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Focusing immune response on key neutralizing epitopes of HIV- 1 through immune complex vaccination

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Focusing immune response on key neutralizing epitopes of HIV- 1 through immune complex vaccination

by

Aditi Agrawal

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics and Genomics

Program of Study Committee:
Michael Cho, Major Professor
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Ames, Iowa

2016

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DEDICATION

Dedicated to my dad, my role model and inspiration.

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ABSTRACT

Since its discovery in 1981, HIV-1 has infected almost 78 million people, and ~39 million people have died. An effective vaccine for HIV-1 remains an utmost priority. In the past several years, tremendous progress has been made in designing different immunogens and employing vaccination strategies, with the common goal of eliciting broadly neutralizing antibodies (bnAbs) and/or T cell responses against HIV infection. Several bnAbs have been isolated from patients, suggesting that the humoral immune system is capable of making such antibodies. Unfortunately, none of the immunogens and vaccination strategies has elicited bnAbs till date, and thus a tremendous amount of work still lies ahead. Here, we evaluated an immune complexing vaccination strategy in rabbits to focus the immune response towards critical neutralizing epitopes on HIV-1 by masking the hypervariable, immunodominant V3 loop on gp120 with a rabbit monoclonal antibody (mAb).

Our findings indicate that although the humoral immune response did not increase in immune complex vaccinated rabbits as compared to gp120 alone group, we still suppressed the V3-specific antibody, as seen by antigen specific antibody titer and fine linear epitope mapping. Also, competition of immune complex sera against V3 loop-neutralizing antibodies (nAbs) confirm repressed antibody activity towards this epitope. Although, immune complex vaccinated rabbits failed to elicit neutralizing antibodies, we have demonstrated the proof of concept and feasibility of this approach. Further evaluation of this strategy using alternate immunogens like BG505 SOSIP gp140 and eOD-GT8 will be carried out in the near future.

CHAPTER I

GENERAL INTRODUCTION

Thesis Organization

This thesis is organized into four chapters. Chapter 1 is a general introduction providing literature review on HIV-1, the disease pandemic, envelope glycoprotein based immunogens and vaccine development against HIV-1. Chapter 2 is the evaluation of an immune complexing strategy for HIV-1 vaccine development. I performed all the experiments, analyzed the results and recorded them. Chapter 3 provides general conclusions and the future directions. Appendix includes “Characterization of a large panel of rabbit monoclonal antibodies against HIV-1 gp120 and isolation of novel neutralizing antibodies against the V3 loop”. This is a manuscript published in PLOS ONE journal. I contributed in planning, carrying out experiments (fig 1, 2, 5 and 6) and evaluation of results. I also assisted in writing the manuscript.

Introduction

Human Immunodeficiency Virus

Human immunodeficiency virus, abbreviated HIV, is the etiologic agent of acquired immunodeficiency syndrome (AIDS), which is a sexually transmitted disease. It belongs to the family *retroviridae*, comprising RNA viruses which can convert their genomic RNA to a DNA for during their “life cycle”, and genus *Lentivirus*, known for their longer incubation periods, as the virus can remain in latent phase in the host for years without progressing into actual disease [1]. The first case of AIDS was reported in US in 1981[2]. HIV was isolated and recognized as causative agent of AIDS in 1983 [3].

HIV has two subtypes: HIV-1 and HIV-2. HIV-1 is the most virulent strain and is responsible for the pandemic throughout the world, while HIV-2 is limited to Western and Central Africa [4]. HIV-1 is divided into the following groups: M (Major), O (outlier), and N (non-major or outlier) and P. Group M accounts for most of the HIV-1 infections, which is further classified into nine major clades: A, B, C, D, F, G, H, J and K; as well as circulating recombinant forms (like CRF A/B, which is a combination of clade A and B) [5].

HIV-1 infects CD4 positive cells like T helper cells, monocytes, macrophages, dendritic cells, etc.; which are important to combating infections [6]. The immune system of the host becomes severely compromised due to rapid depletion of CD4 cells, leading to the condition termed AIDS. During this stage, the patients are prone to opportunistic infections such as Kaposi's sarcoma, lymphoma, tuberculosis, and pneumonia.

Almost 78 million people have been infected with HIV, and ~39 million people have died since the discovery of HIV in 1981. There were nearly 37 million people living with HIV-1 globally (2.6 million children) by the end of 2014 with 2 million new infections every year [7]. HIV-1 is listed as one of the top 10 leading causes of death [8]. Current anti-retroviral therapies can decrease the viral load present in the latent reservoirs but cannot cure or eradicate the virus completely. Therefore, many scientists hypothesize that an effective vaccine is the best way to control the HIV pandemic.

Structure of HIV-1

HIV-1 is an icosahedral shaped retrovirus containing two copies of single stranded positive sense RNA molecules as genetic material. The genetic material of the virus encodes for structural genes (gag, pol, env); regulatory/accessory genes (tat, rev, nef, vpr, vpu and

vpx) and other enzymes required for viral replication (reverse transcriptase, integrase and protease). The virus comprises of an outer envelope glycoprotein, which is made up of two non-covalently linked subunits, gp120 and gp41, which then associate as trimeric spikes on the viral surface as shown in Figure 1. The viral glycoprotein is initially synthesized as a gp160 polyprotein, which undergoes cleavage by a cellular protease into gp120 and gp41 [9].

The envelope gp120 subunit is comprised of an inner domain, an outer domain and a bridging sheet which links the two. It consists of five variable loops (V1-V5), which intervenes five conserved regions (C1-C5). Gp41 consists of an external domain, a transmembrane domain, and a cytoplasmic tail [10]. The viral envelope surrounds the virus core consisting of capsid (p24) and nucleocapsid (p7). The viral matrix (p17) is situated between viral core and envelope. Gp120 initiates the infection by binding to cell surface receptors, and gp41 mediates the fusion of viral and host cell membrane [11-13].

Difficulties in developing an effective vaccine against HIV-1

Despite tremendous efforts and progress in the field, an effective vaccine against HIV-1 still remains an utmost priority and yet an elusive goal. Science and technology have provided in depth knowledge about HIV-1 and the molecular mechanism involved in virus entry, but still there is no vaccine available.

HIV-1 has evolved various immune evasion strategies to overcome the host immune response, which therefore overcomes the effects of drug therapies and vaccines. HIV-1 is known for the extensive amount of genetic variation present in multiple subtypes, clades and circulating recombinant forms due to the error-prone nature of reverse transcription [4,14,15]. HIV-1 strains belonging to same subtypes can differ by 20% in their amino acid

sequence, and that can increase up to 35% within a group [14]. This amino acid variability also presents a major challenge for virus neutralization because different strains have different sensitivities to neutralization based upon sequence differences, with Tier-1 viruses the most sensitive and Tier-3 the most resistant [16]. Neutralizing antibodies (nAbs) are directed towards conserved epitopes like the CD4 binding site (CD4bs) and the co-receptor binding site, which are hidden from the immune system due to conformational masking by flanking variable loops [17] and extensive glycosylation [18,19]. Also, gp120 is easily shed from the surface, causing elimination of conserved neutralizing antibody epitopes and exposure of non-neutralizing epitopes [20]. Different conformations of HIV-1 env can lead to poor interactions with low affinity naïve B cell receptors [21]. Finally, the low number of trimeric spikes (8-12) on the virion can also limit the avidity and antibody based neutralization of the virus [22].

Broadly neutralizing antibodies

Broadly neutralizing antibodies (bnAbs) can neutralize HIV strains belonging to different groups thus providing cross clade neutralization. The passive administration of bnAbs could provide protection in non-human primate models by decreasing viral load and increasing CD4 cell count [23-27]. Therefore, bnAbs have the potential to provide effective protection, and eliciting these antibodies remains crucial goal for stopping the viral pandemic.

However, all the immunogens and vaccine approaches till date have failed to induce bnAbs in vaccinated animals and humans. The ability of HIV-1 to evade the host immune response presents a major hurdle in field of vaccine development as described above. Most

bnAbs demonstrate unusual characteristics like extensive somatic hypermutation from their respective germlines and long heavy chain complementarity-determining region 3 (HCDR3) [28,29] as these antibodies undergo complex affinity maturation pathways and selection. Moreover, exposure of the virus to the host system can provide a different microenvironment for evolution and maturation of such bnAbs, which might be difficult to replicate in vaccine settings. This suggests that vaccination must guide the antibody maturation pathways precisely to elicit such bnAbs.

Broad neutralizing antibodies recognizing different regions on viral envelope

Despite all the hurdles and challenges posed by the virus, approximately 20% of patients [30,31] develop cross-clade neutralizing antibodies (nAbs) during the chronic phase of HIV infection (~2-4 years) [32]. Only rare individuals (~1%) are ‘elite neutralizers’ who develop true bnAbs, suggesting that the humoral immune system can successfully prevent HIV-1 infection [33]. Many bnAbs have been isolated from HIV-1 infected patients. First-generation bnAbs were isolated via phage display and Epstein-Barr virus transformation and had limited neutralization breadth [34,35]. Recently, new approaches like B-cell capture and single cell antibody cloning have isolated next-generation bnAbs, which have more potent and broad neutralizing ability [36-38].

These bnAbs recognize four main regions on the viral envelope: CD4bs, epitopes associated with N-linked glycans on V1/V2 and N-linked glycans on V3 loop and membrane proximal external region (MPER) on gp41 (Figure 2) (as reviewed in [39,40]). Recently, epitopes associated with N-linked glycans on gp120/gp41 bridging regions have been discovered [41-43].

Discovery of bnAbs has provided meaningful insight into conserved neutralization sites. Molecular modeling, co-crystallization studies and electron microscopy studies have provided valuable information regarding bnAb epitopes [29,44]. Understanding how these bnAbs recognize their epitopes has been an asset in designing next-generation vaccine immunogens. Many groups have tried to engineer env-based immunogens and design vaccination strategies with the goal of eliciting bnAbs. To date, no study has elicited such bnAbs (as reviewed in [45]).

Peptide based immunogens

Early immunogens tested in the HIV-1 field were env derived short peptides based on the V3 loop of gp120 [46-49] and MPER in gp41 [50-52]. Unfortunately, vaccination with these immunogens resulted in nAbs against Tier-1 viruses only. We now know that epitopes recognized by bnAbs are highly conformational, and hence peptide based immunogens might never elicit bnAbs.

Epitope scaffolds

Epitope scaffold immunogens containing the V3 loop, V3 glycan epitopes, CD4bs epitopes (b12) or MPER epitopes (2F5, 4E10) engineered on heterologous protein scaffolds were evaluated in the field to elicit conformation-dependent antibodies [53-61].

Unfortunately, results from these immunizations were disappointing and failed to mount an effective neutralizing response in animals.

Gp120 outer-domain (OD) based vaccine immunogens

Gp120 comprises of an inner domain (ID), an outer domain (OD) and a bridging sheet [12]. The immune response elicited by highly immunodominant ID is mostly non-neutralizing [62,63]. Therefore, researchers removed the ID to generate OD immunogens. The majority of past studies using different versions of OD have been unsuccessful [64-69]. Recently, we reported two immunogens, OD and ODx3, designed based on an M group consensus sequence (MCON6). Both immunogens elicited cross-reactive neutralizing antibodies against clade B, C, and AE; and even though the neutralizing breadth was restricted to Tier-1 viruses, the cross reactivity observed was better than reported by previous groups [70].

A new class of engineered outer domain (eOD) immunogens was designed by modifying the CD4bs to enhance the binding of CD4bs bnAbs, like VRC01 and 3BNC60, and their germline precursors [71]. Further optimization gave rise to eOD-GT6, which when multimerized on virus-like nanoparticles could crosslink and activate B cells specific for germline VRC01. Immunogenicity studies were carried with an improved version of this protein (eOD-GT8) in knock-in mice carrying the heavy chain gene for 3BNC60. Mice sera demonstrated strong neutralization only against autologous viruses. Modest neutralization was seen against Tier-2 viruses lacking glycosylation at position 276 [72].

Gp120 core immunogens

To effectively present the CD4bs and induce CD4bs bnAbs, the gp120 core was stabilized by removing the variable loops (V1, V2, V3), and sometimes the amino and carboxy termini are also removed [73,74]. Although humoral immune response against

CD4bs increased, no efficient Tier-2 nAbs responses were observed [73]. Another immunological study with gp120 core proteins lacking major variable loops and masked with N-linked glycans also did not induce cross-clade bnAbs [75]. Furthermore, rabbits immunized with stabilized gp120 trimer bound to CD4 elicited more potent nAbs than the wild type monomer but still failed to induce Tier-2 nAbs [76].

Subunit protein immunogens

The virus env is the sole protein exposed on the surface and therefore the only available target for vaccine design. The safety of recombinant gp160 was tested in uninfected human volunteers. The vaccine was safe and induced T cell responses, but the neutralizing antibodies were limited [77-80]. Recombinant gp120 in particular, has been evaluated extensively as an immunogen in vaccine studies to determine if they provide protection and induce nAbs [77,81-87]. Unfortunately, results from Phase I clinical trials demonstrated that monomeric gp120 elicited nAbs only against laboratory adapted Tier-1 virus strains but not against circulating primary virus isolates [86]. Only a few vaccine trials ever advanced to Phase IIb (Step/Phambili, HVTN 505) and Phase III (VAX003, VAX004, RV144).

The Step/Phambili trials evaluated recombinant Adenovirus vector (Ad) expressing Gag, Pol and Nef and Env genes. The Step trial, also known as Merck 023/HVTN 502, was conducted in America, Australia and Caribbean; and the Phambili trial, also known as HVTN 503, was conducted in South Africa. Both trials were based on promising results in pre-clinical studies in non-human primate models (efficacy and cellular immunity) [88]. Unfortunately, both trials were unsuccessful in decreasing HIV-infectivity and viral plasma

loads in human subjects. The vaccinations for the Phambili trial were halted based on results from the Step trial, which demonstrated increased virus infectivity in some subjects [88-90].

AIDSVAX B/B (Vax004 trial) evaluated high doses of gp120 from two HIV-1 subtype B viruses [91,92] and AIDSVAX B/E (Vax003 trial) evaluated recombinant gp120 from MN and A244 (CRF01-AE) [93]. Unfortunately, these Phase III trials failed to provide protection, and neutralization was primarily limited to the Tier-1 MN strain with weak neutralization detected against primary isolates. This indicated that nAbs raised in response to vaccines might not provide effective protection against a wide range of HIV strains.

The most recent efficacy trial is HVTN 505, which tested a DNA prime with plasmids expressing HIV-1 clade B gag, pol, nef and env protein from multiple clades (A, B and C). The booster consisted of recombinant Ad5 vector expressing HIV-1 clade B gag, pol and env protein as stated. Unfortunately, the viral plasma loads and infectivity remained unaffected, and trial ceased in April 2013 [94].

The only trial to date which decreased the rate of HIV-1 infection, was the RV144 trial conducted in Thailand. Human subjects were primed with recombinant canarypox vector encoding gag, pol, and nef (ALVAC-HIV [vCP1521]) and membrane anchored gp120, and subsequently boosted with gp120-MN and gp120-A244. There was a decrease in HIV-1 infection by 31% in vaccine recipients, but viral plasma loads and CD4+ T cell count remained unaffected [95].

To conclude, subunit protein immunogens were safe and immunogenic but failed to demonstrate cross clade neutralization. Also, humoral immune response was higher when subunit immunogens were immunized in a heterologous prime boost vaccination regimen than alone. One of the major drawbacks of using monomeric gp120 includes shielding of

conserved epitopes by variable loops (e.g hypervariable immunodominant V3 loop) presenting decoy epitopes, which ultimately result in the elicitation of strain specific or non nAbs [96,97]. As many bnAbs bind to more native functional env rather than gp120, functional env might be more relevant to neutralization induction [98]. Therefore, researchers are now pursuing immunogens capable of eliciting neutralizing antibody responses against more resistant Tier-2 viruses. Although little progress has been made, prior research has provided valuable insight into optimization of dose, immunization schedule, choice of adjuvant and vector.

Soluble envelope trimers (Non-native trimers)

Since recombinant viral vectors expressing subunit proteins did not induce bnAbs, researchers required immunogens which better represented the native structure of the virus. Also, antibodies elicited in gp120 immunized animals demonstrated very weak binding to the trimeric env. It is possible that surfaces exposed on monomeric gp120 might be neutralization irrelevant as compared to the native trimer [99,100]. Therefore, trimeric env antigens might be a more useful vaccine immunogens [98].

Soluble env trimers (gp140) represent a potential solution to this problem. Soluble env trimers comprises of gp120 and gp41 ectodomain, which are generated by env truncation removing the gp41 cytoplasmic and transmembrane domains. However, truncation of env results in instability, causing misfolding and subsequent lack of native conformation [101]. The stabilization of soluble trimers has been achieved either by modifying the gp120-gp41 cleavage site to prevent dissociation resulting in uncleaved trimers (gp140_{UNC}) [142] or by fusing the env with trimerization motifs [102-105].

The immunogenicity studies conducted with soluble env trimers have demonstrated better neutralizing antibody responses as compared to gp120, but they were still incapable of eliciting bnAbs [106-110]. In human vaccine trials, these soluble trimers failed to generate antibody responses against heterologous Tier-2 viruses [111,112]. This might be due to misfolding of trimer caused by aberrant disulfide bond formations [113,114]. Also, structural rearrangements in trimer can lead to an open conformation exposing the non-nAb epitopes [115,116].

Native-like trimers

Another approach used by researchers to stabilize the cleaved soluble trimers is the introduction of a disulfide bond (SOS) to covalently link gp120 and gp41 ectodomain [101]. To further improve the trimerization, an Ile-to-Pro mutation (I559P) was introduced in gp41 (together termed ‘SOSIP’) [117]. Immunogenicity studies with these SOSIP trimers from subtype clade A induced better responses than gp120, demonstrating weak neutralization against autologous Tier-2 viruses in rabbits [118,119]. SOSIP trimers were further improved by removing the hydrophobic MPER region by a truncation at position 664 (SOSIP.664) [112,120]. Combining clade A Env , BG505 (derived from an infected infant) [121] with SOSIP.664 resulted in BG505 SOSIP.664 [122]. BG505 SOSIP.664 trimers are true mimics of native viral trimeric spike and have increased solubility and stability, and can bind bnAbs but few non nAbs [112,122,123]. Native-like trimers from B41 (subtype B), ZM197M and DU422 (both subtype C), and other clades were also engineered using the same SOSIP.664 design [124-126]. Immunogenicity studies conducted with BG505 and B41 SOSIP.664 trimers demonstrated autologous Tier-2 nAb responses. However, no heterologous Tier-2

nAbs were seen, and the significant portion of antibodies were directed against the immunodominant V3 loop [127].

In conclusion, these functionally and structurally relevant envelope trimers serve as prototype for next generation of immunogens, but improvements are still needed to minimize the immune response against non bnAb epitopes and to direct the antibody response towards true bnAb epitopes.

Immune complex immunogens

Antigen and antibody complexes, referred to as an immune complexes, have been used as vaccine immunogens to augment the host immune response against various viral and bacterial pathogens, including, infectious bursal disease virus [128,129], hepatitis B surface antigen [130,131], dengue virus [132], ebola virus [133,134], porcine parvovirus [135], equine herpes virus [136], *Clostridium tetani* [137], *Mycobacterium tuberculosis* [138] and *Francisella tularensis* [139].

Augmentation of the host immune response has been mainly attributed to the interaction of antibody and antigen mediated via Fc receptors. The interaction of Fc region of the antibody with the FcRs on antigen presenting cells (APCs) facilitates the uptake, processing and antigen presentation to CD4⁺ T-helper cells, resulting in more effective T cell dependent antibody responses. Deposition of immune complexes on follicular dendritic cells increases the antigen concentration and half-life resulting in enhanced B cell proliferation and development of germinal centers [140-144].

Apart from augmentation of the immune response, antibodies can also alter the antigenicity of epitopes on immunogens. Antibodies can shield non-desired epitopes while

exposing conserved neutralizing epitopes. This is important, as it has been very difficult to direct the immune response towards conserved neutralizing epitopes on HIV-1. Hence, immune complex vaccination could represent a new avenue for vaccine development against HIV-1. The developments in the field for immune complex vaccination strategy are discussed in the next chapter.

Overall Objectives

The main objective of this study was to evaluate an immune complex vaccination strategy for HIV-1 vaccine development. We hypothesize that masking the immunodominant hypervariable V3 loop with mAbs might suppress the immune response against this epitope and focus the response towards the more highly desired broadly neutralizing epitopes (e.g. CD4bs). To test this hypothesis, we made immune complexes of gp120 and anti-V3 loop mAb, immunized rabbits with the immune complexes and performed serological characterization to confirm the hypothesis.

Figures

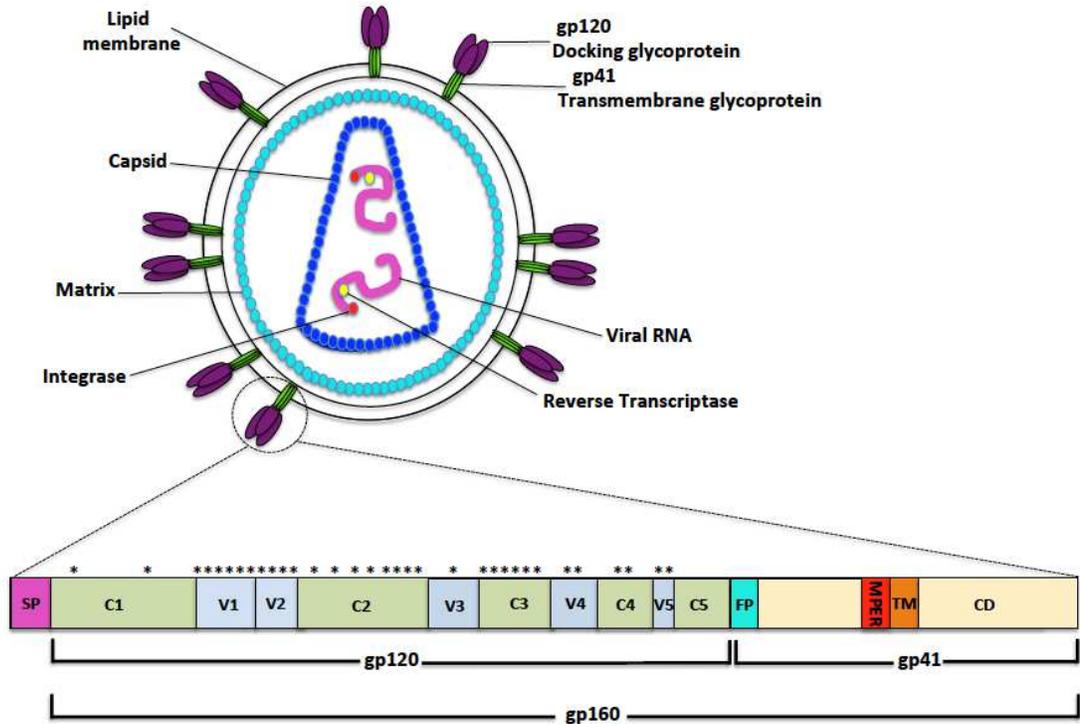


Figure 1. Diagram representing the structure of HIV-1 and envelope glycoprotein domains. Gp120 is made up of 5 constant domains (C1-C5, light green) and five variable loops (V1-V5, light blue). The signal peptide (SP) is pink. Gp41 (beige) comprises of external domain containing MPER (membrane proximal external region, red), transmembrane (TM, orange) and cytoplasmic domain (CD). Asterisks represent putative glycosylation sites on gp120.

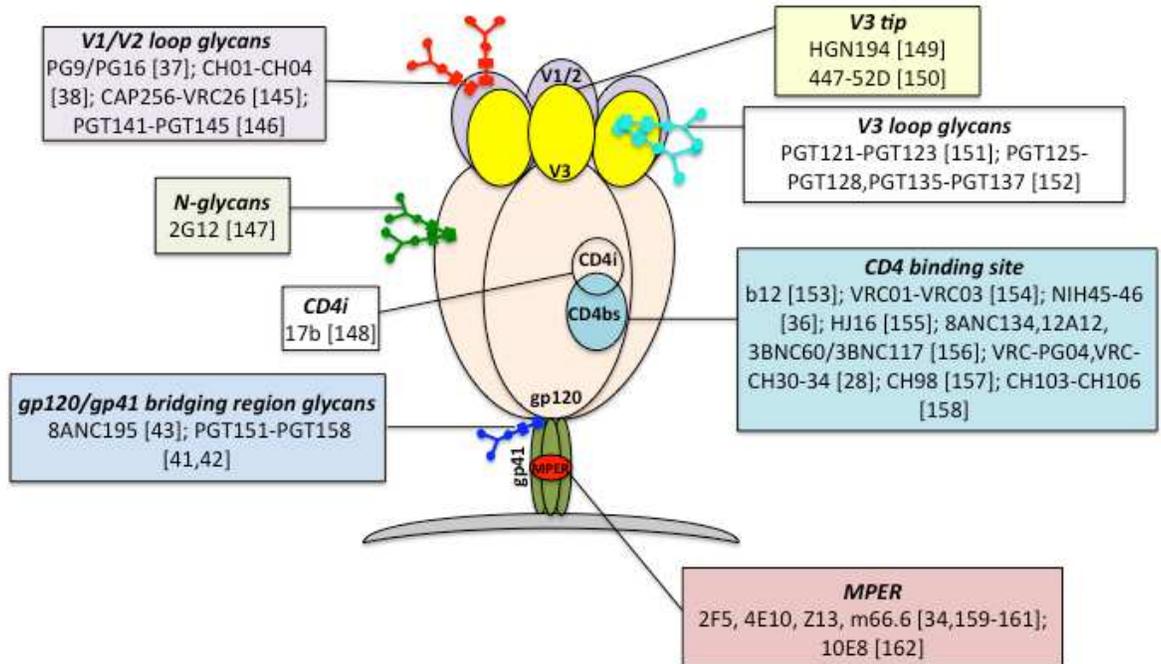


Figure 2. A diagram representing bnAbs discovered from HIV-1 infected patients, recognizing different regions on HIV-1 envelope glycoprotein (Adapted from [39,40]). Branch shaped structures represents glycans on HIV-1 env glycoproteins important for some bnAbs.

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CHAPTER II**EVALUATION OF IMMUNE COMPLEXING VACCINE STRATEGY FOR HIV-1
BY MASKING GP120 V3 LOOP****Abstract**

We evaluated an immune complexing strategy for HIV-1 vaccine development due to its capacity to augment the host immune response and to reduce immune system access to less desired epitopes. We proposed that masking the immunodominant hypervariable V3 loop with mAbs might suppress the immune response against this epitope and focus the response towards the more highly desired broadly neutralizing epitopes (e.g. CD4 binding site). To test our hypothesis, a pilot immunization study was carried out in four rabbits divided in two groups. The first group was immunized with gp120 alone, and the second group was immunized with an immune complex made of gp120 and the recently reported anti-V3 loop rabbit neutralizing mAb 10A37. While not as strong as that observed in gp120 alone, we still observed good humoral responses while suppressing the V3-specific total antibody titer in immune complex immunized rabbits, as seen by antigen specific antibody titer and fine linear epitope mapping. Antibody responses were further evaluated for competition against V3 loop monoclonal nAbs to confirm repressed antibody activity towards this epitope, and neutralizing ability was assessed to see whether the immune response was being focused towards desired critical neutralizing epitopes on HIV-1. Overall, this study demonstrates proof of principle and feasibility of immune complexing vaccination strategy.

Introduction

HIV-1 glycoproteins gp120 and gp41 are the sole antigens exposed on the viral surface and hence the only targets for vaccine development. There have been many attempts at designing vaccines based on subunit protein immunogens using gp120 for eliciting broad and potent neutralizing antibodies (bnAbs) against HIV-1. Unfortunately, immunization with subunit protein antigens alone has resulted in low antibody titers displaying limited neutralization breadth and potency. Also, “rationally-designed” immunogens including eOD-GT6/eOD-GT8 [1,2] and BG505 SOSIP gp140 [3] failed to induce bnAbs despite high hopes and expectations [2,3]. Thus, non-conventional vaccination strategies using novel immunogens that can elicit both cell-mediated and humoral immune responses as well as focus the immune response towards critical neutralizing epitopes, e.g. CD4 binding site (CD4bs), are needed. Therefore, vaccination using immune complexes has particularly drawn attention from its ability to modulate the immune response by shielding non-desired epitopes, and thus directing immune response towards desired conserved neutralizing epitopes [4].

The immune complex vaccination strategy has been somewhat explored for HIV-1. In an earlier study, guinea pigs were immunized with immune complexes made of recombinant gp120 and human mAb A32, an antibody that does not bind to CD4bs but instead increases the exposure of CCR5 binding epitopes on gp120 such that CD4bs is accessible. Although the immune complexes were immunogenic, no cross-clade neutralization was seen [5]. A second group also evaluated immune complex strategy by inoculating mice with different complexes made of gp120 and human mAbs binding different regions on gp120 (like CD4bs, C2 and V2 loop) to better target the neutralizing epitopes on the V3 loop [6-11].

In one study, the authors evaluated the immunogenicity of complexes prepared from gp120_{LAI} with 654-D, an anti-CD4bs neutralizing human mAb, in mice. High anti-gp120 antibody titers were observed as compared to uncomplexed gp120, particularly against the V3 loop due to antibody induced conformational changes. Unfortunately, the sera neutralization ability was limited to homologous viral strains and failed to neutralize heterologous strains [11]

In an attempt to generate cross-reactive neutralization, they later tested the immunogenicity of a complex made up of gp120_{JRFL}/654-D [6]. Since, JRFL strain expresses a V3 sequence from a consensus HIV-1 subtype B as compared to the V3 sequence from the LAI strain, serum from the immune complex immunized mice demonstrated increased neutralization towards homologous and heterologous strains, though the breadth was still limited to neutralization sensitive viruses.

Env glycoprotein gp120 is well known for its extensive glycosylation, which shields conserved neutralizing epitopes thus rendering HIV-1 neutralization resistant [12,13]. Thus, researchers have tried to remove N-linked glycans to improve the neutralizing ability [14,15]. In one study, authors evaluated the immunogenicity of different immune complexes made of gp120s mutants (N448Q and N448E) lacking N-linked glycans and 654-D (N448Q/654-D and N448E/654-D) to improve the antibody titers and potency [7]. Both immune complexes elicited higher antibody levels towards gp120 and the V3 loop as compared to the wild type complex. The elicited nAbs were primarily directed towards V3 loop, and non-nAbs were directed against the ID of gp120. Unfortunately, neutralization was limited to homologous viral strains and failed to demonstrate cross-clade neutralization, similar to what was observed in previous studies.

Thus, all of the immune complex studies elicited high antibody titers against the V3 loop overall. To understand the mechanism behind this immune modulation of V3 loop directed antibody response [9], authors compared the immunogenicity of different complexes made from gp120 and mAbs binding to different regions including the C2 region (1006-30), the V2 loop (2158) or the CD4bs (654-D). Although all three complexes elicited anti-V3 nAbs, the gp120/654-D complex was the most potent in terms of antibody titer suggesting the specificity of a mAb determines the immunogenicity of the complex.

To summarize, while the authors successfully demonstrated the modulation of gp120 antigenic responses by binding select anti-gp120 mAbs, neutralization breadth and potency was limited [8]. This might be due to a major limitation associated with their studies, which is using human mAbs to generate immune complexes for evaluation in mice. This might limit the full activation of the mouse immune system by hindering B-cell activation.

We propose to address this issue by using rabbit mAbs for immune complex vaccination in rabbits. We hypothesized that masking the undesirable, immunodominant epitopes (eg. V3 loop) with mAbs might suppress the immune response against this epitope and indirectly focus the response towards the more highly desired but less immunogenic broadly neutralizing epitopes (e.g. CD4bs) as explained in figure 1A. We initially focused our efforts on minimizing the immune response against the V3 loop. The V3 loop has been known as the principal neutralizing determinant, but elicits type specific nAbs exhibiting limited breadth [16,17]. This phenomenon has been observed consistently in both animals and humans. Furthermore, in our experience, we observed high antibody titers directed towards V3 loop in rabbits when immunized with a consensus sequence derived (MCON6) gp120 only [18], unlike some of the prior immune complexing studies using a specific strain

derived gp120 (Visciano 2008). To test this hypothesis, we performed a pilot study in which four rabbits were immunized with either gp120 alone or gp120 complexed with a recently reported anti-V3 loop rabbit nAb 10A37 [19]. We successfully demonstrated that masking the V3 loop with 10A37 significantly diminished the immune response against this epitope, suggesting the proof of concept and feasibility of this approach.

Material and Methods

Expression and purification of gp120

gp120 protein was expressed and purified using pcDNA*MCON6gp120 plasmid as previously described [18]. pcDNA*MCON6gp120 plasmid used for protein expression was derived from pcDNA-MCON6gp160 construct (kindly provided by Dr. Beatrice Hahn [20]). Briefly, FreeStyle 293-F cells were transfected with pcDNA*MCON6gp120 plasmid using 293fectin (Invitrogen, Life Technologies) as per manufacture's protocol. Four days post-transfection, the 293-F culture medium was harvested and protein was purified using Ni-NTA agarose beads. The eluted fractions of protein were concentrated using Amicon Ultraconcentrator (Millipore) and stored at -80°C until use.

Immune complex formation

Immune complexes were prepared at approximately 1:2 molar ratio of gp120 and 10A37 mAb in PBS by incubating the solution for 2 h at 37 °C. Rabbit mAb 10A37 used in the immune complex formation was produced in house [19].

Preparation of zinc (Zn)-chitosan adjuvant

Zn-chitosan was prepared as described elsewhere [18,21]. Briefly, 2% chitosan solution was prepared by dissolving 2 g of chitosan (Sigma, C3646) in 100 ml of 2% acetic acid followed by autoclave sterilization. Simultaneously, 0.2 M zinc acetate (Sigma, Z4540) solution was prepared and filter sterilized. The 2% (w/v) chitosan solution was diluted 1:1 using deionized water, and the resulting 1% chitosan solution was mixed 0.2 M zinc acetate at 2:3 molar ratio. The suspension was mixed on an end-to-end rocker for 3-4 h at room temperature. The mixture was then sonicated using a Branson Digital Sonifier for 5 min (63% Amplitude). After sonication, the pH of the solution was adjusted to 12.0-12.5 with 10N NaOH. Solution was centrifuged at 1000 x g for 10 min. Supernatant was discarded and the resulting pellet was vortex with 5-10 ml PBS. More PBS was added to this solution (~50 ml) and centrifuged at 1000 x g for 10 min for washing the pellet. This wash was repeated three times. Finally, the pellet was resuspended in PBS (pH 8). This suspension can be stored at room temperature for six months.

On the day of immunization, the final solution prepared above was vortexed followed by centrifuging at 1000 x g for 10 min. The supernatant was discarded and dry weight of the pellet was measured using a weighing balance. Antigen and Zn-Chitosan were mixed at 1:1000 w/w ratio at room temperature for 3 h on an end-to-end rocker prior to immunization [18].

Rabbit immunization and bleeding

Four female New Zealand White rabbits (2.5-3 kg; Charles River) were immunized subcutaneously with either 100 µg of gp120 protein alone or 100 µg of gp120 protein in

complex with 250 µg 10A37 mAb (1:3 molar ratio) on weeks 0, 3, 6 and 9 as shown in Fig. 1B. Due to low antibody titers seen after the first and second immunizations, antigens were supplemented with Zn-chitosan onwards from the third immunization as described above. Rabbits were bled two weeks after each immunization, except after fourth immunization where animals were bled after eight days instead of two weeks (Fig. 1B). All of the studies conducted were approved by IACUC at Iowa State University.

Enzyme-linked immunosorbent assay (ELISA) with protein or overlapping peptides

ELISAs were performed as previously described [18,19,22]. For determination of antibody titers, gp120 and V3 loop peptide were coated at 30 ng and 100 ng per well respectively onto 96-well Nunc-Immuno plates using an antigen coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 3 mM NaN₃, pH 9.6) at 4°C overnight. For linear epitope mapping, 100 ng per well of 15-mer linear overlapping peptides were coated similarly.

The next day, uncoated surfaces were blocked with 200 µl of a blocking buffer (PBS, pH 7.4, containing 2.5% skim milk and 5% calf serum) and incubated for 1 h at 37°C. Wells were subsequently washed five times with a wash buffer (PBS containing 0.1% Tween 20) using a Biotek automated plate washer.

For antibody titer, all rabbit sera were serially diluted in blocking buffer as indicated, and 100 µl was added to each well. For the V3 linear epitope mapping, all the rabbit sera were diluted 1:100 with blocking buffer (except after first immunization, where 1:50 dilution was performed), and 100 µl was added to each well. The plates were incubated for 2 h at 37°C. Wells were washed ten times as described.

Plates were incubated for 1 h at 37°C after addition of 100 µl of secondary antibody (goat anti-rabbit conjugated with horseradish peroxidase (HRP), Pierce; 1:3000 dilution with blocking buffer). Wells were washed ten times again followed by addition of 100 µl TMB HRP-substrate (Bio-Rad). The reaction was stopped after 10 min by addition of 50 µl of 2N H₂SO₄, and the plates were read at 450 nm on a microplate reader (Versamax by molecular devices). All assays were done in duplicate.

The 15-mer overlapping peptide set for gp120 based on an M group consensus sequence (CON-S) was obtained from the NIH AIDS Reagent Program (Cat# 9487). V3 peptide based on the MCON6 sequence (H-TRPNNTRKSIHIGPGQAFYATGEIIGDIRQAH-OH) was synthesized commercially by CHI Scientific (Maynard, MA).

Biotinylation of 10A37 mAb

Biotinylation of 10A37 mAb was performed using EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (Pierce, 21435) according to the manufacture's protocol. Briefly, 10mM Sulfo-NHS-LC-Biotin solution was prepared just before the use. 6.7 µl of 10 mM Sulfo-NHS-LC-Biotin solution was added to the 0.5 mg of 10A37 mAb solution (in 1X PBS). The reaction was incubated for 1 h at room temperature with end-to-end mixing. Excess biotin from the solution was removed by using a Zeba™ Desalt Spin Column. The column was prepared by pre-spinning the column at 1000 x g for 2 min. Storage buffer was discarded, and the column was washed three times with 2.5 ml 1X PBS by centrifuging at 1000 x g for 2 min. The column was placed into a new 15 ml collection tube and the 10A37 mAb solution

was applied on the top. The column was centrifuged at 1000 x g for 2 min. The collected flow-through is the purified biotinylated 10A37 mAb.

Competition assays

Competition assays were performed by modifying the previously described ELISA protocol and as described elsewhere [18,19,22]. As before, gp120 and V3 loop peptide were coated onto 96-well plates at 30 ng and 100 ng per well respectively. Uncoated surfaces were subsequently blocked and washed. Rabbit serum was diluted in 1:5 in blocking buffer. This initial dilution was then subjected to two-fold serial dilutions. B10A37 and 10A3 mAbs [19] were diluted to a concentration of 120 ng/ml in blocking buffer for competition on gp120. For competition on V3 loop peptide, B10A37 was diluted to a concentration of 2 µg/ml. 50 µl of the diluted antibody was added to each well along with 50 µl of the serially diluted rabbit sera. Hence the final mAb concentration was 60 ng/ml and 1 µg/ml in each well, for the competition on gp120 and V3 loop peptides respectively and the starting serum dilution was 1:10. The plate was briefly mixed on a plate shaker and then incubated at 37°C for 90 min. Wells were washed ten times. The plates were incubated for 1 h at 37°C after addition of 100 µl of secondary antibody. For competition assays with B10A37 mAb, the secondary antibody used was anti-streptavidin HRP conjugated (Pierce); diluted 1:10000 in the blocking buffer. For competition assays with 10A3 mAb, rabbit anti-human Fc HRP conjugated (Pierce); diluted 1:3000 in the blocking buffer was used. Plates were again washed ten times. Detection was performed as described above for the standard ELISA.

Neutralization assays

Virus neutralization assays were performed using single cycle HIV-1 pseudovirus infections of TZM-bl cells by as described elsewhere [18,19,23-25]. Briefly, test rabbit sera were heat inactivated by incubating in pre-equilibrated water bath at 56°C for 1 h. 11 µl of heat inactivated rabbit sera were diluted in 100 µl of cell culture media (10% DMEM/FBS) followed by a three-fold serial dilutions. 50 µl of diluted rabbit sera were mixed with 50 µl of 200 TCID₅₀ (50% tissue culture infectious dose) of the viruses. Hence the final dilution range of the rabbit sera was 1:20 through 1:43740. Plates were incubated at 37°C for 1h. After incubation, 1 x 10⁴ TZM-bl cells in 10% DMEM/FBS containing DEAE-dextran (10 µg/ml final concentration) was added to the each well. Plates were incubated at 37°C for 48 h. Following a 48 hr-incubation, 100 µl of culture medium was removed from each well followed by addition of 100 µl of Bright-Glo reagent (Promega, prepared as per manufacturer's protocol). Plates were incubated for 2 min at room temperature cells to allow cell lysis followed by pipetting up and down (at least twice) for complete mixing. 150 µl from each well was transferred to the corresponding well in a 96-well black solid plate. Relative luminescence units (RLU) were measured using a Synerge 2 luminometer. Percent neutralization was determined by calculating the difference in average RLU between test wells (cells plus test sera) and cell control wells (cells only), dividing this result by the difference in RLU between virus control (cells plus virus) and cell-control wells, subtracting from 1, and multiplying by 100.

Results

Comparative antigen-specific antibody titers from gp120 and immune complex immunized animals

Antibody levels were assessed by ELISA against gp120. Animal sera obtained two weeks after each immunization was used, except after the fourth immunization when rabbits were bled 8 days after. Serum collected prior to immunization (designated as “preimmune”) was used as a negative control. As shown in Figure 2, antibody levels increased subsequently with each immunization in both gp120 alone and immune complex groups. Although the antibody titers were weak after the first two immunizations which did not include additional adjuvants (Figure 2A and B), end point titers reached $>10^5$ after the third immunization in gp120 alone group and $>10^4$ in the immune complex vaccinated group when zinc chitosan was used as an adjuvant (Figure 2C). Antibody titers did not further increase after the fourth immunization (Figure 2D).

Antibody responses against the V3 loop in gp120 and immune complex immunized animals

To determine the efficiency of V3 loop masking, antibody levels post second, third and fourth immunizations were assessed by ELISA against a consensus sequence V3 loop peptide. Due to overall low antibody titer after the first immunization (Figure 2A), antibody level against V3 loop was not evaluated for this timepoint. As shown in Figure 3, antibody levels in the gp120 alone group was very low after the second immunization (Figure 3A) but increased over 10 fold after the third immunization (Figure 3B) and remained constant after

the fourth immunization (Figure 3C). As predicted, the antibody titer in immune complex group remained comparatively low throughout the immunization course (Figure 3A-C), suggesting that the V3 loop was efficiently masked.

Linear epitope mapping analysis using V3 loop peptides

To better characterize the immune response, animal sera were evaluated for reactivity against 15-mer linear overlapping peptides spanning the entire length of the V3 loop. The V3 loop peptide (TH33) was used here as a positive control. As shown in Figure 4A, animals from both groups reacted very weakly to the peptides after the first immunization. This is likely due to the overall low antibody titers seen after first immunization (Figure 2A).

Animal sera after the second immunization from both groups also demonstrated very weak reactivity against the peptides (Figure 4B). Only rabbit#2 (R2) from gp120 alone group was slightly reactive to the V3 peptide (OD=0.5). This is surprising as both the animals from the gp120 alone group exhibited weak binding of the V3 loop (Figure 3A), but only R2 showed reactivity in the peptide ELISA.

Animal sera from gp120 alone group after third immunization showed significant reactivity toward V3 loop peptides (Figure 4C). Rabbit #1 (R1) favored peptides 9047, 9049, and V3 (TH33); while R2 showed significant reactivity against 9047 and V3 (TH33) as well as weak binding to 9046. This difference in reactivity might be due to animal-to-animal variation in the immune response. On the other hand, the two rabbits from the immune complex group did not react to any peptides spanning the V3 loop. This result is again corroborated by the weak antibody titer against the V3 loop in immune complex group after the third immunization (Figure 3B).

The reactivity towards the V3 loop peptides increased after the fourth immunization in gp120 only group (Figure 4D). The overall binding pattern was similar to what was observed after third immunization (Figure 4C). Oddly, R2 showed higher reactivity to the V3 (TH33) peptide after the fourth immunization as compared to R1, which is opposite to the after third immunization timepoint (Figure 4C). As expected, animals from the immune complex group did not bind any peptide except weak binding to 9047 (R2) and 9048 (R1). Overall, linear epitope mapping also demonstrated the suppression of antibody response towards V3 loop in immune complex immunized animals.

Competition assays with V3 loop rabbit nAb 10A37

To evaluate the efficient masking of V3 loop and repression of the antibody response towards this epitope, competition assays were performed on gp120 using V3 loop rabbit nAb, 10A37 (Yali Plos one ref). Since 10A37 was the antibody used for immune complexing, it was important to determine whether animal sera elicited antibodies which can compete with 10A37 mAb. To perform the competition, 10A37 was biotinylated (B10A37) to prevent the cross reaction of rabbit antibodies with the human Fc region. Animal sera post second, third and fourth immunizations from the two groups were used (Figure 5).

As shown in Figure 5A, animal sera post second immunization, from both groups did not compete against B10A37. This might be due to the low antibody titers observed against gp120 as well as the V3 loop (Figure 2B and 3A).

Competition increased after third immunization (Figure 5B). R1 from gp120 alone group showed significant competition against B10A37 as compared to R2, which showed weaker competition (Figure 5B). This correlates with the results from linear epitope mapping

post third immunization (Figure 4C) where R1 from gp120 alone group mounted a stronger immune response against 9047, 9049 and whole V3 loop peptides as compared to R2.

Interestingly, R1 from immune complex group exhibited strong competition against B10A37 as compared to R2, which showed almost no competition (Figure 5B).

Similar results were observed after the fourth immunization. But this time, R2 showed strong competition from gp120 only group as compared to R1, which showed no competition (Figure 5C) thus opposite to what was seen after third immunization. This result may not be entirely unexpected, as the strength of the V3 peptide response for these animals also slightly reversed between the third and fourth immunizations (Figure 3C and 4D). However, there was no significant difference in the antibody responses in both rabbits against V3 peptide (TH33) (Figure 4D), so it is somewhat unexpected that only R2 showed strong competition.

Surprisingly, both the animals from immune-complex group demonstrated strong competition (Figure 5C), as we hypothesized there would be no competition in the immune complex animal group. One possibility was that the V3 loop was masked with different efficiency in the two rabbits due to animal-to-animal variation which can affect the stability of the immune complex (Figure 5B). But strong competition in both these rabbits post fourth immunization suggests otherwise (Figure 5C). It is also possible that antibodies elicited in response to the immune complex are binding in the vicinity of 10A37 epitope thus the V3 loop, which therefore prevents the binding of 10A37 mAb due to steric hindrance offered by the competing antibodies. Also, these competing antibodies might exhibit conformational dependent binding, which is not detectable by linear epitope mapping in the immune complex group.

To understand the competition seen in the immune complex group, we performed a similar competition assay with B10A37 but instead on the V3 loop peptide (TH33) rather than gp120 (Figure 6). We considered that the V3 loop might be exposed differently on gp120 as compared to the peptide alone, which might affect the binding of antibodies elicited in the immune complex sera. As we predicted, no competition was seen after second and third immunization (Figure 6A and 6B) in both of the groups. After the fourth immunization, animals from the immune complex group exhibited slightly stronger competition compared to the gp120 only group (Figure 6C), but the competition was weak as compared to that seen on gp120 (Figure 5C). This is in accordance with our prediction that antibodies elicited in the immune complex sera might be binding in the vicinity of either the 10A37 epitope or the V3 loop in general, preventing binding of 10A37. Moreover, this competition was primarily observed on the whole protein (gp120) as compared to smaller peptide (V3 loop) due to the presence of more potential epitopes on the larger protein.

Competition assays with V3 loop rabbit nAb 10A3

We also performed competition assays with a second V3 loop nAb, 10A3, to confirm the repression of antibody response against V3 loop (Figure 7). Since 10A37 and 10A3 have overlapping binding epitopes [19], we expected to see no competition in immune complex group of animals. As expected, immune complex group of animals did not compete after the second, third and fourth immunizations (Figure 7A-C). R1 from gp120 only group possessed stronger competition as compared to R2 post second immunization (Figure 7B). This is similar to the result seen for competition against B10A37 (Figure 5B). After the fourth immunization, R2 from gp120 only group demonstrated significantly increased competition

(Figure 7C). This might be due to the antibody titer increase against V3 loop peptides in R2 post third immunization as seen in Figure 4C. Overall, competition ELISA also demonstrated repressed antibody response towards the V3 loop peptide suggesting proof of this technique.

Neutralizing activity of the immune complex sera

The potency and breadth of animal sera from two groups were assessed using a standard TZM-bl neutralization assay against neutralization sensitive Tier-1 pseudoviruses from different clades and neutralization resistant Tier-2 pseudovirus (Figure 8). Neutralization was only tested for animal sera post fourth immunization. As shown, animal sera from gp120 alone group could effectively neutralize SF162.LS and MW965.26 (Figure 6B and 6C). Weak neutralization was seen against BaL.26 (Figure 6A) and no neutralization was seen against Q23.17 and pBJOX 2000. This is consistent with our previous study [18]. On the other hand, immune complex sera failed to neutralize all tested pseudoviruses. Prior work in this laboratory demonstrated that most of the neutralizing antibodies elicited by the gp120 antigen are towards the V3 loop [18], and thus masking of this epitope likely prevented the induction of such antibodies.

Discussion

In this study, we evaluated an immune complex vaccination strategy for HIV-1 to focus the antibody response towards critical neutralizing epitopes on gp120. We evaluated the differences in the immune response elicited in rabbits vaccinated with gp120 alone versus an immune complex comprised of gp120 and rabbit anti-V3 loop nAb 10A37 using different immunological assays.

While we hypothesized that higher antibody titers would be elicited in the immune complex group from the known augmentation to the immune response, to our surprise, the total antibody titer in gp120 only group was nearly 10 fold higher than the immune complexed group (Chapter 2, Figure 2). The exact reason behind this phenomenon is unknown. However, it is well established that most of the antibody response against gp120 is directed towards the immunodominant V3 loop [16-19,22], and hence masking of a V3 epitope could reduce the total antibody titer observed in the immune complex. Irrespective of this, the total antibody titer in gp120 group remained low throughout immunizations as compared to a previous study where rabbits were immunized at all time points with gp120 on zinc chitosan [18]. In that study, the total antibody titers reached $>10^5$ just after a single immunization, whereas our titers were very low. Following the second immunizations, titers in the original study reached 2×10^6 , while in this study titers remained steady at 1×10^5 . One important thing to note is that in the current study we started using zinc chitosan as an adjuvant only after the second immunization as compared to our previous study where zinc chitosan was used throughout [18]. Lack of adjuvant during the first two immunizations might have also reduced the total antibody immune response.

Apart from the total antibody titer, there was also a clear difference in the immune response against the V3 loop in the two groups. As predicted, the antibody response against the V3 loop was diminished (~ 10 fold) as compared to gp120 only group. This suggests the successful masking of the V3 loop by mAb and the feasibility of an immune complex vaccination strategy. Fine epitope mapping performed by linear peptide ELISA also corroborated our finding that an mAb can modulate the immune response at the epitope level as the immune complex sera did not recognize any V3 loop peptides

throughout the immunizations. On the other hand, gp120 immunized animals showed a strong immune response against V3 loop peptides, which is in agreement with our previous studies [18,22].

Unfortunately, the information provided by linear epitope mapping is limited and cannot be used for assessing antibodies targeting the non-contiguous, conformation dependent epitopes. Although the antibody response in immune complex vaccinated rabbits was diminished against the V3 loop, there still might be antibodies recognizing conformational epitopes not detectable by linear overlapping peptides. As such, we performed competition assays on gp120 with V3 loop neutralizing mAbs 10A37 and 10A3, isolated and characterized in our laboratory [19]. Although immune complex sera did not compete with 10A3, strong competition against 10A37 mAb was seen. This was surprising, as we had predicted there would be no competition for either antibody due to masking of the V3 loop, of which 10A37 and 10A3 share an overlapping epitope. We also observed significant differences in competition amongst the two animals within a group. For example, in the immune complex group, R1 showed strong competition after the third immunization, but both rabbits showed strong competition after the fourth immunization. Whereas in the gp120 only group, both rabbits competed after the third immunization, but only R1 showed competition after the fourth immunization. Animal-to-animal variations in the immune response might account for this difference. Also, such animal-to-animal variations may induce the immune complex to disassociate more quickly in one animal versus the other, thereby permitting the elicitation of 10A37 like antibodies following complex dissolution.

Stronger competition in the immune complex group might also arise from the antibodies binding in the vicinity of the 10A37 epitope or V3 loop, offering steric hindrance to 10A37 mAb preventing its binding. This possibility is corroborated by the lack of competition after the second and third immunizations and the very weak competition seen on the V3 loop peptide as compared to gp120. Variable loops are highly dynamic and in the structural context of the entire gp120 glycoprotein, the V3 loop is likely exposed differently as compared to the truncated peptide presented without the other structural elements of gp120. This can make a huge impact on antibody dynamics. Gp120 immunized animals demonstrated stronger competition against 10A3 as compared to 10A37, which might be due to fact that antibodies binding an N-terminal V3 epitope like 10A3 are more prevalent as compared to those binding more C-terminal epitopes like 10A37 [18,19,22].

Unfortunately, immune complex vaccinated animals failed to demonstrate neutralizing activity against Tier-1 as well as Tier-2 pseudoviruses, while gp120 immunized sera neutralized SF162.LS and MW965.26 (Tier-1 sensitive viruses). We previously demonstrated that most of the neutralizing antibodies in the gp120 immunized animals are directed towards the V3 loop and have limited breadth [18]. Therefore, masking the V3 loop epitope may have caused a significant reduction in the neutralizing activity in immune complex sera.

It is difficult to compare our results with other immune complex studies in HIV-1. Authors from previous studies have masked CD4bs in order to increase the immunogenicity of neutralizing epitopes on V3 loop [6-8,10,11,26]. We, on the contrary, masked the V3 loop to direct the immune response towards other neutralizing epitopes (e.g

CD4bs). While other authors reported higher antibody titers in immune complex group as compared to gp120 only group, which suggested that immune complexes augment the antibody response, we saw the opposite results. They also reported neutralizing activity against Tier-1 sensitive viruses as compared to our study where no neutralization was seen. We hypothesize that it might be more challenging to target the CD4bs as compared to the V3 loop by masking using a single mAb because different mAbs can have different effects on antigenic modulation. Also, difference in the animal model system (mice versus rabbits) and the choice to use species matched versus non-matched mAbs can have an impact on the final results.

Thus, our findings indicate that although the immune complex vaccination strategy is an effective way of modulating immune response against desired epitopes, further improvement is required to achieve final goal of eliciting broadly neutralizing antibodies. These possibilities will be further discussed in the final chapter.

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Figures

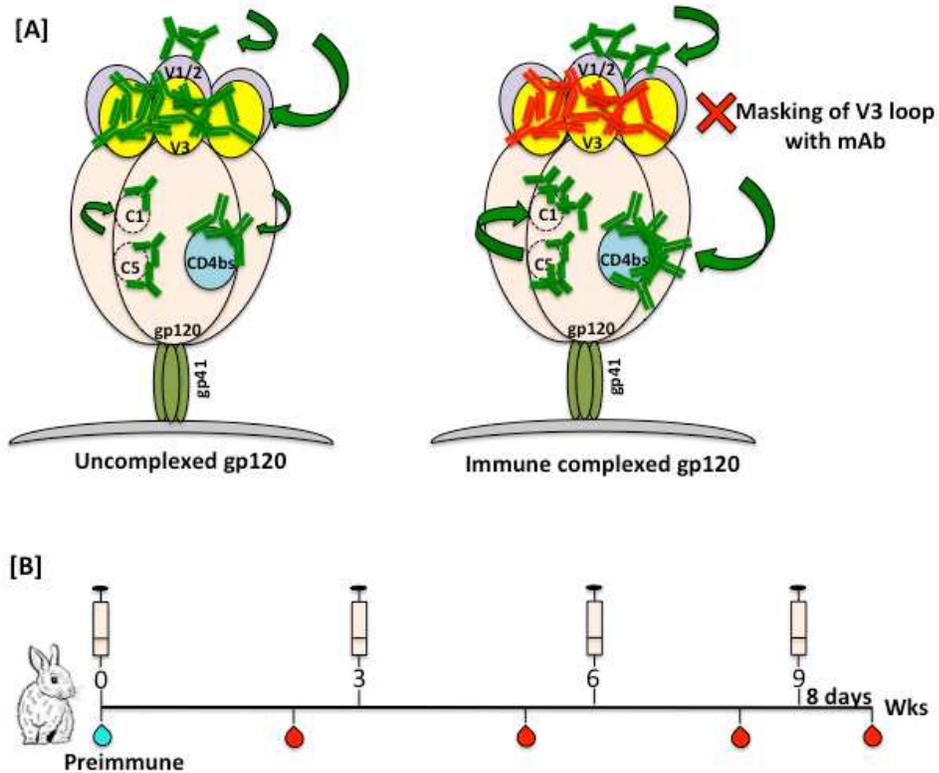


Figure 1. [A] Diagram showing the predicted difference in the immune responses directed against uncomplexed versus immune complexed gp120. When gp120 is uncomplexed, the immune response is directed towards different epitopes on gp120, including the V3 loop, as shown by the green arrows and antibodies. On the right, we hypothesized that when the V3 loop is masked with mAb (shown in red), the immune response will be better directed towards conserved neutralizing epitopes (e.g. CD4bs) and other sub-dominant epitopes, as shown by the larger green arrows. [B] Timeline for rabbit immunization and bleeding.

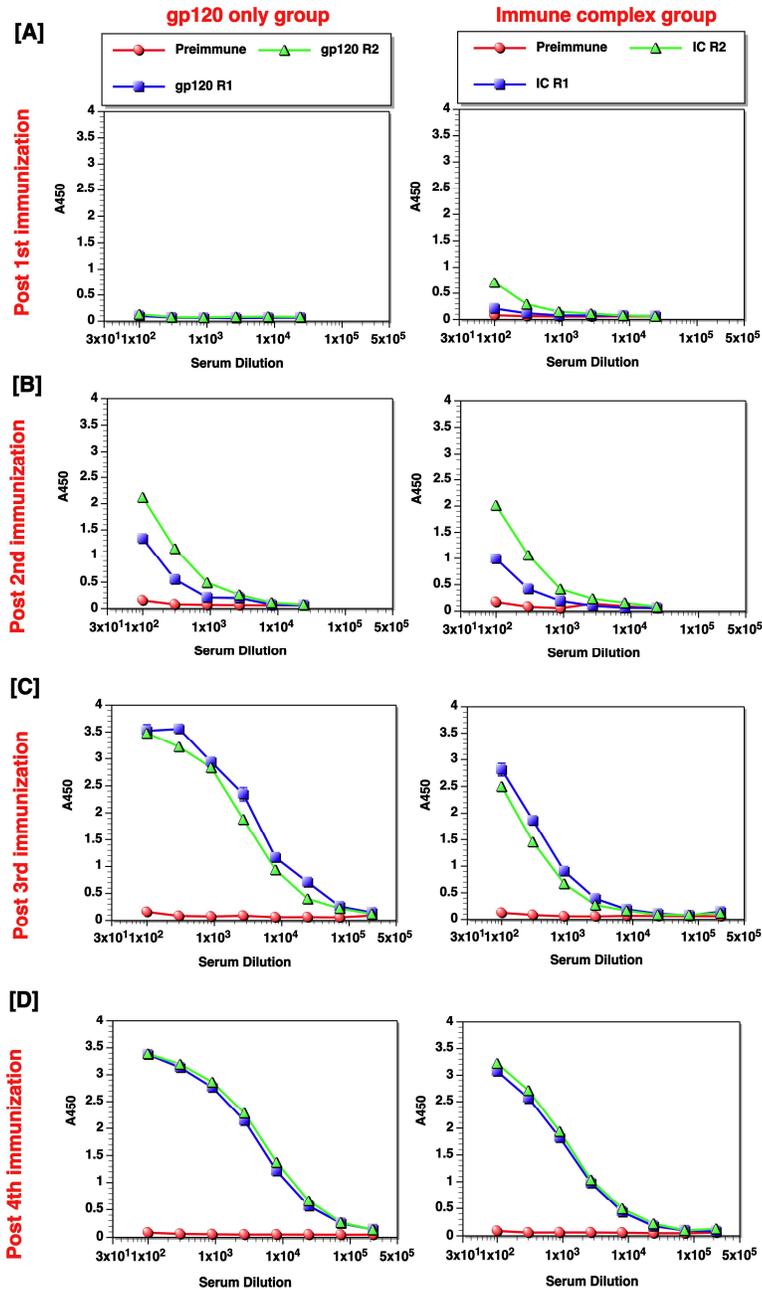


Figure 2. Total antibody titer against gp120 as determined by ELISA. Anti-sera obtained after the [A] first, [B] second, [C] third and [D] fourth immunizations were used. “Preimmune” is the serum sample collected prior to the beginning of immunizations and used here and throughout as a negative control. A450 represents absorbance value at 450nm.

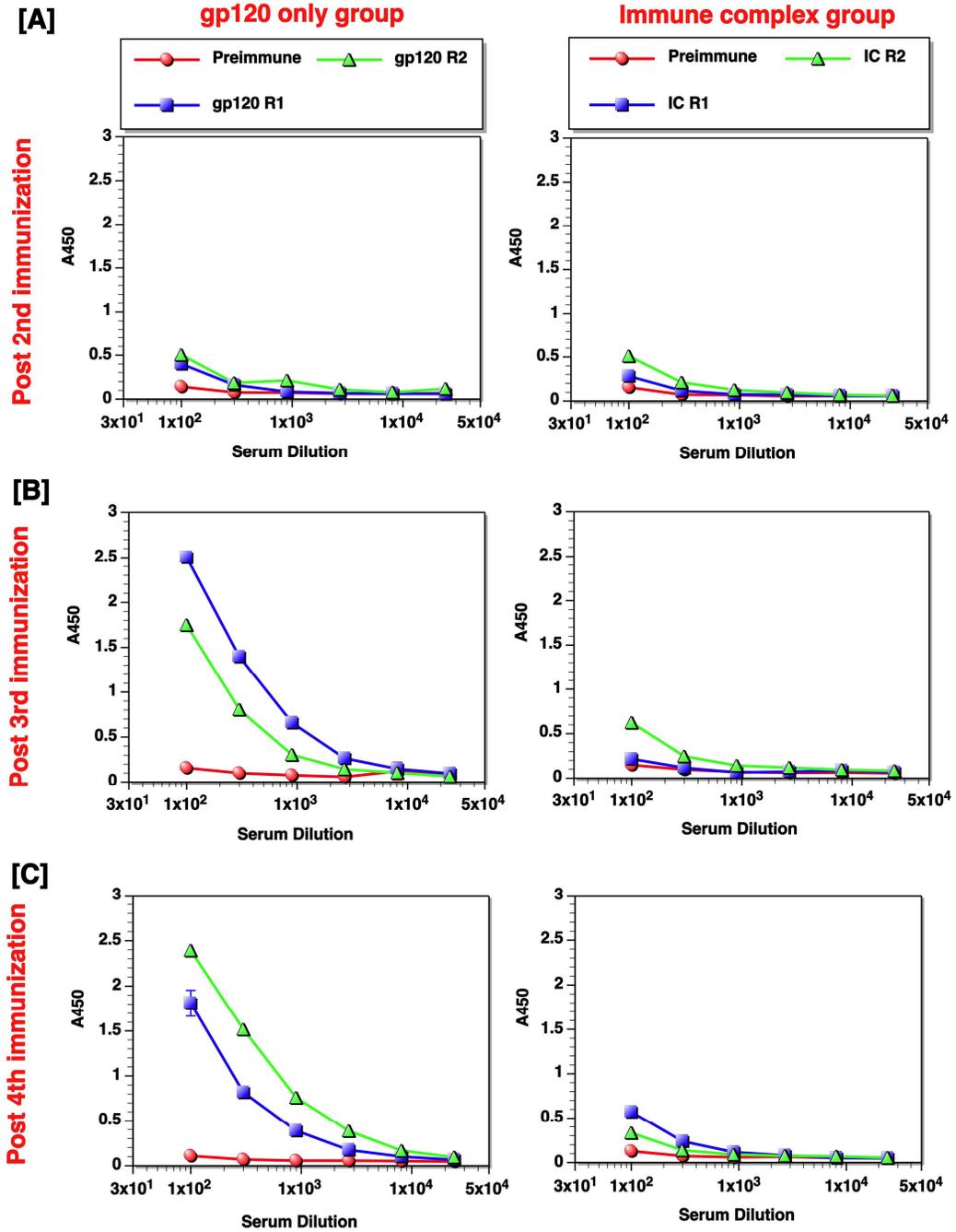


Figure 3. Total antibody titer against the V3 loop as determined by ELISA. Anti-sera obtained after the [A] second, [B] third and [C] fourth immunizations were used.

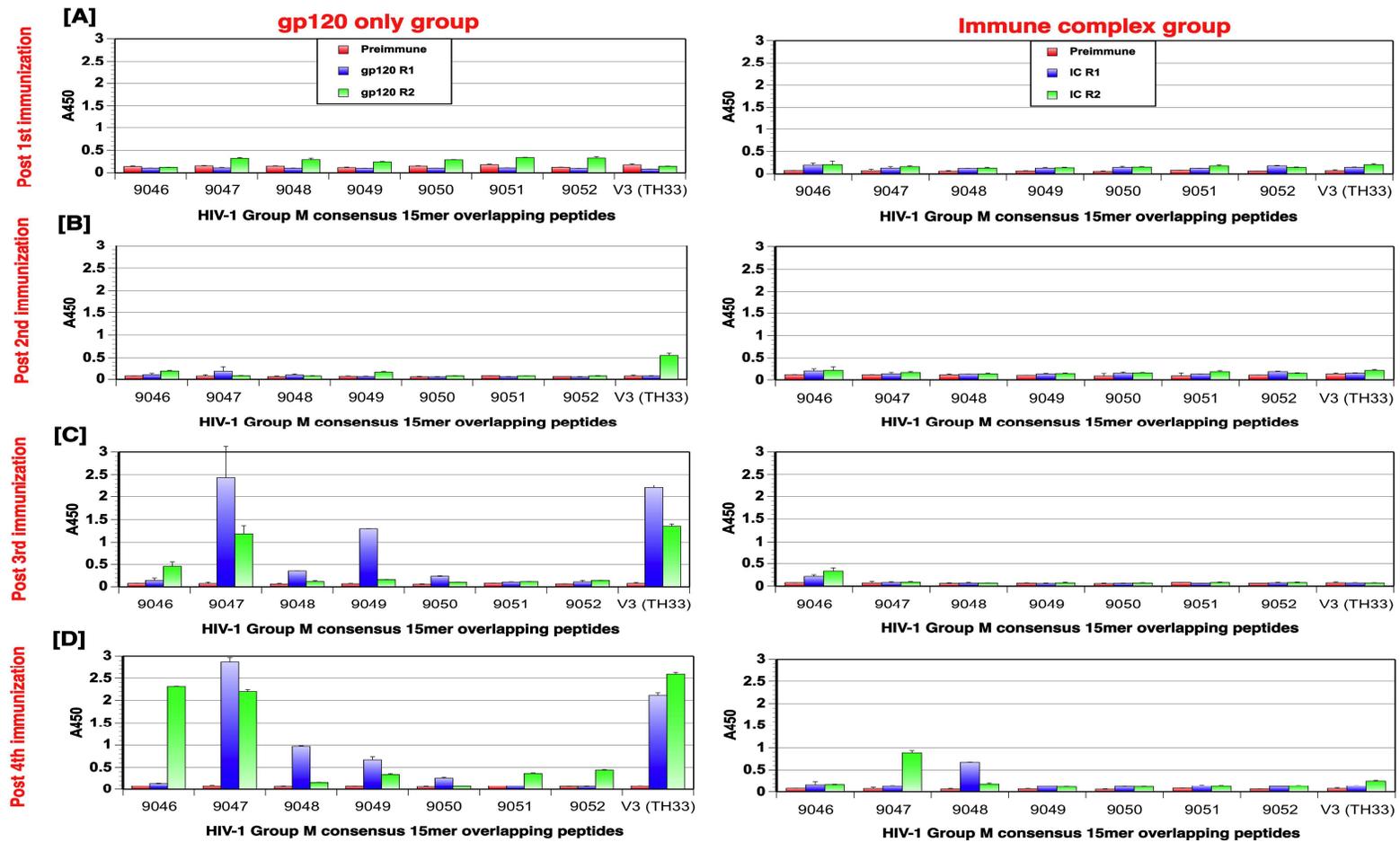


Figure 4. Linear epitope mapping by ELISA using 15mer overlapping peptides. Sera obtained after the [A] first, [B] second, [C] third and [D] fourth immunizations were used.

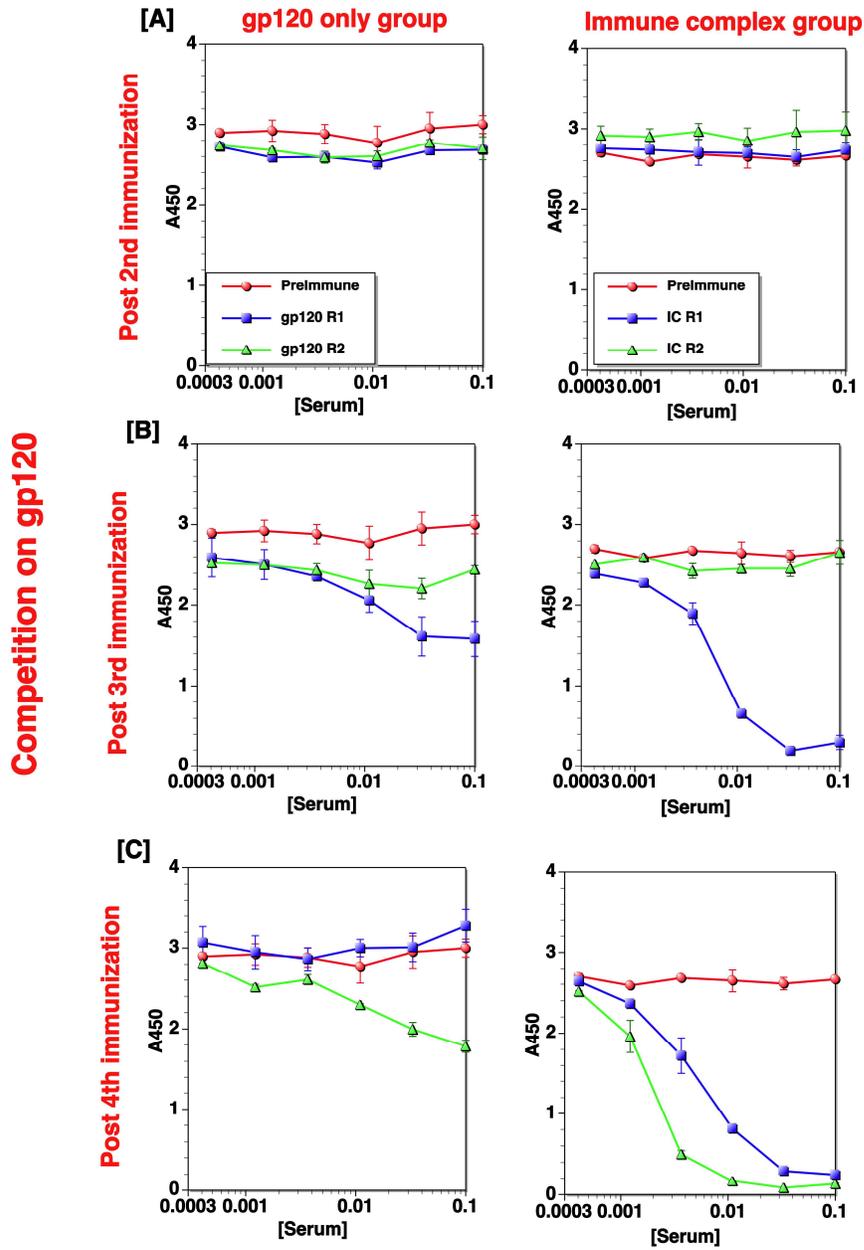


Figure 5. Sera competition against biotinylated 10A37 on gp120. Anti-sera obtained after the [A] second, [B] third and [C] fourth immunizations were used. The value on x-axis represents serum concentration (1/dilution factor).

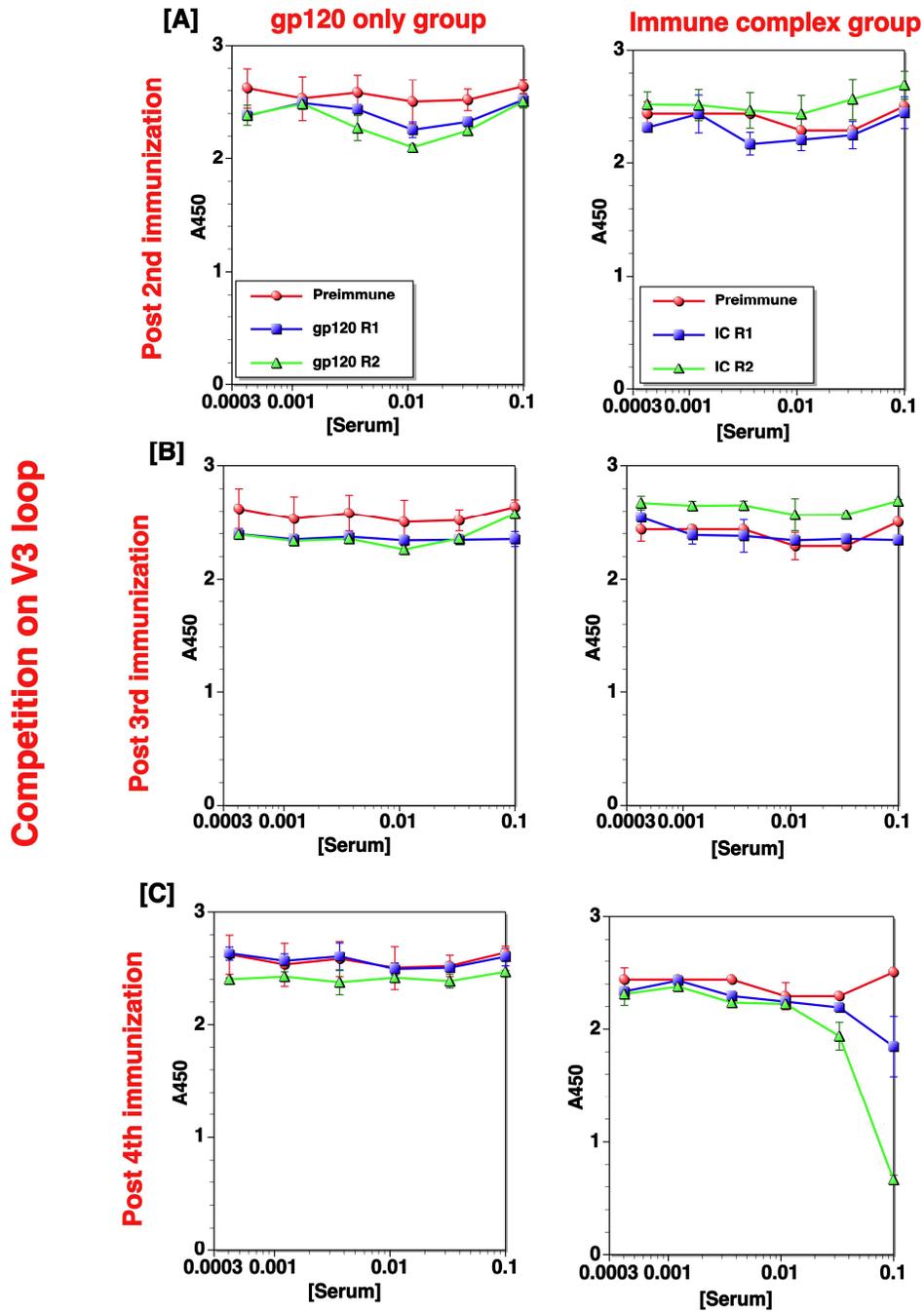


Figure 6. Sera competition against biotinylated 10A37 on V3 loop peptide. Anti-sera obtained after the [A] second, [B] third and [C] fourth immunizations were used. The value on x-axis represents serum concentration (1/dilution factor).

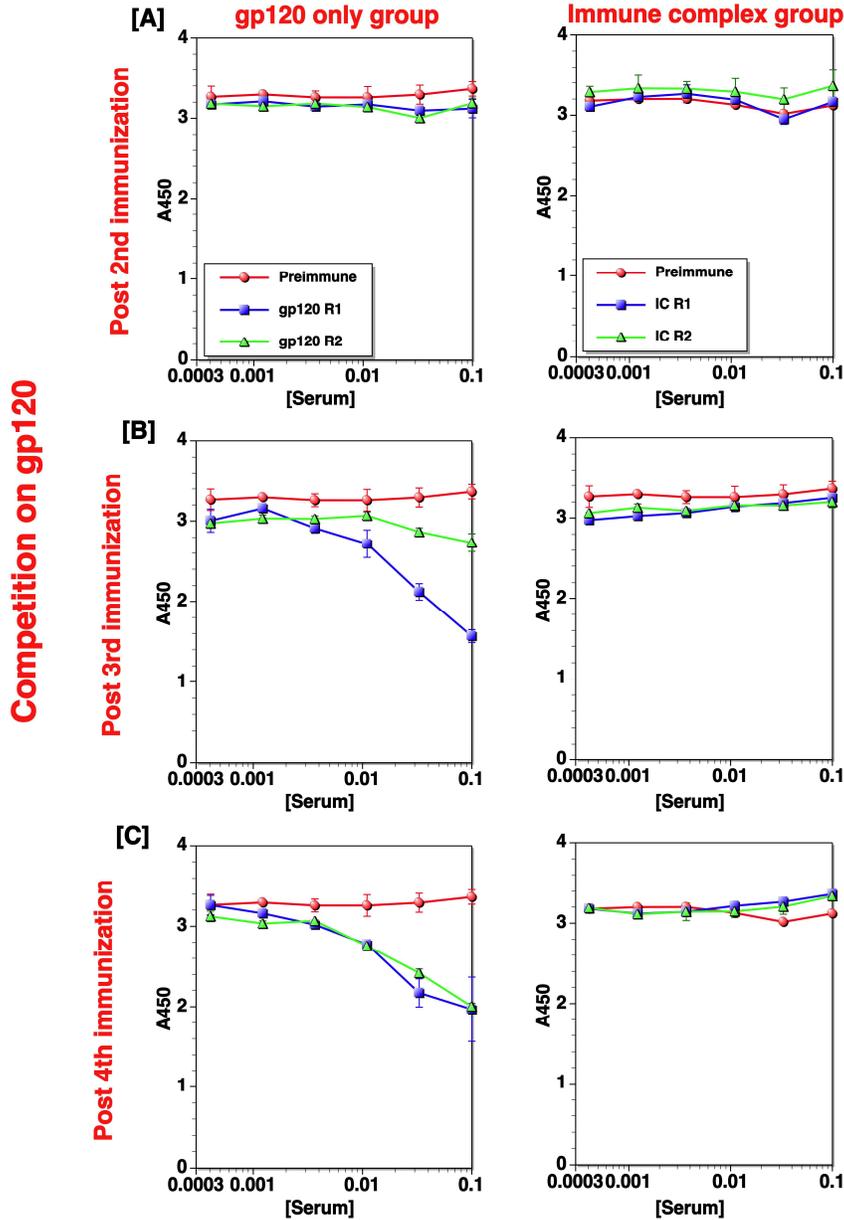


Figure 7. Serum samples after the [A] second, [B] third and [C] fourth immunizations were tested for competition against 10A3. The value on x-axis represents serum concentration (1/dilution factor).

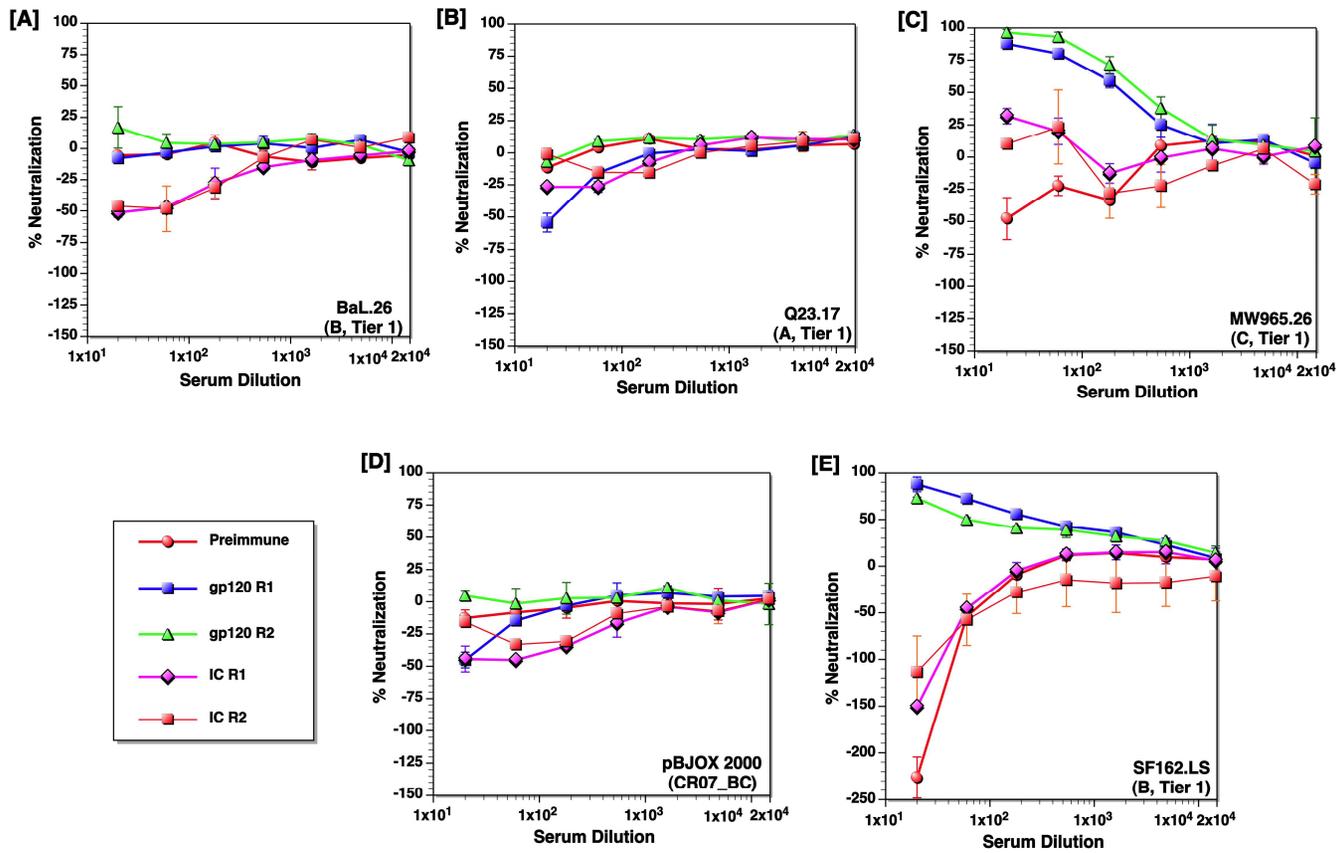


Figure 8. Neutralizing activity of the sera against Tier-1 and Tier-2 viruses from different clades. Serum sample obtained after the fourth immunization was tested.

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CHAPTER III

GENERAL CONCLUSIONS

HIV-1 infection has caused a staggering number of deaths across the globe and created a huge socioeconomic impact. Despite decades of exhaustive research efforts, an effective vaccine against HIV-1 stills remains elusive and a top priority. A universal vaccine targeting HIV-1 has been unsuccessful due to an extensive amount of variation presented by virus to the host immune system [1-3]. In addition to this, HIV-1 has also evolved various immunological evasion strategies, causing the virus to quickly escape the immune system [4-7]. This leaves the host immune system severely impaired, which under such circumstances become highly prone to so called “opportunistic infections”. Discovery of potent anti-retroviral drugs and highly active anti-retroviral therapy (HAART) has made the disease more manageable than before [8]. Unfortunately, current therapies have been unsuccessful at eradicating the virus completely from the host, causing virus levels to rebound in the plasma as soon as the treatment is stopped [9]. Thus, patients are supported on lifelong therapies that lead to potential side effects and enormous health care costs. Thus, an effective vaccine might be the most cost effective way of preventing HIV-1 infection. The discovery of bnAbs in certain HIV-1 infected patients has provided hope that an actual HIV-1 vaccine might be possible.

In the quest for an effective vaccine, researchers across the globe have designed and evaluated various vaccine immunogens based on the structural knowledge of HIV. The envelope glycoproteins gp120 and gp41 are the sole targets available for vaccine design and are widely exploited in various immunogen designs [10]. Unfortunately, all such

immunogens tested in clinical trials were unsuccessful at inducing effective responses against HIV-1. We evaluated a non-conventional, immune complex vaccination strategy for HIV-1 by masking the immunodominant V3 loop on gp120 with a rabbit mAb. Our findings indicate that mAbs can successfully modulate the immune response against desired antigenic epitopes. Although we did not see any neutralizing activity in animal sera, this study provided proof of principle and feasibility of the approach. Further improvement is required to elicit bnAbs by vaccination.

Future Directions

The studies in this thesis evaluated an initial attempt at an immune complex vaccination strategy for HIV-1 vaccine by suppressing the response against immunodominant V3 loop and potentially focusing the immune response towards critical bnAb epitopes. Although we successfully masked the V3 loop as shown by diminished immune response towards this epitope, we have not established where the immune response is actually targeted. Future experiments including extended linear epitope mapping using overlapping peptides covering the entire length of gp120 might provide some answer. Also, competition assays using CD4bs bnAbs can test whether more of the immune response is being directed towards the CD4bs in immune complexed animals versus gp120 alone. There is a possibility that we might not have targeted CD4bs since animal sera failed to neutralize Tier-1 as well as Tier-2 viruses. We therefore might need to mask additional epitopes direct immune response towards conserved sites like CD4bs. One possible solution is masking additional immunodominant epitopes because in the current study we only evaluated one mAb for

making immune complexes and its effect on antigenic modulation. Recently we characterized a large panel of rabbits mAbs isolated from an animal immunized with gp120 alone, which bind to different epitopes [11]. These epitopes include several which were observed to be immunodominant (C1, C2, C5, V5). Immunization with cocktails of different immune complexes targeting different epitopes might be an alternative strategy.

Irrespective of these measures, immune complexing using gp120 might never elicit broadly neutralizing antibodies due to the presence of the large number of immunodominant, but non neutralizing or weakly neutralizing “decoy” epitopes that distract the immune system from mounting a suitable bnAb response. Furthermore, it is suggested that in particular the germline B cell receptors may not effectively interact with the CD4bs bnAb epitope on native env structures such as our gp120 antigen [12]. We would like to evaluate this strategy using alternate immunogens like BG505 SOSIP gp140 [13] and eOD-GT8 [14], which are optimized for better presentation of bnAbs epitopes. Although immunogenicity studies using these immunogens have not yielded impressive results so far [14,15], we propose to improve these immunogens by antibody based antigenic modulation. In addition to the previously mentioned gp120 derived panel [11], we are also currently screening B-cell hybridomas generated from animals immunized with eOD-GT6 and BG505 SOSIP, for finding potential mAbs recognizing these proteins. Thus we have necessary resources to test alternate immunogens with immune complexing in the future.

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APPENDIX**CHARACTERIZATION OF A LARGE PANEL OF RABBIT MONOCLONAL ANTIBODIES AGAINST HIV-1 GP120 AND ISOLATION OF NOVEL NEUTRALIZING ANTIBODIES AGAINST THE V3 LOOP**

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Abstract

We recently reported induction of potent, cross-clade neutralizing antibodies (nAbs) against HIV-1 in rabbits using gp120 based on an M-group consensus sequence. Competing antibodies to VRC01 and PGT121 for binding gp120 were also detected. To better characterize these antibodies, 93 hybridomas were generated, which represent the largest panel of monoclonal antibodies (mAbs) ever generated from a vaccinated rabbit. The single most frequently recognized epitope of the isolated mAbs was at the very C-terminal end of the protein, followed by the V3 loop. Although we did not isolate any VRC01-competing

mAbs, all seven anti-V3 loop mAbs competed with PGT121. Two of the V3 mAbs (10A3 and 10A37) exhibited neutralizing activity. In contrast to 10A3 and most other anti-V3 nAbs, 10A37 was atypical with its epitope positioned more towards the C-terminal half of the loop. To our knowledge, 10A37 is the most potent and broadly neutralizing anti-V3 loop mAb induced by vaccination.

Introduction

A critical problem for developing a vaccine against human immunodeficiency virus type 1 (HIV-1) is the difficulty in inducing broadly neutralizing antibodies (bnAb) against the large number of viral variants that exist [1-3]. The envelope glycoproteins gp120 and gp41 are the sole HIV-1 antigens on the virion surface targeted by nAbs. Therefore, characterizing the immunogenic and structural features of the HIV-1 envelope is important for designing immunogens to elicit bnAbs and to understand the humoral response to HIV-1 infection [4-6].

Monoclonal antibodies (mAbs) have been important tools for probing antigen structures. Recent technology developments for antigen-specific single B cell sorting [7,8], high-throughput clonal memory B-cell cultures [9] and next-generation sequencing (NGS) [10] have enabled isolation of a large number of new bnAbs against HIV-1 from virus-infected patients [11]. Those bnAbs have defined four major targets on the HIV-1 envelope: the CD4 binding site (CD4BS), glycans around N160 along with conserved elements on V1/V2, the base of and glycans around the V3 loop, and the membrane-proximal external region (MPER) of gp41 (as reviewed in [12,13]). Recently, epitopes involving both gp120 and gp41 have been identified as well [14-17].

In contrast to bnAbs isolated from HIV-1 infected humans, envelope-specific mAbs generated from vaccinated subjects, either animals or humans, are limited. Early studies isolated many murine mAbs from immunized animals. However, most did not possess significant neutralizing activity [18-23]. Later, Gao *et al.* reported two mAbs isolated from gp140-immunized mice that cross-reacted with all tested envelope proteins, but neither mAb neutralized primary HIV-1 pseudoviruses [24]. Derby *et al.* isolated six anti-gp120 mAbs from mice immunized with soluble gp140 [25]. These antibodies could neutralize the homologous SF162, and their activities were dependent on the glycosylation patterns of the V1, V2 or V3 loops. However only one anti-V3 mAb displayed cross-clade neutralizing activity, which was dependent on the type of V1 loop present on heterologous viruses. Recently, Sundling *et al.* used a non-human primate (NHP) model to evaluate envelope immunogens that elicited anti-CD4BS antibodies, isolating a panel of functional mAbs from immunized rhesus macaques [26]. However, only eight mAbs were generated that exhibited neutralizing activity against a limited number of mostly tier 1 HIV-1 isolates (*viz.* MN.3, HXBc2, SF162 and MW965.26). The RV144 clinical trial reported an estimated 31% protection efficacy, and protection correlated with the presence of anti-V2 loop antibodies in immunized individuals [27-29]. Most recently, four V2 mAbs were isolated from RV144 participants [30]. These mAbs recognized amino acid residue 169, neutralized tier 1 laboratory HIV-1 strains and mediated antibody dependent cell-mediated cytotoxicity (ADCC). Additionally, two other neutralizing V3 loop mAbs (CH22 and CH23) were isolated from the participants in RV135 trial, which showed limited neutralization against tier 1 strains [31].

Rabbits have been used widely in HIV-1 vaccine studies. Compared to other animal models, rabbits are large enough for collection of sufficient amount of antisera for nAb assessment, and can develop long CDR3s [4,24]. Although rabbits have restricted antibody germline usage, particularly in the heavy chain [32,33], their immune system is similar enough to that of humans [34-38] to allow for assessment of potential nAb induction. Furthermore, rabbit hybridomas can be generated to produce mAbs to determine the specific characteristics of individual antibodies. Recently, Chen *et al.* [39] reported generation and characterization of twelve mAbs from HIV-1 Env-immunized rabbits, three of which exhibited neutralizing activity.

We recently reported induction of potent neutralizing antibodies using soluble gp120 based on M group consensus sequence (MCON6; [40,41]) in rabbits [42]. Although the primary neutralizing epitope appeared to be the V3 loop and that neutralizing activity was largely against tier 1 virus isolates, the cross-clade neutralizing breadth was substantial. Further characterization of antibodies against gp120 in a follow up study revealed that some of them could compete with bnAbs VRC01, PGT121, and PGT126 in binding gp120, suggesting that the antibodies bound at or near the vicinity of epitopes targeted by these bnAbs [43].

Although we did not succeed in inducing “true” bnAbs using our vaccine regimen, it was important to (1) further characterize the nature of potent neutralizing activity against the V3 loop, (2) identify target epitopes of antibodies that competed with bnAbs VRC01 and PGT121/126, (3) determine immunogenic epitopes of antibodies that failed to exhibit neutralizing activity in order to develop better immunogens or immunization strategies, and (4) examine maturation pathways of these antibodies. To achieve these objectives,

antibodies will have to be characterized at a clonal level. In this study, we generated 93 hybridomas from one of the gp120-immunized rabbits that mounted an unusually strong nAb response against a tier 1 Clade AE virus (TH023.6 strain; ID₅₀ >43,740). To our knowledge, this panel of mAbs represents the largest collection of antibodies ever generated from a rabbit against HIV-1 gp120. This report is meant to provide an overview, rather than comprehensive characterization of all of the mAbs generated. It will focus primarily on characterization of the V3 loop mAbs.

Materials and Methods

Rabbit immunizations and hybridoma generation

The gp120 protein vaccine used was derived from pcDNA-MCON6gp160 (kindly provided by Dr. Beatrice Hahn [41]). A more extensive description is detailed in a previous publication [42]. The gp120 protein was purified from cell culture supernatant using tandem affinity chromatography. Three female New Zealand white rabbits (2.5-3 kg; Charles River) were immunized subcutaneously with gp120 formulated with Zn-chitosan on weeks 0, 3, 9, 15 and 27 as previously described [42]. One rabbit (gp120-R2) with the highest nAb titer was boosted at week 65. On week 76, the animal was injected intravenously with 1 mg of gp120 in PBS. Four days later, spleen was collected for fusion. All of the studies conducted were approved by IACUC at Iowa State University (#10-09-6772-LM).

The fusion was performed as previously described [40] with a few minor modifications. Briefly, rabbit splenocytes and the fusion partner cell line 240E-1 (kindly provided by Dr. Katherine L. Knight [40]) were fused at a ratio of 2:1 with 50% PEG 1500

(Sigma-aldrich P7181). The hybridomas were selected by growing in media containing HAT (hypoxanthine, aminopterin, and thymidine) (Sigma-aldrich H0262). Hybridoma supernatants were collected and screened for gp120 binding by ELISA, and for neutralization activity as described below. Hybridomas that were positive for gp120 binding were cloned by limiting dilution, expanded and frozen at -140°C for future use.

ELISA with proteins or overlapping peptides

ELISAs were performed as previously described [42,43] with some modifications. For determination of antibody titer and screening of our hybridoma panel, the indicated proteins were coated onto 96-well Nunc-Immuno plates overnight at 4°C at 30 ng per well using an antigen coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 3 mM NaN₃, pH 9.6). For screening hybridomas for the inner domain peptides (ID-P) and fine epitope mapping of V3 loop positive antibodies, 15-mer linear overlapping peptides were coated onto 96-well Nunc-Immuno Plates overnight at 4°C at 20 pmol per well in the same antigen coating buffer. Uncoated surfaces were blocked with 200 µl of a blocking buffer (PBS, pH 7.4, containing 2.5% skim milk and 5% calf serum) for 1 h at 37°C. Wells were subsequently washed five times with a wash buffer (PBS containing 0.1% Tween 20) using a Biotek automated plate washer. For antibody titer, all rabbit sera were serially diluted in blocking buffer as indicated in figures, and 100 µl was added to each well. For the cross reactivity of our panel of hybridomas and V3 fine epitope mapping, all the hybridoma supernatants were diluted 1:2 with blocking buffer and 100 µl was added to the each well. For the reactivity of hybridomas to ID-P, 100 µl of the hybridoma supernatant was added directly to each well. The rest of the procedure was similar to what has been described before [42,43]. All assays

were done in duplicate. The 15-mer overlapping peptide set for gp120 based on the M group consensus sequence (CON-S) was obtained from the NIH AIDS Reagent Program (Cat# 9487). V3 and V5 peptides based on the MCON6 sequence (H-TRPNNNTRKSIHIGPGQAFYATGEIIGDIRQAH-OH and H-GNNSNKNKTETFRPG-OH, respectively) were synthesized commercially by CHI Scientific (Maynard, MA). Peptides were coated onto wells at 20 pmol per well.

Neutralization assays

Virus neutralization assays were done using single cycle HIV-1 pseudovirus infections of TZM-bl cells as described elsewhere [42,44,45]. Briefly, heat inactivated rabbit sera (56°C for 1hr), hybridoma supernatant or purified IgG was diluted in 100 µl of cell culture media (DMEM supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin). Test samples were diluted over a range of 1:20 to 1:43740 in cell culture medium and pre-incubated with virus (~150,000 relative light unit equivalents) for 1 hr at 37 °C before addition of cells. Following a 48 hr-incubation, cells were lysed and Luc activity determined using a microtiter plate luminometer and BriteLite Plus Reagent (Perkin Elmer). Neutralization titers are the sample dilution (for serum) or antibody concentration (for sCD4, purified IgG preparations and monoclonal antibodies) at which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in cell control wells.

Competition assays

Competition assays were performed by modifying the previously described method [43]. The amount of coating antigen used was 30 ng per well. Briefly, equal amount of hybridoma supernatant was added to equal amount of blocking buffer. This initial dilution was then subjected to two-fold serial dilutions. Monoclonal antibodies were diluted to a concentration of 1 µg/ml in blocking buffer. 50 µl of the diluted antibody was added to each well along with 50 µl of the serially diluted hybridoma supernatant. Hence the final monoclonal antibody concentration during the assay was 0.5 µg/ml in each well, and the starting supernatant dilution was 1:4. In order to test competition at a higher concentration, 50 µl of hybridoma supernatant was added directly with 50 µl of competing antibody (at 1 µg/ml concentration) to result in the 1:2 dilution. Antibodies used for competition included VRC01 [8], and PGT121 [46]. For competition of purified monoclonal antibody to PGT121, 10A37 was diluted to a concentration of 6 µg/ml in blocking buffer and then subjected to two-fold serial dilutions. 50 µl of the diluted PGT antibody was added to each well along with 50 µl of the serially diluted 10A37 antibody. Hence, the final PGT concentration was 0.5 µg/ml in each well, and the starting 10A37 concentration was 3 µg/ml. Another mAb (2C2) that was isolated from a rabbit immunized with C-terminal 54 amino acid of gp41 ectodomain (to be published elsewhere), was used at similar concentrations as a negative control. The rest of the procedure was same as previously described [43].

Cloning of hybridoma antibody genes

Total RNA was extracted from hybridomas using the RNeasy Mini kit (Qiagen) using the Qiacube automated platform. Following extraction, RNA samples were treated with

DNase (Invitrogen) to remove genomic DNA. Samples were subjected to cDNA synthesis using random hexamers and SuperScript III Reverse Transcriptase (Invitrogen). Briefly, 2 μ L of random hexamers (Roche) and 2 μ L of 10 mM dNTPs were added to 22 μ L of DNase treated RNA (equivalent to approximately 3×10^6 cells). The mixture was heated to 65°C for 5 min, then cooled briefly on ice. Subsequently, 8 μ L of 5x First-Strand Buffer, 2 μ L of 0.1 M DTT, 2 μ L of RNaseOUT™ (Invitrogen), and 2 μ L of SuperScript III were added to the mixture. Reaction was incubated at 25°C for 5 min, 45°C for 45 mins, and 70°C for 15 mins. Resulting cDNA was subjected to Ab gene amplification using Platinum Pfx polymerase (Invitrogen) according to manufacturer's recommendations. Primers for Ab gene amplification were based on a previous publication [47]. Primers used for heavy chain amplification were 5'- AGGAATTCTGCAGCTCTGGCACAGGAGCTC-3' and 5'- CTCCGGATCCGTCGACAGGACTCACACCTGAGGAGACGGTGACCA-3'. Primers used for kappa chain amplification were 5'- TATCCGTGCACTCCACCATGGACACGAGGGCCCCCACT-3' and 5'- GTTAGATCTATTCTACTCACGACCTTTGACCACCACCTCGGTCCCTCCGCCGAA-3' or 5'- TCACTGGCGGTGCCCTGGCAGGCGTCT-3' (10A37 only). Cycling conditions were as follows: Initial denaturation at 94°C for 5 mins; followed by 35 cycles of 94°C for 30 sec, 68°C for 1.5 mins; final extension at 68°C for 7 mins; hold at 4°C. Resulting PCR products were directly sequenced. Alternatively, the 10A3 and 10A37 hybridomas were subjected to Ab gene specific cDNA generation and PCR using the SuperScript III One-Step RT-PCR System (Invitrogen), using the primers described.

Heavy and light chain sequence analysis

Heavy and kappa chain sequences were analyzed with IMGT/V-quest [48] to determine germline usage, mutations present, and CDR domain lengths. Protein sequence alignments were performed with Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/).

Expression and purification of 10A3 and 10A37 antibodies

Antibody variable regions were cloned into either the pFUSEss-CHIg-hG1 and pFUSEss-CLIg-hk (human conserved regions, 10A3 heavy and kappa chain respectively, InvivoGen) or pFUSEss-CHIg-rG and pFUSEss-CLIg-rk2 (rabbit conserved regions, 10A37 heavy and kappa chain respectively, InvivoGen) vectors for expression. Heavy chain primers for 10A3 were 5'-ACGTGAATTCGCAGGAGCAGCTGGAGGAGTC-3' and 5'-GACCGCTAGCTGAGGAGACGGTGACCAG-3'. Kappa chain primers for 10A3 were 5'-GGTCGAATTCAGCTCAAGTGCTGACCCAG-3' and 5'-GACCCGTACGTTTGACCACCACCTCG-3'. Heavy chain primers for 10A37 were 5'-ACGTGAATTCGCAGGAGCAGCTGGTGGAGTC-3' and 5'-GCCCACTCGAGACGGTGACCAGGGTGCCTGGGC-3'. Kappa chain primers for 10A37 were 5'-GGCGAATTCAGCCCTTGTGATGACCCAG-3' and 5'-CGAGCTAGCTCGCTCTAACAGTCACCCCTATTG-3'. Restriction sites introduced for subsequent cloning are underlined. The heavy chain PCR product for 10A3 and vector were digested with EcoRI and NheI. The kappa chain PCR product for 10A3 and vector were digested with EcoRI and BsiWI. The heavy chain PCR product for 10A37 and vector were digested with EcoRI and XhoI. The kappa chain PCR product for 10A37 and vector were digested with EcoRI and NheI.

Standard ligation protocols generated the final 10A3 rabbit-human chimera and 10A37 rabbit expression vectors, and sequencing confirmed an in frame variable region fusion.

For 10A3 and 10A37 antibodies purification, heavy and kappa chain constructs were co-transfected into freestyle 293F cells with 293fectin (Invitrogen). The supernatant was collected 5 days after transfection and clarified by centrifugation, followed by immobilized protein A affinity chromatography purification (Pierce). Purified 10A3 and 10A37 was dialyzed in PBS (pH 7.4), aliquoted and then stored at -80°C .

Results

Antibody responses against gp120 booster immunization following an extended resting period

In previous reports, we described antibody responses against monomeric MCON6 gp120 in rabbits following five immunizations over a period of about 29 weeks [42,43]. We selected one of the animals (rabbit #2) that had mounted strong neutralizing activity against Clade AE, tier 1 TH023.6 isolate ($\text{ID}_{50} > 43,740$ in TZM-bl assay), as well as some activity against tier 2 isolates, for long-term evaluation. The animal was allowed to rest for 38 weeks and immunized a 6th time on week 65 (Fig. 1A). A serum sample was collected just prior to immunization (referred to as “pre 6th”) to assess durability of antibody responses and to determine the baseline level, and two weeks post immunization on week 67 (referred to as “post 6th”) to evaluate recall responses.

Antibody levels were monitored by ELISA using the autologous antigen (Fig. 1B). A serum sample collected two weeks after the fifth immunization (on week 29) was used for

direct comparison. A serum from an age-matched, mock-immunized rabbit (designated as “PBS”) was used as a negative control. Results showed that the antibody level declined approximately 7-8 fold during the 38 weeks of resting period after the fifth immunization (estimated half-life of about 12.7 weeks). However, after the sixth immunization, the antibody level increased back to the level achieved after the fifth immunization, which appeared to be the maximum achievable antibody titer. Immunogenic linear epitope profile, as determined by ELISA using 15mer overlapping peptides, was similar to that observed after the fifth immunization (data not shown; [43]).

As described previously [42,43], the potency of neutralizing activity induced in this rabbit was quite substantial, albeit largely against tier 1 viruses. More interestingly, antibodies that could compete with bnAbs VRC01 as well as PGT121 and PGT126 in binding gp120 were observed [43]. Although we did not detect broadly neutralizing activity against tier 2 viruses, we hypothesized that better understanding of gp120-induced antibodies would allow for better design of future immunogens as well as immunization strategies. Our goal was to characterize these antibodies at a monoclonal level. Because such characterizations are time consuming and laborious, we first examined whether the rabbit maintained nAbs as well as VRC01- and PGT121-competing antibodies before undertaking a detailed molecular analysis.

Neutralizing activity of sera from pre- and post-6th immunization was tested against tier 1A viruses from Clade B (MN.3 and SF162.LS) and Clade C (MW965.26). Serum sample after the 5th immunization was also tested for comparison. As expected, neutralizing activity declined against all three viruses during the resting period between the fifth and the sixth immunization (Fig. 1C), similar to the decline observed in antibody titers (Fig. 1A).

Upon the sixth immunization, serum neutralization increased, but remained at a slightly lower level than what was observed after the fifth immunization. It should be noted that significant enhancement of pseudovirus infectivity was observed in mock-immunized serum when used at high concentrations (less than 1:100 dilution; shows up as negative neutralization). The reason for this phenomenon is not yet known. Antibody competition analyses also indicated that antibodies that could compete with bnAbs VRC01 and PGT121 in binding gp120 were also maintained (Fig. 1D), albeit at a slightly lower level compared to the level observed after the fifth immunization [43]. All together, these results indicated that despite slight reductions in neutralizing activity and antibody levels that compete with bnAbs, the overall quality of antibody responses remained largely unchanged despite a prolonged resting period. Based on these results, we decided to further characterize antibody responses by generating mAbs.

Generation of hybridomas and epitope mapping analyses by ELISA

To generate hybridomas, the rabbit was injected intravenously with 1 mg of soluble gp120 in PBS without adjuvants at week 76. Four days later, splenocytes were harvested for generating hybridomas. From ten 96-well plates used for the fusion, 548 hybridomas were generated. Hybridoma supernatants were collected and screened for gp120 binding by ELISA. In total, 95 clones were gp120-specific (17.3% cloning efficiency) although two were lost during propagation.

With a long-term goal of establishing a vaccine-induced “antibodyome” database for gp120, we initiated an effort to define the epitopes recognized by the mAbs by doing ELISA with culture supernatant. A number of envelope-derived protein constructs as well as

peptides were used, including gp120-OD (MCON6 gp120 outer domain we reported recently; [42]), BG505 SOSIP gp140 (soluble, stable trimeric envelope; [49,50]), eOD-GT6 (engineered outer domain that can bind germline BCR of VRC01; [51]), as well as V3 and V5 peptides, both of which were highly immunogenic [43]. Results from these ELISA are summarized in Fig. 2. The mAbs could be divided into four main groups: (1) bind gp120 only, (2) bind gp120 and SOSIP gp140, but not gp120-OD, (3) bind gp120 and gp120-OD, but not SOSIP gp140, and (4) bind all three proteins. Additional hybridomas in group 5, which have not been tested for reactivity to gp140, would belong to either group 1 or group 2. They were not tested further due to shortage of samples and because they were not of significant interest as they tested negative to all other screens. Hybridomas in group 6 exhibited low reactivity to gp120. Most of them reacted only with gp120 and would belong to group 1. However, due to their low antibody levels, some caution is warranted about their antigen-binding profile.

Most likely, the mAbs belonging to group 1 would bind to epitopes in the inner domain of gp120 that would be absent in the gp120-OD and inaccessible on SOSIP gp140 due to its trimeric state. The mAbs belonging to group 2 also likely bind to the inner domain of gp120, but accessible on SOSIP gp140 unlike those belonging to group 1. Based on overlapping peptide ELISA analyses, the rabbit #2 mounted strong antibody responses against several peptides within the inner domain, including those in regions C1 (peptides 8991, 8992 and 9003), C2 (peptides 9033, 9035 and 9036), and C5 (9090, 9094 and 9096). All of these peptides are present and should be accessible to antibodies on SOSIP gp140, except for peptides 9094 (⁴⁸⁹VKIEPLGVAPTKAKR⁵⁰³) and 9096 (⁴⁹⁷APTKAKRRVVEREKR⁵¹¹) in the C5 region (Fig. 3). Peptide 9003

(¹¹⁸PCVKLTPLCVTLNCT¹³²), which lies just upstream of the V1 loop, is situated at the top of the trimeric structure. Peptides 8991 and 8992 (⁷⁰ATHACVPTDPNPQEI⁸⁴ and ⁷⁴CVPTDPNPQEIVLEN⁸⁸, respectively) are on the bottom of the trimer. Peptides 9033, 9035 and 9036 (²⁴⁰KNVSTVQCTHGIKPV²⁵⁴, ²⁴⁸THGIKPVVSTQLLLN²⁶² and ²⁵²KPVVSTQLLNGLA²⁶⁶, respectively) are situated closer to the outer domain, with the latter two peptides encompassing a part of the outer domain (²⁵⁶STQLLNGLA²⁶⁶). To confirm our predictions, many of the mAbs in groups 1 and 2 were further probed with the indicated peptides. Surprisingly, 20 out of 40 (50%) mAbs tested were reactive against peptide 9096, indicating it is extremely immunogenic on soluble gp120. Three mAbs in group 2 (4-2, 6-10 and 8-24) tested positive to both peptides 8991 and 8992. Interestingly mAb 5-37 in group 1 reacted to both peptides 9035 and 9036. However, mAb 9-59 in group 2 tested positive only against peptide 9035, indicating a clear difference in the amino acids residues being recognized and/or the angle of approach in epitope binding.

There were seven mAbs that belonged to group 3, which bound gp120-OD, but not SOSIP gp140. These mAbs might bind epitopes available on monomeric gp120 and gp120-OD, but inaccessible on the trimeric SOSIP gp140. Overall, MCON6 and BG505 share 81% amino acid sequence identity. Thus, another possibility is that the amino acid sequences between MCON6 and BG505 are different at these epitopes. One interesting observation is that mAb 5-12 was able to bind not only V5 loop peptide (GNNSNKNKTETFRPG), but it was the only mAb that could bind eOD-GT6. Surprisingly, amino acid sequence alignment analyses of the region around the V5 loop showed that SOSIP gp140 sequence is actually more homologous to MCON6 gp120 than is eOD-GT6 (Fig. 4). At the present time, it is not known why 5-12 mAb binds to MCON6 gp120, gp120-OD and eOD-GT6, but not SOSIP

gp140. Finally, there were ten mAbs in group 4 that could bind gp120, gp120-OD and SOSIP gp140. Seven of these mAbs bound the V3 loop (1-35, 1-36, 2-37, 8-34-1, 9-13, 10A3 and 10A37) and two mAbs bound the V5 loop peptide (2-5-1 and 9-6). None of the other mAbs tested positive against the V3 loop. Overall, there were only 17 mAbs that were directed against the outer domain of gp120 (about 18%). Thus, mAbs against the V3 loop accounted for about 7.5% of all mAbs recovered and about 42% of all mAbs directed against the outer domain.

Neutralizing activity and competitive binding to or near the neutralizing epitopes

Since the serum from the rabbit showed significant competition against VRC01 and PGT121 (Fig. 1D), we screened all hybridomas for competing activities against these bnAbs. Unfortunately, we did not detect any hybridomas that could compete against VRC01 for gp120 binding, suggesting that such antibodies might be rare. Given that only a small fraction of splenocytes likely have yielded hybridomas, it is probable that B cells expressing VRC01 competing antibodies were not incorporated into our panel. While screening for mAbs that could compete against PGT121, we found seven hybridomas that possessed low, but definite activity (Fig. 5A). Interestingly, all of the competing hybridomas were reactive against the V3 loop peptide (Fig. 2) suggesting that serum competition observed against PGT121 might be due to the strong anti-V3 antibody response. Another mAb, 9-6 from group 4 that did not bind V3 but bound the V5 loop, exhibited no competing activity. Finally, we screened all the hybridomas for neutralizing activity against tier 1 pseudoviruses SF162.LC and MW965.26. Culture supernatants from two V3 peptide positive hybridomas, 10A3 and 10A37, showed strong neutralizing activity (data not shown; see below).

Detailed characterization of anti-V3 loop mAbs.

Since all anti-V3 loop mAbs could compete against PGT121 and two of them exhibited neutralizing activity, all of them were further characterized. First, fine epitope mapping analysis was done using overlapping 15-mer linear peptides that span the entire length of the V3 loop (Fig. 6). The ELISA result demonstrated that peptide 9048 (NNNTRKSIRIGPGQA) was the most immunoreactive segment in the V3 loop, as five mAbs could interact with this peptide. This is consistent with an observation that N-terminal half of the V3 loop is more immunogenic than the C-terminal half [42,43]. mAbs 1-36 and 8-34-1 recognized only peptide 9048. Another pair, 2-37 and 9-13, bound to peptides 9047 (CTRPNNNTRKSIRIG) and 9048. Hybridoma 1-35 was only weakly positive to peptide 9050 (RIGPGQAFYATGDII). The two hybridomas that were identified as having neutralizing activity, 10A3 and 10A37, exhibited totally different peptide recognition patterns. 10A3 reacted most strongly against peptide 9049 (RKSIRIGPGQAFYAT), but also recognized peptides 9047 and 9048. The peptide 9049 has the tip of the V3 crown (GPGQ) almost exactly at the center. This would suggest that mAb 10A3 has the profile of many other anti-V3 loop neutralizing mAbs, such as 447-52D [52], and HGN194 [53]. In contrast to 10A3, 10A37 strongly reacted against peptide 9050 and moderately to peptide 9051 (GQAFYATGDIIGDIR). Out of the seven mAbs, 10A37 was the only one that recognized peptide 9051, indicating that recognition of C-terminal half of the V3 loop is indeed rare. In this regard, 10A37 is a novel V3 loop mAb.

To further characterize V3 mAbs at the molecular level, antibody genes were RT-PCR amplified from hybridomas, sequenced and analyzed using IMGT/V-QUEST database [48]. First, many of the mAbs utilized the same germline V genes (Fig. 7). Four of the

mAbs (10A37, 1-36, 2-37 and 10A3), including the two that exhibited neutralizing activity used V1S45*01 V_H gene. mAbs 8-34-1 and 9-13 were derived from V1S40*01. There were two pairs of mAbs that used the same V_κ gene (V1S36*01 for 10A37 and 1-35, and V1S56*01 for 1-36 and 8-34-1). One interesting observation is that the light chain of mAbs 10A37 and 1-35, both of which bound peptide 9050, were nearly identical. Their LCDR sequences differed by a single amino acid difference in LCDR3. In contrast, HCDR sequences, which were derived from different germlines, were only about 53-56% identical, which may explain why 1-35 bound peptide 9050 only weakly and failed to exhibit neutralizing activity. Additional structure-function analyses of these mAbs would provide critical information that distinguishes their functional properties.

Similar to 10A37 and 1-35, mAbs 1-36 and 8-34-1, both of which only bound peptide 9048, were derived from the same V1S56*01 V_κ gene. Amino acid identities for LCDR1, -2 and -3 were 63%, 100% and 93%, respectively. Even though their V_H genes were derived from different germlines, their HCDR1 and HCDR3 sequences were quite similar (78% and 63%, respectively), suggesting a possible convergent evolution. Conversely, there were some signs of divergent evolution as well. For example, mAbs 10A3 and 2-37, both of which bound peptides 9047 and 9048, originated from the same V1S45*01 V_H germline, but their HCDR1, -2 and -3 sequences were markedly different (only 56%, 33% and 20% identity, respectively). These differences might have enabled 10A3 to bind peptide 9049 strongly and exhibit neutralizing activity. Similarly, mAbs 8-34-1 and 9-13, both of which were derived from the same V1S40*01 V_H germline also exhibited low homology (only 33%, 22% and 50% identity for HCDR1, -2 and -3, respectively). However, their LCDR

exhibited strong similarity despite the fact that they evolved from different germ lines, which might have allowed both mAbs to bind peptide 9048.

Characterization of recombinant neutralizing mAbs

To further characterize the neutralizing activity, recombinant mAbs 10A3 and 10A37 were cloned, expressed and purified. Their potency and breadth were assessed using a standard TZM-bl neutralization assay against a large panel of tier 1 and 2 pseudovirus from different clades (Fig. 8). Recombinant mAb 10A3 neutralized several Clade B and Clade C tier 1A and 1B isolates, as well as one tier 2 virus Clade C virus (TV1.21). Neutralizing potency and breadth of mAb 10A37 was more impressive than 10A3, being able to neutralize several additional viruses. In particular, 10A37 exhibited potent neutralizing activity against Clade AE virus TH023.6, which was shown to be highly susceptible to the immune sera shown in previous report [42]. In addition, 10A37 was able to neutralize tier 2 Clade C virus 25710-2.43, which is one of the twelve virus isolates that belong to the a new “global panel of reference strains” [54]. Unfortunately, 10A37 did not neutralize any of the other eleven viruses from the “global panel” (*i.e.* $IC_{50} > 25 \mu\text{g/ml}$, data not shown). 10A37 and 10A3, combined, were able to neutralize 11 of 15 tier 1A and 1B viruses tested (73%) and only 3/18 tier 2 viruses (17%). However, they were insufficient to recapitulate full neutralizing activity of antibodies present in the immune serum, which was able to neutralize MN.3 [42].

In Fig. 5A, we showed that all of the anti-V3 loop mAbs exhibited ability to compete with PGT121 for binding gp120. To confirm that anti-V3 loop antibodies are indeed able to compete with PGT121, we used recombinant 10A37. As shown in Fig. 5B, 10A37 was able to efficiently compete with PGT121 for binding gp120. These results suggest that PGT121-

competing activity we observed in immune sera could actually be due to anti-V3 antibodies, rather than true PGT121-like antibodies that bind V3 and a glycan immediately adjacent to the C-terminus of V3.

Discussion

The field of HIV-1 vaccine development has been aided immensely by the recent discovery of new bnAbs [11]. Furthermore, deep sequencing analyses have highlighted the complex evolution that the immunoglobulin germline must undergo to generate these rare antibodies [55,56]. Additional antibody structural studies, especially when performed in complex with HIV-1 envelope glycoproteins, have provided valuable insights for generating rationally designed immunogens [49-51]. However, translating the information generated from these studies into designing immunogens that can elicit similar bnAbs has been difficult. A critical fact that needs to be kept in mind is that HIV-1 infected individuals provide an environment where the virus and the immune system responses co-evolve. This dynamic environment generated by chronic virus infection is difficult to replicate using any vaccination regimen. Hence, while characterization of antibody responses in virus-infected individuals is valuable, immunization studies in animal models remain a vital means to evaluate realistic vaccine strategies against HIV-1.

Of all HIV-1 Env immunization studies conducted in animal models, only a limited number of studies have attempted to further characterize antibody responses at a clonal level. Generating mAbs is a labor intensive and time-consuming process. However, they can significantly aid in understanding the immune response as demonstrated in this study. First, mAbs permit precise mapping of B cell epitopes, which would not be possible using polyclonal antisera. This would provide necessary information to establish a comprehensive

map of vaccine-induced antibodyome against HIV-1 envelope glycoprotein, which we believe will be critical for developing a vaccine that can induce bnAbs against the virus. In this study, as an initial attempt to define epitopes recognized by what we believe is the largest panel of anti-gp120 rabbit mAbs generated to date, we examined antibody reactivity against different protein constructs and some of the immunogenic peptides identified from overlapping peptide ELISA [43]. Characterization of epitope targets would greatly aid our understanding of immune responses against HIV-1. This report focused primarily on V3 loop-specific mAbs since two of them exhibited potent neutralizing activity against tier 1 viruses with marked breadth.

The V3 loop has been known as the principal neutralizing determinant for over two decades [57,58]. Despite the fact that nAbs targeting this epitope exhibit only a limited breadth and largely against tier 1 viruses, they are the only ones that could be induced consistently in both animals and humans in a vaccine setting. It should be noted, however, that not all anti-V3 loop neutralizing mAbs exhibit equal potency or breadth; while some only neutralize autologous vaccine strains, others do exhibit marked breadth. Most of the broadly neutralizing V3 loop mAbs characterized to date have been derived from virus-infected human patients, including 447-52D that was reported over 20 years ago [52]. Recently, Hioe *et al.* [58] tested the breadth and potency of seven human anti-V3 loop antibodies. Their study showed that 56/98 (57%) pseudoviruses tested (both tier 1 and 2 isolates from Clades A, AG, B, C and D) could be neutralized by one or more mAbs (using Area Under Curve-methodology). More importantly, 9/24 (37.5%) tier 2 viruses could be neutralized by one or more mAbs. In another study, Corti *et al.* [53] characterized an anti-V3 mAb HGN194 in direct comparison to 447-52D. While 447-52D could neutralize only 88%

of the tier 1 and 4% of the tier 2 viruses tested, HGN194 was able to neutralize all tier 1 and 11% of the tier 2 viruses, suggesting superior breadth.

In contrast to mAbs generated from virus-infected humans, there are far fewer mAbs generated from animals immunized with HIV-1 antigens, especially those that exhibit neutralizing activity. Recently, however, Chen *et al.* reported the isolation of twelve mAbs from a rabbit immunized with a DNA prime-protein boost JR-FL gp120 vaccine regimen [39]. One of the antibodies recovered, R56, targeted the V3 loop and neutralized multiple tier 1 viruses belonging to Clades B, C, AE and AG as well as two tier 2 viruses in standard TZM-bl assays. It is not easy to compare neutralization potency or breadth of different mAbs characterized in different laboratories because not all of the same viruses are tested and the assays are not performed with identical virus stocks. Having said that, the potency of 10A37 seemed to be greater than those of R56. For example, 10A37 neutralized five viruses that R56 could not, including MS208.A1 (Clade A), 6535.3 (Clade B), ZM109F.PB4, 00836-2.5 and 25710-2.43 (Clade C). The neutralization potency (IC_{50}) of 10A37 was also significantly greater than R56 for multiple viruses: TH023.6 (<0.03 vs. $18.57 \mu\text{g/ml}$), SF162.LS (<0.03 vs. 0.1), Bal.26 (0.15 vs. 2.07), Bx08.16 (0.61 vs. 3.18), SS1196.1 (2.97 vs. 7.18). The only virus R56 neutralized, but could not by 10A37 was TV1.21 (Clade C). However, 10A3 could neutralize TV1.21 with greater potency than R56 (5.1 vs. $25.63 \mu\text{g/ml}$). Surprisingly, none of the three rabbit mAbs could neutralize MN.3, which is generally considered to be a highly sensitive tier 1 isolate. While there is a known structural basis for the failure of R56 to neutralize MN.3 [59], the reason for the lack of neutralization by 10A3 and 10A37 remains to be determined. Interestingly, neither 10A3 nor 10A37 neutralized BG505 Δ CT/T332N (data not shown), despite the fact that these two mAbs strongly reacted

to SOSIP gp140 by ELISA (Fig. 2). We speculate that this is possibly due to the V3 loop of SOSIP gp140 being exposed during normal coating of the protein onto ELISA plates. In this regard, a gentler sandwich ELISA protocol might be more appropriate.

One of the advantages of mAbs is that they allow functional separation of antibodies with different properties. As such, one of our objectives was to isolate mAbs that could compete against bnAbs VRC01 or PGT121 in binding gp120. Unfortunately, we were unable to identify any VRC01-competing mAbs. On the other hand, we identified seven mAbs that could compete with PGT121 and these turned out to be specific for the V3 loop. This result indicates that PGT121-competing activity we detected in immune sera [43] is likely due to a high level of antibodies against the V3 loop. This finding highlights a possibility that induction of high titers of V3 loop antibodies might prevent eliciting more effective PGT121-like antibodies. As such, it might be prudent to minimize immunogenicity of the V3 loop in future immunogen design.

One other important benefit of working with mAbs is the ability to evaluate antibody repertoire and function at the molecular level. This is particularly useful when evaluating multiple mAbs that target nearby epitopes at a given region (*e.g.* V3 loop), yet exhibit different functional phenotypes (*e.g.* neutralizing vs. non-neutralizing). Neutralizing mAb 10A37 and non-neutralizing mAb 1-35 are good examples. These mAbs target the same epitope (*i.e.* RIGPGQAFYATGDII), albeit with different affinity. Although their light chains originated from the same germline and are virtually identical in sequence, their heavy chain sequences are quite divergent, which would indicate that the differences in their heavy chains likely account for the phenotypic difference. One could speculate that the heavy chain germline used by 1-35 (IGHV1S7*01) might be poorly primed for efficient affinity

maturation. In contrast, the 10A37 germline might have been better able to undergo affinity maturation, as evidenced in part by the overall preference for IGHV1S45*01 and the closely related IGHV1S40*01 in V3-binding antibodies. It should be emphasized that mAbs we generated likely represent only a small subset of all antibodies that could bind any given epitope. As such, they provide only a snapshot of a long evolutionary pathway. Sequence profiling of all the expressed antibodies in the immunized animal using NGS would provide the means by which to examine differences in the maturation pathways of neutralizing versus non-neutralizing antibodies without the limitations imposed by hybridoma production.

Assuming that all B cells have an equal chance of being fused into hybridomas and that specificity of antibodies have no affect on survivability of the hybridomas, the frequency of hybridomas targeting a given epitope should represent the relative immunogenicity of the epitope. In this regard, the single most frequent epitope recognized by the mAbs is the 9096 peptide (20 of 93 mAbs), which we had previously identified as the single most immunogenic peptide based on ELISA with antisera. The 9096 peptides lies at the very C-terminus of gp120. The next most immunogenic epitope is the V3 loop. Considering that antibodies that bind these epitopes are either non-neutralizing or neutralizing with limited breadth, a better immunogen or a vaccine strategy would be needed to improve focusing immune responses towards epitopes targeted by bnAbs. Although we have not succeeded in inducing bnAbs in this study using MCON6 gp120, the methodologies we have established and the reagents we have generated should facilitate evaluation of antibody responses against other immunogens or vaccine strategies in the future.

Conclusions

In this study, antibody responses against MCON6 gp120 in a rabbit were further characterized at a clonal level using a large panel of monoclonal antibodies (mAbs) generated from the immunized animal. Epitopes were defined using a set of different envelope protein constructs as well as linear peptides. The most immunogenic epitope was at the C-terminal end of the protein, followed by the V3 loop. Two new neutralizing antibodies (nAbs) against the V3 loop were isolated (10A3 and 10A37). While 10A3 was similar to many previously isolated neutralizing mAbs recognizing the N-terminal half of the V3 loop including the crown at the tip, 10A37 was atypical with its epitope positioned more towards the C-terminal half of the loop. 10A37 exhibited potent neutralizing activity with substantial breadth against multiple clades of HIV-1 that exhibit a tier 1A and tier 1B neutralization phenotype. To our knowledge, it is the most potent and broadly neutralizing anti-V3 loop mAb isolated from a vaccinated animal or human to date. Further characterization of its structure as well as the epitope it binds could provide important insights into the neutralization mechanism.

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Figures

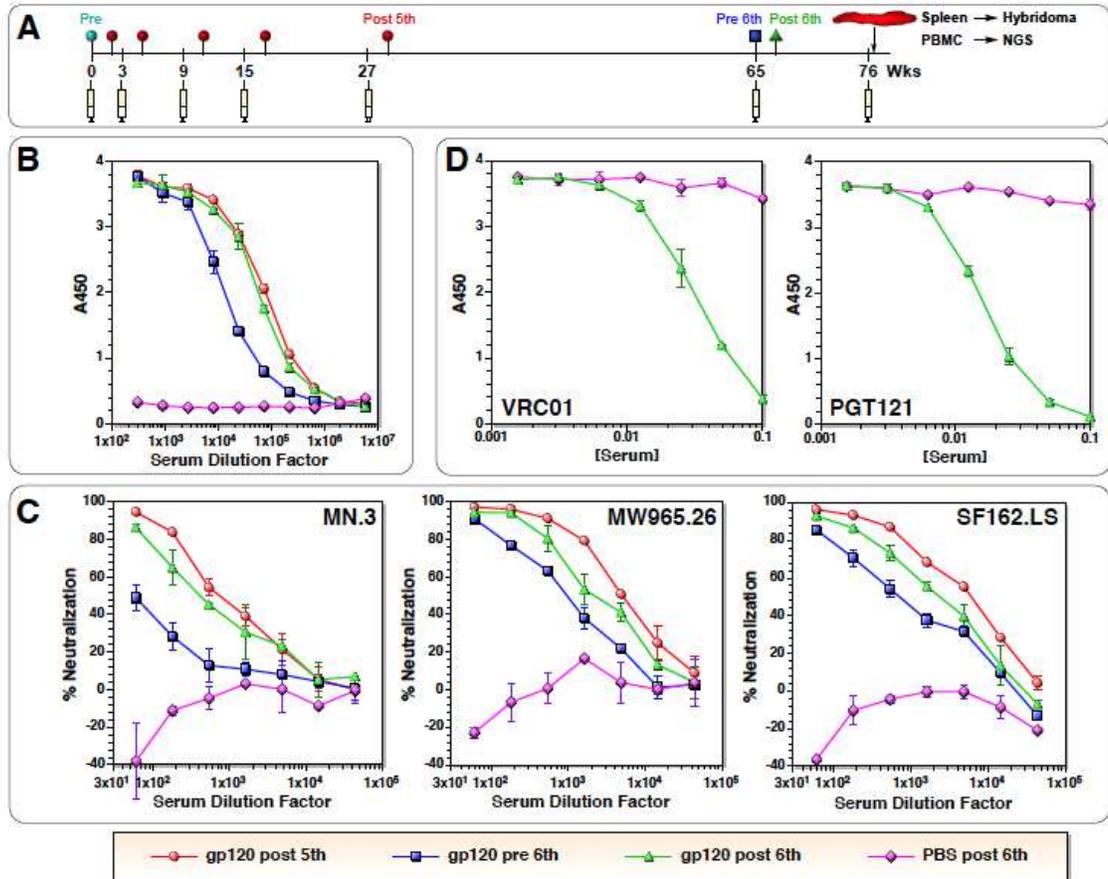


Figure 1. Characterization of antibodies induced after the 6th immunization. (A) Timeline of immunization and sample collection. (B) Comparison of antigen-specific antibody titers after the 5th, before 6th, and after 6th immunization. A serum sample from an age matched, mock-immunized animal, indicated as “PBS”, was used as a negative control. A450 represents absorbance value at 450 nm. The same legend at the bottom is used for all of the panels. (C) Neutralizing activity against MN.3, MW965.26 and SF162.LS. (D) The serum sample after the 6th immunization was tested for competition against VRC01 and PGT121.

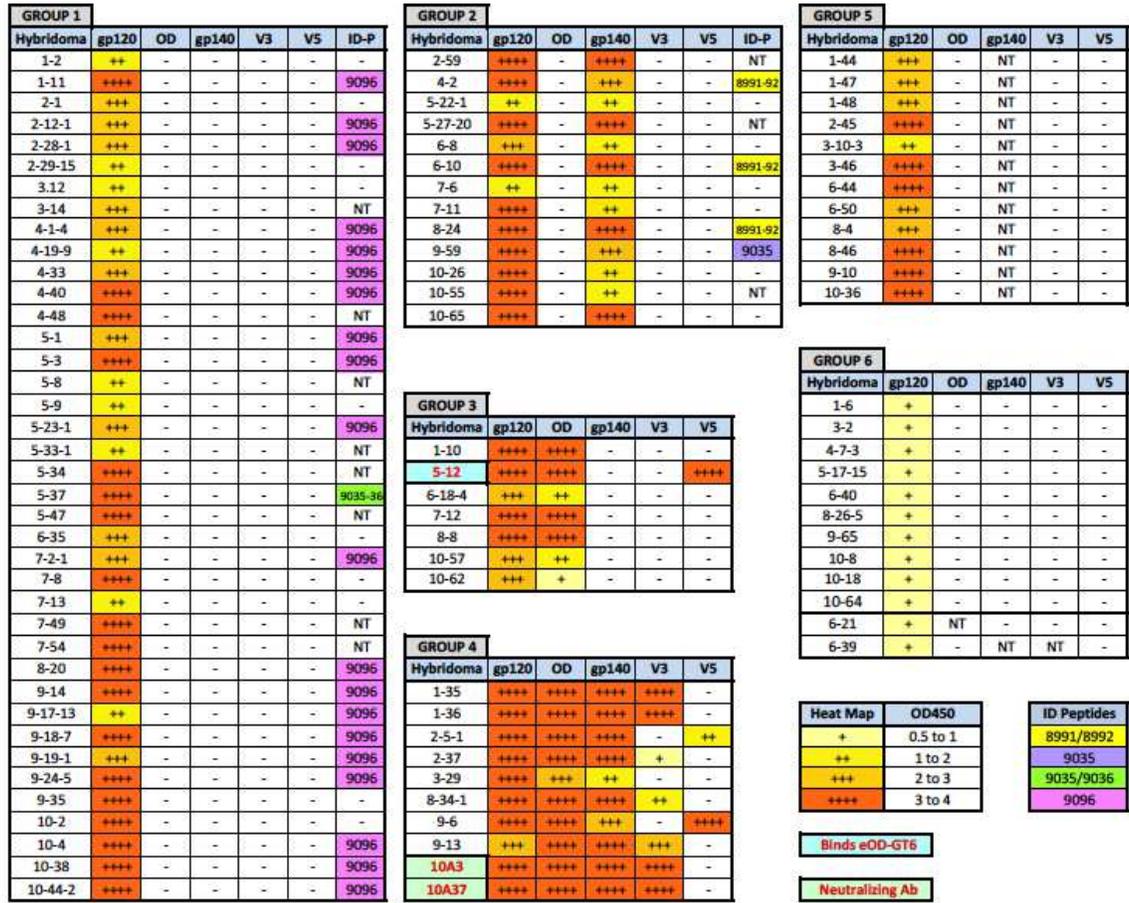


Figure 2. Epitope mapping analyses of hybridomas generated. Hybridomas were evaluated for reactivity against gp120, gp120-OD, BG505 SOSIP gp140, as well as variable loop peptides V3 and V5, and peptides from the inner domain (ID-P). Hybridomas are arranged in groups based on their reactivity to three proteins as discussed in the text.

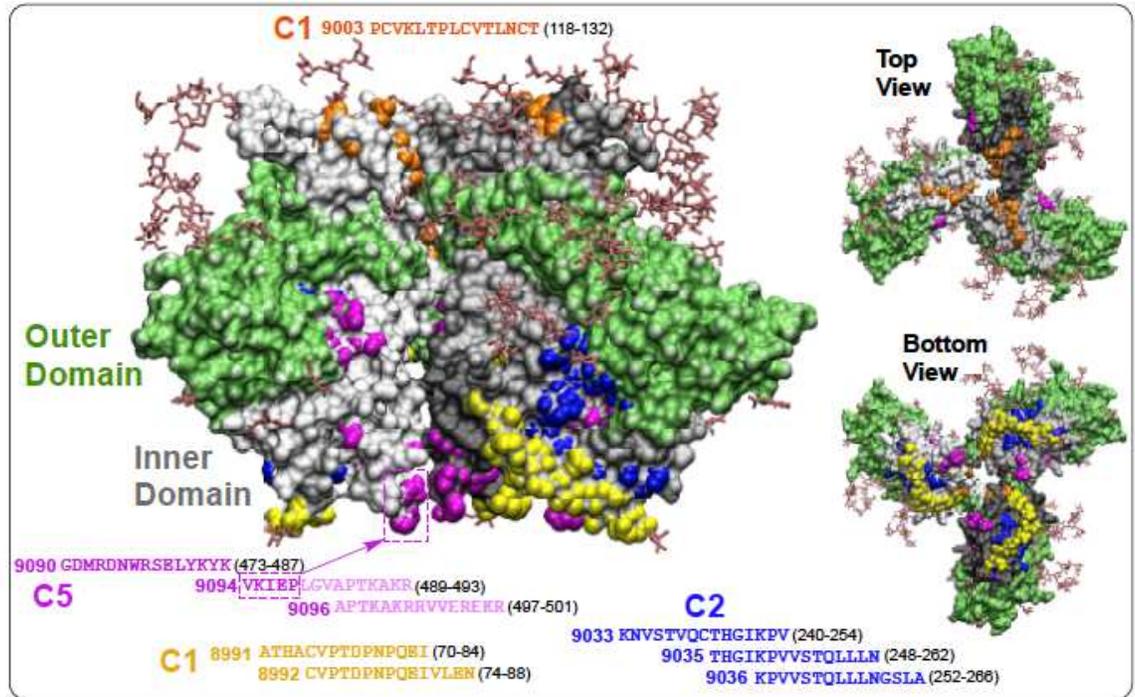


Figure 3. Locations of immunogenic peptides in the inner domain. The crystal structure of trimeric BG505 SOSIP gp140 (pdb: 4NCO) was used to illustrate location of the immunogenic peptides in the inner domain. Only the gp120 portion is shown for clarity. The outer domain is shown in lime and the inner domain is shown in three shades of gray. A part of peptide 9094 and 9096 from the C5 region (indicated in a lighter magenta shade) are not shown in the crystal structure. The arrow points to the position of the five amino acids (VKIEP) on peptide 9094.

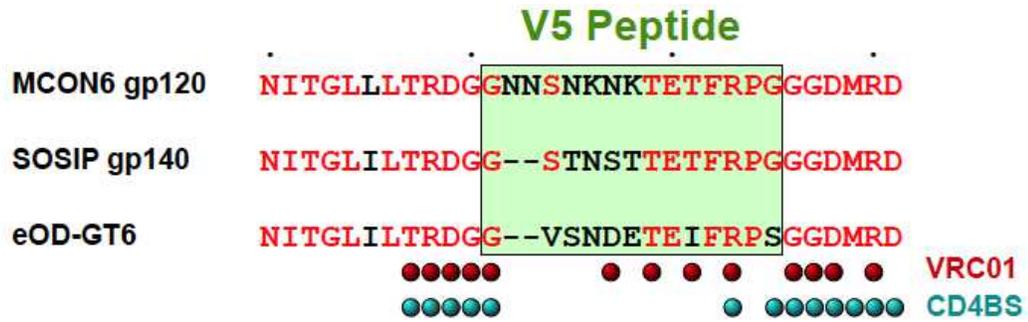


Figure 4. Sequence alignment of a region around the V5 loop. Sequences for the three antigens used for ELISA are shown (MCON6 gp120, BG505 SOSIP gp140 and eOD-GT6). The V5 loop peptide used for ELISA is boxed in. Identical amino acid residues are indicated in red. The residues that make contact with bnAb VRC01 or CD4 are indicated as red or cyan circles.

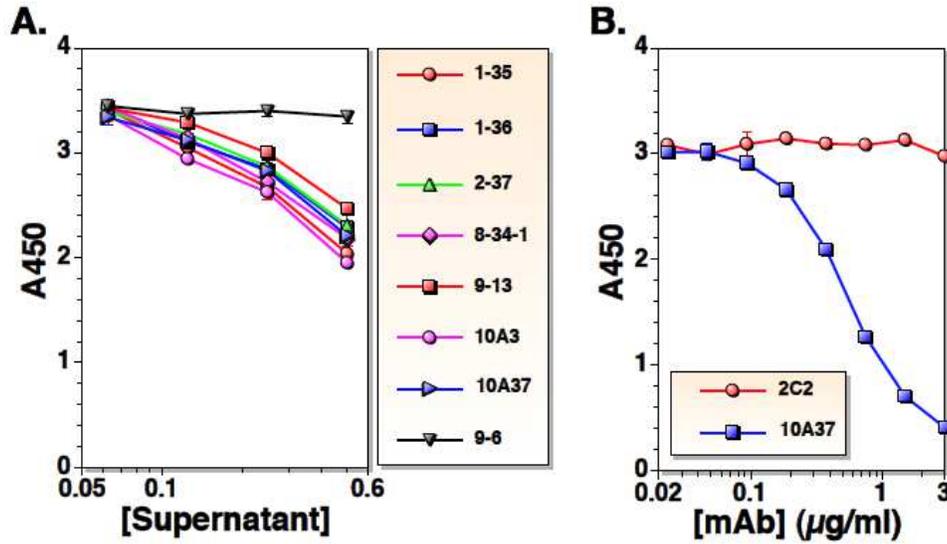


Figure 5. Antibody competition against PGT121. (A) Culture supernatants of hybridomas specific against the V3 loop were evaluated for competing activity against PGT121 for binding gp120. (B) Recombinant mAb 10A37 was used for the competition assay. Anti-gp41, recombinant mAb 2C2 was used as a negative control.

		Peptides							
		9046	9047	9048	9049	9050	9051	9052	V3
V3 Hybridoma	1-35					+			++++
	1-36			++					++++
	2-37		+++	++					+
	8-34-1			++					++
	9-13		++	++					+++
	10A3		+++	+++	++++				++++
	10A37					++++	++		++++
		9046	VEINCTRPNNNTRKS						
	9047	CTRPNNNTRKSIRIG							
	9048	NNNTRKSIRIGPGQA							
	9049	RKSIRIGPGQAFYAT							
	9050	RIGPGQAFYATGDII							
	9051	GQAFYATGDIIGDIR							
	9052	YATGDIIGDIRQAH							
	V3	TRPNNNTRKSIHIGPGQAFYATGEIIGDIRQAH							

Figure 6. Epitope mapping analyses of anti-V3 loop mAbs. The V3 loop-positive antibodies were tested for binding to overlapping 15-mer peptides spanning the entire loop by ELISA. The sequences of the peptides are shown at the bottom.

Chain	mAb	V gene	D gene	J gene	CDR1	CDR2	CDR3	
					56%	56%	53%	
Heavy	10A37	V1S45*01	D8-1*01	J4*01	GFSFSSGFF	IYGGSDNT	CARDAGTSGYIAY-NLW	
	1-35	V1S7*01	D4-1*01	J4*01	GFSLSSSYC	IYAGSSGST	CARAPYSSGWVLYFNLW	
Kappa	10A37	V1S36*01	-	J1-2*01	EDIQRN	GVS	CQSALYTSATDICALF	
	1-35	V1S36*01	-	J1-2*01	EDIQRN	GVS	CQSALYTSASDICALF	
					100%	100%	93%	
					78%	13%	63%	
Heavy	1-36	V1S45*01	N/A	J4*01	GFDLSGYWY	INTGAGD-	CARSFFLW	
	8-34-1	V1S40*01	N/A	J4*01	GFDLSQNWY	IYSIDSSV	CTRSFYFW	
Kappa	1-36	V1S56*01	-	J1-2*01	QTIHNGNN	DAS	CLGGYDCSSADCGAF	
	8-34-1	V1S56*01	-	J1-2*01	QNVFNGNN	DAS	CLGGYDCSSGDCGAF	
					63%	100%	93%	
					78%	33%	47%	
Heavy	2-37	V1S45*01	D4-1*01	J4*01	GFSFSSSY	IYAGSSGTT	CARWDSGGWGPFDLW	
	9-13	V1S40*01	D3-3*01	J4*01	GFSFSSGYD	IYTGNDNP	CARQ-----FDLW	
Kappa	2-37	V1S32*01	-	J1-2*01	QSIYSF--	FAS	CQQTYNF-GAGINSF	
	9-13	V1S46*01	-	J1-2*01	HSVDNINN	DAS	CLGGYDCSTTDCGAF	
					13%	67%	20%	
					56%	33%	40%	
Heavy	10A3	V1S45*01	N/A	J4*01	GFSFSAIA-	I-ATDTGS-	CAR-----NFYLW	
	2-37	V1S45*01	D4-1*01	J4*01	GFSFSSSY	IYAGSSGTT	CARWDSGGWGPFDLW	
Kappa	10A3	V1S15*01	-	J1-2*01	QSVYPNNN	EAS	CLGAYDFTVAEGAAF	
	2-37	V1S32*01	-	J1-2*01	QSIYSF--	FAS	CQQTYNF-GAGINSF	
					38%	67%	33%	
					33%	22%	50%	
Heavy	8-34-1	V1S40*01	N/A	J4*01	GFDLSQNWY	IYSIDSSV-	CTRSFYFW	CDR a.a. Identity
	9-13	V1S40*01	D3-3*01	J4*01	GFSFSSGYD	IYTGNDNP	CARQFDLW	>75%
Kappa	8-34-1	V1S56*01	-	J1-2*01	QNVFNGNN	DAS	CLGGYDCSSGDCGAF	51-75%
	9-13	V1S46*01	-	J1-2*01	HSVDNINN	DAS	CLGGYDCSTTDCGAF	26-50%
					50%	100%	87%	0-25%

Figure 7. Comparison of CDR regions of the anti-V3 loop mAbs. The heavy and light chains of the seven V3 loop-positive mAbs were aligned for analysis.

Comparison was done based on peptide reactivity shown in Fig. 6. Percentages indicate % amino acid identity between the two CDR being compared.

Virus			mAb			
Name	Clade	Tier	10A37	10A3	CH01-31	
Q23.17	A	1B	>68	>45	<0.01	
TH023.6	AE	1A	<0.03	>45	0.1	
MN.3	B	1A	>68	>45	0.75	
SF162.LS	B	1A	<0.03	0.05	0.5	
W61D-TCLA.71	B	1A	>68	<0.01	0.26	
Bx08.16	B	1B	0.61	1.9	0.42	
6535.3	B	1B	0.26	>45	0.22	
BaL.26	B	1B	0.15	1.06	0.09	
SS1196.1	B	1B	2.97	5.51	0.67	
BZ167.12	B	1B	>68	>45	0.13	
MW965.26	C	1A	<0.03	<0.02	3.22	
92BR025.9	C	1B	3.85	39.2	0.57	
ZM109F.PB4	C	1B	6.05	>45	0.05	
ZM197M.PB7	C	1B	>68	>45	0.81	
00836-2.5	C	1B	32.27	>45	>25	
MS208.A1	A	2	18.25	>45	0.02	
BG505ΔCT/T332N	A	2	>68	>45	0.02	
X2278_C2_B6	B	2	>68	>45	0.07	
TRO.11	B	2	>68	>45	0.5	<0.1
25710-2.43	C	2	20.58	>45	0.14	<1
TV1.21	C	2	>68	5.1	0.68	<10
Ce1176_A3	C	2	>68	>45	0.17	<45

Figure 8. Neutralizing activity of anti-V3 loop mAbs 10A3 and 10A37. V3 positive mAbs 10A37 and 10A3 were tested for neutralization against pseudoviruses belonging to different clades and tiers of HIV-1 and their IC₅₀ values were reported. These mAbs were compared to a combination of two mAbs (CH01 and VRC-CH31) that are known to show broad cross-clade neutralization.

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