The Isolation of gp41 Specific Monoclonal Antibodies from the Cervical IgA Repertoire of Highly Exposed Persistently Seronegative (HEPS) Commercial Sex Workers from Nairobi, Kenya using Mammalian Cell Display

By

Ryan G. Gaudet

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DEDICATION

To Courtney, whose love and support were invaluable in both my relocation to Winnipeg and the completion of this degree.

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LIST OF ABBREVIATIONS USED

ADCC	ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY
AIDS	ACQUIRED IMMUNE DEFICIENCY SYNDROME
ART	ANTI-RETROVIRAL TREATMENT
AFC	ANTIBODY FORMING CELL RESPONSE
BHK	BABY HAMSTER KIDNEY CELLS
BNT MAB	BROADLY NEUTRALIZING MONOCLONAL ANTIBODY
CHR	C-TERMINAL HEPTAD REPEAT
CMC	CERVICAL MONONUCLEAR CELL
CMC-R	CERVICAL MONONUCLEAR CELLS FROM A HIV-1 RESISTANT INDIVIDUAL
CDR	COMPLEMENTARY DETERMINING REGION
CT	CYTOPLASMIC TAIL
CTL	CYTOTOXIC T LYMPHOCYTE
DTT	DITHIOTHREITOL
ENV	ENVELOPE
ELISA	ENZYME-LINKED IMMUNOSORBENT ASSAY
EU	EXPOSED YET UNINFECTED
FDA	FEDERAL DRUG ADMINISTRATION
FACS	FLUORESCENCE-ACTIVATED CELL SORTING
FAB	FRAGMENT ANTIGEN-BINDING REGION
FC	FRAGMENT CRYSTALLIZABLE REGION
FR	FRAMEWORK REGION
FP	FUSION PEPTIDE
GAL CER	GLYCOSPHINGOLIPID GALACTOSYL CERAMIDE RECEPTOR
CH	HEAVY CHAIN CONSTANT REGION
DH	HEAVY CHAIN DIVERSITY REGION
JH	HEAVY CHAIN JOINING REGION
VH	HEAVY CHAIN VARIABLE DOMAIN
HEPS	HIGHLY EXPOSED YET PERSISTENTLY SERONEGATIVE
HEK	HUMAN EMBRYONIC KIDNEY CELLS
HIV-1	HUMAN IMMUNODEFICIENCY VIRUS TYPE-1
IGA	IMMUNOGLOBULIN A
IGKC	IMMUNOGLOBULIN KAPPA CONSTANT REGION
VL	LIGHT CHAIN VARIABLE DOMAIN
MPER	MEMBRANE PROXIMAL EXTERNAL REGION
MAB	MONOCLONAL ANTIBODY
MALT	MUCOSA - ASSOCIATED LYMPHOID TISSUE
NHR	N-TERMINAL HEPTAD REPEAT
PBMC	PERIPHERAL BLOOD MONONUCLEAR CELL
PDGFR	PLATELET DERIVED GROWTH FACTOR RECEPTOR
PR	POLAR REGION
R	REPLACEMENT
RT	REVERSE TRANSCRIPTASE
S-IGA	SECRETORY IGA
STI	SEXUALLY TRANSMITTED INFECTION
S	SILENT
SHIV	SIMIAN – HUMAN IMMUNE DEFICIENCY VIRUS
TM	TRANSMEMBRANE DOMAIN

Abstract

The mucosal antibody repertoire of the cervical mucosa in commercial sex workers from Nairobi, Kenya, who are highly sexually exposed to human immune deficiency virus type-1 (HIV-1) but remain persistently IgG seronegative (HEPS), may represent a novel source of broadly neutralizing monoclonal antibodies (mAbs) against HIV-1. Mucosal IgA specific for HIV-1 envelope (Env) subunit gp41 has been suggested as a correlate of protection in HEPS individuals. The in depth studies at both the gene and function level required to confirm their role in HIV-1 resistance are possible only using recombinant monoclonal IgAs. Human mAbs have traditionally been selected from libraries displayed on the surface of microorganisms (phage, yeast). However, due to inherent limitations, such techniques may not be optimal for isolating such rare mAbs from a pool of cervical B cells. We have developed an antibody selection system based on surface display on mammalian cells and used this technology to isolate four novel monoclonal antibodies, against linear epitopes on gp41, from the IgA repertoire of the cervical mucosa in Kenyan HEPS. Furthermore, three of the four mAbs were shown to bind with surface expressed consensus clade B and clade C Env on mammalian cells. Characterization of the variable region cDNA of the two strongest binding mAbs reveals extensive somatic mutations with a bias of replacement mutations clustering in the complementary determining regions (CDR) indicating antigen-driven affinity maturation had occurred. Affinity matured monoclonal IgAs, such as these, may play a role in the identification of new, vulnerable epitopes on HIV-1, or act as a component in a topical microbicide.

1 Introduction

1.1 Global Impact of HIV-1

Despite over 25 years of research, human immunodeficiency virus - type 1 (HIV-1) still represents a major health burden worldwide. During those 25 years, over 25 million people have died from the disease caused by HIV-1, acquired immunodeficiency syndrome (AIDS), and over 33 million people are currently infected [1]. Sub-Saharan Africa has been hardest hit by the pandemic, accounting for nearly two thirds of the individuals living with, and three quarters of all deaths resulting from AIDS [1]. Deplorably, over half of the individuals who acquire AIDS, are infected with HIV-1 before the age of 25, and die within the first 10 years [1]. Due to the great strides made in HIV-1 research and prevention in the past quarter century, the rate of new HIV-1 infections has dropped off in most countries; however, these trends are offset by new infections primarily occurring in regions of Sub-Saharan Africa. Globally there were 2.7 million new HIV-1 infections in 2007; 1.9 million of these cases were reported in Sub-Saharan Africa [1]. Because the majority of new HIV-1 infections arise in such low-income nations, the preponderance of individuals who are becoming infected with the virus do not have access to antiretroviral therapy. Clearly HIV-1 is still a leading global health problem and new innovative research is required to both improve treatment and develop a safe and effective HIV-1 vaccine.

1.2 HIV-1 Virology

AIDS was first described in homosexual men living in the United States in 1981 [2]. French and American scientists later confirmed HIV-1 as the causative agent for AIDS in 1983 and 1984 respectively [3,4]. HIV-1 belongs to the *Lentivirinae* subgroup of *Retroviridae* and has spherically shaped virion with a diameter of approximately 100 nm [5]. The genome consists of two ribonucleic acid (RNA) molecules in a complex with nucleoproteins and surrounded by a lipid bilayer envelope covered with approximately 72 glycoprotein spikes [5].

The HIV-1 genome consists of three major genes: *gag*; *pol*; and *env*. The initial Gag polypeptide is a 55 kD precursor protein that, during maturation, is cleaved into several smaller proteins including: matrix; capsid; p2; p6; and nucleocapsid [5]. The *pol* gene encodes for three enzymes: protease, reverse transcriptase (RT) and integrase [5]. The env gene encodes the glycoprotein spike that decorates the viral envelope. The initial *env* transcript is translated into a precursor protein (gp160) that is glycosylated in the endoplasmic reticulum and has the apparent molecular weight of 160 Kd. Gp160 is then cleaved by host cellular enzymes into two mature Env proteins: gp41 and gp120. Both proteins assemble into trimers coming together at the plasma membrane forming the viral spike [5]. A hydrophobic transmembrane domain allows gp41 to anchor to the viral membrane and interact non-covalently with the extracellular gp120 [5]. HIV-1 also has numerous accessory proteins that play critical roles in the viral lifecycle including, Tat, Rev, Nef, Vpr, Vif, and Vpu.

1.3 Diversity of HIV-1

The extraordinary genetic diversity of HIV-1 has presented an abundance of problems to researchers attempting to generate a universal vaccine. Part of this variability can be attributed to the fast replication cycle of the virus, which can result in the production of up to 10^{10} virions per day [6]. As a retrovirus, HIV-1 uses the enzyme RT to convert its own RNA genome into DNA once inside the host by taking advantage of the host cellular machinery for DNA replication, transcription, and translation. Replicated DNA is converted back into RNA for packaging into progeny virions once translation has occurred. While mutations can arise at any point, they most often occur during reverse transcription due to the high error rate of the viral RT (3×10^{-5} /nucleotide base per cycle) attributable to the lack of proof reading activity [6,7]. Variability is further compounded by the recombination of different types of HIV that infect the same cell [6]. As a consequence of these different mechanisms, many different variants of HIV-1 can be isolated from one individual in just a single day resulting in 1% genetic variation per year. The evolution of quasispecies results in a high rate of antigenic variation and correspondingly, poses an enormous challenge for host immunity.

HIV-1 can be divided into three main types: M, N, and O. Type N, being the least common, is only found in Cameroon, type O is responsible for relatively minor epidemics, while type M is responsible for the current pandemic (6). Based on mutation clock calculations, it is believed that HIV-1 type M crossed from chimpanzee into man in the early 1930's [6]. Following the rapid expansion of the type M population, the virus diversified into 10 subtypes, or clades: A, B, C, D, F_1, F_2,

G, H, J, and K [6,8]. Four of these clades, A, B, C, and D make up the vast majority of new HIV-1 infections. Clade B is the predominant clade found in North America and Europe; clade C, responsible for approximately 50% of infections world wide, is the dominant clade in Southern Africa, India and Nepal; clade A is common in Western Africa; while clade D can be found in Eastern and Central Africa [8]. Different clades of HIV-1 are often found in the same area; for instance, clades A and D are primarily responsible for the HIV-1 epidemic in Nairobi, Kenya [7].

1.4 HIV-1 Life Cycle and Immunopathogenesis

1.4.1 Early Discoveries

Not long after the discovery of AIDS in 1981, Klatzmann et al, (1984) showed that *in vitro*, HIV-1 was able to replicate and cause a cytopathic effect in CD4+ T cells, but not in CD8 T cells [9]. This was supported by the observation that AIDS was associated with the decline of CD4+ T cells in the blood [2]. Additional work by the Klatzmann group demonstrated that it was the CD4 antigen itself that acted as the receptor allowing for viral invasion of these CD4 positive T helper cells [10]. Several years later, using transfection studies of CD4 cDNA, Maddon et al (1986) showed that additional receptors other than CD4 were required on the cell surface for HIV binding [11]. It wasn't until some 10 years later that it CXCR4 was identified as the co-receptor necessary on T cell lines, and CCR5 was identified as the co-receptor necessary on peripheral blood mononuclear cells (PBMC) and macrophages [12,13].

The association of HIV with dendritic cells, initially met with controversy, was first suggested by Knight and Macatonia (1988) [14,15]. The identification of two types of dendritic cells, myeloid, and plasmacytoid, allowed Patterson et al (2001) to demonstrate that myeloid dendritic cells express CD4 and CCR5 supporting HIV binding and replication, while plasmacytoid dendritic cells allow HIV to bind to their DC-SIGN receptor [16]. Binding to dendritic cells allows the virus to be escorted to local lymph nodes where it will encounter the susceptible CD4+ T cells required for viral proliferation.

1.4.2 Typical Course of HIV Infection

HIV infection via the mucosal or parenteral route initially results in high viremia [15]. CD4+ T cells, macrophages and dendritic cells at the site of infection migrate to local lymph nodes delivering virus to CD4+ T cells. The most active site of HIV replication is the CD4 cell rich, mucosa - associated lymphoid tissue (MALT) of the gut [17]. After the initial infection, the viral load declines to a level that can be indicative of the rate at which the individual will progress to AIDS [18]. This decline in viremia has been attributed to both strong cell-mediated immunity and the circulation of HIV-1 neutralizing antibodies [15]. In addition to the neutralizing antibody response, non-neutralizing gp120 and gp41 antibodies may play a role in the decline of the primary infection by either inducing the host complement response, or antibody dependent cellular cytotoxicity [19,20].

Following seroconversion and the partial clearance of the primary infection, there is a long clinically asymptomatic period with fairly stable CD4+ T cell counts

[15]. During this period, CD4 cell death is balanced by a remarkable increase in CD4 cell restoration [21]. Eventually, immune regeneration can no longer offset the cell death caused by the virus, and CD4+ T cell numbers drop below a threshold level of 200 cells/μl [15]. At this point, the immune system of the patient has been depleted to the point where opportunistic infections will likely result in death.

1.4.3 HIV Infection of the Female Genital Tract

Approximately 30-40 % of new HIV-1 infections in women are acquired through vaginal intercourse [22]. Free virions are able to establish mucosal infection of the either the vagina, or the ecto and endo cervix [22]. Initially, the thick mucus layer of the female genital tract is able to trap free and cell-associated virions prolonging the contact time of the virus with the cells of the mucosa [22]. It is at this time that the virus is most susceptible to antiviral substances such as secretory IgA [22].

Infection with HIV at the mucosal surface is very different from parenteral transfer. HIV is able to invade the mucosal epithelium using several different pathways; therebye ensuring that even if one pathway is blocked, the virus is still able to initiate a productive infection. One such pathway is the capture and internalization of HIV virions by Langerhans cell in the epithelium, which then transport the virus to the stroma [22]. Once in the stroma, HIV can infect stromal dendritic cells which then circulate to CD4 cell rich regional lymph nodes where massive viral replication can occur [22]. HIV may also infect intra-epithelial CD4+ T cells directly in the vaginal mucosa, or simply migrate through physical abrasions in the epithelium caused by intercourse [22]. The virus is subsequently is taken up by

lymphatic or venous microvessels and transported to either regional lymph nodes or into peripheral blood [22]. The virus also has the ability to either transcytose through epithelial cells in the basal layer of the vaginal squamous epithelium, productively infect these epithelial cells, be endocytosed into cytosolic compartments, or penetrate the spaces between the epithelial cells [22].

1.5 Structure and Function of the HIV-1 Envelope

1.5.1 Native HIV-1 Envelope Spike

The HIV virion is surrounded by a lipid bilayer envelope covered with gp120-gp41 spike complexes (Figure 1). These complexes initially come together through weak noncovalent interactions at the surface of infected cells and through the budding process, are incorporated into the virus envelope [23]. Experimental evidence, largely based on scanning transmission electron microscopy images of fixed HIV glycoproteins, has suggested that the functional envelope spike (Env) is a heterodimeric trimer complex of gp120 and gp41 (Figure 1) [24]. However, there is also evidence suggesting there are other forms of envelope present on the viral surface. Kuznetsov et al (2003) was unable to demonstrate the presence of uniform trimeric envelope species on HIV virions using atomic force microscopy, while Herrera et al (2005) showed that antibodies unable to neutralize virus in solution, are able to capture virus onto ELISA plates [25, 26]. The appearance of these nonfunctional envelope species is likely the result of inefficient trimerization of the subunits in the Golgi, or the dissociation of gp120 from gp41, leaving uncapped

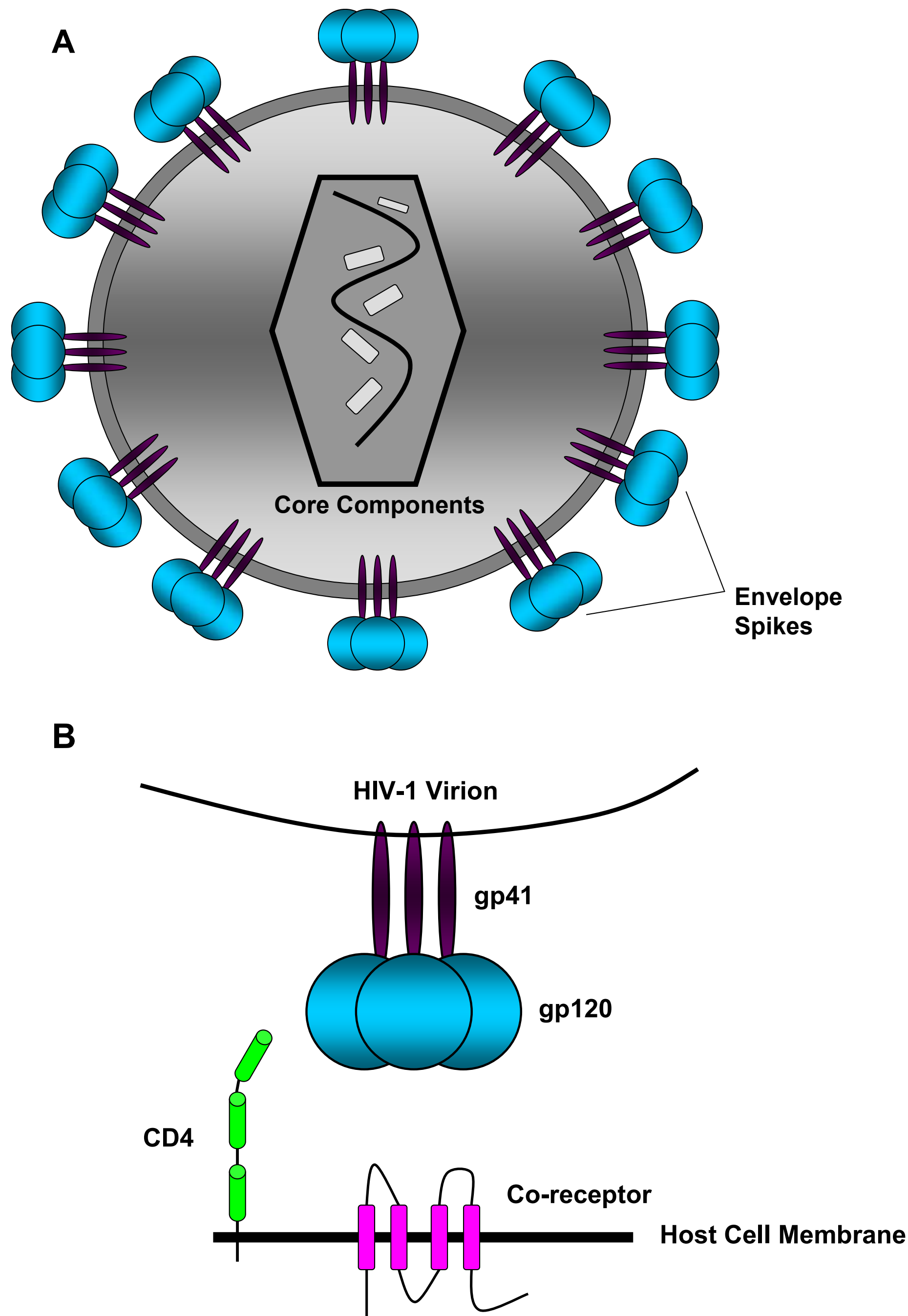

Figure 1. The HIV-1 virion and the envelope glycoprotein trimer complex. A) Simplified depiction of a particle of HIV-1. The Env trimers are the sole target of neutralizing antibodies, while cellular responses may target the core components [29]. B) A single, functional HIV-1 envelope spike composed of gp120 and g41 trimers. The spike interacts with CD4 receptor along with a co-receptor on host cells and initiates virus entry into host cells [29].

gp41 trimers decorating the viral envelope [23, 27]. The HIV spike is extraordinarily diverse differing by up to 35% between viral clades, and up to 20% within each clade [8]. This incredible antigenic diversity allows the virus to evade the host immune response, and has hampered vaccine attempts for decades.

1.5.2 Structure of gp120

The exposed region of Env, gp120, is composed of five relatively conserved regions (C1-C5), and five highly variable loops (V1-V5) [29]. In addition to their variability, the variable loops are highly glycosylated and able to shield the more conserved underlying structures from host antibodies [29]. One relatively conserved region of gp120, the CD4 binding pocket, is located deep within the inner domain [29]. For cell entry, the CD4 molecule on the target cell must bind deep within the CD4 binding pocket resulting in a conformational shift in gp120 that reveals a second conserved site: the coreceptor binding site [29]. The co-receptor binding site associates with either CCR5 or CXCR4 on the target cell initiating a second conformational shift, this time in the gp41 subunit [29].

1.5.3 Structure of gp41

The Env protein gp41, is a 345 amino acid (aa) glycoprotein with a mass of 41 kDa [30]. The structure gp41 is depicted in figure 2. Likely for conservation of function, gp41 is more conserved than gp120 and is not typically subdivided into distinct V and C regions. It can, however, be divided into three major domains: the extracellular domain (aa 512 to 683); the transmembrane domain (TM) (aa 684 to

705); and the cytoplasmic tail (aa 704 to 856) [30] (Figure 2). Numbering of Env aa residues is based on numbering in HXB2 [31]. The extracellular domain, being the only domain accessible to the target cell, can be subdivided into regions that are functionally important in the fusion of the viral and host cell membranes. These include: (i) the fusion peptide (FP), the N terminal hydrophobic region; (ii) the polar region (PR); (iii) two alpha helical structures termed the N-terminal heptad repeat (NHR) and the C-terminal heptad repeat (CHR); (iv) the membrane spanning domain (TM);(v) and a tryptophan rich region next to TM termed the membrane - proximal external region (MPER) (aa 660 to 683) (Figure 2) [30].

The FP corresponds to the first 15 aa of gp41 and through mutational studies, has been shown to be involved in triggering fusion between the viral and target cell membranes [30, 32, 33, 34]. Due to its oblique nature, insertion of FP into the cell membrane causes destabilization of the lipid bilayer permitting membrane fusion [35]. Many enveloped viruses use FPs to mediate membrane fusion following a trigger such as a change in pH or coreceptor binding.

The NHR and the CHR both play an important role in initiating the conformational change in gp41 following gp120 binding to the co-receptor. During this conformational change, three NHRs form a bundle anti-parallel to three helical CHRs, together forming a six-helix bundle [30]. The creation of the six-helix bundle is essential for the formation the pore that permits the entry of the HIV nucleocapsid into the target cell [36].

The TM region of gp41 serves to secure the entire Env spike to the viral membrane. The structure of TM has yet to be elucidated; however, a computer

model proposed by Gallaher et al (1989), has suggested it adopts an α-helical structure [37]. This region is among the most highly conserved between different HIV clades and there is controversy surrounding whether or not it participates directly in the virus-cell fusion process [30].

The MPER, located adjacent to the TM region, is another region of gp41 that is highly conserved among clades of HIV [30]. The structure of MPER was originally predicted as an α-helix by Gallaher et al (1989); however, unlike the TM, additional information has since come out suggesting an alternative structure [37, 30]. Structural analysis of MPER in complex with the monoclonal antibody 2F5 (reviewed later), suggests MPER exists in an extended conformation with a β-turn at its core [38,39]. This discrepancy may be attributable to a conformational shift that occurs in the MPER during virus-cell fusion [40]. The idea that MPER plays a vital role in HIV infection is supported by the following observations: it is highly conserved among isolates [30,41]; epitopes of several of the most potent HIV-1 broadly neutralizing monoclonal antibodies (bNt mAbs) can be found in the MPER [30,41]; mutational studies with MPER have a deleterious effect on virus-cell fusion [30, 42, 43]; and MPER binds with the galactosyl ceramide receptor (Gal Cer) permitting transcytosis of the virus across the mucosal epithelium [4, 30, 44].

Gp41 itself can be found in either three different conformations depending on the stage of the fusion process. Little is known about the pre-fusion structure, however, it is believed that the majority of gp41 domains (the NHR and the CHR) fold together and are largely obscured by gp120 in the native spike [45]. The structure of the fusion intermediate state is also obscure. Based on the crystal

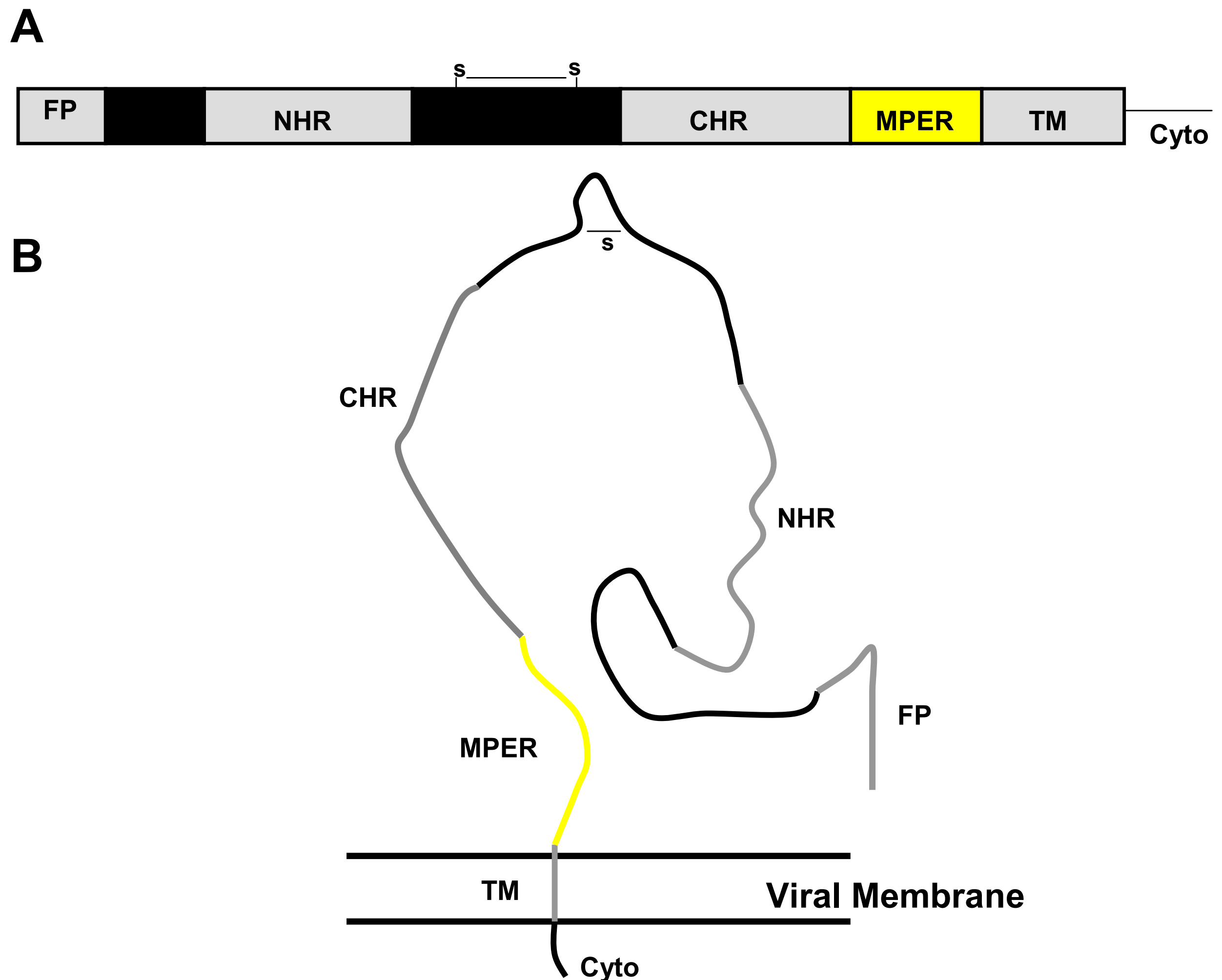

Figure 2. Structure of HIV-1 envelope protein gp41. A, Schematic diagram of linear gp41 with important structural regions indicated; the MPER region is highlighted in yellow, disulfide bonds are indicated with horizontal lines [83]. B, estimated structure of gp41 depicting the orientation of the structural regions indicated in A in native gp41 [83]. The reported immunodominant region is outlined in black between the NHR and CHR and contains the di-sulfide bond.

structure of gp41 peptides with different mAbs, it is has been postulated that gp41 adopts an extended bundle of three α-helixes, partially stabilized by its association with gp120, that is capable of partially inserting FP into the lipid bilayer of the target cell [24].

In contrast to the pre and intermediate fusion states, the structure of the post-fusion state has been well established [24]. As stated above, the conformational shift in gp41 initiated by gp120 binding to the coreceptor allows the CHR and NHR to form the stable six-helix bundle [30]. This model suggests that the action of the unstable extended three-helix rod transitioning to the highly stable six-helix bundle draws the target cell membrane closer to the viral membrane, allowing for complete insertion of FP into the cell [24]. The destabilization of the cell membrane is followed by the formation of a fusion pore by MPER, CT, and possibly portions of TM allowing for the insertion of the nucleocapsid into the target cell [24]. While there are multiple, well-known domains on gp41, the limited surface accessibility of most of these limit the gp41 neutralizing antibody response mainly to the few regions that are surface exposed on the native Env spike.

1.6 Progress Toward an HIV Envelope Based Vaccine

1.6.1 Cell-Mediated or Humoral Immunity

Antibodies are the only host defense capable of providing sterile immunity and providing complete protection from infection. The failure to develop a successful HIV-1 vaccine has to be considered the biggest disappointment in the

field of HIV research during the past 25 years. Many different strategies have been attempted from DNA vector-based vaccines, to vaccines designed to elicit cellular immunity, to Env subunit vaccines designed to elicit humoral immunity. None of these strategies has shown considerable effectiveness in field trials [46,47]. An efficacious HIV vaccine will no doubt require both cell-mediated and humoral immunity against HIV-1, as the majority of successful viral vaccines work by preventing infection using neutralizing antibodies, and stimulating cell-mediated immunity to eliminate any remaining infected cells [92]. Complete or sterilizing immunity from HIV-1 infection will depend on the ability of the vaccine to elicit broadly neutralizing antibodies against HIV-1, in particular at the mucosal surface. The fact that only the presence of neutralizing antibodies has proven capable of preventing HIV/SHIV (simian-human immunodeficiency virus) infection in animal models validates the role of the humoral immune response in providing protection against HIV-1 [48-52]. Furthermore, the most commonly cited goal of CTL or T cell based vaccines is not to induce complete immunity, but to delay the onset of AIDS, for once the virus infects the CD4+ T cell population there is no hope to eradicate the virus from all reservoirs despite antiretroviral treatment [9].

1.6.2 Envelope Vaccines

Researchers have long been aware that ability to elicit humoral immunity is an essential component in the development of any HIV-1 vaccine. Indeed, Env based vaccines of either gp160, or the gp120 subunit were among the first attempts in developing an HIV vaccine in the late 1980’s [53]. Initial results from these trials

seemed promising, as antibodies were produced against gp120 in all recipients, with the majority also showing neutralizing potential [54-59]. However, it was quickly realized that these antibodies demonstrated little activity against naturally occurring HIV-1 strains, and did not protect against HIV infection [29]. Over 20 years later, clinical trials with recombinant gp120 have yet to produce a positive result that validate this approach [29].

Explanations for the failure of the recombinant gp120 vaccines are numerous. The most obvious being that the immunogens used did not faithfully reproduce the native structure of the viral envelope [29]. Other explanations are based on the nature of the viral envelope itself and include: the closed nature of Env hides conserved neutralizing epitopes; the heavily glycosylated nature of gp120 prevents antibody mediated neutralization; the outer V loops of gp120 can tolerate multiple mutations; the inherent genetic variability of *env* among different viral clades; epitopes crucial for virus-cell fusion (i.e CD4 binding site) are inaccessible until the conformational shift mediated by target cell binding; and the fact that gp120 is shed from the virus inducing antibodies to epitopes irrelevant in neutralization [29].

Even with the assortment of mechanisms by which HIV is able to avoid antibody - mediated neutralization, there is still hope that broadly neutralizing antibodies can be elicited by vaccination and provide protection from HIV-1. The isolation of several human monoclonal antibodies (mAbs) that are capable of neutralizing a broad spectrum of HIV-1 isolates has confirmed that there are broadly conserved neutralizing epitopes present on the HIV envelope [29]. MAbs 2F5, 4E10, and Z13 are well studied antibodies that bind to the MPER of gp41 [41,51,61,62], mAb b12 is

able to bind the CD4 binding site of gp120 [63] and mAb 2G12 binds to a conserved carbohydrate residue in the glycan shield of gp120 [88]. Indeed, Hessel et al (2009) has shown that passive transfer of gp41 mAbs 2F5 and 4E10 can provide complete protection against mucosal SHIV challenge in rhesus macaques [144]. Recent studies have also shown that passive transfer of mAbs 2G12 or b12 to macaques provided protection against repeated low-dose mucosal SHIV challenge even at low neutralizing titers [89,90]. Neutralizing titers of these mAbs in macaques were at or below those commonly observed in the sera of HIV-infected individuals against various clades of HIV-1 [90,91]. This suggests that the antibody titers achieved in these studies can be generated in humans provided an appropriate immunogen is used [90].

Several strategies aimed at inducing these neutralizing antibodies by vaccination are being investigated. Fouts et al (2000, 2002) has attempted to generate altered forms of gp120 that expose cryptic neutralizing epitopes by immunizing with a CD4 like molecule bound to gp120 [64,65]. While neutralizing antibodies are generated, it is not clear how relevant these antibodies are to naturally occurring viral isolates [64,65]. Others have attempted to use fusion intermediate complexes to generate antibodies to conserved epitopes only presented during the fusion process [66]. After some initially positive results, this strategy has since fallen out of favor due to poor reproducibility [29]. Pantophlet et al (2004) has hyperglycosylated regions of gp120 in order to mask undesired epitopes generating a gp120 mutant that preferentially binds to mAb b12 [67]. Immunization of this mutant would theoretically concentrate the antibody response

to the b12 epitope (CD4 binding site) [67]. Frey et al (2008), generated a trimeric Env with gp41in the extended fusion intermediate state and showed that it preferentially binds with bNt mAbs 2F5 and 4E10 [68]. Other groups are trying to develop soluble Env constructs that accurately represent the native viral spike [69,70]. The clear correlation between virus neutralization and antibody binding to native Env lends credence to this approach [71, 72].

The obvious potential of using rationally designed immunogens to elicit neutralizing antibodies requires additional investigation; however, the discovery of new bNt mAbs is required for the discovery of novel conserved, neutralizing epitopes on Env to improve the breadth and potency of the antibody response elicited by vaccination. In this regard, the study of the cervical mucosal immune response represents a novel source of B cell diversity.

1.7 Host Resistance to HIV-1

A little over a decade following the emergence of HIV-1 on the global scene, it became apparent that not all individuals exposed to the virus become persistently infected [73]. Definitively establishing the existence of individuals who are protected against HIV-1 proved to be a difficult task, as the only option was to observe individuals who were continuously exposed to the virus yet remained uninfected [74]. One of the first studies to demonstrate the phenomena of HIV resistance was done on a cohort of 424 initially HIV-1-seronegative commercial sex workers in Nairobi, Kenya [73]. Fowke et al (1996) followed these women from

1985 to 1994 and collected data on sexual behaviors, such as frequency of condom usage and the number of different partners, while monitoring their HIV-1 status using standard ELISA and immunoblot techniques [73]. Data analysis revealed that these women had an estimated 64 unprotected sexual exposures to HIV-1 per year [73]. Consequently, an extremely high rate of seroconversion to HIV-1 was observed during the first 2 years of the study [73]. Upon further investigation, they noticed that instead of continuing to rise, HIV-1 prevalence in the cohort reached a plateau, with new incidences of infection virtually non-existent in a small subset of these women (13% of cohort) [73,74]. Despite the fact that the number of annual exposures to HIV-1 has increased over time for these women, they have remained HIV-1 seronegative for more than 20 years [73].

The establishment of additional cohorts of highly exposed persistently seronegative (HEPS) individuals has only added to the drive to understand the mechanisms responsible for providing HIV-1 protection [75]. Since the correlates of immune protection from HIV-1 have yet to be elucidated, understanding how HEPS individuals are protected from HIV-1 is imperative.

Numerous studies on the Nairobi cohort have ruled out the following factors in resistance to HIV-1: a natural resistance to other STIs; mutations or altered gene expression of the CCR2, CCR5, or CXCR4 chemokine receptor; natural killer or lymphocyte activated killer cell activity; innate cellular resistance mechanisms; neutralizing IgG or other factors in the plasma; and the presence of mitogen activating β-chemokines in the plasma [74]. Other studies have focused on the role played by cell-mediated immune response in providing protection against HIV-1 in

the Nairobi cohort [74]. Rowland Jones et al (1998) demonstrated that CTLs with cross-clade specificity to HIV-1 exist in HEPS women [76], while Fowke et al (2000) showed that the CD4+ T cells from HEPS women, when exposed to Env, show increased production of IL-2 when compared with CD4 cells from HIV infected and non-infected individuals [77]. Other groups have postulated a role for HIV specific mucosal IgA in resistance to infection, and it is this theory that will be explored in further detail.

1.8 Role of IgA in Mucosal Immunity in the Genital Tract

1.8.1 IgA in the Genital Tract

An important part of mucosal defence against sexually transmitted pathogens is the presence of secretory IgA (S-IgA) antibodies in the external secretions of the genital tract. S-IgA exists as immunoglobulin dimmers that are protease resistant and have been shown to provide protection from many pathogens including viruses [78]. While not understood in terms of homing or molecular models, the compartmentalization of the immune system of the genital tract can causese antibodies produced locally in cervicovaginal secretions to be absent from the serum antibody response [78]. The induction mechanisms of the immune response in the genital tract have yet to be fully elucidated [78]. However, it is thought that intraepithelial Langerhan's cells present antigens to both B and T lymphocytes at local lymph nodes, or directly to local lymphocytes present in the genital tract mucosa [79]. The observation that vaginal immunization induces a

stronger local antibody response when compared with other mucosal sites, suggests that the activated lymphocytes express yet unidentified adhesion receptors that mediate homing of lymphocytes back to the genital tract from local lymph nodes [79].

1.8.2 Possible Mechanisms of IgA Mediated Protection from HIV-1

While S-IgA antibody mediated immune protection mechanisms have been shown to play an important role the defence against many pathogens, their role in protecting against HIV-1 infection in the genital tract is less clear. One possible mechanism of mucosal antibody mediated protection from HIV-1 infection is by cross-linking virions with non-neutralizing Env specific antibodies; a process referred to as immune exclusion [78]. Cross-linking traps microbes in the thick mucus layer of the genital tract, preventing contact with the mucosal surface and viral promoting clearance [78]. This mechanism has been correlated with protection from several other mucosal pathogens. For example, Burns et al (1996) showed that non-neutralizing IgA mAbs were able to provide protection from rotavirus infection in mice by preventing mucosal attachment [80]. Whether immune exclusion is relevant to HIV infection is unknown [81].

A second mechanism of IgA mediated protection from HIV-1 may be antibody mediated inhibition of viral transcytosis across the epithelial cell layer. Inhibiting transcytosis would prevent the virus from reaching the T cell abundant lamina propria and inhibit viral spread. Alfsen et al (2001), has shown that mucosal S-IgA and not IgG from HIV-1 infected individuals, is able to block HIV-1 transcytosis by

preventing gp41 binding to the glycosphingolipid galactosyl ceramide (Gal Cer) and disrupt the formation of the lipid raft microdomains that mediate transcytosis and endocytosis across the phosphilipid rich cell membrane [82]. Additional immunological studies established that the S-IgA targeted the MPER of gp41, in particular, the conserved ELDKWA hexapeptide that is also the target of the broadly neutralizing gp41 mAb 2F5 as shown by Muster et al (1993) [82,83,41].

A third mechanism of protection may be IgA mediated neutralization of the virus during transcytosis through epithelial cells [84]. Huang et al (2005) used pairs of IgA and IgG gp120 and gp41 neutralizing mAbs to demonstrate that mAbs of the IgA isotype ware able to act intracellularly to inhibit virion production by epithelial cells [84]. They proposed that vesicles containing internalized Env specific IgA intersect in the secretory pathway with post-Golgi vesicles carrying new Env proteins and neutralize virus production [84].

A fourth mechanism whereby IgA may provide some protection from HIV-1 infection is through antibody-dependent cellular cytotoxicity (ADCC). While non-neutralizing antibodies with ADCC activity have been shown to provide protection in other viral models, the role played by IgA induced ADCC in the immune response against HIV-1 is supported by considerably less evidence. Colostral and serum IgA from HIV-1 infected individuals has been shown to lyse gp120 coated cells in the presence of PBMCs *in vitro* [78]. Connick et al (1996), suggested that ADCC plays a role in controlling HIV-1 replication early in acutely-infected individuals, while Baum et al (1996) correlated high levels of ADCC inducing antibodies with slower progression to AIDS [85,86]. Perhaps the strongest evidence comes from Duval et al

(2008), who showed that an anti-gp41/anti-CD89 (IgA Fc receptor) bispecific mAb can direct the destruction of HIV-1 by CD89 positive neutrophils which are highly prevealent in the cervix [87].

1.8.3 IgA in HEPS Subjects

While there is evidence supporting a possible role for mucosal S-IgA in the immune response against HIV-1, the role played by mucosal IgA in mediating resistance against the virus in HEPS individuals has been the subject of much debate. Kaul et al (1999) was among the first to correlate levels of cervical HIV-1 specific IgA to host resistance to infection in the Nairobi Cohort [93]. In that study, HIV specific IgA was observed in 16 of 21 HEPS sex workers, 5 of 19 infected sex workers, and 3 of 28 low risk HIV-1 negative women [93]. HIV-1 specific IgA has also been isolated from cervicovaginal lavage fluids in 50% of female HEPS sex workers in northern Thailand [94], and has been correlated with protection in partners of HIV-1 infected individuals who remain uninfected despite repeated exposure (EU) in Cambodia [95].

It was later shown that IgA purified from the genital tract, saliva, and plasma of HEPS sex workers in Nairobi was able to neutralize a clade B HIV-1 virus isolate [98]. A second study by Devito et al (2002) showed that the IgA in mucosal and systemic compartments of HEPS sex workers in Nairobi showed significantly more cross clade HIV-1 neutralizing ability than the IgA purified from EUs with HIV-1 clade B infected partners [97]. Mucosal and plasma IgA purified from 3 of 6 HEPS

sex workers in Nairobi could also inhibit the epithelial cell transcytosis of a HIV-1 clade B isolate [98].

In addition to differences in total antibody levels, differences in the epitope specificity of serum IgA in HEPS individuals compared with HIV-1 infected individuals has also been observed. In a study of 6 EU individuals, 6 HIV-1 infected women, and 6 healthy controls, Clerici et al (2002) demonstrated that IgA from EU subjects exclusively recognized gp41, while IgA from HIV infected individuals recognized both gp120 and gp41 [99]. Furthermore, the IgA from the infected subjects bound with the immunodominant regions in gp41 (aa 589 – 618 and 642-673), while IgA from EU subjects recognized a restricted epitope (aa 582 – 589) (QARILAV) located in the α - helical region of gp41 that is accessible to antibody only after the binding of gp120 to CD4 on the target cell [99]. Higher levels of IgA specific to gp41 have also been observed in EU subjects when compared with HIV-1 negative individuals in Cambodia, Italy, and in a cohort of intravenous drug users in Vietnam [100].

There is also evidence suggesting IgA does not play a major role in the HIV-1 resistance observed in HEPS individuals. HIV specific IgA could not be detected in the cervicovaginal secretions of 24 EU subjects in California [96]. Ghys et al (2000) could only detect anti-HIV antibodies in the cervix of 7.3 and 29.8% of HEPS sex workers in the Abidjan, Cote d'Ivoire [101], and Lucy et al (2000) detected no significant levels of IgA or IgG in the vaginal secretions of HEPS sex workers in the Gambia [102]. Considerable differences in the exposure levels to HIV-1 may account for the lower levels of HIV-1 specific cervical IgA, as it is estimated that sex workers

in the Gambia have only one unprotected sexual encounter with an HIV-1 infected man per month [102]. Due to these conflicting results, Horten et al (2009) carried out a comprehensive analysis of HIV-1 specific IgA and IgG levels in the cervical lavages of HEPS and HIV-1 infected sex workers in the Nairobi cohort [103]. While HIV-1 specific IgA was found in a number of the resistant women, it was not found at a higher frequency than the HIV-1 infected or uninfected women, suggesting that IgA does not play a significant role in long term protection against the virus [103]. However, because antibody levels were detected using a conventional ELISA against a HIV-1 virus lysate, the study offers no insight into differences in the gp41/g120 binding profile of the different antibody populations, differences in the epitopes recognized, differences in antibody affinity, nor any functional differences between the IgA from HEPS individuals and the IgA from HIV infected individuals as has been shown previously [99].

The conflicting results addressed above are likely due to the difficulties in detecting IgA in the notoriously variable environment of the cervix, differences in antibody detection methods, differences in the antigens used, and variations in the characteristics of the different HEPS populations studied (level of exposure to virus, opportunistic infections, frequency of protected/unprotected intercourse, mode of virus exposure, time of menstral cycle, hormone level, etc.) [104]. Due to these variations, studies at the molecular level using mAbs are required for further characterization cervical IgA [104].

Using a technique called phage display (discussed below) Berry et al (2003) was the first to isolate a HIV-1 neutralizing IgA mAb against gp120 from the cervix

of HEPS sex workers in Nairobi, Kenya [105]. In a recent study, Tudor et al (2009) also used phage display to isolate gp41 specific neutralizing IgA mAbs from the cervix of HEPS sex workers in Cambodia that are able to inhibit the in vitro transcytosis of HIV-1 clade B and C virus isolates [104]. Interestingly, IgG class switched variants of the same mAbs were not as efficient at blocking transcytosis of the virus, indicating a potential role for the IgA constant region [104]. Molecular analysis of the antibody genes revealed extensive somatic mutations and a high replacement to silent (R:S) mutation ratio in the CDR regions suggesting that these antibodies have been affinity matured from repeated exposure to gp41 over many years [104]. Clearly, access to the individual genes of the gp41 specific IgA antibodies provides much stronger evidence of their natural occurrence in the cervix, in contrast to previous studies which demonstrate only the presence of polyclonal IgA in vaginal secretions [104]. The isolation of IgA mAbs from the cervix of HEPS sex workers from other cohorts (i.e Nairobi) is required to confirm their role *in vivo*.

1.9 Monoclonal Antibodies

1.9.1 Antibody Structure

Antibodies are composed of two distinct regions: a variable (V) region and a constant (C) region. The V region contributes to the antigen specificity of the antibody, while the C region, represented by the 5 different isotypes, IgM, IgG, IgA, IgE and IgD, dictates the effector functions of the antibody. The antibody variable

domain is composed of the V regions of the heavy (H) and light (L) chains which come together to form the three dimensional antigen binding site or the paratope [104,106]. Each V region contains three hypervariable loops termed complementary-determining regions CDR1, CDR2, and CDR3 that are largely responsible for antigen binding. The CDR regions are separated by the more conserved framework regions (FR1, FR2, FR3, and FR4). The mature heavy chain transcript is formed by somatic recombination and random assembly of three germline gene segments: variable (VH); diversity (DH); and joining (JH) [104,107,108]. The VH gene can be separated into seven families (VH1-7), each sharing over 80% nucleotide homologies in the framework regions [104,106]. The VH region encodes the CDR1 and CDR2, while the DH and JH both encode for the CDR3. Consequently, there is extraordinarily high diversity in both the composition and length of the H-chain CDR3 (as much as 10^{23} possible sequences) [109]. There are two types of human light chains, Kappa (κ) responsible for approximately 60% of the antibody repertoire, and Lambda (λ), which is responsible for the remaining 40% [104]. Each light chain is composed of rearrangements of the germline VL and JL gene segments forming the CDR1, CDR2, and CDR3. The combination of the rearrangement of the VH, JH, and DH, along with the VL and JL segments, different combinations of heavy and light chains, and somatic mutations in the CDR and FR regions all contribute to the diversity of the human antibody repertoire [104].

1.9.2 Antigen Selection and Affinity Maturation

Affinity maturation refers to the process by which repeated antigenic challenge of B cell clones results in the production of an antibody with progressively higher antigenic affinity [139]. The recombination of un-mutated V,D and J genes determines the initial antigen specificity of the surface antibody. Antigen binding is then optimized via somatic mutations in the FRs and CDRs in an antigen driven process where higher affinity clones are positively selected and amplified [140, 176]. Mutations in the CDRs are more likely to alter the antigen-binding properties of the antibody due to their plasticity and structural location, whereas mutations in the FRs tend to be more structurally destructive [140]. Consequently, antibodies that have been subject to antigen-driven affinity maturation tend to have higher numbers of replacement (R) than silent (S) mutations in the CDRs, and a lower R:S mutation ratio in the FRs [141].

Chang and Casali (1994) were the first to develop a model to estimate the probability that a given VH or VL sequence had been antigen selected. This binomial model compared the R:S ratios of a given VH or VL sequence to the expected R:S value, in the absence of antigen selection, of the most homologous germline gene given random mutation [141]. Lossos et al (2000) later altered this approach by developing a multinomial model that can be applied to any VH or VL gene to estimate the probability that the gene has been subject to antigen-driven affinity maturation [142].

1.9.3 MAb Production Methods

Early methods of producing antibody relied on collecting serum from animals that had been immunized with the antigen of interest [110]. Problems with this method, including the limited supply and polyclonal nature of the antibodies obtained (an undefined mixture of different Abs) were the driving force behind the development of hybridoma technology [110, 173]. This technology, developed in the late 1970's, allows for the immortalization of individual B cells from an immunized lab animal [173]. These cells can be grown *in vitro* to produce large quantities of individual monoclonal antibodies [173].

As useful as hybridoma technology has been, it has several inherent problems that prevent it from fulfilling the vast research and therapeutic potential of monoclonal antibodies. Perhaps the most glaring problem is that hybridoma technology results in the formation of non-human antibodies that, due to their inherent immunogenicity, cannot be administered directly to humans as a therapeutic. A second problem is that because antigen selection initially occurs in the serum of the lab animal there is little to control over the antibody response. Consequently, selection for sensitive molecules, or specific regions of antigens is very difficult [110]. Finally, because the DNA encoding the mAb is not provided, painstaking procedures must be undertaken to convert the antibody to a recombinant form to analyze the VH and VL genes, or to mutate the protein [110].

The development of recombinant antibody technology has largely solved the problems of hybridoma technology. There have been several methods developed for the isolation of fully human recombinant monoclonal antibodies that rely on steps

that mimic the *in vivo* selection process [111]. First, the genetic diversity of VH and VL regions is cloned by PCR from a population of immune or naïve human B cells. Second, the genotype of the antibody (DNA) is coupled to its phenotype (antigen specificity) using a system capable of expressing the library of re-arranged VH and VL genes as functional antibodies. Third, antibodies of interest are selected for their binding properties to a specific antigen. Finally, the amplification and large scale production of the desired antibodies [111, 112].

The predominant method for the expression and selection of recombinant antibodies is called phage display [113]. To a lesser extent, ribosome/mRNA display [114] and microbial display [115] are also used to produce mAbs. Using phage display, Barbas et al (1991) was the first to isolate mAbs against HIV-1 from human B cells by expressing antibody fragments on the surface of filamentous bacteriophages [113]. In that study, Barbas et al (1991) used random combinations of heavy and light chains to produce an antibody library, from wich the HIV neutralizing gp120 specific mAb b12 was isolated, that originated from bone marrow derived B cells of a HIV-1 infected individual [113]. It can be argued that using this approach may yield few antibodies of interest due to the infrequent pairing of the original heavy and light chain combinations. However, as Burton et al (1991) suggests, because the mRNA and not DNA is used to generate the library, the random combinatorial approach represents the current antibody response of the individual donor and original H and L pairings can be observed quite frequently [126]. Phage display is a robust and versatile method that has been used successfully for almost 20 years to produce the majority of human mAbs used today.

Despite the success of the phage, ribosome/mRNA, and microbial cell display systems, improvements in the antibody expression and selection processes can still be made. All of the recombinant techniques outlined above involve the expression of antibodies in a prokaryotic environment rather than the natural environment: the secretory pathway of mammalian cells [112]. This causes a bias in the antibodies produced, as antibody selection occurs not only for antigen specificity, but also for biophysical properties that promote superior expression in the foreign prokaryotic system [112]. Indeed Bowly et al (2007), has shown that a library dipslayed via a prokaryotic system (phage) yields a completely different set of mAbs when the same library is displayed in a eukaryotic system (yest) [174]. Furthermore, severe translational problems and difficulties with protein folding can occur when transferring antibodies selected via phage or microbial cell display to mammalian cell culture systems to express full antibodies [116].

1.9.4 MAb Production via Display on Mammalian Cells

In recent years, antibody selecting systems have been developed that exploit the secretory pathway of mammalian cells. Expressing and selecting antibodies displayed on the surface of mammalian cells ensures that the antibodies produced are less biased by characteristics other than antigen specificity [112]. Another advantage of using a mammalian cell display system is that, provided fresh PBMC samples can be obtained, fluorescence activated cell sorting (FACS) can be used to separate B cells for the presence of surface markers or antigen specificity prior to the cloning of V region diversity. This results in an antibody library that is cloned

from the repertoire of a small subset of desired B cells [117]. Researchers have used this method to clone antibodies from individually sorted B cells from whole blood against influenza antigens, vaccinating antigen, and self proteins [117, 118, 119]. Pre-sorting B cells circumvents the major problem of using mammalian cell display: the amount of cells that can be handled at one limits the size and complexity of the antibody library.

FACS has also been used in the selection of antibodies displayed on the surface of BHK and HEK 293T cells [112, 116]. Ho et al (2006) transiently transfected an expression vector with the human platelet derived growth factor receptor transmembrane domain fused to the C terminal portion of the VH region to promote antibody expression on the surface of 293T cells [116], while Beerli et al (2008) facilitated single chain V region BHK cell surface expression using a Sinbis virus expression system [112]. Both systems utilized FACS to separate a small percentage of antibody decorated cells for antigen affinity and isolated high affinity mAbs from the memory B cells of an immunized individual [112], or from a mutant antibody library generated from a single mAb [116]. Mammalian display systems have yet to be used to isolate antibodies from non-sorted cervical B cells.

1.10 Neutralizing Antibodies and HIV-1

1.10.1 Role of Therapeutic Antibodies

As discussed above, the study of broadly neutralizing monoclonal antibodies (bNt mAbs) against gp120 and gp41 has resulted in the discovery of several

conserved neutralization epitopes present on HIV-1 Env. In addition to providing invaluable structural data on HIV-1, bNt mAbs may also have potential as a therapeutic agent against both HIV-1 infection and AIDS progression. Antibodies have proven their usefulness *in vivo* against viral infections as they have been used as treatments and preventative therapies in humans against hepatitis B virus, cytomegalovirus, varicella zoster virus, rabies virus, and syncytial virus [120-123]. Indeed, over 20 mAbs currently have FDA approval for use in humans as therapeutic agents [120]. Antibody therapy has numerous advantages over traditional small-molecule drugs. These include: a high specificity and affinity for their target; little to no metabolic side effects due to their catabolism to amino acids; a reduced toxicity due to their inability to diffuse across the cell membrane; little to no antigenicity of fully human antibodies; and their long serum half lives allow for infrequent dosing [120]. While therapeutic antibodies currently need to be injected, their infrequent dose regimens could be a viable alternative to the daily pill regimens of many therapies including anti-retroviral therapy (ART) [120].

Whether bNt mAbs can be used successfully against HIV-1 has been the subject of much debate. While passive transfer experiments in animal models has confirmed that bNt mAbs can prevent infection and limit transmission *in vivo* [50, 89, 90, 124], clinical testing in humans has so far been limited and only marginally successful [120]. In order for the development of antibody therapeutics against HIV-1 to move forward, novel mAbs against HIV-1 Env need to be isolated. The discovery of novel Env specific mAbs is required for the identification of: new susceptible target epitopes; novel epitopes conserved numerous HIV-1 subtypes; the effector

functions that provide protection from HIV-1 *in vivo*; and novel combinations of different antibodies capable of provide maximum protection from HIV-1 [120].

1.10.2 MAbs versus gp120

Until recently, the only gp120 specific mAb to show broad neutralizing ability of diverse primary isolates of HIV-1 has been mAb b12. This mAb was selected from an antibody library derived from the bone marrow of an HIV-1 subytype B infected individual that had been asymptomatic for 6 years [125]. This random combinatorial library was displayed on the surface of an M13 bacteriophage and selected for its affinity to gp120 IIIB [125]. Burton et al (1994) converted this fab into a full IgG1 molecule and demonstrated a neutralizing ability of primary virus isolates of more than 75% [126]. Zhou et al (2007) later resolved the crystal structure of b12 with gp120 and confirmed that b12 bound to the conformational dependent CD4 binding site wedged between the non-immunogenic glycan shield and the flexible inner domain [127]. Phage display has yielded numerous other mAbs vs gp120 from subtype B infected individuals that have neutralizing ability [128-131], none however, demonstrate the broad neutralizing ability of b12.

Walker et al (2009) has recently developed the first broadly neutralizing HIV-1 mAbs directed against gp120 since b12 [145]. In this study, memory B cells from an African donor with potent serum neutralizing activity were used to generate two mAbs with remarkable neutralization breadth and potency [145].The two somatic variants, PG9 and PG16, neutralized 127 and 119 out of 162 pseudoviruses respectively in a large multiclade panel [145]. Both mAbs

demonstrated binding to an epitope along the V2 and V3 domain of gp120 only when gp120 was expressed as part of the functional Env spike [145].

1.10.3 MAbs versus gp41

The relatively conserved nature of gp41 suggests it could be a target for bNt mAbs. The major problem with targeting this protein is that in the native spike, gp41 is largely hidden from antibodies by the gp120 trimer [61]. In a comprehensive study of 6 HIV infected individuals, Scheid et al (2009) cloned a total of 433 antibodies from individual B cells sorted for affinity to gp140 [132]. Using ELISA experiments, they determined that 70% of the antibodies bound gp120, and only 30% bound gp41 [132]. Of the 130 gp41 mAbs, zero bound to the MPER containing the 2F5 and 4E10 epitopes, and only 9 bound to the previously reported immunodominant region of gp41 cluster 1 (aa 579-604) [132, 133]. The lack of reactivity against gp41 peptides suggests that the majority of gp41 antibodies from HIV-1 infected individuals recognize conformational dependent epitopes present in the native protein [132].

Binley et al (1996) constructed phage display combinatorial libraries from six HIV-1 positive donors and isolated 23 different mAbs against conformational epitopes on gp41 [134]. All of the antibodies could be mapped to one of three regions on gp41: cluster 1, cluster II (aa 649-668), and cluster III (aa 619-648) [134]. The fact that none of these antibodies showed neutralizing ability against the virus led the authors to conclude that these epitopes are obscured by gp120 on the native spike [134]. These antibodies were likely generated against precursor

envelope proteins, viral debris, or spikes in which the gp120 cap had been stripped away [134].

Many random combinatorial phage display libraries from HIV positive donors have been selected for binding to chimeric gp41 constructs that mimic the various stages of gp41 during the fusion process. This strategy; however, has yielded only a few mAbs with significant cross-clade neutralizing ability. Gustchina et al (2007) screened a combinatorial library for binding to a gp41 construct that displays the N-HR as an α-helix extending from the six-helix bundle [135]. From that library, one Fab was selected (3674) that bound a shallow groove located between two C-HR helices in the six-helix bundle and neutralized primary isolates of clades A, B and C [135]. Two other mAbs showing moderate neutralizing activity, D5 and m46, have been selected for binding to the six-helix bundle, N-HR and C-HR peptides [136, 137].

By far the most intensely studied mAbs against gp41 are 2F5, 4E10, and to a lesser extent, Z13. MAb 2F5 was produced using traditional fusion technology by fusing the PBMCs of a HIV-1 asymptomatic seropositive donor with heteromyeloma cells and screening against gp41 [41]. This antibody and has been shown to recognize a conserved linear epitope (72% of variable isolates) in the MPER region (aa 662-668, ELDKWAS) of gp41 and neutralize a variety of clinical and lab adapted strains of HIV [41]. The broadly neutralizing antibody 4E10 was produced in much the same way as mAb 2F5 and binds an adjacent linear epitope in the MPER (aa 671-681) NWFNITNWLW) [61]. mAb Z13 was selected from a random combinatorial phage display library from an asymptomatic seropositive donor and binds to a

linear epitope in between the 4E10 and 2F5 epitopes on the MPER (aa 669-679, SLWNWFDITN) [61, 138]. To determine how these three bNt mAbs mediate virus neutralization, Song et al (2009) used electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) to examine the conformational changes in the MPER following antibody binding [138]. It was discovered that all three mAbs inhibit MPER related functions in the virus-cell fusion process via different mechanisms [138]. 2F5 binding to gp41 caused a drastic conformational shift in the N terminal hinge region of the MPER exposing the amino acid residues 669 and 670 [138]. 4E10 binding also mediated a conformational shift in the MPER exposing amino acids in the center of the hinge region, 672 and 673 [138]. MAb Z13 does not initiate a conformational shift, but rather rigidifies the MPER preventing the structural rearrangement needed during the fusion process [138].

1.11 Summary/ Statement of Objectives

With the exception of the mAb recently generated by Tudor et al (2009), all of the bNt mAbs against HIV-1have been isolated from peripheral blood or bone marrow derived B cells from HIV-1 seropositive individuals, predominantly infected with clade B virus. To develop antibodies with potency against non-clade B viruses, donors from different geographical regions must be used. The development of the African derived gp120 bNT mAbS PG9 and PG16 validates this approach. To ultimately protect against HIV-1 infection, the virus must be neutralized at the site of initial contact; however, the antibodies that play the most important role at this

site in women, cervicovaginal IgA, has been largely ignored. Furthermore, the majority of the mAbs herein discussed have been developed using comparatively old technology with inherent limitations.

The role played by IgA in resistance to HIV-1 infection is highly controversial. Traditional techniques to measure HIV-1 specific cervical IgA in HEPS individuals have been wildly inconsistent. The isolation of gp41-specific neutralizing monoclonal IgAs from a HEPS cohort in Cambodia has confirmed the presence of HIV-1 neutralizing IgA in the cervix of women from this cohort. In order to elucidate the role played by cervical IgA in the Nairobi cohort, in-depth studies of HIV-1 - specific monoclonal IgAs must be undertaken.

This project was initiated to develop novel HIV-1- specific IgA mAbs from the cervix of Kenyan HEPS individuals using methods that avoid the biases and limitations inherent in other antibody production methods. **We hypothesize that using a recombinant approach, coupled with a mammalian cell tissue culture system, we will be able to retrieve gp41 specific antibodies from the IgA repertoire of cervical mononuclear cells (CMCs) obtained from Kenyan HEPS commercial sex workers.** This work describes the development a mammalian cell display antibody expression and screening system and the role it played in the isolation and characterization of 4 novel gp41 specific monoclonal antibodies derived from the cervical B cell IgA repertoire of two HEPS commercial sex workers in Nairobi, Kenya.

2 Materials and Methods

2.1 Antibody Variable (V) Region Gene Recovery From PBMC and CMC Samples

2.1.1 Study Population

A cohort of commercial sex workers was established through a clinic in the Pumwani area of Nairobi Kenya [73]. Even though the women are encouraged to used condoms and provided with behavioral counseling, it is estimated they have at least 64 unprotected sexual exposures to the HIV-1 virus every year [73]. Initially seronegative women who remain HIV-1 seronegative and PCR-negative for at least 3 years while continuing sex work are classified as HIV-1 resistant [73].

2.1.2 Lymphocyte Collection

For the construction of the cervical mononuclear cell (CMC) antibody library, CMCs from two female commercial sex workers in Nairobi, Kenya were collected. ML 1515 and ML 1356 were selected as lymphocyte donors based on their status as being HIV-1 seronegative despite being highly exposed to the HIV-1 virus through commercial sex. ML 1356 and ML 1515 have continued to test HIV-1 negative since testing began in 1991, and 1992 respectively. CMCs were harvested from the cervix in January 2007 as part of a routine survey using a clinical cytobrush. Samples consisted of on average 1000 to 100 000 lymphocytes (median 10 000).

As proof of principle, a population of B cells known to contain clones specific for HIV-1 was harvested. For construction of this separate antibody library,

peripheral blood mononuclear cells (PBMCs) were harvested from an HIV-1 infected sex worker by Ficoll-Hypaque gradient centrifugation. ML 256, HIV-1 positive since 1985 was selected as a lymphocyte donor based on the high serum titer to both gp120 and gp41.

All samples were washed and re-suspended in buffer RLT (Qiagen) containing 2-mercaptoethanol and stored in liquid Nitrogen. Samples were shipped to Winnipeg using dry ice and subsequently stored at -80°C until ready for use.

2.1.3 RNA Isolation

Total RNA was recovered from each CMC and PBMC cell population using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA was quantified using a ND-1000 Spectrophotometer (NanoDrop®). Typical yields of RNA were 500 to 700 ng for CMC samples and 1500 to 2000 ng for PBMC samples.

2.1.4 cDNA synthesis

cDNA was synthesized from each RNA sample using the Thermoscript RT-PCR system (Invitrogen) with an oligo dT primer according to manufacturer's instructions.

2.1.5 PCR Strategy and Primer Design

Due to the prevalence and importance of IgA in the cervical mucosa, immunoglobulin variable heavy (VH) of the IgA isotype (α) were amplified from the CMC cDNA, while VH regions of the IgG isotype (γ) were amplified from the PBMC

cell population. Immunoglobulin variable light chain genes (VL) Kappa (κ) and Lambda (λ) were amplified from both the CMC and PBMC samples. All PCR reactions were carried out using Expand High Fidelity PCR System (Roche) using 2 μl of cDNA as a template. Immunoglobulin light and heavy chain variable regions encoding each V-domain pool were amplified using panels of sense oligonucleotide primers that hybridize with cDNA in the framework 1 region (Fr-1), (corresponding to the N-terminal amino acid region), of the different antibody families. These were paired with isotype specific anti-sense primers that hybridize with the N terminal amino acid region of constant region 1 (CH1) of either: IgG1-3 (in the case of PBMC cDNA); IgA1,2 (in the case of CMC cDNA as template); IgKappa, or IgLambda. All primers were synthesized by the DNA core Facility (NML). Primers were designed based largely upon sequences available to the public and as in Berry et al (2003) [105].

2.1.6 PCR amplification of IgA and IgG VH regions

Immunoglobulin VH regions 1-6 were independently amplified for both IgA subtypes 1 and 2 from the cDNA CMC template, as well as the three IgG subtypes 1,2a, and 3 from the PBMC cDNA template. Framework 1 gene specific sense primers Fr-1 VH1,3, Fr-1 VH2, Fr-1 VH4, Fr-1 VH4b, Fr-1 VH5, and Fr-1 VH6 were paired with isotype specific antisense primers IgG1 Reverse, IgG2 Reverse, IgG3 Reverse, IgA1,2 Reverse, and IgA1 Reverse (see appendix 1 for all primer sequences).

All PCR reactions were performed in thin walled PCR tubes (FisherBrand) in a total volume of 50 μl containing 600 nM sense and anti-sense primers, 400 μM

each dNTP (Roche), 37μl nuclease free water (Promega), 5 μl 10X Expand High Fidelity Buffer with 15 mM $MgCl_2$ (Roche), and 3.5 units of Expand High Fidelity Enzyme Mix (Roche). Thermal cycling was performed on a GeneAmp PCR system 9700 Thermocycler (Applied Biosystems) consisting of 1 cycle at 94°C for 5 min, followed by 10 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec; then by 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec* (15 sec increase in incubation time with every cycle), and 1 cycle of 72°C for 7 min.

2.1.7 PCR Amplification of Kappa and Lambda VL regions

Amplification of Kappa VL domains 1-5 was done individually using Fr-1 specific sense primers IgK-V1, IgK-V2,4, IgK-V3, and IgK-V5, paired with the corresponding antisense primer specific for the C terminal amino acid region of Fr-4, VJKrev1, VJKrev2,4, VJKrev3, or VJKrev5.

Similarly, amplification of Lambda VL domains 1-10 was done using individual Fr-1 specific sense primers IgLam-V1a, IgLam-V1b, IgLam-V2, IgLam-V3, IgLam-V4, IgLam-V6, IgLam-V7,8, IgLam-V9, and IgLam-V10. The Fr-1 specific sense primers were each paired with a pool of 4 Lambda Fr-4 specific anti-sense primers (200 nM each): VJLamRev1236, VJLamRev4, and VJLamRev5,7. PCR was amplification was done as per section 2.1.6.

2.1.8 Analysis of PCR

A commercial 6X blue/orange loading dye (Promega) was diluted 1:6 in 20 μl of PCR sample and each dispensed into a 100 ml 1% agarose gel in 1X TAE buffer

(Bio-Rad) containing 40 μg ethidium bromide (Invitrogen). 2 μl of the blue/orange loading dye was added to 10 μl of Low Mass DNA Ladder (Invitrogen), and subsequently dispensed into the first well. Samples were run from negative to positive for 1 hour at 100 V using a Bio-Rad PowerPac HC. The gel was visualized under UV light using a Gel Doc EQ (Bio-Rad) and analyzed using Quantity One 1D analysis software 4.6 (Bio-Rad).

2.1.9 PCR fragment Isolation

Immunoglobulin VH and VL DNA of approximately 400 to 700 base pairs were excised from the agarose gel using a sterile scalpel (Feather) and purified using a Qiaquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions.

2.1.10 Extension PCR of VH-γ,α and VL-κ,λ.

A second round of PCR was carried out to add restriction endonuclease sites to the 5′ and 3′ ends of each VH and VL family to facilitate cloning into expression vectors using primers complementary to the termini of the cDNA containing embedded restriction sites. An EcoRV site was added to the 5′ end of both the VH and VL regions, while an Apa1 site was added to the 3′ end of the VH regions and an NcoI site was added to 3′ ends of the VL regions.

As template, 10 to 20 ng of each VH region family was amplified using one of the following sense VH extension primers of appropriate familial specificity: VH1236EXT; VH4bEXT; or VH5ext. Each VH family specific sense primer was paired

with either IgA1,2bpEXT, or IgGbpEXT depending on the source and isotype of the V region being amplified. An EcoRV recognition sequence (GATATC) was encoded approximately 15 bases from the 5′ end of each sense oligonucleotide extension primer, while an ApaI recognition sequence (GGGCCC) was encoded approximately 15 bases from the 3′ end of each anti-sense oligonucleotide extension primer.

Ten to twenty micrograms of Kappa V regions families 1-5 were amplified using KappaFext sense oligonucleotide primer with an EcoRV site approximately 15 bases from the 5′ end, paired with one of the following anti-sense oligonucleotide primers of appropriate familial specificity: VJKRev-1X; VJkRev-2,4X; VJKRev-3X; or VJKRev-5X. Each antisense primer had an NcoI recognition sequence (CCATGG) approximately 10 bases from the 5′ end. Lambda V region families 1-10 were amplified using a pool of two sense oligonucleotide primers (LambdaFext1, and LambdaFext2, 300 nM each) with an EcoRV recognition sequence 15 bases from the 5′ end. The pool of Lambda sense extension primers was paired with a pool of 3 Lambda anti-sense extension primers (VJLRev1236X, VJLRev4X and VJLRev5,7X, 200 nM each) with an NcoI recognition sequence 10 bases from the 5′ end of each primer.

All PCR reactions were performed in thin walled PCR tubes (FisherBrand) in a total volume of 50 μl per well containing 600 nM sense and anti-sense primers, 400 μM each dNTP (Roche), 37μl nuclease free water (Promega), 5 μl 10X Expand High Fidelity Buffer with 15 mM $MgCl_2$ (Roche), and 3.5 units of Expand High Fidelity Enzyme Mix (Roche). Thermal cycling was performed on a GeneAmp PCR system 9700 Thermocycler (Applied Biosystems) consisting of 1 cycle at 94°C for 5

min, 20 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 45 sec, and 1 cycle at 72°C for 7 min. PCR fragments were visualized, extracted, purified and quantified as per sections 2.1.8 – 2.1.9.

2.2 Expression Vector Manipulation

2.2.1 P-dbIgG1 Mutagenesis

To facilitate site specific ApaI mediated cloning into the P-dbIgG1 expression vector (Figure 5, Appendix II), a second ApaI endonuclease recognition sequence (GGGCCC) was removed from the zeocin resistance gene of the vector using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Due to the location of the recognition sequence, three different mutagenesis reactions were performed using the following primer sets, each resulting in three different single base mutations: pIgGmut1F and pIgGmutR (resulting in a GCGCCC); pIgGmut2F, and pIgGmut2R (resulting in GGGCGC); and pIgGmut3F and pIgGmut3R (resulting in GGGCCG).

Next, 2 μl of each P-dbIgG1 mutation were added to chemically competent *E. coli* OneShot ® Top10 cells (Invitrogen) and incubated for 20 min on ice. Cells were heat shocked for 30 sec at 42°C, and incubated for 1 hr at 37°C in 250 μl S.O.C. media (Invitrogen) with shaking at 220 rpm. 40 μl of each transformation was plated on an LB agar plate with 25 μg/ml zeocin (Invitrogen). The mutation with the least effect on the zeocin resistance gene of the plasmid corresponded with the plate showing the highest level of growth. Next, 10 colonies from mutation number 2

were transferred to 3 ml of LB-Lennox media containing 25 μg/ml of zeocin and cultured overnight at 37°C with shaking at 250 rpm. Plasmids were purified using a Qiagen Plasmid Miniprep Kit according to manufacturer's instructions.

2.2.2 P-dbLambda Generation

To ensure optimal expression of VL-Lambda genes, the cDNA encoding the human CH1 Lambda region was used to replace the CH1 Kappa region in the expression vector P-dbKappa (Figure 5). To obtain the Lambda CH1 region, PCR was done using the Expand High Fidelity PCR system as per section 2.1.6 with 2 μl of cDNA from the PBMC sample of ML 256 as the template along with LamConF, and LamConR as the sense and anti-sense primers respectively. A PCR fragment of approximately 350 bp was excised from a 1% agarose gel and purified as per sections 2.1.8-2.1.9.

To permit TA cloning, adenine nucleotides were added to the 5′ and 3′ ends of the PCR fragment. 1 μl of 100 mM dATP (Invitrogen) was added to 100 ng of Lambda-CH1 in a 14 μl reaction volume containing 1 U of *Taq* DNA polymerase (Invitrogen), and 2 μl of 10 X PCR Buffer (Invitrogen). The reaction was incubated for 30 min at 72°C.

For insertion into PCR®2.1 cloning vector (Invitrogen), 4 μl of the A – tailed PCR product was added to 1 μl of PCR®2.1 vector and 1 μl of salt solution (1.2 M NaCl, 0.6M $MgCl_2$) (Invitrogen), and incubated for 5 min at room temperature. 2 μl was then transformed into chemically competent OneShot ® TOP10 *E.coli* cells (Invitrogen) as per section 2.2.1. Cells were plated on LB-agar plates containing 100

μg/ml ampicillin, previously coated with 40 μl of 40 mg/ml X-gal in dimethylformamide (Invitrogen), and incubated overnight at 37°C. 10 white colonies were selected, expanded in 3 ml of LB broth containing 100 μg/ml ampicillin overnight, and processed in a similar fashion as 2.2.1. The ND-1000 Spectrophotometer was used to quantify the DNA and 10 μl of each sample (150 ng/μl) was sent to the Genomics Core Facility within the NML for sequencing along with M13 Reverse (5′CAGGAAACAGCTATGC 3′) and T7 (5′TAATACGACTCACTATAGGG 3′) oligonucleotide primers (Invitrogen). Data was analyzed using DNAStar software.

Extension PCR was done using a single PCR®2.1 vector clone containing the lambda CH1 insert as a template with the Expand High Fidelity PCR System (Roche) as per section 2.1.10. Oligonucleotide primers LamConFext, and LamConRext were used to add an NcoI recognition sequence on the 5′ end and an NheI recognition sequence on the 3′ end of Lambda CH1. The human Lambda CH1 extension PCR product was inserted in the place of the human Kappa CH1 in P-dbKappa following a similar protocol as section and 2.4.

2.3 Restriction Endonuclease Digests

2.3.1 Restriction Endonuclease Digest of VH- α,γ and P-dbIgG1

All VH extension PCR products purified per section 2.1.10 were double digested with EcoRV, and ApaI (New England BioLabs) for cloning into P-dbIgG1 expression vector (Figure 5). Each digest was done in a total volume of 40 μl

containing approximately 500 ng of each VH family, 4 μl of 10X Buffer 4 (New England BioLabs), 4 μl of 10 mg/ml BSA (New England BioLabs), and 50 Units of ApaI restriction enzyme. Each reaction was incubated for 2 hr at 25°C, after which, 40 Units of EcoRV restriction enzyme was added and incubated for an additional 2 hours at 37°C. Next, 5 μg of P-dbIgG1 was digested in a 40 μl total reaction volume under the same conditions. Following digestion of the vectors, 1U of Calf Intestinal Alkaline Phosphatase (Invitrogen) was added to the P-dbIgG1 digestion and incubated for 20 min at 50°C to reduce re-ligation of the vector. Next, 5 μl of blue/orange loading dye was added to each digest and loaded onto a 2% agarose gel as per section 2.1.8. Samples were run for 1.5 hr at 100 V and VH bands of approximately 400 basepairs, and linearized P-dbIgG1 of approximately 4500 basepairs, were excised and purified as per sections 2.1.8-2.1.9.

2.3.2 Restriction Endonuclease Digest of VL-κ,λ, P-dbKappa, and P-dbLambda

All VL-κ,λ extension PCR products purified per section 2.1.10 were double digested with EcoRV, and NcoI (New England BioLabs) for cloning into P-dbKappa and P-dbLambda expression vectors. Each digest was done in a total volume of 40 μl containing approximately 500 ng of each VL family, 4 μl of 10X Buffer 3 (New England BioLabs), 4 μl of 10 mg/ml BSA, 40 Units of EcoRV and 50 Units of NcoI. Each reaction was incubated for 4 hr at 37°C. 5 μg of P-dbKappa and 5 μg of P-dbLambda were digested in a 40 μl total reaction volume under the same conditions. Following digestion of the vectors, 1 U of Calf Intestinal Alkaline

Phosphatase was added and incubated for 20 min at 50°C. Samples were subsequently processed in a similar fashion as 2.3.2.

2.4 Cloning B12 Fab from pComb3X into P-dbIgG1 and P-dbKappa

The phagemid cloning vector pComb3X containing the VH and VL sequence of the gp120 specific antibody B12 was generously donated by Dr. Carlos Barbas III (Scripps Research Institute). The VH and VL regions were amplified by extension PCR as per section 2.1.10 using 10 ng pComb3X as the template with either B12VHFext and IgGbpExt (VH) or 4LFext and 4LRext (VL) as sense and anti-sense primers. Amplified VH and VL regions were purified, digested, and purified a second time as outlined in sections 2.1.7, 2.1.8, and 2.3. Next, 30 ng of digested B12 VH and VL were ligated with linearized Pdb-IgG1 and P-dbKappa in a 10 μl reaction volume containing 2 μl of 5X T4 DNA Ligase Buffer (Invitrogen), and 1 unit of T4 DNA ligase (Invitrogen). Ligations were incubated for 16 hr at 14°C.

Both P-dbIgG1B12, and P-dbKappaB12 ligations were transformed into chemically competent TOP10® *E.coli* cells (Invitrogen) as per section 2.2.1. Cells were plated on LB-amp plates (100 μg/ml) and 10 colonies were selected, expanded, and plasmids isolated following a similar protocol as section 2.2.1. The resulting clones were sequenced using flanking primers B12VHFext and IgG1reverse for P-dbIgG1B12; or 4LFext and 4LRext for P-dbKappaB12 to confirm the presence and orientation of each insert. Clones with the correct insert were expanded using a Qiagen Maxi Prep Kit according to manufacturer's instructions.

2.5 Antibody Secretion System: Generation of ML 256 PBMC IgG Library

2.5.1 Ligation of VH-γ with P-dbIgG1

VH-γ regions digested per section 2.3.1 were pooled and ligated into P-dbIgG1 creating a plasmid library encoding a population of VH genes originating from the PBMCs of the HIV-1 positive sex worker ML 256. The 14 cDNA VH-γ regions that were amplified by two rounds of PCR and subsequently digested by restriction endonucleases, were pooled into seven groups, each consisting of 3.5-4 μg of total DNA. Next, 200 ng of DNA from each pool was ligated with 200 ng of linearized P-dbIgG1 in a 15 μl total volume containing 3 μl 5X T4 DNA Ligase Buffer and 1 Unit of T4 DNA Ligase. Each ligation was incubated separately for 24 hr at 14°C then pooled forming one total ligation. Plasmid DNA was precipitated from the ligation by adding 2.2 volumes 96-100% ethanol (Commercial Alcohols), 0.1 volumes 3M sodium acetate pH 5.5 (Ambion), and 40 μg of glycogen (Roche). Following a 2 hr incubation at -80°C, the ligation was centrifuged for 15 min at 13000 g (4°C) and supernatant discarded. The pellet was washed twice by re-suspension in 500 μl ice cold 70% ethanol followed by gentle agitation and prompt centrifugation for 5 min at 13000 g (4°C). The pellet was air dried for 5 min and re-suspended in 10 μl of nuclease free water (Promega).

2.5.2 Ligation of VL-κ,λ into P-dbKappa and P-dbLambda

The cDNA amplified from four VL-κ families from PBMC sample of ML 256 digested per section 2.3.2 were pooled into 2 groups, while the 9 VL-λ families were pooled into 4 groups. 200 ng of DNA from each group were ligated with 200 ng of either P-dbKappa or P-dbLambda and precipitated as per section 2.5.1.

2.5.3 Plasmid Library Amplification

For amplification, 2 μl from the ML 256 V region ligations into Pdb-IgG1, PdbKappa, and Pdb-Lambda, were each added to 2 vials (6 vials total) of One Shot Electrocompetent TOP10® *E.coli* cells (Invitrogen). Cells were transformed according to manufacturer's instructions with an *E. coli* Gene Pulser® Cuvette (Bio-Rad). Following incubation for 1 hr at 37°C with shaking at 220 rpm in S.O.C media (Invitrogen), 1 μl from each library was plated using a cell spreader on LB plates containing 25 μg/ml of zeocin to assess the diversity of the library. The remaining 250 μl of each transformation was added to 110 ml LB-Lennox broth with 25 μg/ml zeocin in 400 ml Erlenmeyer flasks. Cells were incubated for 18 hr at 37°C with shaking at 250 rpm. Plasmids were isolated with a MaxiPrep Kit (Qiagen) according to manufacturer's instructions.

2.5.4 Determining the Diversity of the ML 256 VH Library

To examine diversity, 15 clones from heavy chain ligation plated in section 2.5.3 were selected and expanded as per section 2.2.1. Plasmids were sequenced using the following set of flanking oligonucleotide sense and anti-sense primers:

SeqF1 and IgG1reverse. Sequence data was analyzed using both DNAstar, and MacVector software.

A restriction digest profile was done on 15 VH clones by digesting 100 μg of each VH region in a total volume of 40 μl with 1 U of AluI restriction enzyme(New England BioLabs) and 4 μl of buffer 4 (New England BioLabs) for 2 hr at 37°C. Digest patterns were visualized on a 1% agarose gel as per section 2.1.8.

2.5.5 Growth and Maintenance of HEK 293F Cells

HEK 293F cells (Invitrogen) were grown in sterile, polycarbonate Erlenmeyer Flasks (VWR) using FreeStyle™ 293 Expression Medium (Gibco) to a density of 2-3 x 10^6 cells/ml at 37°C with 8% CO_2 and shaking at 135 rpm. Cells were passaged every 3 to 4 days to 3 x 10^5 cells/ml. Early passages were frozen in FreeStyle™ 293 Expression Medium with 10% DMSO (Sigma) and stored at -150°C.

2.5.6 Transfection of 293F cells with the ML256 PBMC VH and VL Library

The day of transfection, cells were diluted to 1.1 x 10^6 cells/ml in 28 ml of FreeStyle™ 293 Expression Medium. 80 μl of 293fectin™ (Invitrogen) was diluted in 1 ml of Opti-Mem® media (Invitrogen) and incubated at room temperature for 5 min. 20 μg of the VH P-dbIgG1 library, and 20 ug of VL P-dbKappa/Lambda library diluted in 1ml of Opti-Mem® media was added to the activated 293fectin™ – Opti-Mem® media mixture and incubated for 25 min at room temperature. Immediately following the incubation, The DNA-293fectin™ complexes were added to the previously prepared 293F cells. As a positive control for both expression and

affinity, P-dbIgG1B12 and P-dbKappaB12 were co-transfected into HEK 293F cells following the same protocol.

2.5.7 Re-suspension of the Transfectants into Semi-Solid Media

The day prior to re-suspension, CloneMedia-HEK (Genetix) was allowed to thaw at 4°C. The day of re-suspension, 10 ml of sterile H_2O containing zeocin was added to the media creating a final zeocin concentration of 50 μg/ml. 48 hours post transfection, 50 000 cell aliquots of cell suspension were added to 2 ml of the prepared, semi-solid, CloneMedia-HEK that had been aliquoted into 5 ml Falcon tubes. Following thorough mixing, the suspensions were plated in 6 well tissue culture plates (Genetix) and incubated in humidified air at 37°C with 8% CO_2.

After incubating for 14 to 17 days, colonies could be visually identified and transferred to 96 well tissue culture plates (Corning) containing 200 μl of FreeStyle™ 293 Expression Medium with 50 ug/ml of zeocin. Cells were incubated for another 14 days prior to screening. Individual colonies were considered "clonal" for purposes of experimentation.

2.5.8 Screening Clones for Expression and Reactivity Against gp41 and gp120 Using ELISA

Fourteen to seventeen days following colony picking, culture supernatants from each clone were tested for antibody expression against 100 ng of goat anti-human IgG F(ab')$_2$ fragment specific (Southern Biotech). Antibody specificity was tested in ELISA against 100 ng of recombinant gp120-gp41 mosaic antigen

(Virogen). This antigen consisted of the immunodominant regions of both gp120 and gp41 Env proteins. Antigen was coated overnight at 4°C in 60 μl of Phosphate Buffered Saline (PBS) pH 7.2 onto individual wells of a MaxiSorp 96 well plate (NUNC™). Plates were blocked at 37°C for 2 hr with 0.2% Bovine Serum Albumin (BSA) (Sigma), 0.2% Skim Milk (Promega) in PBS. Plates were washed twice with MilliQ® water (resistivity 18.2MΩ•cm) using a Power Washer 384 (Tecan) automatic washing device. Next, 60 μl of culture supernatant was added and incubated at 37°C for 2 hr, then washed 8X with MilliQ® water as stated previously. Supernatant from cells transfected with B12IgG1/κ was included as a positive control. Next, 60 μl of a 1:2000 dilution of goat anti-human IgG Fc fragment specific polyclonal antibody conjugated to horseradish peroxidase (Southern Biotech) in PBS was added and incubated for 1 hr at 37°C. After 8 washes with water, 60 μl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrate (Roche) was added and incubated for 45 min at room temperature. Colour development was measured at 405 nm by use of a SpectroMax250 Spectrometer (Molecular Devices) and analyzed using SoftMax Pro 4.6 Software (Molecular Devices).

2.5.9 V Gene Recovery from Positive Mammalian Cells

To identify the optimum method of V gene recovery from positive wells of transiently transfected cells, VH and VL genes were isolated using two techniques from two different clones (2G7 and 5B5) that had absorbance values against the gp41-gp120 mosaic antigen of 0.1 or greater in the ELISA. The first strategy was to isolate the RNA from the clone and use RT-PCR to amplify the V regions, while the

second strategy was to amplify the V regions directly from the plasmid DNA of the clone.

To test the first strategy, total RNA was harvested from clone 5B5 using an RNEasy Mini Kit (Qiagen) as per section 2.1.3, clonal mRNA was converted into cDNA using Thermoscript RT-PCR system (Invitrogen) as per section 2.1.4 and the VH and VL regions amplified using the Expand High Fidelity PCR system as per section 2.1.6 using with the following primer sets:

IL2ssVrecovF (sense), GcVrecovR (antisense)

IL2ssVrecovF (sense), KcVrecovR (antisense)

IL2ssVrecovF (sense), LcVrecovR (antisense)

To test the second strategy, clone 2G7 was transferred to 50 μl H_2O, incubated for 10 min at 99.9 °C in a thermocycler, vortexed vigorously, and centrifuged for 15 sec at 10 000 g. 10 μL of supernatant was used directly as template in the PCR reaction outlined above.

Amplified V genes were re-cloned into expression vectors P-dbIgG1, P-dbKappa or P-dbLambda following a similar protocol outlined previously. Plasmids were transformed into Top10 ® *E. coli* cells and 24 colonies from both 5B5 and 2G7 comprising the heavy chain (gamma), light chain (kappa) and light chain (lambda) were selected, expanded, and sequenced as per section 2.5.4.

2.5.10 Screening via Chain Shuffling

One productive VH immunoglobulin sequence was recovered from clone 2G7, while 2 productive VH and 2 productive VL-lambda sequences were recovered

from clone 5B5. Clearly, 5B5 was not clonal. Consequently, to deduce the binding of mAb 5B5, a total of 9 transfections were carried out pairing the 3 productive VH sequences in P-dbIgG1 with each of the 2 VL sequences in P-dbLambda. Transfections were carried out using 293fectin™ in 6 well tissue culture plates (Corning) in a 6 ml total volume following a scaled down version of the protocol outlined in section 2.5.6. Supernatants were screened via ELISA against 100 ng of Goat anti-Human IgG F(ab')$_2$ fragment specific, 100 ng of recombinant, monomericgp120 (Immunodiagnostics) and 100 ng of recombinant, monomeric gp41 (Virogen) following a similar protocol outlined in section 2.5.9.

2.6 Cellular Display System: the Generation of a HIV-1 Resistant Cervical IgA Library

Due to several inefficiencies observed in the antibody secretion method outlined above, a cellular display system was developed to create the IgA CMC library from the HEPS sex workers ML 1356 and ML 1515 (Figure 3).

2.6.1 Creation of P-dbIgG1-TM

To allow for antibody expression on the cell surface, the human platelet derived growth factor receptor (PDGFR) trans-membrane (TM) domain was cloned onto the 3′end of the CH3 region of P-dbIgG1 (Figure 9). The PDGFR TM domain was amplified by PCR from the expression vector pDisplay (Invitrogen) using the Expand High Fidelity PCR system as per section 2.1.10. Oligonucleotide primers PDGFR-rev and Link PCR4-g were used as the sense and anti-sense primers

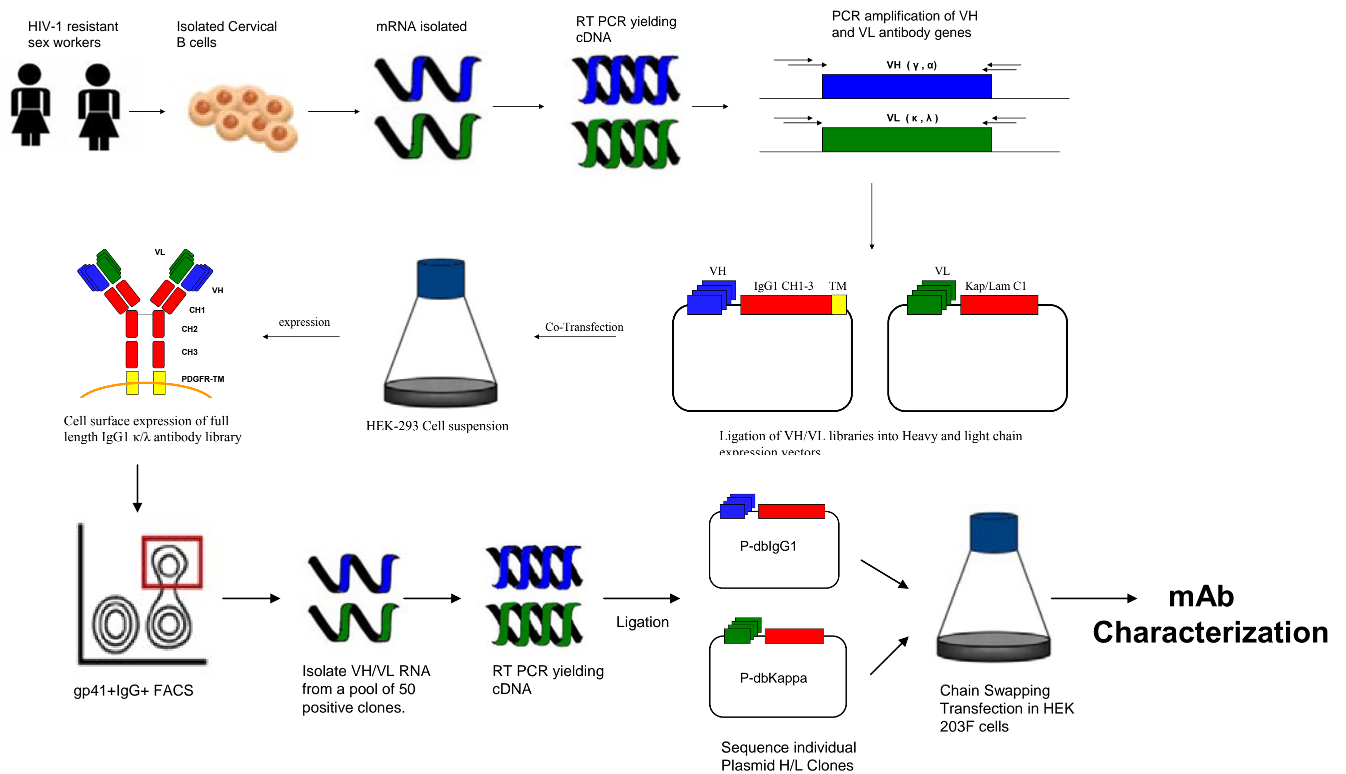

Figure 3. Strategy for the Isolation of gp41 specific mAbs using a mammalian Cell Display system. VH and VL genes were amplified from the cervical B cells from two HIV resistant sex workers from Nairobi, Kenya and cloned into Heavy and Light chain expression vectors. Co-transfection into HEK 203F cells yielded a pool of cells expressing full-length IgG1/Kappa/Lambda antibodies encoding the V regions from the IgA expressing cervical B cells. FACS was done to isolate cells expressing surface IgG that had affinity for gp41. VH and VL genes were recovered from a pool of 50 positive cells and cloned into heavy and light chain expression vectors. The TM region was removed from the heavy chain vector to allow for antibody secretion. 96 clones from both the heavy and light chain ligations were sequenced, and productive heavy and light chain combinations were co-transfected into HEK293F cells. Secreted antibody was tested for expression, cross-reactivity and affinity for gp41.

respectively to add an NheI recognition sequence to the 3′of PDGFR-TM, and the reverse complement of the 3′ terminus of IgG1-CH3 to the 5′ end of the PDGFR-TM. This resulted in the display of VH IgA libraries on IgG1 constant regions.

The IgG1 constant region CH1-3 was amplified off of P-dbIgG1 using the Expand High Fidelity PCR system with IgGconF and Link PCR3-g as the sense and anti-sense oligonucleotide primers respectively. This resulted in the addition of a portion of the 5′ end of the PDGFR-TM domain onto the 3′ end of the CH3 region. Both PCR products IgG1 CH1-3, and PDGFR-TM were isolated by gel electrophoresis as per section 2.1.8, 2.1.9.

To link IgG1 CH1-3 with PDGFR-TM overlap PCR was performed using the Expand High Fidelity PCR system. Fifty micromolar of both PCR products were included in the 50 μl reaction volume as template DNA along with 600 nM of IgGconF and PDGFR-rev as the sense and anti-sense oligonucleotide primers. The reaction was cycled as follows: 94°C for 5 min, 7 cycles at 94°C for 30 sec, 50°C for 45 sec, and 72°C for 2 min, 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min* (15 sec increase in incubation time with every cycle), and one final cycle of 72°C for 7 min. PCR fragments were analyzed by gel electrophoresis as per section 2.1.8 and a band corresponding to approximately 1200 bp (CH1-3 [1050bp] + PDGFR-TM [150 bp]) was excised and purified using a Qiagen Gel Extraction Kit.

The overlap PCR product, as well as the expression vector P-dbIgG1, were double digested using the restriction enzymes ApaI and NheI in NEB buffer 4 and purified using gel electrophoresis following a similar protocol as section 2.3.1. The digested IgG1 CH1-3 – PDGFR-TM PCR product was ligated with the linearized P-

dbIgG1 lacking regions CH1-3 using T4 DNA Ligase following a similar protocol outlined in section 2.4. The resulting ligation was transformed into chemically competent TOP10® *E. coli* cells, plated on LB plates with zeocin (25 μg/ml), 10 clones were selected, amplified, and sequenced with flanking primers PDGFR-rev, and SeqF1 as per section 2.4 to verify the presence and orientation of the insert.

2.6.2 Creation of P-dbIgG1-B12-TM for Positive Control

The B12 VH region was cloned from P-dbIgG1B12 into P-dbIgG1-TM following the protocol outlined in section 2.4.

2.6.3 Ligation of Resistant Cervical (CMC-R) VH-α Genes into P-dbIgG1-TM

VH-α regions amplified by extension PCR, and digested per section 2.3.1 were pooled and ligated into P-dbIgG1-TM creating a plasmid library encoding a population of VH genes from the cervical B cell IgA repertoire of two Kenyan HIV-1 resistant sex workers: ML 1356; and ML 1515. The digested V regions were pooled into 7 groups, ligated into P-dbIgG1-TM and precipitated as per section 2.5.1. Ligations were transformed into electrocompetent *E.coli* TOP10® cells, and isolated using a Qiagen MaxiPrep Kit as per section 2.5.3. Individual Maxipreps were pooled forming one P-dbIgG1-TM library.

2.6.4 Ligation of CMC-R VL-κ,λ into P-dbKappa and P-dbLambda

VL-κ,λ genes from the CMC-R sample were amplified by two rounds of PCR and digested by restriction endonucleases per section 2.3.2 were pooled and ligated

into P-dbKappa or P-dbLambda, then precipitated as per section 2.5.2. Ligations were transformed into electrocompetent *E.coli* TOP10® cells, and isolated using a Qiagen MaxiPrep Kit as per section 2.5.3. Individual Maxipreps were pooled together forming one P-dbKappa and one P-dbLambda library.

2.6.5 Determining the Diversity of the CMC-R Library

Twenty five clones from the P-dbIgG1-TM encoded VH-α library and 10 clones each from the CMC-R P-dbKappa and P-dbLambda VL-κ,λ libraries were selected, expanded, sequenced and analyzed for diversity as per section 2.5.4. DNAstar software and a ClustalW alignment algorithm was used to generate a neighbor joining guide tree for the 21 productive heavy chain sequences isolated.

2.7 Assessment of IgG1/κ,λ Surface Expression on 293F cells

2.7.1 Transfection

Either P-dbIgG1B12-TM and P-dbKappaB12, or PdbIgG1-TM, PdbKappa and PdbLambda encoding the cervical VH-α / VLλ,κ library from ML1515 and 1356 were transfected into HEK 293F cells as per section 2.5.6. As a negative control, 40 ng of P-dbKappa-SVK18 (an irrelevant a light chain sequence) was transfected following the same procedure.

2.7.2 Direct Immunofluorescent Cell Staining

At three different time points following the transfection, cells were counted using a hemacytometer (Reichert) and the viability determined using trypan blue

exclusion (Gibco). Cells were then harvested from the transfections via centrifugation for 5 min at 800 g and re-suspended to a concentration of 2 x 10^7 cells/ml in 2% BSA, 0.1% Sodium Azide (Sigma) (FACS Buffer). Cells were incubated on ice for 10 min and 50 μl aliquots from each transfection (1 x 10^6 cells) were subsequently dispensed into 1.5 ml Eppendorf tubes. One of the following labeled primary reagents was added to each tube and incubated on ice for 50 min with frequent vortexing: 1 μg FITC labeled gp120 (ImmunoDiagnostics); 0.5 μg PE labeled goat anti-human IgG Fc (Southern Biotech); 0.5 μg FITC labeled goat anti-human Kappa (Southern Biotech); and 0.5 μg FITC labeled goat anti-human Lambda (Southern Biotech). Cells were washed three times with 800 μl of FACS buffer and re-suspended in 300 μl of FACS buffer for analysis.

2.7.3 Flow Cytometry Analysis

Cells were stored on ice in the dark for up to an hour prior to analysis. Cells were analyzed using a MoFlo XDP Cell Sorter with the help of Monroe Chan, University of Manitoba, Faculity of Medicine. The percentage of gated cells with fluorescence levels in the positive (R2) region when compared with the negative control was determined for each population.

2.8 Fluorescence–Activated Cell Sorting (FACS)

2.8.1 Transfection

The plasmids encoding the P-dbIgG1-TM VH-α library along with the plasmids encoding the P-dbKappa and P-dbLambda VL-κ,λ libraries were co-transfected into HEK 293F cells as per section 2.7.3. The transfection was incubated for 72 hr at 37°C with 8% CO_2 and shaken at 135 rpm.

2.8.2 Indirect Multi-Colour Immunofluorescent Cell Staining

Following the incubation, cells were harvested, counted, blocked, and re-suspended in FACS buffer at a concentration of 2×10^7 cells/ml as per section 2.7.2. A total of 15 million cells was added in 100 μl aliquots (2 million cells) to eppendorf tubes containing 2 μg of biotin-labeled gp41 (Capital Biosciences) diluted in 5 μl of FACS buffer. Samples were incubated on ice for 1 hr with frequent vortexing and washed twice with FACS buffer. Following re-suspension of the cells in 100 μl FACS buffer, 10 μl of FACS buffer containing 1 μg of R-PE labeled Goat anti-Human IgG Fc, and 1 μg of dylight 488 labeled streptavidin (Jackson ImmunoResearch) was added to each sample and incubated on ice for 1 hr with frequent vortexing. Samples were washed 3 times with FACS buffer and re-suspended in a total of 2 ml FACS buffer at 7.5×10^6 cells/ml.

2.8.3 FACS Isolation of gp41/IgG Double Positive Cells

Cells were dispensed into 5 ml polystyrene round-bottom tubes (Falcon) by passing through a 0.35 μm cell-strainer (Falcon) and analyzed using the MoFlo XDP Cell Sorter. Side scatter plots were used to exclude dead and clumped cells. The gating region was set so that approximately 0.2% of the gated cell population would

fall within the defined parameters. This region would include cells that both express high levels of IgG on their surface, and bind with high affinity to recombinant gp41. In total, 70 000 Dylight 488 (FL1) and R-PE (FL2) positive cells were collected into 2 ml of RNAprotect Cell Reagent (Qiagen). As it was not possible to isolate individual cells, pools of cells were diluted in RNAprotect Cell Reagent to 50, 1000, or 10000 cells/ml and stored at -80°C.

2.9 V Gene Recovery, Selection and Amplification of gp41 Positive Clones

2.9.1 RNA Recovery from 50 Cell Sub-Library

Total RNA was recovered from a 50 cell aliquot of the double positive cells isolated per section 2.8.3 using an RNeasy® Micro Kit (Qiagen) following proper aseptic techniques to avoid RNase contamination. Cells were re-suspended in 75 μl of buffer RLT (Qiagen) with 0.143M β-mercaptoethanol (Bio-Rad). Due to the small cell number, 20 ng of carrier RNA (Qiagen) was added to the sample. One volume of 70% ethanol was added to the sample and transferred to an RNeasy MiniElute spin column and centrifuged for 15 s at 8000 g. The sample was washed by passing 350 μl buffer RW1 (Qiagen) through the column by centrifuging as above. To avoid DNA contamination, 27 Kunitz units of DNase I diluted in 80 μl buffer RDD (Qiagen) was added to the column and incubated at room temperature for 15 min. The sample was then washed once with 350 μl buffer RW1, once with 500 μl buffer RPE (Qiagen), and once with 80% ethanol by centrifuging as above. The column was thoroughly dried by centrifuging with the spin column lid open at 13000 g for 5 min.

RNA was eluted in 14 μl of RNase-free water (Qiagen) by centrifuging for 1 min at 13000 g.

2.9.2 cDNA Synthesis Using Sensi-Script®

RNA was converted to cDNA using Sensi-Script® Reverse Transcription Kit (Qiagen). The entire volume (12 μl) of RNA eluted per section 2.9.1 was added to 8 μl of master mix containing: 1X buffer RT (Qiagen), 0.5mM each dNTP (Qiagen), 1 μM Oligo-dT primer (Invitrogen), 10 Units of Superase•IN RNase Inhibitor (Ambion), and 1 Unit of Sensiscript Reverse Transcriptase (Qiagen). The reaction was incubated at 37°C for 60 min.

2.9.3 VH and VL Amplification from 50 Cell Sub-Library

VH and VL genes were amplified from the 50 cell sub-library of gp41 positive clones using the Expand High Fidelity PCR System. Next, 5 μl of cDNA created in section 2.9.2 was used as the template with each of primer sets outlined in section 2.5.10. Reactions were cycled as follows: 94°C for 5 min, 15 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec* (15 sec increase in incubation time with every cycle), and one final cycle at 72°C for 7 min. PCR products were visualized and purified as stated previously.

2.9.4 Cloning gp41 specific VH and VL Sequences into Expression Vectors

Recovered VH and VL sequences as well as P-dbIgG1, P-dbKappa, and P-dbLambda were digested with appropriate restriction endonucleases and purified as per section 2.3.1. 200 ng of each of the digested VH-α, VL-κ, and VL-λ, regions from the 50 cell sub-library were ligated into 500 ng of linearized P-dbIgG1, P-dbKappa or P-dbLambda following protocols outlined in section 2.5.1.

2.9.5 Plasmid Isolation and Sequencing

Ligations from the sub-library were each transformed into chemically competent TOP10® *E.coli* cells and plated on LB agar with zeocin as per section 2.3.1. A total of 96 clones from the P-dbIgG1 ligation, 48 clones from the P-dbKappa ligation and 48 clones from the P-dbLambda ligation were transferred from the plate into individual wells of two 96 deep well blocks containing 1 ml of LB-Lennox broth with 25 μg/ml of zeocin. Blocks were incubated overnight at 37°C with shaking at 250 rpm. Plasmids were isolated from each well using a Montage Plasmid Miniprep96 Kit (Millipore) following manufacturer's instructions.

Plasmids were sequenced as outlined in section 2.5.4 using SeqF1 as the sense primer, and either IgG1 Reverse, or PdbLiRevSEQ as the anti-sense primer. Sequences from the heavy and light chain clones were aligned using the ClustalW alignment algorithm to generate individual guide trees using DNAStar software. From the heavy chain guide tree, 11 productive immunoglobulin VH sequences were identified. From the light chain guide tree, 13 productive immunoglobulin Vκ sequences were identified.

2.9.6 Chain Shuffling Transfection with V Genes from the gp41 +ve Sub-Library

To identify the VH and VL combinations resulting in functional IgG1-κ antibodies that bind to gp41, each of the 11 VH clones were co-transfected with each of the 13 VL clones into HEK293F cells. In sterile 96 well plates, 1 μg of light chain vector and 0.8 μg of heavy chain vector were diluted into 30 μl Opti-Mem®. 293fectin™ was diluted 1/12 in 5.25 ml Opti-Mem®, incubated for 5 min at room temperature, and added in 33 μl increments to the DNA. DNA-293fectin™ complexes were formed by incubating the reactions for 25 min at room temperature. Each reaction was immediately added to a well of a 24 well tissue culture plate (Corning) containing 1.1 x 10^6 cells in 1 ml of FreeStyle™ 293 Expression Medium. Sterile H_2O was added in between the wells to prevent desiccation. Transfections were incubated for 48 hrs at 37°C and 8% CO_2 with shaking at 250 rpm.

2.9.7 Screening the Chain Shuffling Transfection via ELISA

Antibody expression and affinity for gp41 was determined for each VH/VL combination using a standard ELISA. 200 ng of recombinant gp41, 200 ng of goat anti-human IgG $F(ab')_2$, and 200 ng of BSA were coated onto individual wells of a MaxiSorp 96 well plate and blocked as per section 2.5.9. 60 μl of supernatant from each transfection was added to the wells and incubated for 2 hrs at 37°C. Plates were then processed as per section 2.5.9 and the absorbance at 405 nm was determined.

2.9.8 Selection and Amplification of gp41 Specific Antibodies

Four mAbs with the highest OD value against gp41, H3L2, H4L9, H6L9, and H9L2 were chosen from the 144 different VH/VL combinations tested. The four VH regions encoded in P-dbIgG1, and the two VL regions encoded in P-dbKappa were transformed into chemically competent One Shot TOP10® *E.coli* cells as per section 2.3.1. Glycerol stocks for each plasmid were made by diluting 200 µl bacterial culture in 800 µl of 33 % glycerol and stored at -80°C. Plasmids were expanded and isolated using the Qiagen Plasmid MaxiPrep as per manufacturer's instructions.

<u>2.10 Large Scale Antibody Expression</u>

2.10.1 Large Scale Transfection of gp41 mAbs

HEK 293F cells were grown to 150 ml volumes in 500 ml flasks and transfected with 300 µg of light chain expression vector, and 150 µg heavy chain expression vector for each of the four mAb combinations following a scaled up version of the protocol outlined in section 2.5.6. Cells were incubated for 5 days at 37°C with 8% CO_2 and shaking at 135 rpm.

2.10.2 Concentration and Purification of gp41 MAbs

Supernatants from the four transfections were concentrated 10 to 15 times using a Centriprep Centrifugal Filter Device (Millipore) with an Ultracel® 30 kDa cut-off membrane. Supernatants were then mixed 1:1 with PBS pH 7.2 and loaded onto a 1 ml Protein A column (GE Healthcare) that had been pre-equilibrated with

10 volumes of PBS. Flow through was collected and passed over the column twice more to collect any residual antibody. The column was washed with 10 ml PBS and eluted with 10 ml 0.5 M acetic acid pH 3.0 in 1ml fractions into 1.5 ml microfuge tubes containing 100 μl of 1.0 M Tris-HCl pH 8.0. The fractions were pooled and dialyzed three times against PBS at 1500 g and concentrated to a final volume of 1.5 ml using the centriprep device. Each sample was filter sterilized by passing through a low protein binding 0.2 μm filter (Millipore).

2.11 Characterization of the gp41 MAbs

2.11.1 Measurement of Antibody Specificity for gp41

ELISA titrations of the 4 gp41 mAbs in 0.1% BSA versus 200 ng of gp41, gp120, and BSA were performed using standard ELISA techniques as per section 2.5.9.

2.11.2 Measurement of Antibody Reactivity with Denatured/Reduced gp41

Recombinant gp41 was heat - denatured by incubating in H_2O at 99.9°C for 20 min in a thermocycler, then diluted 1/10 in PBS for coating. Additionally, recombinant gp41 was reduced by incubating for 20 min in 0.05 M DTT (dithiothreitol) (Invitrogen) then diluted 1/10 in PBS for coating. The process was repeated for recombinant gp120. ELISA titrations in 0.1% BSA for the 4 gp41 mAbs were performed versus 200 ng of denatured/reduced gp41, while ELISA titrations

for IgG1B12 were performed versus 200 ng of denatured/reduced gp120 as per section 2.5.9.

2.11.3 Assessment of Antibody Binding to HIV-1 Envelope Spike (gp160) using Flow Cytometry

HEK 293F cells were grown to 30 ml volumes and transfected with 30 μg of one of the following HIV-1 envelope expression vectors as per section 2.5.6: consensus clade B Env expression vector (pConBgp160-opt); or consensus clade C Env expression vector (pConCgp160-opt). P-dbKappa (SVK18) (irrelevant light chain expression vector) was also transfected as a negative control. Both HIV-1 Env expression vectors were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Beatrice Hahn. Vectors contained the consensus clade B or C Env amino acid sequence ligated into the pcDNA3.1 (-) expression vector and optimized for expression in mammalian cells. Both vectors had been amplified using a Qiagen MaxiPrep kit as outlined previously. Cells were incubated for 48 hr at 37°C with 8% CO_2 and shaking at 135 rpm.

Following incubation, 1 x 10^6 cell aliquots were washed once with FACS buffer, and re-suspended in 0.1 or 10 μg/ml of mAb H3/L2, H4/L9, H6/L9, H9/L2, or G75. Cells were incubated on ice for 1 hr with frequent vortexing and washed three times with FACS buffer. Cells were re-suspended in 75 μl FACS buffer with 6.7 μg/ml R-PE labeled Goat Anti-Human IgG and incubated for one hour on ice. Cells were washed 3 more times and re-suspended in 300 μl FACS buffer. 10 000 cell

aliquots were analyzed as per section 2.7.3 and the median R-PE fluorescence was determined each for cell population.

2.11.4 Assessment of Antibody Binding to HIV-1 Envelope Spike (gp160) using Fluorometric Microvolume Assay Technology (FMAT)

Transiently transfected HEK293F cells expressing consensus HIV-1 envelope clade B, clade C or SVK18 were prepared as per section 2.11.6. Cells were then counted and the viability determined using trypan blue exclusion. All three cell populations were washed twice with 1% BSA and re-suspended at 5 x 10^5 cells/ml.

Six micron polystyrene beads (Spherotech) were passively coated with either recombinant gp41 or BSA as follows: 200 µl of beads were added to 1 ml PBS (pH 7.2), vortexed, centrifuged at 13000 g for 1 min, and supernatant discarded. The beads were washed a second time in PBS following the same procedure. Beads were then re-suspended in 1 ml PBS containing 100 µg of recombinant gp41 or BSA and tumbled overnight at 4˚C. Beads were washed twice as stated above, re-suspended in 1 ml 1% BSA, and tumbled for 2hr at room temperature. Finally, the beads were washed two more times and re-suspended in 1 ml of 0.1% BSA.

To run the assay, either 2 x 10^4 of the transfected cells, or 1 x 10^4 antigen coated beads were added in 40 µl of dilution buffer (1% BSA in PBS) to each well of a FMAT 8100 HTS 96 well black-clear bottom plate (Applied Biosystems). Next, 50 µl of dilution buffer containing 0.4 ug/ml of FMAT Blue goat anti-human IgG (Fc) (Applied Biosystems) was then added to each well. Then, 15 µl of dilution buffer containing 1/2 serial dilutions (8 µg/ml —› 0.016 ug/ml) of the following purified

primary antibodies were added to the wells: H3/L2; H4/L9; H6/L9; H9/L2; B12; or G75. Following the simultaneous addition of the beads/cells, secondary, and primary antibodies, the plates were incubated in the dark for 4 hr at room temperature and analyzed using the Applied Biosystems 8200 Cellular Detection System (8200 cds). Event counts were measured to determine positive binding. A minimum of 50 events was set as the cut-off for a positive sample, and titers were recorded as the lowest mAb concentration with an event count over 50.

2.11.5 Sequence Analysis of gp41 mAbs

Nucleotide and amino acid sequences of the VH and VL genes (5′ end of framework 1, to the 3′end of framework 3) from the 4 novel gp41 mAbs generated herein were compared with known human germ line VH and VL genes using IMGT/V-Quest software :
(http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanIg) [143]. Heavy and light chain framework (FR) and complementary determining regions (CDR) sequences were analyzed using V-Quest software and the R:S mutation ratio for each region (CDR1-2, or FR1-3) were calculated using the following equation:

R:S = (r /s)

Where r = total number of replacement mutations in CDR1,2 or FR1-3

And s = total number of silent mutations in CDR 1,2 or FR1-3.

The multinomial model developed by Lossos et al (2000) was used to compare the observed R:S value of each VH and VL sequence to the expected R:S value of the closest related germline sequence given random mutation [142].

Germline FR and CDR sequences along with the number of observed R and S mutations in each region were input into the JAVA applet available at: http://www-stat.stanford.edu/immunoglobulin [142].

Using this model, the probability (*Pm*) could be calculated that the observed excess or scarcity of replacement mutations in the CDRs or FRs respectively occurred by random mutation and not by antigen-driven affinity maturation.

3 Results

3.1 Generation of an Antibody Secretory Library from HIV-1 positive PBMCs

3.1.1 V Gene Amplification and Library Generation

To investigate the ability of a mammalian cell antibody secretion system to isolate HIV-1 Env specific antibodies, an antibody library was initially developed from the bulk, unselected V genes of a B cell population known to contain clones specific to HIV-1 Env. The PBMCs of the HIV-1 positive sex worker ML256 were chosen as the source for the B cells based on a the high serum titer to both gp120 and gp41 (Figure 4A, B). VH genes from families 1-5 of the IgG isotype, and VL genes of the Kappa and Lambda isotype were amplified from these PBMCs (Figure 4C) and cloned into expression vectors of the appropriate isotype designed for the secretion of soluble antibody (Figure 5A-C). Supernatants from the co-transfection of heavy and light chain plasmids encoding either the V gene library or B12, were screened for expression of IgG, IgKappa, IgLambda, and reactivity to recombinant gp120 and gp41. As indicated in Figure 5D, heavy chain and light chain expression was achieved for both the antibody library and B12, while the IgG1/Kappa B12 transfection maintained antigen affinity for gp120. The Diversity of the PBMC antibody library was assessed by AluI digest (Figure 6B), and by sequence alignment using the clustalW alignment algorithm (Figure 6A). A diverse selection of VH digestion patterns suggests a diverse VH library (Figure 6A), while Figure 6B demonstrates that 4 different VH families were represented in the 15 clones that

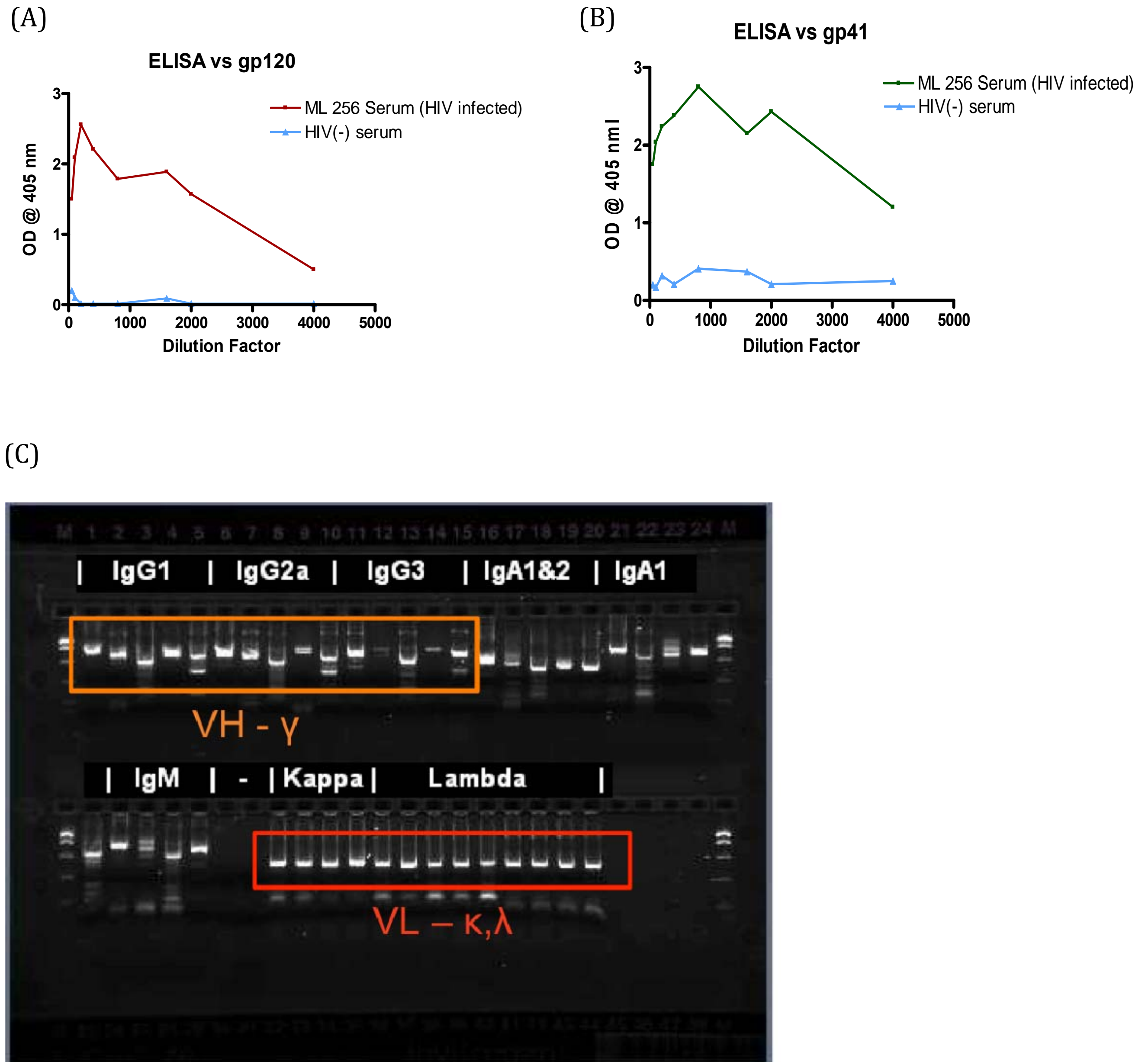

Figure 4. Cloning the IgG/κ,λ PBMC antibody repertoire from ML 256 (HIV-1 positive). Antibody serum titer of ML 256 against recombinant gp120 (A) and recombinant gp41 (B) compared with HIV-1 negative serum. Amplification of antibody V regions from the PBMCs of ML 256 (c). VH domains 1-6 of the IgG1-3 isotype (orange), VLλ domains 1-10 and VLκ domains 1-5(red) were extracted from the gel and cloned into expression vectors.

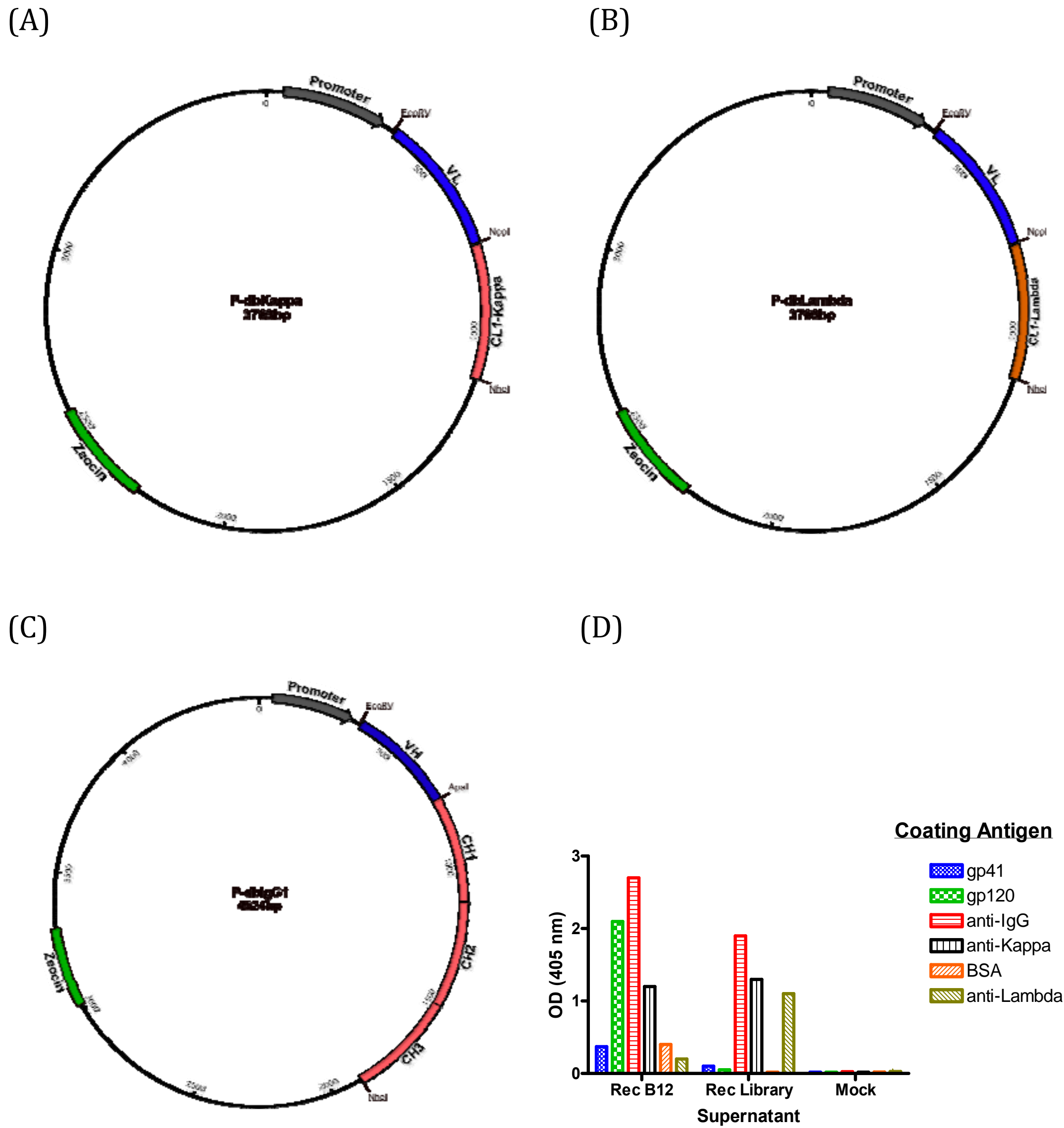

Figure 5. Secretory antibody expression system. P-dbKappa (A), P-dbLambda (B) and P-dbIgG1 (C) expression vectors. ELISA of antibody expression and specificity of supernatant when 293F cells were transfected with B12 VH/VL cloned into P-dbIgG1/P-dbkappa, or the ML256 PBMC VH/VL library cloned into PdbIgG1/P-dbKappa/P-dbLambda (D). Supernatants were tested for IgG, Igκ, and Igλ expression, as well as reactivity with recombinant gp120 and gp41.

(A)

```
                       FR-1            CDR1          FR-2          CDR2                       FR-3                          CDR3
clone 12   ISVQLLESGGGLIEPGGSLRLSCAVSGFSFGNAWMSWVRQAPGKGLEWVGLIKSNTDGGTTEYGAPVRGRFTISRDDSKNSLYLQMNSLKTEDTAVYYCTTARRWELG-------PDFWGQGTLVTV
clone 10   ISVQLLESGGGLVQPGGSLRLSCVGSGFVFSDFAISWVRQAPGKGLEWVSAFSG--NRDATWYADTVKGRFSISRDNSKNAVFLQMNSLRVEDTAIYYCAKESPYRSGSRE--YYFDSWGQGILVTV
clone 9    ISVQLLESGGGLVKPGGSLRLSCAASGFTFSDYYMSWIRQAPGKGLEWVSYLSY--SSNTIYYADSVQGRFTFSRDNAKNSLYLQMNSLRDDDTAVYFCARQKEPG-------TVFDYWGQGTLVTV
clone 8    ISVQLLESGGGLVKPGGSLRLSCAASGFTFSDHYMNWIRQAPGKGLEWVSYISS--SGTTIYYADSVKGRFTISRDNAKNSLYLQINILRAEDTAVYYCAKEGVGDGYNLGYYYFLDVWGKGTAVTV
clone 7    ISVQLLESGGGVVQPGGSLRLSCAASGFTFRDYGIHWVRQAPGKGLEWVSYIRH--HGREMYYADSVKGRFTISRDNSKNTLSLQMNSLRVDDTAVYFCAKDYDWA---------LDIWGQGTRVTV
clone 5    ISVQLLESGGGVVQPGGSLRLSCAASGFTFSSYWMTWVRQAPGKGLEWVANIEQ--DGTQKYYVDSVKGRFTISRDNAKNSLYLQMNSLRVEDTAVYYCARGLLGD-----------YWGQGTLVTV
clone 3    ISVQLLESGGRLVQPGGSLRLSCVGSTFTFTRYAMSWVRQVPGRGLEWVAAVSS--DGAHTFYPDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK-TPAKLLTHY--YYMDVWGKGTTVTV
clone 2    ISVQLLESGGGLVQPGGSLRLSCAASGFTFSSYWMTWVRQAPGKGLEWVANIKQ--DGSEKFYLDSVKGRITLSRDNAKNSLYLQMNSLRAEDTAVYHCARGLLGD-----------YWGQGTLVTV
           **********  .. *********  * * *     . *.** **.*****               *   *.** . *** .**.. **.* *. .***.* *.                   **.*  ***

clone 14   ISVQLLESGPGLVKPSQTLSLTCAISGDSVSNNTSAAWNWIRQSPSRGLEWLGRTYYRSKWYNDYAVSVKSRITINPDTSKNQFFLQLNSVTTEDTAVYYCARDCSNSWKWLD-PLWGQGTLVTV
clone 11   ISVQLLESGPGLVKRSQTLSLTCAISGDCVSSNS-AAWNWIRQSPSRGLECLGRTYYRSKWDNDYAVSVKSPITINPDTSKNQFFLQLNSVTPEDTAVYYCARDGSNSWYWFDNPLWGQGTLVTV
           ************** ************* ** ** *************** ********* ********* ******************** *********** **** * * * *********

clone 15   ISVQLLESGPGLVKPSETLSLTCTVSGGLWRQAVYWNWIRQPPGGVSLERMGYIYYSAKDTHYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCASALSGGGDFEYWGQGTLVSV
clone 4    ISVQLLESGPGLVKPSETLSLTCTVSGGS-ISSYYWNWIRQPPG-KGLERMGYIYYSG-STHYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCASGGFGGYDFEYWGQGTLVSV
           ****************************      **********   *** *******  ****************************************   ** *************

clone 1    ISKKPGESLKISCKGSGYSFGNYWIGWVRQMPGRGLEWMGIIDPGDSETRYSPSFQGQVTMSVDRSSGTAFLQWNTLGASDTAMYYCTRYPRSSCGDSCSWDVQFWGQGTLVIV
clone 6    ISKKPGESLKISCKGSGYSFGNYWIGWVRQMPGRGLEWMGIIDPGDSETRYSPSFQGQVTMSVDRSSGTAFLQWNTLGASDTAMYYCTRYLRSSCGDSCSWDVQFWCQGTLVIV
clone 13   ISKKPGESLKISCKGSGYSFTSYWIGWVRQMPGRGLEWMGIIDPGDSETRYSPSFQGQVTMSVDRSSGTAFLQWNTLXASDTAXYYCTRYKRSSTGDSCSWCVQFWGQGTLVIV
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(B)

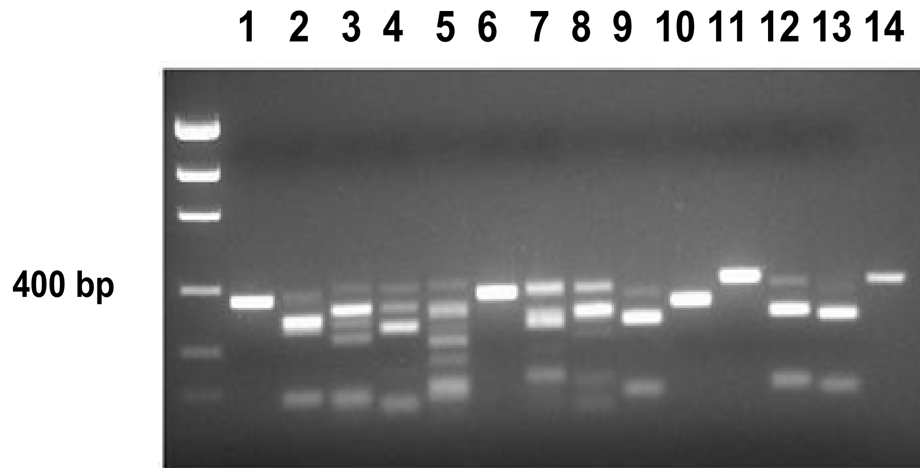


Figure 6. Diversity of the constructed ML 256 PBMC library. 15 clones were selected from the IgG vector library and analyzed by sequencing (A) and AluI digest (B). Clones were aligned based on their heavy chain variable region domain. Greatest diversity is seen in the CDR3 region (red) of the selected clones. Asterisks indicate totally conserved amino acids, while periods indicate fairly conserved amino acids.

that were sequenced and, as expected, diversity was primarily localized to the three CDR regions, in particular CDR3.

3.1.2 The Screening and Selection of Clones from the ML 256 PBMC Library

In order to isolate individual mAbs from the cells expressing the ML 256 antibody library, the cells had to be separated into individual clones in semi-solid media and screened for antibody expression and binding to the Env proteins. Following co-transfection of the heavy and light chain plasmids, the cells were re-suspended in semi-solid media with zeocin selection. 1500 Clones were selected, expanded and screened for antibody expression and reactivity to a recombinant gp120/gp41 mosaic antigen in an ELISA. Figure 7A summarizes the antibody expression profiles of the 1500 clones tested. The 9 clones with the highest antibody expression and binding to the gp120/41 mosaic antigen are shown in Figure 7C. The top two clones, 2G7 and 5B5 were selected for further sub-cloning.

3.1.3 V Gene Recovery and Expression

Due to their limited lifespan, further characterization of the 2 clones selected required that their V genes be recovered from and cloned back into the expression vectors. Two determine the optimal method of V gene recovery, two methods were explored: V gene amplification using the plasmid DNA in the clones as a template in the PCR reaction; or V gene amplification using cDNA synthesized from the recovered RNA as a template. Figure 8 A,B indicates that only 1 VH sequence could

(A)

Clones Screened	Expression Positive	gp41/120 +ve (OD > 0.1)
1500	150	12

(B)

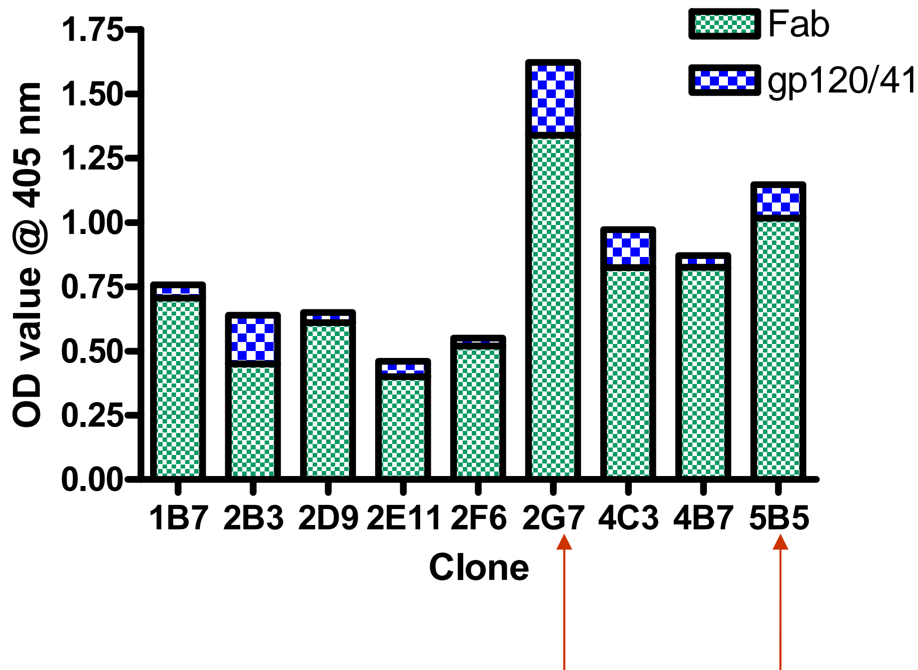


Figure 7. Initial Screening of 1500 293F clones transfected with the ML256 PBMC library. Results summary (A) and corrected ELISA OD values (OD value against BSA subtracted from all values) of the top 9 clones for antibody expression (Fab) and affinity to a recombinant gp120/41 mosaic antigen (B). Two clones, 2G7, and 5B5 were selected for V gene recovery based on their expression levels and OD values against gp120/41.

be recovered from clone 2G7 using plasmid DNA as the template, while 2 VH (5B5a, 5B5b) and 2 VL sequences (VL1,VL2) were recovered from clone 5B5 when the RNA isolation method was used to recover the V genes.

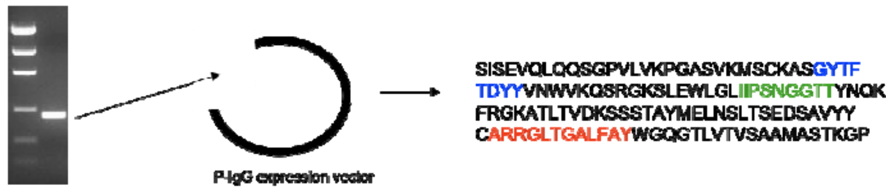
Recovered V genes were co-transfected into HEK-293F cells in various heavy and light combinations including B12 VL (4L) and G75 VL (anti-PA antibody) as controls (Figure 8C). Supernatants were screened for expression of Fab, and reactivity against monomeric, recombinant gp120 and gp41. The OD values of each supernatant vs. BSA were subtracted as background. One novel combination, 5B5a/VL2 had a corrected OD value vs. gp41 of approximately 0.5 (Figure 8C).

3.2 Generation of a Mammalian Cell Antibody Display System: HIV-1 Resistant CMC IgA Antibody Library

3.2.1. Amplification of V genes and Library Generation

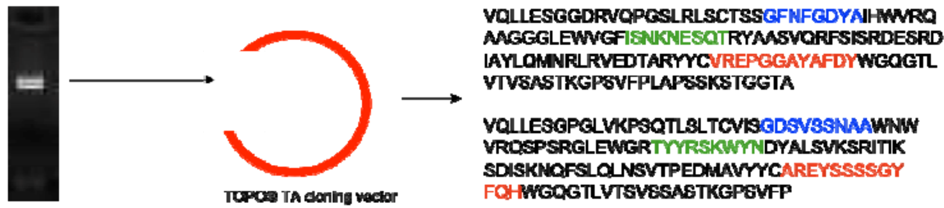
A novel strategy was developed to isolate gp41 mAbs from cervical IgA B cells using mammalian cell display (Figure 9). VH families 1-6 were amplified from the cervical IgA repertoire of two Kenyan HEPS: ML 1356 and ML 1515 (Figure 9A,B). VH-α and VL-kappa/lambda bands (400 bp) were cloned into antibody expression vectors. VL-kappa/lambda regions were ligated into the kappa/lambda expression vectors used previously (Figure 5A, B). Due to the inefficiencies of the antibody secretion system (summarized in Figure 7A), a new vector was generated by inserting the human PDGFR-TM domain onto the C terminus of the IgG CH3 in P-dbIgG1 creating P- dbIgG1-TM (Figure 9C).

(A) DNA V gene Recovery Revealed 1 VH Sequence in Clone 2G7



*Unable to recover light chain sequence of 2G7

(B) RNA V gene Recovery Revealed 2 VH Sequences in Clone 5B5



*Recovered 2 VL-λ sequences from clone 5B5

(C)

Chain Shuffling Transfection

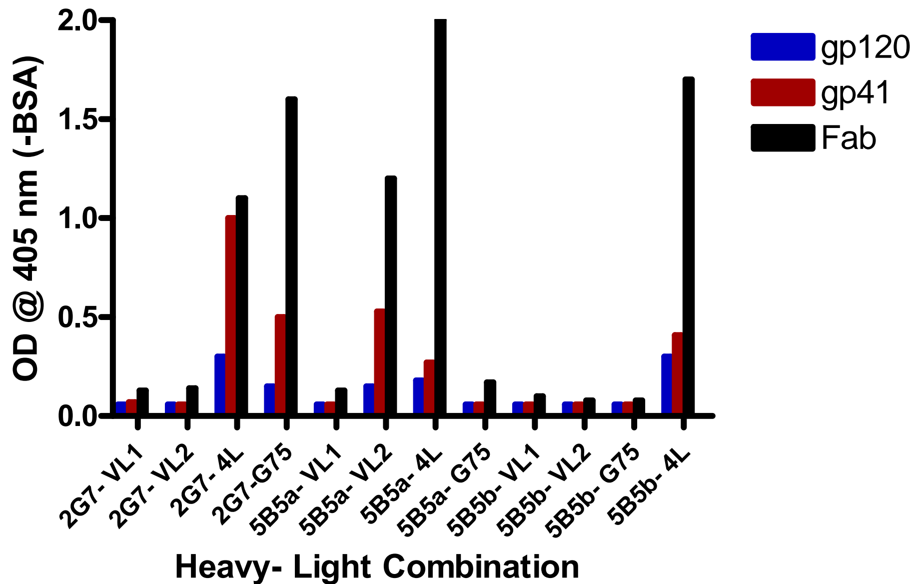
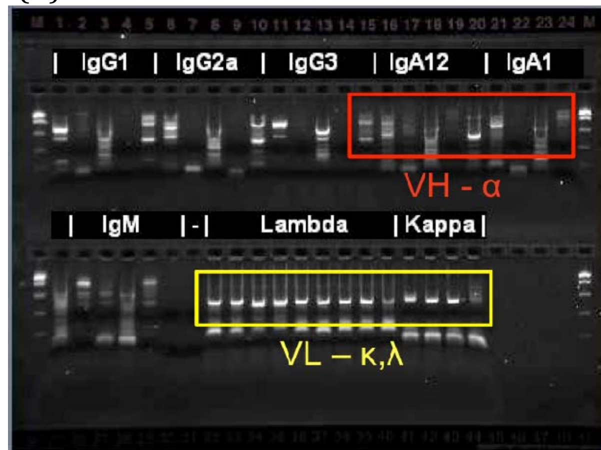


Figure 8. V gene recovery and chain shuffling from clone 2G7 and 5B5. One productive VH sequence was recovered from 2G7 using the DNA method (A), while two productive VH and VL sequences were recovered from clone 5B5 using the RNA method (B). Chain shuffling transfection using 3 novel sequences reveals one novel combination (5B5a / VL2) with a corrected OD value against gp41 of 0.5 (C).

(A)

Isotype	V Family	1515 (ML)	1356 (ML)
IgA1,2	1,3,5		X
IgA1,2	2		
IgA1,2	4	X	X
IgA1,2	5		
IgA1,2	6	X	
IgA1	1,3,5	X	X
IgA1	2		X
IgA1	4	X	
IgA1	5	X	
IgA1	6	X	

(B)



(C)

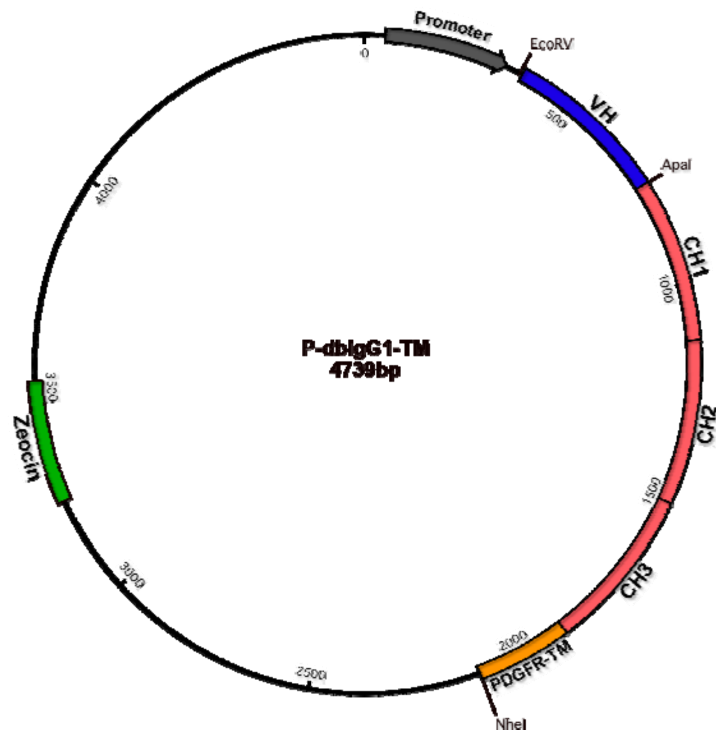


Figure 9. Creation of the VH – α antibody library from the CMCs of ML 1356 and ML 1515. Summary of the different V families that were successfully isolated for each IgA isotype from ML1515 or ML1356 (A). Agarose gel electrophoresis of PCR amplified antibody V regions from ML1515 (B). The human PDGFR-TM region was cloned into P-dbIgG1 to create the cell surface IgG antibody expression vector P-dbIgG1-TM (C).

Co-expression of this vector with P-dbKappa/P-dbLambda permitted antibody expression on the cell surface of mammalian cells, providing a better link of the antibody's phenotype to its genotype.

Once the VH-α regions were cloned into P-dbIgG1-TM, the diversity of this library was assessed by transforming *E.coli* cells, selecting, and sequencing 24 clones, and aligning the sequences using a ClustalW alignment algorithm creating a guide tree (Figure 10). Of the 24 clones selected, 20 had productively re-arranged heavy chain sequences. 13 distinct VH re-arrangements (a minimum of 40 nucleotide differences) were identified from the 20 productive sequences which were comprised of VH families 3,4 and 5,

3.2.2 Antibody Expression on the Cell Surface in Transient Transfection

P-dbIgG1-TM encoding the CMC VH-α library or B12 VH, was co-transfected with P-dbKappa/Lambda encoding the CMC VL library or B12 VL into HEK-293F cells to assess whether functional IgG was displayed on the cell surface. Transfected cells were stained with R-PE labelled anti-IgG, or FITC labelled anti-kappa antibodies and analyzed by flow cytometry (Figure 11). The R2 region in Figure 11B and C when compared with 8A, indicates that IgG is expressed on the cell surface of a significant portion of the transfected cells. Figure 11E and F, when compared with 8D, indicates that IgKappa is localized to the cell surface by its association with IgG. For both cases, expression of B12 is slightly higher than the CMC library reflecting the fact the library contained a pool of V genes.

To assess the functionality of cell surface antibodies, cells were stained with

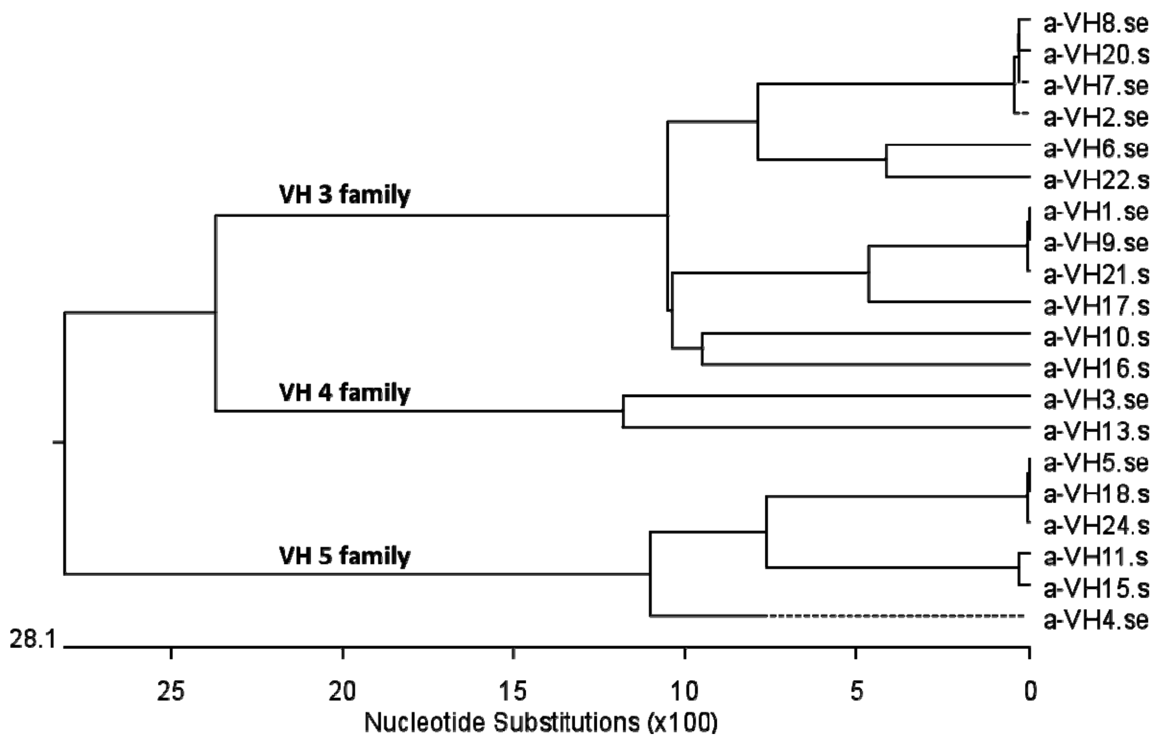


Figure 10. The Diversity and V gene composition of the CMC-R VH - α library in P-dbIgG1-TM. 24 colonies from the heavy chain library were isolated, sequenced, aligned using the ClustalW alignment algorithm and displayed as a neighbor joining guide tree. Sequences were made up of VH region domains 3, 4 and 5. 13 distinct sequences (greater than 40 nucleotide substitutions) were isolated from the 20 clones that had productively re-arranged heavy chain genes.

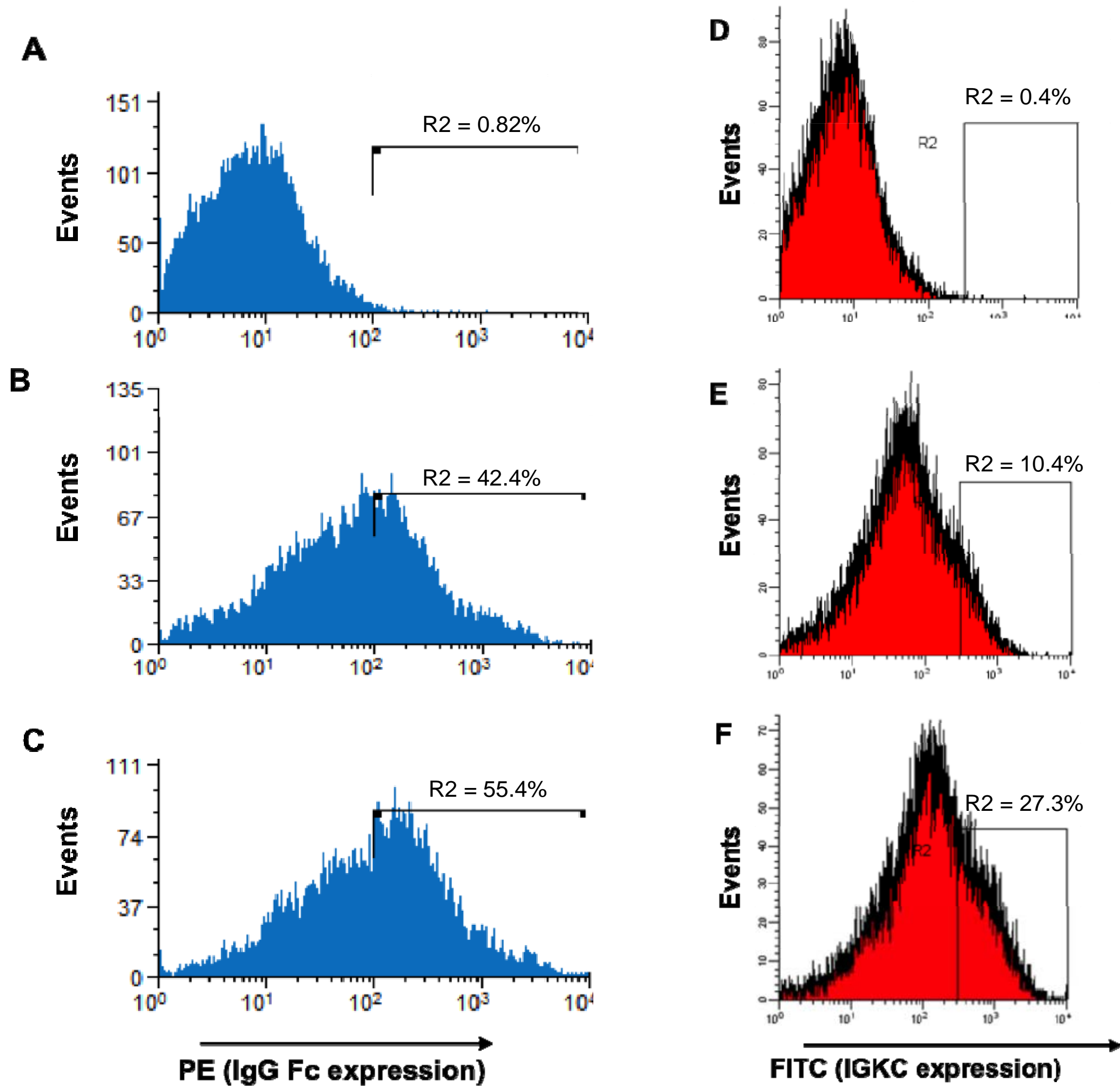


Figure 11. Assessment of antibody expression on the cell surface. Cells were transfected with: an irrelevant light chain expression vector (A,D); the cervical VH-α, VLλ,κ library from ML1515/1356 encoded into P-dbIgG1-TM, P-dbKappa and P-dbLambda respectively (B,E); or P-dbIgG1B12-TM/ P-dbKappa4L (C,F). Cells were stained with either R-PE anti-human IgG Fc (A-C) or FITC anti-human IGKC (D-F). The percentage of gated cells with fluorescence levels in the positive region (R2) when compared with the negative control (A, D), are listed.

either recombinant gp41 (CMC library) or recombinant gp120 (B12) and analyzed by flow cytometry. The R2 region in Figure 12D indicates that when IgG1/kappa B12 is transiently expressed on the cell surface, it retains its ability to bind to gp120. As expected, the CMC library displayed low reactivity with recombinant gp41 (Figure 12B).

3.2.3 FACS Analysis of Transiently Transfected CMC Library

Once it was determined that the recombinant antibody library was expressed as functional IgG on the surface of transiently transfected mammalian cells, FACS was done to collect only those clones displaying antibodies recognizing recombinant gp41. Scatter-gated fluorescence was done on the two transfectants (SVK18, or the IgG/Kappa/Lambda library), to eliminate dead cells, clumped cells, and cellular debris. Cells were gated at R1 and total of 45.2% of the total cell population expressing the antibody library were analyzed by FACS (Figure 13B). Gated cells were sorted for expression of IgG Fc and binding to biotinylated gp41, detected using R-PE labelled anti-human IgG, and dylight 488 labelled streptavidin respectively (Figure 13C,D). The obvious shift in fluorescence of the cells expressing the antibody library (Figure 13D) when compared with the SVK18 transfectants (Figure 13C) illustrates that while the majority of cells from the library transfectants expressed IgG on their surface, only a small fraction had affinity for recombinant gp41. A region comprising 0.2% of the gated antibody library transfectants was used as the sort window (Figure 13D).

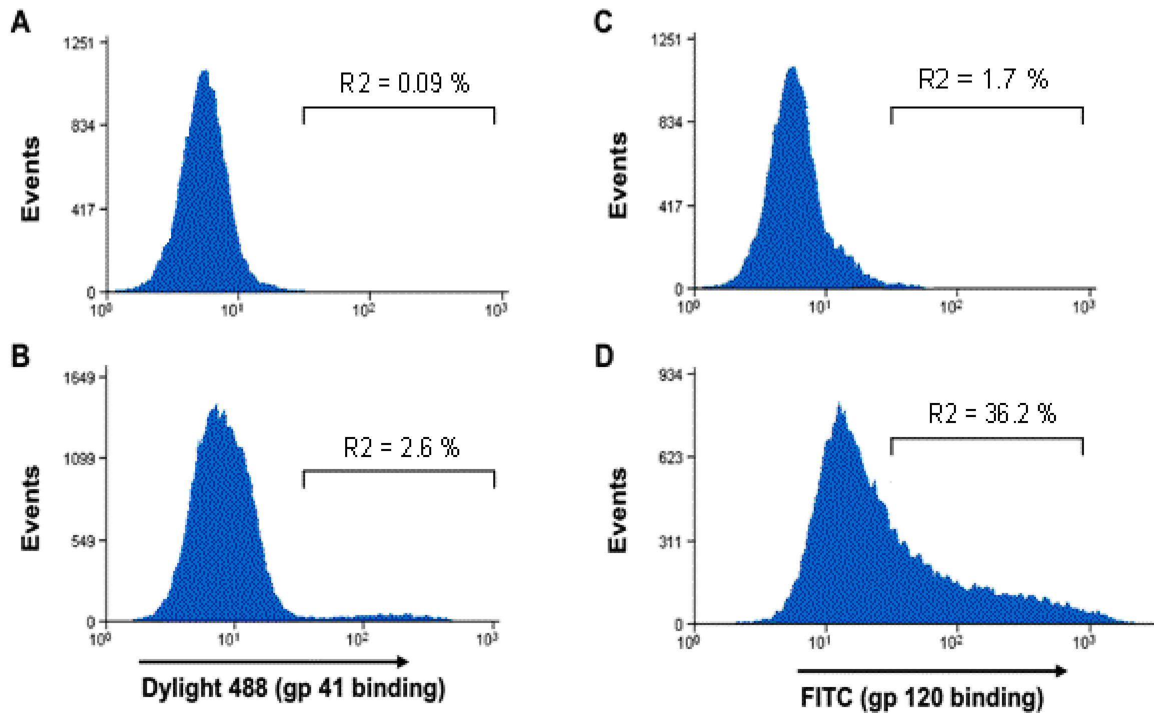


Figure 12. Affinity of cell surface antibody for gp120 and gp41. Cells were transfected with either: an irrelevant light chain expression vector (A,C); the cervical IgA VH-α, VLλ,κ library from ML1515/1356 encoded into P-dbIgG1-TM, P-dbKappa and P-dbLambda (B); or P-dbIgG1B12-TM/ PdbKappaB12 (C). Cells were stained with either FITC labeled gp120 (C,D) or biotin labeled gp41 followed and detected using dylight 488 labeled streptavidin. The percentage of cells in the positive region (R2), when compared with the negative controls (A,C), are indicated for each sample. Results indicate that surface displayed B12 has affinity for gp120, and a small minority of surface displayed antibodies from the CMC library have affinity for g41.

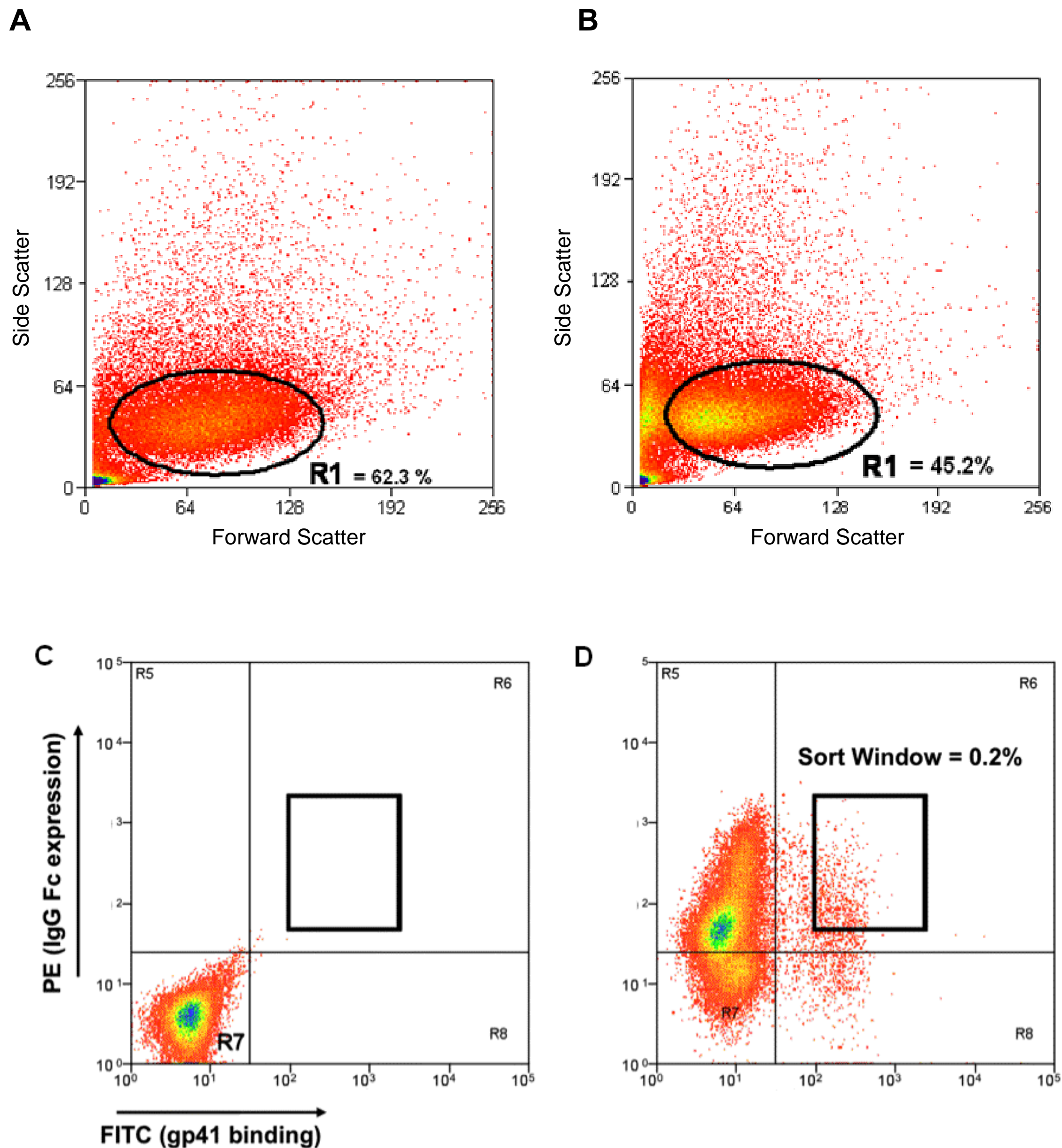


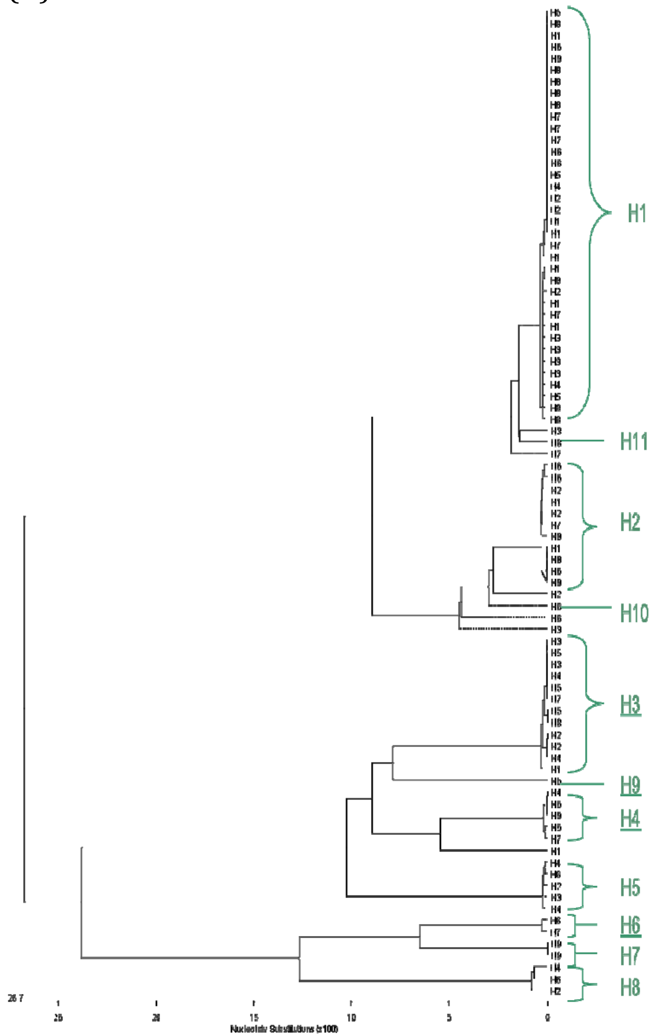
Figure 13. Isolation of gp41 specific antibodies by FACS. Cells were transfected with the negative control (SVK18) (A,C) or the CMC plasmid library (B,D). Scatter-gated fluorescence analysis was done on both samples (A,B) to exclude dead cells, clumped cells, and cellular debris. Live cells were gated on R1 for both samples. For sorting, cells were stained for the presence of cell surface IgG, and affinity to recombinant gp41. The sort window was set to collect the top 0.2% of cells positive for both variables from the CMC library (D) for V gene recovery.

3.2.4 VH and VL Recovery and Sub-Cloning

A 50 cell aliquot was removed from the cell population sorted for affinity to gp41 and expression of cell surface IgG. From this 50 cell sub-library, VH and VL genes were recovered from total RNA and cloned into the expression plasmids P-dbIgG1, P-dbKappa or P-dbLambda. The resulting three plasmid sub-libraries were transformed into *E.coli* cells and either, 96, 84, or 12 clones were selected from the P-dbIgG1, P-dbKappa, or P-dbLambda transformations respectively and sequenced. Productive VH and VL sequences were aligned using the ClustalW alignment algorithm to generate heavy and light chain guide trees. A total of 94 of the 96 P-dbIgG1 clones had productively re-arranged VH sequences, while 66 of the 84 P-dbKappa and none of the 12 P-dbLambda clones that were selected had productively rearranged VL sequences. Figure 14 shows the genetic diversity of the 94 VH and 66 VL sequences. The VH guide tree is composed of 16 different VH sequences, while the VL guide tree is composed of 15 different sequences. To test which combinations produced functional antibody, 11 of the P-dbIgG1-VH and 13 of the P-dbKappa-VL clones were selected from the guide tree (Figure 14). When one of the chosen clones was composed of related sequences with individual point mutations, a single clone was selected.

Each of the 11 P-dbIgG1-VH clones were transfected with each of the P-dbKappa-VL clones in a chain swapping experiment to identify which VH/VL combinations produced a functional antibody with affinity for gp41. Supernatants from each transfection were tested for reactivity against recombinant gp41 and BSA using an ELISA. Figure 15 identifies the 4 heavy chain clones that resulted in gp41

(A)



(B)

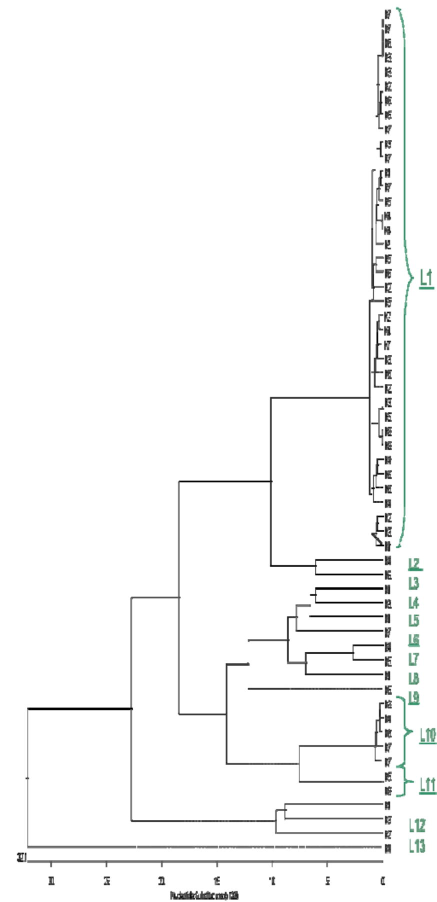


Figure 14. Analysis of recovered VH and VL genes from sorted gp41 positive cells. V genes were recovered from a 50 cell sub-library from sorted gp41 positive cells and cloned into P-dbIgG1/Kappa/Lambda vectors. 94 clones from the VH sub-library (A) and the 66 clones from the VLκ sub-library (B) were analyzed. Sequences were aligned using the ClustalW alignment algorithm to generate heavy and light chain guide trees. The 11 productive VH sequences (A) and 13 productive VL sequences (B) recovered from the sub-library and used in subsequent chain shuffling experiments are indicated in green. Underlined sequences are those that were found to have affinity for gp41 in subsequent chain experiments.

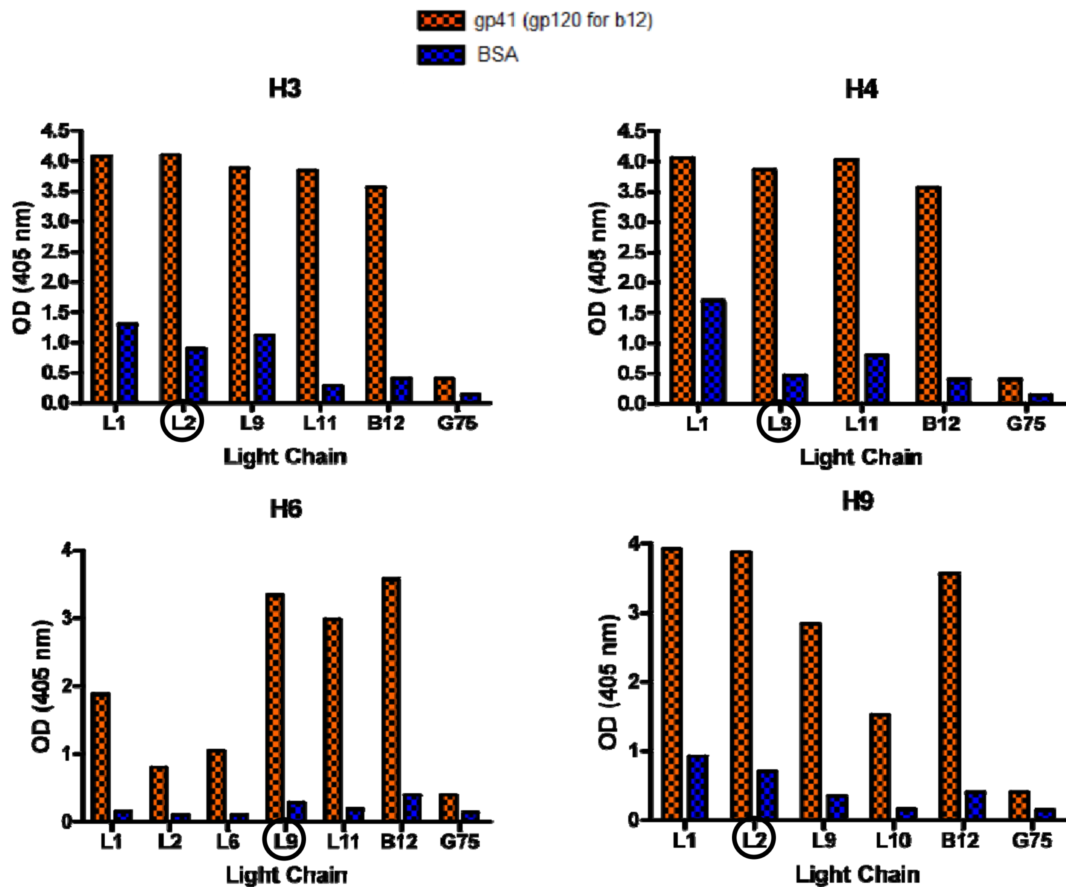


Figure 15. Identification of productive VH/VL combinations via chain shuffling. The 11 recovered VH genes were each paired with the 13 recovered VL genes in a chain shuffling transfection in HEK 293F cells. Of the 143 different combinations, 16 produced an antibody with affinity for recombinant gp41. Shown are the 4 productive VH sequences that had affinity for gp41 when paired with the indicated light chains. B12 and G75, a chimeric PA specific mAb previously characterized by our lab, were included as positive and negative controls. The light chains selected as the best partner for each heavy chain is indicated with a circle. These four combinations were selected for further characterization.

specific antibodies when paired with the indicated light chain clones. Supernatant from B12 and G75 transfections were included as positive and negative controls respectively. Promiscuous light chain pairing was observed for each heavy chain clone. The light chain partner resulting in the highest OD value against gp41 and the lowest OD against BSA was selected for each of the heavy chain clones for furthercharacterization. The following combinations were selected: H3/L2, H4/L9, H6/L9, H9/L2.

Figure 16 shows the sequence alignment of the 4 heavy chain sequences and the 6 light chain sequences that produced a gp41 specific antibody in the chain-swapping experiment. Each sequence is distinct and the diversity between sequences is most prevalent in the three CDR regions (Figure 16).

3.3 Characterization of gp41 Specific MAbs

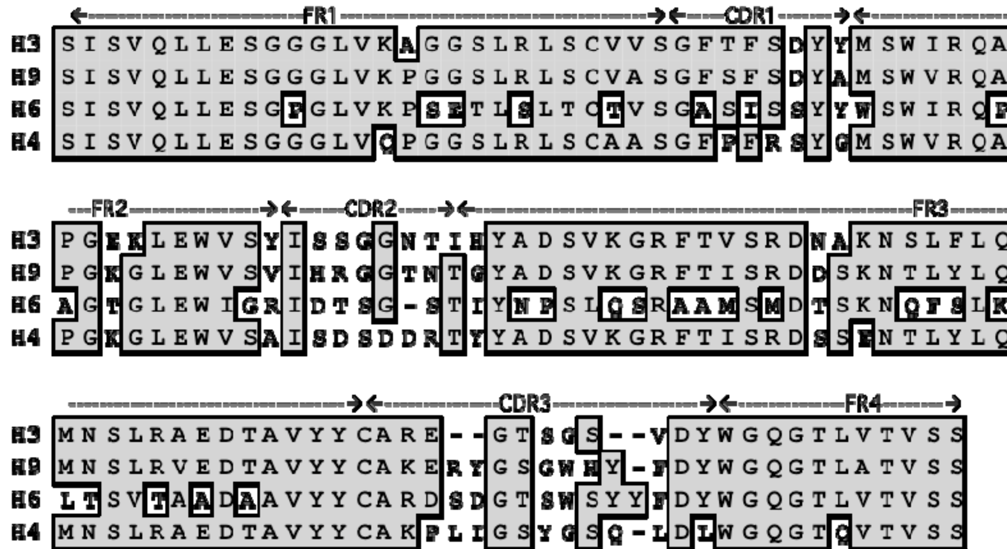
3.3.1 Evaluation of MAb Specificity for gp41

To assess the specificity of the four gp41 mAbs, each were expressed, purified and titrated against gp41, gp120 and BSA (Figure 17). All four mAbs show similar binding curves to gp41 and demonstrate little cross-reactivity with either gp120 or BSA (Figure 17).

3.3.2 Determination of the Nature of the MAb Epitopes

To determine if the 4 mAbs bind to conformational or linear epitopes, each mAb was titrated against gp41 that was either denatured using heat or reduced

(A)



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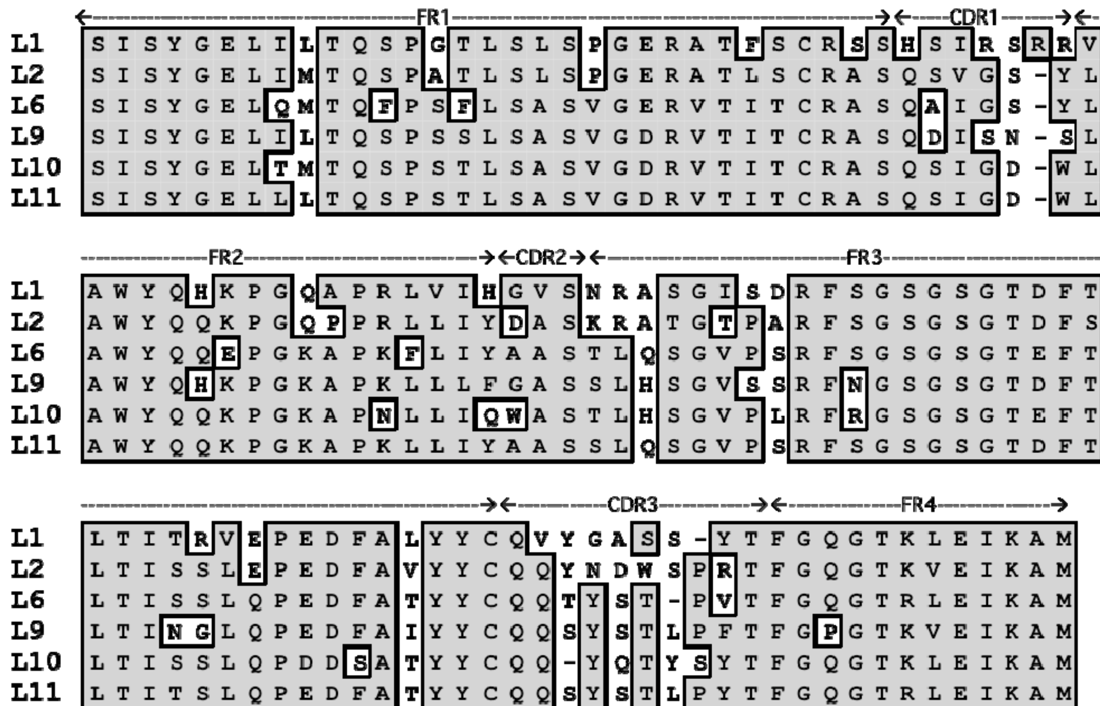

Figure 16. Sequences of recovered gp41 specific VH (A) and VL (B) clones. Regions of similarity are indicated with grey boxes. As expected, diversity is most prevalent in the three CDR regions.

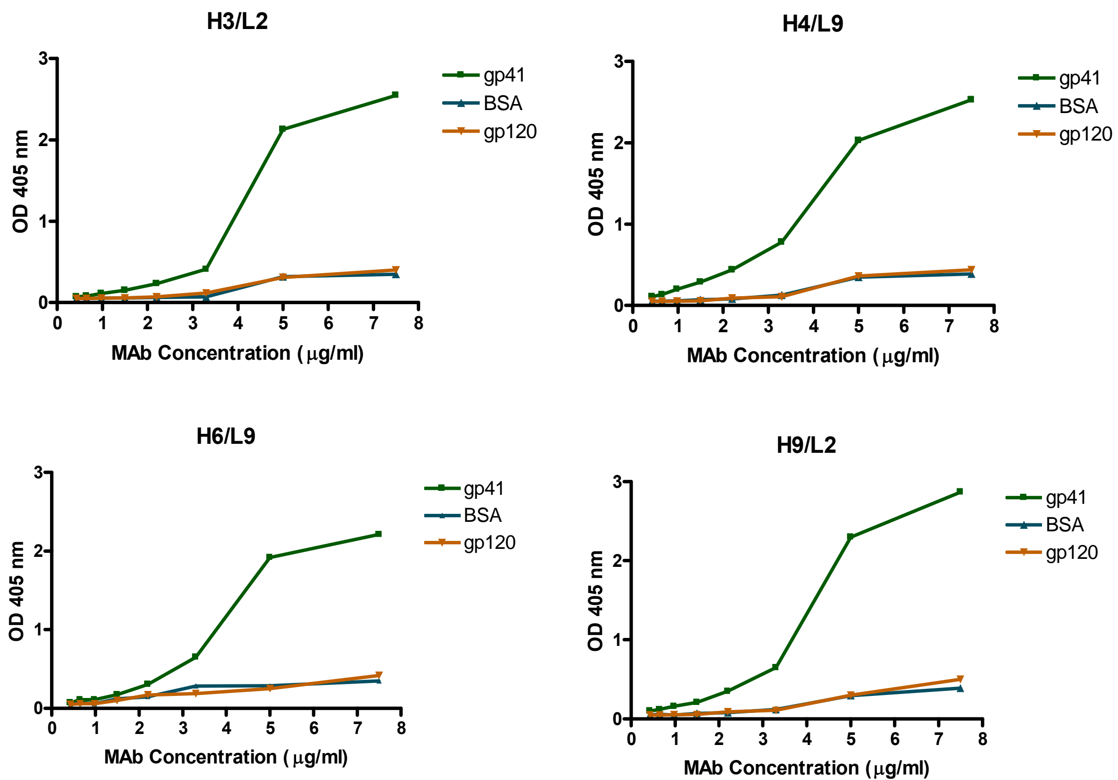

Figure 17. ELISA titrations of the gp41 mAbs against gp41, gp120, and BSA. OD values suggest all four mAbs demonstrate little reactivity with both negative controls BSA and gp120.

using DTT. The conformational b12 antibody and recombinant gp120 was included as a control. DTT treatment eliminated b12 binding to reduced gp120; however, all four gp41 mAbs maintained some level of binding to reduced gp41 (Figure 18B). H3/L2 and H4/L9 were affected the most by DTT treatment as binding was reduced by approximately 30% and 50% respectively (Figure 18B). Heat treatment alsocompletely eliminated b12's affinity for gp120; however, as was seen with DTT treatment, all four gp41 mAbs maintained affinity for heat-denatured gp41. Again, H3/L2 and H4/L9 were the most affected by heat denaturation as binding was reduced by approximately 45% and 55% respectively (Figure 18C). The differences in gp41 binding suggest that all four mAbs bind to predominantly linear epitopes. However, the reduced binding of H3/L2 and H4/L9 suggest that at least part of the epitope recognized by these antibodies has a conformational component.

3.3.3 Binding of MAbs to HIV-1 Envelope Spike

While each mAb was shown to bind to recombinant, monomeric gp41, the binding ability of each against gp41 in its native conformation as the transmembrane portion of the viral spike is more important in identifying any *in vivo* effector functions the mAbs may possess. Figure 19 A shows the flow cytometry results when HEK-293F cells expressing either SVK18 or HIV-1 Env from clade B or C were stained with each of the four novel gp41 mAbs and detected using R-PE labelled anti-human IgG Fc. Examining the median fluorescence value for each population indicates that three of the four mAbs bind to cell surface HIV-1 envelope clade B and clade C (Figure 19B). Only H6/L9 showed low reactivity against the Env

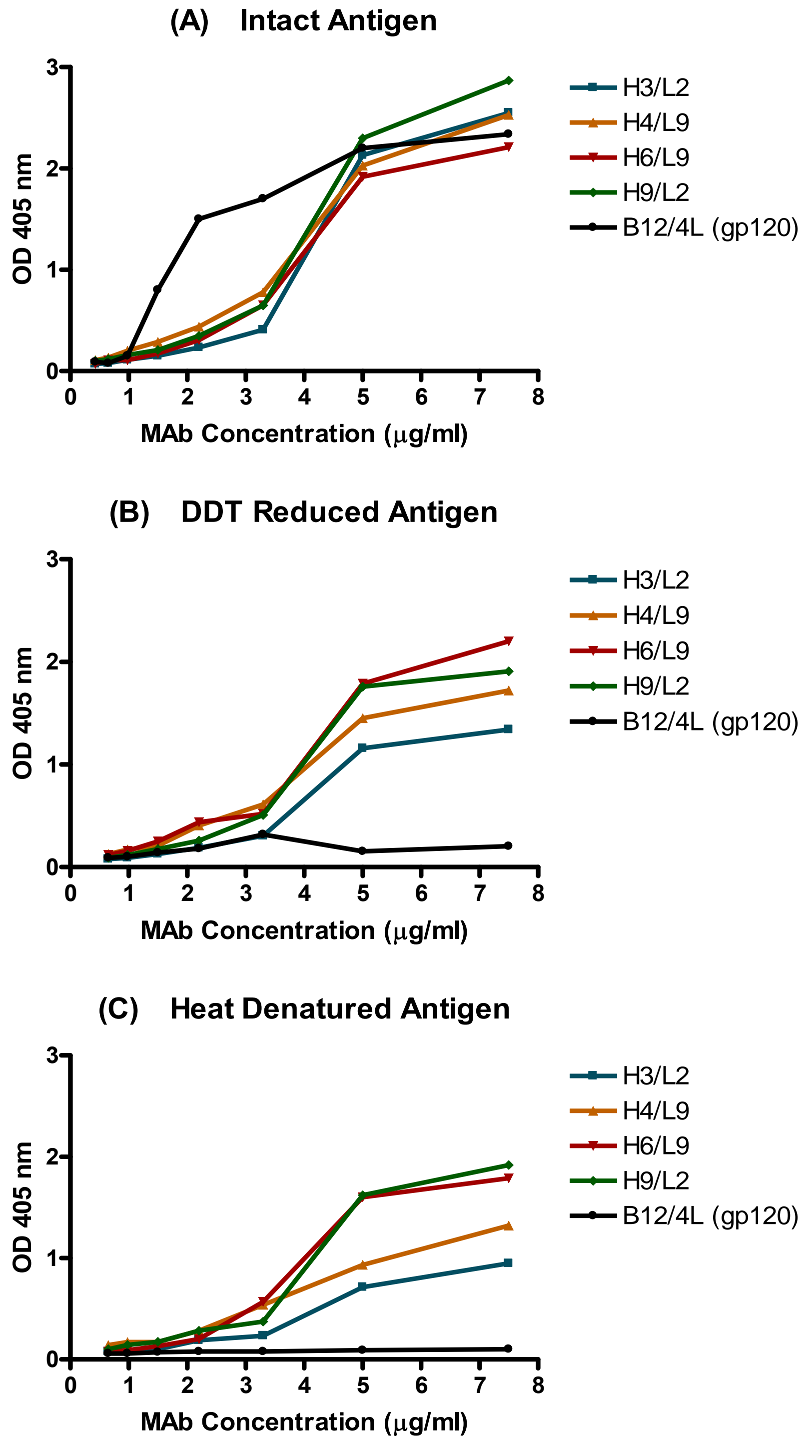

Figure 18. ELISA titrations of the 4 gp41 mAbs against intact gp41 (A), gp41 reduced using DTT (B), and heat denatured gp41 (C). B12/4L was included as a control due to the conformational epitope it recognizes on gp120. OD values for the gp41 mAbs against the denatured/reduced gp41 compared with those of B12, suggest each novel mAb recognizes a predominantly linear epitope on gp41.

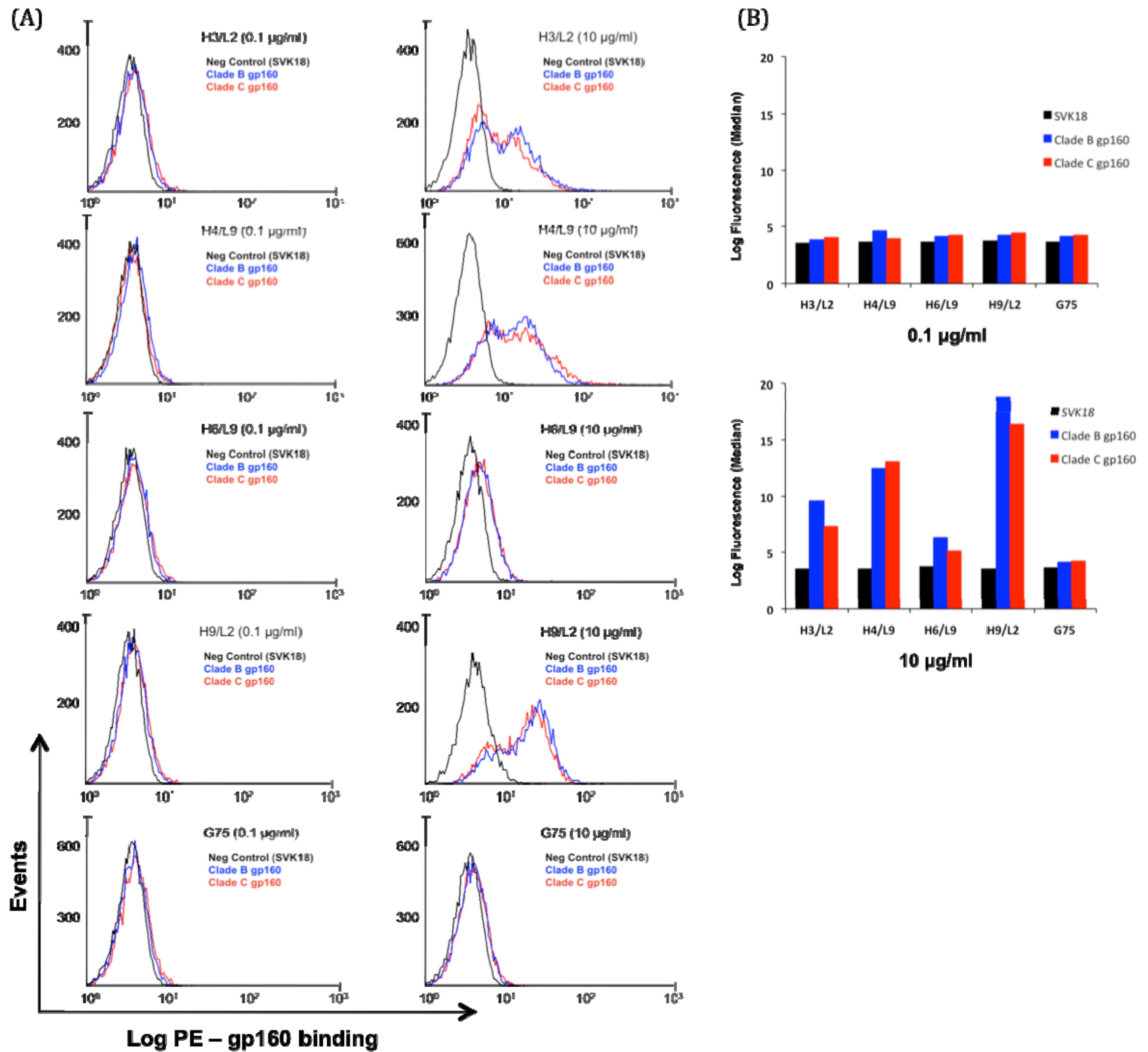

Figure 19. Binding of gp41 mAbs to HIV-1 envelope expressed on the surface of 293F cells. (A) Flow cytometry histograms showing binding profile the four novel gp41 mAbs to HIV-1 gp160 expressed on the cell surface. Cells were transfected with an irrelevant light chain expression vector SVK18, HIV-1 clade B consensus Env expression vector, or HIV-1 clade C consensus Env expression vector. Cells were stained with 0.1 and 10 µg/ml of the four gp41 mAbs, and the anti-PA antibody G75 as a negative control. (B) The median log fluorescence of each antibody tested is indicated for each for the different cell populations.

from clade B or C (Figure 19). H3/L2, H9/L2, and H6/L9 all showed reactivity against cell surface expressed Env, with H9/L2 showing the strongest affinity (Figure 19 A,B). All three mAbs showed similar reactivity against consensus clade B and clade C indicating that the epitopes recognized by these mAbs are conserved among both clades. To confirm these results, purified mAbs were titrated against the same transfectants, in addition to recombinant gp41 coated beads, and analyzed using the ABI cellular detection system. Examining each titration curve indicated that high concentrations of primary antibody caused a "hook-effect", as identified in Corbett et al (2007) where too much antibody saturates the system and inhibits antibody binding (Figure 20) [172]. The binding of all four gp41mAbs to surface Env was considerably less than mAb b12 (Figure 20). All four mAbs had lower endpoint titers against gp41 coated beads when compared with the Env transfectants (Figure 20). Mabs H4/L9 and H9/L2 showed the highest affinity for surface Env with an endpoint titer of 0.5 μg/ml. Confirming the flow cytometry results, clone H6/L9 was not positive against surface Env. Also consistent with results observed in Figure 19, all clones showed similar reactivity for surface Env from both clade C and clade B (Figure 20).

3.3.4 Molecular Characterization of gp41 Specific MAbs

Molecular analysis was carried out on the VH and VL genes comprising the novel mAbs specific for gp41 using IMGT software (Table 1). All sequences were highly mutated from their germline predecessors (Table 1). The VH genes all differed in their D(H) gene usage; however, all sequences did contain the same J

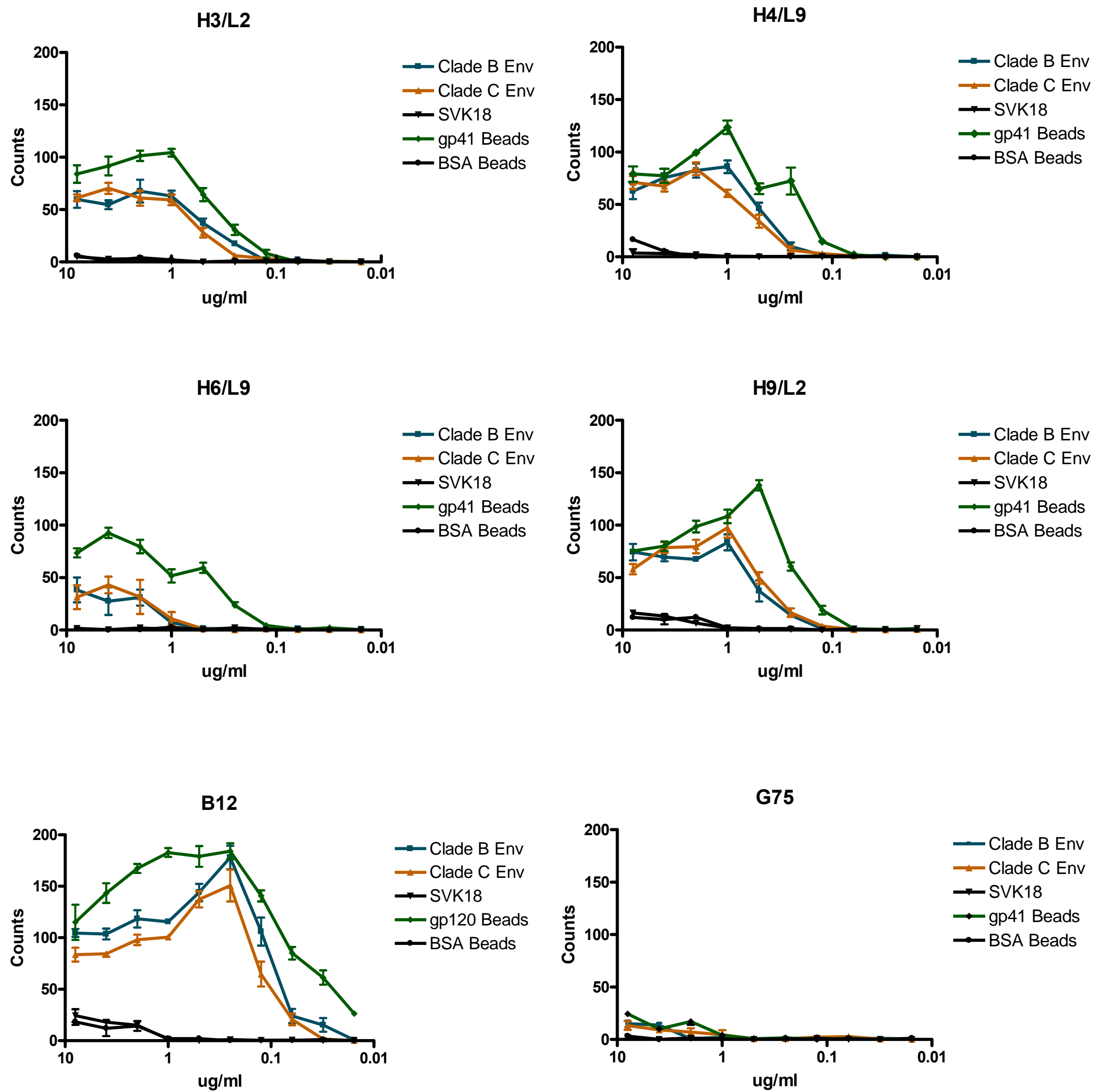

Figure 20. Titration of MAbs vs. HIV-1 envelope spike (clade B and C) using FMAT technology. HEK 293F cells transfected with clade B or clade C HIV-1 envelope expression vectors, or SVK18, were incubated with varying dilutions of the four gp41 specific mAbs, IgG1B12, or G75 and labeled using FMAT blue labeled goat anti-human IgG. Six-micron polystyrene beads coated with recombinant gp41 or BSA are included in place of transfected cells for comparison purposes. Data is expressed as the number of fluorescent events (counts) per well, with a positive result being defined as a count greater than 50 events per well. Results are the mean value of three independent experiments.

gene (J4*02). Analysis of somatic mutations in the FR and CDR regions of the VH sequences revealed that, as is the case with affinity-matured antibodies, H4 and H9 have replacement (R) to silent (S) nucleotide change ratios (R:S) that were higher in the CDRs when compared with the FRs (Table 1). H4 and H9 also had a higher percentage of absolute mutations occur in the CDRs (42%, 38%) when compared with H3 and H6 (17%, 18%). The P_M value for H4 (0.0006) and H9 (0.009) suggests that there is an extremely low probability that the high R:S ratio observed in the CDRs of these sequences occurred by chance through random mutation of the original germline gene (Table 1). This suggests that H4 and H9 have been antigen selected through multiple rounds of affinity maturation resulting in an accumulation of R mutations in the CDRs and S mutations in the FRs.

Analysis of the VL genes indicates that all sequences have diverged considerably from their germline predecessors with L1 and L9 showing the lowest homology (86% and 88%) (Table 1). Examining the P_M value suggests that only clones L1, L10 and L11 have been antigen-selected. Both clones L2 and L9 have low numbers of mutations occurring in the CDR, with approximately 18% and 7% of the total number of mutations occurring in these regions.

(A) Characteristics of Recovered gp41 Specific VH Genes

Clone	Germline gene	Identity (%)	DH	JH	Mutations FR	Mutations CDR 1,2	R:S FR	R:S CDR 1,2	P_M
H3	IGHV3-11*01	92	IGHD2-2*01	IGHJ4*02	19	4	2.8	1.0	0.729
H4	IGHV3-23*01	93	IGHD3-16*01	IGHJ4*02	12	9	0.8	8.0	0.0006
H6	IGHV4-4*07	92	IGHD6-13*01	IGHJ4*02	18	4	3.5	1.0	0.457
H9	IGHV3-23*03	93	IGHD6-19*01	IGHJ4*02	13	8	1.6	7.0	0.009

(B) Characteristics of Recovered gp41 Specific VL Genes

Clone	Germline gene	Identity (%)	JL	Mutations FR	Mutations CDR 1,2	R:S FR	R:S CDR 1,2	P_M
L1	IGKV3-20*01	86	IGKJ2*01	25	9	2.1	8.0	0.004
L2	IGKV3-11*01	92	IGKJ1*01	14	3	1.8	2.0	0.288
L6	IGKV1-9*01	94	IGKJ5*01	11	2	1.8	∞	0.175
L9	IGKV1-NL1*01	88	IGKJ3*01	28	2	2.1	∞	0.544
L10	IGKV1-5*03	91	IGKJ2*01	16	5	7.0	∞	0.018
L11	IGKV1-39*01	91	IGKJ5*01	17	7	1.0	6.0	0.010

Table 1. Gene characteristics of gp41 specific VH (A) and VL (B) sequences. Each rearranged VH or VL chain is listed with its most homologous germline gene, identity of each JH and DH segment, number of total mutations occurring in the framework (FR) regions, and in the complementary determining (CDR1,2) regions, and the replacement (R) to silent (S) nucleotide mutation ratio for each region. A higher R:S value in the CDR regions is indicative of antigenic selection. The P_M value represents the probability, calculated using a multinomial model [142] that the observed excess of R mutations in the CDRs and S mutations in the FRs occurred by chance through random mutation. A P_M value of < 0.05 suggests that the V sequence has been subject to antigen driven affinity maturation.

4 Discussion

4.1 Introduction

Human monoclonal antibodies against HIV-1 envelope proteins continue to play an important role in HIV-1 research. The structural characterization of gp120 and gp41, the identification of epitopes conserved among different HIV-1 subtypes, and the discovery of neutralizing epitopes on Env can all be attributed to the use of gp120 and gp41 specific monoclonal antibodies. Furthermore, bNt mAbs will likely play a major role in any topical microbicide yet to be developed, as only the passive transfer of gp120 and gp41 specific mAbs has proven capable of providing complete protection against SHIV mucosal viral challenge in macaques [48-52, 89-91, 144].

Efforts to produce novel bNt mAbs against HIV-1 have been hampered by inherent methodological limitations that have prevented the isolation of unique antibodies against gp120 or gp41. These limitations include: a considerable bias towards antibodies generated against clade B virus; a bias towards antibodies selected for biophysical properties advantageous for expression in prokaryotic systems; a lack of antibodies representing the repertoire of B cells at the site of initial contact with the virus; and a bias towards antibodies isolated from HIV positive individuals that, by definition, have been unsuccessful in preventing HIV-1 infection.

This paper describes the generation and characterization of 4 novel mAbs against the HIV-1 envelope protein gp41 using methods designed to limit the biases prevalent in previous production methods. A potential novel source of bNt mAbs

against HIV-1, the cervical B cell repertoire from HEPS sex workers in Nairobi, Kenya was used as the source for the antibody library. The characterization and gene analysis of these four IgA gp41 specific mAbs confirms that there are HEPS commercial sex workers in Nairobi that have cervical IgA specific for the HIV-1 envelope protein gp41 despite repeatedly testing seronegative.

4.2 Advantages of the Cell Display System.

Initially, an antibody secretion system was developed from the IgG antibody repertoire of the PBMCs of a HIV positive donor with a high serum titer to gp120 and gp41. Using the serum IgG repertoire from this individual eliminated the question of whether the isolated B cells contained clones specific for HIV-1 Env. This strategy was based on the secretion of recombinant antibody by transiently transfected HEK 293F cells. Cells were re-suspended in semi-solid media with antibiotic selection, clones selected, expanded, and screened against gp120 or gp41.

Our hypothesis was that this system would be an efficient and effective method of isolating monoclonal antibodies of desired specificity from an immune antibody library. In practice; however, this system proved to be inefficient, time consuming, and rife with limitations. Consequently, it was concluded that this system was not the optimal choice for the far more challenging task of isolating Env specific mAbs from the CMC IgA repertoire of HIV-1 resistant individuals. The ineffectiveness of the antibody secretion system can be traced to four major problems: First, because the phenotype or binding specificity of the antibody was not physically linked to its' genotype, individual cells could not be screened for

binding. Cells had to be grown for over 2 weeks for clonal expansion to have occurred to the point where the clone was visible and could be isolated. A second problem was that because both vectors contained the same resistance gene (zeocin), clones were initially selected for the presence of either the heavy or light chain expression vector, not both. Ideally, the initial selection of clones would have included only those that contained both vectors and secreted a functional antibody.

This leads to the third major problem with this system: screening the sub - clones was both time consuming and not high-throughput. Once a clone was chosen from the semi-solid media and transferred into a 96 well plate, the clone had to be cultured another 2 to 3 weeks prior to screening. The total number of clones screened was limited by practical limitations of cell culture, and when dealing with a random combinatorial library, a high number of clones must be screened to isolate an antibody with the desired specificity. The final problem with this strategy is that due to the transient nature of the transfection, the expression of antibody at the point when the clones were ready to be screened was very low. Clones were screened well over a month after the initial transfection. A month is well beyond the culture time recommended for transiently transfected cells, as cells begin to loose the plasmids even in the presence of a selective agent. Consequently, the amount of recombinant antibody present in the culture supernatant at the time of screening was not sufficient to give a positive signal on the ELISA.

To isolate mAbs from the CMCs of HIV-1 resistant donors, a new strategy was developed that would eliminate the problems posed by the antibody secretion system. This system permitted antibody expression directly on the cell surface of

transfectants, providing a physical link of the antibodies phenotype to its genotype, allowing single cells to be individually screened for antigen binding only 48 hours post transfection. To mediate surface expression, the human PDGFR-TM region was cloned onto the C terminal end of IgG1-CH3 encoded in the secretory vector P-dbIgG1, creating P-dbIgG1-TM. Flow cytometry analysis of HEK 293F cells co-transfected with P-dbIgG1-TM and P-dbKappa indicated high expression levels of IgG1 on the cell surface. Significantly, analysis also demonstrated the presence of IGKC on the cell surface. Due to the fact that P-dbKappa does not encode for the TM region, the presence of IGKC on the cell surface indicated that a functional IgG1/kappa antibody was expressed on the cell membrane, and orientated away from the cell. As expected, surface expression of IgG Fc and IGKC was higher in the b12 transfectants than the library transfectants. As mentioned above, IGKC surface expression requires the VH region to associate with a complementary VL region to form a functional antibody. Because only a portion of the VH and VL combinations generated by the antibody library will be productive, surface expression of IGKC is lower in the library transfectants when compared with cells expressing native heavy and light chain pairs. Flow cytometry analysis of IgG1/Kappa b12 transfectants confirmed that the surface expressed antibodies maintained antigen affinity.

The ability to screen individual cells for antigen binding permitted the use of FACS to separate the positive from negative clones. This method is very high-throughput, as 10 million cells were screened in minutes for both antibody expression and antigen specificity. VH and VL genes were recovered from a 50 cell aliquot of positive clones, inserted into excretory expression vectors, and 96 clones

sequenced. Only 16 different heavy chains were identified in the pool of 96 sequences. This indicated that the sorting process substantially decreased the complexity of the library, as an experiment carried out to prior to sorting identified 13 distinct sequences in a total of 20 productive re-arrangements. Contrary to what was anticipated, the frequency of the observed heavy and light chain sequences recovered from the 50 cell aliquot did not appear to correlate with gp41 specificity. This is evident by the fact that sequence H1, making up 40% of the recovered sequences, did not show reactivity with gp41 when paired with any of the recovered light chains. It is possible that the native VL sequence for H1 was not recovered from the 50 cell pool.

Ideally, B cells recovered from the cervix of the HIV-1 resistant donors would have been sorted for affinity to gp41 prior to the construction of the library. This would have increased the chances of generating a high affinity clone by eliminating irrelevant VH and VL sequences, and increasing the likelihood of observing the natural heavy and light chain pairs. This method has been used to generate antibodies either form single sorted B cells [117-119], or from a surface displayed antibody library generated from pre-sorted B cells [112,116]. Due to technical and geographical limitations, isolated CMCs could not be maintained in the live state necessary for sorting; consequently, the library was generated from the total CMC population. The fact that 4 novel gp41 specific mAbs could be isolated from non-sorted B cells speaks to both the feasibility of the methods used, and possibly, to the prevalence of IgA antibodies specific for gp41 in the cervix of these HEPS women.

Due to the nature of HIV infection, it was important that the antibody library from the HEPS donors be generated from the repertoire of the current antibody forming cell response (AFC) against HIV-1 [146]. Consequently, the antibody library was generated from the mRNA of B cells found at the site of initial contact with the virus. The fact that activated plasma cells have 1000 times more mRNA than resting memory B cells [146] suggests that it is likely this antibody library is representative of the AFC in the cervical mucosa within these Kenyan HEPS individuals.

4.3 Nature of the gp41 Epitope Recognized by the Four Mabs

Each of the four gp41 mAbs isolated from the CMCs of two Kenyan HEPS sex workers maintained affinity for heat-denatured gp41, as well as DDT reduced gp41. This suggests that each antibody binds to a predominantly linear epitope present on gp41. Two of the clones, H3/L2, and H4/L9, had their relative antigen affinity affinity via ELISA drop by approximately 50% against denatured/reduced gp41, suggesting that these two clones bind to epitopes with a partial conformational determinant. These results are in contradiction to several studies suggesting that the majority of antibodies raised against gp41 recognize conformational determinants. In a comprehensive study of serum IgG from six HIV infected individuals, Scheid et al (2009) found that the majority of gp41 antibodies isolated bound to conformational epitopes [132]. Furthermore, of the 25 gp41 specific Fabs isolated from HIV-1 infected donor by Binley et al (1996), not one bound to a linear epitope on gp41 [134]. The predominance of conformational antibodies in the serum of HIV-1 infected individuals was confirmed by serological studies, as titers

against reduced/denatured gp41 were on average 1 log lower than titers against the intact protein [134].

While conformational antibodies make up the majority of the antibody response against gp41, the only bNt mAbs isolated against gp41 bind to linear epitopes. Clones 2F5, 4E10, and Z13 are broadly neutralizing antibodies that bind to linear epitopes present on the MPER of gp41 [41,61,138]. The nature of the gp41 epitope optimal for HIV-1 neutralization has not been definitively established. In the case of gp120, the rigidification of the trimer prevents antibodies to linear epitopes from neutralizing the virus due the lack of a “correct” fine specificity for the functional spike [149]. This lowers the ability of antibodies raised against monomeric forms of gp120 common in viral debris, from reacting with the trimer to neutralize the virus [149]. Indeed, the majority of the anti-HIV serum antibody response is elicited by monomeric gp120 and unprocessed gp160 found in viral debris [27,151]. However; In the case of gp41, the occurrence of bNt mAbs that recognize linear epitopes on gp41 suggests that linear antibodies may play an important role in the response against HIV-1.

The ability of antibodies to neutralize HIV-1 has been correlated with their ability to bind with the native envelope spike [72, 152, 153, 155]. As a result antibody binding to the native, functional HIV envelope spike appears to be a prerequisite for antibody mediated virus neutralization [150, 154, 156]. In this regard, clones H3/L2, H4/L9, and H9/L2 were shown to bind to two clades of Env on the surface of HEK 293F cells using flow cytometry and confirmed by using the ABI cellular detection system.

Simply binding to an Env species on the virus; however, does not in itself accurately predict the neutralization potential of a given antibody. This is because several non-neutralizing Env specific antibodies have been shown to capture infectious HIV-1 virions in a highly specific manner [25, 26, 157-160]. Moore et al (2006) showed that these non-neutralizing antibodies were binding with alternative non-functional forms of Env present on the infectious virion [156]. This study showed that both gp41 timers from which gp120 has been shed, and gp120/gp41 monomers decorate the surface of viral particles [156]. The presence of uncapped gp41 trimers on the surface of HIV-1 virions has been observed previously [23, 27]. The gp120/gp41 monomer is likely formed during Env synthesis prior to oligomerization, while gp120 is most likely shed from the gp41 stump following virion maturation [156]. Furthermore, uncleaved gp160 precursor molecules have been shown to make up a substantial portion of the surface Env expressed by HIV infected cells [161]. This non-functional Env spike can be recognized by both neutralizing and non-neutralizing antibodies specific for gp120 and gp41 [161].

Given the considerable heterogeneity in the surface expressed Env of HIV-1, it is not possible to conclusively say that mAbs H3/L2, H4/L9, and H9/L2 bind with the functional Env spike from the data presented. Future work includes conducting neutralization studies to determine if these gp41 mAbs bind with functional Env and mediate virus neutralization. Without the neutralization data; however, we can say that these 3 mAbs will likely have the ability to bind directly to infectious virus by binding to functional or non-functional Env species on the viral membrane. Even if the mAbs bind to non-functional Env species, antibody coating of non-functional Env

can have consequences distinct from antibody mediated neutralization [156]. Non-neutralizing antibodies have been shown to induce Fc receptor-mediated phacocytosis and inhibit HIV-1 infection of macrophages [162]. In addition, antibody coating in the absence of neutralization may explain the high concentrations of immune complexes in HIV positive individuals with low serum neutralization titers [163,164]. Virus coating with non-neutralizing antibodies may encourage trapping of these complexes on the surface of follicular dendritic cells by prolonging their persistence [165, 166]. Furthermore, because these mAbs were generated from the IgA repertoire of the cervical mucosa, the ability to bind directly to the virus may have an increased correlation with protection attributable to the ability of mucosal IgA to trap virus in the mucosa by virion cross-linking and immune exclusion [78, 80].

4.4 Molecular Analysis of the gp41 Specific MAbs

Antigen specificity is largely encoded by the 6 CDRs, 3 on the VH and 3 on the VL. X-Ray diffraction studies have shown that the CDR3 of the VH region is integral to antigen binding in most mAbs due to its position at the center of the antigen binding site [147]. The role played by the light chain in the antigen specificity of an antibody is variable. Some antibodies require a specific VL region to maintain both functionality and antigen specificity, while antibodies in which the VH region is largely responsible for antigen binding can incorporate many different light chains [125,134]. While examining the HIV-1 mAbs isolated from phage display libraries, both Burton et al (1991) and Binley et al (1996) observed that the same heavy chain

could be paired with different light chains and maintain antigen specificity [125,134]. This light chain promiscuity suggests that the heavy chain plays a dominant role in antigen binding to gp120 and gp41. Accordingly, chain swapping experiments conducted with the heavy and light chains isolated from the present antibody library also displayed light chain promiscuity, as all four gp41 specific heavy chains could be paired with at least 3 and as many as 5 different light chains and maintain affinity to gp41. This finding is also in agreement with the previously described role of the heavy and light chain for the gp41 clones 69 and 77 described by Tudor et al (2009), and for the bNt mAb 2F5 [104,148].

Extensive antigen-driven maturation is essential for the development of neutralizing antibodies against HIV-1. The majority of HIV-1 bNt mAbs have a high frequency of somatic mutation associated with a marked increase in replacement mutations indicating antigen-driven affinity maturation has occurred [168]. Accordingly, the VH and VL of all four mAbs generated herein were shown to have extensive somatic mutations when compared with the most homologous germline sequence. Consistent with antigen-driven affinity maturation, clones, H4/L9 and H9/L2 have high replacement to silent nucleotide exchange ratios in the CDRs compared with the FRs. Furthermore, when the multinomial model developed by Losos et al (2000) to predict the probability of antigenic selection was applied to the these clones, both had P_m values that suggested there was an extremely low probability that the observed excess of R mutations in the CDRs and S mutations in the FRs occurred by random mutation [142]. While this model does not take into account intrinsic biases caused by mutation hotspots in the VH and VL, it does

provide a good approximation of the degree of antigen-driven affinity maturation experienced by a given antibody gene [142, 169]. Alternatively, these individuals may be polymorphic in the VH locus and their V genes may be inherently different at the DNA level in the germline [105]. To eliminate this possiblility, VH sequences need to be examined from chromosomal DNA.

It has been suggested that HIV has evolved a protective mechanism that shields its most vulnerable epitopes, including the CD4 binding site and the MPER, by exploiting "holes" in the human germline antibody repertoire [167]. This was proposed by Xia et al (2009) who showed that germline predecessors of bNt mAbs b12, 2G12 and 2F5 lacked measurable binding to Env, while the predecessors of weakly neutralizing mAbs showed high affinity to Env [167]. Consequently, the predecessors of bNt mAbs may be unable to bind strongly enough to Env during the initial stages of infection to initiate their development via antigen-driven affinity maturation [167]. This suggests that the continued B cell exposure to viral antigens that occurs during a chronic HIV-1 infection is an important factor in the development of a broadly neutralizing antibody response [167]. Discouragingly, by the time the antibody response has undergone sufficient antigen-driven affinity maturation to target these vulnerable epitopes, the infection is far too advanced for the antibodies to have any significant effect. However, if HIV seronegative individuals are subject to repeated exposed to HIV antigens at mucosal sites, it could result in the generation of affinity-matured HIV-1 specific mucosal IgA antibodies at the site of initial contact with the virus [104]. The presence of affinity-matured IgA at mucosal sites may provide the best chance at preventing initial infection, as once

the virus has travelled through the mucosal epithelium, the chances of eliminating the infection are remote.

The study population where these antibodies have the best chance at being found are the HEPS individuals. These individuals are repeatedly exposed to viral antigens at mucosal sites through unprotected intercourse with HIV positive individuals (estimated at 64 viral encounter per year in the Nairobi cohort [73]). Accordingly, Tudor et al (2009) isolated several IgA antibodies from the cervix of HEPS sex workers from Cambodia, specific for gp41, that show extensive somatic mutations and appear to have undergone antigen-driven affinity maturation [104]. These mAbs were also able to block epithelial cell transcytosis and neutralize CD4+ T cell infection, suggesting that they are specific for susceptible epitopes on gp41 [104]. As mentioned above, the gp41 mAbs generated, herein, from the cervical IgA repertoire of HEPS women in Nairobi appear to have been somatically mutated, with two of the clones showing strong evidence of being subject to extensive antigen-driven affinity maturation. Whether or not these mAbs bind with susceptible epitopes on gp41 and play a role in HIV-1 resistance is yet to be determined.

4.5 Path Forward

Attempts to produce all four gp41 mAbs on a large scale have so far been unsuccessful. This is most likely due to inefficiencies in the vector expression system, as it was not designed for large scale transfections, and problems with antibody purification. However, efforts to modify the expression/purification

system to allow for large scale expression of the 4 mAbs continues. Testing all 4 mAbs in standard HIV-1 CD4+ T cell neutralization assays is of the highest priority. Furthermore, because the mAbs were isolated form the IgA repertoire of cervical B cells, transcytosis assays also need to be performed. Once any *in vivo* effector functions have been identified for the 4 mAbs, epitope mapping should be carried out. Due to the linear nature of the epitopes recognized by the four mAbs, an ELISA against a set of gp41 peptides should elucidate the gp41 epitope recognized by each. Furthermore, competition ELISAs with known gp41 mAbs such as 2F5, and 4E10 are underway. Identifying the epitopes important in eliciting protective IgA affinity-matured antibodies is critical to the development of a mucosal vaccine capable of eliciting a local antibody response that prevents HIV-1 infection at the point of entry.

Attempts to generate microbicides with the goal of preventing HIV-1 transmission have so far been unsuccessful. Efforts to generate the next generation of microbicides based on antiretroviral agents is underway. This approach, however, is likely to become harmful to women over the long term due to antiretroviral drug induced changes in vaginal and virological parameters that could lead to increased rates of viral infection [104, 170]. Natural agents, such as affinity-matured HIV-1 neutralizing IgA antibodies, may be among the safest and most effective components of an effective microbicide. These antibodies, provided they possess potent effector functions, would prevent free virus from invading the mucosa, and prevent viral release from infected cells [104]. Indeed, vaginal administration of the bNt mAb b12 has prevented vaginal transmission of SHIV in

macaques [171]. The characterization of existing affinity matured Env specific IgA mAbs, and the discovery of additional clones is necessary for the continued progress of antibody based HIV-1 microbicides.

4.6 Conclusion

A mammalian cell display system has been developed for the generation of fully human recombinant monoclonal antibodies. Using this system, four novel gp41 monoclonal antibodies were isolated from the IgA repertoire from cervical B cells obtained from two HEPS sex workers from Nairobi, Kenya. All four mAbs bind to predominantly linear epitopes on gp41, and three of the clones demonstrated affinity for HIV-1 Env clade B and clade C expressed on the surface of transfected cells. The two clones showing the highest affinity for Env also appear to have undergone extensive antigen-driven affinity maturation. The presence of these gp41 specific antibodies in the cervix of HEPS sex workers from Nairobi confirms there are HEPS commercial sex workers in Nairobi that have cervical IgA specific for the HIV-1 envelope protein gp41 despite repeatedly testing seronegative. Further characterization of the effector functions of these mAbs is required to determine their role in HIV-1 resistance.

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Appendix I

PRIMER	SEQUENCE 5′ - 3′
Fr-1 VH1,3	CAGGTGCAGCTGCTCGAGTCTGG
Fr-1 VH2	CAGGTCACCTGGCTCGAGTCTGG
Fr-1 VH4	CAGGTGCAGCTGCTCGAGTCGGG
Fr-1 VH4b	CAGGTGCAGCTACTCGAGTGGGG
Fr-1 VH5	AAAAAGCCCGGGGAGTCTCTGARGA
Fr-1 VH6	CAGGGTACAGCTGCTCGAGTCAGG
IgA1,2 Reverse	CTAGTGACCTTGGGGCTGGTCGGGGATGC
IgA1 Reverse	AGTTGAACTAGTTGGGCAGGGCACAGTCAC
IgG1 Reverse	CATGTACTAGTTTTGTCACAAGATTTGGG
IgG2a Reverse	TCGACACTAGTTTTGCGCTCAACTGTCTT
IgG3 Reverse	TGTGTGACTAGTGTCACCAAGTGGGGTTTT
IgM Reverse	CTCACACTAGTAGGCAGCTCAGCAATCAC
IgK-V1	TACGGGGAACTCCAGATGACCCAGTCTCC
IgK-V2,4	TACGGGGAACTCGTGATGACYCAGTCTCC
IgK-V3	TACGGGGAACTCGTGWTGACRCAGTCTCC
IgK-V5	TACGGGGAACTCACACTCACGCAGTCTCC
IgLam-V1a	TACGGGGAACTCGTGBTGACGCAGCCGCCCTC
IgLam-V1b	TACGGGGAACTCGTGCTGACTCAGCCACCCTC
IgLam-V2	TACGGGGAACTCGCCCTGACTCAGCCTCCCTCCGT
IgLam-V3	TACGGGGAAGCTCGAGCTGACTCAGCCACCCTCAGTGTC
IgLam-V4	TACGGGGAACTCGTGCTGACTCAATCGCCCTC
IgLam-V6	TACGGGGAACTCATGCTGACTCAGCCCCACTC
IgLam-V7,8	TACGGGGAACTCGTGGTGACYCAGGAGCCMTC
IgLam-V9	TACGGGGAACTCGTGCTGACTCAGCCACCTTC
IgLam-V10	TACGGGGAACTCGGGCAGACTCAGCAGCTCTC
VjKRev1	TTTGATTTCCACCTTGGTCCC
VjKRev2,4	TTTGATCTCCAGCTTGGTCCC
VjKRev3	TTTGATATCCACTTTGGTCCC
VjKRev5	TTTATCTCCAGTCGTGTCCC
VjLamRev1236	GCCTAGGACGGTCASCTTGGTSCC
VjLamRev4	GCCTAAAATGATCAGCTGGGTTCC
VjLamRev57	GCCGAGGACGGTCAGCTSGGTSCC
VH12346EXT	GCACTTGTCACGAATTCGATATCCGTGCAGCTGCTCGAGTCDGG
VH4bEXT	GCACTTGTCACGAATTCGATATCGCAGGTGCAGCTACTCGAGTGGG
VH5EXT	GCACTTGTCACGAATTCGATATCGAAAAAGCCCGGGGAGTCTCTGARGA
IgGbpEXT	GCGCAGACGGGAAGACCGATGGGCCCTTGGTGGA
IgA12bpEXT	GCGCAGACGGGAAGACCGATGGGCCCTTGGTGGAGCTGGTCGGGGATGC
KappaFext	GCACTTGTCACGAATTCGATATCGTACGGGGAACTCVHRHTSACNCAGTCTCC
LambdaFext1	GCACTTGTCACGAATTCGATATCGTACGGGGAASYYV
LambdaFext2	GCACTTGTCACGAATTCGATATCGCTGCTGACTCAGCCACC
VjKRev1-X	AGCCACAGTACCCATGGCTTTGATTTCCACCTTGGTCCC
VjKRev2,4-X	AGCCACAGTACCCATGGCTTTGATCTCCAGCTTGGTCCC
VjKRev3-X	AGCCACAGTACCCATGGCTTTGATATCCACTTTGGTCCC
VjKRev5-X	AGCCACAGTACCCATGGCTTTAATCTCCAGTCGTGTCCC
VJL1236RevX	AGCCACAGTACCCATGGGCCTAGGACGGTCASCTTGGTSCC

VJL4RevX	AGCCACAGTACCCATGGGCCTAAAATGATCAGCTGGGTTCC
VJL57RevX	AGCCACAGTACCCATGGGCCGAGGACGGTCAGCTSGGTSCC
VJL1236-link1	ACCAAGSTGACCGTCCTAGGCCAGCCCAAGGCTGCCCCCTCGGTC
VJL4-link1	ACCCAGCTGATCATTTTAGGCCAGCCCAAGGCTGCCCCCTCGGTC
VJL57-link1	ACCCAGCTGATCATTTTAGGCCAGCCCAAGGCTGCCCCCTCGGTC
VJL1236-link2	GGGGGCAGCCTTGGGCTGGCCTAGGACGGTCASCTTGGTSCC
VJL4-link2	GGGGGCAGCCTTGGGCTGGCCTAAAATGATCAGCTGGGTTCC
VJL5,7-link2	GGGGGCAGCCTTGGGCTGGCCGAGGACGGTCAGCTSGGTSCC
IgGconF	CCTCCACCAAGGGCCCATCGGTCTTCCCCCTGG
Link PCR3-g	GTCCTGGCCCACAGCATTCTCTCTCCCCTGTTGAAGCTCTTTGTGAC
PDGFR-rev	AGATTACTATGCTAGCCTAACGTGGCTTCTTCTGCCAAGC
Link PCR4-g	AGCCTCTCCCTGTCTCCGGGTAATGCTGTGGGCCAGGACACGC
IL2ssVrecovF	GCACTAAGTCTTGCACTTGTCACG
KcVrecovR	CGGGAAGATGAAGACAGATGGTGC
LcVrecovR	CGGGAACAGAGTGACCGAGGGGGC
GcVrecovR	GGGAAGACCGATGGGCCCTTGGTGGA
PdbLiRevSEQ	GTATCTTATCATGTCTGGCCAGCTAGC
LamConF	CAGCCCAAGGCTGCCCCCTCGGTC
LamConR	TGAACACTTCGTAGGGGCAACTGTTTCCTCCAC
4LFext	GCACTTGTCACGAATTCGATATCGGAGCTCACGCAGTCTCCAGGC
4LRext	TTATCATGTCTGGCCAGCTAGCCTAACACTCTCCCCTGTTGAAGC
B12VHFext	GCACTTGTCACGAATTCGATATCCCTCGAGCAGTC
pIgGmut1F	GGGGGGTTGGCGCTGACTAGTCAAAA
pIgGmut1R	CTAGTCAGGGCCGCAACCCCCCCAA
pIgGmut2F	GGGGGGTTGGGGCGCTGACTAGTCAAA
pIgGmut2R	TTTTGACTAGGTCAGCGCCCCAACCCCCC
pIgGmut3F	GGGGGGTTGGGGCCGTGACTAGTCAAACA
pIgGmut3R	TGTTTTGACTAGTCACGGCCCCAACCCCCC
LamConFext	ATGCGATTCATACCATGGCAGCCCAAGGCTGCCCCCTCGGTC
LamConRext	ATGCGATTCATAGCTAGCTGAACACTTCGTAGGGGCAACTGTTTCCTCCAC
SeqF1	ATGTACAGGATGCAACTCCTGTCTTGC

* Underlined portions of sequence indicate the location of the restriction endonuclease site indicated in the text

Appendix II

Plasmid	Backbone	Isotype	Restriction Enzymes	Mammalian Resistance	Alterations
P-dbIgG1	pFuse	IgG CH1-3	EcoRV, ApaI	Zeocin	2nd ApaI site mut
P-dbKappa	pFuse	IGKC	EcoRV, NcoI	Zeocin	N/A
P-dbLambda	pFuse	IGLC	EcoRV, NcoI	Zeocin	N/A
P-dbIgG1-TM	pFuse	IgG CH1-3	EcoRV, ApaI	Zeocin	PDGFR-TM at 3′ CH3