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DEFINING C3-V4 NEUTRALISATION EPITOPES ON HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 SUBTYPE C ENVELOPE GLYCOPROTEINS

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the of Master of Science in Medicine.

Johannesburg, 2011

Declaration

I, Constantinos Kurt Wibmer declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signed:  26 - 08 -2011

Dedication

As this is my first dedication, previously having nothing worth dedicating, I dedicate this work to my parentals Esther and Franz. They have poured everything into my education, and their sacrifices have not gone unnoticed. Thank you.

Acknowledgements

First and foremost I would like to thank my primary supervisor Dr. Elin Gray, who nurtured in me the ability to think laterally and without whom I would not call myself a scientist today. I would also like to thank Dr. Penny Lane for her insight and general daily 'dillyness', as well as my HOD Prof. Lynn Morris for additional supervision and for providing me with the space and funding to study at her unit. In addition to these I will be forever indebted to my companion and fellow MSc student Jinal Bhiman, who forced me to tie up the loose ends when I never really cared to.

Furthermore I extend my gratitude to everyone else past and present (staff and students) at the NICD AIDS Virology Unit for all their help in not only this work but also other projects I took on at our lab. I would like to acknowledge the bursary funding received for this project from the National Research Foundation, the Poliomyelitis Research Foundation, and the Wits Postgraduate Merit Award.

Publications from this work

Elin S. Gray, M. Anthony Moody, Constantinos Kurt Wibmer, Xi Chen, Dawn Marshall, Joshua Amos, Penny L. Moore, Andrew Foulger, Jae-Sung Yu, Bronwen Lambson, Salim Abdool Karim, John Whitesides, Georgia D. Tomaras, Barton F. Haynes, Lynn Morris, and Hua-Xin Liao (2011). Isolation of a Monoclonal Antibody That Targets the Alpha-2 Helix of gp120 and Represents the Initial Autologous Neutralizing-Antibody Response in an HIV-1 Subtype C-Infected Individual. Journal of Virology Vol. 85, No. 15, P. 7719-7729.

Presentations from this work

Early autologous neutralising antibodies targeting epitopes in the C3 and V4 or V5 regions of HIV-1 clade C envelope proteins are apparent on monomeric gp120

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Presented at the University of the Witwatersrand Faculty of Health Sciences Research Day (22nd September 2010), and the 3rd Cross-Faculty Postgraduate Symposium (27th-29th October 2010).

Early Autologous Neutralising Epitopes in C3-V4 and β14+V5 are readily adsorbed with monomeric gp120

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Presented at the AIDS Vaccine Conference, Bangkok, Thailand (11th-15th September 2011).

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List of Abbreviations

AIDS	- Acquired Immune Deficiency Syndrome
BSA	- Bovine Serum Albumin
CAPRISA	- Centre for the AIDS Programme of Research in South Africa
CCR5	- C-C motif Chemokine Receptor type 5
CD4	- Cluster of Differentiation 4
CDRH3	- Complementarity Determining Region-3 (Heavy chain)
CRF	- Circulating Recombinant Form
CXCR4	- C-X-C motif Chemokine Receptor type 4
DC-SIGN	- Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non- integrin
DMEM	- Dulbecco′s Modified Eagle′s Medium
DNA	- Deoxyribonucleic Acid
ELISA	- Enzyme-Linked Immunosorbent Assay
F_{ab}	- Fragment antigen binding
FBS	- Foetal Bovine Serum
F_c	- Fragment crystallisable
gp120	- HIV envelope glycoprotein (120 kilodalton)
gp41	- HIV envelope glycoprotein (41 kilodalton)
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	- Human Immunodeficiency Virus Type-1
HRP	- Horseradish Peroxidase
ID_{50}	- Inhibitory Dilution (at 50% inhibition)
IgG	- Immunoglobulin G
LB	- Lauria Bertoni
MPER	- Membrane Proximal External Region
mRNA	- messenger Ribonucleic Acid
PAGE	- Polyacrylamide Gel Electrophoresis
PBS	- Phosphate Buffered Saline
PCR	- Polymerase Chain Reaction
PEI	- Polyethyleneimine
pPPI4	- plasmid PPI4
RLU	- Relative Light Units
sCD4	- soluble CD4
SDS-PAGE	- Sodium Dodecyl Sulphate
SIV	- Simian Immunodeficiency Virus
SOC	- Super Optimal broth with Catabolite repression
Tris	- tris(hydroxymethyl)aminomethane
C1, C2, C3, C4, C5	- Conserved regions of gp120
V1, V2, V3, V4, V5	- Variable loop regions of gp120
V1-V2	- The entire V1 and adjoining V2 region
C3-V4	- The entire C3 and adjoining V4 region
C3+V5	- Discontinuous but structurally adjacent regions C3 and V5
β14+V5	- Discontinuous but structurally adjacent regions β14 and V5

List of Symbols

α	- alpha
β	- beta
Δ	- delta
μ	- mu
°	- degree
®	- registered trademark
™	- trademark

Amino Acid Abbreviations

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

Abstract

The rational design of an HIV-1 vaccine immunogen able to induce potent, cross-reactive, neutralising antibodies remains one of the single greatest challenges in the field of vaccine research today. Roughly a dozen broadly neutralising monoclonal antibodies have been isolated to date, and their epitopes represent important vaccination targets. Interestingly, apart from three that identify over-lapping epitopes in gp41, all of the broadly neutralising monoclonal antibodies target epitopes apparent on different conformations of gp120 (including the epitopes of PG9/PG16). Thus the gp120 monomer remains the most ideal template for immunogen design. Recently, epitopes in the C3-V4 region of gp120 have been shown to be major targets for early strain-specific neutralising antibodies in subtype C infected individuals. Autologous neutralising antibodies identify vulnerable sites on the envelope, and understanding the nature of antigenic "hotspots" on gp120 will help to guide rational vaccine design. This study sought to confirm in four individuals that the C3-V4 epitope was in fact apparent on monomeric gp120, and thereafter to better characterise the nature of viral escape from these antibodies. Using magnetic beads coated with one of 16 different recombinant gp120 proteins it was confirmed that the C3-V4 response was aimed at a monomer-specific epitope in all four cases. In two instances these antibodies were shown to contribute to autologous neutralisation, while in a third the existence of quaternary structure specific antibodies that could not be adsorbed with monomeric gp120 made this link impossible. In the forth instance transfer of the C3-V4 region was shown to expose a normally occluded epitope in the CD4 binding site. This research also provided evidence for other epitopes for autologous neutralising antibodies in C3, overlapping with the CD4 binding site and V5. Lastly, by introducing relevant escape mutations into the parental recombinant gp120s and then comparing the ability of these proteins to adsorb out anti-C3 antibodies, it was shown that while these mutations conferred complete resistance to neutralisation they did not prevent the antibodies from binding to their respective epitopes. The extensive characterisation of C3-related epitopes such as those described in this research should no doubt contribute to the rational design of a gp120 based vaccine immunogen aimed at eliciting broad and potent neutralising antibody responses.

Chapter One: Introduction

Although the HIV/AIDS epidemic appears to be stabilising, recent statistics released by the World Health Organisation still counts approximately 33 million people living with HIV across the globe as of 2008. Two thirds of these people are located in sub-Saharan Africa and an astonishing 17% (5.7 million people) live in the Republic of South Africa (Joint United Nations Programme on HIV/AIDS and World Health Organization, 2009). Every year roughly three million people are infected with HIV and two million people die from AIDS related illnesses. Although efficient antiretroviral programmes are dramatically increasing the life expectancy of HIV-infected people, an effective means for preventing the spread of infection is not yet generally available. After a recent trial conducted in South Africa, the success of an intra-vaginally applied microbicide gel in preventing infection was reported (Abdool Karim et al., 2010). No doubt with much more research, microbicide gels will afford a valuable means with which to control infection. However a microbicide gel serves only as a single barrier and once an HIV virion has crossed the mucosa it is no longer effective. Also the success of such a product correlates significantly with strict adherence. Pre-exposure chemoprophylaxis is another prevention strategy shown to significantly reduce transmission rates in people at high risk for HIV infection (Grant et al., 2010). However this strategy is also dependent on strict adherence, and may further compound the problem of drug resistance for people already on anti-retroviral treatment. A vaccine able to induce a potent immune response offers a far more practical solution to the HIV/AIDS epidemic. Once a target cell is infected, the virus integrates into the host's genome and sets up a viral reservoir which serves as a constant source of new viruses. To successfully clear this infection, vaccination may need to induce both neutralising antibodies and an effective cytotoxic immune cell response (Bramwell and Perrie, 2005). It is of potential significance though that most antiviral vaccines today work primarily by inducing neutralising antibodies (Pantaleo and Koup, 2004). In fact promising work in macaques has shown that the passive transfer of neutralising antibodies is able to block infection by chimeric SIV-HIV viruses (Mascola et al., 2000; Parren et al., 2001). However the elicitation of broadly cross-reactive neutralising antibodies has proven to be a daunting task, both in natural infection and after conventional vaccination strategies. Resolving the structure of the HIV envelope glycoprotein sub-units and their interaction with neutralising antibodies has helped researchers to understand some of the requirements for a good vaccine immunogen (Calarese et al., 2003; Song et al., 2009; Zhou et al., 2010; Zhou et al.,

2007). Unfortunately much is still unknown about the nature of accessible, immunogenic epitopes on the envelope surface. This is even further compounded by subtype specific differences in HIV antigenicity (Gnanakaran et al., 2007). Although there are at least nine different viral subtypes, clade C is the most predominant and accounts for roughly half of all global infections (Hemelaar et al., 2006). This is largely due to the fact that subtype C makes up the majority of infections in the world's most heavily burdened countries in sub-Saharan Africa and sub-Central Asia. As a consequence the South African population is an ideal research base from which to study the diverse viral antigenicity and subsequent host responses generated by one of the largest epidemics in modern history.

HIV-1 Envelope Glycoprotein Synthesis

The envelope glycoprotein is preceded by a long (approximately thirty amino acids) hydrophobic signal peptide with a short polar N-terminal cap (Wain-Hobson et al., 1985). The signal peptide is responsible for co-translational translocation of the envelope glycoprotein into the rough endoplasmic reticulum. It is then cleaved off from the final protein structure (Ellerbrok et al., 1992). The secreted glycoprotein is covered by approximately 30 potential N-linked glycans that make up a third of its 160 kDa net weight (Geyer et al., 1988; Wain-Hobson et al., 1985). These glycans are added during translation and are important for proper folding of the envelope glycoprotein (Land, Zonneveld, and Braakman, 2003). They are almost exclusively oligomannose in structure, with a low proportion being processed further to complex glycans (Geyer et al., 1988). Once properly folded and glycosylated, the envelope glycoprotein is transported to the Golgi complex and cleaved by host cellular proteases to yield non-covalently associated gp120-gp41 dimers (Stein and Engleman, 1990). The pro-protein convertases and in particular the serine protease furin are implicated in recognition and processing of the gp120-gp41 cleavage motif R-X-K/R-R (Moulard and Decroly, 2000). The gp41 monomer is membrane embedded and contains the membrane fusing machinery (Chan et al., 1997). Trimeric complexes consisting of three gp41 and three gp120 non-covalently linked sub-units localise to lipid rafts on the host membrane, which serve as the sites for viral assembly and eventual budding of nascent virions (Campbell, Crowe, and Mak, 2001). The glycoprotein trimeric complex represents the sole target for neutralising antibodies.

Structure of the gp120 monomer

Population sequence analysis traditionally divides the HIV-1 envelope gene into five conserved regions (Figure 1A) separated by five interjecting variable regions (Figure 1B) (Modrow et al., 1987). Within the conserved gp120 amino acid sequence are usually nine disulphide bonds, and four of these cystine pairs traditionally define the stems of variable loops V1, V2, V3, and V4 (Leonard et al., 1990). However there exist inter-clade structural differences such an additional disulphide bond found in the V4 loop of CRF_AE (McCutchan et al., 1996; Mokili et al., 2002; Trkola et al., 1995).

The crystal structure of gp120 derived from several strains has been elucidated in complex with a soluble CD4 receptor and various co-receptor binding site directed monoclonal antibody fragments (Diskin, Marcovecchio, and Bjorkman, 2010; Huang et al., 2007; Huang et al., 2005; Kwong et al., 1998; Pancera et al., 2010a). Crystal structures of gp120 have also been obtained in complex with various anti-CD4 binding site antibodies (Chen et al., 2009; Zhou et al., 2010; Zhou et al., 2007). In addition the crystal structure of an unliganded SIV_{mac} gp120 has also been determined (Chen et al., 2005). To facilitate crystal formation these structures lack the hyper-variable V1-V2 domain, and in most instances portions of the N-terminal and C-terminal ends and V3 loop. However, overall they divide the gp120ΔV1-V2 core into three conserved structural domains (Figure 1C). These are the inner, outer, and bridging sheet domains (Kwong et al., 1998). The outer domain is the most solvent exposed in the context of the trimer and forms the primary neutralisation determinant on gp120 (Zhou et al., 2007). The inner domain is highly conserved and represents the region of gp120 that is shielded from solvent at the trimer interface. The bridging sheet is composed of elements in C1, C2, and C4, forming a mini domain that makes up part of the CD4 and co-receptor binding sites. A recent crystal structure was the first to include the N- and C- terminal structures (Figure 1D). These 79 amino acids define a previously unidentified seven stranded β-sandwich containing predominantly gp41 contacting residues (Pancera et al., 2010a). This structurally invariant region provides an immobile face for gp120-gp41 non-covalent interactions. In contrast by comparing the liganded and unliganded structures it seems that radically different conformations are possible in the inner domain (Figures 2).

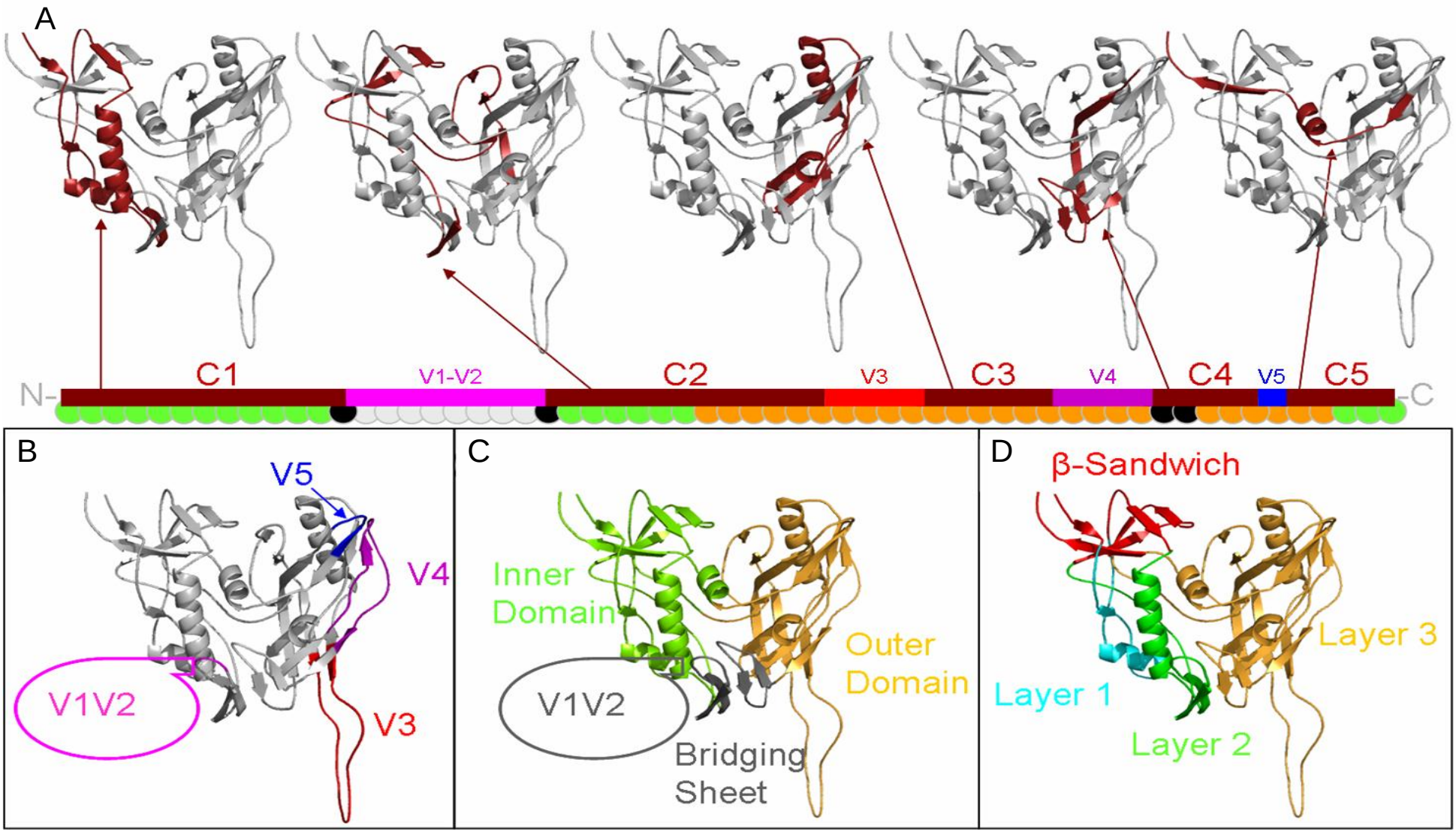


Figure 1: Structure of the HIV-1 envelope glycoprotein sub-unit gp120

(A) A linear schematic of the HIV-1 envelope sequence is shown where the conserved regions C1-C5 are indicated in maroon, and interjecting variable sequences V1-V2, V3, V4, and V5 are shown in pink, red, purple, and blue respectively. The location and secondary structure of each of the conserved regions within the gp120 molecule are indicated by ribbon diagrams. That part of the sequence that makes up the inner domain (green), outer domain (orange), the V1-V2 domain (grey), or the bridging sheet (black) is indicated by coloured circles. **(B)** Ribbon diagram showing the location of variable loop structures on the gp120 molecule, coloured as in (A). **(C)** Sub-domain structure of the gp120 monomer is shown and coloured as in (A). **(D)** Ribbon diagram depicting the layered topology of the gp120 molecule. Layers one, two, and three are coloured cyan, green, and orange respectively. Protein structure images for this and all subsequent figures (unless otherwise stated) were created from the superimposed pdb files 2B4C and 3JWD using Swiss-PdbViewer programme (Guex and Peitsch, 1997) and Pymol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA (http://www.pymol.org).

Of course the unliganded SIV gp120 core has a greater conformational freedom and the crystal structure that was resolved may not reflect the native conformation of the HIV-1 gp120 protein monomer. Nevertheless it implies that multiple conformations are possible within a single gp120 monomer, and these rearrangements potentially mask the receptor binding sites (Chen et al., 2005). From the seven stranded beta sandwich three topological layers extent outward by means of flexible loop structures. Layers one and two of the inner domain serve to stabilise the CD4 bound conformation through extensive aromatic hydrophobic packing interactions (Kassa et al., 2009). Surprisingly many of the amino acids that are important for stabilisation of the envelope oligomer are found in the layer two associated alpha helix, which is not surface exposed in the CD4 bound conformation (Finzi et al., 2010). In addition this alpha helix displays the greatest conformational plasticity between liganded crystal structures (Chen et al., 2009). Therefore current thinking implicates layer two as the primary structure involved in the refolding of gp120 between liganded and unliganded conformations.

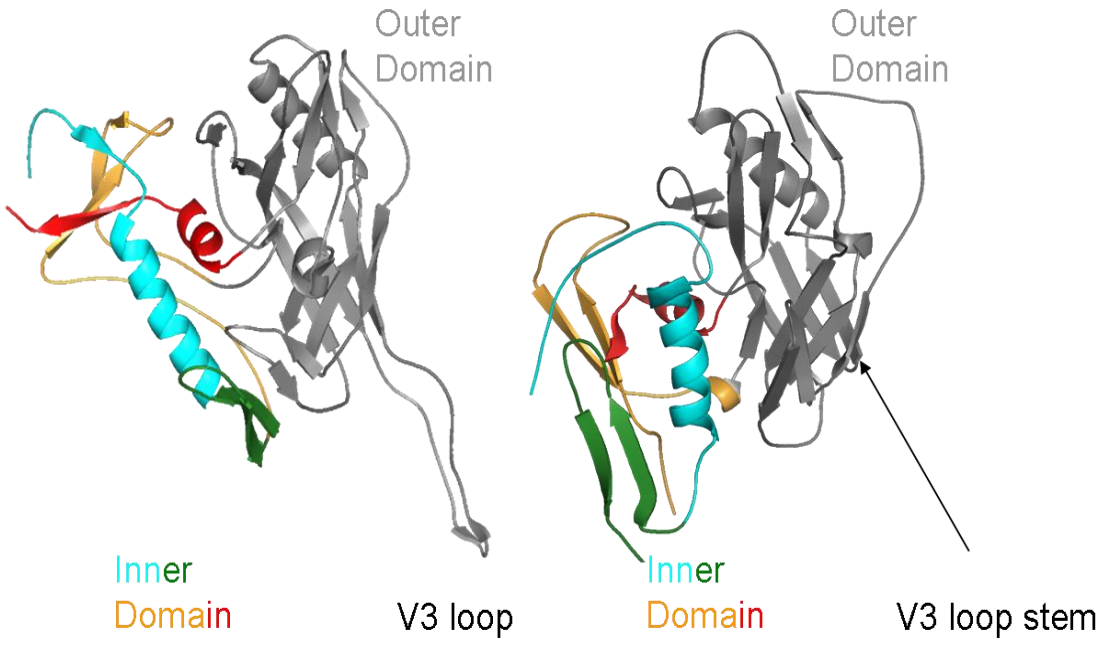


Figure 2: Conformational plasticity of the inner domain of gp120
The liganded crystal structure of the HIV-1 gp120 molecule is shown on the left, while the unliganded SIV gp120 is shown on the right. The outer domain is coloured grey, while the inner domain is in multi-colours. C1 is coloured blue, β2 and β3 of the bridging sheet are coloured green, elements of the inner domain in C2 are coloured orange, while those in C5 are coloured red. The crystal structure of the SIV gp120 core has a truncation of the V3 loop not present in the HIV liganded structure. Figures were created from the pdb files 2B4C and 2BF1.

The CD4 binding site

The crystal structure of HIV-1_{HXB2} in complex with soluble CD4 has mapped the CD4 binding site to a complex, discontinuous surface on gp120 (Kwong et al., 1998). CD4 contact residues reside in conserved regions of the bridging sheet domain, C2, C3, C5, and the stem of V5 (Figure 3). By scattering contact residues between several mobile secondary structures, binding of the host CD4 receptor to gp120 results in an unfavourable entropy ($-T\Delta S$) change of 44.2 kcal/mol (Kwong et al., 2002). It is the highly specific nature of the interface and thus the highly favourable enthalpy of binding (-54.62 kcal/mol) that renders the interaction feasible. While the average contribution of entropy for standard antibody-protein interactions is approximately 7 kcal/mol, the average entropy of binding for CD4 binding site directed antibodies is 23.1 kcal/mol (Kwong et al., 2002). This means that potential neutralising antibodies targeting the CD4 binding site must have significantly favourable enthalpies to compensate for gp120 reorganisation. The CD4 binding site is further protected from antibodies by its occlusion both in monomeric gp120 and the native trimer. The contact surface exists in a crevice between the inner and outer domains. At the base of this recession a hydrophobic pocket is formed, and its interaction with Phe^{43} at the distal most tip of CD4 is crucial to favourable binding (Kwong et al., 1998). On the native trimer the large V1-V2 loop and an array of flexible glycans have been implicated in occluding the CD4 binding site (Binley et al., 2010; Pinter et al., 2004; Wyatt et al., 1995; Zhu et al., 2001). Despite these defence mechanisms almost all HIV-1 infected individuals develop antibodies that target the CD4 binding site of gp120, but most possess limited or no neutralisation potential (Gray et al., 2009). The epitopes for these antibodies are not accessible on the native trimer, and these B-cell specificities are probably induced by less constrained conformations of monomeric gp120 released into the blood from cellular debris or shed from the virus surface.

The co-receptor binding site

In addition to CD4, HIV-1 must also directly bind to a chemokine receptor such as CCR5 or CXCR4 in order to efficiently infect host cells. The binding of gp120 to co-receptors occurs via a separate discontinuous region to CD4 attachment however, the conformational rearrangements induced by CD4 are an essential precursor to this interaction (Lapham et al., 1999; Trkola et al., 1996; Wu et al., 1996). The co-

receptor binding site is thus similar to the CD4 binding site in that it is composed of multiple mobile regions of gp120 protected by a conformational mask (Figure 3). Residues that interact with the co-receptor are found around the bridging sheet, C4, and V3 (Cormier et al., 2001; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998). The V3 loop is highly immunogenic and HIV infected individuals often develop high titre antibodies to this region, but these are only capable of neutralising laboratory adapted isolates because the V3 epitope is occluded on primary isolates by the V1-V2 domain (Krachmarov et al., 2005; Pinter et al., 2004; Zhu et al., 2001). Reorganisation of trimeric gp120 following CD4 docking re-orientates the V3 loops in a drastically exposed conformation toward the host membrane allowing the V3 crown to bind the second extracellular loop of CCR5 (Huang et al., 2005). CD4 independent isolates have been isolated *in vitro* that constitutively present an exposed co-receptor binding site and are thus highly neutralisation sensitive (Kolchinsky et al., 2001). Thus it seems that CD4 dependence may have evolved as a mechanism to protect the co-receptor binding site from the immune system (Edwards et al., 2001). The V3 loop is a very basic structure, and charge changes particularly at positions 302, 304, and 315 are associated with viral tropism to either CCR5 or CXCR4 expressing cells. Amino acid numbering of gp120 described here and across this document corresponds to the numbering detailed by the HXB2 liganded crystal structure (Kwong et al., 1998).

Adhesion receptors

In addition to binding receptors on T-cells that mediate infection, HIV-1 virions are also able to bind other cellular receptors. Specific interactions with certain membrane proteins facilitate the rapid transport of infecting virions to various lymphoid tissues. A dense cluster of conserved oligomannose glycans on the outer domain of gp120 may have a conserved functional role in creating a specific binding site for DC-SIGN receptors and other C-type lectins expressed on the surface of dendritic cells (Hong et al., 2007). Through this interaction HIV virions are passively transported by dendritic cells from the mucosal tissue at the site of infection to secondary lymphoid organs rich in T-cells (Geijtenbeek et al., 2000). Lectin interactions have also been implicated in facilitating the CD4 independent endocytic infection of astrocytes (Liu et al., 2004). A tri-peptide loop in the V1-V2 domain comprised of residues 182-184 mediates binding to α4β7 integrins on T-cells and natural killer cells, and facilitates transport to the gut-associated lymphoid tissue (Arthos et al., 2008).

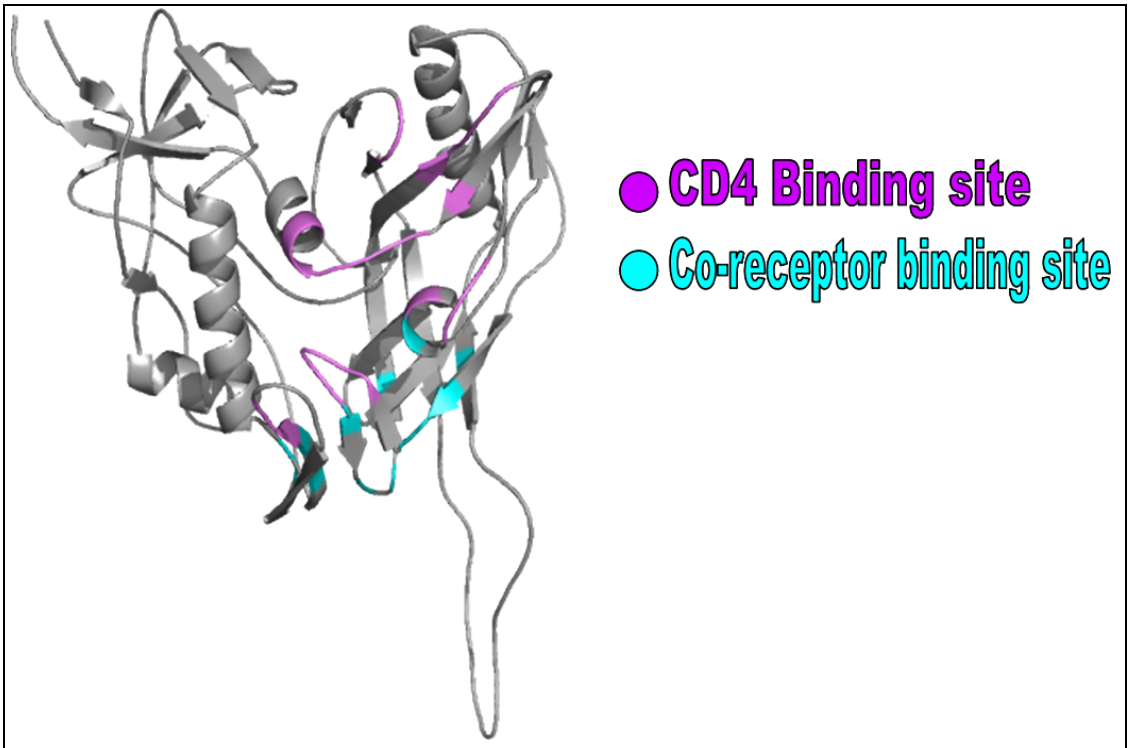


Figure 3: The complex receptor binding sites of HIV-1 gp120
A ribbon diagram of the HIV-1 envelope gp120 molecule depicting the CD4 binding site in purple and co-receptor binding site in cyan. The remaining gp120ΔV1-V2 molecule is shown in grey.

The role of Asn-linked glycans

The envelope glycoproteins are extensively glycosylated by N-linked glycans (Figure 4). Since the same carbohydrate moieties are post-translationally attached to host cell glycoproteins, these glycans act as a non-immunogenic barrier that shields the underlying peptide structure from the immune system. This divides the outer domain of gp120 further into a generally neutralisable and immunologically silent face based on epitope accessibility (Wyatt et al., 1998). A particularly dense patch of glycans on the outer domain of gp120 is comprised of only high molecular weight mannose-rich glycans (Zhu et al., 2000). They are resistant to mannosidases as a result of tight glycan packing, and are present in the context of both monomeric and native gp120 (Doores et al., 2010). The remaining glycans bordering the receptor binding sites and trimer interface are considerably more variable depending on the expression context. While some believe that the native envelope glycoproteins are covered in exclusively low molecular mass mannose-rich glycans, there is also convincing evidence to suggest that the viral trimer is dotted in an array of complex and hybrid glycans (Binley et al., 2010; Doores et al., 2010). Viruses grown in N-acetylglucosaminyltransferase I negative cells, which are unable to create hybrid or

complex glycans from mannosidase processed glycans, are ten-fold more sensitive to neutralisation by sCD4 (Binley et al., 2010). This suggests that an important role does exist for the few complex/hybrid glycans associated with the native trimer in protection of conserved epitopes. Glycans bordering the neutralisable face have been implicated in protecting the receptor binding sites and V3 loop from neutralising antibodies. Specifically glycans at N197 (V1-V2 stem), N301 (V3 stem), and N386 (V4 stem) have been implicated in protecting the CD4 and co-receptor binding sites (Gray et al., 2007b; Koch et al., 2003; Sanders et al., 2008b; Wu et al., 2009). As with many glycoproteins the glycans on gp120 have been shown to be essential for proper folding of the molecule (Li et al., 1993). Furthermore removal of the few glycosylation sites in gp41 prevents the gp160 protein from being properly processed and efficiently transported in the Golgi apparatus (Fenouillet and Jones, 1995). Thus the extraordinary glycosylation of HIV-1 glycoproteins is highly conserved due to their multifaceted functional role.

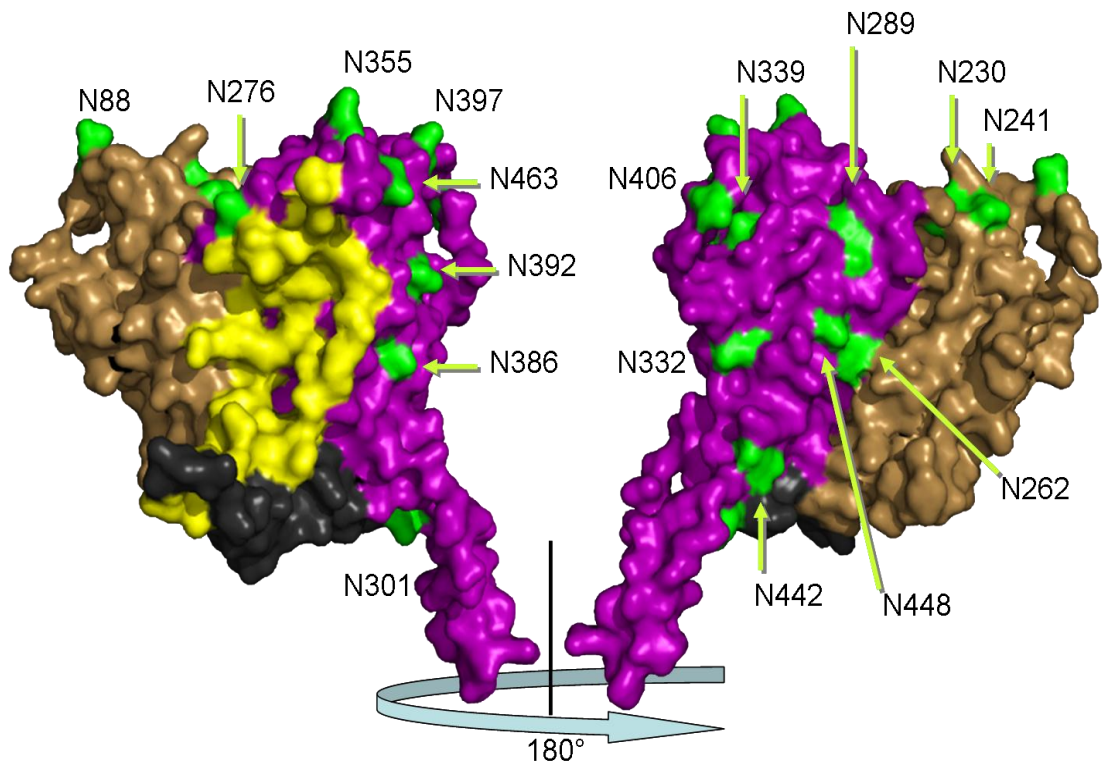


Figure 4: Extensive glycosylation of gp120
A surface model of gp120 showing the CD4 binding site (yellow) and asparagine residues potentially linked to glycan (green). The inner domain, outer domain, and bridging sheet are coloured brown, purple, and black respectively. The molecule is rotated around 180° to show all potential glycosylation sites associated with an HIV-1 subtype C consensus gp120 monomer.

Neutralising antibodies

Most HIV-1 infected individuals develop binding antibodies to immunodominant epitopes in gp41 within the first month of HIV-1 infection, followed to varying degrees by anti-gp120 antibodies (Tomaras et al., 2008). These antibodies have no neutralising potential and often target the positively charged co-receptor binding site and V3 loop. Autologous neutralising antibodies begin to develop within the first year of infection (Gray et al., 2007a; Tomaras et al., 2008). By definition they are not cross-reactive and target epitopes defined by variable structures such as those contained in V1-V2, V4, and V5. They develop to high titres, and exert enough immune pressure to force complete replacement of the viral population *in vivo* (Wei et al., 2003). Autologous neutralising antibodies develop in sequential waves targeting multiple distinct epitopes throughout the course of natural infection. This continual selective pressure drives chronological waves of escape across the envelope glycoprotein (Moore et al., 2009). However very few autologous antibody targets have been identified thus far, and their possible influence on the development of broadly cross-reactive neutralising antibodies later on in infection has yet to be elucidated.

Recent data suggests that approximately 25% of all HIV-1 infected patients go on to develop a moderately broad neutralising antibody response much later after infection (Doria-Rose et al., 2009; Sather et al., 2009). The lower frequency of these broadly cross-reactive antibodies in HIV-1 infected individuals when compared to the diverse autologous neutralising antibody response, as well as the later development of these antibodies, suggests that their highly conserved epitopes are less immunogenic than those recognised by strain-specific neutralising antibodies. This presents a considerable obstacle for vaccine design aimed at inducing broadly cross-reactive neutralising antibodies. An even smaller subset of the population (~1% of all HIV-1 infected individuals) classified as elite neutralisers develop exceptionally potent and broadly cross-neutralising antibodies (Simek et al., 2009). These antibodies probably best represent the kind of neutralising antibody response that must be induced by a vaccine immunogen. By identifying monoclonal antibodies with epitopes in these conserved regions, researchers hope to better understand the requirements of a vaccine immunogen able to elicit such antibodies.

Broadly neutralising antibodies targeting the receptor binding sites

The broadly cross-reactive neutralising antibody b12 recognises the CD4 binding site of gp120 and neutralises just under half of all primary isolates (Zhou et al., 2007). The crystal structure of F_{ab}b12 bound to the gp120 core reveals that it makes contacts primarily with the structurally rigid components of the outer domain, and thus does not incur an unfavourable entropic penalty upon contact of its epitope (Kwong et al., 2002; Zhou et al., 2007). The b12 antibody has an 18 amino acid long CDRH3 with a tryptophan at its tip which buries into the hydrophobic pocket normally recognised by Phe^{43} of CD4 (Saphire et al., 2001). Although this is longer than the average CDRH3 length in human antibodies interacting with proteins, it is not uncommon for antibodies targeting viral pathogens or glycans structures (Collis, Brouwer, and Martin, 2003). Crystal structures of gp120 bound to multiple CD4 binding site antibodies reveals that although they recognise only slightly divergent epitopes, the conformation of gp120 required for antibody interaction is not compatible with the predicted structure of the envelope trimer (Chen et al., 2009). F_{ab}F105 binding to gp120 opens up the bridging sheet like a book, significantly displacing the V1-V2 stem and exposing the hydrophobic face of the α1-helix in the inner domain. Binding of the F_{ab}b13 is similar to F_{ab}b12 in that it recognises an epitope in the outer domain however the angle of binding is 17° divergent and this results in a 152° displacement of the V1-V2 stem. In both cases the repositioning of the V1-V2 domain results in a predicted clash at the trimer interface and this is proposed to be the reason as to why these antibodies are non-neutralising.

Recently several other CD4 binding site directed antibodies have been discovered (Wu et al., 2010). Antibodies VRC01, VRC02, and VRC03 were all isolated from the same clade B infected individual (Wu et al., 2010). VRC03 binds to the same epitope as b12 but has significantly broader cross-neutralising potential. It has undergone extensive somatic mutation with 30% of its heavy chain residues and 20% of its light chain residues diverging from the germline sequence. VRC01 and VRC02 are clonally related and recognise the complete CD4 binding site, with an almost identical enthalpy and entropy of binding when compared to CD4. They are able to neutralise more than 90% of all viral isolates at remarkably low IgG concentrations and are the most broadly neutralising naturally occurring antibodies ever discovered.

Furthermore they have also undergone extensive affinity maturation of a magnitude comparable to VRC03. HJ16 has a similar neutralisation breadth to b12, but recognises a distinct epitope that is not reliant on the Asp^{368} residue in the CD4 binding loop important for most CD4 binding site antibodies described to date (Corti et al., 2010). It almost exclusively neutralises primary isolates, and in particular neutralises those viruses that are insensitive to neutralisation by b12. In fact a combination of antibodies HJ16 and b12 is able to neutralise more than two thirds of all HIV-1 isolates. The HJ16 antibody binds a recently identified epitope adjacent to the b12 epitope and significantly dependent on residues Asp^{474}, Met^{475}, and Arg^{476} in the α5-helix that joins the inner and outer domains (Pietzsch et al., 2010). The epitope has been designated CD4/DMR or more broadly $gp120_{core}$, and is potentially present in a large fraction of all cross-reactive plasma (Scheid et al., 2009).

The isolation of co-receptor binding site directed F_{ab} fragments with high neutralisation potential initially hinted at the possibility of exploiting this region in vaccine design (Moulard et al., 2002). Often these CD4 induced antibodies have long CDRH3 loops that are sulphated in an effort to mimic the sulphated N-terminus of chemokine receptors (Choe et al., 2003). However in the context of IgG these molecules lose their neutralisation potency due to the steric constraints imposed by the host membrane following CD4 engagement (Labrijn et al., 2003).

Broadly neutralising antibodies targeting gp41

Three anti-gp41 broadly neutralising antibodies (2F5, 4E10, and Z13) have been identified that target an overlapping epitope in the membrane proximal ectodomain region (MPER) of gp41 (Zwick et al., 2001). In addition to binding between host and viral membranes and thus sterically obstructing the fusion process, these antibodies also act to immobilise a hinge mechanism in the MPER which normally bends the viral membrane towards the host membrane after receptor engagement (Song et al., 2009). Two of these antibodies (2F5 and 4E10) again have unusually long CDRH3 regions with hydrophobic residues at their apex which are not involved in peptide binding but rather are essential for neutralisation by interacting with the viral membrane (Alam et al., 2009; Julien et al., 2010). Such auto-reactive antibodies are usually down-regulated in the context of a healthy immune system (Haynes et al., 2005).

Broadly neutralising antibodies dependent on glycans

The broadly neutralising antibody 2G12 recognises a mannose rich glycan arrangement on the immunologically silent face of gp120 (Sanders et al., 2002; Scanlan et al., 2002). This antibody has an unusual proline at residue 113 in the heavy chain framework at the base of the F_{ab} domain. This facilitates an exchange of heavy chain variable domains allowing them to pair with the opposite arm light chain variable domains in the context of a single immunoglobulin (Calarese et al., 2003). Thus 2G12 creates a third antigen combining site at the variable domains heavy chain juncture, and this increases the binding avidity and thus allows for efficient recognition of carbohydrate. More recently, by mapping the targets of plasma from individuals with broadly neutralising antibodies, a novel target dependent on the glycan at Asn^{332} (also important for 2G12) has been identified (Walker et al., 2010).

While antibodies to the V3 domain are for the most part non-neutralising due to steric occlusion, two antibodies named PG9 and PG16 (which are somatic variants of each other) have been identified that bind to a novel epitope in the V2-V3 domains of HIV-1 (Walker et al., 2009). These antibodies neutralise roughly 80% of all viral isolates and are very potent. This discovery was novel in that the V1-V2 domain was previously thought to only elicit type-specific neutralising antibodies (Kimura et al., 2009). PG9 and PG16 have the longest CDRH3 loops of all the above discussed monoclonal antibodies, with twenty eight amino acids forming a unique tyrosine sulphated sub-domain (Pejchal et al., 2010). Again, about fifteen to twenty percent of the heavy and light chain variable regions have been affinity matured (Pancera et al., 2010b). Although both antibodies display much sequence similarity, they recognise distinct but overlapping epitopes. PG16 is more heavily reliant on amino acids in V3 (Walker et al., 2009). While both antibodies are reliant on the glycan at Asn^{160} for proper epitope formation, PG16 is more sensitive to the precise glycosylation pattern of the envelope trimer (Doores and Burton, 2010). Collectively this data suggests that broadly neutralising antibodies appearing later in infection probably result from the affinity maturation of either autologous neutralising antibodies or other non-neutralising antibodies. This provides a new argument for identifying novel targets for autologous neutralising antibodies, and understanding the role they play in the development of broadly cross-reactive neutralising antibodies.

A novel target for autologous neutralisation in the outer domain

Along with the V1-V2 loops the solvent exposed outer domain is the primary target for neutralising antibodies. Recent work from our laboratory identified a novel target for autologous neutralising antibodies in the outer domain comprised of amino acids in the V4 loop and adjacent C3 region (Moore et al., 2008). The outer domain represents a conformationally stable β-barrel structure stabilised by inter-sheet hydrogen bonding as well as disulphide bonds at the base of V4, V3, and connecting C3 to C4 (Sanders et al., 2008a; Zhou et al., 2007). Most proximal to the bridging sheet the β-barrel structure is narrower and bulges out only to form the CD4 binding loop. At the opposite end of the β-barrel the amphipathic α2-helix is nestled between loop structures in C2, V4, and V5. These two alpha helices (the α2 and α3 helices) associated with the outer domain are contained exclusively within the C3 region of gp120 (Figure 5). Between the α2- and α3-helices in the C3 region is a highly variable β-sheet (β14). This sheet interacts with β-sheets in V4(β18) and V5(β24), and serves to bind these three structures together.

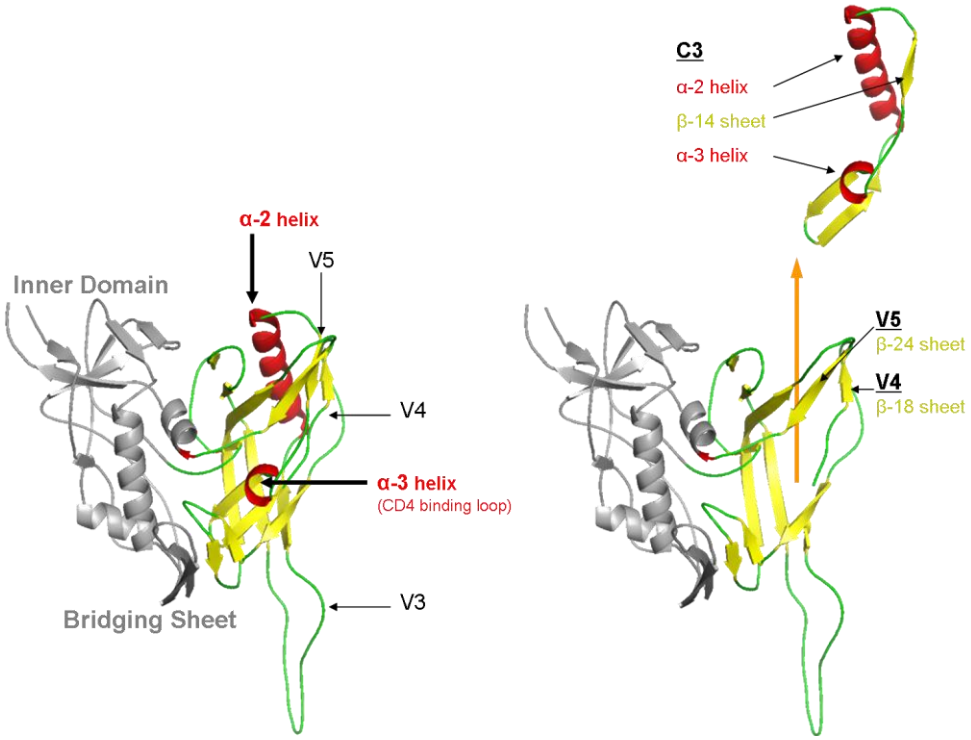


Figure 5: The outer domain forms a β-barrel that wraps around the α2-helix
The outer domain is coloured according to secondary structure with α-helices, β-sheets, and connecting loop structures coloured red, yellow, and green respectively. The C3 region contains the conserved CD4 binding loop, as well as the more variable α2-helix flanked by V4 and V5. Variable anti-parallel β-sheet structures β14(C3), β18(V4), and β24(V5) interact closely with each other.

Subtype specific selective pressures

Different regions of the HIV-1 envelope are subject to varying selective pressures in different clades (Choisy et al., 2004). In particular the V3 loop is more conserved in subtype C viruses when compared to subtype B viruses, while the α2-helix of subtype C is under greater selective immune pressure (Gaschen et al., 2002). The α2-helix has a more conserved amphipathic structure in subtype C when compared to other clades, with amino acids at positions 336 and 346 (Figure 6) more likely to be hydrophilic in subtype C, suggesting greater exposure of the helix (Gnanakaran et al., 2007). By superimposing the crystal structure of a recently published subtype C gp120 with the previously determined subtype B gp120 structures, and a recently crystallised subtype A gp120, the α2-helix of the clade C glycoprotein can be seen to be relatively more solvent exposed (Figure 7). In addition the V4 loop is generally shorter in subtype C when compared with subtype B and overall the α2-helix also shows a strong correlation of charge compensation with V4, where the α2-helix is more positively charged and the V4 more negative (Gnanakaran et al., 2007).

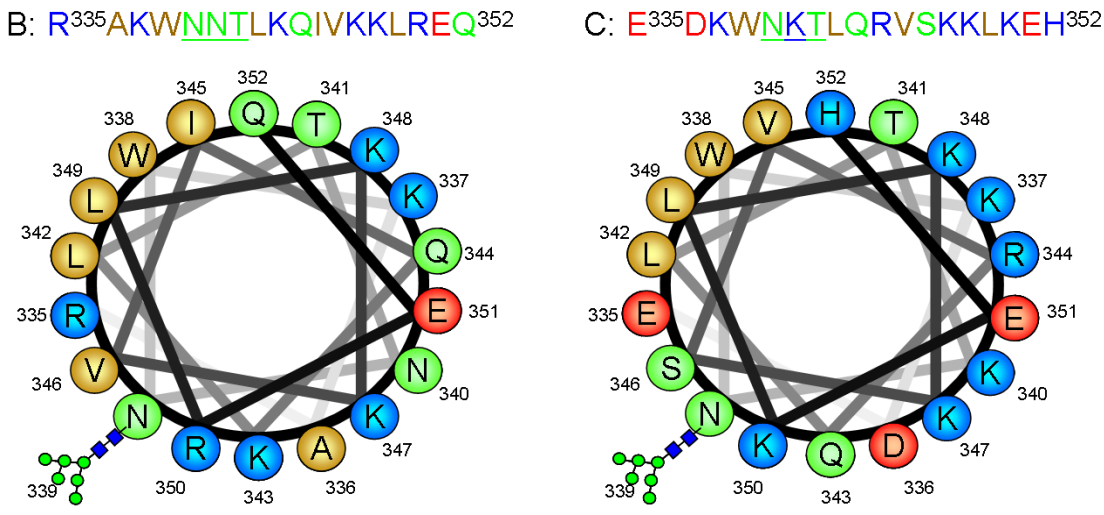


Figure 6: The subtype C α2-helix is more amphipathic
Helical wheel plot diagrams of the α2-helix from consensus subtype B gp120 and consensus subtype C gp120 sequences. The α2-helices are shown winding into the page from C-terminus (position 352) to N- terminus (position 335). Hydrophobic, non-charged polar, positively charged, and negatively charged amino acids are coloured brown, green, blue, and red respectively. The N-linked glycosylation site associated with the α2-helix is indicated with the symbol and underlined in the sequence.

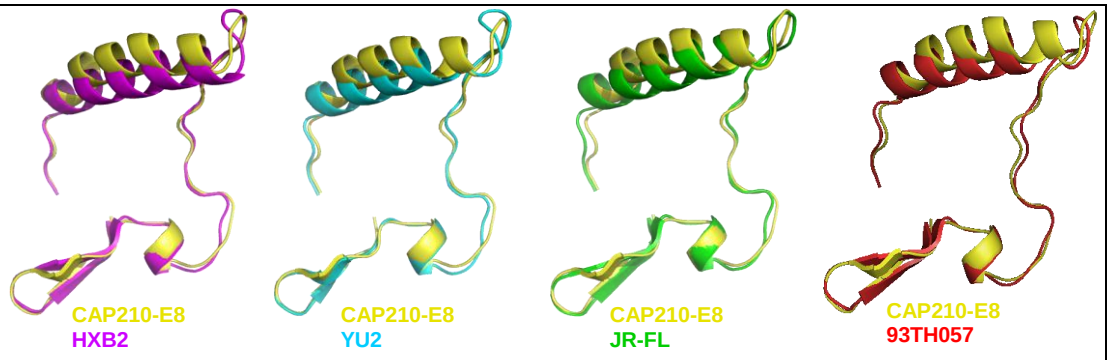


Figure 7: The subtype C α2-helix is more solvent exposed on monomeric gp120
The crystal structures of the HIV-1 gp120 C3 region from subtype B isolates HIV-1_{HXB2} (purple), HIV-1_{YU2} (blue), and HIV-1_{JR-FL} (green), as well as the subtype A isolate HIV-$1_{93TH057}$ (red) were all superimposed on the subtype C structure of HIV-1_{CAP210} (yellow) using the Swiss-PdbViewer programme (Guex and Peitsch, 1997). Structures were obtained from the pdb files 1G9M, 2QAD, 2B4C, 3NGB, and 3LQA.

Acquisition of length in the V4 loop is associated with resistance to autologous neutralising antibodies, and it could be speculated that part of this role involves escape via epitope occlusion from antibodies targeting the α2-helix. However, transfer of the α2-helix alone from a neutralisation sensitive donor virus to a neutralisation resistant recipient virus often does not alter the neutralisation phenotype of the latter (Rong et al., 2007). In contrast, by transferring the entire C3-V4 region it was possible to completely transfer neutralisation sensitivity to early autologous plasma, suggesting that the entire region was required to reconstitute these types of neutralisation epitopes (Moore et al., 2008). In this original study four individuals from the CAPRISA_002 Acute Infection Cohort (described below) were identified as containing anti-C3-V4 autologous neutralising antibodies: CAP45, CAP63, CAP84, and CAP88 (Appendix A). They were identified by creating heterologous chimeric viruses for the C3-V4 region as described above. The neutralisation epitopes recognised by CAP88 antibodies appeared to be directed towards the C3 region alone. Transferring the HIV-1_{CAP88} C3 region into a resistant heterologous virus resulted in the complete transfer of neutralisation sensitivity, while transferring the V4 region alone did not confer any neutralisation sensitivity. Similarly by transplanting the HIV-1_{CAP63} V4 region only, but not the C3 region alone, partial sensitivity to CAP63 plasma was transferred. This suggested that the epitopes recognised by CAP63 antibodies were more dependent on residues in V4 than in C3. In addition the use of viruses chimeric for other regions also allowed for the identification of other epitopes

for autologous neutralising antibodies in V1-V2 for participants CAP45 and CAP88, and in V5 for participant CAP84. Each chimera was tested for the acquisition of an unnaturally sensitive neutralisation phenotype by testing them against heterologous serum samples from multiple HIV-1 infected individuals. Since almost all HIV-1 infected individuals develop anti-V3 and anti-CD4 binding site antibodies, if the chimeric viruses adopted an exposed conformation they should become sensitive to heterologous plasma samples. None of the chimeras became universally sensitive to these unrelated plasma samples, suggesting that their neutralisation was mediated by strain-specific antibodies (Moore et al., 2008).

Escape from autologous neutralising antibodies

A second study from our lab looked at escape from autologous neutralising antibodies, and included participants CAP45 and CAP88 (Moore et al., 2009). The authors showed that the virus infecting CAP88 isolated at one month post infection was sensitive to the anti-C3 antibodies in CAP88 plasma, while the virus isolated from six months post infection was not. Sequence analysis identified three amino acids in the α2-helix under selective pressure. The acquisition of a potential N-linked glycosylation site at position (339 normally found in most viruses) and a charge change from glutamic acid to lysine at either position 343 or position 350. The potential glycosylation site acquired at position 339 was highly conserved by six months post infection, while the charge changes at positions 350 and 343 were each present in 50% of the population, suggested that their roles in escape were mutually exclusive. Amino acids at positions 343 and 350 have been previously identified as being under considerable selection pressure in subtype C viruses (Rong et al., 2007). They map to the same face of the helix as position 339, and are in a position to interact directly with V4 (Figure 8A). Removing the glycan at position 339 in a six month clone resulted in the partial restoration of neutralisation to plasma from an earlier time frame (Figure 8B, purple line). Once the charge change at position 350 was also incorporated, the six month virus became completely sensitive to earlier plasma (Figure 8B, pink line).

Escape mutations to neutralising antibodies in CAP45 plasma were also identified in a similar way. CAP45 had been previously shown to develop an antibody response to V1-V2 and C3-V4 (Moore et al., 2008). Of those residues identified as being under

positive selection, one amino acid in V1 (N147S) and two in V2 (R186K/E190aG[1]) were back mutated in a clone isolated at 12 months post infection. In so doing the later virus became partially sensitive to neutralisation by earlier serum samples (Moore et al., 2009). This was consistent with the previously identified anti-V1-V2 activity in CAP45 plasma. Surprisingly back-mutation of two residues in V5 (E460K, and G462D) made the later virus almost completely sensitive to plasma from all earlier time points. It was hypothesised that these two mutations must confer escape to the anti-C3-V4 antibodies, since back-mutation of all the remaining positively selected mutations in the escaped virus could not restore any sensitivity to earlier plasma samples (Figure 8B).

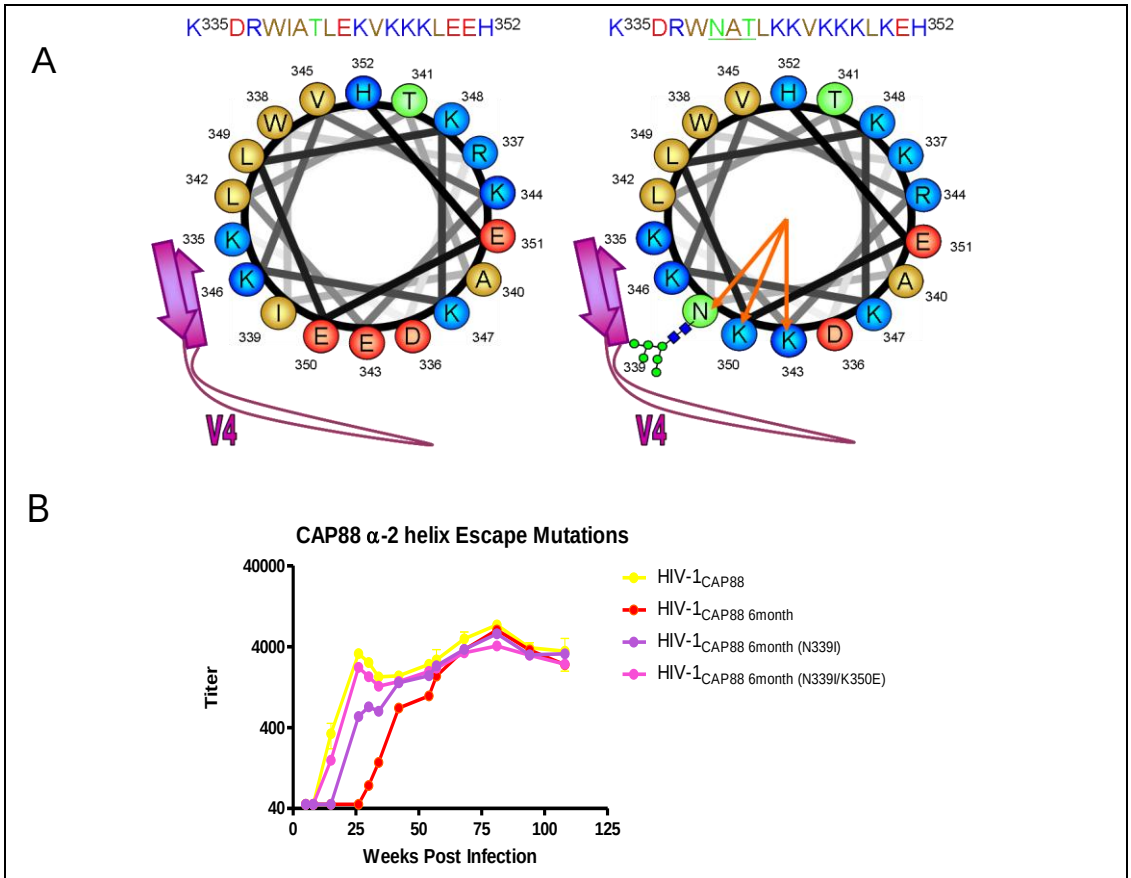


Figure 8: Escape mutations to CAP88 antibodies map to the α2-helix
(A) Helical wheel plot diagrams showing the CAP88 α2-helix winding into the page from C-terminus to N-terminus. The wild-type helix is shown on the left, while the escaped helix is shown on the right. Amino acids under positive selection at six months post infection are indicated with the orange arrows. The location of the V4 loop is shown in purple, and the adjacent N-linked glycosylation site is indicated. Amino acids are coloured as in Figure 6. **(B)** Escape from CAP88 neutralising antibodies (adapted from Moore *et al*., 2009). Titres against the one month infecting clone (yellow), six month clone (red), N339I back-mutant (purple), and N339I/E350K double mutant (pink) are shown.

[1] Referred to as R181K/E186G in the Moore *et al.*, 2009 study based on HIV-1_{CAP45} numbering

Project Aim

There is very little research published to date on autologous neutralising antibodies, with most of the work focused on identifying and characterising broadly cross-reactive antibodies. Autologous neutralising antibodies report which regions of the native envelope trimer are exposed for antibody mediated neutralisation. Furthermore they may provide important clues as to the origins of broadly neutralising antibodies. Given the recent identification of the C3-V4 region as a complex neutralisation epitope within gp120, the aim of this project was to better define the nature of this epitope in hope that it will contribute to the knowledge necessary to develop an HIV vaccine.

Main Objective

To determine whether C3-V4 autologous neutralising antibody epitopes are contained wholly within monomeric gp120, and to further characterise the nature of escape from the antibodies targeting this site.

Specific Objectives

- To determine whether the autologous neutralising antibodies in serum samples from CAPRISA_002 participants CAP45, CAP84, CAP63, and CAP88 could be adsorbed using autologous monomeric gp120.
- To ascertain whether these same serum neutralising antibodies could be adsorbed using chimeric gp120 containing an autologous C3-V4 region grafted into a heterologous gp120 backbone.
- To assess the effect of escape mutations introduced into wild-type autologous monomeric gp120 on the adsorption of anti-C3-V4 directed neutralising antibodies.

Chapter Two: Materials and Methods

The CAPRISA_002 Acute Infection Cohort

The cohort, enlisting 245 women at high risk for HIV infection, has been described previously (Gray et al., 2007a; van Loggerenberg et al., 2008). Sixty two women became infected with HIV and were enrolled into the study. They have been extensively monitored including CD4 count, viral load, and regular plasma/serum sampling. Although the primary aim was to document the evolution of the virus and host immune response during the course of acute infection, the majority of the participants have been successfully enrolled for more than three years and some as long as five.

The PPI4 expression vector

The pPPI4 expression vector was developed by Progenics Pharmaceuticals Inc. (New York, U.S.A.), and contains several elements designed to enhance recombinant gp120 expression as described previously (Binley et al., 2000; Trkola et al., 1996). These include a cytomegalovirus major immediate-early promoter-enhancer for high level constitutive expression, a tissue plasminogen activator signal peptide for efficient secretion of soluble gp120, and a bovine growth hormone polyadenylation signal to stabilise the transcribed mRNA.

Creating histidine tagged gp120 expressing plasmids

The gp120 coding regions of four subtype C viruses were amplified from plasmids containing the full length envelope genes. The envelope clones and their GenBank accession numbers are: CAP45.2.00.G3J (EF203960), CAP63.2.00.A9J (EF203973), CAP84.2.00.32J (EF203963), and CAP88.2.00.B5J (EF203927). Each of the four viruses represents the relatively homogenous infecting population circulating in four individuals of the CAPRISA cohort approximately one month post-infection, prior to the development of neutralising antibodies. They are thus sensitive to all host autologous neutralising antibodies. For each of these distinct envelope clones, a set of chimeric pseudoviruses was previously engineered (Moore et al., 2008). For instance, the C3-V4 region of HIV-1_{CAP88} (sensitive to CAP88 plasma) was grafted into the HIV-1_{CAP63} (resistant to CAP88 plasma) envelope backbone to produce the heterologous chimera HIV-$1_{CAP63\text{-}88(C3\text{-}V4)\text{-}63}$. This chimeric virus was then tested for any acquired sensitivity to CAP88 plasma, which confirmed the role of the C3-V4 region as a target for CAP88 neutralising antibodies. Additional chimeric

pseudoviruses were also created in this study (described below). From some of these new chimeric envelope encoding plasmids, the previously engineered C3-V4 chimeric plasmids, and the parental sequences, the gp120 coding regions were amplified. In addition several mutations were also introduced into the gp120 constructs (described below). A summary of the parental, chimeric, and mutant template sequences used for gp120 production has been provided in Figure 9. A summary of the chimeric pseudoviruses created for this study is shown in Figure 10.

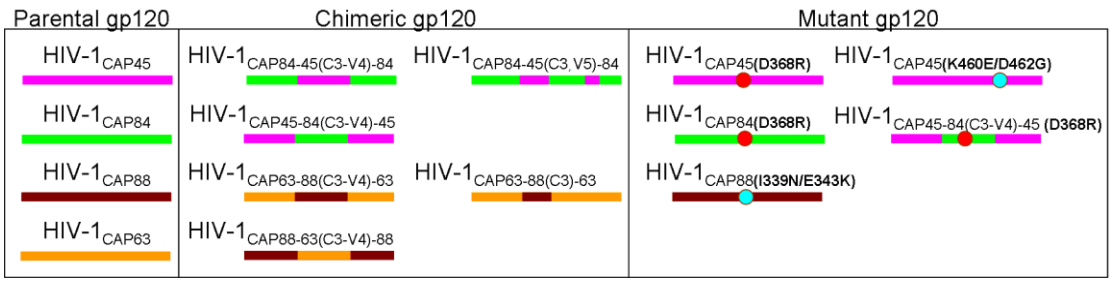


Figure 9: Template sequences used for gp120 production
A schematic summary of the different template sequences used for gp120 production in this study. The gp120 coding sequence corresponding to HIV-1_{CAP45}, HIV-1_{CAP63}, HIV-1_{CAP84} and HIV-1_{CAP88} is coloured pink, yellow, green, and brown respectively. The red dots indicate those gp120 constructs into which the D368R mutation was introduced, while the blue dots indicate the gp120 constructs into which previously identified escape mutations were introduced.

Primers KpnIEnvC and BstBI-HT-re were used to amplify all gp120 coding regions. Sequences for these as well as all subsequent primers used in this study are listed below (Table 1). The PCR protocols included thirty five cycles, with an annealing temperature of 50 °C for thirty seconds. Denaturation and extension times and temperatures varied according to the manufacturer's recommendations for each of the different high fidelity enzymes used. Although primer melting temperatures and actual annealing temperatures used varied by as much as 10 degrees, no non-specific binding was expected given the nature of the plasmid DNA template. A codon optimised plasmid encoding the CAP63 gp120 and chimeric CAP63-88(C3)-63 gp120 was obtained from Dr. Liao (Duke Human Vaccine Institute, North Carolina, U.S.A.) to facilitate a greater level of gp120 production. In order to PCR amplify these gp120 encoding genes for downstream sub-cloning, the primers CAP63(Codop)-KpnI-fo and CAP63(Codop)-Histag-BstBI-re were designed using the online OligoCalc tool (Kibbe, 2007). These were used in the same manner as the KpnIEnvC and BstBI-HT primer pair described above.

All PCR products were treated with 10 U - 20 U DpnI at 37 °C for one to two hours to digest the methylated template DNA, and then purified with the Qiagen PCR purification kit (Hilden, Germany) using the protocol provided. The KpnIEnv and CAP63(Codop)-KpnI-fo primers introduced a KpnI cut site into the N-terminus of gp120, while the BstBI-HT-re and CAP63(Codop)-Histag-BstBI-re introduced a BstBI cut site, six histidine residues (HisTag) preceded by a flexible Gly-Gly-Ser linker motif, and two stop codons into the C-terminus of gp120. This allowed for the directional cloning of PCR products in to the mammalian expression vector pPPI4. PCR products were digested with 50 U KpnI for one hour at 37 °C, and then 20 U BstBI for one hour at 65 °C in the appropriate buffers supplied. Following each digestion the PCR product was again purified using the Qiagen kit. Approximately 2 μg of the destined expression vector pPPI4 (harbouring an irrelevant gp120 gene) was digested simultaneously. The linearisation of the vector, and liberation of its associated gp120 open reading frame was monitored on a 1% agarose gel in Tris-Acetate buffer (supplemented with 0.5 μg/mL ethidium bromide), and the digestion times were manipulated accordingly.

The enzymatically digested vector and PCR products were gel purified using the Qiagen gel extraction kit and protocol provided. All DNA concentrations in this study were determined using a NanoDrop^{TM}-1000 Spectrophotometer (Thermo Scientific, Wilmington, U.S.A.). A ratio of 3:1 PCR product to linearised vector was then incubated overnight at 16°C in the presence of Invitrogen T4-phage DNA ligase (Carlsbad, U.S.A.) in the buffer supplied. Ligation products were used to transform Escherichia coli XL10-Gold chemically competent cells produced by Stratagene (La Jolla, USA) in the presence of β-mercaptoethanol according to the 42 °C heat shock method provided. Cells were recovered in a nutrient-rich super optimal broth with catabolite suppression (SOC) medium for one hour at 37 °C with shaking (225 Hz) and plated onto Luria Bertani (LB) media supplemented with 100 μg/mL ampicillin at 37 °C overnight to selectively isolate single colonies. These were subsequently cultured in 50 μL Luria Bertani broth supplemented with 300 μg/mL ampicillin for one hour, and then screened for positive transformants by PCR using the KpnIEnv/BstBI-HTre or CAP63(Codop)-KpnI-fo/CAP63(Codop)-Histag-BstBI-re primer pairs. Successful transformants were selected for plasmid extraction and confirmation of the inserted gene by DNA sequencing.

Table 1: List of primers used in this study	
PCR primers	
KpnIEnvC	GTCTATTATGGGGTACCTGTGTGGAAAGAAGC
BstBI-HT-re	CCCACTAAATCAAAAAGGAGAGTGGTGGAGTCAGAAAAAGGCGGATCCCACCACCACCACCACCACTGATGATTCGAAGCGAGG
CAP63(Codop)-KpnI-fo	ACGGGGTACCCGTGTGGCGCGAGGCCAAGACCACC
CAP63(Codop)-Histag-BstBI-re	CAGATTCGAATCATCAGTGGTGGTGGTGGTGGGATCCGCCGCGCTTCTCGCGGCCCACC
V1-fwd	GTGTAAAGTTGACCCCACTCTG
V1-rev	CAGAGTGGGGTCAACTTTACAC
V2-fwd	CAGGCCTGTGTTATGGTTGAGG
V2-rev	CCTCAACCATAACACAGGCCTG
84-45V5fo	GATGGAGGGAAAACAGACAGGAATGACACAGAGATATTCAGACCTGCAGGAGG
84-45V5re	GACATTCCTGTCTGTTTTCCCTCCATCACGTGTCAGCAGTAGTCCCGTGATAC
84-45β14flankfo	TTCCTTAATAGAACAATAGAATTTGAACCACCCTCAGGAGGGGATCTAG
84-45β14flankre	GGGTGGTTCAAATTCTATTGTTCTATTAAGGAAGTGTTCCTTTAATTTTTCCTT
T7	TAATACGACTCACTATAGGG
EnvAdir	CACCGGCTTAGGCATCTCCTATGGCAGGAAGAA
EnvM	TAGCCCTTCCAGTCCCCCCTTTTCTTTTA
Sequencing primers	
T7	TAATACGACTCACTATAGGG
tPA-fo	CCATGCCCGATTCAGAAGAGGCGCCAG
EnvAdir	CACCGGCTTAGGCATCTCCTATGGCAGGAAGAA
KpnIEnvC	GTCTATTATGGGGTACCTGTGTGGAAAGAAGC
E175	TTTAGCATGTGATGCACAAAATAG
EnvB	AGAAAGAGCAGAAGACAGTGGCAATGA
A589	GTGTAAAGTTAACCCCACTCTG
A590	AATCGCGAAACCAGCCGGCGCACAAT
Br	AATTTCTAGGTCCCCTCCTGA
Df	AGCACATTGTAACATTAGT
E55	GCCCCAGACTGTGAGTTGCAACAGATG
E85	GTCCCTCATATCTGCTCCTCCAGGTCTG
EnvArev	TGCTGCTCCCAAGAACCCAA
gp41Fo	GCCAGTGGTATCAACTCAAC
BstBI-HT-re	CCCACTAAATCAAAAAGGAGAGTGGTGGAGTCAGAAAAAGGCGGATCCCACCACCACCACCACCACTGATGATTCGAAGCGAGG
Nr	GGTGAGTATCCCTGCCTAACTCTA
E210	TAACAAATTGGCTGTGGTATATAA
E240	ATAATGATAGTAGGAGGCTTGATAGGC
MPERseq	CACAAGCACAATATACAGGTTACTTGAAGACTCGCAAAACCAGCAGG
EnvM	TAGCCCTTCCAGTCCCCCCTTTTCTTTTA
PPI4-re	GGCAAACAACAGATGGCTGGCAACTAGAAG
63Codop-PLCV-re	GCAGTTCAGGGTCACGCACAGG
63Codop-C1HED-fo	CAGATGCACGAGGACATCATCTCC
63Codop-PVVSTQ-fo	GTGGTGTCCACCCAGCTGCTG
63Codop-NNAKT-re	CGATGATGGTCTTGGCGTTGTTGG
63Codop-FFYC-fo	CGAGTTCTTCTACTGCAACACCTCC
63Codop-PCRIKQ-re	CTGCCACATGTTGATGATCTGCTTGATG
Mutagenesis Primers	
CAP45-(K460E/D462G)-fo	CACGTGATGGAGGGGAAACAGGCAGGAATGACACAGAG
CAP45-(K460E/D462G)-re	CTCTGTGTCATTCCTGCCTGTTTCCCCTCCATCACGTG
CAP88-I339N-E443K-fo	ACTAAAGACAGATGGAACGCAACTTTAAAAAAGGTAAAGAAGAAA
CAP88-I339N-E443K-re	TTTCTTCTTTACCTTTTTTAAAGTTGCGTTCCATCTGTCTTTAGT
CAP45D368Rfo	CACCCTCAGGGGGGCGTCTAGAAGTTACAACAC
CAP45D368Rre	GTGTTGTAACTTCTAGACGCCCCCCTGAGGGTG
CAP84.32-D368Rfo	CCAGTCTCAGGAGGGCGTCTAGAAGTTACAACACATAGC
CAP84.32-D368Rre	GCTATGTGTTGTAACTTCTAGACGCCCTCCTGAGACTGG

*Primers designed specifically for this study are shaded grey

Creating chimeric pseudovirions

Plasmids encoding wild-type and C3-V4 chimeric envelope constructs for pseudovirion production have been engineered previously (Moore et al., 2008). However this project required the generation of several additional chimeras. Primers 84-45V5fo, 84-45V5re, were used in conjunction with EnvAdir and EnvM to create the chimeric HIV-$1_{CAP84\text{-}45(C3+V5)\text{-}84}$ pseudovirus construct from the HIV-$1_{CAP84\text{-}45(C3)\text{-}84}$ encoding plasmid. Primers 84-45β14flankfo and 84-45β14flankre (again with EnvAdir, EnvM, and plasmid HIV-$1_{CAP84\text{-}45(V5)\text{-}84}$) were used to create the HIV-$1_{CAP84\text{-}45(\beta14+V5)\text{-}84}$ chimera. Primers A589 and A590 were used with the EnvAdir/EnvM combination to introduce the HIV-1_{CAP45} V1-V2 loop into the HIV-$1_{CAP84\text{-}45(C3\text{-}V4)\text{-}84}$ chimera. Both early and escaped V1-V2 sequences were introduced this way. A schematic describing the construction of these chimeras is shown in Figure 10. The HIV-$1_{CAP84\text{-}45(C3+V5)\text{-}84}$ pseudovirus was selected for gp120 production (as described above). The primer pair EnvAdir/EnvM was used to amplify full length envelope genes which were then cloned into the pcDNA 3.1D-TOPO expression vector from Invitrogen using the protocol provided. Ligation products were used to transform ultra-competent cells as described above and positive transformants were selected by PCR using a primer specific for the vector (T7) as well as EnvM. Chimeric envelopes were then confirmed by DNA sequencing (described below).

Pseudoviruses were produced in mammalian 293T cells grown in DMEM supplemented with 10% FBS, 50 μg/mL gentamycin, and 25 mM HEPES buffer. This was done by co-transfecting envelope genes with the pSG3ΔEnv HIV-1 subtype B backbone plasmid. Transfections were executed with FuGENE®6 (described above) using 80 ng/cm^2 of each plasmid and a 3:1 ratio of FuGENE®6 to plasmid DNA. After 48 hours the cell culture supernatant was filtered through 0.45 μm and adjusted to 20% FBS, before being stored at -70 °C for later use in the neutralisation assays. The pseudovirion particles that are produced express functional gp160 trimers on their envelope surfaces, but do not encode a functional envelope gene and are thus only capable of a single round of infection. Pseudoviruses at various dilutions were co-incubated in a 96-well flat bottom plate with 10 000 TZM-bl reporter cells per well to a total volume of 250 μL for 48 hours at 37 °C. TZM-bl cells are engineered to constitutively express CD4, CCR5, & CXCR4, and encode a

luciferase gene under the transcriptional control of the long terminal repeat element of HIV-1. After 48 hours 150 µL was removed from each well and the cells were lysed with 100 µL of a luciferin containing Bright-Glo™ Luciferase Assay Buffer (Promega, Fitchburg U.S.A.) for two minutes. Finally 150 µL of lysate was transferred to black F96 MicroWell Plates manufactured by NunC (Roskilde, Denmark), and the relative light units (RLU) produced measured in a VICTOR^{3}™ Multilabel Counter 1420 (PerkinElmer, Waltham, U.S.A.).

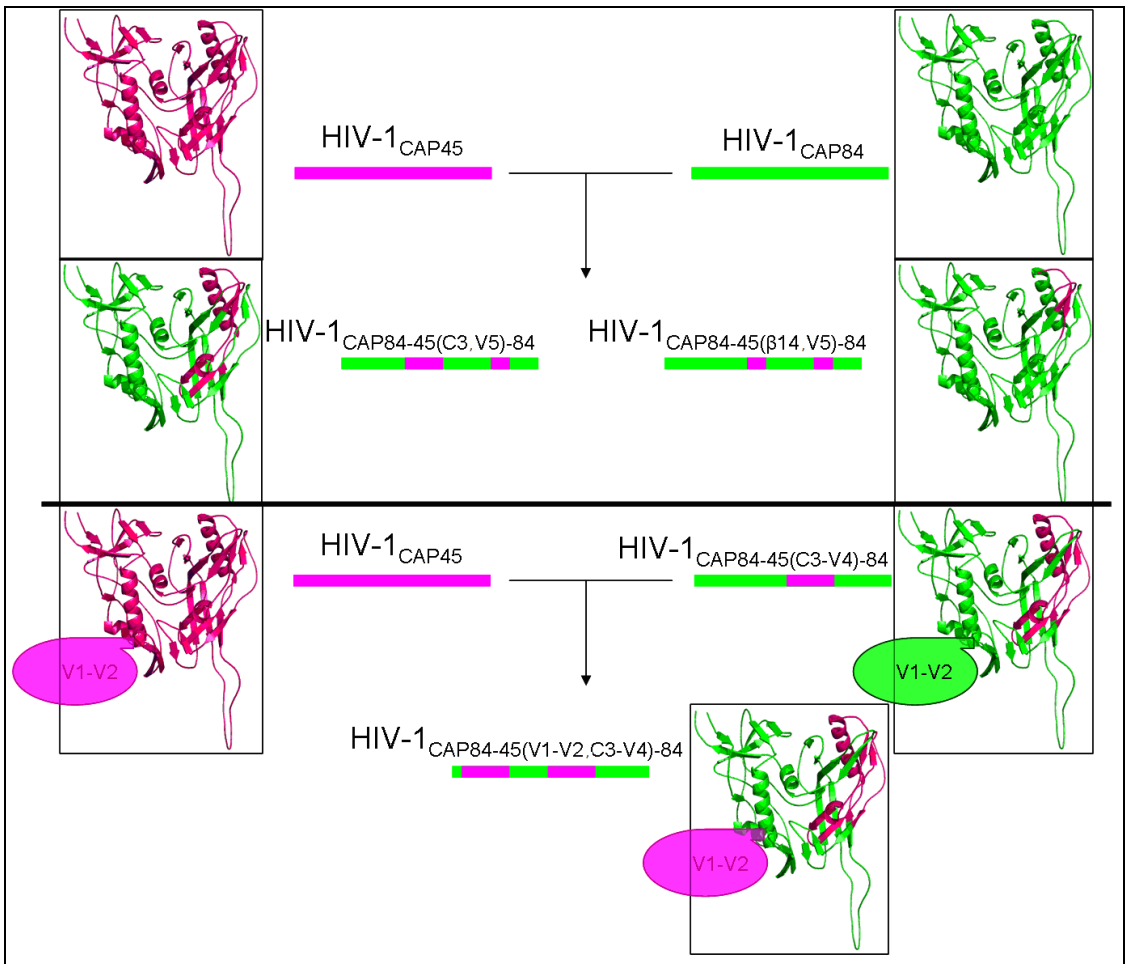


Figure 10: New chimeras created for this study
A schematic diagram describing how various chimeric pseudoviruses were engineered for this study. As in Figure 9 the gp120 coding sequence corresponding to HIV-1_{CAP45} was coloured pink, while the HIV-1_{CAP84} coding sequence was coloured green. Structures are included to illustrate how genetically distant regions of the envelope may come together in the context of a gp120 monomer to provide a single, discontinuous neutralisation epitope. The location of the flexible V1-V2 loop in the CD4 bound conformation of gp120 is not known, and approximated with the coloured bubbles.

Mutagenesis

Amino acid changes used to map antibody specificities and evaluate the effects of known escape mutations were introduced with the QuikChange™ site-directed

mutagenesis kit from Stratagene (La Jolla, USA). Primers for this purpose were designed based on the manufacturer's recommendations. The primer pairs CAP45-(K460E/D462G)-fo / CAP45-(K460E/D462G)-re and CAP88-I339N-E443K-fo / CAP88-I339N-E443K-re were used to introduce known escape mutations into the V5 loop and α2-helix of HIV-1_{CAP45} gp120 and HIV-1_{CAP88} gp120 respectively. The primer pairs CAP45D368Rfo / CAP45D368Rre and CAP84.32-D368Rfo / CAP84.32-D368Rre were designed to introduce the D368R mutation into the CD4 binding loop of HIV-1_{CAP45} gp120, HIV-$_{1CAP45\text{-}84(C3V4)\text{-}45}$ gp120, and HIV-1_{CAP84} gp120. Mutagenesis reaction products were digested with DpnI at 37 °C for two hours to remove the methylated template sequence, and then used to transform chemically competent cells as described above. Up to eight colonies were selected from each plate for plasmid extraction and DNA sequencing.

DNA sequencing

Plasmids were extracted from LB broth cultures using either the Qiagen Miniprep kit or the Sigma Midiprep kit (St.Louis, U.S.A.) depending on the amount of plasmid required for downstream experiments. Extractions were done according to the protocol provided with the exception that triple the volume of culture advised was used for each extraction to increase yields. Plasmids were sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems (Foster City, U.S.A) and the Half-Dye Mix from Bioline (London, U.K.) in a 20 μL reaction using an in-house optimised reaction mix. Primers T7, tPA-fo, EnvAdir, KpnIEnvC, E175, EnvB, A589, A590, Br, Df, E55, E85, EnvArev, gp41Fo, BstBI-HT-re, Nr, E210, E240, EnvM, PPI4-re, and MPERseq were used in varying combinations to sequence either gp120 or full length Env. Primers 63Codop-PLCV-re, 63Codop-C1HED-fo, 63Codop-PVVSTQ-fo, 63Codop-NNAKT-re, 63Codop-FFYC-fo, and 63Codop-PCRIKQ-re were designed using OligoCalc to sequence the sub-cloned codon optimised plasmids. Sequencing reaction products were precipitated out with 120 mM sodium acetate in 100% ethanol at 2000 x g for thirty minutes. The pellet was rinsed with 70% ethanol at 2000 x g for five minutes, and then dried for three minutes at 63 °C and resuspended in 10 μL Hi-Di™ Formamide from Applied Biosystems. The sequences were resolved on an Applied Biosystems 3100 automated genetic analyser, and analysed using Sequencher v4.5 from Genecodes (Ann Arbor, U.S.A.).

Expression of recombinant gp120

A polyethyleneimine (PEI) expression protocol was used for the transfection of 293T cells. A similar protocol has been described previously (Kirschner et al., 2006). Polyethyleneimine with an average molecular weight of 25 kDa from Polysciences (Warrington, U.S.A.) was prepared to a 1 mg/mL solution in sterile water and filtered through 0.22 μm. The 293T cells were seeded at 40 000 cells/cm^2 24 hours prior to transfection to achieve approximately 80% confluency. To optimise the transfection protocol different ratios of PEI:DNA (160 ng/cm^2 plasmid DNA) were used to transfect a codon optimised gp120 encoding gene derived from HIV-1_{Du151}. The plasmid DNA was pre-incubated with PEI in Invitrogen serum-free DMEM at 1/20th of the final cell culture volume for 45 minutes. Cells were transferred to serum-free DMEM and the transfection reaction mixture was added. After five to six hours the medium was changed to DMEM supplemented with 10% FBS, and 50 μg/mL gentamycin. The level of gp120 expression using PEI was compared with the commercial transfection reagent FuGENE®6 from Roche Applied Science (Mannheim, Germany). These reactions were also set up according to the manufacturer's guidelines with varying ratios of DNA:FuGENE®6 (using 160 ng/cm^2 plasmid DNA) in DMEM supplemented with 10% FBS, and 50 μg/mL gentamycin.

From each transfection procedure cell culture media containing expressed soluble gp120 was collected after 48 hours and added to 96-well ELISA plates pre-coated overnight at 4 °C in a carbonate-bicarbonate buffer (pH 8.5) with D7324, a sheep anti-gp120 antibody produced by Aalto (Dublin, Ireland). After one hour incubation at 37 °C the bound HIV-1 envelope proteins were detected using the ELISA protocol described below. Once an optimal transfection protocol was identified it was scaled up to 500 mL culture volumes to increase expression levels for subsequent adsorption experiments, and the gp120 containing cell culture media was collected at three to four subsequent 48 hour intervals. Cellular debris was pelleted at 4210 x g for 20 minutes, and the supernatant was stored at -70 °C for later purification.

Purification of recombinant gp120

Cell culture supernatants containing the same recombinant gp120 proteins were pooled together in 2 L batches and filtered though 0.22 μm. The filtrate was then passed through a *Galanthus nivalis* derived lectin affinity chromatography matrix

(from Sigma) three times to ensure retention of the major glycosylated fraction. The protein bound beads were washed with roughly twenty column volumes of PBS containing 0.5 M sodium chloride to displace any non-specifically bound proteins, and then soaked in 50 mL of 1 M methyl-α-D-mannopyranoside in PBS for one hour to elute the bound glycoproteins. The elution was concentrated in PBS to less than 2 mL and then passed through a Q-sepharose anion exchange matrix (GE Healthcare, Little Chalfont, U.K.) at 0.3 µL/min in PBS. This process was automated on the Amersham AKTAprime™ (now GE Healthcare, Little Chalfont, U.K). Unwanted proteins were bound to the matrix and the column flow through containing histidine tagged recombinant gp120 was collected and concentrated to 5 mg/mL using the Vivaspin 20 centrifugal concentrators from Sartorius Stedim (Aubagne, France). Aliquots were supplemented with the cOmplete™ protease inhibitor cocktail from Roche (Penzburg, Germany) and stored at -20 °C. Due to the relatively variable expression levels of gp120 derived from different viral strains, the same recombinant proteins were expressed on numerous occasions and different batches were each assessed qualitatively by SDS-PAGE and ELISA (described below). All protein concentrations were determined using a NanoDrop™-1000 Spectrophotometer (Thermo Scientific).

Qualitative assessment of recombinant gp120

Protein purity was assessed via SDS-PAGE. 20 µg of purified sample was run on an 8% polyacrylamide gel and then stained overnight with Coomasie blue. The relative proportion of gp120 to other protein species in the sample was calculated on the Bio-Rad Molecular Imaging® Chemi Doc XRS™ using the Quantity One software (Hercules, U.S.A.). Purity of 85% gp120 and above was tolerated for downstream applications. Since the introduction of heterologous protein sequence into the chimeric gp120 may deform overall protein structure, wild-type and chimeric gp120 was evaluated for conformational integrity. This was done in the context of ELISA by assessing their ability to bind monoclonal antibodies directed towards known discontinuous epitopes. Treated 96-well plates were coated with 4 µg/mL gp120 overnight at 4 °C in a carbonate-bicarbonate buffer (pH 8.5). Plates were then washed thrice with PBS (0.05% Tween20) and the remaining uncoated surfaces blocked with 200 µL PBS (5% milk) for one hour at 37 °C. All subsequent reaction steps were conducted in 100 µL reaction volumes at 37 °C, and preceded by an

identical PBS (0.05% Tween20) wash step. First, plates were incubated with serial dilutions of monoclonal antibodies at concentrations between 10 µg/mL and 128 pg/mL. As a positive control the HIV-1 positive serum sample BB105 was used at 1 in 5 serial dilutions starting at 1:100. For the second and third steps plates were incubated first with 166.67 ng/mL biotin labelled, anti-human, goat polyclonal sera (KPL, Gaithersburg, U.S.A.), and then with 500 ng/mL anti-biotin, goat polyclonal HRP-conjugates (Calbiochem, Darmstadt, Germany). Finally plates were incubated at room temperature with 100 µL volumes of the 1-Step™ Ultra TMB-ELISA (3,3',5,5'-tetramethylbenzidine) substrate from Thermo Scientific for approximately three minutes and the resulting colour reaction stopped with 25 µL of 1M H_2SO_4 and read at 450 nm on a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale U.S.A.).

Adsorption of monomer-specific serum antibodies

Recombinant gp120 was coated in 400 µg batches to 200 µL of MyOne™ tosylactivated Dynabeads® from Invitrogen as per the manufacturer's recommendations. A covalent interaction between the magnetic beads and gp120 monomers provides a stable complex that can then be used to bind out gp120 specific antibodies. All prior and subsequent incubation periods were carried out at 37 °C with continuous rotary motion to prevent clumping. Beads coated with gp120 and blocked with BSA were washed thrice with 1 mL serum-free DMEM to remove all previous buffers, and then divided into two equal volumes. The first of these was re-suspended in 200 µL of the relevant serum sample diluted between 1:20 and 1:150 depending on the expected titres. After one hour incubation the adsorbed serum was recovered and used to re-suspend the second volume of gp120 coated beads. Following this the adsorbed serum was again recovered and separated from any remaining matrix particles by centrifugation. The supernatant was stored at 4 °C for use in downstream applications. The adsorbed sera was assayed for the successful depletion of binding antibodies by ELISA (as described above). A drop in absorbance values when compared to serum adsorbed only with blank beads (negative control) correlated with the adsorption of gp120 specific antibodies (Figure 11A). The depletion of neutralising antibodies was measured by a neutralisation assay (as described below). Again, a drop in neutralisation titres when compared to the negative control was a result of neutralising antibody depletion (Figure 11B).

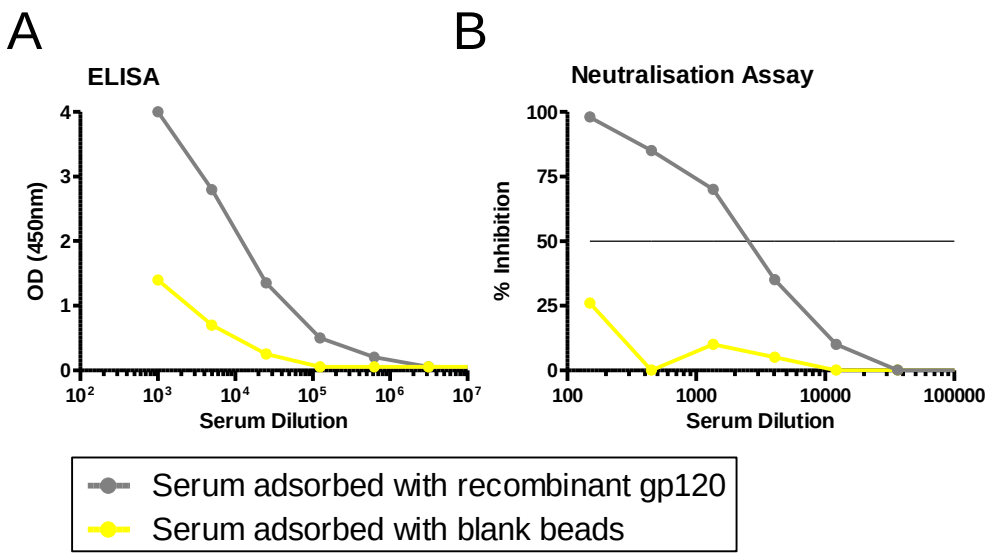


Figure 11: Mock adsorption data
(A) Mock ELISA results from an adsorption experiment. Serum/plasma adsorbed with blank beads only is coloured black, while serum adsorbed with recombinant gp120 is coloured grey. Adsorption of gp120 binding antibodies results in a reduced OD. **(B)** Mock results for a neutralisation assay using serum/plasma from an adsorption experiment. The graph is coloured as in (A). Adsorption of gp120-specific neutralising antibodies results in a drop in the neutralisation titre (measured as ID_{50}).

Neutralisation assay

Neutralisation was measured in a single round of infection assay that allows for the assessment of antibody mediated inhibition of viral entry into host cells. This neutralisation assay was adapted from the previously described method (Montefiori, 2009). Briefly, monoclonal antibodies or serum/plasma samples were serially diluted down a 96-well plate, and co-incubated in a total volume of 100 µL for one hour at 37 °C with pseudovirus at a concentration previously determined to yield 40 000 relative light units (RLU). To this 50 µL of TZM-bl cells were added to a final concentration of 10 000 cells per well, and incubated for 48 hours at 37 °C (5% CO_2). Following the two day incubation period cells were digested in the Luciferase Assay Buffer and resulting RLUs measured in the VICTOR^{3}™ Multilabel Counter as described above. To save on sample the assay was adapted such that diluted antibody/serum, virus, and TZM-bl cells were co-cultured in a total of 70 µL per well reducing the amount of gp120 adsorbed serum input required. A feeding step twenty four hours post-infection (adding 130 µL), and a reduction in the volume of cell culture media removed (100 µL) at forty eight hours post-infection (prior to the addition of the luciferin containing buffer) was also included.

Chapter Three: Results

Optimisation of polyethyleneimine based transfection system

Genes can be delivered into eukaryotic cells using a variety of tools from retroviruses to liposomal reagents and cationic lipids or polymers (Bonetta, 2005). The latter are often incorporated into commercial chemical transfection reagents, and act as a bridge allowing foreign DNA to associate with the cell membrane. Polyethyleneimine (PEI) has been previously described as a vehicle for gene delivery (Boussif et al., 1995). It is also significantly more economical when compared to commercially available transfection reagents (Kirschner et al., 2006).

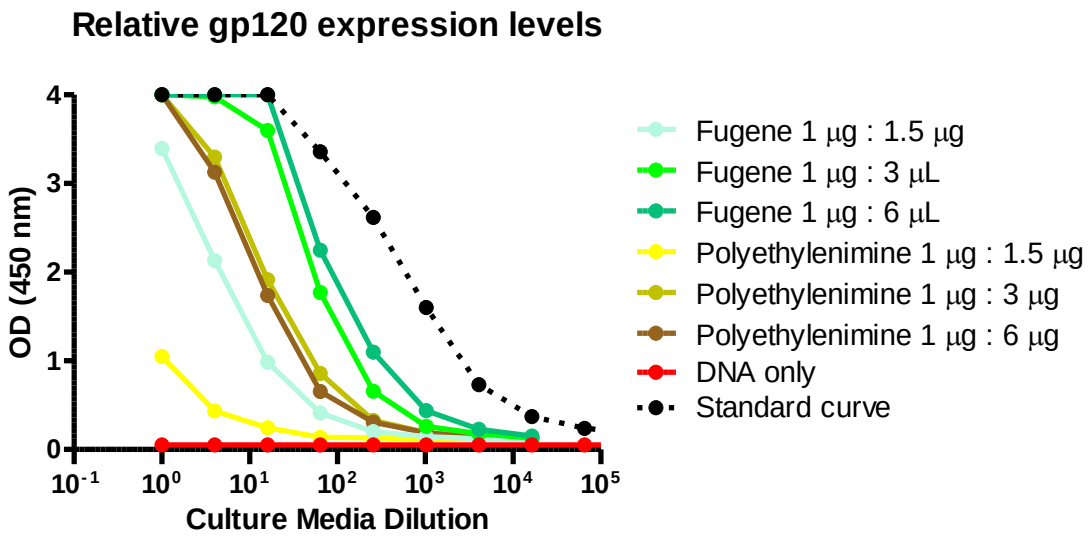


Figure 12: A comparison of PEI and Fugene®6 as gene delivery reagents
ELISA data showing the relative gp120 expression levels of 293T cells transfected with different ratios of either PEI or FuGENE®6. Optical Density readouts were compared to a standard curve of known gp120 concentrations serially diluted 1 in 4 from 40 µg to 2.44 ng. The relative amount of gp120 produced for each transfection ratio used is shown.

Table 2: Quantity of gp120 produced

Fugene®6	1 µg DNA : 1.5 µL Fugene®6	450 ng/mL
	1 µg DNA : 3 µL Fugene®6	7 500 ng/mL
	1 µg DNA : 6 µL Fugene®6	17 100 ng/mL
Polyethyleneimine	1 µg DNA : 1.5 µg Polyethyleneimine	30 ng/mL
	1 µg DNA : 3 µg Polyethyleneimine	1 500 ng/mL
	1 µg DNA : 6 µg Polyethyleneimine	1 300 ng/mL
	1 µg DNA	0 ng/mL

To reduce the cost of recombinant gp120 expression for this study a polyethyleneimine based transfection system was explored. We evaluated the effect of different PEI : DNA ratios on recombinant gp120 expression levels, and compared the data to the yields obtained with FuGENE®6, the commercial transfection reagent traditionally used in our laboratory for gene delivery in a ratio of 3 μL FuGENE®6 : 1 μg DNA. The level of gp120 produced after transfection was quantified by ELISA (Figure 12). A standard curve was constructed by capturing previously expressed recombinant gp120 of known concentrations onto the ELISA plate. Transfection without a transfection reagent (DNA only) was included as a negative control. As expected the transfection with DNA only did not produce any detectable levels of gp120 expression. The level of expression increased significantly by increasing the ratio of FuGENE®6 : DNA, and similarly decreased dramatically when decreasing this ratio (Table 2). A ratio of 3 μg PEI : 1 μg DNA was found to be optimal, and produced significantly more protein than the lower 1.5 μL FuGENE®6 : 1 μg DNA ratio. Using more PEI did not significantly enhance the transfection efficiency. Thus the PEI : DNA transfection ratio of 3 : 1 was shown to be a far more economical alternative for protein expression when compared to the FuGENE®6 expression system previously used, and was adopted for the remainder of the study.

Qualitative analysis of purified recombinant gp120

This research project required expression of sixteen different recombinant gp120 molecules derived from four distinct viral isolates, their chimeras, and mutants. Each protein was isolated from cell culture media to an estimated 91.7% ± 3.7% purity, as measured with the Bio-Rad Molecular Imaging® Chemi Doc XRS™ (Figure 13). The transfection efficacy of 293T cells using PEI was inconsistent within the context of the same envelope construct, but certain gp120 expression constructs (and in particular HIV-1_{CAP63} derived gp120) consistently produced less protein. Poor gp120 expression correlated with less efficient purification. This was attributed to increased carryover of glycosylated FBS proteins that then saturated the ion exchange column, contaminating gp120 collected in the flow through. The HIV-$1_{CAP63\text{-}88(C3\text{-}V4)\text{-}63}$ derived gp120 was least pure, but produced the best adsorption data from this study (Figure 20, discussed below). Therefore we reason that the level of purity obtained for all recombinant gp120s was sufficient for the purposes of these adsorption studies.

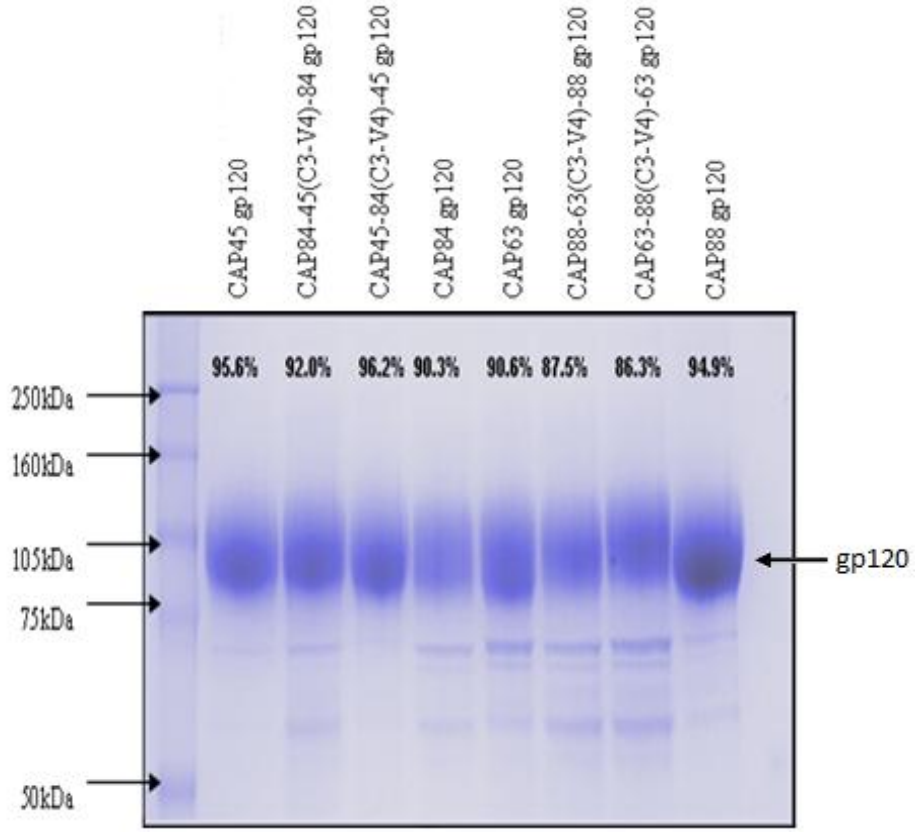


Figure 13: Purity of recombinant gp120 produced in this study
Coomasie blue stained SDS-PAGE showing eight of the sixteen recombinant proteins produced in this study. The relative purity of each protein was calculated as a percentage of the total protein content in each lane using Quantity One software.

As a result of the primers used to amplify the recombinant gp120 molecules, each of the recombinant proteins had a common N-terminal and common C-terminal sequence including a histidine tag (T^{499}KSKRRVVESEK^{510}GGSHHHHHH^{519}). A full sequence alignment of the parental recombinant gp120s used in this study is shown in Figure 14. These polyhistidine tags provide a large, flexible surface distinct from biologically relevant sites on monomeric gp120. At high pH histidine deprotonates to become available for coupling to the tosyl activated beads used in the downstream adsorption studies. Despite the insertion of heterologous sequence into the protein termini, recombinant gp120 proteins produced in this study were able to bind monoclonal antibodies that recognise discontinuous, conformationally dependent epitopes (Figure 15). Antibodies b12 and b6 contact the CD4 binding site (Pantophlet et al., 2003), A32 recognises a discontinuous epitope in C1-C2-C4 (Boots et al., 1997), and 2G12 binds a unique glycan epitope (Scanlan et al., 2002). In support of these data the recently published crystal structure of a liganded gp120 core with full N- and C- terminal domains showed that resides 40-497 are responsible for intra-

monomer interactions, and variation outside of these residues (residues 1-39 and 487-511) in all probability do not impact on the protein's overall conformation (Pancera et al., 2010a). Since the CD4 binding site overlaps the C3 region and the base of V5, it was expected that the binding of monoclonal antibodies b12 and b6 to gp120 chimeric for the C3 and/or V5 regions would differ from the binding to parental gp120 molecules. This was indeed the case for these CD4 binding site antibodies (Figure 15). In some instances these differences could be explained by previous work that had mapped the binding site of these two antibodies. Position 471 in the HIV-1_{CAP84} backbone is occupied by an alanine, while in HIV-1_{CAP45} this residue is a glycine (Figure 14). Alanine scanning mutagenesis has shown that anything bulkier than a glycine at this position significantly inhibits the binding of b12, while the more common glycine residue is less favourable for b6 interactions (Pantophlet et al., 2003). This provides some explanation as to why b12 (Figure 15, yellow line) does not bind well to the HIV-1_{CAP84} parental gp120 or chimeric gp120 with the HIV-1_{CAP84} backbone, while b6 (Figure 15, purple line) binds very well to these proteins. Furthermore the T467I mutation in V5 significantly affects b6 binding, explaining differences in the binding of this antibody to the V5 chimeras.

Asp^{368} is part of the CD4 binding loop and critical to HIV-1 entry. Substitution of this residue with an arginine abolishes binding to CD4 as well as most anti-CD4 binding site antibodies, including b6 and b12 (Li et al., 2007). As seen in Figure 14, b12 and b6 were unable to bind the three recombinant gp120s with this mutation. The antibody 2G12 is subtype B specific and unable to neutralise the viruses used in this study. The binding to 2G12 detected by ELISA may have resulted from low affinity interactions with high mannose structures contributing to the 2G12 epitope, or with another binding site for 2G12 formed by alternate gp120 processing. Such binding sites have already been described for gp160 (Crooks et al., 2011). As the A32 epitope does not involve variable residues in C3, V4, or V5, the ability of this antibody to bind all of the chimeric gp120s resembled binding to the parental gp120 proteins from which the backbone was derived. Pooled HIV-1 positive plasma (such as BB105) contains gp120 specific cross-reactive antibodies (such as anti-V3 antibodies) and was used in all ELISAs as a positive control. Overall, binding of these monoclonal antibodies to at least one discontinuous epitope on each recombinant protein confirmed proper folding of all gp120s expressed in this study.

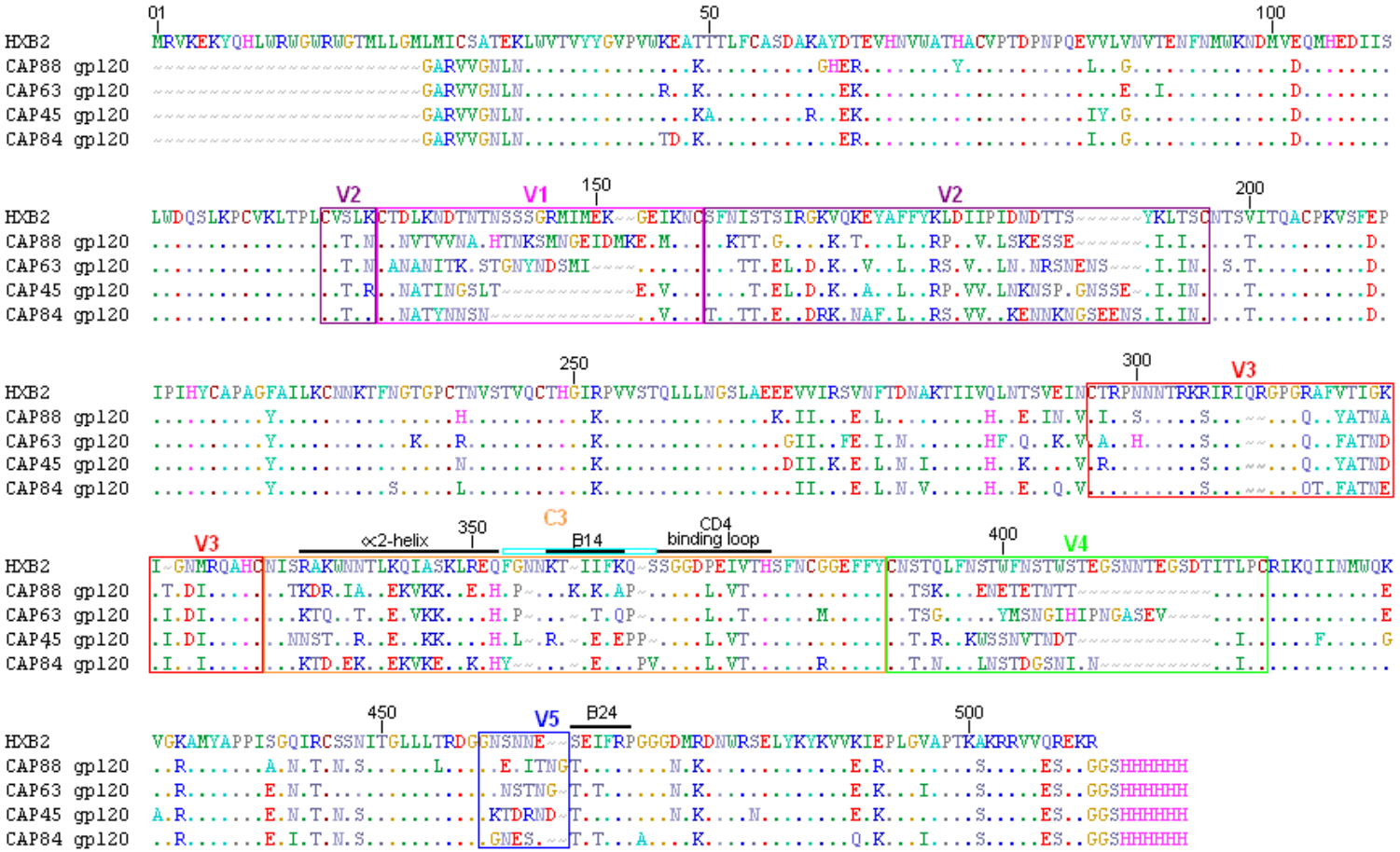


Figure 14: Sequence alignment of the four parental recombinant gp120 proteins used in this study
Sequences were aligned with the HIV-1_{HXB2} gp120 sequence. Residues making up the variable loops V1-V5 are boxed in pink, purple, red, green, and blue respectively. The C3 region is boxed in orange and the α2-helix, β14-sheet, β24-sheet, and CD4 binding loop are highlighted. The β14 and flanking region described later is highlighted in cyan. Dots indicate amino acids identical to the HIV-1_{HXB2} sequence, while the ~ symbol is used when an amino acid is missing from one of the five sequences shown.

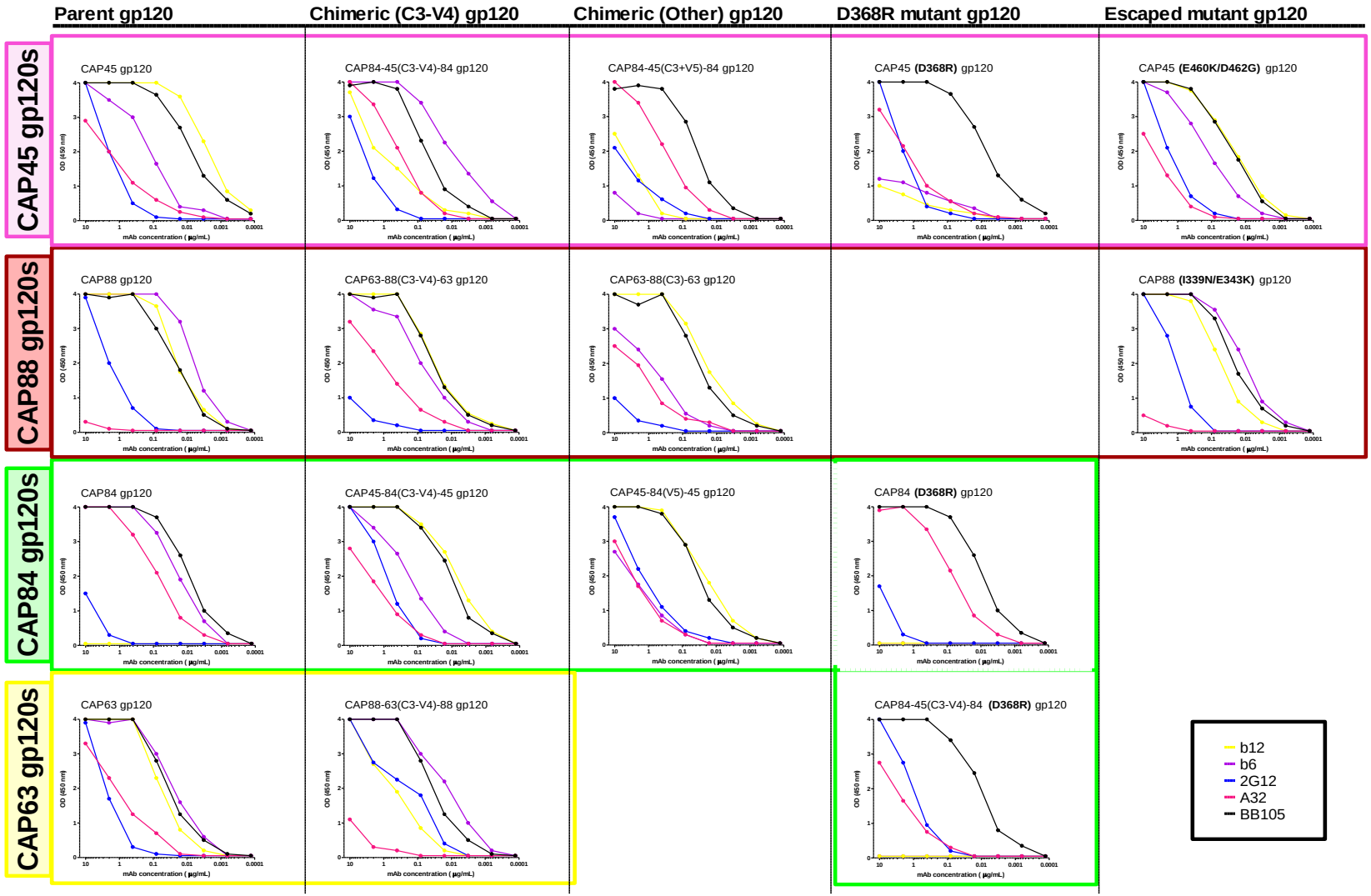


Figure 15: Binding of monoclonal antibodies to recombinant gp120 expressed in this study

ELISA data showing the binding of the various gp120 proteins produced in this study to monoclonal antibodies targeting complex discontinuous epitopes. Anti-CD4 binding site antibodies b12 and b6 are shown in yellow and purple respectively. A32 is shown in pink while 2G12 is shown in blue. The HIV-1 positive pooled plasma sample BB105 is shown in black, and the dilutions that were used are described in the materials and methods.

Adsorption of neutralisation activity by autologous gp120

CAP88

Previous studies in our laboratory showed that the plasma from participant CAP88 taken at 26 weeks post-infection contained anti-C3 autologous neutralising antibodies (Moore et al., 2009). At this time point the anti-C3 antibodies had reached peak titres while a second response targeting the V1-V2 region had just begun to appear (Figure 16A). This latter response went on to peak at 81 weeks post-infection. At 54 weeks post-infection the declining anti-C3 response and the increasing anti-V1-V2 antibodies were at approximately identical titres. In this study we showed that using magnetic beads coated with HIV-1_{CAP88} derived monomeric gp120 it was possible to completely adsorb all gp120 specific antibodies from plasma taken at 26 weeks post-infection, as measured by ELISA (Figure 16B). The removal of these gp120 binding antibodies resulted in the complete depletion of autologous neutralising antibodies from this plasma sample (Figure 16C). Therefore we can conclude that all the neutralising antibodies in this plasma sample targeted epitopes apparent on monomeric gp120.

These same HIV-1_{CAP88} gp120 coated magnetic beads were then used to adsorb all of the gp120 specific antibodies from plasma taken at 54 weeks post-infection (Figure 16D). By depleting these antibodies it was possible to significantly decrease the ID_{50} value from a titre of 1:1025 to a titre of 1:200, effectively reducing the neutralisation titres by 81% (Figure 16E). The residual neutralisation activity not adsorbed by monomeric gp120 at this later time point might be due to the antibodies in this sample that target the V1-V2 loop. Anti-V1-V2 neutralising antibodies isolated to date typically recognise epitopes only present on the native trimer, and cannot be adsorbed out of sera/plasma samples using monomeric gp120 (Kimura et al., 2009; Walker et al., 2009). It has been speculated that these antibodies may bind epitopes that link long flexible loop components of adjacent monomers (such as V2 and V3), or that the V1-V2 loop domain simply does not adopt its native conformation in the context of monomeric gp120 (Walker et al., 2009).

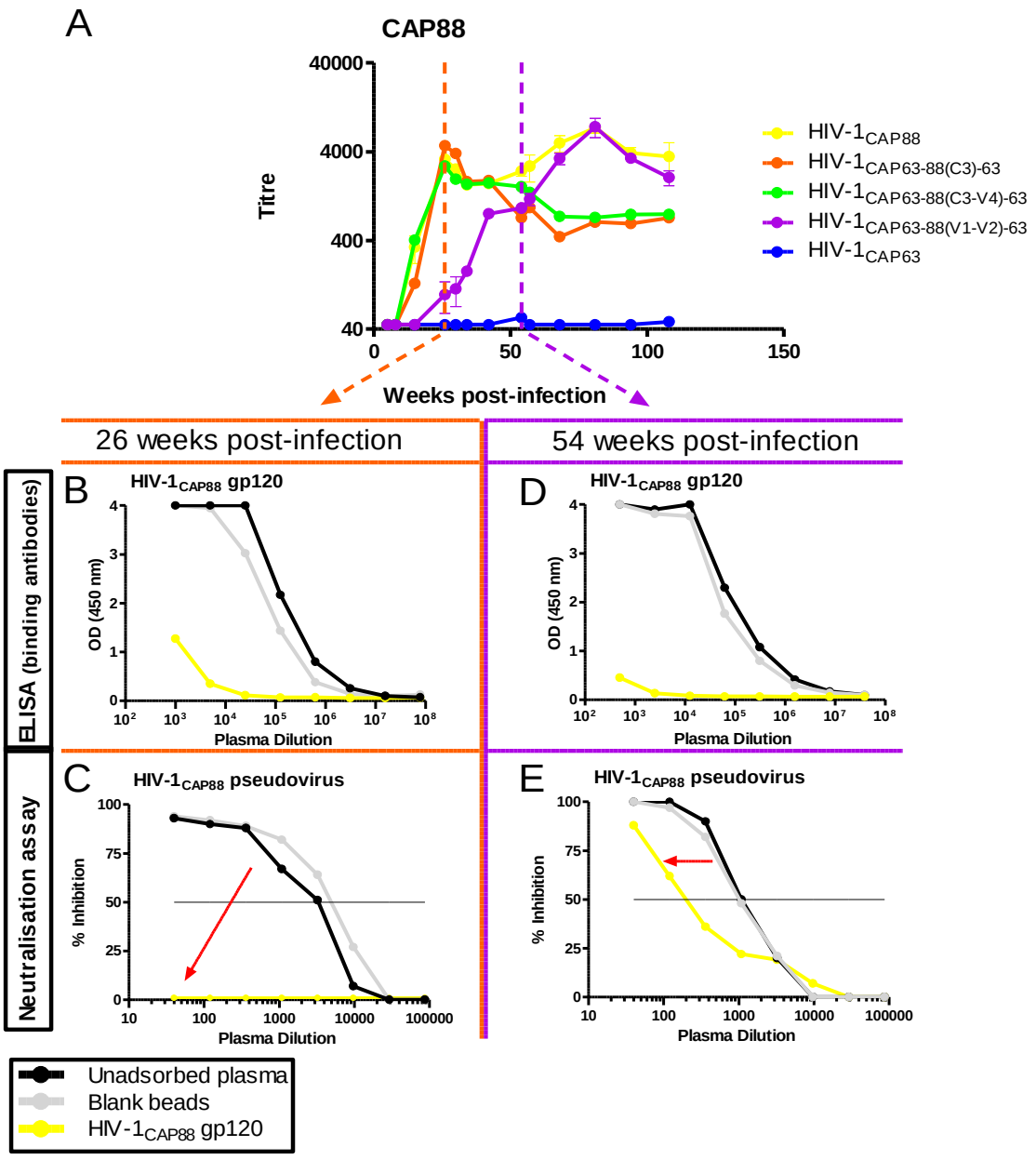


Figure 16: Adsorption of CAP88 antibodies using monomeric g120
(A) Development of neutralising antibody titres in CAP88 over time (modified from (Moore et al., 2009). Titres against the autologous HIV-1_{CAP88} and heterologous HIV-1_{CAP63} are shown in yellow and blue respectively. Titres against chimeric viruses incorporating autologous C3, C3-V4 or V1-V2 regions are shown in orange, green, and purple respectively. The two time points (26 and 54 weeks post infection) used for the adsorption studies are indicated with the dotted lines. **(B)** Adsorption of CAP88 gp120 binding antibodies at 26 weeks post infection measured by ELISA. Plasma adsorbed with blank beads or autologous gp120 coated beads are shown in grey and yellow, while untreated plasma is shown in black. A reduction in the OD at 450 nm correlates with adsorption of gp120 specific antibodies. **(C)** Complete adsorption of CAP88 neutralising antibodies from 26 weeks post-infection, coloured as in (B). A reduction in ID_{50} and loss of neutralisation activity (indicated with the red arrow) correlates with the adsorption of neutralising antibodies **(D)** Adsorption of CAP88 gp120 binding antibodies at 54 weeks post-infection, coloured as in (B). **(E)** Partial adsorption of CAP88 neutralising antibodies from 54 weeks post-infection, coloured as in (B).

CAP45

Preceding studies have also shown that neutralisation titres in participant CAP45 plasma peak at 43 weeks post-infection (Moore et al., 2009). This coincided with peak neutralisation titres against chimeras carrying either autologous C3-V4 or V1-V2 regions (Figure 17A). Magnetic beads coated with HIV-1_{CAP45} recombinant gp120 were also able to adsorb out all the CAP45 gp120 binding antibodies from this time point (Figure 17B), resulting in a concurrent 68% ± 8% drop in the neutralisation titres at this time point (Figure 17C) as calculated by the reduction in ID_{50}. It was hypothesised that similarly to the CAP88 adsorption experiment, incomplete adsorption of CAP45 neutralisation titres using monomeric gp120 was a consequence of anti-V1-V2 antibodies that require the native envelope quaternary structure.

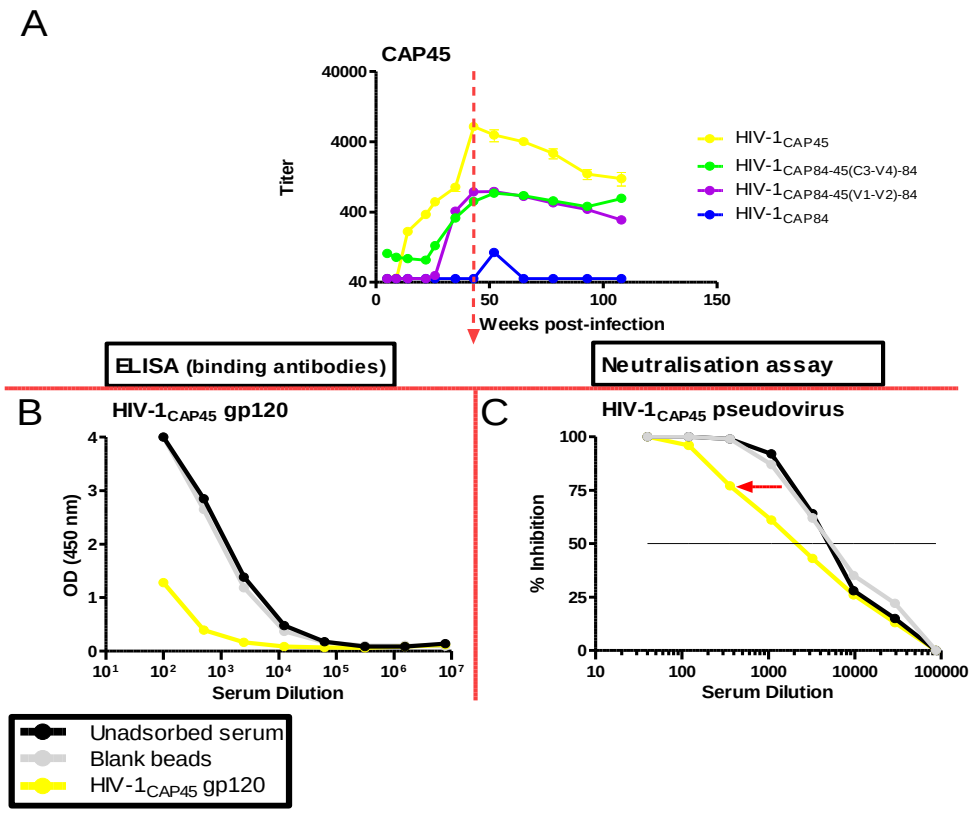


Figure 17: Adsorption of CAP45 antibodies using monomeric g120
(A) The development of CAP45 neutralising antibody titres over time (Moore et al., 2009). Titres against the autologous HIV-1_{CAP45} and heterologous HIV-1_{CAP84} are shown in yellow and blue respectively. Titres against chimeric viruses incorporating autologous C3-V4 or V1-V2 regions are shown in green and purple respectively. The time point used for the adsorption studies is indicated with the dotted line. **(B)** Adsorption of CAP45 gp120 binding antibodies measured by ELISA. Plasma adsorbed with blank beads or autologous gp120 coated beads are shown in grey and yellow, while untreated plasma is shown in black. **(C)** Partial adsorption of CAP45 neutralising antibodies from 43 weeks post-infection, coloured as in (B). The reduction in neutralising activity is indicated with the red arrow.

CAP84

The development of autologous neutralising antibodies in CAP84 has not been as extensively mapped as it has with CAP88 and CAP45, making it difficult to pinpoint the time post infection at which anti-C3-V4 antibody titres had peaked. Previous data suggested the presence of antibodies in CAP84 serum targeting the HIV-1_{CAP84} C3-V4 and V5 regions (Figure 18A), with titres for both antibody specificities peaking at 37 weeks post-infection, and remaining fairly constant through to 54 weeks post-infection (Moore et al., 2008). Therefore both these time points were chosen for the adsorption studies. Using magnetic beads coated with monomeric gp120 derived from HIV-1_{CAP84} it was possible to adsorb almost all the binding antibodies detectable by ELISA at both 37 weeks (Figure 18B) and 54 weeks (Figure 18D) post-infection. Similarly, recombinant gp120 was able to adsorb out all the neutralising antibodies in both these serum samples (Figures 18C and 18E) with the level of depletion similar irrespective of the time point used. Given the proximity of the V5 loop to C3-V4 it is possible that these two specificities are related, or perhaps even the same epitope. As shown by the dotted green curve in Figure 18A, removal of the autologous C3 region (the reverse chimera) impacted negatively on the titres of both these antibody specificities, suggesting that they are dependent on the C3 region (Moore *et al.*, 2008). Nevertheless the results of these adsorption studies suggest that both these antibody responses (whether related or not) target epitopes that were apparent on monomeric gp120.

CAP63

Unfortunately CAP63 progressed to AIDS very rapidly, making extensive mapping impossible. Despite this earlier studies have shown that antibodies targeting the HIV-1_{CAP63} C3-V4 region were present at peak titres from 20 through to 37 weeks post-infection (Moore et al., 2008). A portion of this activity appeared directed towards the V4 alone, as the HIV-$1_{CAP88\text{-}63(V4)\text{-}88}$ chimera was also sensitive to CAP63 antibodies (Figure 19A). Removal of the autologous C3-V4 region or the autologous V4 alone (reverse chimeras) had similar effects on autologous neutralisation titres. This data suggests that the C3 and V4 alone do not entirely encompass this particular epitope, or that another neutralisation specificity began to appear simultaneously alongside the anti-C3-V4 response. Using magnetic beads coated with monomeric gp120 derived from HIV-1_{CAP63} almost all the gp120 binding antibodies in CAP63 serum from 20 weeks (Figure 19B) and 37 weeks post infection (Figure 19D) could be adsorbed out.

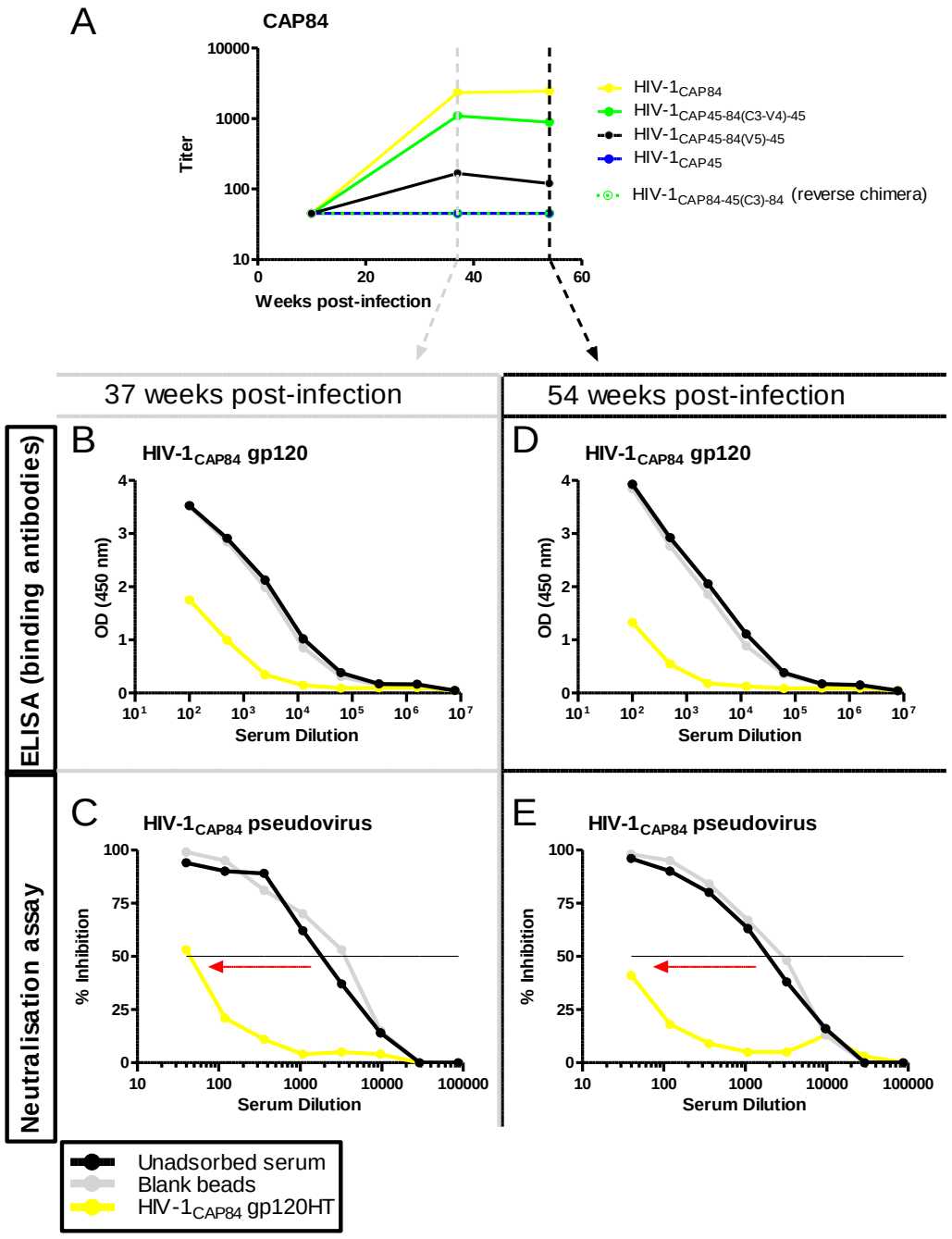


Figure 18: Adsorption of CAP84 antibodies using monomeric gp120
(A) The development of CAP84 neutralising antibody titres over time (modified from (Moore et al., 2009). Titres against the autologous HIV-1_{CAP84} and heterologous HIV-1_{CAP45} are shown in yellow and blue respectively. Titres against chimeric viruses incorporating autologous C3-V4 or V5 regions are shown in green and black respectively. Titres against the reverse chimera incorporating a heterologous C3 region are shown with the dotted green line. The time points used for the adsorption studies are indicated with the dotted grey (37 weeks) and black (54 weeks) lines. **(B)** Adsorption of CAP84 gp120 binding antibodies from 37 weeks post-infection, measured by ELISA. Plasma adsorbed with blank beads or autologous gp120 coated beads are shown in grey and yellow, while untreated plasma is shown in black. **(C)** Adsorption of CAP84 neutralising antibodies from 37 weeks post-infection, coloured as in (B). The reduction in neutralising activity is indicated with the red arrow. **(D)** Adsorption of CAP84 gp120 binding antibodies from 54 weeks post-infection as measured by ELISA, coloured as in (B). **(E)** Adsorption of CAP84 neutralising antibodies from 54 weeks post-infection, coloured as in (B). The reduction in neutralising activity is indicated.

By adsorbing these antibodies it was possible to deplete 50% ± 7% of the neutralisation titres at both 25 weeks (Figure 19C) and 37 weeks (Figure 19E) post-infection. The level of depletion was similar irrespective of the time point used. Despite the drop in ID_{50} titres (from ~1:5500 to ~1:2750) the partial depletion of neutralisation activity does not reflect in the neutralisation curves, which are plotted on a log scale. There was also a minor adsorption of antibodies using blank beads only (negative control) relative to the unadsorbed serum sample in this and subsequent experiments. This was attributed to the incomplete inactivation of free tosyl groups when the beads were 'blocked' with BSA, or some level of non-specific binding of serum antibodies to the BSA coated beads.

In summary, the autologous neutralising activities in plasma/serum from each of the four participants could be depleted to varying degrees using monomeric gp120. All the neutralisation activity in CAP88 plasma (within the first 6 months of infection) and CAP84 sera was directed towards epitopes contained entirely within monomeric gp120. However, adsorption of a later plasma sample from CAP88 resulted in only partial adsorption of the total neutralisation activity. This coincided with the development of anti-V1-V2 antibodies. Since all known anti-V1-V2 antibodies target quaternary epitopes it is most probable that these CAP88 antibodies could not be adsorbed by gp120, and were responsible for the residual neutralisation activity. Similarly the neutralisation activity in CAP45 sera could not be completely adsorbed with monomeric gp120, and this was also attributed to anti-V1-V2 antibodies that developed alongside the C3-V4 antibody response. Although CAP63 ID_{50} titres dropped following adsorption with monomeric gp120, the serum samples still retained considerable neutralisation potency. As was the case with CAP45 and the later sample from CAP88 it is possible that the residual neutralisation activity in the gp120 depleted serum was the result of a trimer specific antibody outside of C3-V4. However it may also be possible that the CAP63 C3-V4 epitope was one that requires the native trimer, or is not well reconstituted in the context of monomeric gp120 and could thus not be properly adsorbed. To confirm that the neutralising antibodies adsorbed by monomeric gp120 are in fact targeting the C3-V4 region, gp120s chimeric for each of the four C3-V4 regions were expressed for adsorption studies. The adsorbed sera were then assayed against the chimeric viruses to verify that each of the C3-V4 epitopes were in fact apparent on a single gp120 monomer.

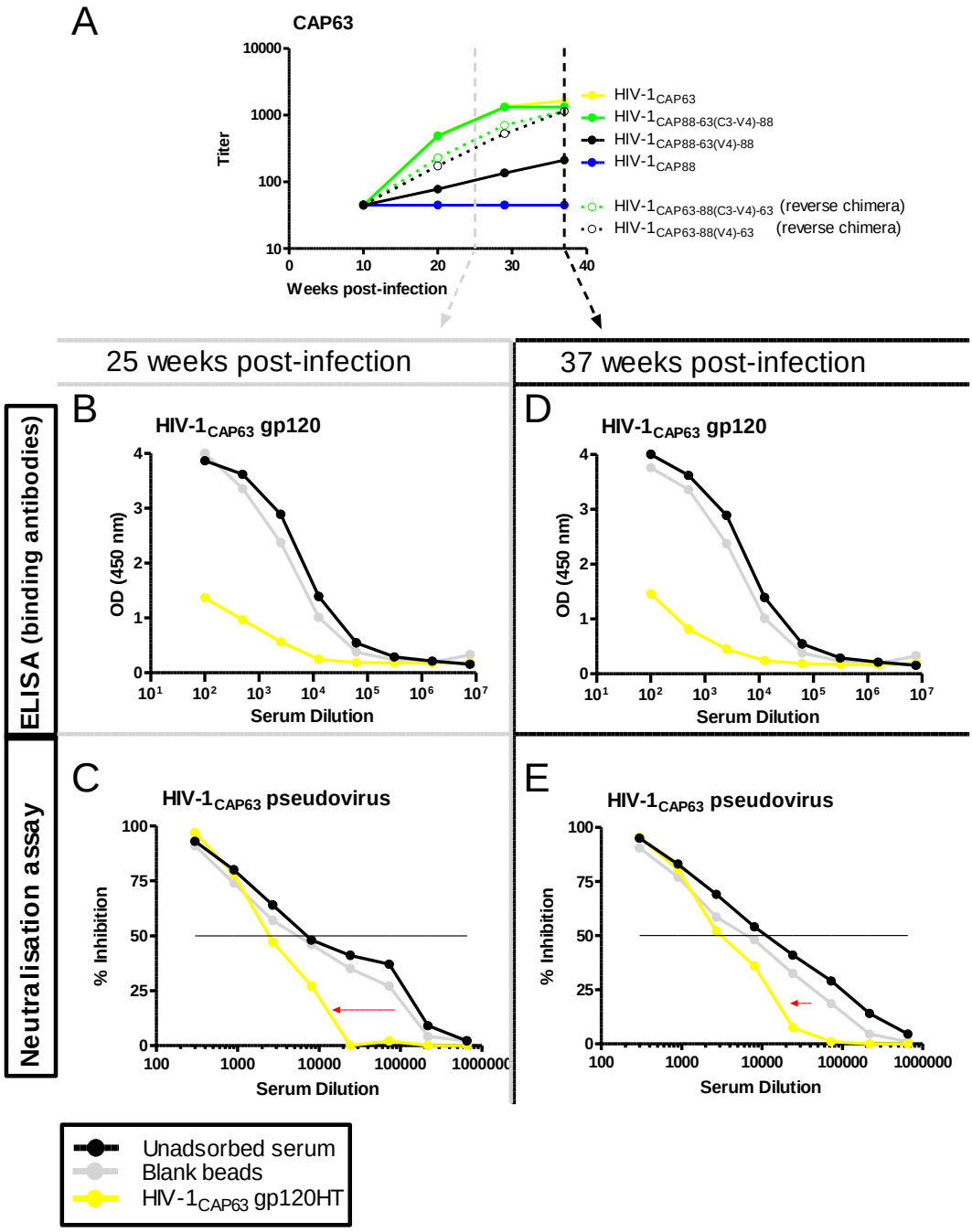


Figure 19: Adsorption of CAP63 antibodies using monomeric gp120
(A) The development of CAP63 neutralising antibody titres over time (Moore et al., 2009). Titres against the autologous HIV-1_{CAP63} and heterologous HIV-1_{CAP88} are shown in yellow and blue respectively, while titres against chimeric viruses incorporating autologous C3-V4 or V4 regions are shown in green or black respectively. Titres against the reverse chimeras, incorporating either a heterologous C3-V4 or V4 region, are shown with the dotted green and black lines. The time points used for the adsorption studies are indicated. **(B)** Adsorption of CAP63 gp120 binding antibodies at 25 weeks post-infection measured by ELISA. Plasma adsorbed with blank beads or autologous gp120 coated beads are shown in grey and yellow, while untreated plasma is shown in black. **(C)** Adsorption of CAP63 neutralising antibodies from 25 weeks post-infection, coloured as in (B). The reduction in CAP63 neutralisation titres is indicated with the red arrow. **(D)** Adsorption of CAP63 gp120 binding antibodies at 37 weeks post-infection measured by ELISA, coloured as in (B). **(E)** Adsorption of CAP63 neutralising antibodies from 37 weeks post-infection, coloured as in (B). The reduction in CAP63 neutralisation titres is indicated.

CAP88 neutralising antibodies can be entirely adsorbed with the C3 region of gp120

Of the four participants with neutralising antibodies targeting C3-V4, the CAP88 response was unique in that it did not require the autologous V4 for efficient neutralisation (Moore et al., 2008). Rather, the variable component of this epitope appeared to be contained almost entirely within the C3 region (Figure 16A). Escape from these antibodies was mediated entirely by two mutations in the α2-helix (Moore et al., 2009). Although one change resulted in the addition of a glycan which might alter glycan packing and sterically occlude more distant epitopes, the second mutation resulted in a charge change at a solvent exposed surface, suggesting that the α2-helix was in fact the antibody target (Figure 8). For the purposes of this study gp120s from the chimeric pseudoviruses HIV-$1_{CAP63\text{-}88(C3\text{-}V4)\text{-}63}$ and HIV-$1_{CAP63\text{-}88(C3)\text{-}63}$ were assessed for their ability to adsorb out the neutralising antibodies in CAP88 plasma from 26 weeks post-infection. Heterologous gp120 from the resistant HIV-1_{CAP63} virus that was used to make the backbone was also expressed and served as a negative control.

Both autologous and heterologous parental gp120s, as well as both the C3 and C3-V4 chimeric gp120 proteins were able to adsorb out all of the cross-reactive binding antibodies in CAP88 plasma capable of binding HIV-1_{CAP63} derived heterologous gp120 in ELISA (Figure 20A). These cross-reactive antibodies are non-neutralising and likely recognise conserved epitopes such as those in V3 or the CD4 binding site. However, the heterologous HIV-1_{CAP63} gp120 was unable to significantly deplete the binding signal generated in ELISA against any of the other gp120s (Figures 20B, 20C, and 20D - blue line). This was explained by the inability of the heterologous gp120 to adsorb out any of the HIV-1_{CAP88} specific autologous binding antibodies, such as the C3-V4 neutralising antibodies. While both chimeric proteins could completely adsorb out the binding antibodies specific for the C3 and C3-V4 chimeras (Figures 20B - orange line, and 20C - green line), they could both only partially deplete the binding signal generated against the autologous gp120 (Figure 20D - orange and green lines). This residual signal could be attributed to other autologous binding antibodies in CAP88 serum specific to epitopes outside the C3-V4 region. These antibodies would have to be non-neutralising (discussed below), and probably recognise epitopes that overlap with other variable regions such as V1-V2.

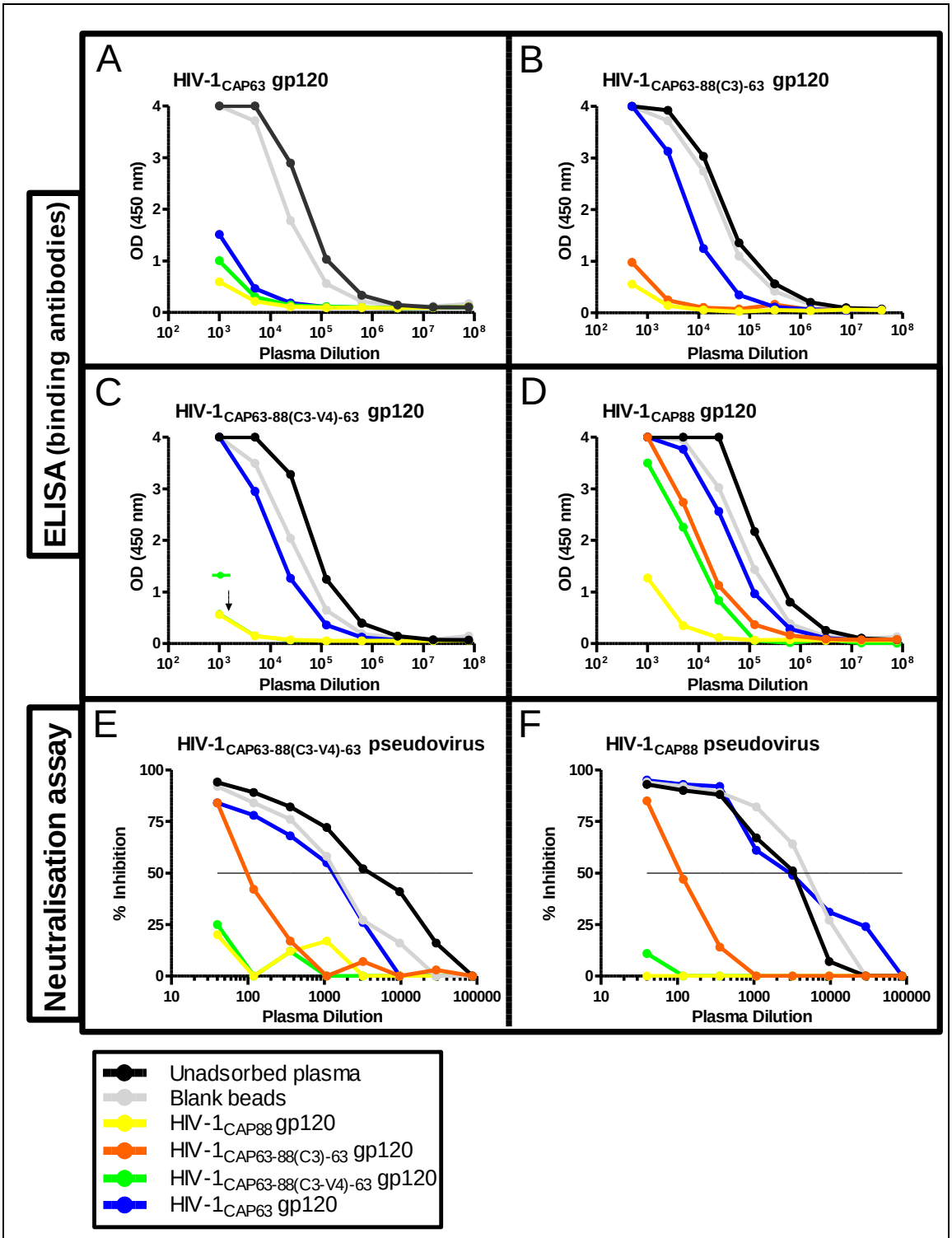


Figure 20: Adsorption of CAP88 antibodies using chimeric gp120 proteins
CAP88 plasma from 26 weeks post-infection was adsorbed with blank uncoated beads (grey), heterologous HIV-1_{CAP63} gp120 (blue), autologous HIV-1_{CAP88} gp120 (yellow), and chimeric gp120 from HIV-$1_{CAP63\text{-}88(C3)\text{-}63}$ (orange) or HIV-$1_{CAP63\text{-}88(C3\text{-}V4)\text{-}63}$ (green), and then compared to the unadsorbed plasma sample (black). Adsorbed plasma samples were tested by ELISA for binding to **(A)** heterologous gp120, **(B)** C3 chimeric gp120, **(C)** C3-V4 chimeric gp120, and **(D)** autologous gp120. They were also tested for their residual neutralisation activities against both the **(E)** HIV-$_{1CAP63\text{-}88(C3\text{-}V4)\text{-}63}$ chimeric virus, and the **(F)** autologous HIV-1_{CAP88} virus.

While the heterologous gp120 could not absorb any of the neutralising antibodies in CAP88 serum (Figures 20E, and 20F - blue line), both the autologous (yellow line) and C3-V4 chimeric (orange line) gp120 proteins were able to completely deplete the CAP88 serum sample of its neutralising activity against both C3-V4 chimeric (Figure 20E) and autologous (Figure 20F) viruses. The C3 protein was slightly less efficient at adsorbing out these neutralising antibodies, but nevertheless was still able to drop the ID_{50} titres against both the wild-type and chimeric viruses by 84.1% and 94.8% respectively. Thus it appears that access or binding to the CAP88 C3 epitope is influenced to some extent by the adjacent V4 loop. Since all of the neutralising activity targeting the C3-V4 chimeric virus could be adsorbed by the autologous and C3-V4 chimeric proteins, the CAP88 C3-V4 epitope must be apparent on monomeric gp120. Furthermore since the C3-V4 chimeric gp120 could adsorb out all of the autologous neutralising antibodies in CAP88 plasma at 26 weeks post infection, this region must serve as the sole neutralisation target at this particular time point.

C3-V4 antibodies were completely adsorbed with monomeric gp120

C3-V4 chimeric gp120s from the remaining three viruses (HIV-1_{CAP45}, HIV-1_{CAP84}, and HIV-1_{CAP63}) were also assessed for the ability to adsorb out autologous neutralising antibodies. Heterologous gp120s from the resistant viruses were again used as negative controls. In all three experiments the binding ELISA data showed a similar pattern as the CAP88 data discussed above. All recombinant proteins were able to successfully deplete out the cross-reactive binding antibodies in serum samples from CAP45 (Figure 21A), CAP63 (Figure 21B), and CAP84 (Figure 21C), while only the chimeric and autologous proteins were able to deplete the C3-V4 specific binding antibodies in these same samples (Figures 21D, 21E, and 21F - green and yellow lines). It was solely the autologous gp120s that were able to efficiently deplete all the autologous binding antibodies (Figures 21G, 21H, and 21I - yellow lines).

Despite these similarities when compared to the adsorption of CAP88 binding antibodies, there were considerable differences when comparing the adsorption of CAP45, CAP63, and CAP84 specific neutralising antibodies to the CAP88 experiment (Figure 22). In these three individuals the C3-V4 specific neutralising antibodies could again be completely adsorbed with autologous and chimeric gp120, as was evident when testing the adsorbed samples against the C3-V4 chimeric viruses (Figures 22A,

22B, and 22C - green and yellow lines). However unlike the CAP88 adsorption experiment, the chimeric proteins did not adsorb any neutralisation activity when assayed against the parental viruses (Figures 22D, 22E, and 22F - green lines). Thus the complete adsorption of the C3-V4 responses had no effect on the adsorption of autologous neutralisation activity in these three individuals. This might suggest the presence of other antibody specificities that prevented complete adsorption of autologous neutralisation. Similarly inclusion of the autologous C3-V4 may have rendered the chimeric pseudoviruses sensitive to only a subset of the neutralising antibodies targeting C3, with other antibodies in the samples targeting epitopes overlapping C3 but requiring other components of the envelope adjacent to C3, such as the V5 loop. Lastly it is possible that the C3-V4 antibodies do not contribute at all to autologous neutralisation. Therefore to attempt to explain this data, a second set of mutant/chimeric viruses and recombinant proteins was engineered.

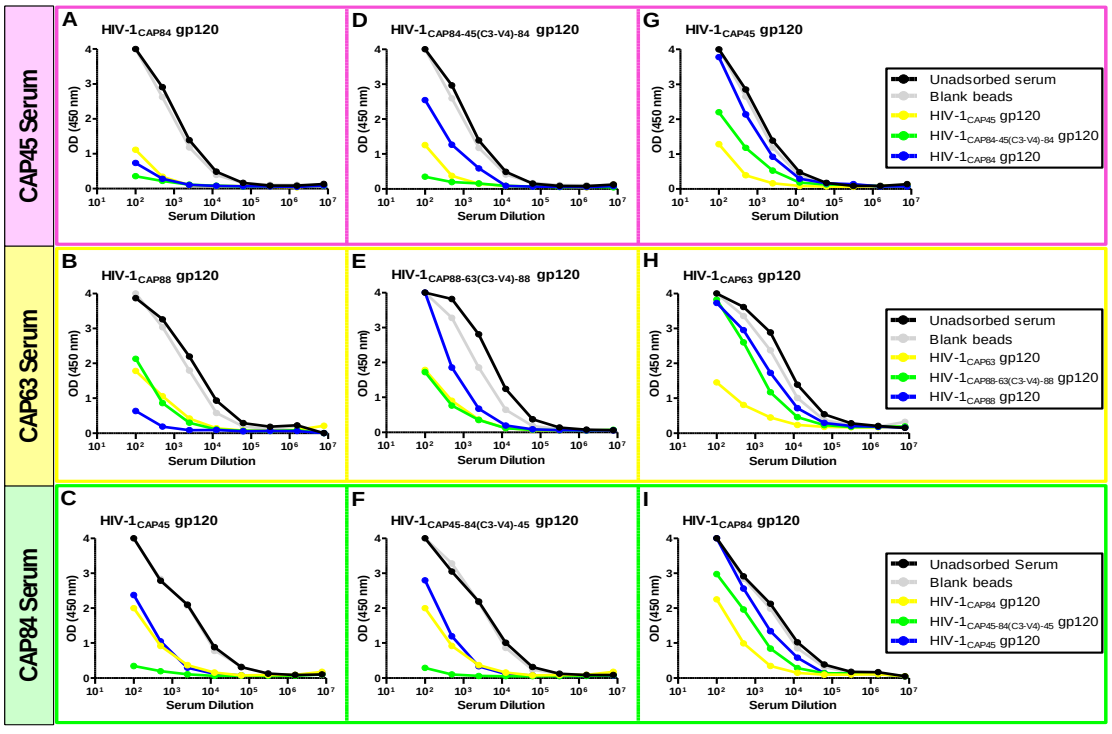


Figure 21: ELISA data showing the adsorption of CAP45, CAP63, and CAP84 binding antibodies using monomeric gp120

Adsorption of binding antibodies from CAP45 serum taken at 43 weeks post-infection **(boxed in pink)**, CAP63 serum taken at 37 weeks post-infection **(boxed in yellow)**, and CAP84 serum taken at 37 weeks post-infection **(boxed in green)**, as measured by ELISA against heterologous gp120 (A, B, and C), C3-V4 chimeric gp120 (D, E, and F), or autologous gp120 (G, H, and I). Each serum sample was adsorbed with either autologous gp120 (yellow), chimeric gp120 (green), or heterologous gp120 (blue), and compared to unadsorbed serum (black) and serum adsorbed with blank beads only (grey).

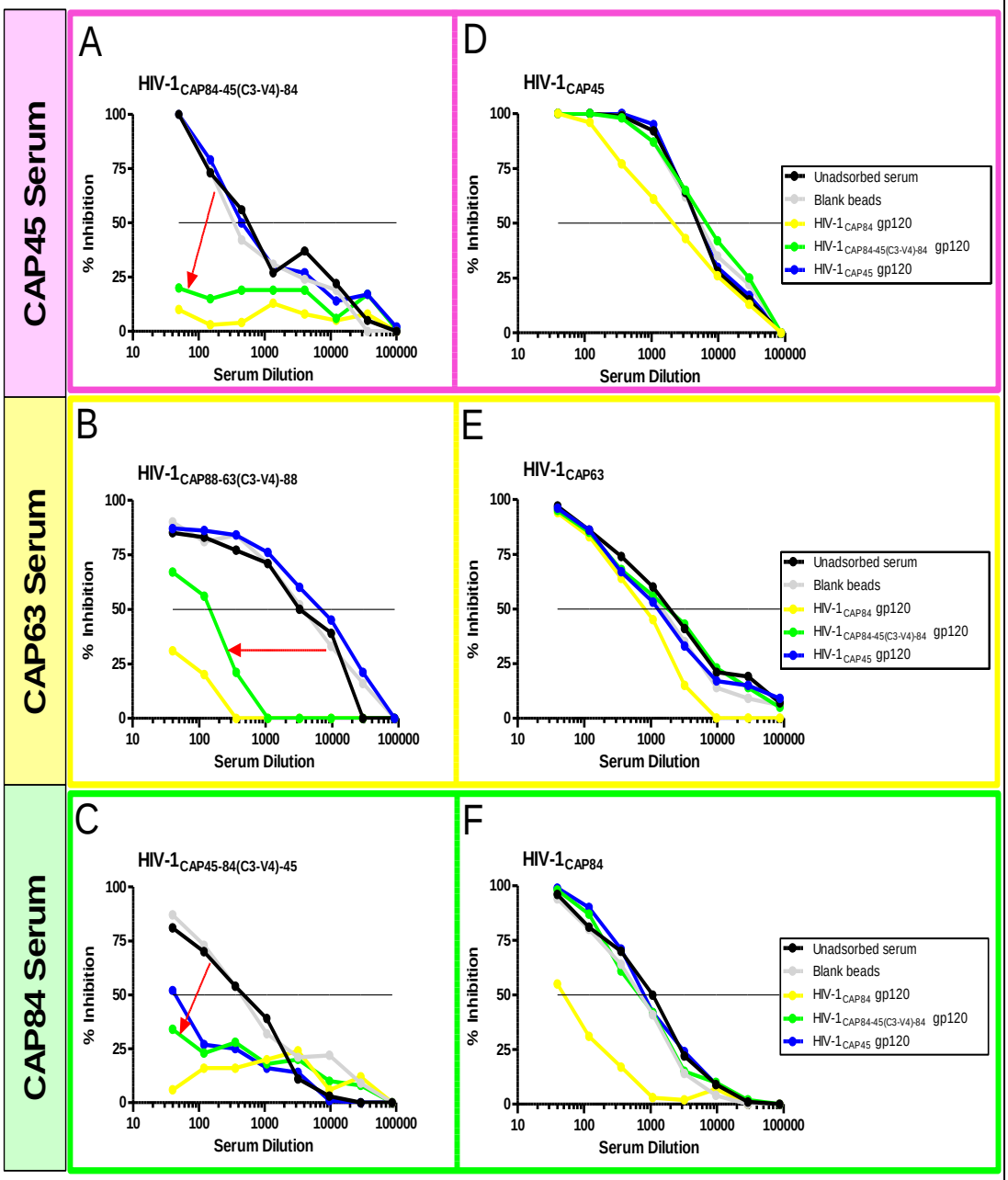


Figure 22: Adsorption of C3-V4 specific neutralising antibodies from participants CAP45, CAP63, and CAP84

Monomeric gp120 was used to adsorb out the neutralising activity in serum samples from CAP45 taken at 43 weeks post-infection **(boxed in pink)**, CAP63 taken at 37 weeks post-infection **(boxed in yellow)**, and CAP84 taken at 37 weeks post-infection **(boxed in green)**. The adsorbed material was assayed against either the C3-V4 chimeric viruses (A, B, and C) or the autologous viruses (D, E, and F) for residual neutralisation activity. Serum was adsorbed with either autologous gp120 (yellow), chimeric gp120 (green), or heterologous gp120 (blue), and compared to unadsorbed serum (black) and serum adsorbed with blank beads only (grey). The neutralisation activities adsorbed with the chimeric gp120s are indicated with the red arrows.

C3-V4 chimeric envelope glycoproteins may expose epitopes in the CD4 binding site

When CAP84 serum was adsorbed using magnetic beads coated with monomeric gp120 it was discovered that heterologous gp120 was able to adsorb out the antibodies in CAP84 serum responsible for the neutralisation of the chimeric virus HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ (Figure 22C). Despite this, both heterologous and chimeric gp120 could not adsorb any of the CAP84 autologous neutralising antibodies (Figure 22F). This data might be explained if the HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ chimeric viruses adopted a more exposed conformation, rendering certain epitopes for normally non-neutralising antibodies more accessible on the native trimer. The exposed epitope would have to be one that is common to both autologous and heterologous gp120. This implicated the functionally conserved receptor binding sites, which are accessible on the native trimer, and partly overlap with the C3 region of the envelope. To test whether the receptor binding sites had become exposed to CAP84 non-neutralising antibodies on the chimeric viral envelope, both parental and chimeric viruses were tested against a range of monoclonal antibodies (Figure 24).

Only monoclonal antibodies with detectable binding to gp120 from the viruses concerned were used for the neutralisation experiments (Figure 15 and Figure 23). 17b and 1.9E were the only co-receptor binding site antibodies able to bind all three gp120 proteins tested, and only when incubated in the presence of sCD4 (Figure 23). Therefore in order to neutralise viruses using co-receptor binding site directed antibodies, viruses were first pre-incubated for one hour at 37 °C with sCD4 at a concentration previously determined to inhibit viral entry by 40%. None of the anti-co-receptor binding site antibodies were able to neutralise either parental or chimeric viruses in the presence of soluble CD4 (Figure 24A). Similarly the normally non-neutralising anti-CD4 binding site antibody b6 could not neutralise any of these viruses (Figure 24B). It was particularly significant though, that the chimeric pseudovirus was 2-20 fold more sensitive to neutralisation by CD4-IgG2 (IC_{50} of 0.22 μg/mL) than the parental viruses HIV-1_{CAP45} and HIV-1_{CAP84} (IC_{50}s of 0.41 μg/mL and 4.63 μg/mL respectively). CD4-IgG2 is a fusion protein combining the F_c immunoglobulin domain with the two most terminal domains of CD4. It was perhaps even more striking that the chimeric virus was neutralised at relatively low

concentrations of soluble CD4 (9.55 µg/mL). The highly neutralisation sensitive T-cell laboratory adapted strain HIV-1_{SF162} was used as a positive control, and was sensitive to neutralisation by all co-receptor and CD4 binding site antibodies tested. Based on these data it was concluded that the HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ chimeric pseudovirus had a more exposed CD4 binding site. To confirm whether this exposure was responsible for the neutralisation of the chimeric virus by CAP84 serum, a D368R mutation was introduced into the parental and chimeric recombinant gp120s (Figure 25). This mutation is positioned in the CD4 binding loop of C3 and is known to abrogate the binding of CD4 resulting in a non-functional virion. Furthermore since anti-CD4 binding site antibodies typically interact with the CD4 binding loop, the D368R mutation also abrogates the binding of most anti-CD4 binding site antibodies (Li et al., 2007).

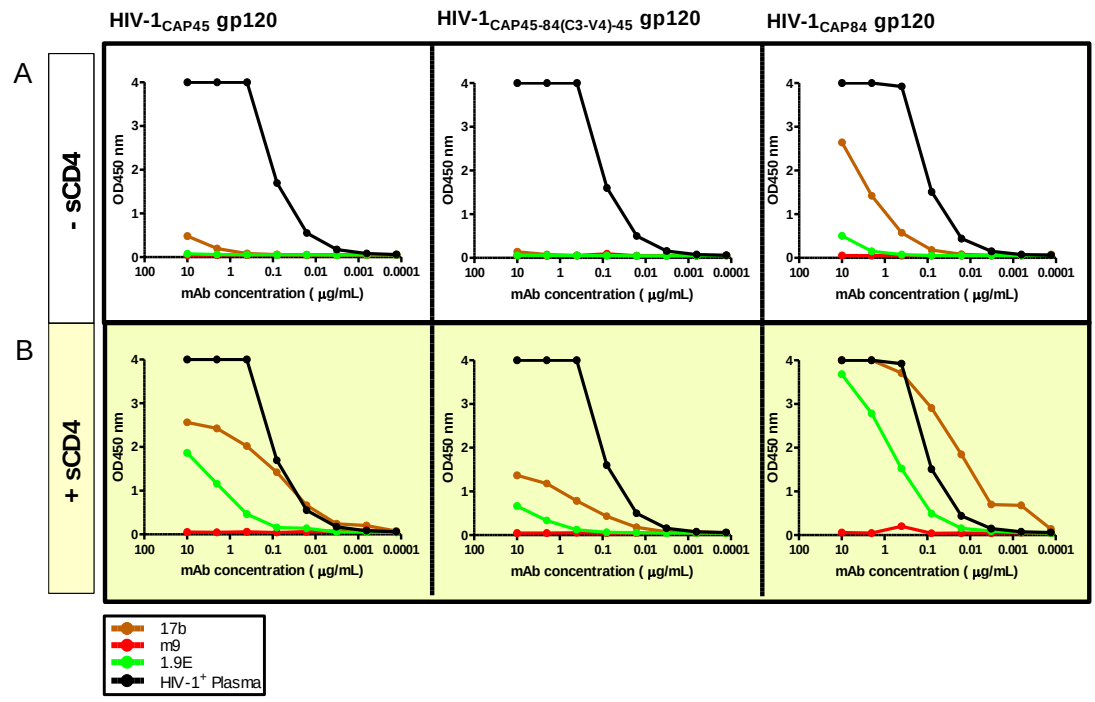


Figure 23: Binding of monoclonal antibodies to the co-receptor binding site is sCD4 dependent
(A) Monomeric gp120 from HIV-1_{CAP45}, HIV-1_{CAP84}, and the chimeric virus HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ was tested for binding to antibodies m9 (red), 1.9E (green), and 17b (brown). **(B)** The experiment shown in (A) was repeated in the presence of saturating concentrations of sCD4.

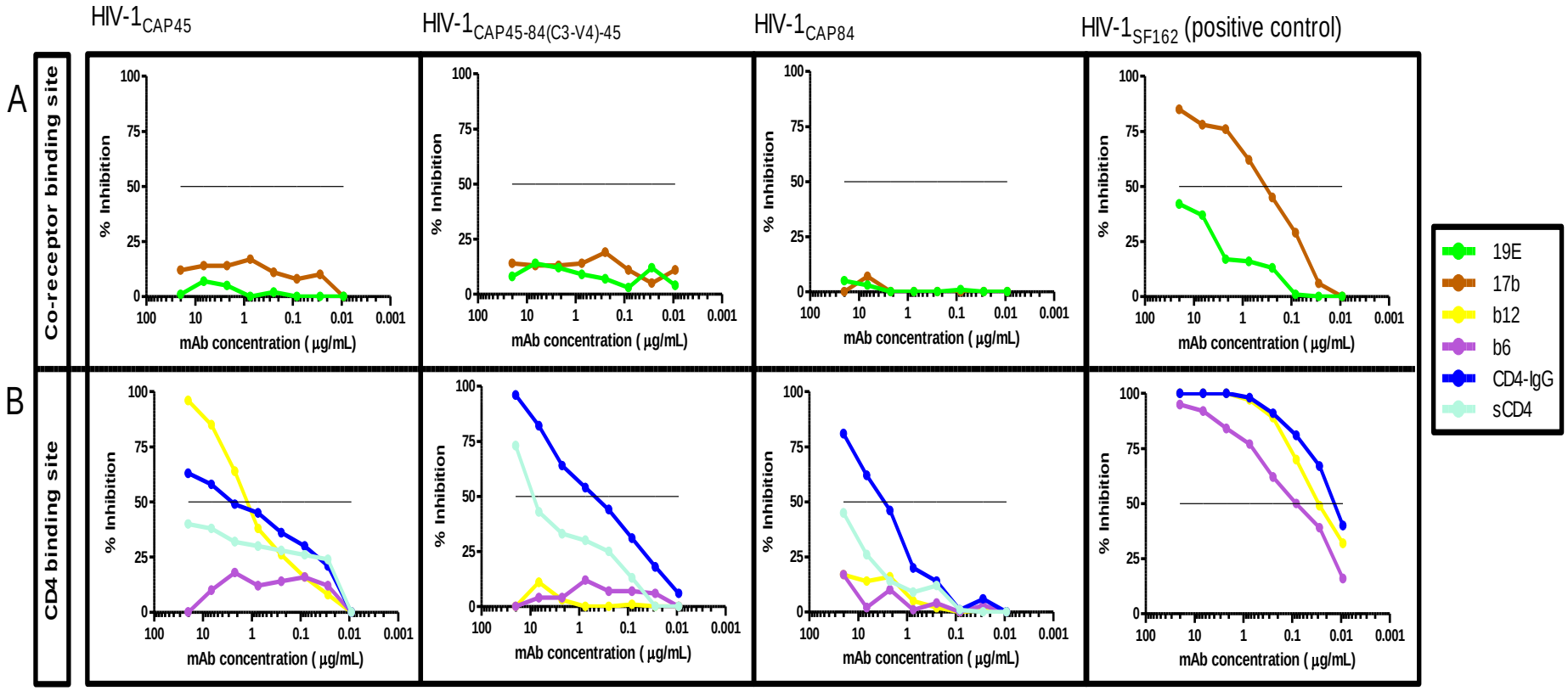


Figure 24: Exposure of the CD4 binding site in a C3-V4 chimeric virus leads to enhanced neutralisation by CD4-IgG2 and sCD4
(A) Neutralisation of HIV-1_{CAP45}, HIV-1_{CAP84}, and the chimeric virus HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ using monoclonal antibodies 17b (brown), and 1.9E (green) in the presence of sCD4. **(B)** Neutralisation HIV-1_{CAP45}, HIV-1_{CAP84}, and the chimeric virus HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ using soluble CD4 (cyan), and monoclonal antibodies b12 (yellow), b6 (purple), and CD4-IgG2 (blue) with epitopes in the CD4 binding site.

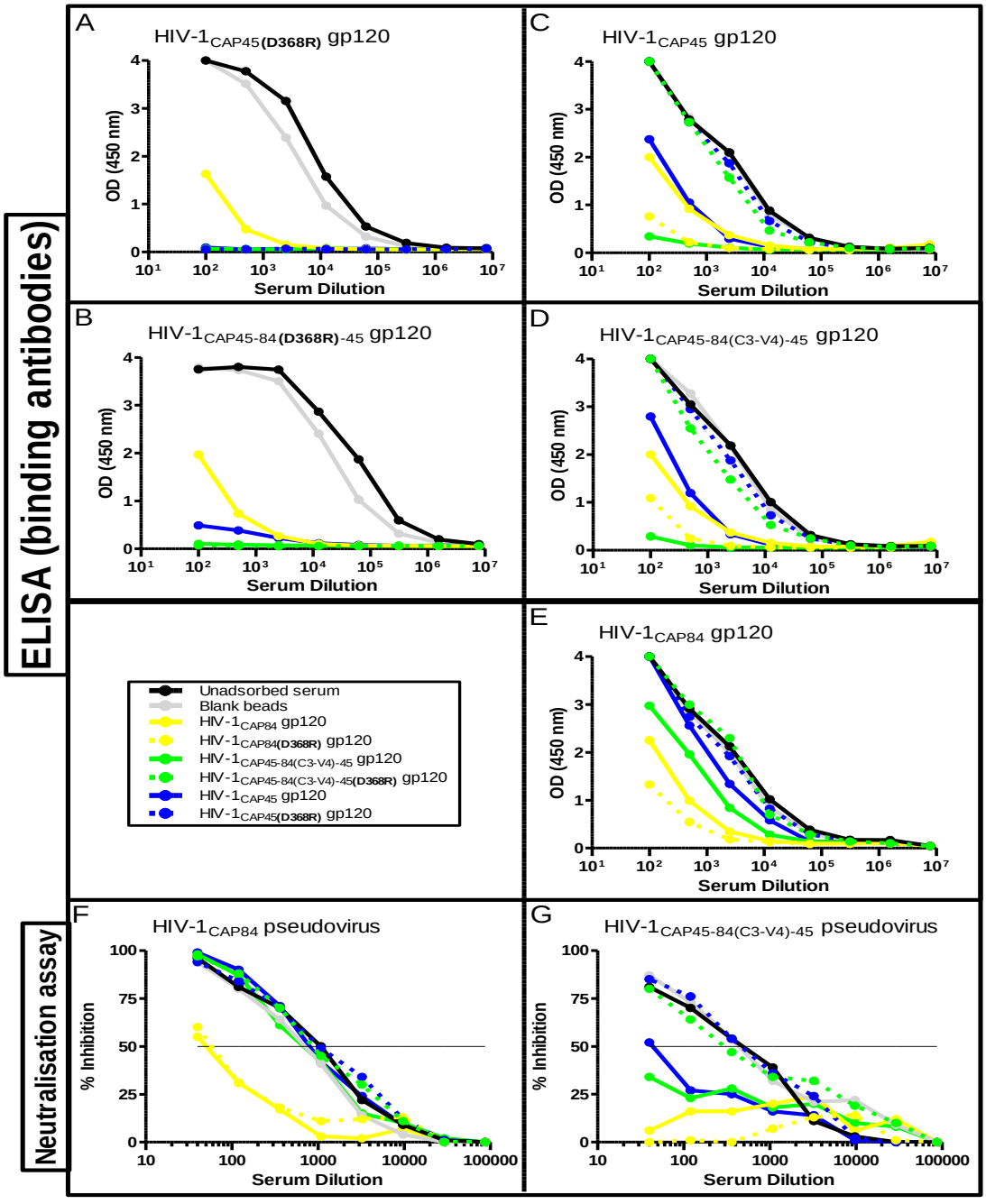


Figure 25: Poor adsorption of anti-C3-V4 antibodies in CAP84 serum by monomeric gp120 containing the D368R mutation

CAP84 serum was adsorbed with wild-type gp120s (solid lines) as well as the D368R mutant gp120s (dotted lines) from heterologous HIV-1_{CAP45} (blue), autologous HIV-1_{CAP84} (yellow), and the C3-V4 chimeric virus HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ (green). Residual binding antibodies were detected in ELISA against either **(A)** heterologous gp120 with the D368R mutation, **(B)**, heterologous gp120 **(C)**, C3-V4 chimeric gp120 with the D368R mutation **(D)** C3-V4 chimeric gp120, and **(E)** autologous gp120. Adsorbed sera tested for the ability to neutralise both the **(F)** autologous and **(G)** C3-V4 chimeric pseudoviruses.

Recombinant gp120s derived from HIV-1_{CAP45} or HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ carrying the D368R mutation were able to efficiently adsorb out all the binding antibodies specific for the mutant proteins (Figures 25A and 25B - dotted blue and green lines), but this adsorption did not affect the binding signal generated against the parental (Figures 25C and 25E) and chimeric (Figure 25D) proteins in ELISA. Thus despite the adsorption of all gp120 binding antibodies not affected by the D368R mutation, a saturating ELISA signal was still generated to the wild-type proteins from exclusively anti-CD4 binding site antibodies. Conversely autologous gp120 derived from HIV-1_{CAP84} with the D368R mutation was able to completely adsorb out all the gp120 binding antibodies in CAP84 serum (Figures 25C, 25D, and 25E - dotted yellow line).

Similarly while serum adsorbed with the heterologous HIV-1_{CAP45} gp120 and chimeric HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ gp120 depleted all of the neutralisation activity against the HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$ chimeric virus (Figure 25G - solid green and blue lines), when the D368R mutation was introduced into these two proteins they were no longer able to adsorb out this activity (Figure 25G - dotted green and blue lines). Since the only difference between the serum adsorbed with either wild-type gp120s or mutant gp120s was the absence or presence of anti-CD4 binding site antibodies, these antibodies must be responsible for the neutralisation of the C3-V4 chimeric virus. Consistent with the ELISA data HIV-1_{CAP84} autologous gp120 with the D368R mutation retained the ability to deplete the anti-CD4 binding site antibodies responsible for the neutralisation of HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$, and was just as efficient as the wild-type autologous protein at adsorbing the neutralising antibodies specific for HIV-1_{CAP84} (Figures 25F and 25G - yellow lines). Thus neutralisation of the C3-V4 chimera is mediated by antibodies to the CD4 binding loop, but these antibodies can still be adsorbed by autologous gp120 with the D368R mutation. Therefore it must be concluded that these antibodies bind significantly to other surrounding residues in the autologous protein that are not present in the heterologous gp120.

CAP45 antibodies target an epitope in β14+V5 that is apparent on monomeric gp120

Adsorption of CAP45 antibodies targeting C3-V4 did not affect autologous neutralisation titres, but adsorption of all gp120 binding antibodies partially depleted this activity. To explain this data a more detailed map of the antibody specificities in

CAP45 serum within the first year of infection was constructed. The viral quasi-species in participant CAP45 showed very little envelope sequence variability (Moore et al., 2009). In fact after one year of infection there were only eight amino acid substitutions that were positively selected for across the whole of HIV-1_{CAP45} gp160 (Appendix B). Of these mutations, three in V1-V2 have been shown to correlate with escape from V1-V2 directed antibodies, while an additional two in V5 were shown to mediate escape from a potent neutralising antibody with an unknown specificity, presumed to be related to the anti-C3-V4 response (Moore et al., 2009). Transferring the HIV-1_{CAP45} V5 into the resistant HIV-1_{CAP84} heterologous backbone did not confer neutralisation sensitivity to participant CAP45's neutralising antibodies (Moore et al., 2008). The remaining three mutations were in C1, V3, and V4 and had no effect on neutralisation sensitivity when back mutated in the 12 month clone (Moore et al., 2009). To attempt an explanation of the neutralisation escape mediated by the two V5 mutations, and potentially account for the escape from anti-C3-V4 antibodies, we created a chimera incorporating the autologous C3 and V5 regions (HIV-$1_{CAP84-45(C3+V5)-84}$) and assessed its sensitivity to neutralisation by CAP45 serum. The resulting chimera generated neutralisation titres that were consistently lower when compared to the wild-type virus, but showed a distinctly similar pattern over time (Figure 26 - red line). This was in contrast to the C3-V4 chimera which showed titres similar to the V1-V2 chimera (Moore et al., 2009). A C3+V5 chimeric gp120 was then generated for adsorption studies. Given the saturating levels of neutralisation despite a 1 in 20 dilution of CAP45 serum in the C3-V4 adsorption experiments, the serum was diluted further to 1:150 for the C3+V5 adsorption studies. Adsorption of binding antibodies by the C3+V5 chimeric protein was similar to that of the C3-V4 chimeric protein in that it was able to deplete out all the cross-reactive and C3+V5 binding antibodies specific for the chimeric protein, but could not deplete out all of the autologous binding antibodies in CAP45 serum (Figures 21, 27A and 27B). However distinct differences were apparent in the ability of the C3+V5 chimeric gp120 to adsorb out the CAP45 autologous neutralising antibodies.

In addition to adsorbing out all the C3+V5 specific antibodies responsible for neutralising HIV-$1_{CAP84-45(C3+V5)-84}$ (Figure 27C – red line), the C3+V5 chimeric gp120 was also able to adsorb some of the autologous neutralising antibodies (Figures 27D - red line), something the C3-V4 chimeric gp120 was unable to achieve (Figure 22).

However the C3+V5 chimeric gp120 could not deplete any of the neutralising antibodies targeting the C3-V4 chimeric pseudovirus HIV-$1_{CAP84\text{-}45(C3\text{-}V4)\text{-}84}$ and vice versa, suggesting that these two epitopes are distinct from each other on monomeric gp120 (Figures 27C - green line, and 27E - red line). Furthermore the autologous gp120 and the C3+V5 chimeric gp120, but not the C3-V4 chimeric gp120 were also able to deplete out all of the neutralisation activity targeting the twelve month clone with the two back-mutations in V5 (HIV-$1_{CAP45.52wks.07\ V5(E460K/G426D)}$) implicating these mutations in mediating escape from the C3+V5 specific antibodies but not C3-V4 specific antibodies (Figure 27F). To further define the C3+V5 neutralisation epitope we constructed a chimera containing the autologous V5, as well the autologous β14 and flanking regions (highlighted in cyan in Figure 3). Neutralisation titres against this chimera closely matched the titres against the C3+V5 chimeric pseudovirus, with minor differences possibly being explained by an improper conformation of the epitope in the context of β14+V5 only, or a diverse B-cell response to the C3+V5 epitope (Figure 26 - grey line). Thus, the C3+V5 epitope was further defined as an epitope in β14+V5, immediately adjacent to the CD4 binding site. Since the β14+V5 epitope overlaps the CD4 binding site, it is possible that antibodies targeting this site are reliant on residues in the CD4 binding loop. To determine whether this was the case we attempted to adsorb out CAP45 anti-β14+V5 antibodies using autologous HIV-1_{CAP45} gp120 with and without the D368R mutation (Figure 28). Both these proteins were able to adsorb out the CAP45 neutralising antibodies to similar extent, indicating that anti-β14+V5 antibodies were not sensitive to the D368R mutation (comparison of the solid and dotted yellow lines).

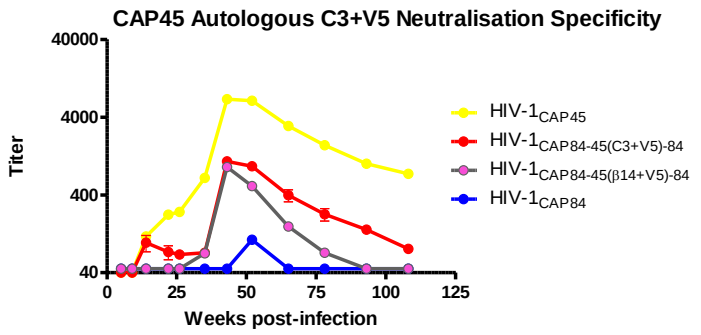


Figure 26: Development of anti-β14+V5 neutralising antibodies in CAP45 serum
The neutralisation titres in CAP45 serum was plotted at each time point against either the autologous virus (yellow), heterologous virus (blue), C3+V5 chimeric virus (red), or β14+V5 chimeric virus (dark grey filled in pink).

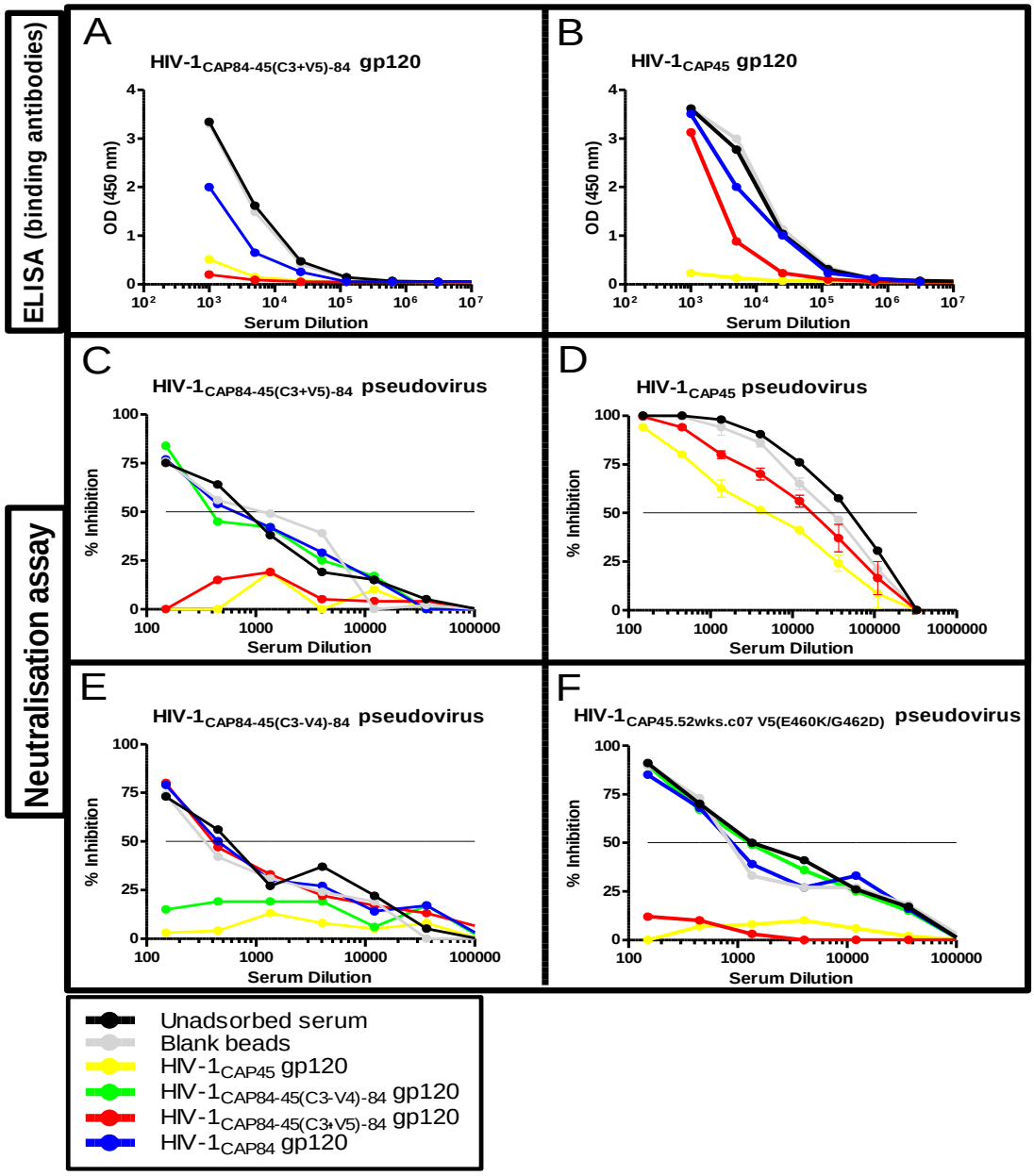


Figure 27: Adsorption of C3+V5 neutralising antibodies using monomeric gp120
CAP45 serum was adsorbed with either blank beads (grey), autologous gp120 (yellow), heterologous gp120 (blue), C3-V4 chimeric gp120 (green), or C3+V5 chimeric gp120 (red), and compared to unadsorbed serum (black). Adsorption of binding antibodies to either the **(A)** C3+V5 chimeric protein, or **(B)** autologous protein was assessed by ELISA. Adsorbed serum was tested for residual neutralisation activity against either the: **(C)** C3+V5 chimeric virus, **(D)** infecting autologous virus, **(E)** C3-V4 chimeric virus, or **(F)** autologous virus isolated from twelve months post-infection with back-mutations in two residues in V5 (E460K/G462D).

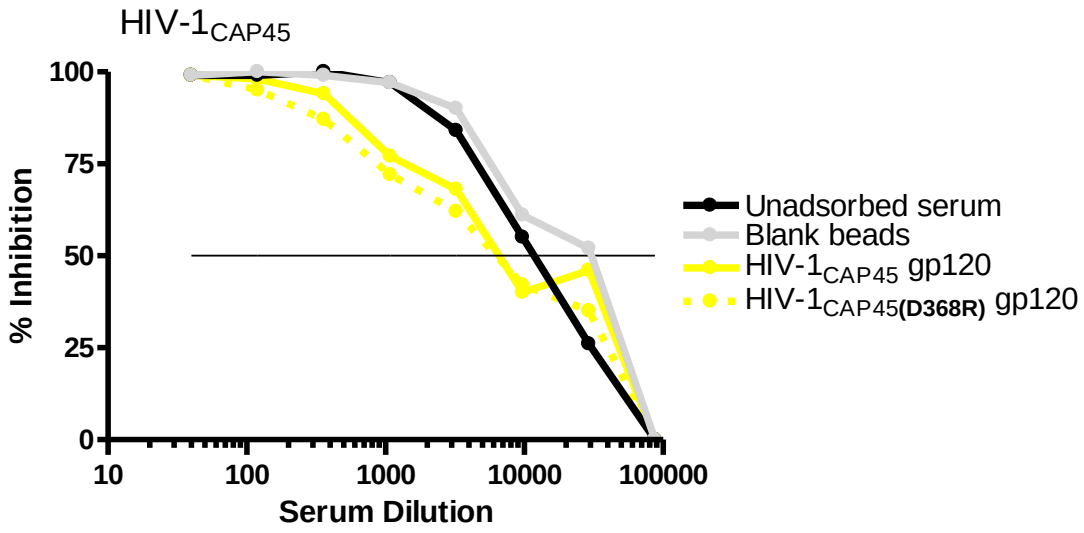


Figure 28: The β14+V5 epitope is distinct from the CD4 binding site epitope
Adsorption of participant CAP45 serum antibodies from 43 weeks post-infection using autologous gp120 with either the wild-type aspartic acid or mutant arginine amino acid at position 368 (colouring is the same as in Figure 22).

In summary, a third target for neutralising antibodies in CAP45 sera was identified, the titres of which peaked at roughly the same time as the previously identified C3-V4 and V1-V2 antibody specificities. This third group of antibodies targeted an epitope made up of the juxtaposed β14 sheet (in C3) and V5 loop, adjacent to the CD4 binding site. Furthermore all of the neutralisation activity directed towards this region could be completely adsorbed with monomeric gp120. The complete adsorption of these antibodies had a significant effect on autologous neutralisation titres, but did not account for all the neutralisation activity in the CAP45 sample. Two mutations in V5 previously identified as conferring escape from CAP45 sera were shown to mediate sensitivity to these antibodies. However this antibody specificity was distinct from the previously identified anti-C3-V4 response to which the V5 mutations were thought to have been produced. Since the only other mutations previously shown to mediate escape from CAP45 antibodies were in V1-V2, we attempted to use V1-V2+C3-V4 chimeric virions to determine whether V1-V2 escape mutations were responsible for mediating escape from anti-C3-V4 neutralising antibodies.

Mutations in V1 mediate escape from antibodies with epitopes in C3-V4

Apart from the two mutations in V5 that have now been shown to confer resistance to CAP45 autologous neutralising antibodies targeting the HIV-1_{CAP45} β14+V5 region, only three other mutations have previously been shown to affect neutralisation sensitivity to CAP45 sera. Two of these changes were in V2 while a third was in V1 (Moore et al., 2009). To determine whether these mutations were responsible for conferring resistance to the CAP45 anti-V1-V2 antibodies they were introduced into the HIV-$1_{CAP84\text{-}45(V1\text{-}V2)\text{-}84}$ heterologous chimera (Figure 29A - dotted lines). Introduction of the V1 associated S139N mutation, which shifts a potential N-linked glycosylation site by two amino acids, failed to confer any neutralisation resistance to antibodies targeting the V1-V2 region (dotted grey line). Conversely introduction of the two V2 associated mutations (K186R/G190aE) conferred complete resistance to these antibodies (dotted brown line). It has been shown previously that the HIV-$1_{CAP45.52wks.c07\ V1(N139S)}$ back mutant (which inherently has both V2 associated escape mutations) was still sensitive to earlier CAP45 serum antibodies (Moore et al., 2009). Therefore this data suggested that the S139N mutation in V1 mediates escape from antibodies targeting epitopes outside of V1-V2.

To examine whether the V1 associated mutation conferred resistance to CAP45 antibodies targeting the C3-V4 region we created pseudoviruses chimeric for both the C3-V4 and V1-V2 regions (HIV-$1_{CAP84\text{-}45(V1\text{-}V2+C3\text{-}V4)\text{-}84}$), and assessed their neutralisation sensitivity to CAP45 sera. Titres against the V1-V2+C3-V4 (Figure 29B - pink line) chimera were greater than those against either the C3-V4 (Figure 29B - green line) or V1-V2 (Figure 29A - purple line) chimeric viruses alone, compatible with an accumulation of these two neutralisation specificities. Interestingly introduction of the S139N mutation into the V1-V2+C3-V4 chimeric virus dropped these neutralisation titres to a magnitude similar to the titres generated against the HIV-$1_{CAP45.52wks.c07\ V2(R186K/E190aG)}$ back mutant (Figure 29B - dotted grey line). By introducing both V1 and V2 associated changes into the V1-V2+C3-V4 heterologous chimera these titres dropped even further, becoming almost negligible (Figure 29B - dotted black line). Cumulatively this data suggested that the S139N mutation was involved in mediating escape from anti-C3-V4 autologous neutralising antibodies.

Although the V1-V2 stem exists in a region distinct from C3-V4, the structure of the V1-V2 domain has not yet been resolved. Repositioning of a large glycan structure at position 139 may thus serve to sterically occlude C3-V4 epitopes, possibly by influencing the overall conformation of V1-V2 or glycan packing in the context of the native trimer. In support of this data it has recently been shown that two glycans in the V2 region confer resistance to neutralisation by b12, an anti-CD4 binding site antibody with an epitope overlapping the C3 region (Utachee et al., 2010; Wu et al., 2009).

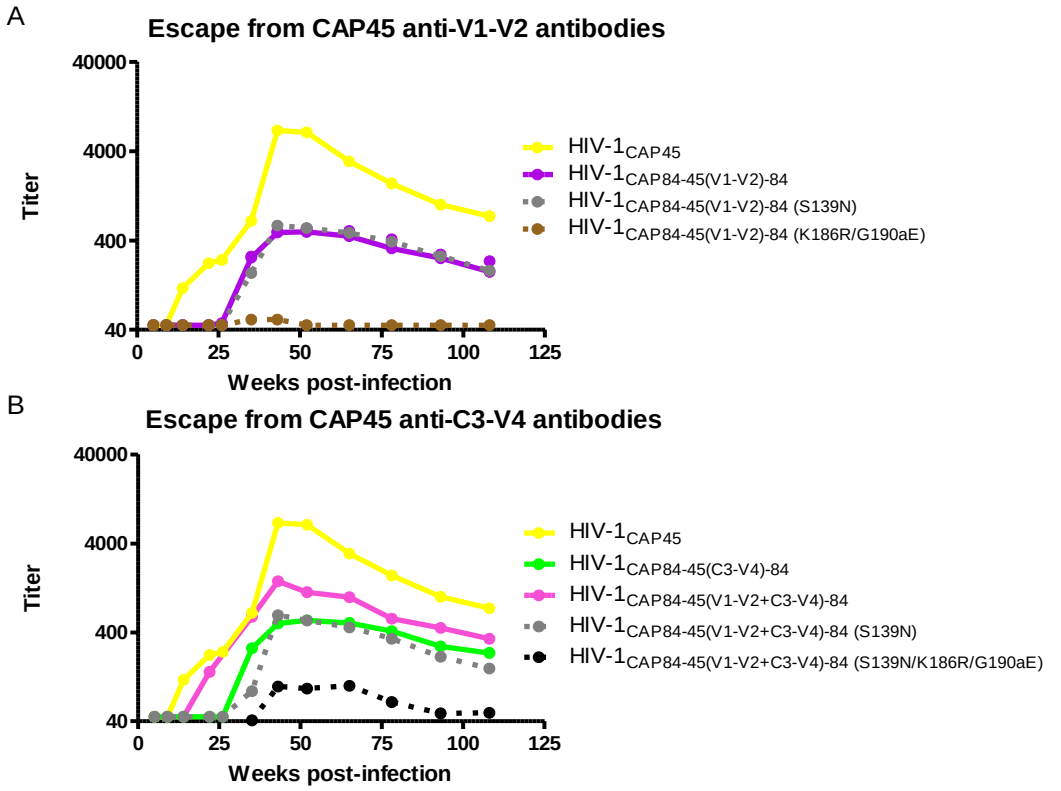


Figure 29: Escape mutations in V1-V2 potentially affect the C3-V4 epitope
Longitudinal development of CAP45 neutralisation specificities and the concurrent development of HIV-1_{CAP45} escape mutations in V1-V2 with time. Titres generated against the autologous HIV-1_{CAP45} virus is shown in yellow. **(A)** Development of titres against the wild-type V1-V2 heterologous chimera (purple) and mutant V1-V2 chimeras with either the S139N (dark grey) or K186R/G190aE escape mutations (brown) are shown. **(B)** Development of titres against the wild-type C3-V4 (green) or V1-V2+C3-V4 (pink) chimeric viruses, and mutant V1-V2+C3-V4 chimeras with either the S139N (dark grey) or S139N/K186R/G190aE (black) escape mutations are shown.

Complete escape from neutralising antibodies does not correlate with complete loss of binding

The introduction of two mutations into the α2-helix of HIV-1_{CAP88} (I339N/E350K) rendered the virus completely resistant to anti-C3 autologous neutralising antibodies (Moore et al., 2009). Similarly the introduction of two mutations in the V5 loop of HIV-1_{CAP45} (K460E/D462G) conferred complete resistance to neutralisation by an antibody that recognises a discontinuous epitope in β14 and V5. However the mechanism through which HIV-1 is able to escape these antibodies has yet to be elucidated. To this end recombinant gp120 proteins incorporating the described escape mutations were expressed for subsequent adsorption experiments (Figures 30 and 31). Mutant gp120s were assessed for the ability to adsorb out neutralising antibodies in CAP88 plasma from twenty six weeks post-infection and CAP45 serum from forty three weeks post-infection, when compared with the wild-type autologous gp120s. In both instances although the escaped gp120s were able to adsorb out all the binding antibodies specific for the escaped proteins (Figures 30A and 31A - dotted lines), they were unable to completely deplete the signal generated by autologous antibodies recognising the wild-type proteins (Figures 30B and 31B - dotted lines). The residual signal generated by antibodies that could not be adsorbed by the mutant gp120s must therefore be the result of epitopes directly affected by these escape mutations.

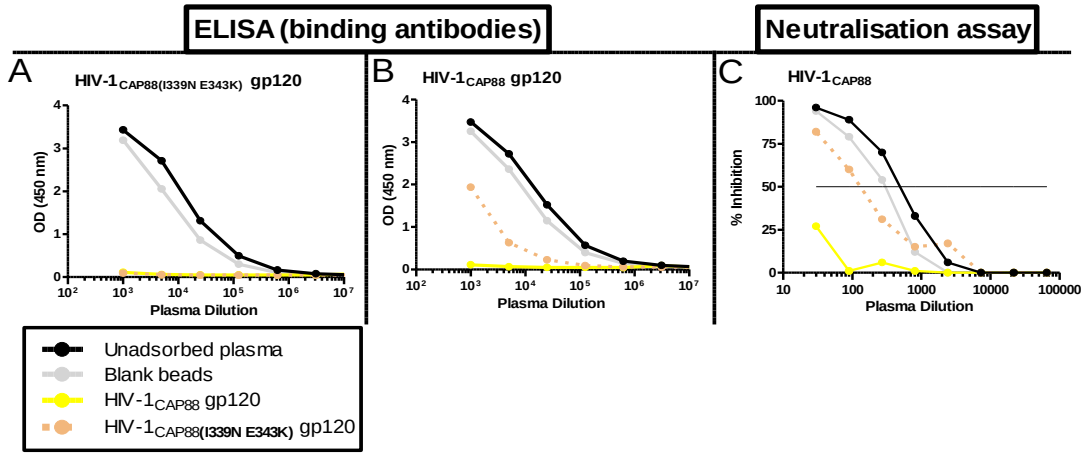


Figure 30: CAP88 anti-C3 antibodies bind to escaped gp120
CAP88 plasma from 26 weeks post-infection was adsorbed with either blank beads (grey), beads coated with autologous gp120 (yellow), or beads coated with autologous gp120 containing the escape mutations I339N and E343K (peach), and compared with unadsorbed plasma (black). Adsorbed plasma was assayed for residual binding antibodies to either **(A)** escaped gp120, or **(B)** wild-type autologous gp120, as well as **(C)** residual autologous neutralising antibodies against HIV-1_{CAP88}.

As expected HIV-1_{CAP88} gp120 with the escape mutations I339N and E343K could not deplete the autologous neutralisation activity in CAP88 plasma to the extent that wild-type gp120 could (Figure 30C - dotted line). Nevertheless it was still able to partially deplete some of these anti-C3 neutralising antibodies. Similarly, although HIV-1_{CAP45} gp120 with the escape mutations K460E and D462G was also unable to adsorb out the autologous neutralising antibodies to the degree that the wild-type protein could, it was still able to significantly deplete the neutralisation activity in CAP45 serum (Figure 31C). This partial adsorption of autologous neutralisation by gp120 carrying the K460E and D462G mutations may be explained by adsorption of CAP45 antibodies targeting the C3-V4 epitope. To rule out this particular scenario, the ability of escaped HIV-1_{CAP45} gp120 to deplete exclusively anti-C3+V5 titres was measured by assessing the ability of mutant gp120 to deplete the antibodies responsible for the neutralisation of the HIV-$1_{CAP84\text{-}45(C3+V5)\text{-}84}$ chimeric and HIV-$1_{CAP45.52wks.c07(E460K/G462D)}$ back mutated viruses Again the mutant gp120 was able to partially deplete the neutralising activity against these two viruses (Figures 31D and 31E). Thus although mutations in the α2-helix of HIV-1_{CAP88} and the V5 loop of HIV-1_{CAP45} mediate complete escape from neutralising antibodies, they do not completely abrogate the ability of these antibodies to bind their respective epitopes.

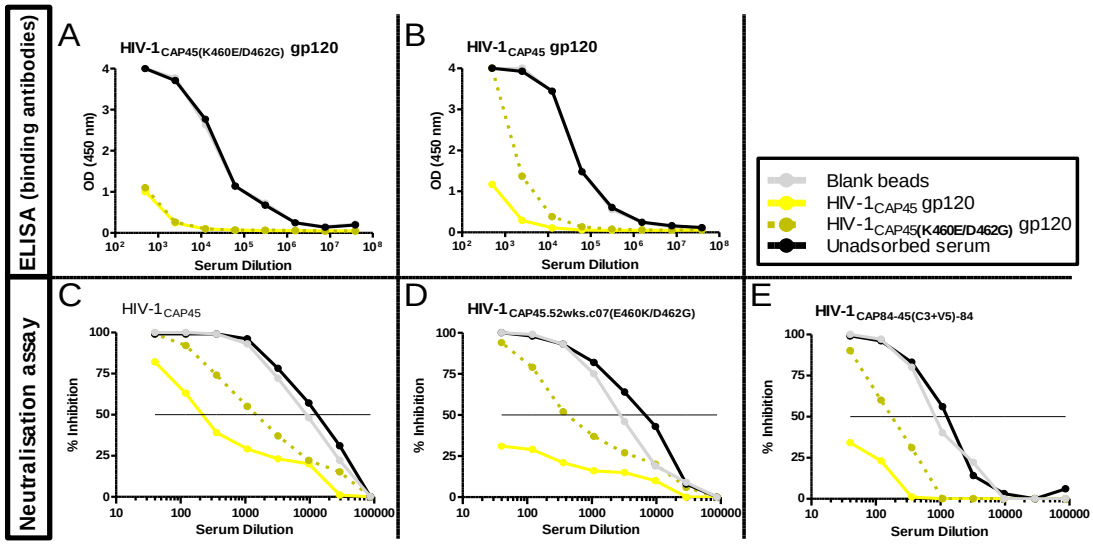


Figure 31: CAP45 anti-C3+V5 antibodies bind to escaped gp120
CAP45 serum from 43 weeks post-infection was adsorbed with blank beads (grey), wild-type autologous gp120 (dark pink), or escaped mutant gp120 (light pink), and compared to unadsorbed serum (black). Adsorbed sera was assayed for residual binding antibodies to either **(A)** escaped gp120, or **(B)** wild-type autologous gp120, as well as residual autologous neutralising antibodies against either **(C)** HIV-1_{CAP45}, **(D)** HIV-$1_{CAP45.52wks.c07(E460K/G462D)}$, or **(E)** HIV-$1_{CAP84\text{-}45(C3+V5)\text{-}84}$.

Chapter Four: Discussion

With respect to the native envelope trimer the HIV-1 gp120 monomer is the most well understood component of the viral infection machinery. Given the ever increasing wealth of knowledge on the structure of this glycoprotein and its interactions with broadly neutralising antibodies, it represents the best starting point for the rational design of an HIV-1 vaccine. To understand exactly how this building block needs to be manipulated into an effective immunogen will require an in depth understanding of the types of immunodominant epitopes contained within its structure. This study has conclusively shown that the newly discovered C3-V4 epitope is one that is contained in its entirety on monomeric gp120, and thus represents an important aspect of gp120 antigenicity to consider during immunogen development.

After it was originally identified as a complex discontinuous epitope, anti-C3-V4 antibodies were found in four individuals from the CAPRISA cohort (Moore et al., 2008). The region was identified as a neutralisation target through the use of chimeric viruses. This approach has in the past been shown to have its caveats, for instance transplanting the V1-V2 region from one virus to another has been shown to modulate the exposure of distal epitopes in gp120 and gp41, rendering the chimeric viruses sensitive to normally non-neutralising antibodies (Ching and Stamatatos, 2010; Pinter et al., 2004). If binding antibodies are present in the study sample with epitopes that are normally occluded on the native trimer, but become more accessible in the context of a chimeric envelope, then those antibodies will have the ability to potently neutralise the chimeric virus, but not either of the parental strains. It has been shown previously that most HIV infected individuals develop non-neutralising antibodies to epitopes in the receptor binding sites (Gray et al., 2009). The use of C3-V4 chimeras in previous studies was justified by the fact that these chimeric viruses remained resistant to heterologous serum from multiple individuals (Moore et al., 2008). Data from this study showed that transplanting the C3-V4 region may expose epitopes in the CD4 binding site not normally accessible on the native trimer. However, although the HIV-1$CAP_{45\text{-}84(C3\text{-}V4)\text{-}45}$ chimeric pseudovirus had a more exposed CD4 binding site, it did not become universally sensitive to all non-neutralising antibodies targeting the CD4 binding site. Rather, it gained sensitivity to exclusively the CAP84 antibodies. The fact that autologous HIV-1_{CAP84} gp120 with the D368R mutation was still able to deplete the CAP84 anti-CD4 binding site antibodies suggests that while neutralisation of the chimera was mediated by conserved elements in the CD4 binding

loop, the antibodies were also dependent on isolate specific residues in more variable regions. CAP84 developed an anti-V5 neutralisation response that coincided with the development of anti-C3-V4 antibodies (Moore et al., 2008). Furthermore all the neutralisation activity in CAP84 serum within the first year of infection was entirely reliant on an autologous C3 region. Given the juxtaposition of the V5 and C3-V4 regions it is plausible that these two responses are in fact one in the same. Potentially by adopting a slightly more exposed conformation the C3-V4 chimeric pseudovirus indirectly acquired neutralisation sensitivity to the CAP84 autologous neutralising antibodies. Such antibodies might recognise a conserved element in the CD4 binding loop and variable components in the immediately adjacent C3 and/or V4 regions transferred to the chimera, as well as the other variable residues in the V5 loop (Figure 32D). Rather than wholly exposing the CD4 binding site to a myriad of cross-reactive antibodies, it seems that this chimera exposed an epitope only partially in the CD4 binding site but still for the most part reliant on exposed variable regions normally accessible to neutralising antibodies.

This study showed that a discontinuous epitope in the C3+V5 regions (and more specifically the β14+V5 regions) is exposed on the viral trimer and serves as a potential target for autologous neutralising antibodies. This epitope is apparent on monomeric gp120 and lies adjacent to the conserved CD4 binding site. While the CD4 binding site is protected from neutralising antibodies by entropic masking and occlusion by glycans and/or V1-V2, the initial site of CD4 attachment must remain constant on the outer domain to facilitate efficient recognition of the host receptor. Antibodies that bind to this site in the outer domain do not need to suffer the large entropic penalties of binding normally induced after CD4 docking (Kwong et al., 2002). Indeed it is this accessible site of initial CD4 attachment that has been identified as a potential vaccine target (Figure 32). The broadly neutralising antibody b12 binds to this epitope (Figure 32B), and isothermal titration calorimetry data has implicated the newly discovered VRC03 antibody in recognising this same epitope (Wu et al., 2010). Both these antibodies are significantly affected by the D368R mutation, which abrogates their binding to monomeric gp120. Conversely, while both the CAP45 and CAP84 antibodies appear to target an epitope that overlaps with the CD4 binding site (and possibly the CD4 binding loop), their binding to autologous gp120 was unaffected by the D368R mutation.

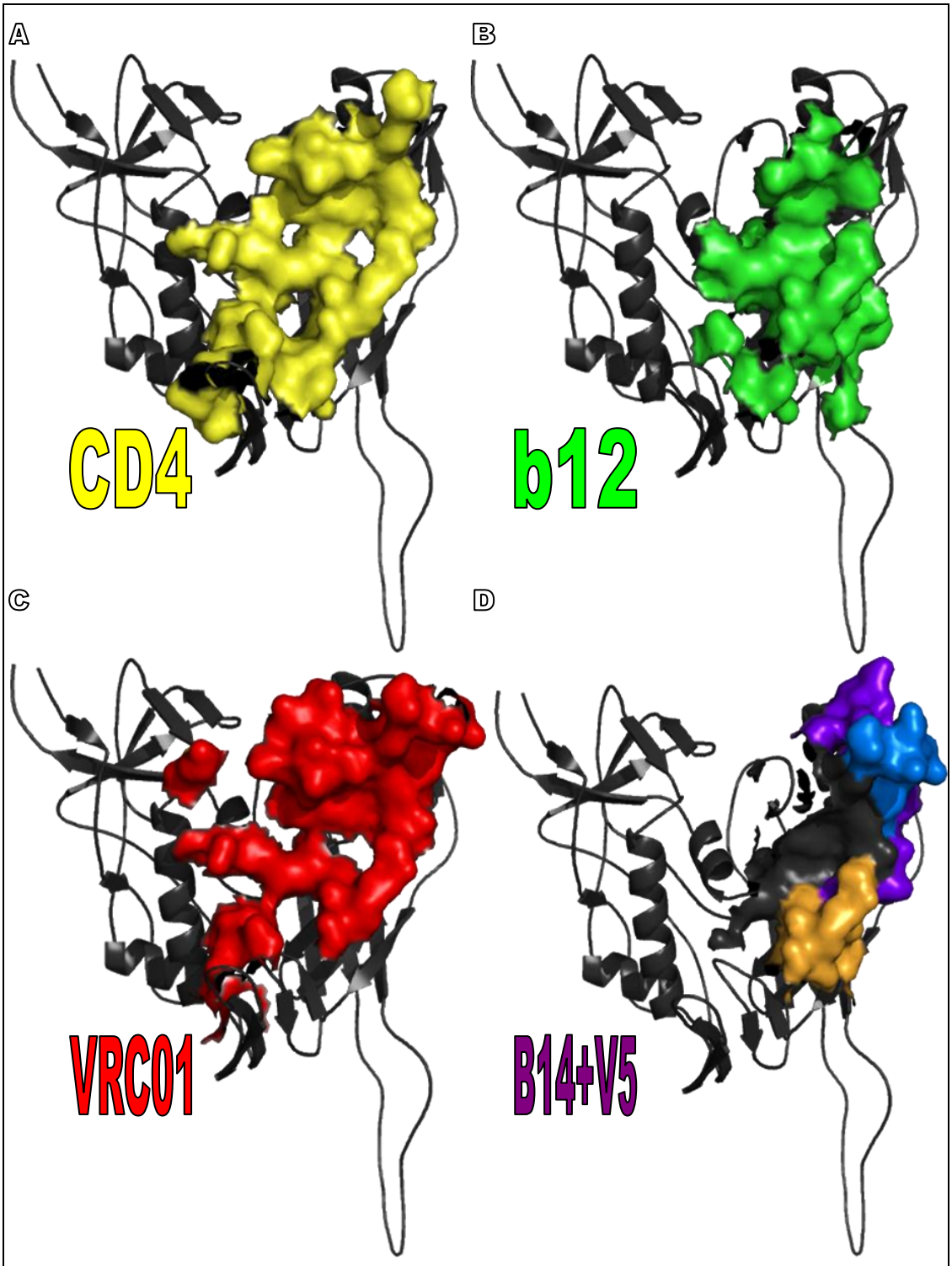


Figure 32: C3+V5 epitopes overlap with epitopes of known broadly neutralising antibodies targeting the CD4 binding site

Ribbon diagrams of gp120 coloured grey. The surface areas contacted by **(A)** CD4, **(B)** b12, and **(C)** VRC01are shown. **(D)** The surface area of gp120 potentially recognised by anti-C3+V5 antibodies. The β14 sheet and adjoining areas are coloured purple, while the V5 loop and CD4 binding loop are coloured blue and orange respectively. Structures were created in Pymol from the pdb files 2B4C and 3JWD.

No doubt it is this reliance on adjacent variable structures that greatly limits the neutralisation breadth of these antibodies. Thus it seems by placing a small but highly immunogenic variable loop structure such as V5 immediately adjacent to the recessed CD4 binding site, the HIV-1 envelope is able to avoid a broadly cross-reactive B-cell response by redirecting antibody paratopes to less essential regions. However, the V5 loop is also an important component of the binding site for the most potent and broadly cross-reactive neutralising antibodies discovered to date, VRC01 and VRC02. Of the 11 amino acids that make up the V5 loop and adjoining β24 sheet, eight are contacted by VCR01 (Figure 32C) through direct interaction with both main chain and side chain atoms (Zhou et al., 2010). The C3+V5 epitope may be seen as the enemy, one that distracts the immune system from developing broad and potent neutralising antibodies. However in the context of affinity maturation the C3+V5 epitope may actually be seen as a fortuitous gift to the vaccine developer, an immunogenic epitope adjacent to the CD4 binding site containing elements in both V5 and C3 that may initiate a B-cell response with the potential to mature and develop broad cross-reactivity such as that exhibited by antibodies VRC01 and b12 (Figure 32D). A greater understanding of the frequency and nature of β14+V5 or related epitopes, and their relationship with later cross-neutralising antibodies should significantly aid in the development of a vaccine immunogen able to elicit a broadly neutralising antibody response to the CD4 binding site.

The contribution of the C3-V4 response in CAP45 serum is harder to elucidate. Removal of C3-V4 binding antibodies did not impact at all on the overall autologous neutralisation titres. This may result from the contemporaneous existence of other neutralising antibodies with epitopes in C3+V5 and V1-V2, the combined titres of which have a potency of neutralisation equal to the unadsorbed serum sample. It was originally thought that given their position relative to C3-V4 two mutations in V5 conferred neutralisation resistance to the C3-V4 epitope. Here it was shown that these mutations resulted in viral escape from antibodies recognising an epitope in β14+V5. While two escape mutations in V2 confer complete resistance to antibodies targeting V1-V2, it appears the relocation of a glycosylation site by two amino acids in V1 confers resistance to the C3-V4 neutralisation epitope. As a direct result of the extremely dense glycosylation sequon clustering of HIV-1, often a potential N-linked glycosylation site is not utilised because of the steric interference from a neighbouring

glycan. The down regulation of one site by another is particularly significant when the sequons are separated by five or less amino acids (Poon et al., 2007). In the context of the HIV-1_{CAP45} V1 loop the glycan implicated in escape moves from position 137 to position 139. This shifts it to a more favourable position for actual N-linked glycosylation between the more conserved V1 flanking glycans at position 133 and 146 (Figure 33A). Thus the S139N mutation may actually result in the acquisition of a glycan (rather than the repositioning of one) which is sterically constrained enough to provide the escape for an epitope in the distant C3-V4 region. As has been documented by the non-neutralising antibody b13 and the broadly neutralising antibody b12, which recognise almost identical overlapping epitopes in the CD4 binding site, the precise angle of antibody binding to its antigen is a critical determinant of its ability to neutralise (Chen et al., 2009). Resistance to the b12 antibody can be conferred by introducing a glycosylation site into V2 at position 197 or position 186, potentially occluding the angle of approach for b12 (Wu et al., 2009). The observation that the accessibility of epitopes in C3-V4 might actually be restricted by glycans in V1-V2 is an important consideration when constructing C3-V4 heterologous chimeras in future instances. The HIV-1_{CAP84} V1-V2 domain is equivalent in length to the HIV-1_{CAP45} V1-V2 domain, and the V1 loop differs in sequence by less than 30% with a very similar glycosylation pattern. This allowed for the acquisition of neutralisation sensitivity by the C3-V4 chimera despite the presence of a heterologous V1-V2 loop (Figure 33B).

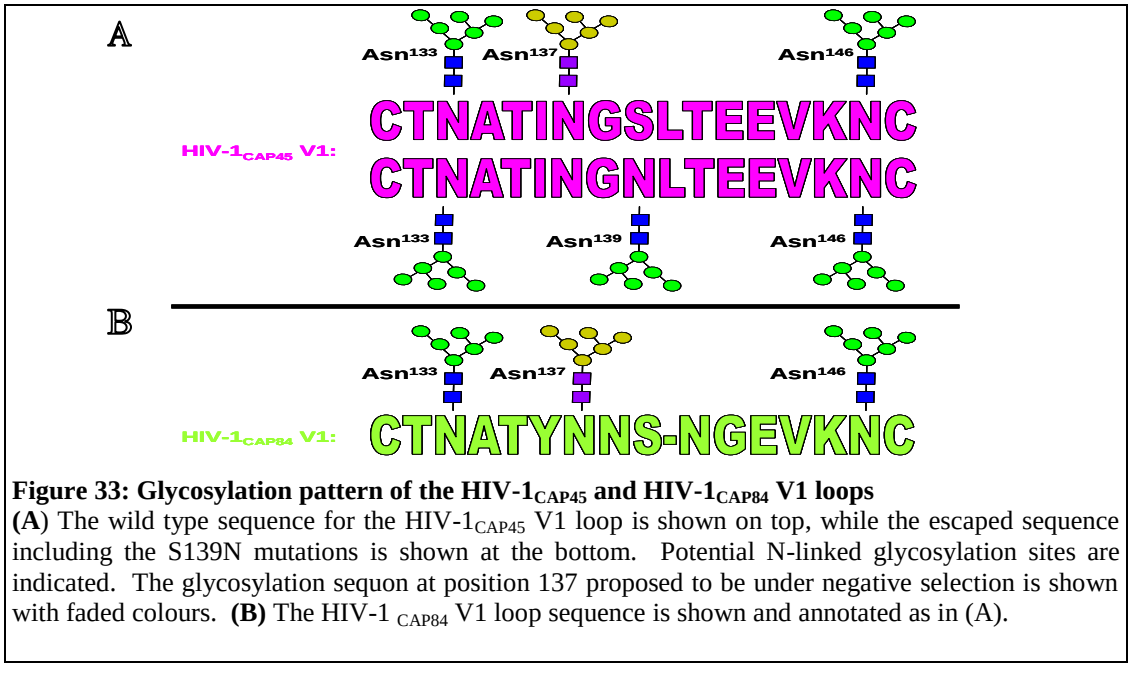


Figure 33: Glycosylation pattern of the HIV-1_{CAP45} and HIV-1_{CAP84} V1 loops
(A) The wild type sequence for the HIV-1_{CAP45} V1 loop is shown on top, while the escaped sequence including the S139N mutations is shown at the bottom. Potential N-linked glycosylation sites are indicated. The glycosylation sequon at position 137 proposed to be under negative selection is shown with faded colours. **(B)** The HIV-1_{CAP84} V1 loop sequence is shown and annotated as in (A).

The CAP63 C3-V4 response is as equally puzzling as the CAP45 C3-V4 response in that adsorption of these antibodies also failed to impact on autologous neutralisation titres. Furthermore despite the fact that all the C3-V4 responses were apparent on monomeric gp120, it appears the HIV-1_{CAP63} C3-V4 epitope was not as well adsorbed by monomeric gp120. Partial transfer of neutralisation sensitivity could be obtained using V4 alone and so it appears that the epitope was predominantly in V4, although C3 was an important contributing factor. The V4 loop is already a large plastic structure, and monomeric gp120 is obviously far more dynamic in solution than in the native trimer. So even if this epitope was wholly contained within monomeric gp120 it may not be well represented outside of the more conformationally constrained viral envelope spike. Since complete adsorption of the C3-V4 epitope did not affect autologous neutralisation titres it is possible that this epitope, like the one presented in HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$, is the result of an exposure of a conserved sequence motif. However it can also be argued that antibodies targeting the quaternary structure in the CAP63 sample adsorbed of its anti-gp120 activity are still able to produce an almost saturating neutralisation signal, as was the case with the CAP45 adsorbed sera. From the limited longitudinal data available for participant CAP63, replacement of both V4 and C3-V4 with heterologous sequence resulted in an equivalent loss in titre. This loss in titre was not complete at any time point tested and suggested that as in the case of CAP45, a second antibody specificity in CAP63 sera developed alongside the anti-C3-V4 antibodies. Like the CAP45 antibodies targeting V1-V2, this second specificity would also target the native envelope trimer since it cannot be adsorbed by monomeric gp120, and as such it would be responsible for the saturating neutralisation signal generated by gp120 adsorbed sera.

Collectively the data generated from this study cautions the use of heterologous C3-V4 chimeric viruses to discern autologous neutralisation epitopes. With that being said these chimeras can provide important clues as to the nature of neutralising antibody targets when taken in conjunction with longitudinal escape data. If neutralisation specificities develop simultaneously and with almost identical titres in a single individual, this may mean that current strategies using back-mutations in escaped viruses could underestimate the number of antibody specificities that drive neutralisation escape of HIV-1 viruses during early infection.

Data from this and other studies on CAP88 plasma put forward a strong case for the presentation of the α2-helix of subtype C envelope glycoproteins as a direct target for autologous neutralising antibodies (Moore et al., 2009). Adsorption data from this study suggests a role for V4 in this epitope. The HIV-1_{CAP63} V4 loop is eight amino acids longer than the HIV-1_{CAP88} V4 loop and has a distinctly different glycosylation pattern (Figure 14). It is possible that the α2-helix epitope is partially occluded by V4 in the context of the HIV-$1_{CAP63\text{-}88(C3)\text{-}63}$ C3-chimera. Alternatively it is possible that these antibodies interact with atoms in the V4 loop or that binding is complemented by V4 specific glycans. These potential interactions would be non-essential for antibody binding but may serve to fasten the antibody into place after it has docked with the α2-helix. Their absence may thus promote faster complex dissociation and result in poor adsorption of the autologous neutralising antibodies. Though six months may be limited time for antibody affinity maturation, there exists yet another possible explanation for the involvement of V4 in the C3 epitope. It has become increasing clear as of late that one can no longer assume that a particular antibody response is the product of a single antibody. The recent isolation of the potent neutralising antibodies PG09-PG16 and VCR01-VCR02 reveal that they exist as clonally related antibodies with slight differences in epitope recognition (Walker et al., 2009; Wu et al., 2010). This information advocates the possibility that there are two or more clonally related C3-V4 directed antibodies in the CAP88 plasma sample, one of which is more reliant on V4 than the other.

It is fascinating that neutralisation escape mutations did not correlate with complete loss of antibody binding. These autologous neutralising antibodies bind epitopes that are accessible on monomeric gp120 even in the context of escape. In the context of the native trimer the effect of escape mutations may influence intermolecular interactions and significantly manipulate the conformation of an adjoining neutralisation epitope. Once gp120 is liberated from the native trimer its inherently plastic structure may still allow for proper formation of such an epitope. Similarly the acquisition of glycan involved in escape from CAP88 anti-C3 antibodies and CAP45 anti-C3-V4 antibodies may only serve to occlude the neutralisation epitopes in the context of the completely glycosylated native trimer. In an alternative scenario, escape mutations such as those in the V5 of HIV-1_{CAP45} may actually lie within the antibody binding site. These mutations may simply serve to lower the affinity

between an antibody and its antigen to a point that the binding is no longer tight enough to promote neutralisation of the free virion. Given either circumstance these observations are of great significance in the context of vaccine design. If antibodies continue to bind their respective epitopes even after viruses have escaped their neutralising activities, these antibodies may continue to affinity mature towards multiple variants of the same epitope and eventually might develop greater neutralisation breadth. Alternatively these antibodies may actually exert some form of immune pressure on the virus such as low level neutralisation not detectable in current neutralisation assays, or antibody-dependent cell-mediated cytotoxicity. Evidence for this may exist in CAP45. The viral population in this individual at 8 months post infection adopted the mutations S139N, K186R and D462G, effectively escaping all antibodies targeting the V1-V2, C3-V4, and β14+V5 epitopes. Yet despite complete escape from these antibodies, the viral population four months later acquires the additional escape mutations G191aE and K460E, suggesting that some immune pressure is still being exerted on the virus *in vivo* despite no detectable neutralisation *in vitro*. By studying the evolving interactions between antibodies and their antigens with time it may be possible to gain exciting insight as to how an immune response might be guided towards the development of potent and broadly neutralising antibodies. C3 related neutralisation epitopes such as those in the α2-helix and β14+V5, together with other non-neutralising epitopes adjacent to the CD4 binding site (such as those potentially exposed by C3-V4 chimeras), represent a major component of the autologous neutralising antibody response targeting monomeric gp120 and potentially immunogenic epitopes for a gp120 based vaccine immunogen.

Autologous neutralising antibodies are far more common than broadly cross-reactive neutralising antibodies, and highly immunogenic strain specific epitopes overlapping with the CD4 binding site provide significant obstacles towards eliciting broadly neutralising anti-CD4 binding site antibodies. Conversely these epitopes may also serve as immunogens to elicit anti-CD4 binding site precursor antibodies. Once an antibody is partially reliant on the CD4 binding site, it may then be possible to affinity mature these antibodies to more conserved epitopes. Such a strategy has already been proposed for eliciting PG09-PG16 like antibodies (Pancera et al., 2010b). Understanding the structure and immunogenicity of these autologous neutralisation epitopes and designing strategies to shift B-cell responses to more conserved epitopes

will contribute greatly to the rational design of a vaccine better able to elicit neutralising antibodies with broad cross-reactivity. Future work will aim at isolating monoclonal antibodies from sequential time points in selected individuals with broadly cross-reactive sera, which together with contemporaneous HIV-1 envelope sequence data should promote a better understanding of how these type-specific antibodies became broadly neutralising. Overall there has been a major shift in research focus of late towards isolating monoclonal antibodies to specific sites on the envelope. As this study has shown, the isolation of specific monoclonal antibodies is greatly complicated by the diverse nature of the B-cell response within a single individual. Isolating a single specificity is most economically possible with a highly specific reagent, such as the RSC3 protein engineered for the isolation of VRC01, VRC02, and VRC03 (Wu et al., 2010). When the epitope is not known, or only poorly defined, the best reagent in these instances remains a gp120 monomer such as those used in this study. By better understanding which antibody specificities can be adsorbed with monomeric gp120 and how the virus escapes from them (such as the data generated in this study) researchers would be better able to design more specific reagents for the isolation of a single B-cell response.

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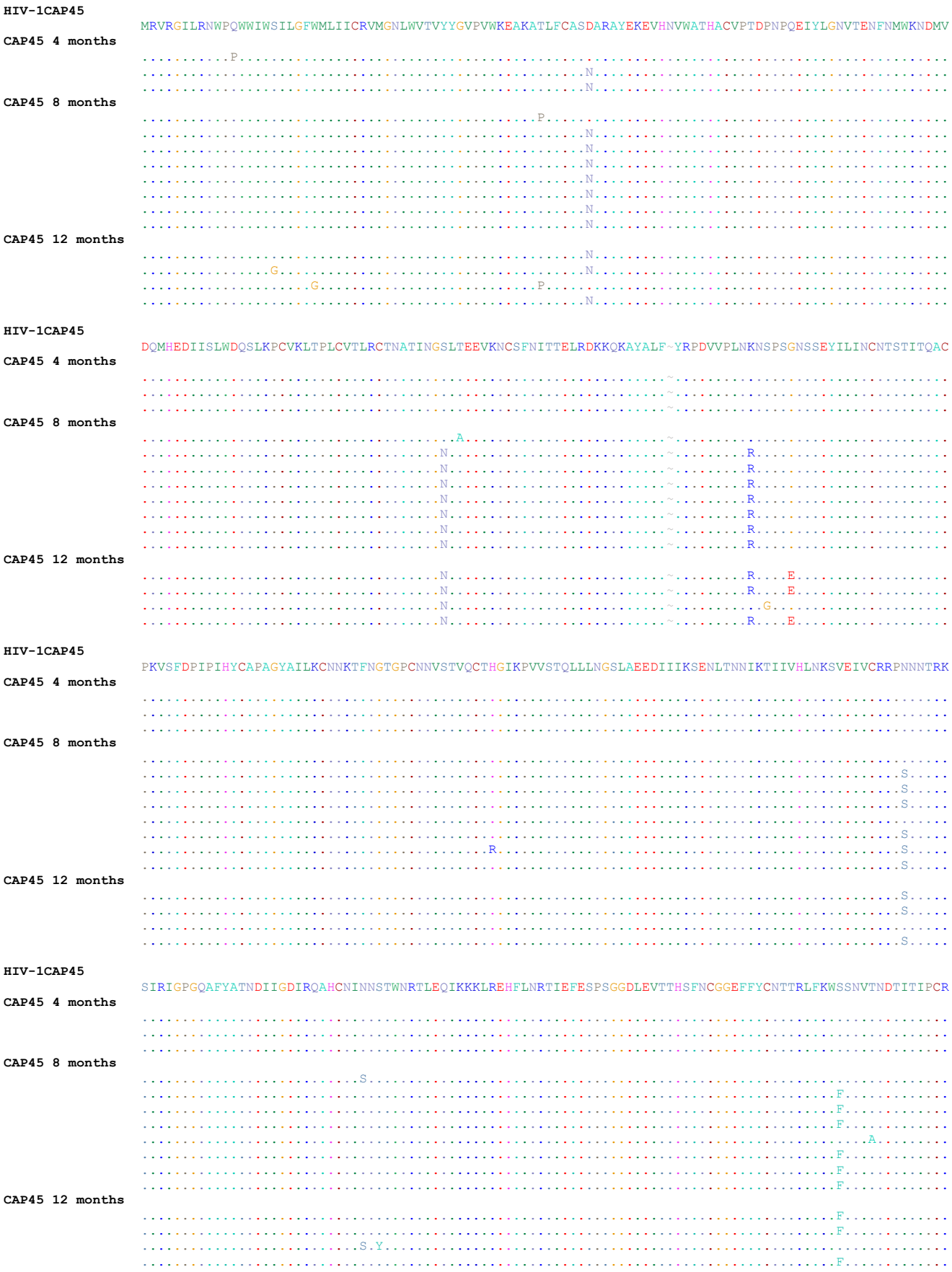
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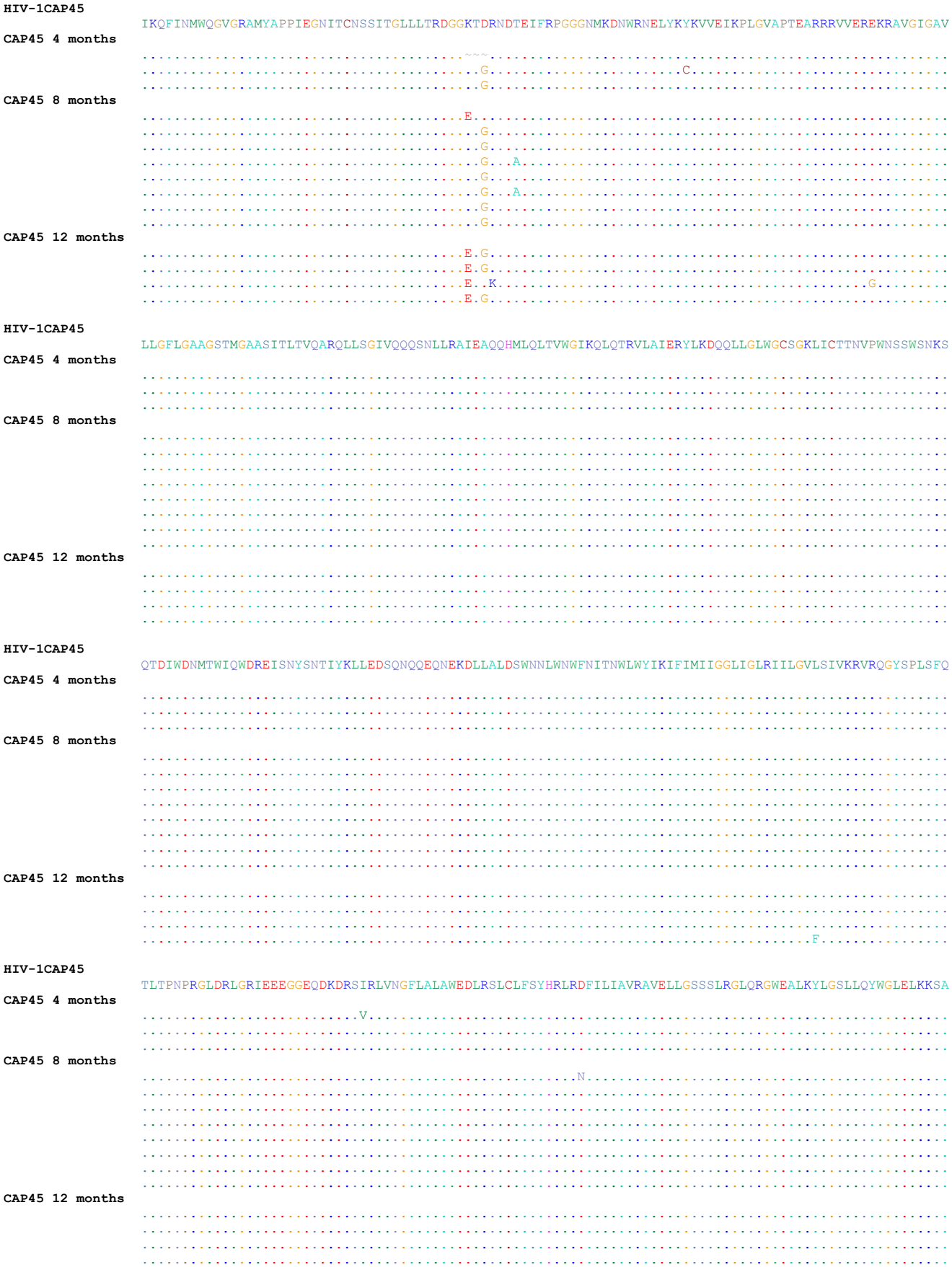
Chapter Six: Appendix

Appendix A: Transfer of neutralisation sensitivity using chimeric pseudoviruses (Moore *et al.*, 2009)

The V1-V2, V4, V5, C3, and C3-V4 regions were transferred from autologous neutralisation sensitive donor viruses (red) to heterologous neutralisation resistant recipient viruses (blue). Chimeras are shown in dotted lines and coloured to match the recipient virus. Serum ID_{50} titres are shown on the y-axis, while the time point post-infection at which they were obtained is shown on the x-axis. Shaded boxes indicate a transfer of sensitivity to the chimeras.



Appendix B-1: Sequence alignment of the HIV-1_{CAP45} envelope gene as it evolves in individual CAP45 with time.



Appendix B-2: Sequence alignment of the HIV-1_{CAP45} envelope gene as it evolves in individual CAP45 with time.



Appendix B-3: Sequence alignment of the HIV-1_{CAP45} envelope gene as it evolves in individual CAP45 with time.

Examiner Comments – Examiner 1

In this research study, Mr. Wibmer studies the C3-V4 epitopes in the human immunodeficiency virus (HIV-1) gp120 envelope glycoprotein that serve as targets for autologous neutralizing antibodies during natural infection. Four strain-specific neutralizing antibody responses are characterized by testing the sensitivity of envelope glycoprotein chimeras to neutralization and by using defined envelope glycoproteins to adsorb the neutralizing antibodies from the sera. In some cases, escape from neutralization occurred while serum antibodies still were shown to bind the escape gp120 glycoprotein, indicating that quantitative changes in binding or in the consequences of antibody binding may be involved in neutralization escape. The work provides interesting and novel insights into autologous neutralization and subsequent virus escape. The studies are carefully conducted and controlled, and the results are interpreted rigorously in the context of current information on the structure of the HIV-1 envelope glycoproteins. The dissertation is well-written and organized in a clear and logical fashion. In summary, this work represents a high level of effort and conforms to the highest standards of quality.

Examiner Comments – Examiner 2

TITLE
Defining C3-V4 Neutralisation Epitopes on Human Immunodeficiency Virus Type-1 Subtype C Envelope Glycoproteins

CANDIDATE
C K Wibmer

Dissertation Overview

The development of a vaccine against the Human Immunodeficiency Virus (HIV) remains a global healthcare priority and one of the biggest challenges facing modern biomedical science. Much research has focused on the HIV-1 Envelope protein (Env) as the basis of an immunogen that can elicit an effective antibody-based antiviral response: it plays a critical role in the viral life cycle by mediating attachment to and infection of host $CD4^+$ lymphocytes and macrophages, and is the only component of intact, infectious virions exposed to circulating antibody. While a robust antibody response against Env is normally generated in the HIV-infected individual, these antibodies are, in the acute phase of infection at least, mostly type-specific and do not react efficiently with diverse viral species that are generated during the natural course of infection. In recent times, however, several antibodies that are able to neutralise a large diversity of viruses potently have been isolated from HIV-infected individuals. It appears that these (so-called broadly neutralising) antibodies, which have highly unusual features such as abnormally long CDR loops involved in epitope binding, are derived by extensive affinity-maturation of germline precursors, and emerge only during advanced stages of infection. Understanding the evolution of antibody responses against the HIV Envelope protein is thus a critical component of defining candidate immunogens and vaccination regimens for the development of an HIV vaccine. In this study, C Wibmer used various experimental approaches to investigate autologous neutralising antibody responses in a set of HIV-positive serum samples. In previous studies, the HIV-1 gp120 C3-V4 region had been identified as an important target for antibodies elicited early during infection. This study builds on from this data by studying the contribution of the C3-V4 region to autologous neutralisation responses in an extended set of HIV-positive serum samples, by testing the transferability of neutralisation sensitivity to heterologous gp120s, and by describing how the C3-V4 context and certain mutations influence sensitivity to neutralisation. In doing so, the thesis makes some important observations regarding the type of immunogen that might be required to elicit a broad and potent antibody response, and the challenges implicit in generating such a molecule.

General Comments

The experimental approaches undertaken in this study are laborious and technically challenging, and the successful execution of these have provided a platform for what I consider to be a very good thesis. Through these, the candidate has mastered a set of fundamental molecular research methodologies, the principle requirement for graduating at M.Sc. level. Beyond this, the candidate has also begun to make some important novel observations on the nature of antibody responses present in HIV-infected patient sera. Scientific writing conventions have been adhered to closely, the layout, formatting and referencing is consistent with those prescribed for scientific theses and the data is presented, for the large part, clearly, concisely and accurately. I found the author's insights into a complex research field, and his ability to convey these insights in a scientifically cogent and stylistically mature manner, particularly impressive. As such, my comments are essentially minor in nature, and my recommendation is that the candidate's M.Sc. thesis be approved once these are addressed to the satisfaction of the candidate's supervisor/HOD. Congratulations on an excellent piece of work.

Specific Comments - Styles/Grammar/Typographical Errors

1. Page viii (List of Abbreviations): change "120kDa" to "120 kDa". In general, a space should always exist between a number and its corresponding unit description. This error occurs throughout the thesis (eg. Page 24, paragraph 1, line 10: change "20U to "20 U"), and should be corrected in each case accordingly.

2. Page x (Abstract), Line 14: change "...was in fact one apparent..." to "...was in fact apparent".

3. Page 3, 2nd paragraph, line 5: insert space between "structure" and "(Ellerbrok et al., 1992)".

4. Page 7, 1st paragraph, line 6: change "...results in an unfavourable entropy value in the magnitude of -44.2kcal/mol..." to " results in an unfavourable entropy change of -44.2 kcal/mol..."

5. Page 7, 1st paragraph, line 8: change "...that rescues the interaction." to "...that renders the interaction thermodynamically feasible".

6. Page 8, 1st paragraph, line 11: change "The *in vitro* isolation of CD4-independent...isolates...are highly neutralisation sensitive..." to "CD4 isolates isolated *in vitro* constitutively present an exposed co-receptor binding site and are highly neutralisation sensitive...".

7. Page 9, Figure 3: Provide a reference and PDB ID for the gp120 structure shown. Do the same for Figures 4 and 5.

8. Page 13, 3rd paragraph, line 2: change "an overlapping epitope the membrane..." to "...an overlapping epitope **in** the membrane...".

9. Page 23, top line: insert "of" between "some" and "these".

10. Page 23, Figure 9 legend: change "A Schematic summarising..." to "A schematic representation of..." or "A schematic summary of...".

11. Page 24, 2nd paragraph, line 5: change "over night" to "overnight".

12. Page 26, 1st paragraph, line 10/11: change "A schematic describing..." to "A schematic representation of..."

13. Page 28, 1st paragraph, line 2: change "manufacturers'" to "manufacturer's".

14. Page 29, 1st paragraph, line 16: change "manufacturers" to "manufacturer's"

15. Page 32, line 5: change "co-cultured" to "co-incubated". (Strictly speaking there is no culturing until cells are added)

16. Page 35, 1st paragraph, line 2: change "...system explored" to "...system **was** explored".

17. Page 35, 1st paragraph, line 8: change "...to the ELISA plate" to "...**on** the ELISA plate".

18. Page 36, line 14: change ".....on the proteins overall conformation..." to "...on the protein**'s** overall conformation..."

19. Page 43: There is a font size difference between the headings ("CAP84" and "CAP63")

20. Page 48, Figure 20C and D: Unless there is descriptive significance in the different designations "HIV-1CAP63-88(**C3-V4**)-63" vs "HIV-1CAP63-88(**C3/V4**)-63", these should be standardised and appropriate changes made in the corresponding legends. Such differences occur serially in several other figures in the Results section (eg compare Figure 20D and F; Figure 25D and G, Figure 27A and E and others).

21. Page 51, Figure 22 Legend, line 5: delete one copy of "...was adsorbed..."

22. Page 52, 1st paragraph, lines 3-5: the author refers to the ability of heterologous gp120-adsorbed CAP84 serum samples to neutralise chimeric HIV-$1_{CAP45\text{-}84(C3\text{-}V4)\text{-}45}$, and refers the reader to Figure 22A and 22B. However, unless I have misinterpreted the data, Figures 22A and B show data derived using CAP45 and CAP63 sera.

23. Page 55, Figure 22: Some of the figure headers are printed in boldface, while others are in regular print (compare Figure 25A and 25C, and Figure 25B and D)

24. Page 55, Figure 25 Legend: it is stated that "Residual binding antibodies were detected in ELISA against (A) heterologous gp120, (B) heterologous gp120 with the D369R (sic) mutation, (C) C3-V4 chimeric gp120 (D) C3-V4 chimeric gp120 with D368R mutation...".

    a. Change "D369R" to D368R".
    b. The header on Figure 25C appears to indicate that data shown describes the binding of adsorbed CAP84 serum to the **heterologous HIV-1_{CAP45} gp120**, which is in contrast with the legend stating that the target is **C3-V4 chimeric gp120.** Similarly, Figure 25D indicates the target as being **HIV-$1_{CAP45\text{-}84(C3/V4)\text{-}45}$** (no mutation inferred), while the legend describes the target as "**C3-V4 chimeric gp120 with the D368 mutation**".

    Clearly there is some disconnect between the figure legend and information presented. I have noticed this in several figures (eg Figure 27, in which the legend refers to 27A and 27 B as describing "...binding antibodies to either (A) autologous protein or (B) C3+V5 chimeric protein..." respectively, while the figures themselves are headed conversely). The author should take time to rectify this meticulously, as it does cause a fair amount of confusion in trying to interpret what are fairly data-rich results.

25. Page 56, 2nd paragraph, line 13: "Figure 25G – yellow lines" should surely be "Figure 25F – yellow lines"?

26. Page 59, Figure 27 and associated text: references are made to "C3V5", "C3-V5" or "C3 + V5". Unless there are qualitative differences between these (which do not appear to be alluded to in the text), these should be standardised.

27. Page 61, 1st paragraph, line 14: "...as shown in Moore *et al.*, 2009,..." is, by convention, not an appropriate format for citation. The author(s) name(s) should appear in parenthesis along with the year of publication, and this is normally inserted after the statement referring to the cited work. The statement is perhaps better formulated as: "...as shown previously by Moore and colleagues (Moore et al., 2009),...". Check for this throughout the document please.

28. Page 69, 2nd paragraph, lines 7/8: "Here it was shown that these mutations escaped antibodies..." is perhaps better rephrased as "Here it was shown that these mutations resulted in viral escape from antibodies ...."

Specific Comments – Content/Technical

1. The author describes the cloning of recombinant gp120 proteins containing polyhistidine tags (page 22). Normally these are used to facilitate purification via metal chelate affinity chromatography, for immobilisation on certain divalent metal ion-charged surfaces or for immunochemical detection. However, from what I was able to discern, the gp120 purification approach described in this study involved a combination of lectin affinity- and ion-exchange chromatography, and I was unable to find any other application referred to that made use of His-tags. Accordingly, it would be informative to elaborate on the reasons for cloning His-tagged gp120s.

2. Page 35, 2nd paragraph: the author states that "Poor expression levels correlated with an increase in non-specific binding of FBS proteins during the chromatographic steps...". I'm unsure as to why low levels of gp120 would cause an increase in the binding of serum-derived proteins to the lectin. Is the author alluding to a competitive effect, and if so, would this not only occur when the level of lectin-binding protein loaded exceeds the capacity of the resin?

3. Page 37, 2nd paragraph: the author states that the "low level of binding to 2G12 detected by ELISA is presumably non-specific...". However, in some instances shown in Figure 17, the 2G12 signals generated actually exceed those of other antibodies used to confirm conformational integrity by virtue of their (very specific) binding mechanism(s). (eg compare antibody binding to CAP45-84(V5)-45 gp120 and to CAP88-63(C3-V4)-88 gp120). The question is, on what basis then, does one discriminate between "specific" and "non-specific" binding events?

4. For all gp120-binding and pseudovirion neutralisation assays, a question one might naturally be inclined to ask relates to the reproducibility of the data. How has the data been replicated, and have any statistical methods been applied to establishing the significance of the observed changes? Referring to Figure 25 for example (specifically panels C, D and E), it remains unclear to me why the binding of CAP84 serum depleted with wild-type CAP84 gp120 is consistently higher than the corresponding serum depleted with CAP84 (D368R). Are these differences significant? If so, why, and if not, why is there a consistent trend across all 3 binding experiments?

List of Corrections

Reviewer one had no corrections for this dissertation

Reviewer two – Style, Grammar, and Typographical errors

1. A space was inserted between all numbers and their corresponding unit descriptors with the exception that a space was not added before the percent symbol (%) or the degree symbol (°), when the latter was not preceding the symbol for Celsius (C).
2. The word "one" was deleted from the sentence.
3. A space was inserted between "structure" and "(Ellerbrok et al., 1992)".
4. The sentence was altered as suggested.
5. The sentence was altered as suggested.
6. The sentence was changed to: "CD4 independent isolates have been isolated *in vitro* that constitutively present an exposed co-receptor binding site and are thus highly neutralisation sensitive".
7. In the figure caption for Figure 1 we state that: "Protein structure images for this and all subsequent figures (unless otherwise stated) were created from the superimposed pdb files 2B4C and 3JWD using Swiss-PdbViewer programme and Pymol", to avoid unnecessary repetition.
8. The sentence was altered as suggested.
9. The sentence was altered as suggested.
10. The sentence was altered as suggested.
11. The sentence was altered as suggested.
12. The sentence was altered as suggested.
13. The sentence was altered as suggested.
14. The sentence was altered as suggested.
15. The sentence was altered as suggested.
16. The sentence was altered as suggested.
17. The sentence was altered as suggested.
18. The sentence was altered as suggested.
19. All figure headings were changed to the same font size.
20. In all instances where two adjacent regions 'x' and 'y' were transferred together, this is referred to as 'x-y'. Similarly in all instances where to discontinuous regions 'x' and 'z' were transferred together, this is referred to as 'x+z'. Where this was in error it was corrected accordingly. In addition this was further clarified in the abbreviations section.
21. The sentence was altered as suggested.
22. The sentence was corrected so that it referred to Figures 22C and 22F.
23. All graph headings were changed to boldface.
24.
    a. The sentence was altered as suggested.
    b. All figure captions referring to the incorrect graphs were changed.
25. The parentheses were changed to contain: "Figures 25F and 25G - yellow lines".
26. Corrected in point 20 (above).
27. Any sentence include a citation was changed so that the citation appeared in parentheses at the end of the sentence.
28. The sentence was altered as suggested.

Reviewer two – Content/Technical

1. The reviewer was correct in noticing that histidine tags were added to the recombinant gp120 proteins, but not used for subsequent protein purification. These tags were in fact added to facilitate coupling to the magnetic beads used for the adsorption studies. At high pH a polyhistidine tail is deprotonated, making it a good nucleophile. This provides a good surface distinct from other biologically relevant regions on monomeric gp120 (like the receptor binding sites) for covalent binding to the tosyl-activated beads. We did attempt purification with Ni-agarose matrix to make full use of the tags, but found this method of purification to be inferior to the lectin affinity column.
   To explain this in the dissertation the following sentence was added to page 36, paragraph 1, lines 5-8: "These polyhistidine tags provide a large, flexible surface distinct from biologically relevant sites on monomeric gp120. At high pH histidine deprotonates to become available for coupling to the tosyl activated beads used in the downstream adsorption studies".
2. We have noted as a common trend in our laboratory that the worse our expression levels the less pure a final product actually is. This is independent of the construct used. We hypothesise the reason for this to be in part related to a competitive effect. When recombinant gp120 expression is low, this allows for a greater number of glycosylated FBS proteins with lower affinity for the lectin affinity column to be adsorbed from the cell culture supernatant and eluted with gp120. For the subsequent chromatographic step this mannose elution is loaded onto a Q sepharose ion exchange column, and purified gp120 is collected in the flow through. If the sample contains a higher ratio of FBS proteins to recombinant gp120, then a larger fraction of the sample must be retained by the matrix. As the column becomes more and more saturated the FBS proteins begin to compete with each other for binding to the matrix, and there is a greater likelihood that some proteins will escape into the flow through and contaminate the gp120 sample. Some potential corrective measures may be to reduce the lectin column capacity, or increase the Q sepharose column capacity, but ideally this would require foresight as to whether or not a protein will express poorly.
   To explain this theory the following sentence was added to page 35, paragraph 2, lines 7-10: "Poor gp120 expression correlated with less efficient purification. This was attributed to increased carryover of glycosylated FBS proteins that then saturated the ion exchange column, contaminating gp120 collected in the flow through".
3. We agree that perhaps the choice of wording here was not entirely clear Rather than suggesting that binding to 2G12 was random and non-specific, we were suggesting that this binding was in fact to glycans but not specifically to the 2G12 epitope as it is defined in the literature. It is possible that this binding is to some portion of the 2G12 epitope that exists in subtype C, but is not enough to confer neutralisation sensitivity. Alternatively this binding may be explained by the formation of alternate binding sites for 2G12 as a product of other recombinant gp120 glycosylation pathways. For instance, a variant of gp160 produced in natural infection that is glycosylated in the endoplasmic reticulum is covered in high mannose glycans and exhibits at least two binding sites for 2G12.
   To clarify this statement the sentence was altered to read: "The binding to 2G12 detected by ELISA may have resulted from low affinity interactions with high mannose structures contributing to the 2G12 epitope, or with another binding site for 2G12 formed by alternate gp120 processing. Such binding sites have already been described for gp160 (Crooks et al., 2011)".
4. All results from the adsorptions and neutralisation assays were repeated and confirmed, however as there is a small degree of variation between experiments an accurate comparison can only be made when run in parallel with the same sample. This is the final data that went into each of the figures shown. That being said after several repeats we can say with much conviction that the differences observed between each assay are not significant. In the example provided, the reviewer asks why binding of CAP84 serum depleted with wild-type CAP84 gp120 is consistently higher than the corresponding serum depleted with CAP84 (D368R), and whether or not this difference is significant. The answer is that this difference is very small and not significant given the variation of the experiment.

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5 August 2011

Postgraduate Office
Faculty of Health Sciences
University of the Witwatersrand
Johannesburg

Award of Degree MSc(MED): Mr. Constantinos Kurt Wibmer

Thesis Title: Defining C3-V4 Neutralisation Epitopes on Human Immunodeficiency Virus Type-1 Subtype C Envelope Glycoproteins

Both myself and his main supervisor, Dr Elin Gray have reviewed the examiners reports and the corrected thesis of Mr. Constantinos Kurt Wibmer and we are satisfied that all the corrections and suggestions made by the examiners have been satisfactorily and adequately addressed.

We now support the award of the degree of MSc(Med) to Mr. Constantinos Kurt Wibmer at the next graduation ceremony.

Yours sincerely

Prof. Lynn Morris
HEAD:AIDS Research Unit

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of the Witwatersrand,
Johannesburg

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Investigator: **Constantinos Kurt Wibmer (student no 0403095E)**

Project title: **Defining C3V4 neutralisation epitopes on HIV-1 subtype C envelope glycoproteins.**

Reason: This is a wholly laboratory study using stored samples collected under HREC(Medical) clearance numbers M051140 and M040202. No new human specimens will be collected.

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