris was removed by centrifugation and the envelope protein was immunoprecipitated with HIV envelope specific monoclonal antibodies (D7324, b12, b6, F425-b4e8, 5B11, 8C7, or 7D3 depending on virus isolate) or serum from SIV infected animals. Protein A and G beads were added and incubated overnight at 4°C. The protein was eluted by heating in loading buffer (containing dithiothreitol) for 10 min at 100°C and resolved by SDS-PAGE. The envelope

band was confirmed by western blot and cut to use directly in glycan analysis. Envelope isolation was performed by Katie Doores.

2.2.18. Cytokine release assay on PBMCs

Monocytes were isolated from healthy individual blood, and differentiated into macrophages (Miller *et al.* 2008). Incubation with different gp120 glycoforms at a 0.2 mg/mL concentration in X-vivo media was performed for two days at 37°C. Cytokines released in the supernatant were then analysed using a BioPlex magnetic bead custom kit (BioRad) on a Luminex 200 platform. Standard curves were generated as multipoint logarithmic. The cell assays and cytokine analysis were performed by Drew Sayce (University of Oxford).

2.2.19. Cytokine release assay on U-937 cells

U-937 cells were grown in RMPI-1640 media supplemented with 10% FCS. Cells were differentiated into macrophages by adding 20 nM of Phorbol 12-myristate 13-acetate (PMA) for 48%. Cells were then washed with PBS. Incubation with different gp120 glycoforms at a 50 μg/mL concentration in RMPI-1640 media was performed for 48 hours at 37°C. Cytokines released in the supernatant were then analysed using a Cytokine Array detection kit, using a Fujifilm Las-1000 Intelligent DarkBoxII to visualize the membranes. Spot intensity was determined using the ImageJ software.

Chapter 3

Biogenesis of the non-self glycans of HIV-1 gp120

The HIV-1 envelope glycoprotein gp120 is one of the most highly glycosylated protein found in nature (Myers *et al.* 1992), with carbohydrates accounting for half the molecular weight of the entire glycoprotein (Pantophlet *et al.* 2006; Leonard *et al.* 1990). The number of glycosylation sequons varies for different viral isolates, with a median of 25 N-linked glycosylation sites (Korber *et al.* 2001; Zhang *et al.* 2004). Glycosylation of recombinant monomeric gp120 has previously been shown to diverge from host-cell glycosylation by exhibiting unprocessed oligomannose-type glycans (section 1.2.4) (Leonard *et al.* 1990; Zhu *et al.* 2000; Cutalo *et al.* 2004). This cluster of ectopically expressed ER glycans provides a window for immunological recognition and forms the epitope for a few known broadly neutralising anti-carbohydrates antibodies against HIV, such as IgG1 2G12 and PGT128 (section 1.4.4) (Scanlan *et al.* 2002a; Calarese *et al.* 2005; Pejchal *et al.* 2011; Walker *et al.* 2011).

The persistence of this mannose patch indicates that not all gp120 N-linked glycans are processed equivalently in the ER. It has been proposed that the dense clustering of glycans on gp120 outer domain might form a protected patch of unprocessed oligomannose glycans which are maintained as a result of their inaccessibility to ER and Golgi α1→2mannosidases (Scanlan *et al.* 2007). In this chapter, the biosynthetic origin of gp120 mannose patch is

analysed through kinetic analysis of gp120 glycans processing by the first enzyme involved in oligomannose trimming in the ER, ER class I α1→2mannosidase.

3.1. Analysis of monomeric gp120$_{BaL}$ glycosylation

3.1.1. HIV-1$_{BaL}$ gp120 expression

HIV-1$_{BaL}$ is a clade B, 2G12-sensitive isolate (Binley *et al.* 2004a). Recombinant, monomeric gp120$_{BaL}$ was transiently expressed in HEK 293T cells. A Ni-affinity resin was used to purify the C-terminal His-tagged glycoprotein, and was followed by size exclusion chromatography (Figure 3.1.A), with the different fractions assessed by SDS-PAGE (Figure 3.1.B).

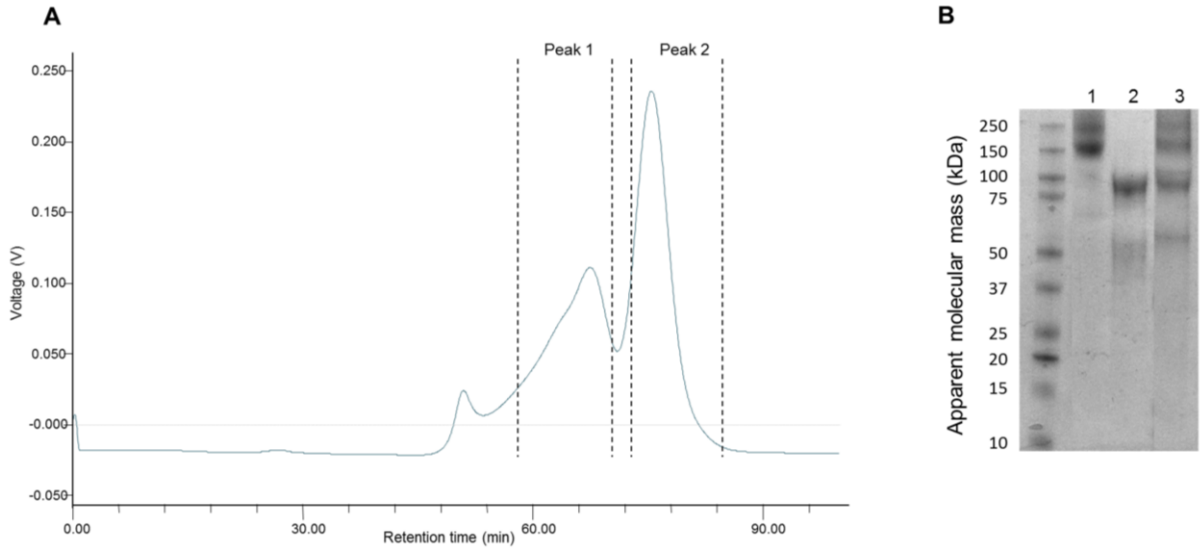


Figure 3.1. Expression and purification of gp120$_{BaL}$
HEK 293T cells were transfected with the gp120$_{BaL}$ sequence inserted in the pHLsec vector. **(A)** Size-exclusion chromatogram of metal-affinity purified gp120$_{BaL}$. The two main peaks, 1 and 2, correspond to an aggregate of gp120 and the monomer respectively. **(B)** SDS-PAGE analysis of gp120$_{BaL}$: lane 1 corresponds to the gp120$_{BaL}$ 'peak 1' fraction collected after size-exclusion chromatography purification, lane 2 to the gp120$_{BaL}$ 'peak 2' fraction collected after size-exclusion chromatography purification, and lane 3 to metal-affinity purified gp120$_{BaL}$

The gp120$_{BaL}$ protein sequence (residues 1-499) exhibits 23 potential N-linked glycosylation sites (Figure 3.2). On average, two thirds of potential *N*-linked glycosylation sites are occupied for host glycoproteins. However the occupancy rate has been observed to

be much higher for viral glycoproteins, including gp120 (Leonard *et al.* 1990). Whereas the deglycosylated protein molecular weight is 56.5kDa, the apparent molecular weight observed by SDS-Page analysis confirms that the majority of N-linked glycosylation sites are occupied (Figure 3.1.B).

```
MDAMKRGLCCVLLLCGAVFVSPSTEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHN 60
VWATHACVPTDPNPQEVELENVTENFNMWKNNMVEQMHEDIISLWDQSLKPCVKLTPLCV 120
TLNCTDLRNATNGNDTNTTSSSREMMGGGEMKNCSFKITTNIRGKVQKEYALFYELDIVP 180
IDNNSNNRYRLISCNTSVITQACPKISFEPIPIHYCAPAGFAILKCKDKKFNGKGPCSNV 240
STVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSENFADNAKTIIVQLNESVEINCTRPNNN 300
TRKSIHIGPGRALYTTGEIIGDIRQAHCNLSRAKWNDTLNKIVIKLREQFGNKTIVFKHS 360
SGGDPEIVTHSFNCGGEFFYCNSTQLFNSTWNVTEESNNTVENNTITLPCRIKQIINMWQ 420
KVGRAMYAPPIRGQIRCSSNITGLLLTRDGGPEDNKTEVFRPGGGDMRDNWRSELYKYKV 480
VKIEPLGVAPTKAISSVVKHHHHHH
```

Figure 3.2. Protein sequence of recombinant gp120$_{BaL}$
The Asn residues present in a glycosylation sequon (Asn-Xxx-Ser/Thr, Xxx any amino acid except Pro) are highlighted in bold and underlined. The oligomannose glycans that take part in the 2G12 epitope – N295, N332, N339, N386, N392 – are highlighted in green. The numbering is based on the HxB2 isolate sequence. The signal peptide sequence is highlighted in blue.

3.1.2. HIV-1$_{BaL}$ gp120 glycosylation profile

The gp120$_{BaL}$ N-linked glycans were released with PNGase F in-gel digestion, and analysed by Matrix Assisted Laser Desorption Ionisation Time Of Flight Mass Spectroscopy (MALDI-TOF MS). A typical glycosylation profile obtained for desialylated glycans released from recombinant gp120$_{BaL}$ is illustrated in Figure 3.3.A. To assess the reproducibility of the method employed here and throughout the thesis, the glycosylation of five different batches of gp120$_{BaL}$ were analysed by MALDI-TOF MS. The average glycan abundance are reported in Figure 3.3.B.

Two different subpopulations of glycans were observed. Firstly, a subset of unprocessed, oligomannose-type $Man_{5\text{-}9}GlcNAc_2$ glycans, which are biosynthetic intermediates that are typically not displayed on mature glycoproteins, was observed (Table 3.1, 45% of total N-linked glycans). Secondly, 55% of the remaining glycans were complex-type glycans, characteristic of the HEK 293T cell glycosylation machinery.

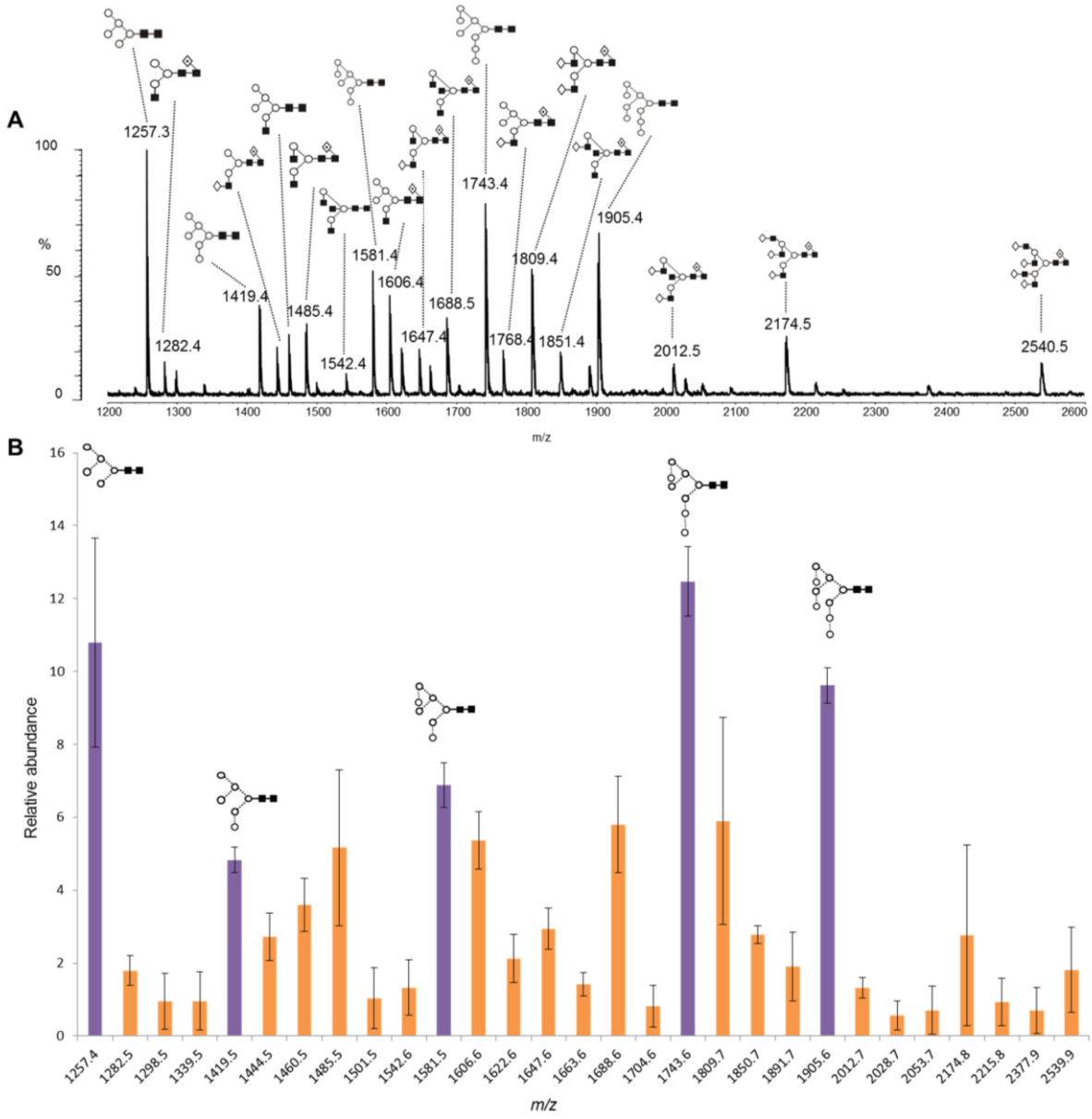


Figure 3.3. Monomeric gp120$_{BaL}$ glycosylation
(A) MALDI-TOF MS spectrum of PNGase F released N-linked glycans ($[M+Na]^+$ ions) from recombinant gp120$_{BaL}$ expressed in HEK 293T cells and treated with neuraminidase, with the chemical structure of the main glycans depicted. **(B)** Average abundance for each N-linked glycans released from five different batches of monomeric gp120$_{BaL}$ and observed by MALDI-TOF MS ($[M+Na]^+$ ions). A 95% confidence interval is indicated for each peak. The oligomannose series and complex-type glycans are highlighted respectively in purple and orange.

Table 3.1. Abundance of gp120$_{BaL}$ N-linked glycans[†]

	% oligomannose ($Man_xGlcNAc_2$)							Complex
	Total	**Man_5**	**Man_6**	**Man_7**	**Man_8**	**Man_9**	**Man_{6-9}**	
gp120$_{BaL}$	45	11	5	7	12	10	34	55

[†]Abundances obtained for desialylated PNGase F-released N-linked gp120$_{BaL}$ glycans.

3.2. Remodelling of $gp120_{BaL}$ glycosylation

To further analyse the biosynthetic origin of this patch, two different glycoforms were generated by disrupting different key steps of the glycosylation pathway, resulting in gp120 glycoforms closely representing biosynthetic intermediates of the ER and the IC/*cis*-Golgi based on the known localisation of the processing enzymes (Figure 1.5).

3.2.1. ER gp120 glycoform

The first glycoform was obtained by expressing $gp120_{BaL}$ in HEK 293T cells in the presence of kifunensine, an inhibitor of ER α-mannosidase I as well as type I Golgi α-mannosidases (Elbein *et al.* 1990). As a result, $Man_9GlcNAc_2$ is the predominant glycan displayed on gp120 (Figure 3.4.A). This glycoform thus mimics an early ER gp120 glycoform.

3.2.2. IC/medial-Golgi gp120 glycoform

The second glycoform, representing an IC/*cis*-Golgi gp120, was obtained by expressing $gp120_{BaL}$ in GnT I-deficient HEK 293S cells. Glycan processing in this cell line is stalled at the $Man_5GlcNAc_2$ biosynthetic intermediate (Reeves *et al.* 2002). The amount of $Man_5GlcNAc_2$ glycans observed (Table 3.2, 67%) correlates with the usual amount obtained in GnT I-competent 293T cells expressed gp120 (11%) added to the population of complex glycans (55%). Additionally to the $Man_{5-9}GlcNAc_2$ oligomannose range observed, a fucosylated structure, $Man_5GlcNAc_2Fuc_1$ is also present (Figure 3.4.B., *m/z* 1403.6). Fucosylation of the reducing-terminal GlcNAc in the α1→6 position is performed by the Golgi-resident α1→6-fucosyltransferase (FUT8), and can occur in a GnT I-independent manner (Crispin *et al.* 2006).

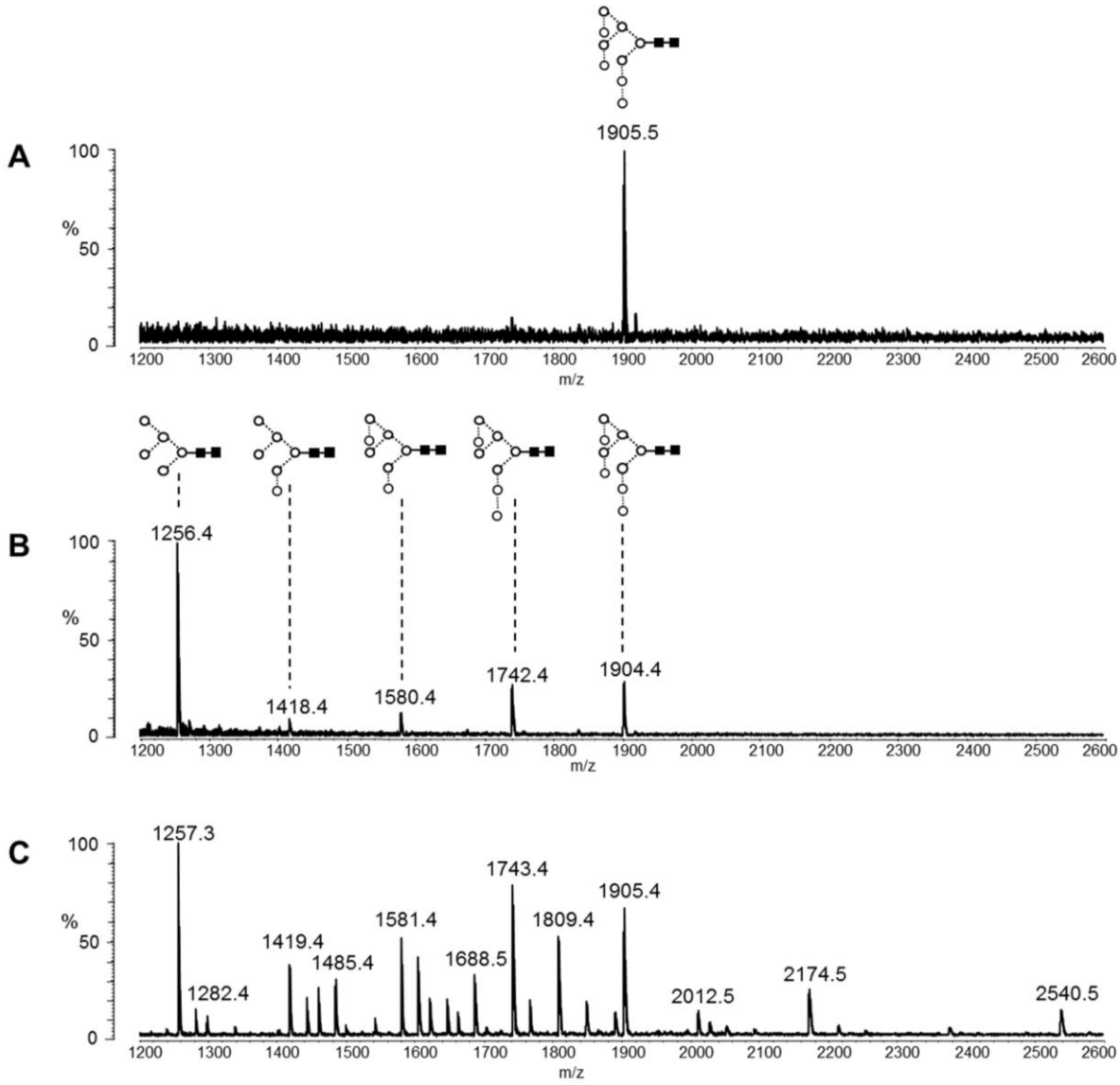


Figure 3.4. Remodelling of monomeric $gp120_{BaL}$ glycosylation
MALDI-TOF MS spectra of PNGase F-released N-linked glycans ($[M+Na]^+$ ions) from **(A)** recombinant $gp120_{BaL}$ expressed in HEK 293T cells in the presence of kifunensine, **(B)** recombinant $gp120_{BaL}$ expressed in GnT I-deficient HEK 293S cells and **(C)** recombinant $gp120_{BaL}$ expressed in HEK 293T cells.

Table 3.2. Abundances of released N-linked glycans for different $gp120_{BaL}$ glycoforms[†]

gp120[‡]	% oligomannose ($Man_xGlcNAc_2$)						Complex
	Total	Man_5	Man_6	Man_7	Man_8	Man_9	
(A) ER glycoform	100	-	-	-	1	99	-
(B) IC/Golgi glycoform	99	67	2	3	14	13	1
(C) wild-type	45	11	5	7	12	10	55

[†]Abundances obtained for desialylated N-linked glycans released from gp120.
[‡]Notation corresponds to the one used in Figure 3.4: (A) gp120 expressed in 293T cells in the presence of kifunensine, (B) gp120 expressed in GnT I-deficient 293S cells, (C) gp120 expressed in 293T cells.

3.3. Biosynthetic origin of gp120 mannose patch

A key feature of mammalian glycan biosynthesis is an extensive heterogeneity in the complex-type glycan structures seen in the late Golgi apparatus and on mature glycoproteins. This diversification occurs only after the oligomannose-type glycans have been trimmed in a largely linear and sequential pathway in the ER and early Golgi compartments. However, on gp120, a population of *N*-linked glycans are highly resistant to α-mannosidase processing and therefore persist through the secretory pathway as unprocessed oligomannose-type glycans ($Man_{5-9}GlcNAc_2$). The first enzyme to process $Man_9GlcNAc_2$ is the ER-resident class I α1→2 α-mannosidase which removes a single D-mannose residue from the D2 arm of $Man_9GlcNAc_2$ to generate D1,D3-$Man_8GlcNAc_2$. Thus, differential kinetics of processing of $Man_9GlcNAc_2$ glycans by this enzyme would help to explain the presence of a residual population of unprocessed oligomannose glycans.

3.3.1. Determination of ER α-mannosidase I activity

The ER α-mannosidase I catalytic domain (residues 236-699) had previously been cloned in the pHLsec vector and transiently expressed in HEK 293T cells (Dunlop *et al.* 2010b). ER α-mannosidase I hydrolysis of the $Man_9GlcNAc_2$ glycan was conducted on the ER gp120 glycoform and the PNGase F-released glycans analysed by MALDI-TOF MS (Figure 3.5).

Three different amount of enzyme were used, and all spectra show a trimming of the $Man_9GlcNAc_2$ into the $Man_8GlcNAc_2$ glycan, with less hydrolysis observed with 1 μg of enzyme, and comparable results for both 2.5 μg and 5 μg of enzyme per 50 μg of glycoprotein.

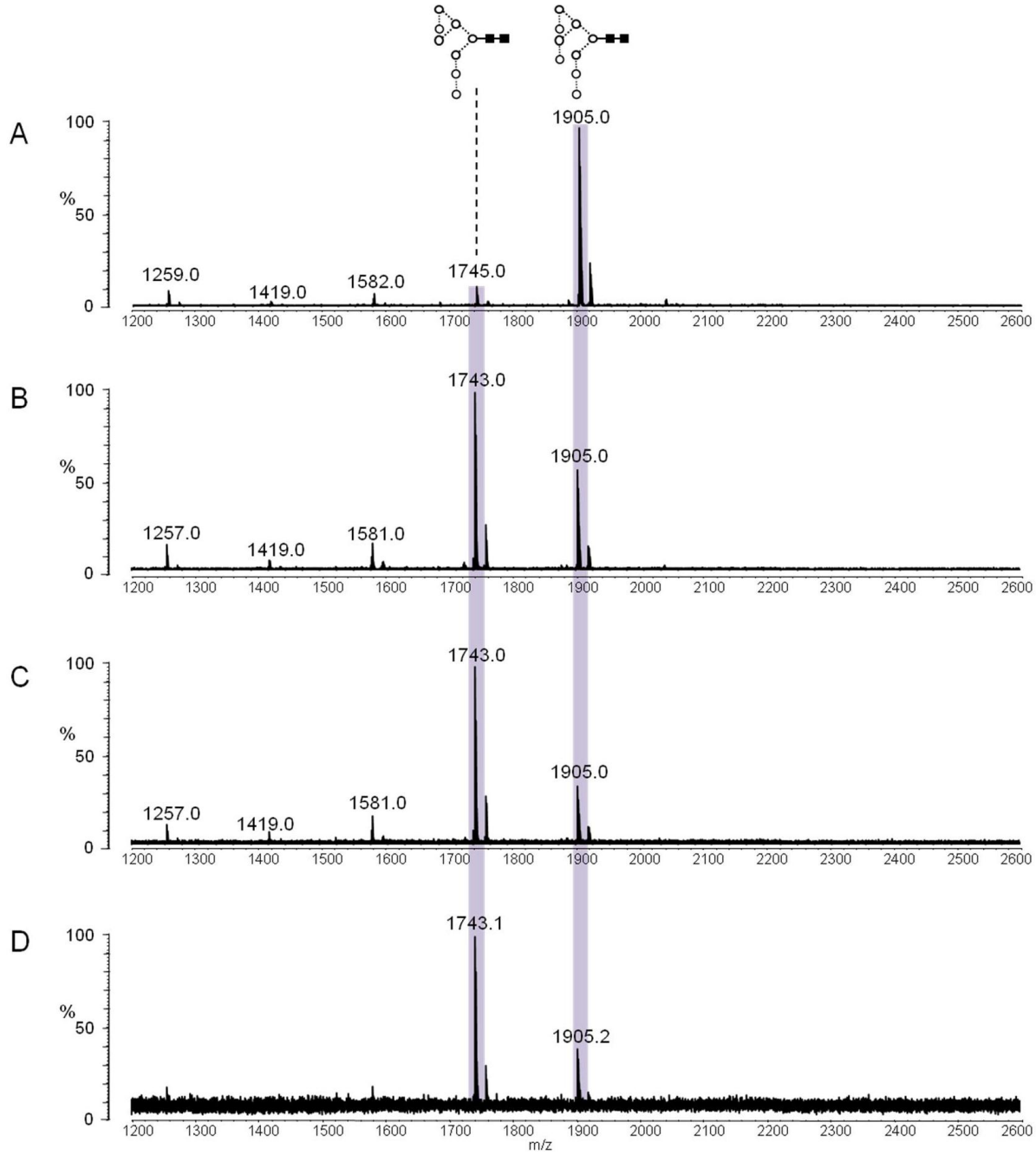


Figure 3.5. ER α-mannosidase I hydrolysis of $Man_9GlcNAc_2$
MALDI-MS analysis of PNGase F-released glycans from 50 μg of ER $gp120_{BaL}$ glycoform **(A)** before treatment with ER α-mannosidase I, showing a dominant peak corresponding to $Man_9GlcNAc_2$; after treatment for 30 minutes at 37°C with **(B)** 1 μg; **(C)** 2.5 μg; **(D)** 5 μg of ER α-mannosidase I, all showing a dominant peak corresponding to $Man_8GlcNAc_2$.

3.3.2. ER α-mannosidase I kinetic hydrolysis of ER $gp120_{BaL}$ glycoform

The ER $gp120_{BaL}$ glycoform was subjected to hydrolysis by ER α-mannosidase I at 37°C over a period of 4 hours, in the presence of 1 μg or 5 μg of enzyme (Figure 3.6). Both

amounts of enzyme leave a residual population of protected and inaccessible $Man_9GlcNAc_2$ glycans (25% of total N-linked glycans). After exhaustive digestion overnight, 11% of total N-linked glycans were left as unprocessed $Man_9GlcNAc_2$ glycans.

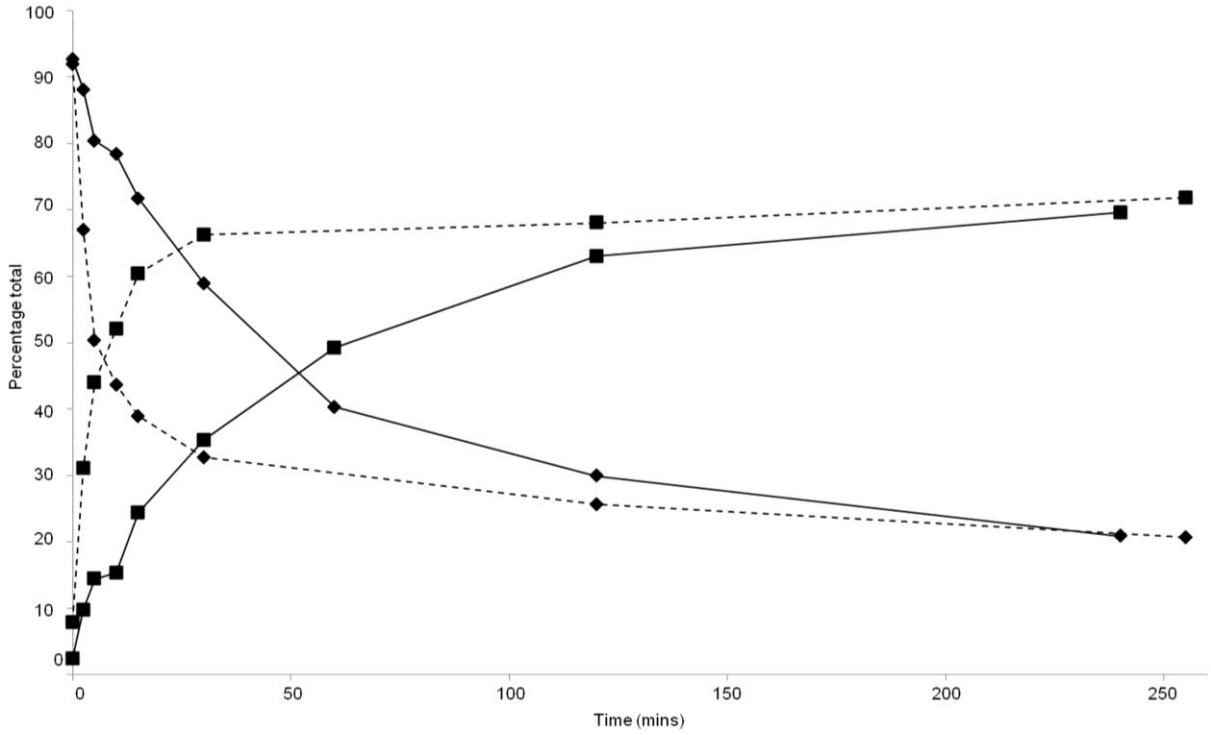


Figure 3.6. ER α-mannosidase I kinetic hydrolysis of $Man_9GlcNAc_2$
The abundances of PNGase F-released N-linked glycans were determined by MALDI-TOF MS analysis. The hydrolysis of the ER gp120 glycoform was monitored in the presence of 1 μg (solid line) or 5 μg (dashed line) of ER α-mannosidase I. The abundance of the $Man_9GlcNAc_2$ (♦) and $Man_8GlcNAc_2$ (■) glycans were recorded over 250 min of digest at 37°C.

To further assess kinetically the hydrolysis process of gp120 oligomannose glycans, the lowest amount of enzyme required was chosen (i.e. 1 μg for each 20 μg of gp120 used for each time point), and repeated the analysis twice (Figure 3.7). The residual population of $Man_9GlcNAc_2$ glycans (around 25% of total N-linked glycans) was still evident. The decrease in $Man_9GlcNAc_2$ correlates with an increase in the $Man_8GlcNAc_2$ species, and also a small amount of $Man_7GlcNAc_2$. This is consistent with a previous study showing that $Man_7GlcNAc_2$ was formed following longer incubation time with ER α-mannosidase I (Herscovics *et al.* 2002).

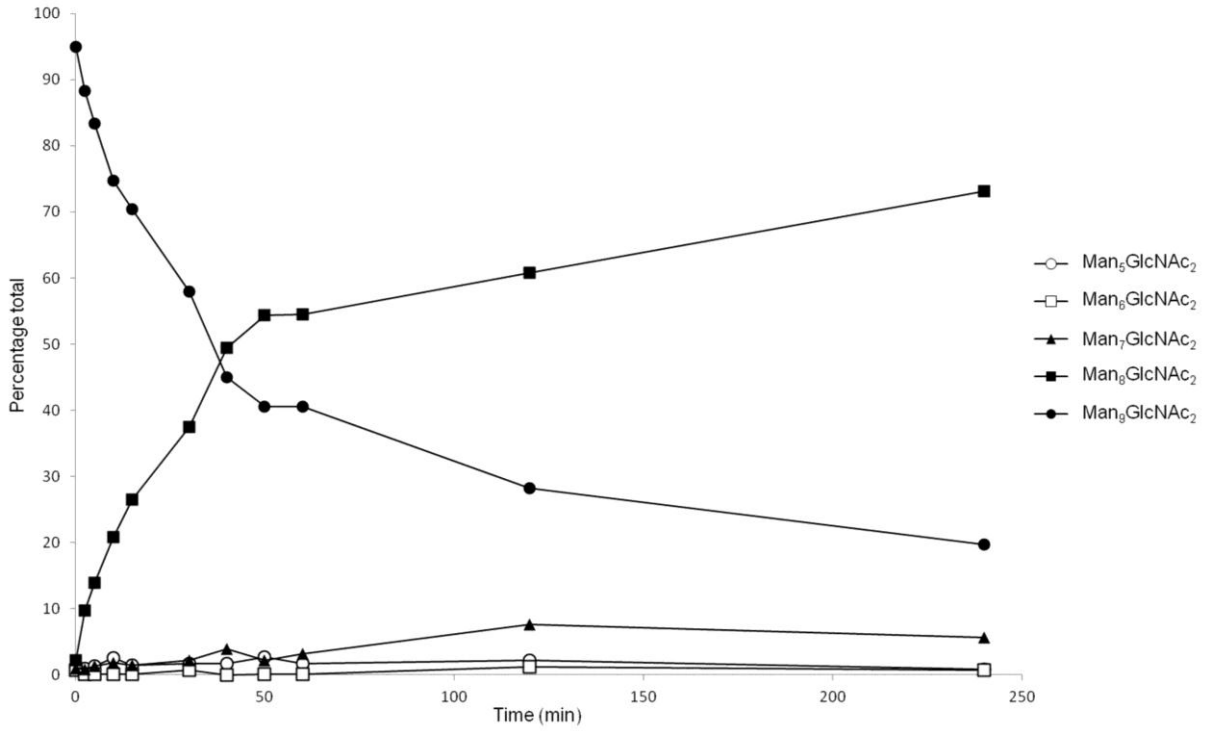


Figure 3.7. ER α-mannosidase I kinetic hydrolysis of the ER gp120$_{BaL}$ glycoform
The hydrolysis of the ER gp120 glycoform was monitored in the presence of 1 μg of ER α-mannosidase I at 37°C.The abundances of the released N-linked glycans were determined by MALDI-TOF MS analysis. The average abundances of the glycans $Man_9GlcNAc_2$ (♦), $Man_8GlcNAc_2$ (■), $Man_7GlcNAc_2$ (▲), $Man_6GlcNAc_2$ (□) and $Man_5GlcNAc_2$ (○) for two experiments are depicted.

3.3.3. ER α-mannosidase I kinetic hydrolysis of gp120$_{BaL}$ produced in GnT I-deficient cells

Two hypotheses could explain the remaining population of unprocessed $Man_9GlcNAc_2$ glycans on mature gp120: (i) some glycans are inaccessible to ER α-mannosidase I; (ii) gp120 transit time in the ER (or location within the lumen) is not long enough for all its glycans to be processed by glycosylation enzymes. The fact that oligomannose glycans persist despite exhaustive digestion suggest that the proposition (i) might better explain the presence of the mannose patch.

To further test this hypothesis, an IC/*cis* Golgi gp120$_{BaL}$ glycoform was subjected to hydrolysis by ER α-mannosidase I. The glycans on gp120$_{BaL}$ expressed in GnT I-deficient cells have already been processed by ER and Golgi mannosidases, and a population of around 13% of unprocessed $Man_9GlcNAc_2$ is observed (Figure 3.4). The hydrolysis was conducted in

parallel to the ER $gp120_{BaL}$ hydrolysis, at 37°C over a period of 4 hours, in the presence of 1 µg of ER α-mannosidase I for each 20 µg $gp120_{BaL}$ time point (Figure 3.8). The initial $Man_9GlcNAc_2$ population was left unprocessed by ER α-mannosidase I, indicating that these glycans are largely inaccessible to the enzyme.

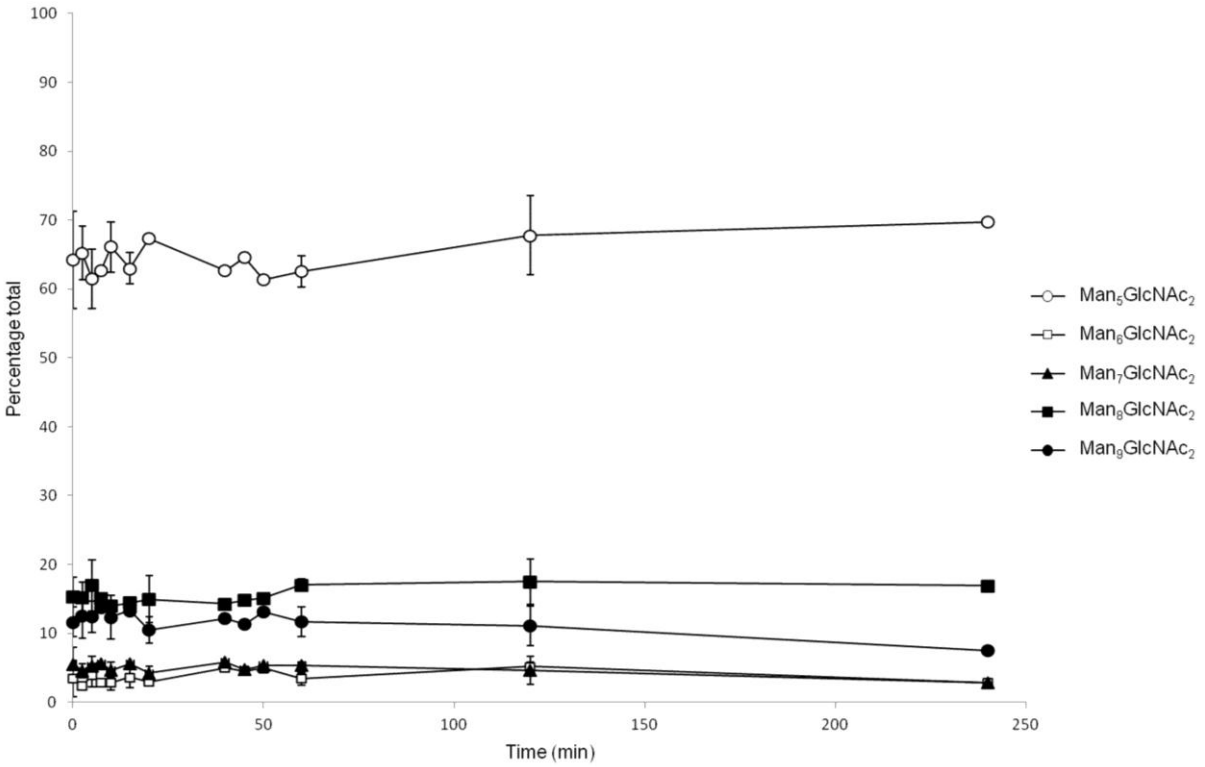


Figure 3.8. ER α-mannosidase I hydrolysis of the IC/*cis*-Golgi $gp120_{BaL}$ glycoform
The hydrolysis of the IC/Golgi $gp120_{BaL}$ glycoform was monitored in the presence of 1 µg of ER α-mannosidase I. The abundances of the released N-linked glycans were determined by MALDI-TOF MS analysis. The experiment was repeated three times, and the average abundance of glycans $Man_9GlcNAc_2$ (◆), $Man_8GlcNAc_2$ (■), $Man_7GlcNAc_2$ (▲), $Man_6GlcNAc_2$ (□) and $Man_5GlcNAc_2$ (○) are depicted. Standard deviations are indicated for each time point.

3.3.4. Biphasic mannosidase kinetics leads to the emergence of the oligomannose patch

The ER and IC/*cis*-Golgi $gp120_{BaL}$ glycoforms were subjected to hydrolysis by recombinant ER α-mannosidase I. Approximately 60% of the $Man_9GlcNAc_2$ glycans present on the ER $gp120_{BaL}$ glycoform were rapidly trimmed – initial rate per mole of enzyme was 0.1 $mol.s^{-1}$ after 2.5 minutes – whereas the remaining glycans were processed at a much slower rate (approximately 100-fold), of 8.10^{-4} $mol.s^{-1}$ after 2 hours (Figure 3.7). A significant

population of $Man_9GlcNAc_2$ was left unprocessed (25% after a 4-hour hydrolysis, 11% after an overnight digest).

This residual $Man_9GlcNAc_2$ population is also evident on the IC/*cis*-Golgi gp120 glycoform. The $Man_9GlcNAc_2$ glycans remaining on this glycoprotein are entirely resistant to ER α-mannosidase I (Figure 3.8).

The observed differential kinetics of α-mannosidase hydrolysis gives rise to a bifurcation in glycan processing within monomeric gp120, whereby more rapidly processed glycans are trimmed to smaller oligomannose or complex-type glycans whereas the more protected, slowly processed glycans persist as larger oligomannose-type glycans. The protection of glycans is thought to arise from their dense clustering on the outer domain of gp120. Alternatively, the size of the processing enzymes could be another factor. The rate(s) of α-mannosidase hydrolysis are responsible for the bifurcation in glycan processing between monomeric gp120 and host glycosylation.

3.4. Oligomannose glycans as a marker of steric occlusion

The presence of unprocessed glycans has previously been described for several glycoproteins (Crispin *et al.* 2004b; Suzuki *et al.* 2003; Suzuki *et al.* 2009), including the influenza haemagglutinin (Mir-Shekari *et al.* 1997) and suggests that, as observed with HIV-1 Env, steric occlusion of glycans on viral envelope glycoproteins lead to bifurcation in glycan processing.

Recently, the structure of the Semliki Forest Virus, an alphavirus belonging to the *Togaviridae* family, has been reported. The two envelope glycoproteins E1 and E2 are cleaved from a common precursor and assemble as trimer of heterodimers to form a glycoprotein cage on the virion surface (Mancini *et al.* 2000). Whilst E2 is involved in receptor binding, E1, the transmembrane protein, is responsible for the fusion of the viral and

host cell membranes. The protection of E1 glycans, shielded underneath E2 surface, could lead to their lack of processing in the ER/Golgi compartments due to glycan/protein steric occlusion.

To test this hypothesis, the glycosylation profiles of E1 and E2 were analysed by ion-mobility mass spectrometry (Figure 3.9). E1 buried glycans contained a significant amount (70%) of unprocessed, oligomannose-type glycans (predominantly $Man_{5-7}GlcNAc_2$), whereas accessible glycans present on the E2 glycoprotein were more processed towards complex-type glycans (75%). This suggests that E1 glycans are less accessible to glycosylation enzymes in the ER and Golgi compartments, perhaps as a result of membrane proximity and steric occlusion from the E2 glycoprotein.

3.5. Conclusion

Unprocessed, oligomannose-type $Man_{5-9}GlcNAc_2$ glycans are biosynthetic intermediates that are not typically displayed on mature glycoproteins (Crispin *et al.* 2004a). However, $gp120_{BaL}$ exhibits a patch of underprocessed $Man_{5-9}GlcNAc_2$ glycans (Table 3.1, 45% of $Man_{5-9}GlcNAc_2$), in agreement with previous site analysis glycosylation studies performed on recombinant monomeric gp120 (Leonard *et al.* 1990; Go *et al.* 2008; Go *et al.* 2009). Complex-type glycans are usually displayed by host glycoproteins, and are specific of the cell from which they derive. Recombinant monomeric $gp120_{BaL}$ exhibit 55% of such complex-type glycans, typical from HEK 293T cells glycosylation (Table 3.1). The complex-type glycans present on gp120 are situated close to the receptor binding sites on gp120 neutralising face, thus preventing antibodies from accessing the conserved receptor and co-receptor binding sites, as well as modulating infectivity (Binley *et al.* 2010). Most unprocessed immature oligomannose-type glycans are found on gp120 outer domain so-called

'immunologically silent face' and form the basis of the epitope of 2G12 and other anti-carbohydrates broadly neutralising antibodies such as PGT128 and PGT145.

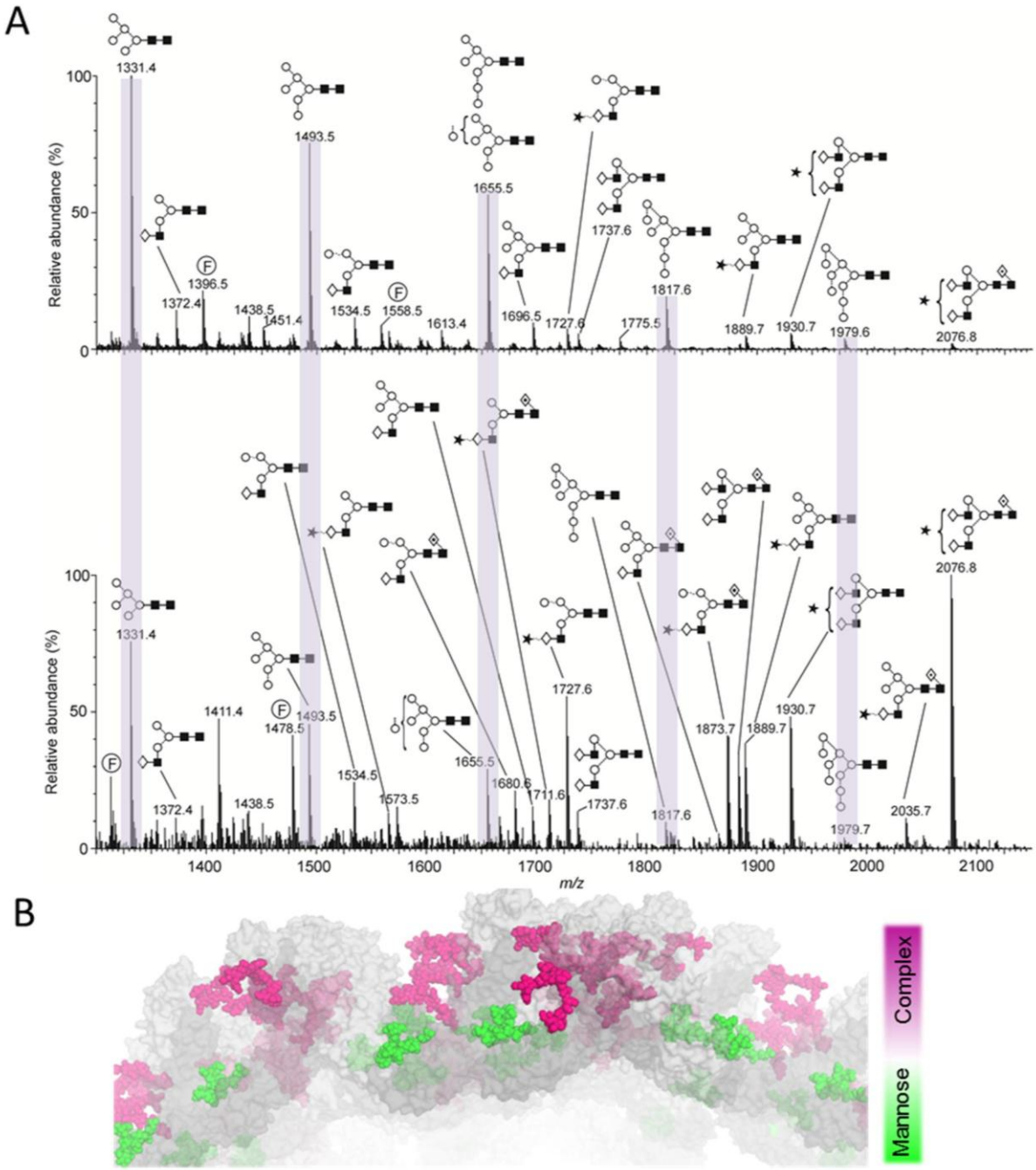


Figure 3.9. Differential glycosylation of Semliki Forest Virus envelope glycoproteins
(A) Ion-mobility spectra of PNGase F-released N-linked glycans ($[M+H_2PO_4]^-$ and $[M-H]^-$ ions) from E1 and E2 glycoproteins. The oligomannose series is highlighted in purple. Fragment ions are indicated by an encircled "F". **(B)** Molecular modelling of the envelope glycoprotein spikes, with E1 depicted at the bottom and E2 at the top. Oligomannose-type glycans are shown in green, and complex-type glycans in pink. Glycosylated model was constructed from the cryo-electron microscopy study of Semliki Forest Virus (Mancini *et al.* 2000) (Max Crispin and Thomas Bowden, manuscript in preparation).

Chapter 4

Glycosylation of viral envelope glycoprotein

The viral envelope is a trimer of gp120/gp41 heterodimers. The steric consequences of this additional quaternary structure further impacts glycan processing, as highlighted by a study showing that SIV envelope spikes glycans were less accessible than monomeric gp120 to treatment by endo- and exo-glycosidases (Means *et al.* 2000). The first step towards the determination of the chemical structure of viral envelope glycans was achieved in 1988, with the analysis of the N-linked glycosylation of envelope glycoprotein isolated from cultures of HIV infected cells (Geyer *et al.* 1988). An intracellular fraction, isolated from lysates of infected lymphoblastoid H9 cells, was shown to be comprised mainly of oligomannose glycans, whereas another fraction isolated from culture supernatant (shed gp120) displayed a wide array of oligomannose-type glycans as well as a set of fucosylated and sialylated bi/tri/tetraantennary complex-type glycans. Two years later, a study conducted on cell-associated gp120 showed that gp120 displayed a wide range of oligomannose, hybrid, and complex glycans, with 16% of bi/tri/tetra-antennary with bisecting GlcNAc residues and sialylated complex glycans (Mizuochi *et al.* 1990). A wide range of lectin-binding studies have confirmed these results on both monomeric and viral trimeric gp120 (Alexandre *et al.* 2010; Zou *et al.* 2011), however the precise chemical structure of viral glycans had not yet been determined. In this chapter, the N-linked glycosylation of the envelope spikes of infectious viral particles is reported, together with commonly used virus-like particles.

4.1. Env glycosylation of PBMC-derived virus

4.1.1. Glycosylation profile of recombinant monomeric gp120$_{JRCSF}$

HIV-1$_{JRCSF}$ is a clade B, 2G12-sensitive isolate that bears 23 potential N-glycosylation sites (Binley *et al.* 2004a). HIV$_{JRCSF}$ gp120 (residues 33-503) was cloned in the pHLsec vector and transiently expressed in HEK 293T cells. Expression of the protein was confirmed by western-blot analysis and in-gel PNGase F digestion was used to release the sugars from gp120 isolated from culture supernatant.

The N-linked glycosylation profile of gp120$_{JRCSF}$ (Figure 4.1) exhibits a significant proportion of oligomannose-type glycans (29% of total N-linked carbohydrates) and a large array of complex-type glycans (71%).

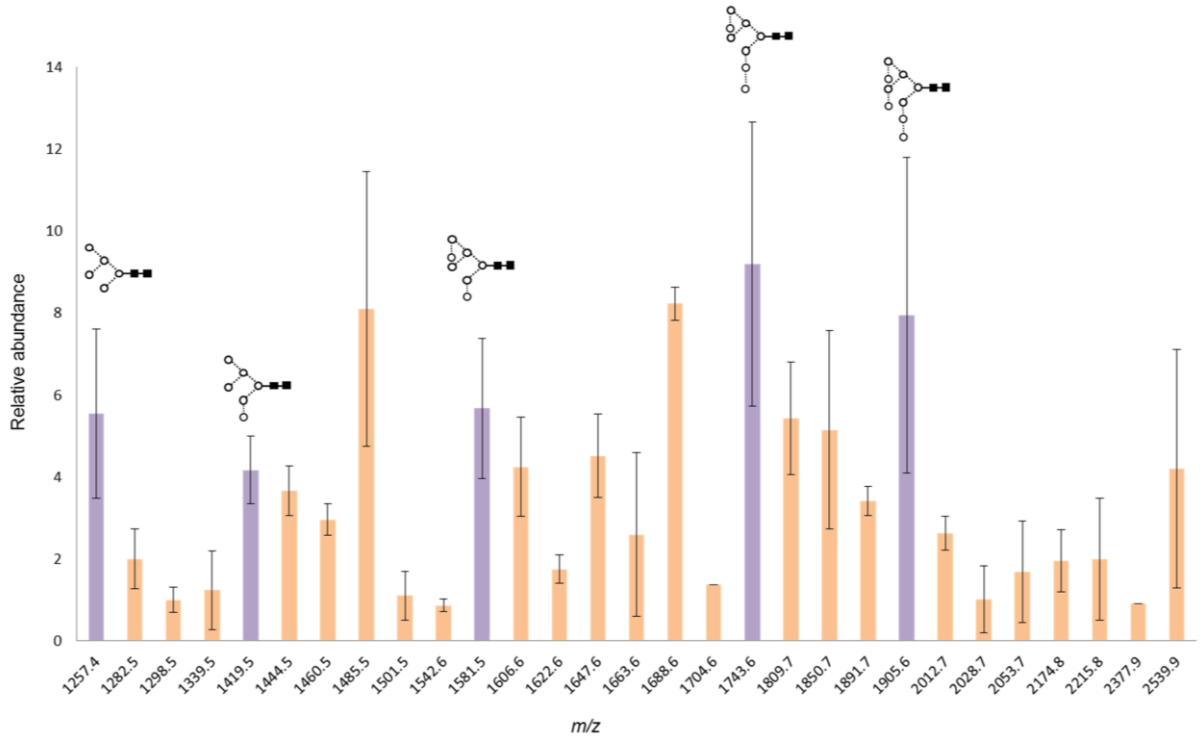


Figure 4.1. Monomeric gp120$_{JRCSF}$ glycosylation
Average abundance of desialylated N-linked glycans ($[M+Na]^+$ ions) released from three different batches of monomeric gp120$_{JRCSF}$ and observed by MALDI-TOF MS. A 95% confidence interval is indicated for each peak. The oligomannose series and complex-type glycans are highlighted respectively in purple and orange, and the chemical structure of the oligomannose-type glycans is depicted.

4.1.2. Increase in oligomannose content for virion-associated gp120$_{JRCSF}$

PBMCs were infected with HIV-1$_{JRCSF}$ (Figure 4.2). The MALDI-TOF MS spectrum for recombinant, monomeric gp120$_{JRCSF}$ (Figure 4.3.A) was compared to the one derived from the glycans isolated from infectious PBMC-derived virus (Figure 4.3.B). The viral Env glycosylation profile showed a higher abundance of oligomannose-type glycans (79%, *m/z* 1257, 1419, 1581, 1743, 1905), together with a population of sialylated, complex-type glycans (21%, *m/z* 1809, 2012, 2174, 2539).

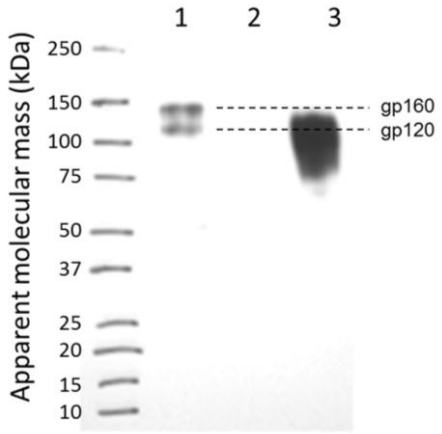


Figure 4.2. Viral envelope glycoprotein isolation
HIV-1$_{JRCSF}$ virus stocks were grown and titered on CD8$^+$-depleted PBMCs, and virus particles were pelleted by ultracentrifugation. The envelope glycoproteins were immunoprecipitated with HIV envelope specific monoclonal antibodies, and the envelope band was confirmed by western blot analysis. Lane 1 corresponds to the immunoprecipitated envelope fraction, lane 2 to the purification flow-through and lane 3 to recombinant, monomeric gp120 control. The separate bands corresponding to gp120 and gp160 are indicated. Virus preparation and Env isolation presented in this chapter was performed by Katie Doores.

Overall, the HIV$_{JRCSF}$ envelope derived from PBMCs shows an extended array of oligomannose-type glycans, with a three-fold increase in Man$_{5\text{-}9}$GlcNAc$_2$ species (Table 4.1). Thus, α-mannosidase processing during the early steps of viral glycoprotein assembly and secretion is greatly limited on the native virus in comparison to monomeric gp120. This is highlighted by the three-fold increase in Manα1→2Man terminating glycans.

Furthermore, the decrease in complex-type glycans abundance demonstrates that further along the glycosylation pathway, the envelope spikes show a partial resistance to

processing by GnT I or by subsequent Golgi-resident enzymes. Moreover, in contrast to monomeric gp120, complex-type glycans isolated from viral envelope spikes form a restricted set of sialylated bi/tri/tetra-antennary glycans.

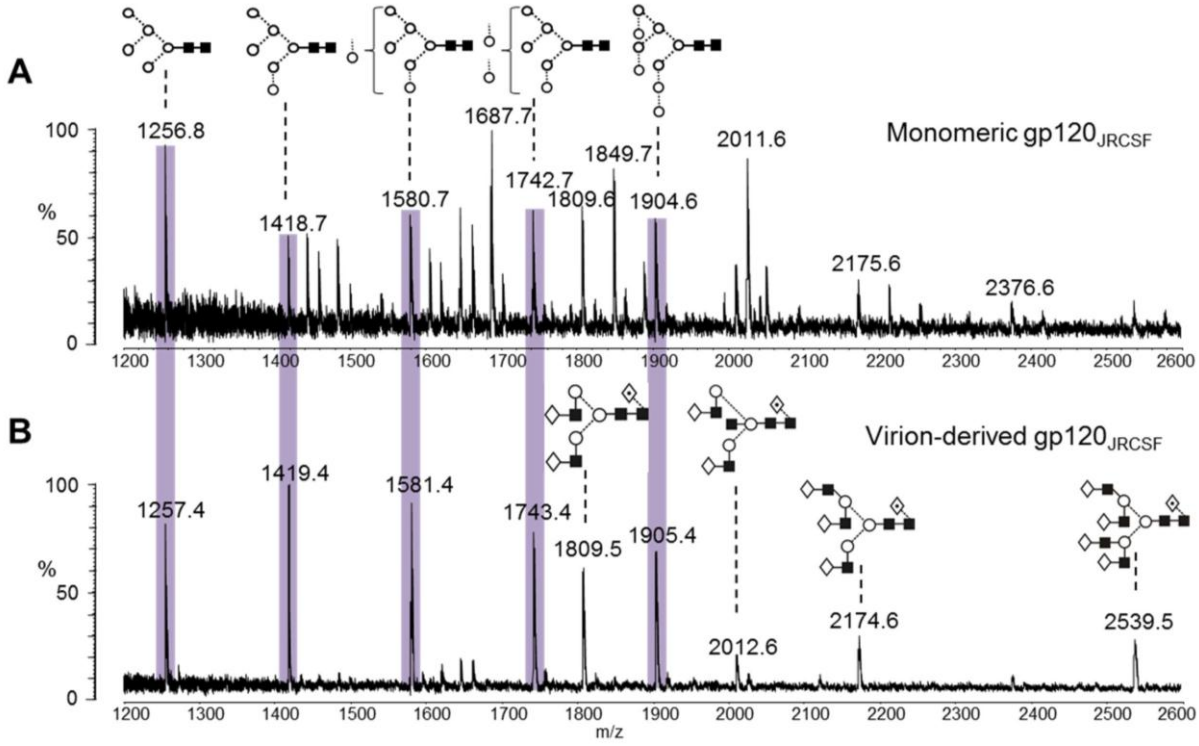


Figure 4.3. Mannosidase processing is limited on the native trimer
MALDI-TOF MS analyses of the desialylated N-linked glycans ($[M+Na]^+$ ions) released from **(A)** recombinant monomeric $gp120_{JRCSF}$ expressed in HEK 293T cells, **(B)** $gp120_{JRCSF}$ isolated from virus obtained by infection of human PBMCs with HIV_{JRCSF}. The oligomannose series is highlighted in purple.

Table 4.1 Abundance of PNGase F-released N-linked glycans obtained from gp120[†]

gp120	% oligomannose ($Man_xGlcNAc_2$)							Complex
	Total	Man_5	Man_6	Man_7	Man_8	Man_9	Man_{6-9}	
Monomeric	29	7.7	2.5	5	7	7	21.5	71
Viral	79	12	20	19	15	13	67	21
Fold increase[‡]	2.7	1.6	8	3.8	2.1	1.9	3.1	0.3

[†]Abundances obtained for desialylated N-linked glycans released from gp120.
[‡]Values represent the increase in glycan population for viral gp120 compared to monomeric, recombinant gp120.

4.1.3. Glycosylation profiles of antibodies used to isolate the viral envelope

A cocktail of anti-gp120 antibodies (D7324, b12, b6, F425-b4e8, VRC01, VRC03 and PGV04) was used to immunoprecipitate the envelope glycoprotein. MALDI-TOF MS

glycosylation profiles of b6, b12, F425-b4e8, and D7324 are given in Figure 4.4, showing a profile typical of antibody glycosylation, with fucosylated and partially galactosylated structures at *m/z* 1485, 1647 and 1809. None of the predominant $Man_3GlcNAc_4Fuc$ species is present on the viral gp120 mass spectra (Figure 4.3.B), confirming the lack of antibodies glycan contamination in that the gp120 glycosylation profile obtained in the previous section.

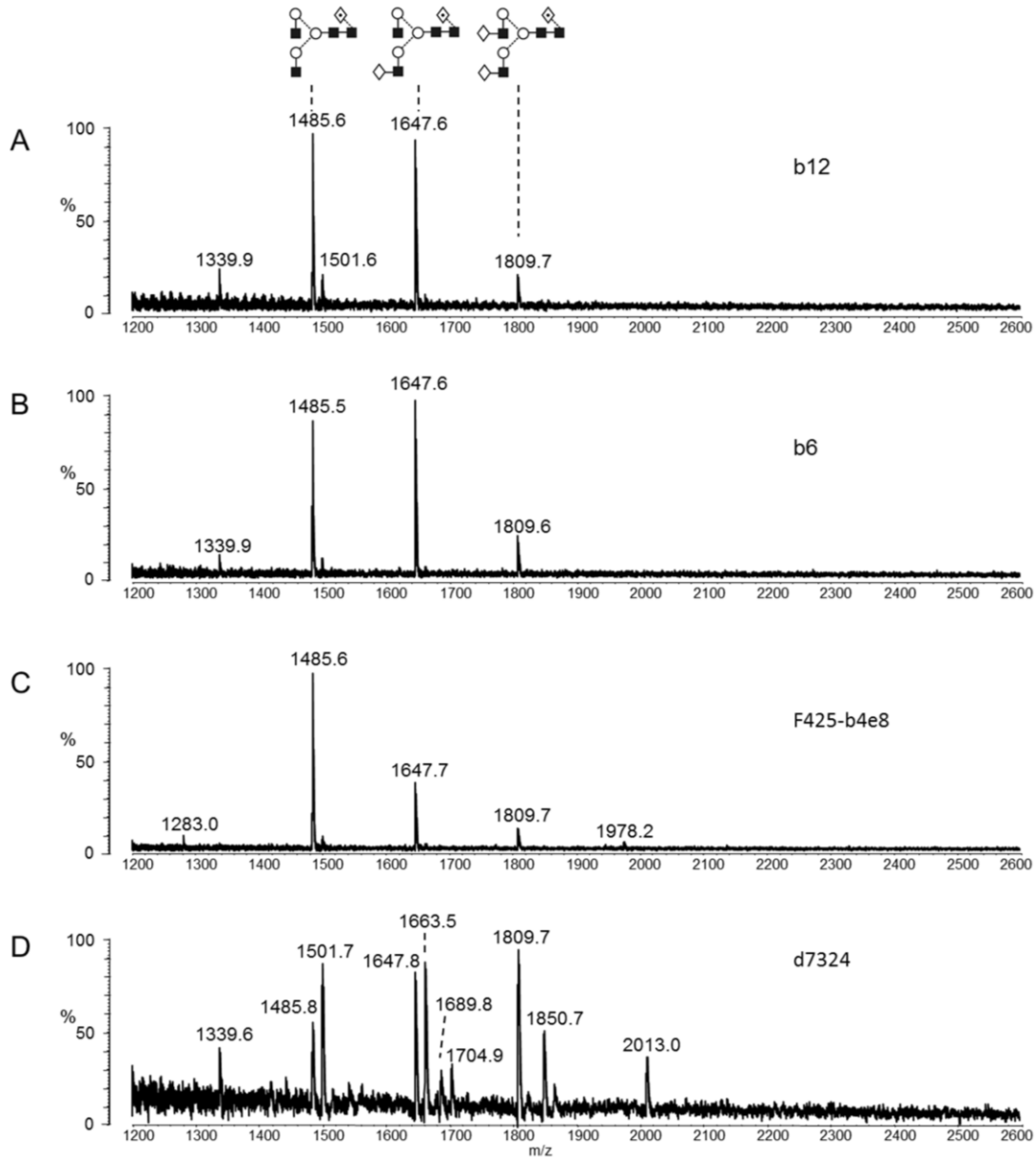


Figure 4.4. Glycosylation profiles of antibodies used for immunoprecipitation of the viral envelope

MALDI-TOF MS analyses of the desialylated N-linked glycans ($[M+Na]^+$ ions) released from **(A)** b12, **(B)** b6, **(C)** F425-b4e8, **(D)** d7324. The antibodies b12 and b6 target the CD4 binding site, and would thus only recognize proper conformation of gp120. F425-b4e8 is a V3-loop antibody, and d7324 targets a peptide epitope within the C5 region.

4.1.4. A subset of sialylated complex-type glycans present on viral Env

Unlike neutral glycans such as oligomannose glycans, sialylated carbohydrates usually decompose by loss of sialic acid or carbon dioxide when ionized by positive ion MALDI. The removal of the negatively charged sialic acid moieties occurs within the ion source and also as a post-source fragmentation (Harvey 1999). A method to study sialylated glycans is therefore to remove the sialic acid residues and analyse the desialylated spectra.

MALDI-TOF MS analyses of $gp120_{JRCSF}$ envelope isolated from PBMC-derived virus, both before and after desialylation, showed that nearly all complex-type glycans present on gp120 are sialylated (Figure 4.5).

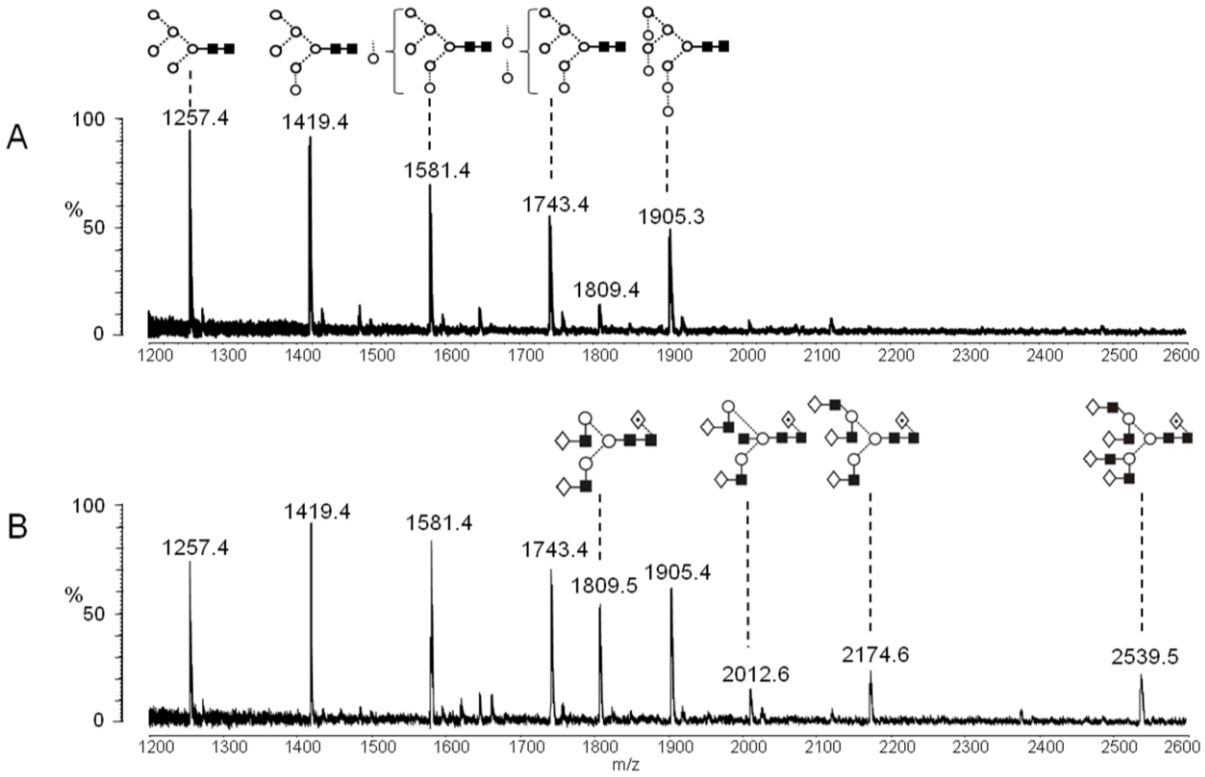


Figure 4.5. Sialylated glycans on viral Env
MALDI-TOF MS analyses of the released N-linked glycans ($[M+Na]^+$ ions) from gp120 isolated from virus obtained by infection of human PBMCs **(A)** before desialylation, and **(B)** after desialylation with acetic acid. The series of branched, fucosylated complex-type glycans at *m/z* 1809, 2012, 2174 and 2539 correspond to the neutral derivatives of sialylated bi, tri and tetra-antennary glycans.

The presence of a this set involves glycan processing by several glycosylation enzymes, including α-mannosidase II, fucosyltransferase (FUT), N-acetylglucosaminyltransferases (GnT) II, IV, and V, galactosyltransferases (GalT) and sialyltransferases (ST). In particular, reactions with α-mannosidase II, FUT 8, GnT II, GalT and ST are going to completion according to the viral glycosylation profile, and could be due to a slow retention time of the glycoproteins in the ER/Golgi (Willey *et al.* 1988), resulting in the presence of highly processed complex-type glycans on the surface of gp120.

4.2. Cross-clade conservation of Env glycans

4.2.1. Conservation of virion-associated gp120 glycosylation profile

Analyses of gp120 derived from virus prepared by infection of PBMCs with viruses from clade A (92RW009), clade B (JRCSF), and clade C (93IN905) showed a predominantly oligomannose glycan composition (Figure 4.6, Table 4.2, 62–79% $Man_{5-9}GlcNAc_2$), with a distribution similar to that of PBMC-derived $gp120_{JRCSF}$.

The series of branched, fucosylated complex-type glycans at *m/z* 1809 (11–24%), 2012 (1.5–5%), 2174 (3–5.5%) and 2539 (3–4.7%), corresponding to the neutral derivatives of sialylated bi, tri and tetra-antennary glycans, were also conserved in all three spectra.

This demonstrates that in marked contrast to the underlying protein epitopes, there is a high degree of conservation of the surface exposed glycan structures, with a set of unprocessed oligomannose-type glycans, and an additional conserved set of highly processed, fucosylated, sialylated bi-, tri- and tetra-antennary complex-type glycans.

Table 4.2. Abundances of released N-linked glycans from different isolates[†]

gp120	% oligomannose ($Man_xGlcNAc_2$)							Complex
	Total	Man_5	Man_6	Man_7	Man_8	Man_9	Man_{6-9}	
92RW009	64	10	20	11	6.8	16	54	36
JRCSF	79	12	20	19	15	13	67	21
93IN905	62	19	15	11	10	7.5	43	38

[†]Abundances obtained for desialylated N-linked glycans released from gp120.

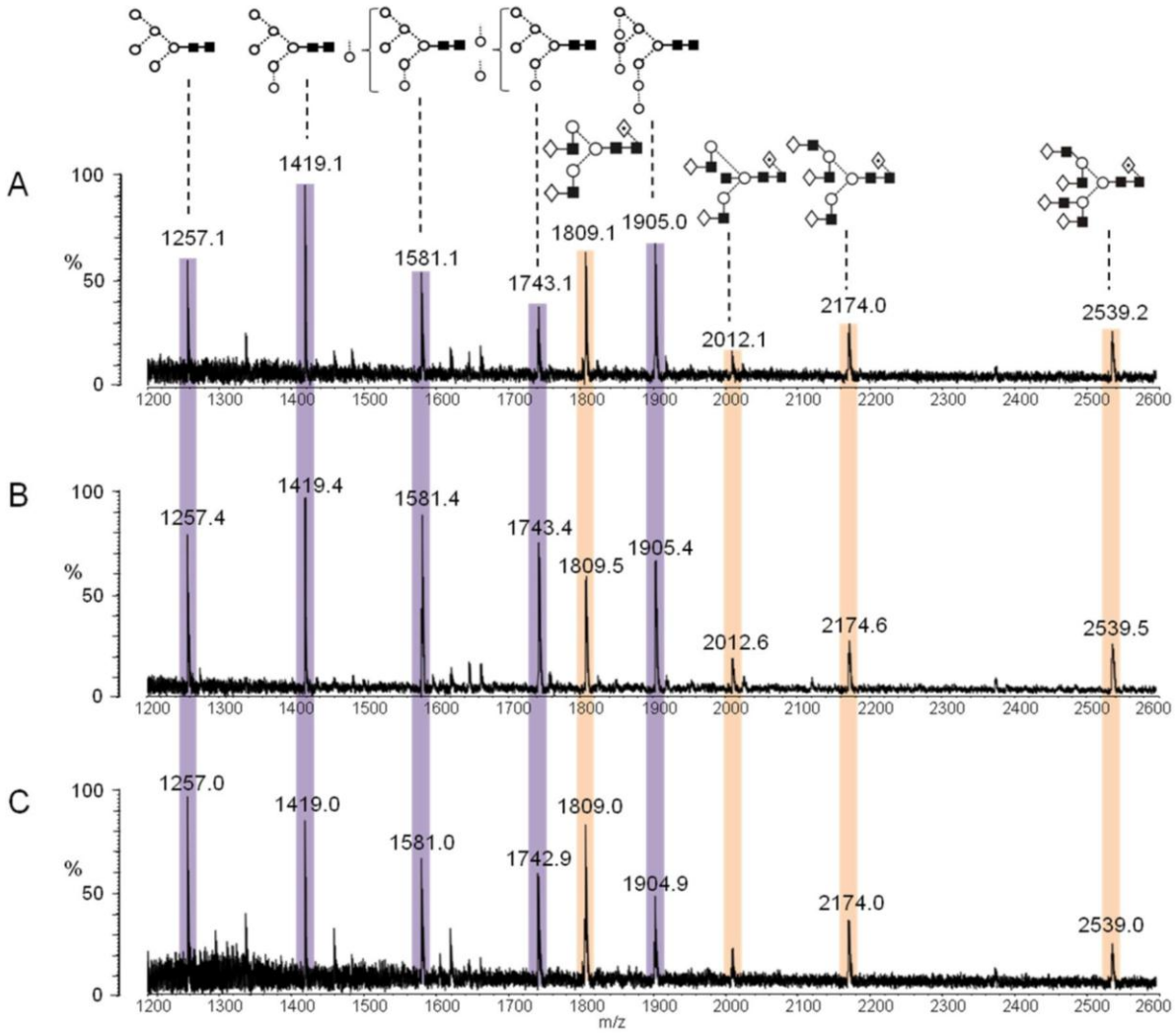


Figure 4.6. Cross-clade analysis of viral envelope glycosylation
MALDI-TOF MS analyses of desialylated N-linked glycans ($[M+Na]^+$ ions) released from **(A)** $gp120_{92RW009}$, **(B)** $gp120_{JRCSF}$, **(C)** $gp120_{93IN905}$ isolated from virus obtained by infection of human PBMCs. The oligomannose series and a subset of sialylated, complex-type glycans are highlighted respectively in purple and orange.

4.2.2. Variation in glycan abundance within the oligomannose series

The relative abundance of glycans within the oligomannose series differs slightly between isolates from different clades (Figure 4.7). The ratio of oligomannose-type glycans that terminate with Manα1→2Man linked mannose residues, compared with those that do not, is higher for gp120 derived from HIV-$1_{92RW009}$ (clade A, 5.4) and HIV-1_{JRCSF} (clade B, 5.6) compared to that of HIV-$1_{93IN905}$ (clade C, 2.3). A likely explanation for this difference in glycan processing, in clade C envelope, is the absence of key glycosylation site(s) which

reduce the density of the intrinsic mannose patch and consequently increase the processing of adjacent Manα1→2Man termini. Notably, the oligomannose glycan attached to Asn295 is absent in most clade C isolates, including HIV-$1_{93IN905}$, and is critical for efficient neutralisation by a number of mannose-specific ligands, including 2G12. This phenomenon is analysed in further detail in Chapter 5.

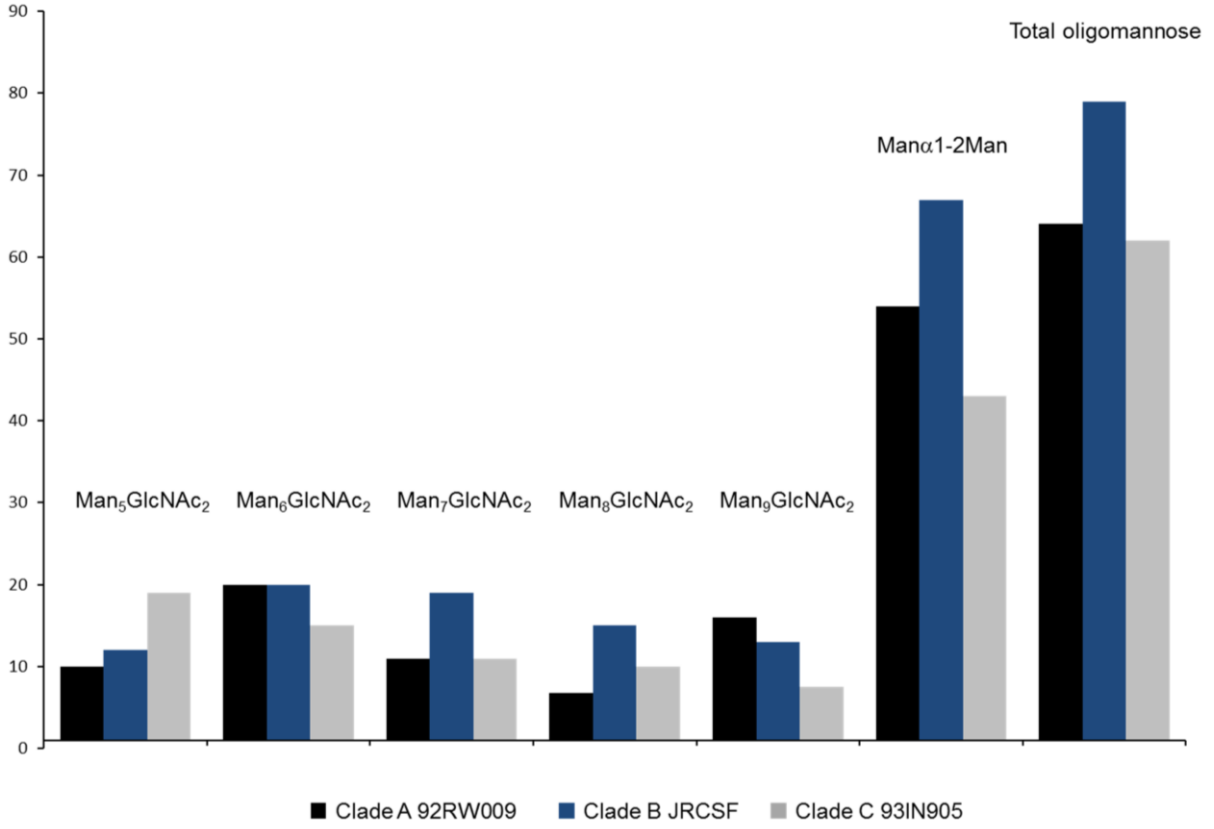


Figure 4.7. Comparison of abundance of oligomannose-type glycans
Abundances of $Man_{5-9}GlcNAc_2$, Manα1→2Man terminating glycans ('Manα1-2Man'), and total oligomannose-type glycans are represented in black for the clade A HIV-$1_{92RW009}$ isolate, in blue for clade B HIV-1_{JRCSF}, and grey for clade C HIV-$1_{93IN905}$. Values were obtained from the data presented in Table 4.2. Whilst the overall oligomannose content is similar for gp120 derived from HIV-$1_{93IN905}$ and HIV-$1_{92RW009}$, the abundance of Manα1→2Man terminating glycans is higher for $gp120_{92RW009}$ than for $gp120_{93IN905}$, consistent with the respective 2G12 sensitivity and resistance observed for these two isolates (Binley *et al.* 2004a).

4.3. Glycosylation analysis of viral and pseudoviral Env

Although HEK 293T cells are not naturally infected by HIV-1, they are routinely employed to produce pseudoviral particles that can then be used for neutralisation assays or

immunisation studies. It is therefore crucial to determine the glycosylation of Env from pseudoviral particles and study the differences and similarities with viral Env glycosylation.

4.3.1. Infectious molecular clone Env glycosylation

Envelope glycoprotein was isolated from a replication-competent virus prepared in HEK 293T cells using an infectious pLAI-JRCSF Env molecular clone (Figure 4.8).

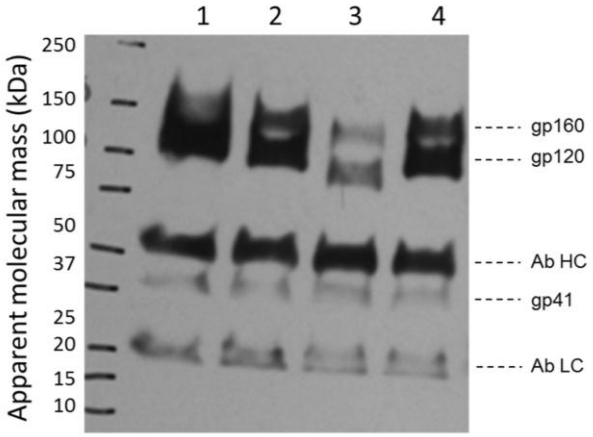


Figure 4.8. Envelope glycoprotein isolation for an infectious molecular clone
Fully replicative HIV_{JRCSF} virus capable of multiple round infections was made by transfection with a single plasmid construct (pLAI-JRCSF Env). The envelope band, immunoprecipitated as previously described, was confirmed by western blot analysis. Lane 1 corresponds to transfection in 293T cells, lane 2 in 293T with the presence of kifunensine, lane 3 in GnT I-deficient 293S cells, and lane 4 in GnT I-deficient 293S cells in the presence of kifunensine. The bands corresponding to gp160, gp120, gp41, as well as the antibodies's heavy chain (HC) and light chain (LC), are indicated.

The profile obtained for gp120 glycosylation (Figure 4.9.A) shows a more even distribution between oligomannose (Table 4.3, 56%) and complex-type glycans (44%) compared to the glycosylation profile obtained for gp120 isolated from virus produced in PBMCs. The complex-type glycans are predominantly of the bi- or tri-antennary type with variable galactosylation and fucosylation typical for the HEK 293T cells.

In addition to cleaved gp120, uncleaved, non-functional gp160 was also detected. The analysis of gp160 glycosylation (Figure 4.9.B) revealed less efficient processing by the Golgi α-mannosidase IA-C, with an elevated population of Manα1→2Man linked oligomannose

glycans compared to gp120 (Table 4.3, 68% $Man_{6-9}GlcNAc_2$ for gp160 compared to 46% for gp120). This suggests that uncleaved gp160 adopts a quaternary arrangement with more occluded glycans compared to cleaved gp120/gp41. Indeed, lack of cleavage of gp160 might be attributed to misfolding, rendering it less accessible to proteases as well as glycosidases. On the other hand, gp120 results from a proper cleavage and is thus more accessible and processed.

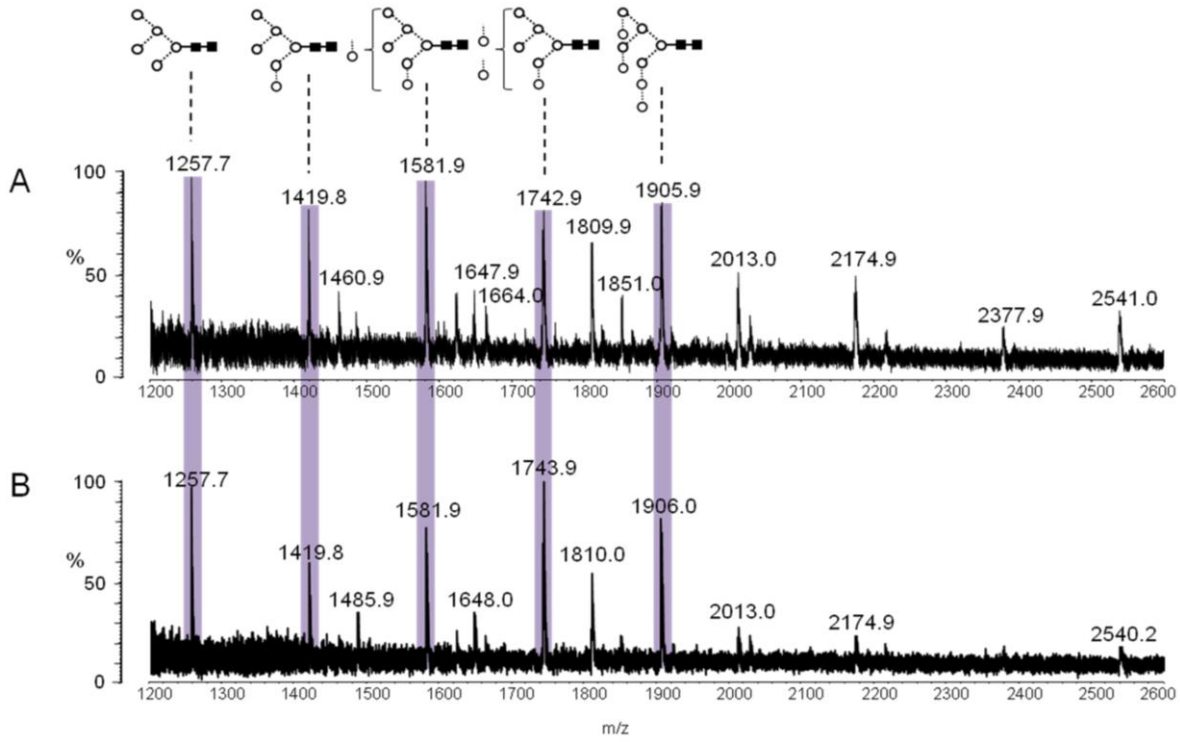


Figure 4.9. Glycosylation of gp120 and gp160 derived from infectious molecular clone
MALDI-TOF MS analyses of desialylated N-linked glycans ($[M+Na]^+$ ions) released from **(A)** gp120 and **(B)** gp160 isolated from replication competent viral particles generated by transfection of HEK 293T cells with pLAI-JRCSF Env molecular clone.

Table 4.3. Abundances of released N-linked glycans from infectious molecular clone envelope glycoproteins[†]

glycoprotein	% oligomannose ($Man_xGlcNAc_2$)							Complex
	Total	Man_5	Man_6	Man_7	Man_8	Man_9	Man_{6-9}	
gp120	56	10	9	13	10	14	46	44
gp160	83	15	6	14	26	22	68	17

[†]Abundances obtained for desialylated released N-linked glycans.

4.3.2. Pseudoviral particles envelope glycosylation

Pseudoviral particles, generated by co-transfection of HEK 293T cells with plasmids carrying the JRCSF envelope gene and the HIV-1 backbone (Figure 4.10), are also routinely used for neutralisation assays and immunisation studies.

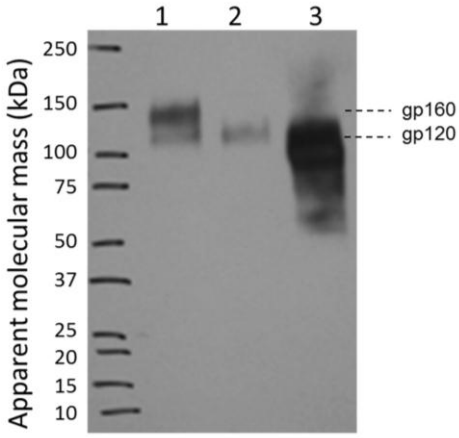


Figure 4.10. Envelope glycoprotein isolation for pseudoviral particles
HEK 293T cells were transfected with plasmids carrying the reporter gene expressing the functional envelope clone (pSVIII-JRCSF) and the virus backbone (pSG3ΔEnv) at a ratio of 1:2. The envelope band was confirmed by western blot analysis. Lane 1 corresponds to envelope immunoprecipitated from the pseudoviral pellet fraction, lane 2 to envelope immunoprecipitated from the supernatant after the virus had been removed by ultracentrifugation ('soluble non-virion associated fraction'), and lane 3 to recombinant, monomeric gp120 control. The position of the analysed gp120 and gp160 bands is indicated.

MALDI-TOF MS analysis of isolated gp120 envelope glycans (Figure 4.11.A) revealed an exclusively oligomannose population with peaks at *m/z* 1257, 1419, 1582, 1743, 1905 ($[M+Na]^+$ ions) corresponding to the mannose series $Man_{5\text{-}9}GlcNAc_2$. Most notably, the $Man_5GlcNAc_2$ glycan, found only as a minor species on monomeric gp120 (Table 4.1, 7.7%), or at a comparable level as the other oligomannose structures on viral envelope (Table 4.1, 12%), is observed as the single most abundant species (Table 4.4, 37%) on gp120 derived from pseudoviral particles.

Similar to the infectious molecular clone system, an uncleaved, non-functional gp160 band was also isolated and analysed by MALDI-TOF MS (Figure 4.11.B). It showed the

presence of higher levels of oligomannose glycans and Manα1→2Man terminating glycans (Table 4.4).

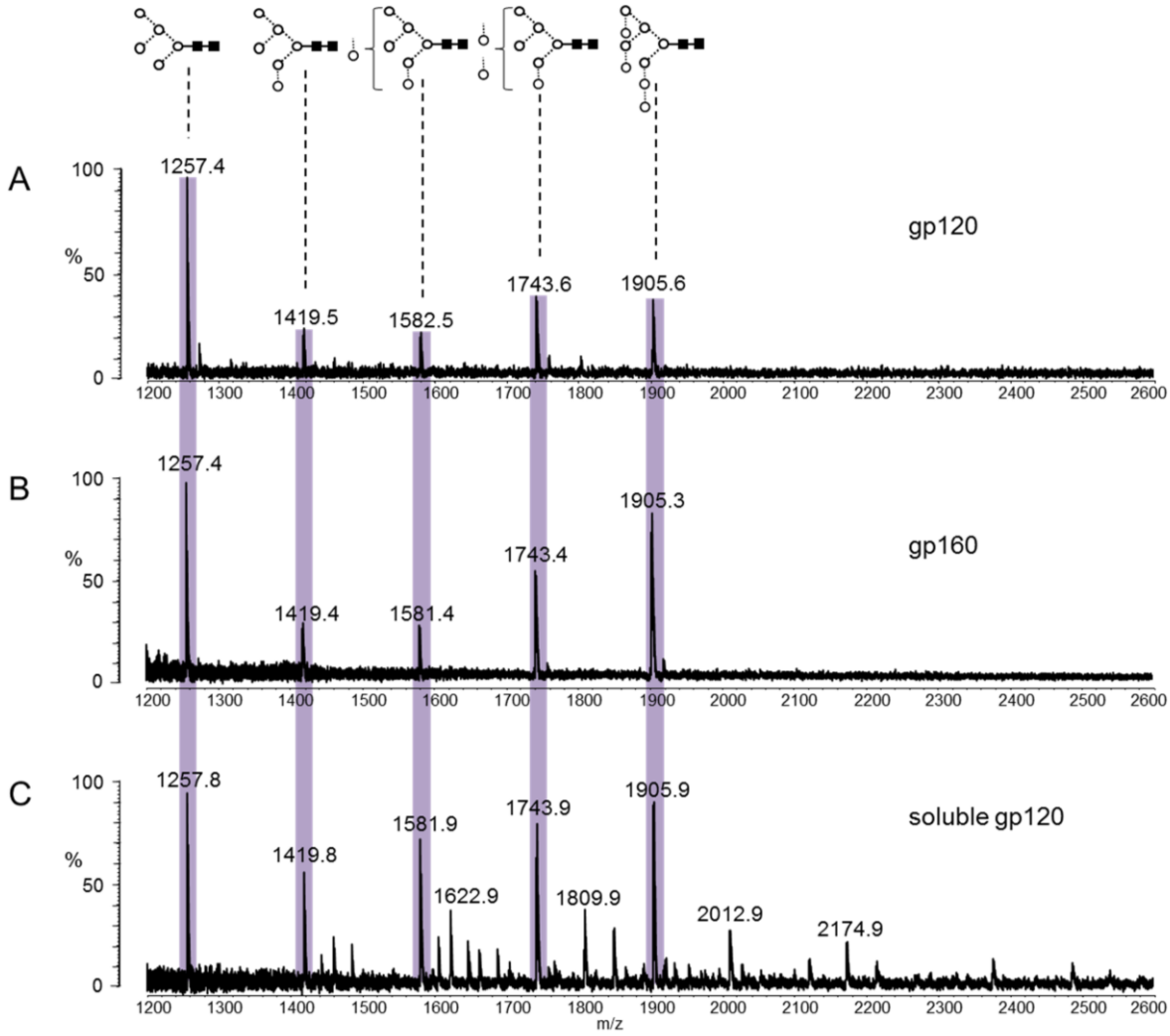


Figure 4.11. Glycosylation of envelope glycoprotein derived from pseudoviral particles MALDI-TOF MS analyses of desialylated N-linked glycans ($[M+Na]^+$ ions) released from **(A)** gp120, **(B)** gp160 and **(C)** non-virion, soluble gp120 isolated from pseudoviral particles generated by transfection of HEK 293T cells with pSVIII-JRCSF and pSG3ΔEnv plasmids in a 1:2 ratio.

A distinct gp120 glycoform (Figure 4.11.C), shed in the supernatant, and proposed to be derived solely from cleaved functional trimers (Crooks *et al.* 2011), shows a decrease in α1→2-mannosidase trimming, together with a population of residual, complex-type glycans (Table 4.4, 26%). This resembles the glycosylation of gp120 isolated from functional virus. Moreover, the 26% complex-type glycans seen in this shed gp120 were accompanied by a

corresponding reduction in the $Man_5GlcNAc_2$ peak compared to virion-associated gp120 (Table 4.4, 17% compared to 37%). This indicates that this species does not evade processing by GnT I and subsequent Golgi-resident glycosidases and glycosyltransferases.

Table 4.4. Abundances of released N-linked glycans from pseudoviral particles envelope glycoproteins[†]

glycoprotein	% oligomannose ($Man_xGlcNAc_2$)							Complex
	Total	Man_5	Man_6	Man_7	Man_8	Man_9	Man_{6-9}	
gp120	99	37	6.5	9	21	25	62	1
gp160	100	31	10	10	20	29	69	-
gp120sup[‡]	74	17	7	13	17	20	57	26

[†]Abundances obtained for desialylated released N-linked glycans.
[‡]soluble gp120 obtained from culture supernatant, non-virion associated.

To determine the residual amount of complex-type glycans present on viral envelope, pseudoviral particles were expressed in HEK 293T cells in the presence of swainsonine, a Golgi α-mannosidase II inhibitor. As a result, the heterogeneity of processed complex-glycans is reduced, allowing a $GlcNAc\beta1\rightarrow2Man_5GlcNAc_2$ intermediate to be observed as a trace population (Figure 4.12, peak at *m/z* 1460), and confirming that the complex-type glycan population on the pseudoviral envelope comprises less than 2% of total N-linked glycans.

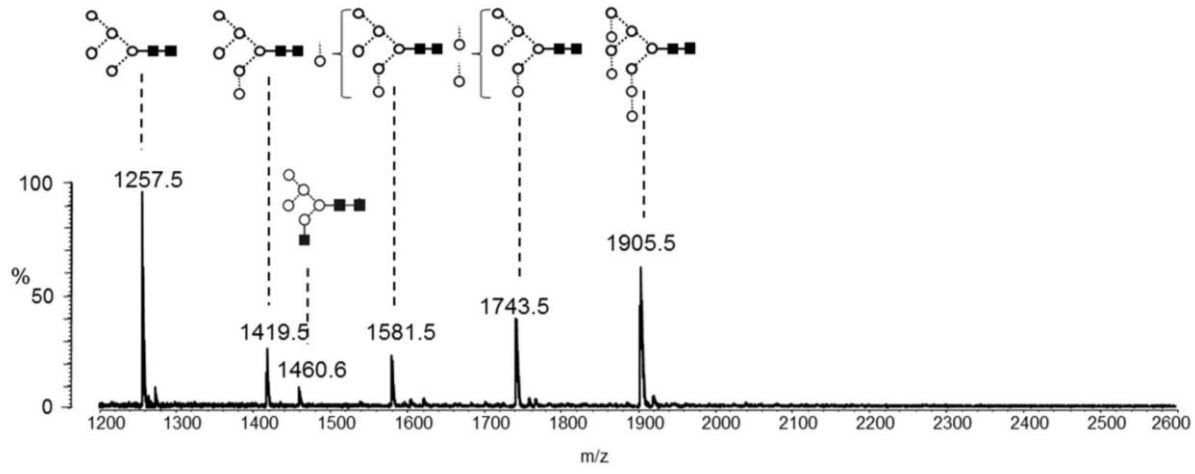


Figure 4.12. Glycosylation of envelope glycoprotein derived from pseudoviral particles in the presence of swainsonine
MALDI-TOF MS analysis of desialylated N-linked glycans ($[M+Na]^+$ ions) released from gp120 isolated from pseudoviral particles generated by transfection of HEK 293T cells with pSVIII-JRFL and pSG3ΔEnv plasmids in a 1:2 ratio in the presence of swainsonine (20 μM).

To assess the importance of the HIV backbone in envelope glycosylation, pseudoviral particles were prepared with another backbone plasmid, pNL4-3ΔEnv. The MS profile of gp120 isolated from these pseudoviral particles was similar to that produced using pSG3ΔEnv as the backbone plasmid (Figure 4.13). This indicates that the backbone plasmid used for pseudoviral preparation does not influence envelope glycosylation.

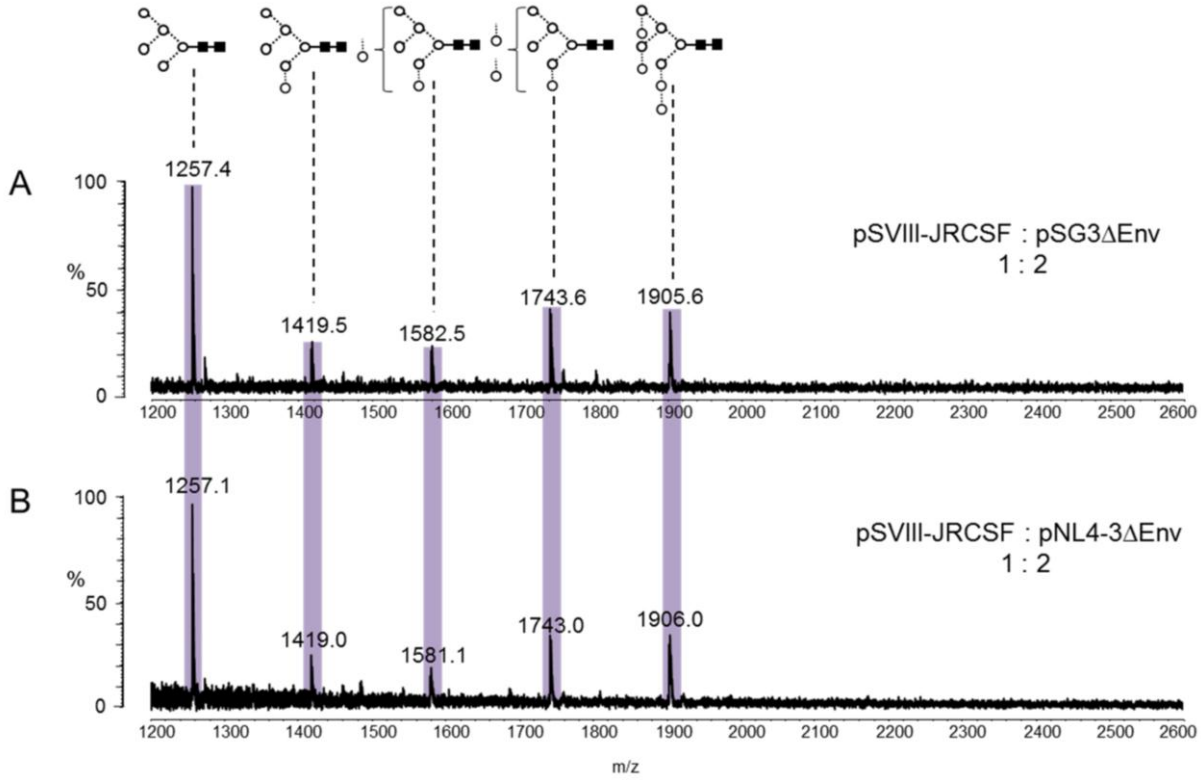


Figure 4.13. Influence of backbone plasmid on gp120 glycosylation
MALDI-TOF MS analyses of desialylated N-linked glycans ($[M+Na]^+$ ions) released from gp120 isolated from pseudoviral particles generated by transfection of HEK 293T cells with **(A)** pSVIII-JRCSF and pSG3ΔEnv plasmids in a 1:2 ratio, and **(B)** pSVIII-JRCSF and pNL4-3ΔEnv plasmids in a 1:2 ratio.

4.3.3. Influence of envelope expression levels

A reduced envelope expression level was observed in the one-plasmid infectious molecular clone system, compared to the two-plasmid pseudoviral particles production system (Figure 4.14), and this lower level of Env expression was found to correlate with a reduction in oligomannose abundance.

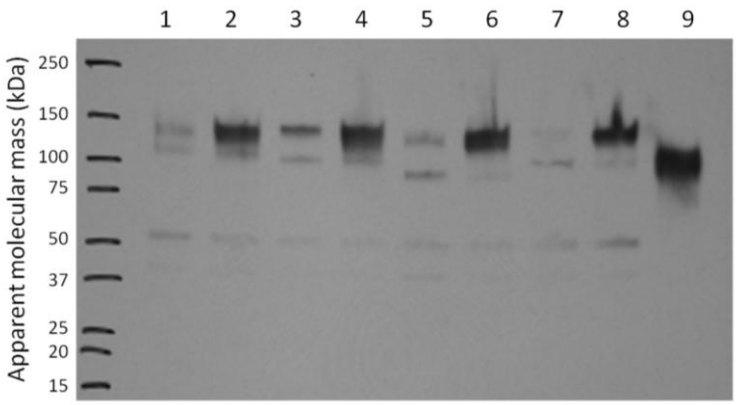


Figure 4.14. Comparison of envelope expression levels for viral and pseudoviral production systems
Infectious molecular clone (IMC) and pseudoviral particles (PV) were produced as described previously. Western blot analysis showing in lane 1 IMC and lane 2 PV both produced in 293T cells; lane 3 IMC and lane 4 PV both produced in 293T cells in the presence of kifunensine; lane 5 IMC and lane 6 PV both produced in GnT I -/- 293S cells; lane 7 IMC and lane 8 PV both produced in GnT I -/- 293S cells in the presence of kifunensine, and lane 9 recombinant, monomeric gp120 control.

To assess whether the envelope levels would influence glycosylation processing of virion-associated gp120, pseudoviral particles were generated using ratios that would reduce envelope production and the glycosylation of gp120 was analysed (Figure 4.15). Decreasing the ratio of envelope:backbone plasmids to 1:10 instead of the 1:2 ratio previously used resulted in a slightly lower abundance of oligomannose-type glycans (Figure 4.15.B, 85% of $Man_{5-9}GlcNAc_2$ glycans instead of the 99%). Further decrease in the ratio to 1:40 lead to similar oligomannose abundance (Figure 4.15.C, 84% of $Man_{5-9}GlcNAc_2$ glycans). These results suggest that the high expression level of envelope glycoprotein observed in the pseudoviral expression system could be linked to a lack of processing of envelope glycans in the Golgi compartments.

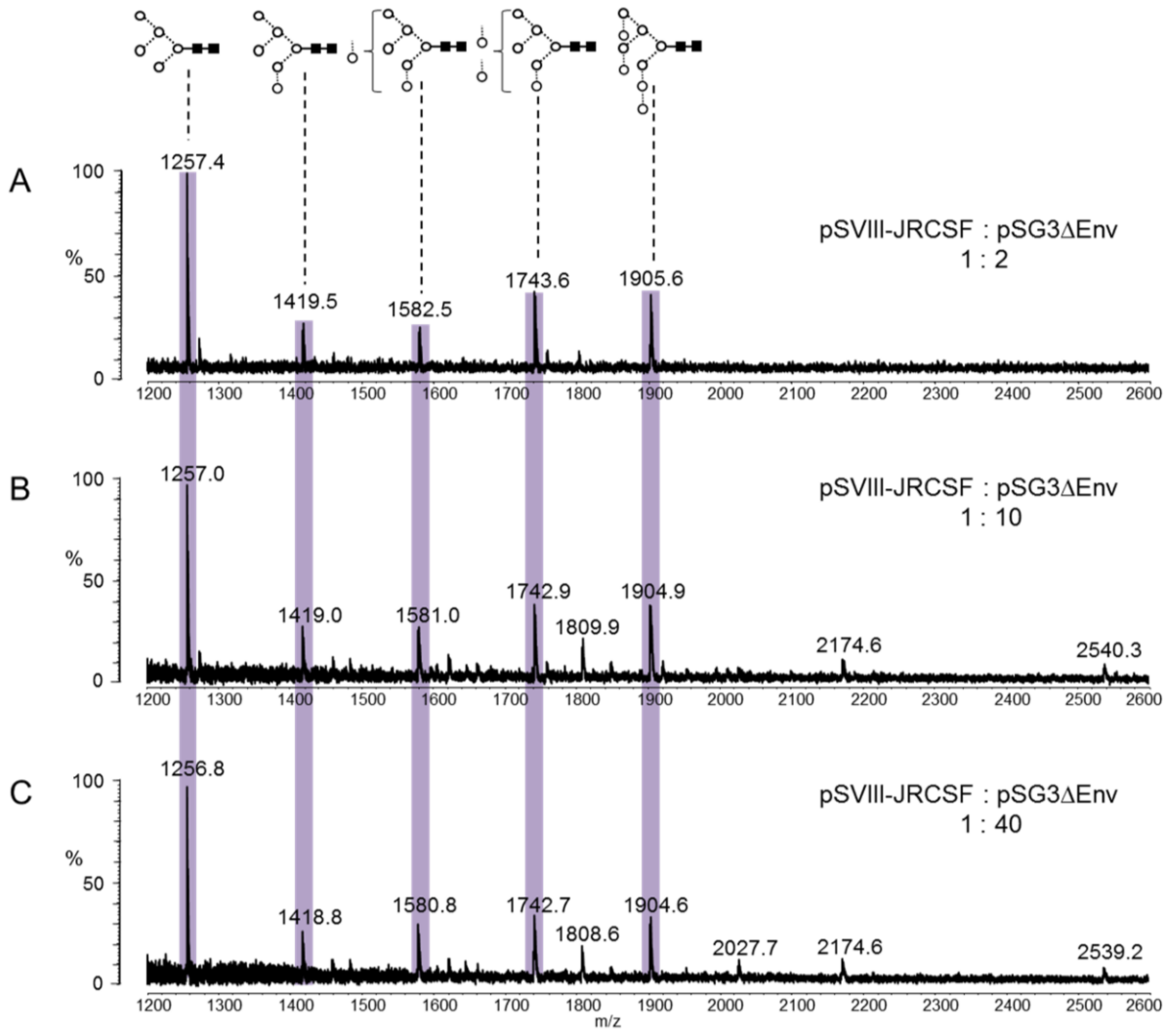


Figure 4.15. Influence of envelope expression level on gp120 glycosylation processing
MALDI-TOF MS analyses of desialylated N-linked glycans ($[M+Na]^+$ ions) released from gp120 isolated from pseudoviral particles generated by transfection of HEK 293T cells with plasmids pSVIII-JRCSF and pSG3ΔEnv in a **(A)** 1:2, **(B)** 1:10, and **(C)** 1:40 ratio.

4.3.4. Cross-clade glycosylation analysis in pseudoviral system

MALDI-TOF MS analyses of envelope N-linked glycans derived from pseudoviral particles produced using clade B (HIV-1_{JRFL}), clade A (HIV-$1_{92RW020}$), clade C (HIV-1_{DU422}), or SIV (mac 239) envelope gene plasmids, showed similar profiles composed predominantly of oligomannose-type glycans (Figure 4.16). In all cases, $Man_5GlcNAc_2$ was by far the most abundant glycan structure detected, indicating that evasion from Golgi-resident GnT I is a conserved feature of pseudoviral systems.

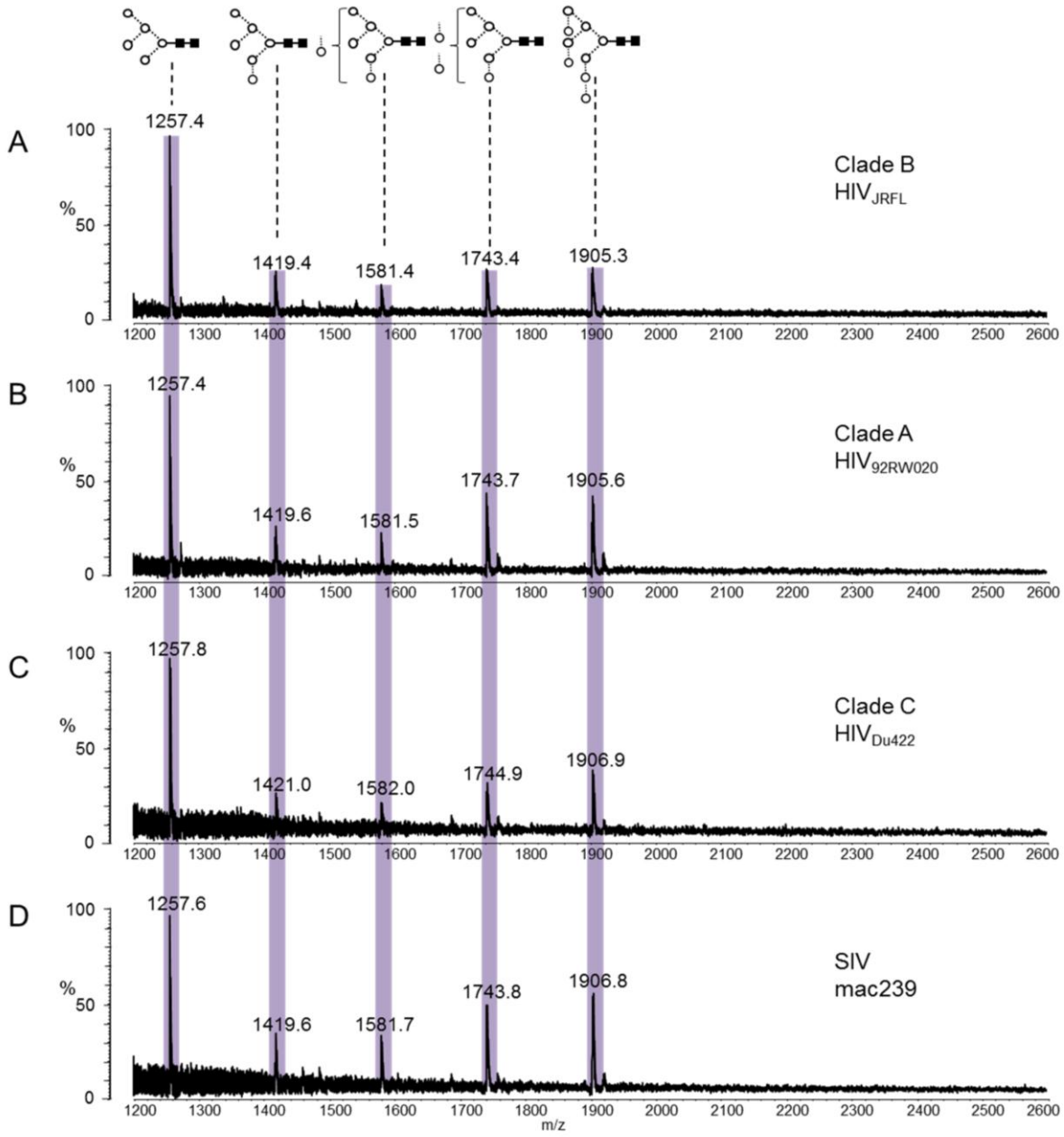


Figure 4.16. Cross-clade glycosylation conservation in pseudoviral system
MALDI-TOF MS analyses of desialylated N-linked glycans ($[M+Na]^+$ ions) released from gp120 isolated from pseudoviral particles generated by transfection of HEK 293T cells using pseudotyped envelope plasmid from **(A)** clade B HIV_{JRFL}, **(B)** clade A $HIV_{92RW020}$, **(C)** clade C HIV_{Du422}, **(D)** SIV_{mac239}.

4.4. Conclusion

Our analysis of N-linked glycans from gp120 isolated from virions produced in PBMCs showed that oligomannose-type glycans are a key feature of viral envelope

glycoproteins, together with a conserved set of highly processed, sialylated bi/tri/tetra antennary complex-type glycans.

Overall, the distribution of oligomannose-type glycans for the PBMCs-derived virus is similar to the one observed for the single-plasmid infectious pLAI-JRCSF molecular clone, and the shed material from the pseudoviral system, with the presence of complex-type glycans, but without an elevated $Man_5GlcNAc_2$ peak. A common feature of glycosylation of envelope isolated from pseudoviral particles is the high abundance of the $Man_5GlcNAc_2$ glycan. When interpreted in the context of the biosynthetic pathway, gp120 derived from pseudoviral particles exhibits a lack of processing by GnT I or any of the subsequent Golgi-resident glycan processing enzymes. The localisation of GnT I to the *medial*-Golgi (Burke *et al.* 1992) suggests that pseudoviral glycoproteins exit the classical secretory pathway from the *medial*-Golgi compartment (Figure 1.7). This data is consistent with recent reports that pseudoviral particles contain a large proportion of Endo-H sensitive, non-functional envelope glycoprotein (Crooks *et al.* 2011).

Viral envelope glycoproteins exhibit a further level of divergence in their glycosylation compared to host cell glycosylation and monomeric glycosylation. Processing by α-mannosidase during the early steps of viral glycosylation assembly and secretion is limited to a greater extent on the native virus than on monomeric gp120. This would be compatible with a model where envelope oligomerisation occurs in cellular compartments with active α1-2 mannosidases, and where structural constraints, such as glycan-glycan and glycan-protein interactions at the trimer interface, alter HIV glycan processing.

In summary, the glycosylation of HIV envelope glycoproteins diverges from typical host-cell glycosylation on at least three levels. Firstly, the clustering of N-linked glycans gives rise to an intrinsic mannose patch, observed both on monomeric and trimeric gp120. Secondly, the steric constraints of trimerisation result in an additional population of

oligomannose glycans observed on viral Env. Finally, in pseudoviral systems, the majority of envelope glycoproteins bypass Golgi-resident enzymes responsible for complex glycan biosynthesis and protein cleavage, leading to an unusual elevation of $Man_5GlcNAc_2$ and non-functional envelope gp160. The intrinsic and trimer-associated mannose patches give rise predominantly to oligomannose-type glycans on virion-associated gp120 that is conserved regardless of the virus production system, the envelope expression level, 2G12 sensitivity, or the envelope sequence.

Chapter 5

Persistence of oligomannose population despite glycosylation site variation

The population of unprocessed oligomannose glycans, which forms the basis of the epitope of anti-carbohydrate broadly neutralising antibodies such as 2G12 and PGT128, was shown to persist due to a lack of processing by ER and Golgi mannosidases caused by steric hindrance from neighbouring glycans. However, the consequences of removing one or several key glycosylation site(s) on neighbouring glycan processing have not been demonstrated, and are important to understand antibody resistance. In this chapter, the effect of elimination of one or several glycans within this cluster on the processing of neighbouring Manα1→2Man motifs is studied. This analysis is conducted on recombinant gp120 from two different 2G12-sensitive HIV strains, HIV-1_{BaL} and HIV-1_{JRCSF}, which exhibit different levels of oligomannose glycans, as well as gp120 isolated from pseudoviral particles.

5.1. Glycoform epistasis for recombinant material

Several hypotheses could explain how the loss of one or more glycan(s) might influence antibody binding affinity, including the change in processing of the remaining glycans that could be altered by the absence of one or several glycan(s), as remaining glycans

would be more accessible to glycosylation enzymes such as mannosidases in the ER and the Golgi.

To determine the effect of reducing the density of oligomannose-type glycans on the processing of neighbouring structures within the intrinsic mannose patch, and consequently better understand the antibody resistance of certain isolates, gp120 mutants were generated with glycosylation site deletions. The glycans important for 2G12 binding are N295, N332, N339, N386, N392, with the glycan at position N332 being crucial for binding to other broadly neutralising antibodies such as PGT128 (Scanlan *et al.* 2002a; Walker *et al.* 2011).

5.1.1. Mannose patch mutations on gp120$_{BaL}$

The wild-type and the mutant glycoproteins were expressed in HEK 293T cells, and the glycans released by PNGase F were subsequently analysed by MALDI-TOF MS (Figure 5.1). Analysis of the abundance of released N-linked glycans (Table 5.1) showed a decrease in the overall oligomannose-type glycan population upon removal of one or two key glycosylation sites. More particularly within the oligomannose series, it was observed that that whilst the respective levels of $Man_{5\text{-}7}GlcNAc_2$ remained untouched by removal of one or two N-linked glycans, the previously less processed $Man_{8,9}GlcNAc_2$ abundances were most affected. A drop is observed in particular for the $Man_9GlcNAc_2$ peak compared to wild-type glycosylation, that cannot be accounted for by deletion of one (and respectively) two glycosylation sites alone. This suggests that removal of one glycan affects processing of adjacent glycans.

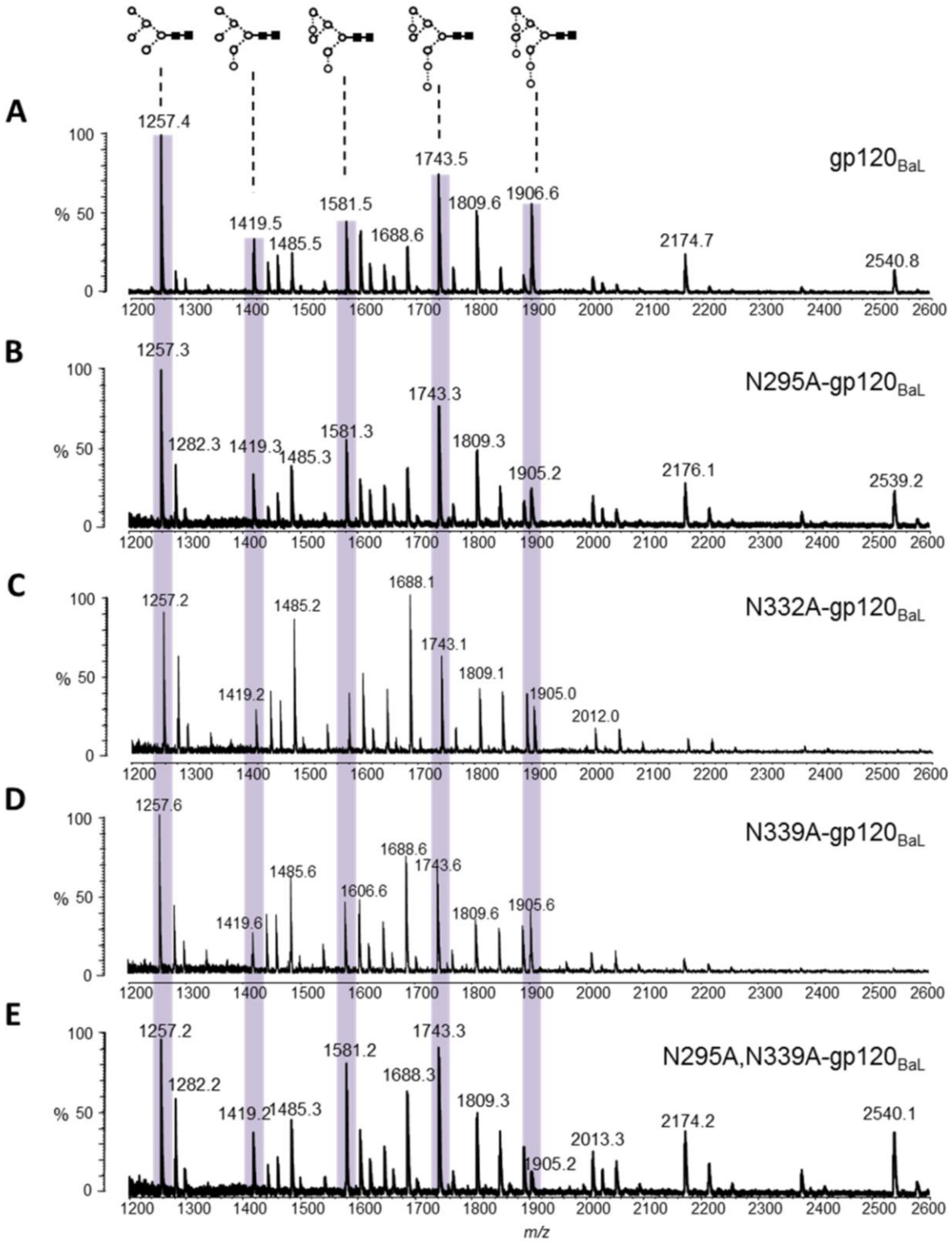


Figure 5.1. Glycoform epistasis for $gp120_{BaL}$
MALDI-TOF MS analyses of the desialylated PNGase F-released glycans from **(A)** $gp120_{BaL}$, **(B)** $gp120_{BaL}$ with a N295A mutation, **(C)** $gp120_{BaL}$ with a N332A mutation, **(D)** $gp120_{BaL}$ with a N339A mutation, **(E)** $gp120_{BaL}$ with a N295A,N339A double mutation. The oligomannose series is highlighted in purple.

Table 5.1. Abundances of released N-linked glycans obtained from recombinant monomeric gp120$_{BaL}$ wild-type and mutants[†]

gp120	% oligomannose ($Man_xGlcNAc_2$)						Complex	Mannose content relative to WT[‡]
	Total	Man_5	Man_6	Man_7	Man_8	Man_9		
(A) Wild-type	45	11	5	7	12	10	55	1
(B) N295A	40	13	4	7	12	4	60	0.88
(C) N332A	31	11	3	5	8	4	69	0.69
(D) N339A	31	11	3	5	7	5	69	0.69
(E) N295A, N339A	36	12	3	9	12	<1	64	0.80

[†]Abundances obtained for desialylated N-linked glycans released from gp120 described in this study.
[‡]Values represent the increase in oligomannose population ($Man_{5-9}GlcNAc_2$) for mutant glycoproteins compared to wild-type gp120$_{BaL}$.

5.1.2. Mannose patch mutations on gp120$_{JRCSF}$

Single mutants of gp160$_{JRCSF}$ had already been developed in the pSVIII plasmid, and in particular the N295A, N332A, N339Q, and N392Q mutants have been shown to retain b12 binding and thus proper gp120 conformation (Scanlan *et al.* 2002a). The gp120$_{JRCSF}$ insert was cloned in the pHLSec vector. The single mutation N295A, N339Q and N392Q were introduced on WT-gp120$_{JRCSF}$. The double mutant N295A,N339Q was prepared by inserting the N339Q point mutation into the N295A-gp120$_{JRCSF}$ clone. The proteins were transiently expressed in HEK 293T cells, run on an SDS-PAGE gel, and the protein bands were subjected to in-gel PNGase F digestion. MALDI-TOF MS analysis of the released glycans was performed (Figure 5.2).

The glycosylation profile of gp120$_{JRCSF}$ was compared to that of a gp120$_{JRCSF}$ variant with the glycan at position N295 removed (N295A). The deletion of the Asn295 glycosylation site does not significantly reduce the relative abundance of oligomannose (32%) to complex-type glycans significantly but does lead to a perturbation in the relative levels of glycans within the oligomannose series (Table 5.2). A decrease in the abundance of $Man_9GlcNAc_2$ is observed together with an increase in the relative abundances of $Man_{5-8}GlcNAc_2$. This effect was observed in the other single mutants with glycan deletion at Asn339 or Asn392.

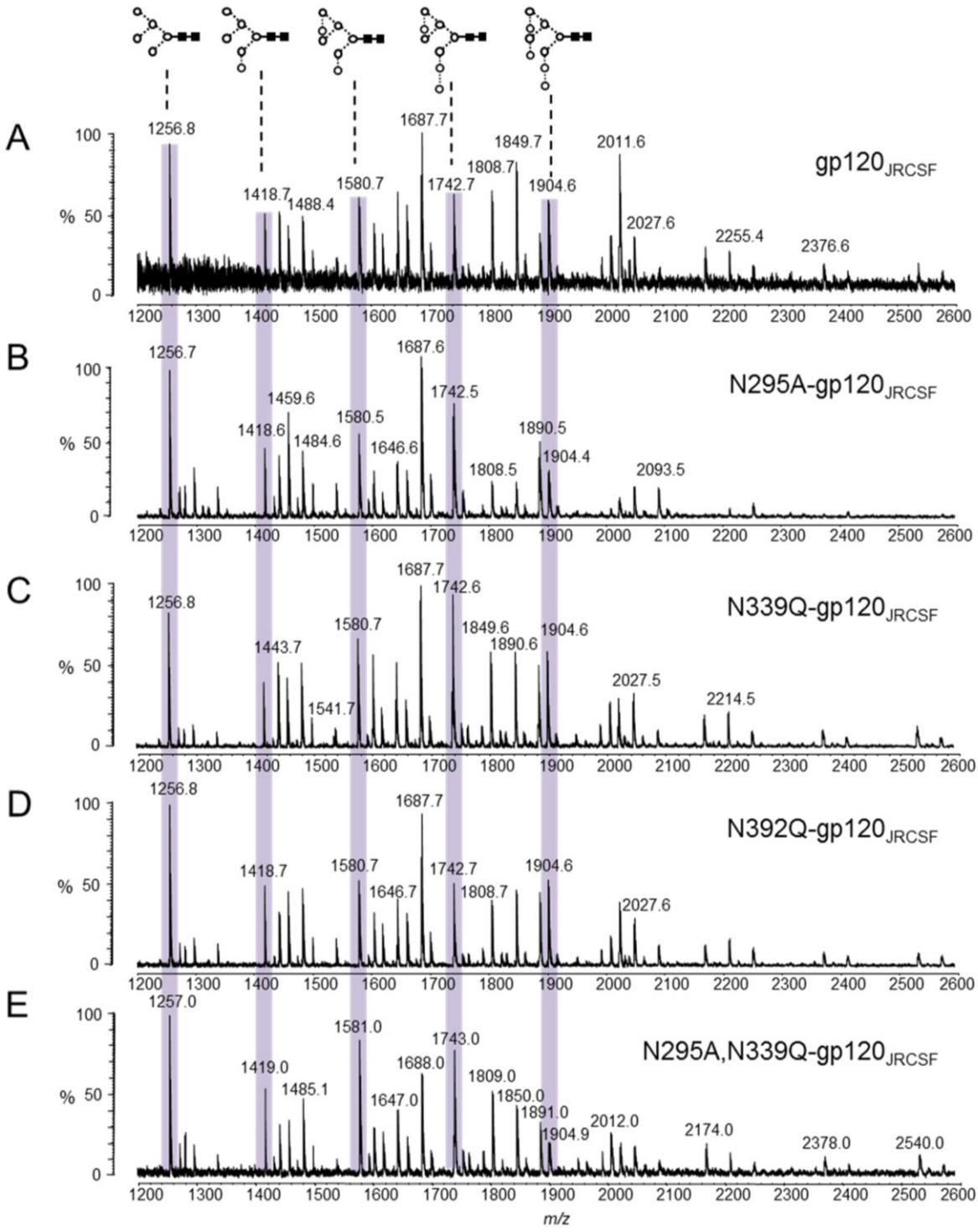


Figure 5.2. MALDI-TOF MS analysis of gp120$_{JRCSF}$ wild-type and mutants
MALDI-TOF MS analyses of the desialylated PNGase F-released glycans from **(A)** gp120$_{JRCSF}$, **(B)** gp120$_{JRCSF}$ with a N295A mutation, **(C)** gp120$_{JRCSF}$ with a N339Q mutation, **(D)** gp120$_{JRCSF}$ with a N392Q mutation, **(E)** gp120$_{JRCSF}$ with a N295A,N339Q double mutation. The oligomannose series is highlighted in purple.

The additional N339Q mutation, though known to affect 2G12 binding, did not further affect the total oligomannose composition of the N295A-gp120 mutant (32%) but did lead to a further redistribution of oligomannose species (Table 5.2).

Table 5.2. Abundance of released N-linked glycans obtained from $gp120_{JRCSF}$ wild-type and mutants[†]

$gp120_{JRCSF}$	% oligomannose ($Man_xGlcNAc_2$)						Complex	Mannose content relative to WT[‡]
	Total	Man_5	Man_6	Man_7	Man_8	Man_9		
(A) wild-type	32	5	4	6	9	8	68	-
(B) N295A	32	8	5	5	9	5	68	1
(C) N339Q	30	6	3	6	8	7	70	0.94
(D) N392Q	31	9	4	5	6	7	69	0.96
(E) N295A, N339Q	32	9	4	8	8	3	68	1

[†]Average abundances obtained for desialylated N-linked glycans released from $gp120_{JRCSF}$ described in this study.
[‡]Values represent the increase in oligomannose population ($Man_{5\text{-}9}GlcNAc_2$) for mutant glycoproteins compared to wild-type $gp120_{JRCSF}$.

Two trends became evident from this mutagenesis study (Figure 5.3): (i) a decrease in $Man_9GlcNAc_2$ population, which would be expected from removal of one or more oligomannose glycans that form the 2G12 epitope, and (ii) the increase in abundance of the more processed $Man_5GlcNAc_2$ peak, indicating that neighbouring glycans are become slightly more accessible to mannosidase enzymes in the ER and the Golgi. For $gp120_{BaL}$, which exhibits elevated levels of oligomannose-type glycans on the wild-type protein, the trend (i) is observed, together with an overall decrease in oligomannose-type glycans abundance. However, wild-type $gp120_{JRCSF}$ display lower levels of oligomannose-type glycans, and, as such, a mutation within the mannose patch does not lead to further decrease of the overall oligomannose abundance, but rather to a redistribution of glycans within the oligomannose series, and an increase in the more processed $Man_5GlcNAc_2$ glycan highlighted.

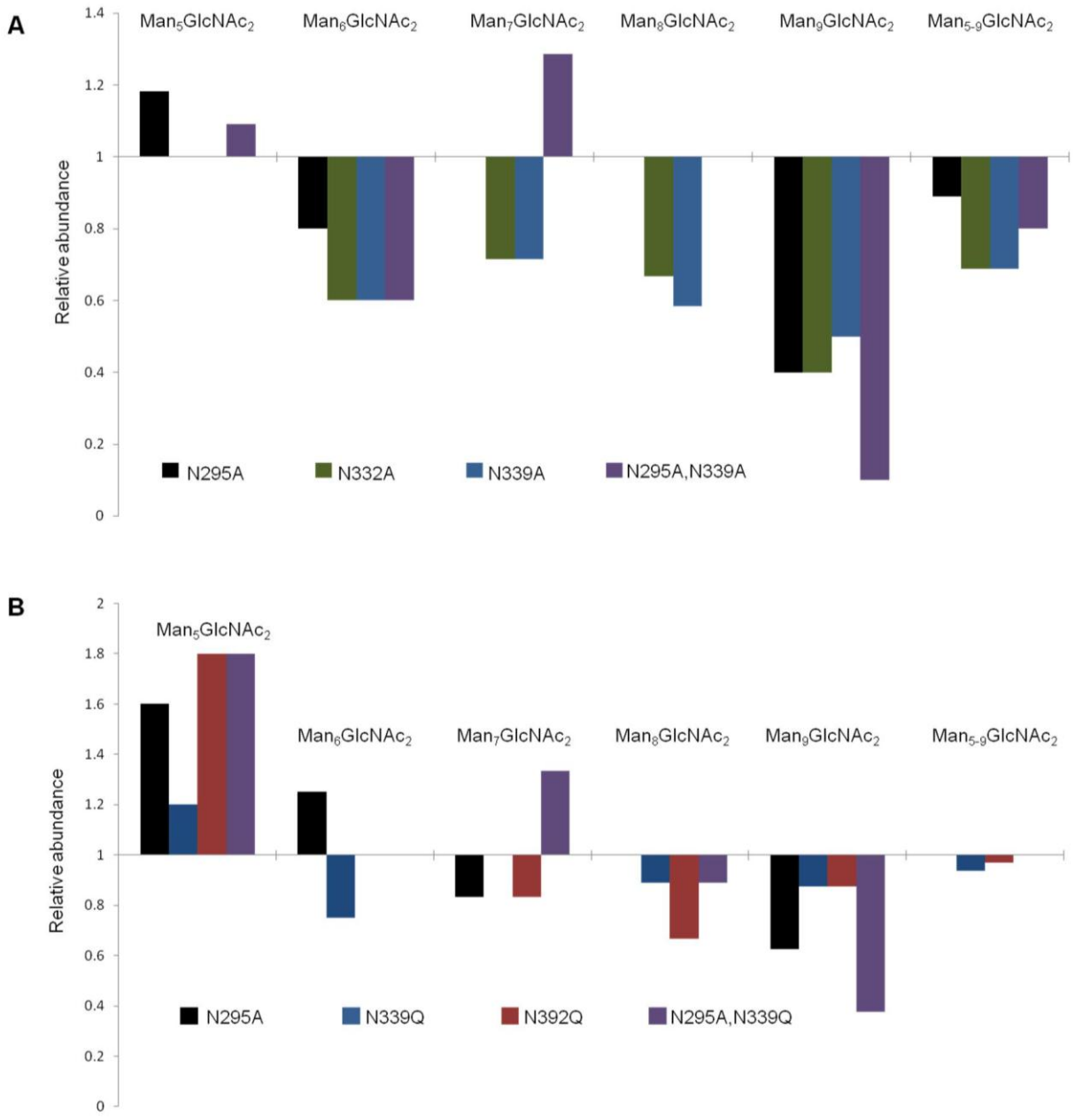


Figure 5.3. Abundances of oligomannose glycans on $gp120_{BaL}$ and $gp120_{JRCSF}$ mutants relative to wild-type

Ratio of abundances of $Man_{5-9}GlcNAc_2$ glycans compared to wild-type abundances for (**A**) $gp120_{BaL}$ single mutants N295A (black), N332A (green), N339A (blue) and the double mutant N295A,N339A (purple), and (**B**) $gp120_{JRCSF}$ single mutants N295A (black), N339Q (blue), N392Q (red) and the double mutant N295A,N339Q (purple). Values were obtained from the data presented in Table 5.1 and Table 5.2.

5.2. Persistence of oligomannose glycans in 2G12-resistant mutants

Although pseudoviral Env glycosylation differs from viral glycosylation in the level of the $Man_5GlcNAc_2$ species and related complex-type glycans abundance, the population of $Man_{6-9}GlcNAc_2$ glycans observed in this two systems are similar, and consequently pseudoviral particles can be used to study gp120 oligomannose content.

2G12 binds equivalently to both monomeric and trimeric gp120, suggesting that most of the additional Manα1→2Man terminating glycans found on the native envelope are unrelated to the 2G12 epitope, and that the monomeric mannose patch is intrinsic to the monomer and generated independently of the mechanism that prevents viral glycan processing on viral envelope.

To test this hypothesis, mutant pseudoviruses were generated with glycosylation site deletions that disrupt the 2G12 epitope: N295A and N332A. Analysis of the N-linked glycans from these envelopes shows a modest decrease in the abundance of $Man_9GlcNAc_2$ glycans, consistent with the loss of single glycans required for productive 2G12 recognition, but the overall profile remains largely unperturbed (Figure 5.4).

These results are further confirmation both of the mutagenesis work done on monomeric gp120, and of the cross-clade analysis performed with PBMCs and show that the overall composition of the glycan shield is unperturbed by sequence variation.

In agreement to the results obtained in the previous section, a redistribution of glycans within the oligomannose series was observed upon loss of one glycosylation site, whilst keeping the overall oligomannose population constant. An increase in the less processed $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ glycans is observed (Figure 5.5), as would be expected on the more sterically constrained trimers.

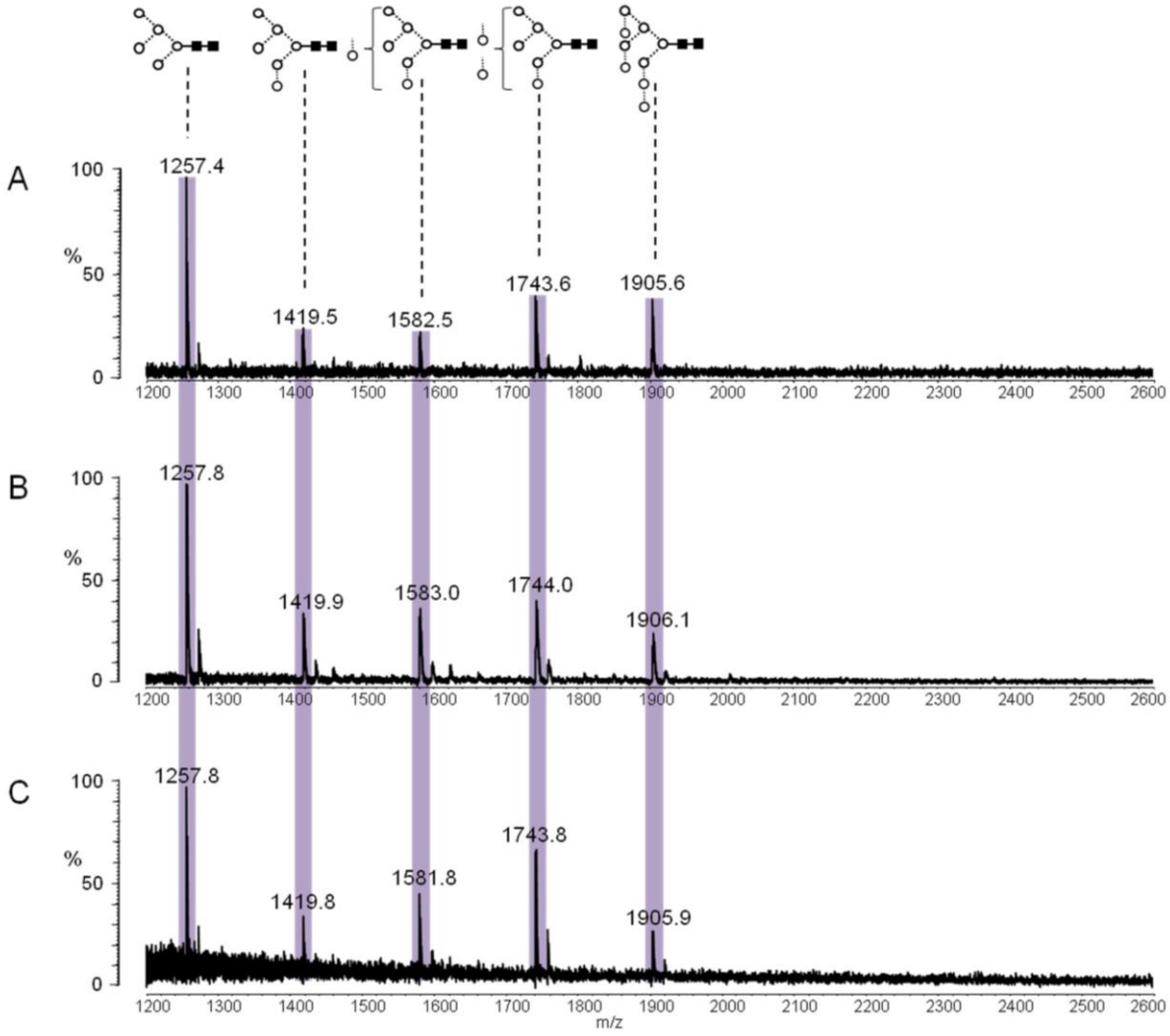


Figure 5.4. N-linked glycans analysis of pseudovirus mutants
MALDI-TOF MS analyses of desialylated N-linked glycans ($[M+Na]^+$ ions) released from gp120 isolated from pseudoviral particles generated by transfection of HEK 293T cells using pseudotyped envelope plasmid from **(A)** JRCSF-WT, **(B)** JRCSF-N295A, **(C)** JRCSF-N332A.

Table 5.3. Abundances of released N-linked glycans from pseudoviral particles envelope glycoproteins mutants[†]

gp120	% oligomannose ($Man_xGlcNAc_2$)							Complex
	Total	Man_5	Man_6	Man_7	Man_8	Man_9	Man_{6-9}	
JRCSF WT	99	37	6.5	9	21	25	62	1
JRCSF N295A	97	43	15	15	17	7	54	3
JRCSF N332A	99	31	14	18	25	11	68	1

[†]Abundances obtained for desialylated released N-linked glycans.

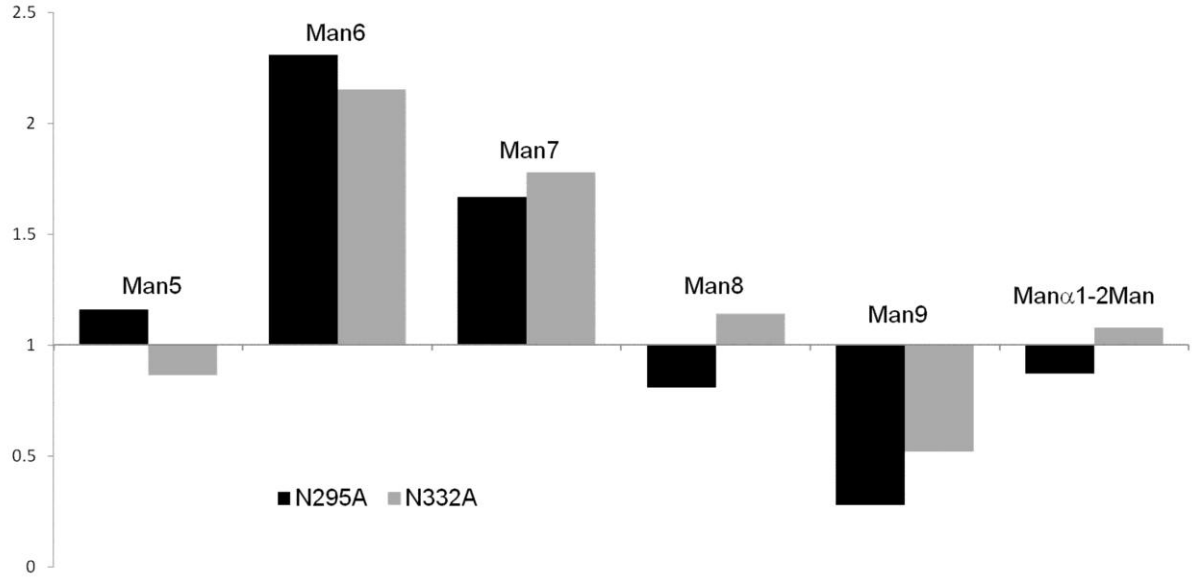


Figure 5.5. Pseudovirus mutants
Ratio of $Man_{5\text{-}9}GlcNAc_2$ glycans within the oligomannose series for the two pseudovirus mutants JRCSF-N295A and JRCSF-N332A compared to the wild-type envelope glycosylation. Values were obtained from the data presented in Table 5.3.

5.3. Conclusion

The stability of gp120 mannose patch was assessed by mutagenesis on two HIV-1 strains. Using a recombinant gp120 model of the mannose cluster that forms the epitope of the mannose-binding antibody 2G12 and PGT128, it was shown that elimination of glycans within this cluster alters the processing of neighbouring Manα1→2Man motifs. In the case of $gp120_{BaL}$ which exhibits 45% of oligomannose-type glycans, removal of one key glycosylation site leads to a drop into this particular population, mainly due to a decrease in the less processed $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ structures. Whilst the overall level of oligomannose glycans remains constant for HIV_{JRCSF} gp120, the pattern of terminal branching is similarly significantly altered across the 'intrinsic' mannose patch following removal of individual glycan sites. This result was confirmed in a pseudoviral system, where loss of one N-linked glycosylation site did not affect the total overall glycan composition, but rather induced a subtle redistribution within the oligomannose series. The reduction in

$Man_9GlcNAc_2$ species can explain the loss of binding of 2G12 to gp120 lacking one or two key glycosylation sites, as observed for clade C isolates.

This result indicates that the consequence of a mutation of a glycosylation site may have a subtle but widespread effect on the antigenic structure of the glycoprotein surface, and consequently on ligand interactions. This secondary effect on glycan processing may augment the antigenic effect of point mutations in virions that arise under selection pressure from agents targeting viral carbohydrates.

Chapter 6

Creation of a library of gp120 glycoforms: a toolkit to study gp120 interactions with the immune system

Two main conserved glycan sub-populations are present on viral envelope glycoprotein, characterised in Chapter 4: one composed of oligomannose-type glycans and one composed of highly processed sialylated complex-type glycans. In this chapter, recombinant monomeric gp120 glycosylation is remodelled to mimic these different conserved components of viral Env. By using selected cell lines, enzyme inhibitors, or recombinant glycosylation enzymes, specific glycan populations can be obtained on monomeric gp120, contributing to the formation of a library of gp120 glycoforms.

The construction of this glycan engineered library should allow for more sensitive elucidation of the role of HIV glycans in immune interactions. Examples of preliminary studies employing these modified gp120 are shown for vaccine and immunological assays. For example the interaction between gp120 sialylated glycans and immune receptors on macrophages, such as siglecs, was assessed and secreted cytokines were measured. Finally, an oligomannose-enriched gp120 glycoform was used to assess the immunogenicity of the $Man_5GlcNAc_2$ glycan, which is amongst the predominant glycans observed on viral Env glycosylation.

6.1. Monomeric gp120 as a mimic of viral Env glycosylation

The different glycan populations present on viral Env can be obtained on recombinant gp120 by expressing gp120 in selected cells lines, in the presence of relevant inhibitors, or by direct remodelling of the glycans with recombinant glycosyltransferases, as described in the next sections and in Figure 6.2. The glycosylation of recombinant gp120 consequently obtained exaggerate particular features of viral Env glycosylation.

6.1.1. Oligomannose-enriched gp120

Remodelling of $gp120_{BaL}$ glycosylation to obtain glycoforms bearing oligomannose-type glycans is detailed in Chapter 3 (section 3.2). Monomeric $gp120_{BaL}$ expressed in HEK 293T cells in the presence of kifunensine exhibits predominantly $Man_9GlcNAc_2$ glycans, whilst $gp120_{BaL}$ expressed in a GnT I-deficient HEK 293S cell line bears predominantly $Man_5GlcNAc_2$ glycans, together with the monomeric mannose patch of $Man_{6\text{-}9}GlcNAc_2$ glycans (Figure 3.4).

6.1.2. Hypersialylated gp120

Recombinant monomeric $gp120_{BaL}$ expressed in HEK 293T cells exhibit a wide range of complex-type glycans (55% of total N-linked glycans on average), including nongalactosylated glycans and glycans bearing α2-3 linked sialic acids. In order to mimic the conserved set of bi-, tri- and tetra-antennary sialylated type glycans observed on viral Env, galactose and α2-6 linked sialic residues were added to monomeric gp120 that had previously been treated with neuraminidase (Figure 6.1.A).

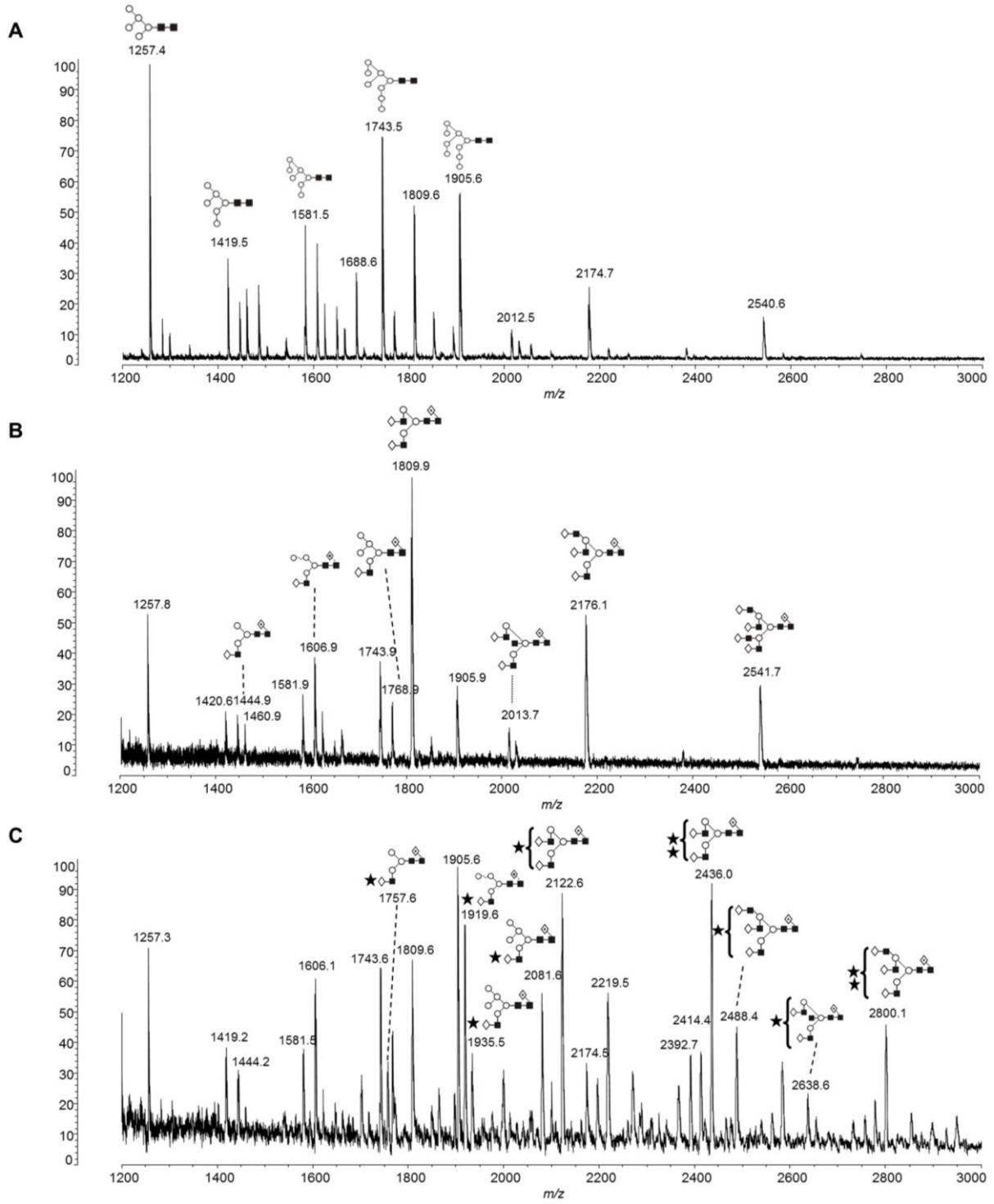


Figure 6.1. Hypergalactosylated and hypersialylated gp120 glycoforms
MALDI-TOF MS analyses of PNGase F-released N-linked glycans from **(A)** neuraminidase treated $gp120_{BaL}$, **(B)** $gp120_{BaL}$ subsequently treated with B4GalT I, and **(C)** $gp120_{BaL}$ subsequently treated with ST6Gal I. The sodium adduct(s) of mono- and bi-sialylated glycans are observed. The galactosylated and sialylated glycan structures are indicated respectively on profile B and C.

The mouse β1,4-galactosyltransferase I (B4GalT I) catalytic domain (residues 127-399), inserted in the pHLsec vector, was transiently expressed in HEK 293T cells. Neuraminidase-treated monomeric $gp120_{BaL}$ was subjected to B4GalT I in the presence of UDP-Galactose, and the PNGase F-released glycans analysed by MALDI-TOF MS (Figure 6.1.B). This reaction converted most of $gp120_{BaL}$ complex-type glycans into galactosylated glycans, with the predominant glycan peaks at *m/z* 1809, 2175 and 2540 corresponding to the neutral glycan structures obtained on desialylated viral gp120 (Figure 4.3.B).

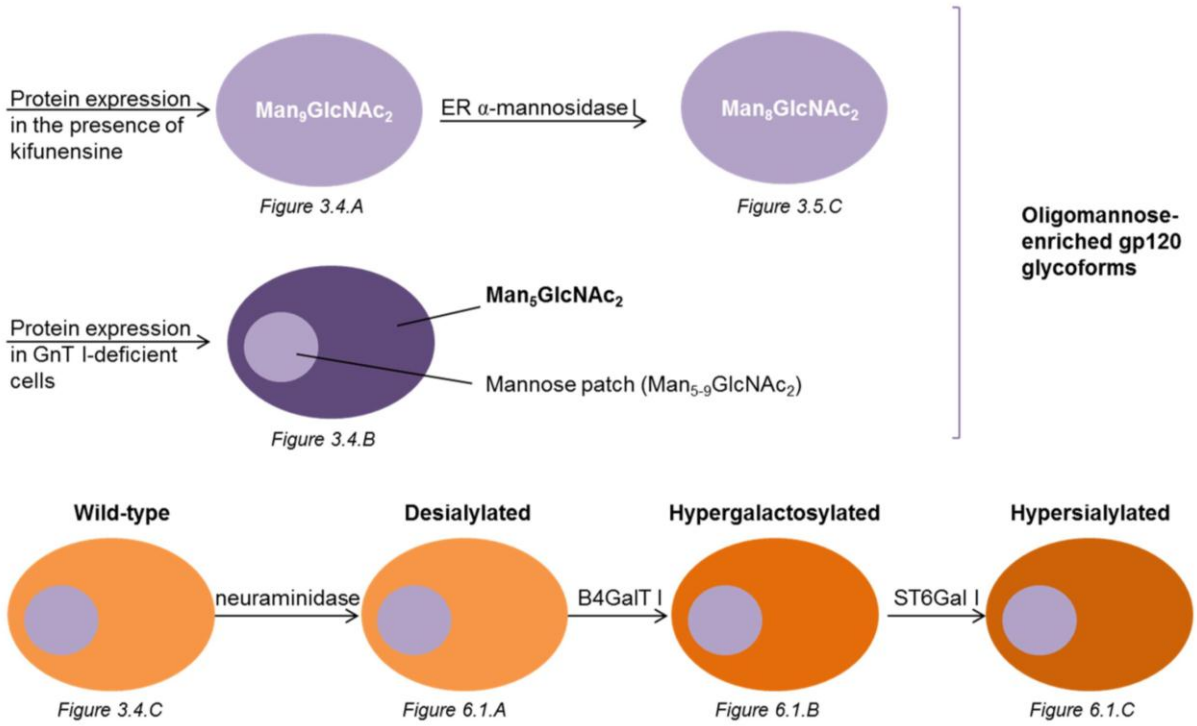


Figure 6.2. Library of gp120 glycoforms
Oligomannose-enriched glycoforms, such as gp120 bearing predominantly $Man_9GlcNAc_2$, $Man_8GlcNAc_2$ or $Man_5GlcNAc_2$, can be obtained by directly interfering with the cell's glycosylation machinery, and respectively by using the ER α-mannosidase I inhibitor kifunensine, or a cell line deficient in the GnT I enzyme, which performs the first obligate step towards complex-glycan diversification. A desialylated gp120 glycoform, useful for sialic acid-related studies, can be obtained by submitting gp120 to neuraminidase. The hypergalactosylated and hypersialylated gp120 glycoforms, that exaggerate viral Env glycosylation, can be obtained by treatment of gp120 with the recombinant glycosylation enzymes B4GalT 1 and ST6Gal I. All the glycoforms (except the $Man_9GlcNAc_2$-gp120) will bear a residual population of $Man_{5-9}GlcNAc_2$ glycans that constitute gp120 mannose patch (highlighted in light purple).

The rat α2,6-sialyltransferase (ST6Gal I) catalytic domain, inserted in the pHLsec vector, was transiently expressed in HEK 293T cells. Hyper galactosylated $gp120_{BaL}$ obtained previously was treated with ST6Gal I in the presence of CMP-sialic acid, and the PNGase F-released glycans were analysed by MALDI-TOF MS (Figure 6.1.C). The sodium adduct of several sialylated glycans were observed, confirming the obtention of an hypersialylated gp120 glycoform, a mimic of viral Env glycosylation that could be used in future cell assay aiming to assess the immunomodulatory effect of $gp120_{BaL}$ complex-type glycans.

6.2. HIV-1 Env complex glycan interactions with immune cell receptors

Whilst the nature, abundance and roles of gp120 oligomannose glycans have been extensively studied, the importance of the subset of sialylated glycans is not yet well-understood. Sialic acid binding lectins (Siglecs) are receptors expressed on many immune cells that modulate key intracellular signalling pathways (Crocker *et al.* 2007). A recent study demonstrated that monomeric gp120 α2-3 linked sialylated glycans bind to several siglecs, including sialoadhesin (α2-3 specific) – which was also shown to promote HIV infection of macrophages (Zou *et al.* 2011). However glycoproteins naturally derived from $CD4^+$-T cells exhibit mainly terminal α2-6-linked sialic acids. A particular siglec that is highly specific for α2-6-linked sialic acids is CD22 (siglec-2), which is expressed on B cells and assists in dampening immune response to "self" by down-regulating B-cell receptor activation. More generally, the α2-6 linked sialic acid motif has emerged as a key negative regulator of adaptive immunity: for example, intravenous immunoglobulin (IVIG) treatment, used for wide array of autoimmune diseases, is believed to act via binding of α2-6 sialylated antibody Fc glycans to immune receptors. To characterise the extent of potential immunosuppressive effects mediated by HIV gp120 sialylated glycans binding to siglecs, a preliminary assay was

performed, assessing the modulation in cytokine production upon interaction of engineered gp120 glycoforms with macrophages.

6.2.1. Preliminary assay on macrophages differentiated from PBMCs

Monocytes were obtained from healthy individuals' blood, and differentiated into macrophages. The cells were analysed for siglec expression by FACS (Figure 6.3), which showed positive expression of siglecs 5, siglec 7, and siglec 9.

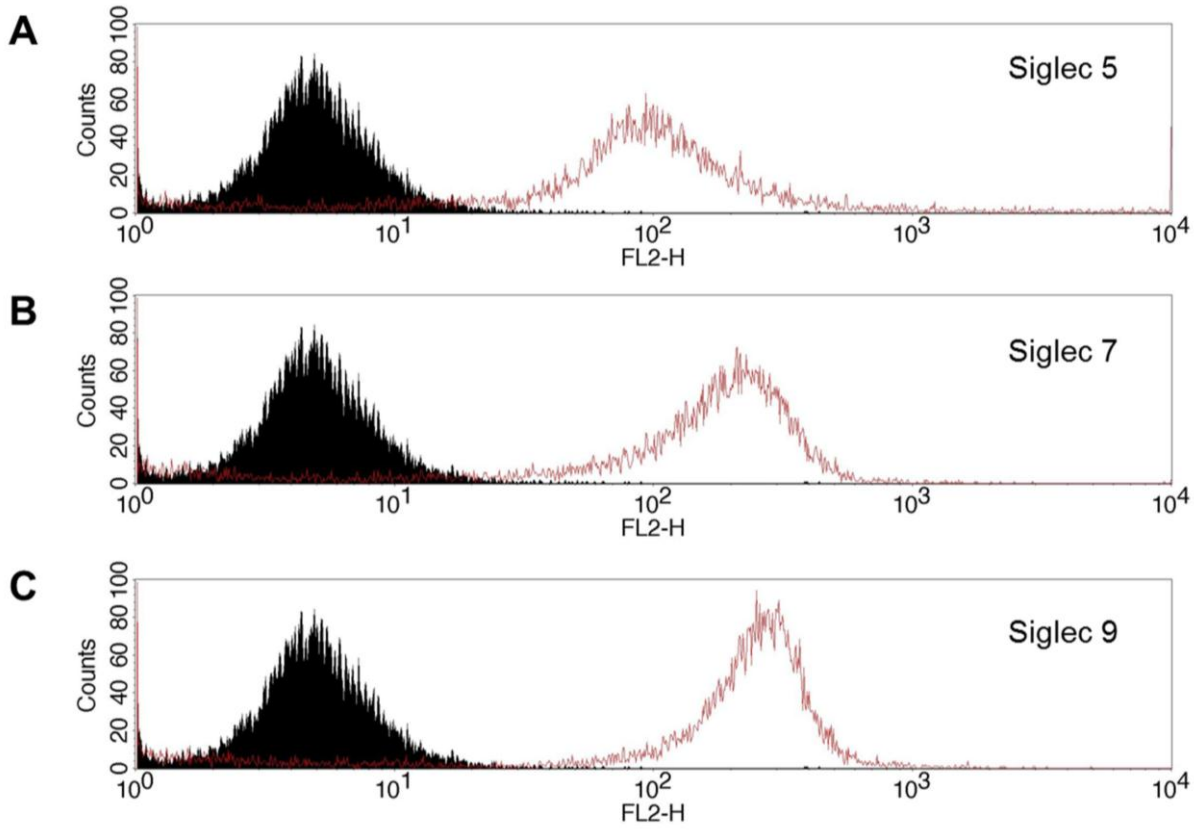


Figure 6.3. Siglecs expressed on macrophages
FACS analysis of macrophages stained with PE-labelled anti-siglec antibodies, showing the cell-surface expression of **(A)** siglec 5, **(B)** siglec 7, and **(C)** siglec 9.

The macrophages were treated with two different gp120 glycoforms for two days. The glycoform 'gp120-neur' was obtained by treating recombinant monomeric gp120 expressed in

HEK 293T with neuraminidase, whilst 'gp120-endo' was obtained by treating gp120 with EndoH, leading to the removal of two-third of oligomannose-type glycans. The level of cytokines released by macrophages treated with the gp120-neur and the gp120-endo glycoform were determined (Figure 6.4).

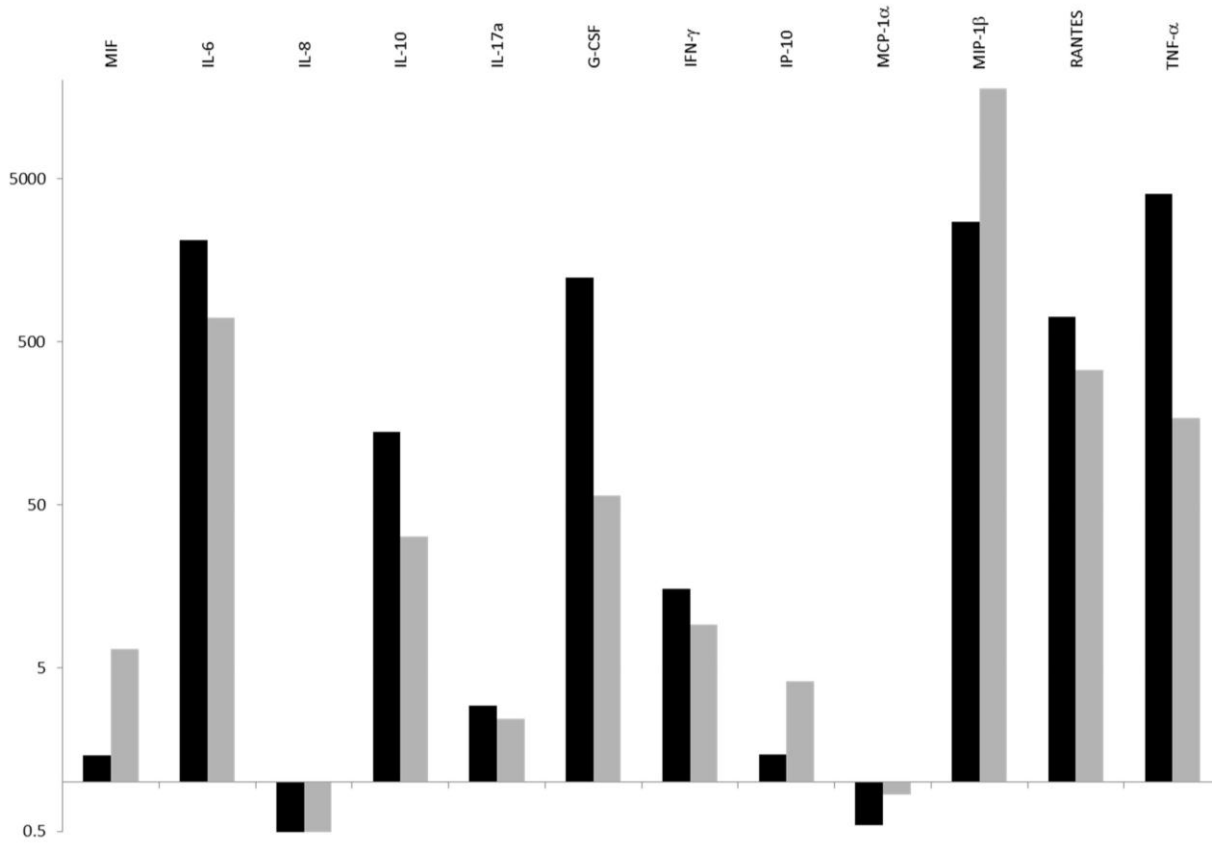


Figure 6.4. Cytokines released by gp120-treated macrophages
The ratio of cytokines expressed by macrophages treated with gp120 glycoforms over the untreated control is plotted as a log scale. The gp120 displaying oligomannose-type glycans, but no terminal sialic acid residues (gp120-neur) is shown in black. The gp120 exhibiting sialic acid residues, but less oligomannose-type glycans (gp120-endo) is shown in grey. The following abbreviations are used: migration inhibitory factor (MIF), Interleukin (IL), granulocyte colony-stimulating factor (G-CSF), Interferon (IFN), IFN-inducible protein 10 (IP-10), monocyte chemoattractant protein 1(MCP-1), macrophage inflammatory protein 1 (MIP-1β), Regulated on activation, normal T expressed and secreted (RANTES, or CCL5), tumor necrosis factor (TNF-α). The relative levels of cytokine expression were obtained using a Luminex-based multiplex assay (cell assay and cytokine analysis done by Drew Sayce).

When comparing both samples, gp120-neur treatment resulted in up-regulation of IL-6, IL-10, G-CSF, IFN-γ, RANTES and TNF-α. These cytokines could be up-regulated either

as a direct consequences of loss of sialic acid, or as a lack of mannose that would usually down-regulate them.

The only cytokine that was dramatically up-regulated by gp120-endo (i.e. less mannose, more sialic acid) was MIP-1β, suggesting that mannose play a part in the down-regulation of this cytokine. This chemokine is secreted together with MIP-1α, IP-10 (which is also up-regulated by gp120-endo), and RANTES, by $CD8^+$ T-cell as part of noncytolytic inhibition of viral replication (Cocchi *et al.* 1995; Freel *et al.* 2010; Saunders *et al.* 2011), and down-regulation of MIP-1β expression by mannose moieties would assist HIV to evade immune system.

6.2.2. Preliminary assay on macrophages derived from a monocyte cell line

In order to understand the impact of sialylated glycans, the influence of desialylated and hypersialylated gp120 glycoforms on cytokine regulation were then assessed on PMA-differentiated U-937 cells. FACS analysis was conducted to determine the pattern of siglecs expression on these cells (Figure 6.5). The cells were positive for siglec 3 (CD33), siglec 5 and siglec 7 expression, and to a lesser extent siglec 9. Furthermore, PMA-treated U-937 cells have previously been reported to express sialoadhesin (Hartnell *et al.* 2001).

The differentiated macrophages were then treated with the two different gp120 glycoforms for two days. The levels of cytokines released were then obtained and compared against an untreated cells control. The major cytokines for which a differential effect was observed are reported in Figure 6.6.

A group of cytokines composed of GM-CSF, TNF-α, G-CSF, IL-6, MIP-1β, IL-1β and s-ICAM-1, was found to be up-regulated after gp120 treatment. Unlike other members of the siglec family, the cytoplasmic tail of sialoadhesin does not display any ITIM domain.

However, sialoadhesin might be involved in recognition of sialylated gp120 and further induction of pro-inflammatory cytokines such as TNF-α and IL-6 (Klaas *et al.* 2012).

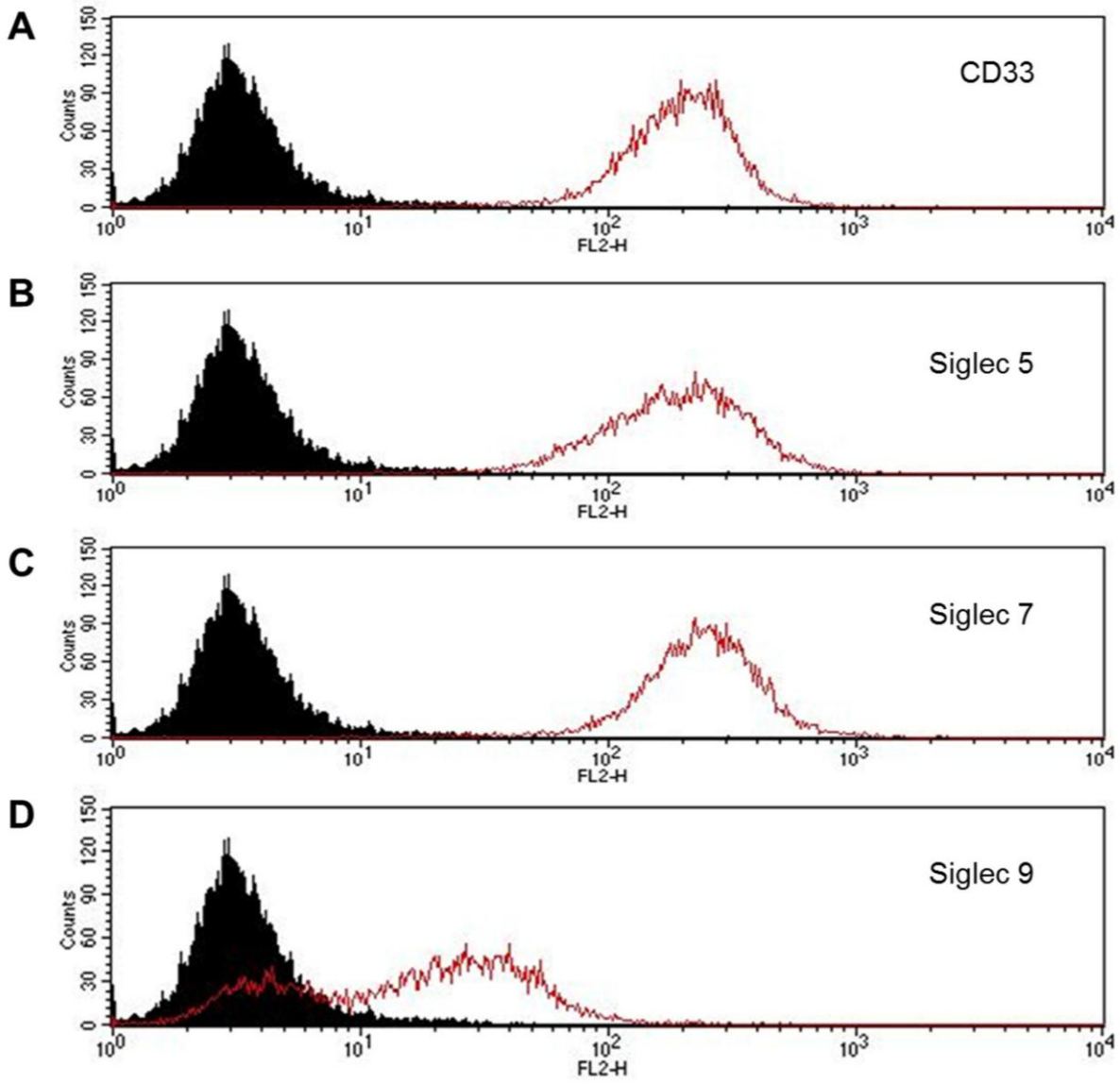


Figure 6.5. Siglecs expressed on PMA-differentiated U-937 cells
FACS analysis of macrophages stained with PE-labelled anti-siglec antibodies, showing the cell-surface expression of **(A)** siglec 3 (CD33), **(B)** siglec 5, **(C)** siglec 7, and **(D)** siglec 9.

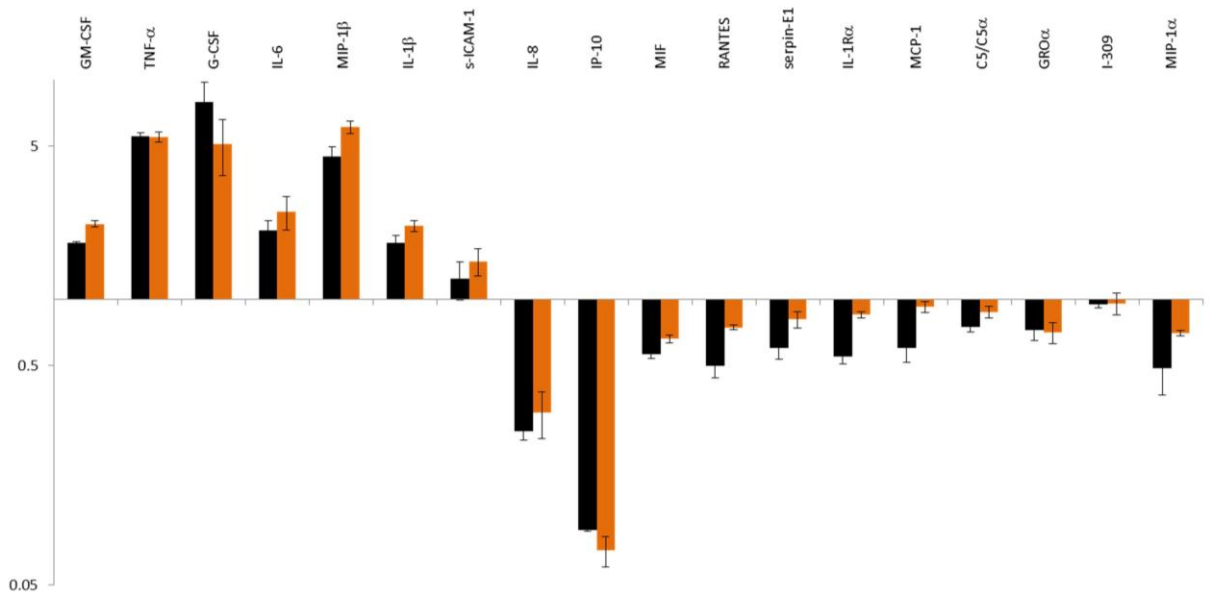


Figure 6.6. Cytokines released by gp120-treated macrophages
The ratio of cytokines expressed by macrophages treated with gp120 glycoforms over the untreated control is plotted as a log scale. The desialylated gp120 glycoform is shown in black, the hypersialylated gp120 glycoform is shown in orange. The following abbreviations are used: granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF-α), interleukin (IL), macrophage inflammatory protein 1 (MIP-1β), intercellular adhesion molecule 1 (ICAM-1), IFN-inducible protein 10 (IP-10), migration inhibitory factor (MIF), regulated on activation, normal T expressed and secreted (RANTES, or CCL5), monocyte chemoattractant protein 1(MCP-1), growth regulated oncogene (GRO). The values were derived from the intensity observed on the membrane with the Image J software.

6.2.3. Modulation of cytokine production upon gp120 stimulation

The cytokine storm that appears during HIV acute infection has been reported based on plasma samples, and includes up-regulation of cytokines such as TNF-α, IFN-γ , IP-10, MCP-1, IL-6, IL-8 and IL-10 (Stacey *et al.* 2009). It has also been shown that macrophages infected with HIV *in vitro* overexpress β-chemokines such as MIP-1α, MIP-1β, RANTES and MCP-1. Whilst both assays reported in the previous sections showed an up-regulation of TNF-α, IL-6, G-CSF, and MIP-1β, they showed different trend of IP-10, MIF and RANTES regulation. Both assays showed a slight down-regulation of IL-8 and MCP-1.

The PBMCs- and U-937-based assays are both in agreement with previous studies showing that gp120 induces IL-6 expression through the NF-κB pathway on astrocytes

derived from human brain tissues (Shah *et al.* 2011), and that gp120 stimulated the expression of IL-6, TNF-α and IL-1β. Likewise, both assays show up-regulation of G-CSF, MIP-1β and TNF-α. However, moderate down-regulation of IL-8 is observed, consistent with a study conducted on U-937 cells which showed that IL-8 down-regulated HIV-1 replication (Tiemessen *et al.* 2000).

However, the regulation of several cytokines diverge in the two assays. High IP-10 levels during the first stages of HIV infection have been shown to correlate with increased viremia and rapid disease progression (Jiao *et al.* 2012), by stimulating HIV replication in PBMCs and macrophages (Lane *et al.* 2003). IP-10 is up-regulated in the PBMCs assay, but down-regulated in the U-937 assay. Up-regulation of MIF, as observed in the PBMCs assay, has been evidenced in HIV-infected individuals, and especially during acute infection (Delaloye *et al.* 2012). Moreover, it has previously been shown that HIV mannose residues induced secretion by PBMCs and macrophages of the anti-inflammatory IL-10 cytokine (Ameglio *et al.* 1994; Borghi *et al.* 1995; Gessani *et al.* 1997; Shan *et al.* 2007), consistent with the expression levels shown in Figure 6.4.

The PBMC-based assay consequently appears to be the more reliable, and should be preferred in future experiments. It is impossible to draw a conclusion regarding the differential effect of sialylated glycans on cytokine production. In future experiments, additional controls such as LPS, IFN-γ and TGF-β could be included to assess the influence of gp120 glycans on activatory and inhibitory cytokine production.

6.3. Immunisation with oligomannose-enriched gp120

HIV envelope glycoproteins are covered by an extensive array of glycans that are synthesized by the host cell. The isolation of 2G12 and the characterisation of its epitope have led to a variety of approaches to design an immunogen that would mimic monomeric gp120

glycosylation (Lee *et al.* 2004a; Scanlan *et al.* 2007; Dunlop *et al.* 2008; Wang *et al.* 2008; Luallen *et al.* 2009). However, eliciting broadly neutralising antibodies towards these self-carbohydrates still remains a challenge (Astronomo *et al.* 2008; Joyce *et al.* 2008; Luallen *et al.* 2008; Morner *et al.* 2009; Astronomo *et al.* 2010b; Dunlop *et al.* 2010b; Kabanova *et al.* 2010; Luallen *et al.* 2010; Marradi *et al.* 2011; Clark *et al.* 2012).

Viral glycosylation of HIV envelope glycoproteins was shown to differ from host cell glycosylation, and even from the glycosylation of monomeric gp120 previously used in immunisation studies and clinical trials. These results, and in particular the predominance and cross-clade conservation of oligomannose-type glycans such as $Man_5GlcNAc_2$ on the viral envelope, should therefore guide the design of an immunogen that more closely resembles viral, trimeric gp120 glycosylation.

6.3.1. Immunogen design and formulation

The large abundance of the unprocessed $Man_5GlcNAc_2$ glycan on the viral envelope makes it an attractive target for vaccine design as it is not usually displayed by host glycoproteins. Viral gp120 glycosylation was closely mimicked by expressing gp120 in GnT I-deficient HEK 293S cells (referred to as 'gp120-Man5' in this chapter). Recombinant gp120 produced in this cell line displays predominantly the $Man_5GlcNAc_2$ glycan (67%) and the additional population of $Man_{6\text{-}9}GlcNAc_2$ glycans corresponding to the mannose patch (Section 3.2.2.).

Furthermore, two different approaches were used to improve the global immunogenicity and to induce a T-cell response that would otherwise be lacking with carbohydrate-only immunogens. The introduction of highly immunogenic motifs, such as nitroaryl groups, has been shown to terminate self-tolerance towards weakly immunogenic antigens in fields such as auto-immunity (Aulak *et al.* 2001) and cancer (Berd 2002). For

instance, nitration of murine TNF-α has been used to render the protein more immunogenic, and induce a long-lasting IgG response in mice (Gruenewald *et al.* 2008; Grunewald *et al.* 2009). In the particular case of HIV, where all bnAbs isolated to date share key features, such as a high level of somatic hypermutation, as well as long CDR H3 domains, the induction of a T_H response would be essential to trigger such events in an immunisation setting. Therefore, nitration of the gp120-Man5 peptide backbone, leading to 'NIT-gp120-Man5', was carried out in order to induce an immune response from T-helper cells and dendritic cells, as well as to help overcome self-tolerance.

In parallel, a toll-like receptor (TLR) agonist, monophosphoryl lipid A (MPLA), was used as an adjuvant. MPLA, a TLR4 agonist, is derived from lipopolysaccharide (LPS) but with reduced inflammatory toxicity and has previously been shown to foster durable antibody and antiviral T_H1-dependent cytotoxic T cell responses against self carbohydrate antigens (Baldridge *et al.* 2000; Thoelen *et al.* 2001; Mata-Haro *et al.* 2007; Casella *et al.* 2008).

6.3.2. Vaccination schedule

The study was conducted on 6 animals per group, with the first group receiving gp120-Man5 with MPLA, the second group NIT-gp120-Man5 with MPLA, the third group gp120-Man5 only, and the fourth group NIT-gp120-Man5 only. The immunisation schedule is detailed in Figure 6.7.

6.3.3. Serum binding affinity towards the immunogen

Sera collected from each bleed – weeks 0, 1, 2, 4, 6, 8, 12 – were assayed by ELISA.

	Adjuvant (MPLA)	Nitrated gp120
Group 1 gp120-Man5, MPLA	✓	
Group 2 NIT-gp120-Man5, MPLA	✓	✓
Group 3 gp120-Man5		
Group 4 NIT-gp120-Man5		✓

	Injection	Blood Collection
Week 0	✓	5mL
Week 1		2mL
Week 2	✓	5mL
Week 4	✓	5mL
Week 6	✓	5mL
Week 8		5mL
Week 12		Terminal bleed

Figure 6.7. Immunisation schedule
The study was conducted on 24 New Zealand white female rabbits split in four groups. Animals were injected intramuscularly at two sites (quadriceps) on weeks 0, 2, 4 and 6. Each animal received 16.5 μg of gp120 in 500 μL of PBS per dose. Animals from Group 1 and Group 2 received 20 μg of MPLA in 500 μL water per dose additionally.

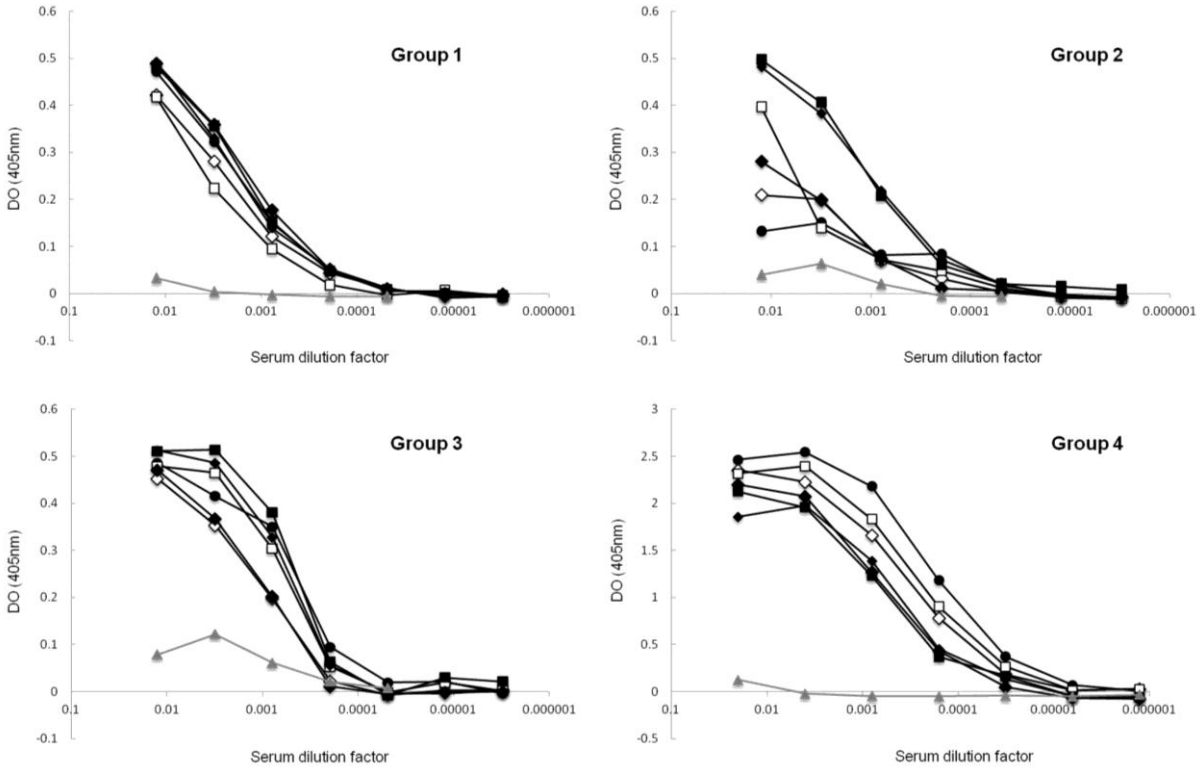


Figure 6.8. Serum binding gp120-Man5
ELISA plates were coated with gp120-Man5 overnight, and serial dilution of serum from week 8 was added. Detection was done using an anti-rabbit alkaline-phosphatase conjugated secondary antibody, read at 405 nm. The average absorbance obtained for the pre-bleed is indicated in grey. Group 1 corresponds to gp120-Man5 with the adjuvant MPLA; group 2 to NIT-gp120-Man5 with MPLA; group 3 to gp120-Man5; and group 4 to NIT-gp120-Man5.

The immunogen, gp120-Man5 from the HIV_{BaL} strain, was captured onto an ELISA plate overnight. The binding affinity of week 8 sera is shown in Figure 6.8 and the antibody titres obtained at half binding for week 2, 4, 6, and 8 sera are shown in Figure 6.9.

Sera from week 8 exhibited overall the higher antibody titers, with the following sera dilution obtained at half-binding: 1:660 for group 1, 1:1000 for group 2, 1:2500 for group 3, and 1:3300 for group 4. The results from this section show that the non-adjuvanted groups (ie. groups 3 and 4) exhibit a stronger immune response against the immunogen. Moreover, a slight increase in antibody titres was observed for inoculation with nitrated gp120.

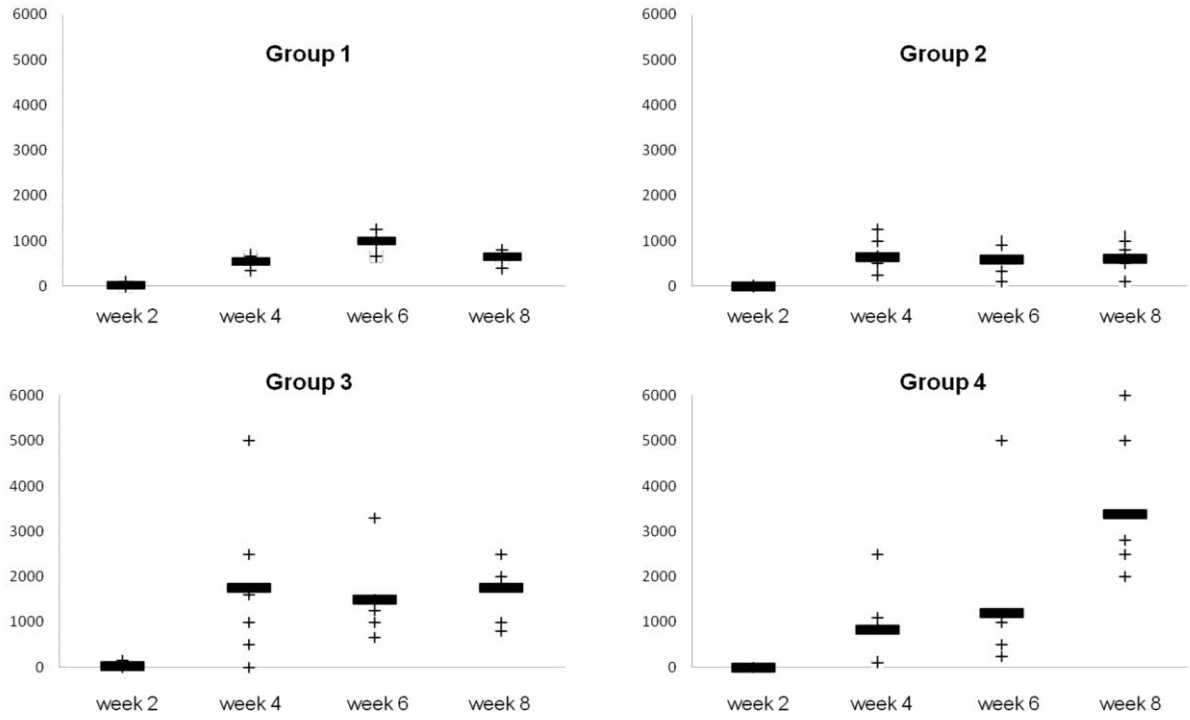


Figure 6.9. Antibody titers at OD_{50}
Antibody titres obtained at half binding for each group, for week 2, 4, 6 and 8 sera, assayed by ELISA againt gp120-Man5. Group 1 corresponds to gp120-Man5 with the adjuvant MPLA; group 2 to NIT-gp120-Man5 with MPLA; group 3 to gp120-Man5; and group 4 to NIT-gp120-Man5.

6.3.4. Cross-clade serum binding affinity

Next, the breadth of the antibody response was assessed by ELISA against gp120 from the clade B HIV_{Hxb2} (Figure 6.10) and the clade C HIV_{DU422} (Figure 6.11) strains. Week 8 sera showed similar binding affinity to $gp120_{BaL}$ and $gp120_{Hxb2}$. However, a 10-fold reduced affinity was observed on average for binding to the clade C $gp120_{Du422}$.

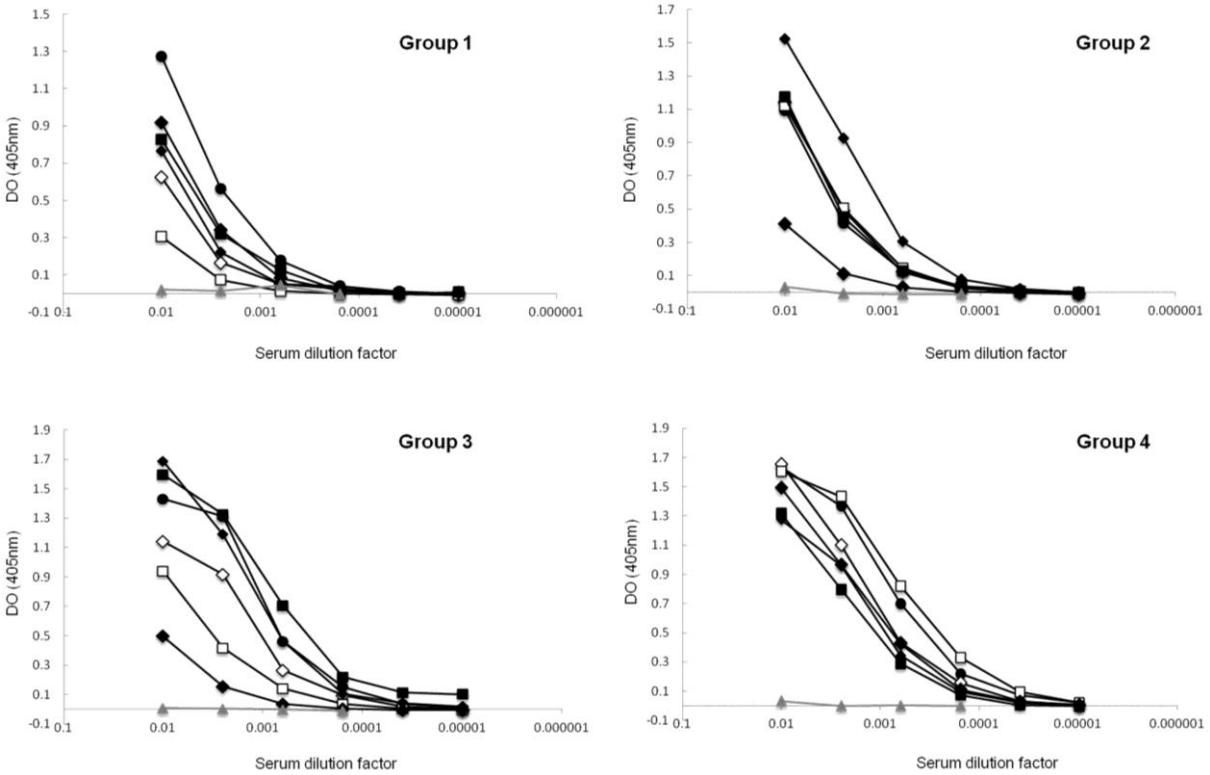


Figure 6.10. Serum binding to gp120 from the HxB2 strain
ELISA plates were coated with $gp120_{Hxb2}$ overnight, and a serial dilution of serum from week 8 was added. Detection was performed using an anti-rabbit alkaline-phosphatase conjugated secondary antibody, read at 405 nm. The average absorbance obtained for the pre-bleed is indicated in grey. Group 1 corresponds to gp120-Man5 with the adjuvant MPLA; group 2 to NIT-gp120-Man5 with MPLA; group 3 to gp120-Man5; and group 4 to NIT-gp120-Man5.

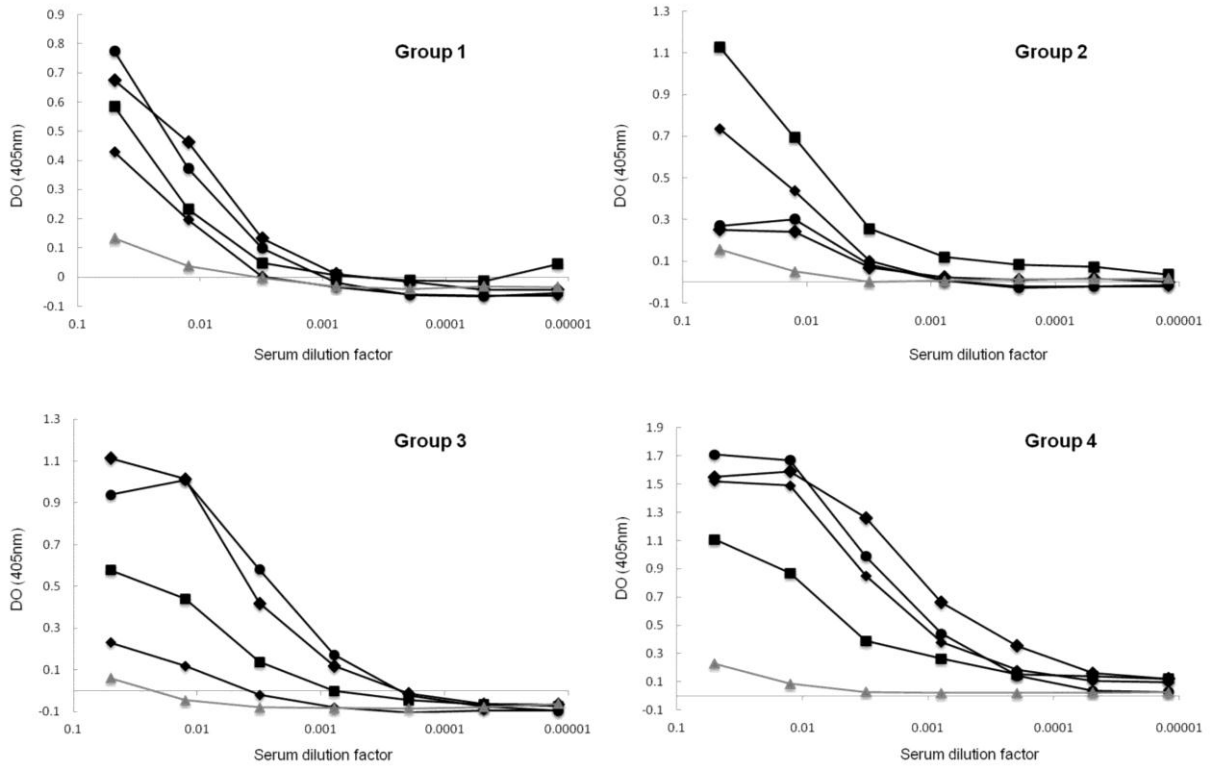


Figure 6.11. Serum binding to gp120 from the Du422 clade C strain
ELISA plates were coated with $gp120_{Du422}$ overnight, and a serial dilution of serum from week 8 was added. Detection was performed using an anti-rabbit alkaline-phosphatase conjugated secondary antibody, read at 405 nm. The average absorbance obtained for the pre-bleed is indicated in grey. Group 1 corresponds to gp120-Man5 with the adjuvant MPLA; group 2 to NIT-gp120-Man5 with MPLA; group 3 to gp120-Man5; and group 4 to NIT-gp120-Man5.

6.3.5. Neutralisation assays

The sera from week 8, which showed the highest antibody titres, were tested against a panel of HIV-1 pseudoviruses with the Monogram Neutralising Antibody Assay (Figure 6.12).

As observed with the preliminary ELISA binding studies, sera from group 3 and group 4 showed the most potent ability to bind to gp120 and thus prevent HIV infection. However, the inhibition was mainly restricted to the clade B isolate HIV_{SF162} and surprisingly did not act on the strain used in the immunogen development, HIV_{BaL}.

		Vaccine strain	Tier 1, clade B		Tier 2 clade B	Tier 2, multi-clade				control
		BaL	SF162	NL4-3	JRCSF	94UG103	92TH021	93IN905	IAVI-C22	aMLV
GROUP 1	Pre-bleed	N	N	N	N	N	N	N	N	N
	1	N	30	N	N	N	N	N	N	N
	2	N	10	N	N	N	N	N	N	N
	3	N	N	N	N	N	N	N	N	N
	4	N	30	N	N	N	N	N	N	N
	5	N	10	N	N	N	N	N	N	N
	6	N	30	N	N	N	N	N	N	N
GROUP 2	Pre-bleed	N	N	N	N	N	N	N	N	N
	1	N	N	N	N	N	N	N	N	N
	2	N	30	N	N	N	N	N	N	N
	3	N	30	N	N	N	N	N	N	N
	4	N	30	N	N	N	N	N	N	N
	5	N	N	N	N	N	N	N	N	N
	6	N		N	N	N	N	N	N	N
GROUP 3	Pre-bleed	N	N	N	N	N	N	N	N	N
	1	10	270	N	N	N	N	N	N	N
	2	10	90	N	N	N	10	N	N	N
	3	N	90	N	N	N	N	10	N	N
	4	10	90	N	N	N	N	N	N	N
	5	N	30	N	N	N	N	N	N	N
	6	N	270	N	N	N	N	N	N	N
GROUP 4	Pre-bleed	N	N	N	N	N	N	N	N	N
	1	N	270	N	N	N	N	N	N	N
	2	N	30	N	N	N	N	N	N	N
	3	N	N	N	N	N	N	N	N	N
	4	N	30	N	N	N	N	N	N	N
	5	N	10	N	N	N	N	N	N	N
	6	N	90	N	N	N	N	N	N	N

Figure 6.12. Neutralisation screen of week 8 sera against HIV-1 pseudoviruses
Sera from week 8 from each group were screened against a panel of pseudoviruses including clade B isolates (BaL, JRCSF, Sf162, NL4-3), clade A (94UG103, 92TH021) and clade C (93IN905, IAVI-C22). A negative control (aMLV, murine leukemia virus) was used. Positive results are highlighted in green – indicating at least 50% of inhibition of infection of an HIV strain (and an IC_{50} at least three-fold higher that the IC_{50} obtained against aMLV). Serum was tested for inhibition of HIV infection at dilution 1:10, 1:30, 1:90 and 1:270. The values indicated correspond to the highest serum dilution giving a positive call (i.e. at least 50% of inhibition of HIV infection). Group 1 corresponds to gp120-Man5 with the adjuvant MPLA; group 2 to NIT-gp120-Man5 with MPLA; group 3 to gp120-Man5; and group 4 to NIT-gp120-Man5.

6.4. Conclusion

Both branches of the immune system response were preliminary assessed in this chapter. HIV's interactions with the innate immune system has previously been evidenced, as for instance guanidine-uridin-rich ssRNA fragments of the HIV genome have been shown to interact with TLR7/8 and induce production of IFN-α and other cytokines (Heil *et al.* 2004). The assays performed here aimed to evaluate the influence of gp120 on cytokine production

in macrophages, and to assess the differential regulation due to sialic acid or mannose moieties. HIV-1 expression in latently infected cells is up-regulated by pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, TNF-β, and GM-CSF (Poli *et al.* 1994; Poli *et al.* 1990; Folks *et al.* 1989; Folks *et al.* 1987). Accordingly, IL-6 and TNF-α were up-regulated upon gp120 interaction with PBMCs-derived macrophages (Figure 6.4).

The adaptive immune system response to an oligomannose-enriched gp120 immunogen was also analysed. Four groups composed of 6 rabbits each were immunised with gp120 bearing mainly the $Man_5GlcNAc_2$ glycan. Groups 2 and 4 received a nitrated version of gp120-Man5, and groups 1 and 3 were co-administered with MPLA as an adjuvant. ELISA data (Figure 6.8) showed stronger binding for serum from the non-adjuvanted groups, as was confirmed with the pseudovirus neutralisation assays (Figure 6.12). Overall, nitration of gp120 was shown to slightly improve antibody titers as assessed by ELISA assays, but this trend was not evident in the pseudovirus neutralisation screening. Moreover, the use of MPLA as an adjuvant did not lead to an increased antibody response. Future immunisation studies could employ pseudoviral particles or recombinant trimer that are more immunogenic than gp120 glycoprotein (Beddows *et al.* 2005) and also an alternate choice for adjuvant or adjuvant formulation.

In future experiments, recombinant trimers could also be engineered with the techniques presented in this chapter to mimic even more closely viral Env glycosylation. The enhanced clustering of glycans on trimers compared to monomeric gp120 would allow further study of their influence in immune regulation, as well as guide immunogen design. Another interesting part would be to understand how antibody production is affected by the binding of gp120 to B-cell siglecs such as CD22. It has previously been shown that binding of tumour-produced mucins to CD22 on B-cells inhibits signal transduction through the B-cell receptor (Toda *et al.* 2009). Therefore, a mouse immunisation study could be conducted to monitor

antibody production for different gp120 glycoforms, including desialylated and hypersialylated gp120.

Chapter 7

Discussion

Mammalian N-linked glycosylation is strictly compartmentalised and follows a unidirectional and highly ordered processing pathway (Figure 1.7), which usually gives rise to a wide range of glycans structures that are typical of the cells from which the glycoprotein is derived. However, gp120 was shown to exhibit several degrees of protein-directed glycan processing, and a model for gp120 glycosylation is proposed, based on the results from Chapter 3 and Chapter 4, highlighting the divergences observed in glycan structures between host glycoproteins, recombinant gp120, viral Env and pseudoviral Env (Figure 7.1).

Protein-directed glycosylation had previously been reported for recombinant monomeric gp120 (Leonard *et al.* 1990; Cutalo *et al.* 2004; Go *et al.* 2008; Go *et al.* 2009), which bears several unprocessed oligomannose-type glycans that form the basis of the bnAbs 2G12 and PGT128 epitopes. This 'intrinsic mannose patch' was observed on the two isolates studied in this thesis, HIV-1_{BaL} and HIV-1_{JRCSF}, and its origin attributed to lack of processing by ER α-mannosidase I. The presence of a dense cluster of glycans on gp120 outer domain is thought to decrease glycosidase accessibility to glycan structures and result in the presence of unprocessed oligomannose-type glycans on gp120 surface. Whilst the mannose patch is a characteristic feature of monomeric gp120 glycosylation, the overall oligomannose-type

glycan abundance is likely to be strain-dependent, as observed for the two isolates studied in Chapter 3 and further observed in Chapter 5.

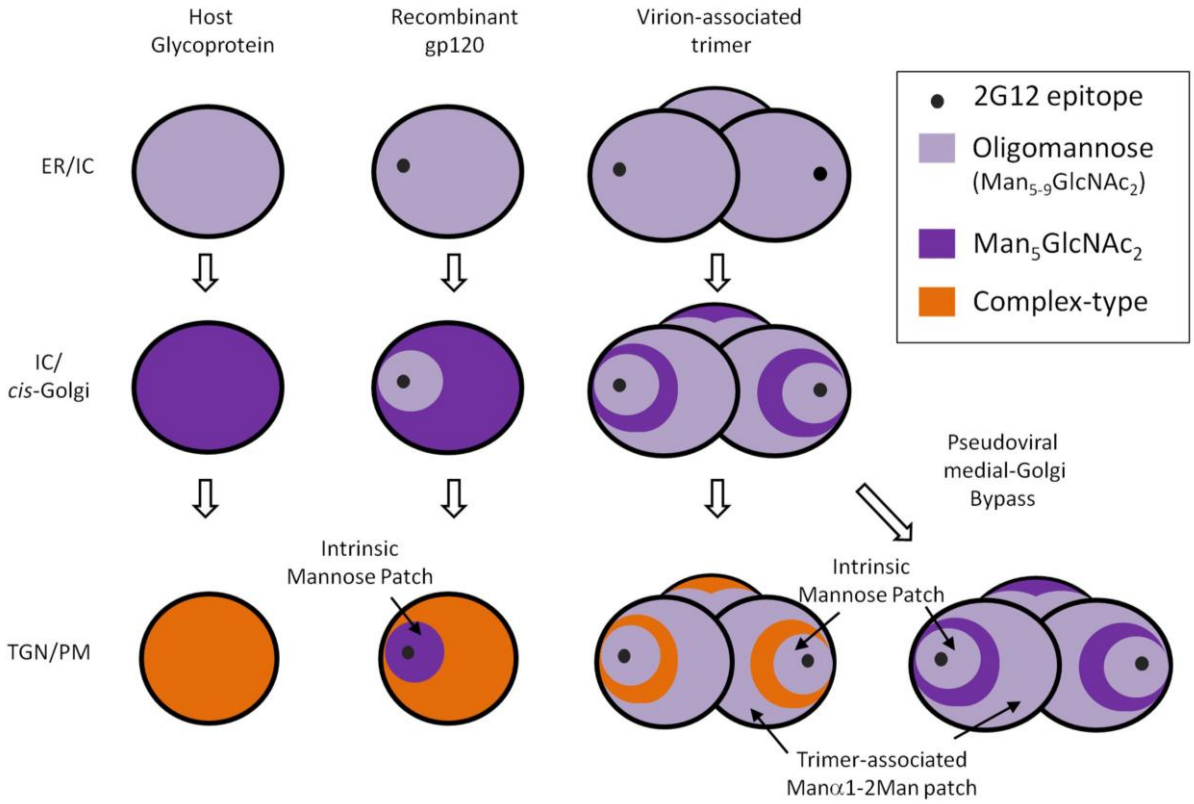


Figure 7.1. Envelope glycoproteins glycosylation model
Whilst gp120/gp41 glycans are processed by the host cell glycosylation machinery, the chemical composition of monomeric gp120 and native trimeric HIV Env glycans was shown to exhibit markedly constrained, protein-directed glycan processing when compared to that of the host cell. Following removal of terminal α-linked glucose residues in the ER, folded glycoproteins contain exclusively oligomannose glycans. During transit through the ER, intermediate compartment (IC) and *cis*-Golgi apparatus, Manα1→2Man termini are removed by ER α-mannosidase I and Golgi mannosidases IA-C to yield $Man_5GlcNAc_2$. However, the oligomannose cluster intrinsic to monomeric gp120 limits glycan processing on both monomeric and oligomeric gp120. The steric consequences of trimerisation further limit Manα1→2Man trimming leading to an additional 'trimer-associated' population of $Man_{5-9}GlcNAc_2$. The exposed $Man_5GlcNAc_2$ glycans on gp120 that passage through the full extent of the Golgi apparatus and *trans*-Golgi network (TGN) to the plasma membrane (PM) are processed by GnT I and subsequent enzymes to form complex-type glycans. However, envelope glycoprotein that does not follow this route to the plasma membrane is characterized by an elevated abundance of $Man_5GlcNAc_2$ and reduced furin cleavage. Thus the intrinsic mannose patch, which includes the 2G12 epitope, persists from the earliest stages of glycan processing whilst other elements of the glycan shield exhibit variably processed glycans depending on oligomerization state and, at least in the case of pseudoviral gp160/gp120, cellular trafficking.

Likewise, and to a further extent, trimeric HIV Env glycans exhibit markedly constrained, protein-directed glycan processing when compared to that of the host cell. The 2G12 and PGT128 epitopes are contained within a conserved $Man_{5-9}GlcNAc_2$ patch that is intrinsic to the monomer of gp120 and which is present on both monomeric and trimeric gp120. The oligomannose-type glycans found on recombinant monomeric gp120 constitute a population that is independent of envelope trimerisation. However, a much higher level of these Manα1→2Man terminating glycans was observed on the virus trimeric spike, indicating that these additional structures derive from a mechanism separate from the one leading to the formation of the monomer-intrinsic mannose patch. This suggests that given the extensive opportunities for glycan-glycan and glycan-protein interactions at the trimer interface, these structural constraints do alter HIV glycan processing.

The fundamental divergence between host and viral envelope glycosylation occurs at the biosynthetic transition from precursor oligomannose to mature complex-type glycans. The obligate committed step in the biosynthesis of complex-type glycosylation is the addition, by GnT I, of a single β1-2linked GlcNAc residue to $Man_5GlcNAc_2$. When this step is successfully achieved, that is for around 20-40% of Env N-linked glycans, the carbohydrates were further trimmed and fully processed into sialylated bi/tri/tetra antennary complex-type glycans.

A common feature of glycosylation of envelope isolated from pseudoviral particles is the presence of the $Man_5GlcNAc_2$ peak observed as the single most abundant glycan structure. When interpreted in the context of the biosynthetic pathway, gp120 derived from pseudoviral particles exhibits a lack of processing by GnT I or any of the subsequent Golgi-resident glycan processing enzymes. Evasion of GnT I processing is sufficient to result in the purely oligomannose range of glycans observed on pseudoviral particles. However, GnT I is fully functional in pseudovirus-producing cells, as complex-type glycans are observed on

soluble, non-virion associated gp120 isolated from culture supernatant. This lack of processing by Golgi-resident GnT I is consistent with higher abundance of uncleaved gp160 observed in pseudoviral system, as furin is also localised in the Golgi compartment.

A recent study has proposed that different envelope glycoforms were present on pseudovirion membrane (Crooks *et al.* 2011). It was proposed that the major component of envelope population was uncleaved monomeric gp160 bearing exclusively oligomannose glycans, thus avoiding processing by Golgi-resident glycosylation enzymes, and a residual number of fully functional trimeric gp120/gp41 spikes were covered by both complex- and oligomannose-type glycans. This study is in agreement with our results, where the membrane-associated gp120 was predominantly oligomannose, and the shed material resemble functional spike glycosylation.

The oligomannose-type glycan population on viral Env is also the target of bnAbs. Amongst the anti-carbohydrate bnAbs isolated, two main groups can be distinguished: antibodies targeting gp120 outer domain and involving the N332 glycan (PGT121-123, PGT125-128, 2G12, PGT135-137) and antibodies targeting a quaternary epitope involving gp120 V1/V2 loops and the N160 glycan (PG9 and PG16). The former have been shown to bind to oligomannose-type glycans, though their specificity is slightly different – 2G12 binds to Manα1→2Man linked oligomannose within $Man_9GlcNAc_2$ D1 arm, PGT135 bind preferentially to $Man_8GlcNAc_2$ D2/D3 arm, and PGT128 binds to moieties closer to the core $Man_9GlcNAc_2$ structure as well as D1 arm. Interestingly, PGT121 has been shown to recognise a sialylated bi-antennary complex glycan (Mouquet *et al.* 2012).

The high abundance of $Man_{5-9}GlcNAc_2$ structures on gp120 isolated from virus derived from PBMCs is consistent with the presence of anti-oligomannose antibodies in sera from HIV-1 infected individuals (Walker *et al.* 2010b). Furthermore, the conservation of the overall abundance of oligomannose-type glycans upon mutation of one or two key

glycosylation sites, as well as between isolated from clade A, B, and C, as shown in Chapter 4 and Chapter 5, indicates that oligomannose-type glycans are a conserved feature of viral glycosylation, and therefore a potential template for immunogen design.

The gp120 asparagine residue at position 332 is conserved between 73% of primary isolates, explaining the breadth of neutralisation of PGT128. However for antibodies such as 2G12 and PGT131-135 that recognise additional and less conserved glycans, a limited breadth of neutralisation is observed, and the deletion of one or several glycosylation site(s) impacts more on their neutralisation ability. Interestingly, it has been shown recently that two clade C infected individuals had developed neutralisation sensitivity to anti-N332 bnAbs over the course of infection (Moore *et al.* 2012), however the acute virus was found not to be glycosylated at this position. Glycan repositioning from 334 to 332 was due to immune escape from early strain-specific antibodies that targeted the highly immunogenic V3 region. In the particular subtype C, the N332 glycan is less conserved (66%), compared to all chronic virus (84%), probably facilitating transmission and early viral outgrowth. These results are extremely important for a vaccine strategy, whereby eliciting N332-specific antibodies might not stop the infection to establish, but would reduce the viral load and disease progression.

Finally, from the perspective of viral transmission and pathogenesis, it is proposed that the oligomannose glycans are required for the interaction with DC-SIGN on peripheral dendritic cells which promote the capture and dissemination of HIV in the early stage of infection. The broad conservation of the oligomannose array reported here suggests that such interactions are highly efficient for any isolate or clade of HIV.

HIV-1 Env glycans are not solely used by the virus as a mean of protection from the immune system, by repositioning, adding or deleting carbohydrates (Rao *et al.* 2010; Zhang *et al.* 2004). HIV envelope glycosylation is composed of a patch of oligomannose glycans, that take part in enhancing infectivity, and some highly processed and sialylated complex-type

glycans, potentially involved in interaction with immunosuppressive receptors such as siglecs and subsequent modulation of the host immune response. However, it has been shown that the immune system was exploiting the very same carbohydrates to develop a broadly neutralising response against the virus, and recent progress made in bnAbs isolation and characterisation, combined with the analysis and engineering of viral Env glycosylation will guide future immunogen design.

List of abbreviations

2-AA	Anthranilic Acid (2-aminobenzoid acid)
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCDC	Antibody-Dependent Complement-Dependent Cytotoxicity
ADCP	Antibody-Dependent Cell Phagocytosis
ADCVI	Antibody-Dependent Cell-mediated Viral Inhibition
AICD	Activation-Induced Cell Death
AID	Activation-Induced cytidine Deaminase
AIDS	Acquired Immuno Deficiency Syndrome
AP	Alkaline Phosphatase
APC	Antigen Presenting Cell
APOBEC	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like
B4GalT I	β1,4Galactosyltransferase I
BAFF	B-cell Activating Factor
BCR	B Cell Receptor
bnAb	broadly neutralising Antibody
BSA	Bovine Serum Albumin
CA	Capsid
CD4BS	CD4 Binding Site
CDR	Complementary-Determining Regions
CHO	Chinese Hamster Ovary
CLR	C-type Lectin Receptor
CRD	Carbohydrate Recognition Domain
CRF	Circulating Recombinant Form
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DCIR	Dendritic Cell Inhibitory Receptor
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DHB	2,5-dihydroxybenzoic acid
DR5	Death Receptor 5
DMEM	Dulbecco's Modified Eagles Medium
ELISA	Enzyme-linked immunosorbent assay
Endo H	Endoglycosidase H
Env	Envelope glycoprotein
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
ESCRT	Endosomal Sorting Complex Required for Transport
FACS	Fluorescence-activated cell sorting
Fc	Crystallisable fragment
FCS	Fetal Calf Serum
FcγR	Fcγ receptors
FITC	Fluorescein isothiocyanate
FUT	Fucosyltransferase
Gal	Galactose
GalT	Galactosyltransferase

GALT	Gut-Associated Lymphoid Tissues
Glc	Glucose
GlcNAc	N-acetylglucosamine
GnT	*N*-acetylglucosaminyltransferase
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
IC	Intermediate Compartment
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IN	Integrase
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
LC	Langerhan Cell
LPS	Lipopolysaccharide
LTR	Long Terminal Repeat
MA	Matrix
Man	Mannose
MALDI-TOF MS	Matrix assisted laser desorption ionisation – time of flight mass spectrometry
MHC	Major Histocompatibility Complex
MPER	Membrane-proximal external region
MR	Macrophage receptor
NB-DNJ	*N*-Butyl-deoxynojirimycin
NC	Nucleocapsid
Neu5Ac	N-acetylneuraminic acid
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEI	Polyethyleneimine
PIPES	Piperazine-*N,N′*-bis(2-ethanesulfonic acid)
PM	Plasma Membrane
PMA	Phorbol 12-myristate 13-acetate
PNGase F	Peptide *N*-glycosidase F
PNPP	p-Nitrophenyl Phosphate
PR	Protease
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RRE	Rev Response Element
RT	Reverse Transcriptase
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SHIV	Chimeric virus encoding HIV Env, Tat, Rev with an SIV backbone
Siglec	sialic acid binding, immunoglobulin-like lectins
SIV	Simian Immunodeficiency Virus
SP1 and 2	Spacer peptide 1 and 2
ST6Gal I	α2,6sialyltransferase I
TAR	Trans-Activating Response

TCR	T-Cell Receptor
TGF	Tumor Growth Factor
TGN	*Trans*-Golgi Network
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UDP	Uridine Diphosphate

Appendix A

Expression of recombinant CD22

To study the binding interaction between gp120 glycoforms and several members of the siglec family, soluble domain constructs of CD22 were cloned and expressed into 293T cells. CD22 (siglec 2) structure is composed of 7 Ig-like domains. The intracellular domain is composed of 6 tyrosine-based signalling motifs, 4 of which are ITIMs. It is expressed on the surface of B cell at the time of Ig gene rearrangement, and lost when mature B cells differentiate in plasma cells. It is believed to act as a negative regulator of B-cell activation, as it participates in the threshold for signalling via B cell receptors via association with the B-cell receptor (BCR). CD22, like the other siglecs, is masked by *cis* glycans, but when the B cell contacts another cell, CD22 can redistribute to the interaction region, allowing *trans* interactions. CD22 is homomultimerized via protein and Sia interactions – sialylation could thus lead to sequestration of CD22 from the BCR.

The siglec N-terminal V-set domain is responsible for sialic acid binding and specificity, and it was also shown that the adjacent C2 domain is required for activity (Crocker *et al.* 2007). There is currently no crystal structure of CD22 available, however the crystal structures of the terminal V-set domain of sialoadhesin (siglec 1) (Zaccai *et al.* 2007), and the two N-terminal domains of siglec 5 (Zhuravleva *et al.* 2008) and siglec 7 (Dimasi *et al.* 2004) led us to design several soluble CD22 constructs including but not limited to these

two N-terminal domains. The full-extracellular domain construct, composed of CD22 7 Ig-like domains, was successfully expressed in HEK 293T and GnT I deficient 293S cells (Figure A.1.).

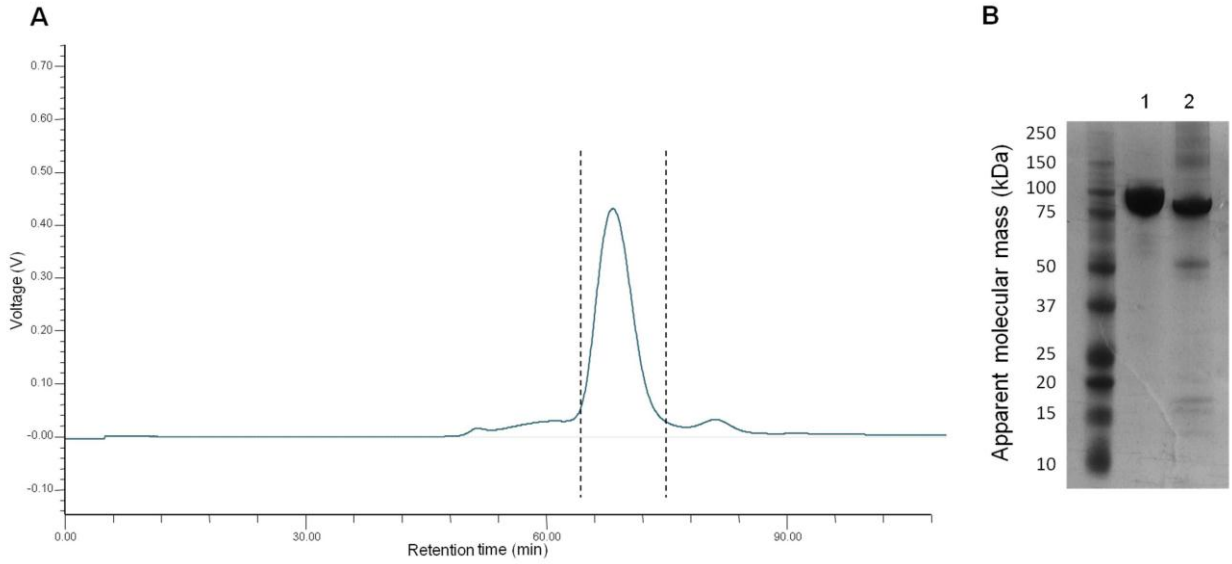


Figure A.1. Expression of the recombinant CD22 protein
(A) Size-exclusion chromatogram of metal-affinity purified CD22 expressed in HEK 293T cells. The peak corresponding to the collected fraction is indicated. **(B)** SDS-PAGE analysis of CD22: lane 1 corresponds to size-exclusion chromatography-purified CD22 expressed in HEK 293T, lane 2 to metal-affinity purified CD22 expressed in GnT I -/- HEK 293S cells. A shift, corresponding to the lack of glycan processing beyond the $Man_5GlcNAc_2$ intermediate in the GnT I-deficient cell line is observed.

The soluble CD22 glycoprotein expressed contains 12 potential N-linked glycosylation sites, and as such its molecular weight varies depending on the cell line in which it was expressed (Figure A.1.B). Disulfide bonds within and between each of the 7 Ig-like domains enhance protein stability. Both the protein expressed in GnT I -/- cells and a deglycosylated protein have been submitted for crystallographic analysis.

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