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Design and evaluation of immunogens based on gp41 membrane-proximal external region for development of a vaccine against human immunodeficiency virus type 1

Saikat Banerjee
Iowa State University

Design and evaluation of immunogens based on gp41 membrane-proximal external region for development of a vaccine against human immunodeficiency virus type 1

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Saikat Banerjee

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Program of Study Committee:
Michael Cho, Major Professor
Cathy Miller
Bradley Blitvich
Michael Wannemuehler
Amy Andreotti

Iowa State University

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DEDICATION

This dissertation is dedicated to my mom, my first teacher.

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ABSTRACT

Since its discovery in 1981, HIV-1 has infected ~78 million people and killed ~39 million people. Developing an HIV-1 vaccine remains one of the top priorities in the fight against this devastating pandemic. The modest efficacy showed by the recent RV144 trial suggests that achieving this goal might be possible. As the search for an effective vaccine continues, the induction of antibodies that can neutralize a large number of antigenically distinct viruses from different clades remains a major goal. In recent years, a number of broadly neutralizing antibodies (bnAbs) that target the HIV-1 envelope have been isolated from virus-infected patients, offering hope for vaccine development. Of the different targets on the HIV-1 envelope, the membrane-proximal external region (MPER) of gp41 has been recognized as an attractive candidate for vaccine development. Besides playing a critical role in virus fusion, this domain also contains highly conserved linear epitopes recognized by some of the broadest neutralizing antibodies like 4E10 and 10E8. However, the lack of MPER structural details poses a significant challenge in designing MPER-based vaccines. In our attempts to induce MPER targeting bnAbs, we have designed and evaluated immunogenic properties of multiple antigens in rabbits. Our findings demonstrate that the immunogenicity of the MPER is strongly influenced by the presence or the absence of neighboring domains. Although we have not yet succeeded in inducing 4E10-/10E8-like antibodies, we have made significant progress towards targeting 4E10/10E8 epitope.

CHAPTER 1

GENERAL INTRODUCTION

Dissertation Organization

This dissertation is divided into six chapters. Chapter 1 presents the "General Introduction" describing the history of HIV-1 and the current pandemic, the course of HIV-1 infection, components of the HIV-1 genome and the importance of HIV-1 envelope function for both virus infection and host immune response. This is followed by an overview of the current status of HIV-1 vaccine development and detailed discussion of gp41-based vaccine development approaches.

Chapter 2 is presented as a manuscript in preparation titled "Effect of polyethylene glycol-based silencing of gp41 envelope cluster II immunodominant epitope on MPER immunogenicity." This manuscript describes an attempt to prevent immune responses against a non-neutralizing, immunodominant epitope on a soluble gp41 antigen. The effect of this immuno-silencing on MPER immunogenicity is further evaluated. The contribution of each author is as follows: Habtom Habte provided the gp41-54Q antigen; Saikat Banerjee performed the PEGylation, antigenic characterization of the PEGylated antigens, rabbit immunizations and characterization of antibody responses; Saikat Banerjee and Michael Cho wrote and revised the manuscript.

Chapter 3 is presented as a manuscript in preparation titled "Immunological characterization of putative gp41 fusion intermediates of HIV-1." This manuscript evaluates MPER immunogenicity in the context of antigens designed to mimic the fusion intermediate form of gp41. The contribution of each author is as follows: Saikat

Banerjee generated HR1-AA-54Q and HR1-EE-54Q antigens while Heliang Shi generated the HR1-Δ10-54K and HR1-Δ17-54K antigens; Saikat Banerjee and Heliang Shi immunized the rabbits, and performed assays for antibody titers and linear epitope mapping; in addition, Saikat Banerjee performed the antigenic and structural characterization of all antigens, and analyzed all anti-MPER antibody responses using PepScan assays; Saikat Banerjee and Michael Cho wrote and revised the manuscript.

Chapter 4 is presented as a manuscript in preparation titled "Immunological characterization of a gp41-MPER antigen containing the native transmembrane domain of HIV-1." This manuscript attempts to characterize MPER immunogenicity in the context of a membrane bound antigen using a liposomal delivery system. The contribution of each author is as follows: Yali Qin cloned the gp41-54TM antigen; Saikat Banerjee produced the protein antigen, developed and characterized the liposomal delivery system, conducted rabbit immunizations and characterization of antibody responses; Saikat Banerjee and Michael Cho wrote and revised the manuscript.

Chapter 5 is presented as a manuscript in preparation titled "Characterization of MPER-targeting rabbit hybridomas generated using a novel prime-boost immunization". This manuscript evaluates the ability of two prime-boosts approaches to elicit MPER targeting antibodies. One rabbit is further used for hybridoma generation and three different hybridomas are characterized. The contribution of each author is as follows: Heliang Shi provided the MPER28x3 antigen; Yali Qin cloned 54CT DNA; Saikat Banerjee, Yali Qin and Hojin Moon performed animal immunizations; Saikat Banerjee generated the gp41-54TM proteoliposomes, and characterized all antibody responses. Hojin Moon generated and maintained rabbit hybridomas; Saikat Banerjee screened and

characterized all rabbit hybridomas; Saikat Banerjee and Michael Cho wrote and revised the manuscript.

Finally, Chapter 6 summarizes the overall conclusions of each chapter along with future directions. The dissertation also contains an appendix section containing a recently published manuscript co-authored by Saikat Banerjee. The work in this manuscript is relevant to the work described in the dissertation and hence has been provided for reference.

Literature Review

The HIV-1 Pandemic

In 1981, following the deaths of young homosexual men from rare opportunistic infections and malignancies, a new disease named Acquired Immune Deficiency Syndrome (AIDS) was identified[1,2]. While this disease was originally dubbed as the "gay plague", reports of heterosexual transmission of AIDS by the CDC in 1983 strongly challenged this misconception[3]. In the same year, the cause of this disease was traced to a retrovirus that is now known as Human Immunodeficiency Virus Type 1 (HIV-1)[4-7]. Interestingly, a morphologically similar virus named HIV-2 was identified in patients in western Africa in 1986[8]. However, HIV-2 was found to be antigenically distinct from HIV-1 and more closely related to other viruses that were collectively known as simian immunodeficiency viruses (SIVs)[9,10]. While SIVs were non-pathogenic in their natural hosts, simian versions of HIV-1 and HIV-2 were soon discovered in chimpanzees[11] and sooty magabeys[12], suggesting that cross-species lentiviral infection from different primates might have led to the emergence of AIDS-like disease in these animals. Later

reports further suggested that HIV-1 and HIV-2 evolved as a result of zoonotic virus transfer from primates in Africa[13].

Based on distinct cross-species transmission events, HIV-1 has been divided into four lineages named M (major), O (outlier), N (non-M, non-O) and P (putative/pending the identification of further human cases). Since its discovery in 1990, Group O has been found to represent less than 1% of the total HIV-1 infections and is restricted to Cameroon and its neighboring countries. Group N (discovered in 1998) has been found only in Cameroon, and Group P was recently discovered in a Cameroonian woman in France in 2009. In comparison, the first-known lineage, Group M, has infected millions of people across the globe and represents the pandemic form of HIV-1. These groups are further divided into clades (or subtypes) based on their phylogenic relationship based originally on subgenomic regions of individual genes and later modified to include multiple subgenomic regions or full-genome sequences (reviewed in[14]). This newer analysis has identified circulating recombinant forms (CRFs) found in more than one individual or unique recombinant forms (URFs) found in a single individual. These recombinant forms probably originated in individuals infected with multiple subtypes. For example, Group M is divided into at least 9 different clades (A, B, C, D, F, G, H, J and K) along with many CRFs and URFs. Traditionally, these subtypes and CRFs are associated with specific geographic regions (reviewed in [14-16]), but recent cases of non-native subtype transmission within a certain geographic region due to immigration have been reported[17].

As of 2013, there are about 35 million HIV-1 infected individuals according to the report from the Joint United Nations Program on HIV/AIDS

(http://www.unaids.org/sites/default/files/media_asset/UNAIDS_Global_Report_2013_en_1.pdf). Most of these infections are acquired through mucosal surfaces in adults, but this virus can also be transmitted through percutaneous, and perinatal routes[18,19]. While the administration of antiretroviral therapy drug therapy has been effective in controlling the infection in affected individuals (reviewed in[20,21]), it is believed that a successful HIV-1 vaccine is critical for preventing new infections.

HIV-1 envelope

While HIV-1 primarily infects both CD4+ macrophages and T cells, the overall depletion in T cell population leads to immunopathogenesis that renders the host susceptible to secondary infection (reviewed in[22,23]). HIV-1 binds and infects these cells using the envelope glycoprotein. Initially, each envelope glycoprotein is expressed as a gp160 precursor that is heavily glycosylated in the Golgi complex[24] and is cleaved by a furin-like protease into two subunits, gp120 and gp41[25,26]. These two subunits associate non-covalently to form trimeric, heteroduplex spikes on the virus surface (Fig. 1).

The primary function of gp120 is to bind the host cell receptor (CD4) and co-receptors (*viz*. CCR5 and CXCR4), while the role of gp41 is to mediate fusion between viral and cellular membranes[27-29] (Fig. 2). Following docking of the viral spike to CD4, the gp120 subunit undergoes local as well as quaternary structural rearrangement to position the V1/V2 and V3 loops for co-receptor binding[30-33]. This "opening-up" of the gp120 domain in-turn triggers conformational changes in the gp41 subunit. The gp41 subunit stores the free energy needed for membrane fusion in a metastable, pre-fusion conformation. Changes in gp120 transform gp41 into a fusion-active intermediate state

represented by the insertion of N-terminal fusion peptide domain into the host-cell membrane. During the final steps of fusion process, interactions between two heptad repeat regions, HR1 and HR2, lead to the formation of a hairpin structure that pulls the virus and host cell membrane together and merges them while forming the highly stable six-helix bundle[27,28].

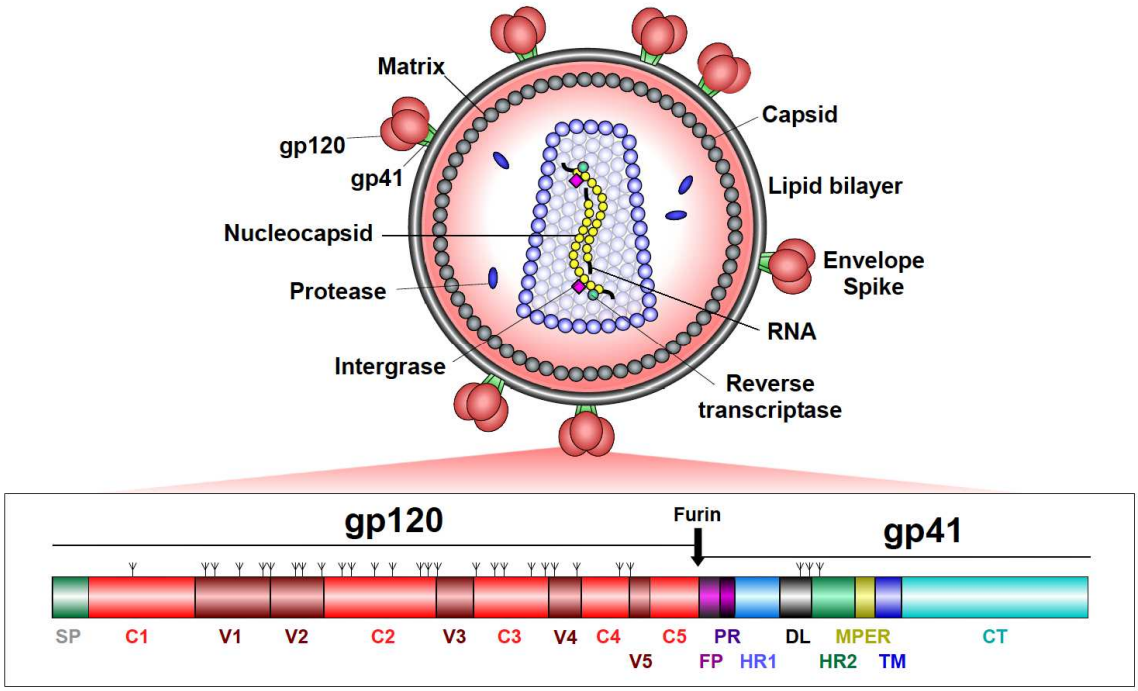

Figure 1: Structure of the mature HIV-1 virion showing the different domains of the gp120 and gp41 subunits. These two domains are generated by furin cleavage of the gp160 envelope protein. The heavily glycosylated gp120 subunit contains a signal peptide (SP) followed by five constant (C1-C5), and five variable (V1-V5) regions. The gp41 protein consists several domains in the order (N- to C-terminus)- fusion peptide (FP), polar region (PR), heptad repeat 1 (HR1), immunodominant loop (DL), heptad repeat 2 (HR2), membrane proximal external region (MPER), transmembrane (TM) and cytoplasmic tail (CT).

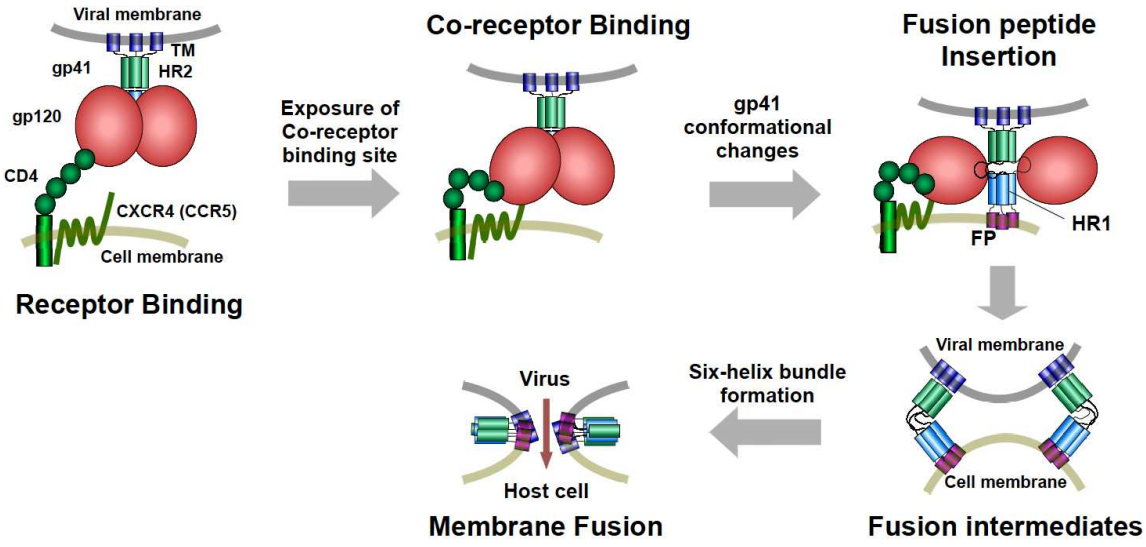

Figure 2: Role of HIV-1 envelope in virus fusion.

While the envelope glycoprotein plays the crucial role of mediating virus fusion to the host, it is equally important for the host humoral immune responses. HIV-1 specific antibodies can be detected as early as the first week after infection[34]. This initial antibody response is non-neutralizing, first targeting the gp41 and followed by anti-gp120 antibodies within a few weeks. After several months, some autologous neutralizing antibodies are developed[35-39]. Interestingly, these antibodies inadvertently drive virus evolution to generate escape mutants[38-40]. Ultimately, this continuous co-evolution of virus and the host immune system results in the development of some cross-clade neutralizing antibodies in about 2-4 years[41]. High levels of these cross-clade neutralizing antibodies have been detected in about 20% of the infected individuals[36,42-45]. However, 1% of the infected population (called "elite neutralizers") can elicit neutralizing antibodies effective against hundreds of different viral strains belonging to different HIV-

1 clades[46], termed broadly neutralizing antibodies (bnAbs). Typically, the neutralizing breadth and potency of sera from elite neutralizers is due to the synergistic and/or complementary activity of multiple neutralizing antibodies and/or a few different bnAbs[47-52]. While these bnAbs might not suppress virus replication completely due to viral escape[38,39], they might help by sustaining low viral loads in infected patients, especially since mutational escape may decrease virus fitness[53]. However, passive transfer of bnAbs confers protection against HIV-1 infection[54-58]. These findings have established a general consensus that a successful HIV-1 vaccine must have components for eliciting bnAbs[59,60].

The isolation of potent bnAbs from infected patients has improved greatly over the last five years. This has been possible due to the use of flow cytometry based B cell capture using novel recombinant, soluble HIV-1 envelopes and development of single cell antibody cloning techniques[61-64]. Epitope mapping of these highly potent bnAbs has redefined the sites of vulnerabilities on the envelope glycoprotein into five major categories (Fig. 2, from a recent review by [65]). These are the (1) CD4 binding site, glycan associated (2) V1/V2 loops and (3) V3 loop, (4) membrane proximal external region (MPER) and the newly identified (5) glycan associated bridging region between gp120 and gp41. In addition, one bnAb, 2G12[66], has been reported to target a glycan-only epitope on gp120[67]. Regardless of category, all bnAbs function either by blocking virus binding or by inhibiting the conformational changes required for virus fusion. Additionally, sequence and structural characterization of these bnAbs have highlighted some of the unique features that might contribute to their ability to neutralize multiple virus strains. First, these antibodies contain a high percentage of somatic hypermutation

(as high as 40% amino acid substitutions), especially in the variable heavy chain region genes[50,63,68-74], along with insertions or deletions in their complementarity determining regions (CDRs)[70,75]. This suggests that their *in vivo* maturation might be a complex process. Second, several antibodies have especially long heavy chain CDR3 (HCDR3) (between 20-34 amino acids). These long HCDR3s are thought to interact with V1/V2 and V3 loops by penetrating the glycan shield, bind the gp120/gp41 bridging region and aid in accessing the gp41 MPER domain[76]. Finally, several of these antibodies show autoreactivity or polyreactivity[48,70,72,73,77,78], suggesting that these might be induced as a result of dysfunctional B cell tolerance in patients with prolonged chronic HIV-1 infection[64,79,80].

As discussed above, bnAbs are effective against large numbers of virus strains from multiple HIV-1 clades because they typically target epitopes that are more conserved and functionally important. However, they are generated only after multiple years post-infection. This is probably due to the fact that HIV-1 has evolved multiple ways to hinder the elicitation of effective neutralizing antibodies. First, HIV-1 causes major changes in B cell development and function during infection due to pathological effects on lymphoid tissues that harbor the B cells, as well as the lack of T cell help in later stages. These changes include hypergammaglobulinemia, enhanced polyclonal activation, increased plasmablast differentiation and memory B-cell exhaustion[81]. Second, a recent study suggests that interaction of viral proteins with B-cell surface molecules during the early stages of infection might delay the initial humoral response against HIV-1 by directly suppressing B cell activation and proliferation[82]. The authors suggested that B cell interactions with gp120 envelope can induce immunosuppressive

cytokine TGF-β1 production, expression of inhibitory receptor FcRL4 and decreased expression of the CD80 costimulatory molecule involved in antigen presentation. Third, the HIV-1 envelope provides multiple challenges for the recognition of the vulnerable conserved sites that are targeted by bnAbs. HIV-1 uses high rates of genetic mutation[83] and recombination[84] to generate impressive sequence diversity in its envelope glycoproteins. This sequence diversity is further complicated by the presence of extensive and variable glycan shielding that can be readily shifted to prevent virus neutralization[85,86]. Conformational masking of the receptor binding site[30] and steric occlusion of the co-receptor binding site[87] are some of the other mechanisms employed by the virus to evade host antibody response. Gp41 based studies have also revealed that MPER epitopes targeted by bnAbs are only transiently exposed[88-92]. HIV-1 also uses uniquely low envelope spikes density as means to prevent inter-spike bivalent binding by antibodies[93,94]. Furthermore, spike structure inherently prevents intra-spike antibody binding. These mechanisms ensure that antibodies elicited against HIV-1 envelope bind through monovalent interactions, have low potency and allow easy virus escape through mutation. Interestingly, a recent study demonstrated that engineered antibody-based molecules capable of bivalent intra-spike crosslinking can show a 100-fold increase in neutralizing potency against multiple viruses[95]. The envelope glycoprotein contains immunodominant decoy epitopes like the V3 loop on gp120 that give rise to non-neutralizing or strain-specific neutralizing antibodies[96,97]. Antibody response is also diverted due to the presence nonfunctional forms like monomeric spikes, gp41 stumps, and uncleaved gp160 that are also displayed on the virus surface[98,99].

HIV-1 vaccine trials

Out of the hundreds of vaccine trials conducted to date (reviewed in [100]), only five have advanced to Phase IIb and Phase III clinical trials; these are the VAX003, VAX004, STEP/Phambili, RV144 and HVTN505 trials.

The world's first phase III trials, VAX003 and VAX004, were conducted between 1999 and 2003, and tested bivalent recombinant, gp120 envelopes delivered with alum[101,102]. While the VAX004 trial used envelopes derived from two different subtype B viruses, VAX003 used envelopes derived from subtypes B and E. However, both trials failed at inducing neutralizing antibodies and did not reduce HIV-1 acquisition in the vaccinated groups.

The STEP and the Phambili Phase IIb trials (conducted between 2005 and 2007) were conducted in America/Australia and South Africa respectively[103,104]. Both trials tested an identical adenovirus serotype 5 based delivery and expression of HIV-1 group specific antigen (*gag*), polymerase (*pol*), and negative regulation factor (*nef*) genes. However, no protection was demonstrated, and these trials were stopped following the observation that certain groups of immunized individuals showed higher chances of infection compared to placebo groups[105,106].

The most recently concluded trial, HVTN505 (conducted between 2009 and 2013), used a recombinant DNA vector prime and a recombinant adenovirus serotype 5 boost. The priming vector coded for HIV-1 clade B Gag, Pol and Nef proteins along with Env proteins (subtypes A, B and C)[107]. The boosting adenovirus coded for Gag and Pol from subtype B and Env from subtypes A, B, and C. This vaccination too failed to confer any significant protection or reduce viral loads in infected patients.

The only trial to display vaccine-based protection against HIV-1 is the RV144 trial that was conducted in Thailand between 2003 and 2006[108]. This trial combined two previously failed vaccines into a prime-boost immunization. Priming antigen used was a recombinant HIV-1-canarypox vector that delivered *env, gag* and *protease* (ALVAC-HIV [vCP1521]) and was followed by alum-based boosting with bivalent recombinant gp120 envelope proteins belonging to subtypes B and E (AIDSVAX B/E). This study reported 31% efficacy in preventing virus acquisition upon vaccination. Further analysis has revealed that while the vaccine did not induce any neutralizing antibodies, IgG antibodies elicited against the V1/V2 loops of gp120 correlated with reduced risks, and protection might be mediated by antibody-dependent cell-mediated cytotoxicity (ADCC). In contrast, generation of envelope specific IgA correlated with risk enhancement[109-111].

The small success against HIV-1 in the RV144 trial is a significant milestone that has provided much needed hope for the field of HIV-1 vaccine development. Based on the findings of these trials, it is now agreed that a successful HIV-1 vaccine should induce both humoral and cell mediated immunity to (a) block or reduce virus infection by antibody mediated mechanisms, and (b) control infections caused by virus breakthrough using cytotoxic T lymphocyte mediated responses[100,112,113]. Multiple efforts to design such vaccine components that meet these goals are being carried out (recently reviewed in [114]).

Gp41-based vaccine development

Designing antigens that can induce bnAbs in animals is a major goal for HIV-1 vaccine development. The virus envelope glycoprotein is the only known target of

neutralizing antibodies. As discussed earlier, bnAbs are directed against several critical epitopes on the envelope. Three of these sites of vulnerabilities; the CD4 binding site, the V1/V2 loop glycan epitopes and the V3 loop glycan epitopes; are present on the gp120 subunit, while a fourth site consists of the gp120-gp41 interface. Multiple studies have focused on designing gp120-based vaccines to induce similar bnAbs (reviewed in [115-117]). However, such efforts have been met with several challenges including high sequence variability, extensive (up to 50%) and variable glycosylation, presence of decoy immunodominant epitopes and the highly conformational nature of the epitopes capable of eliciting broad and potent neutralizing antibodies[39,85,86,96,97].

In comparison, the gp41 subunit contains a short, ~22 amino acid long domain located between the heptad repeat 2 (HR2) and the transmembrane (TM) domains. This highly conserved domain, named the membrane proximal external region (MPER), is the only bnAb-targeted epitope present on gp41 (reviewed in[118]). Apart from being highly conserved and unglycosylated, the MPER is recognized by five bnAbs isolated to date[119-123]. Two of these, 2F5, and m66.6, bind the N-terminus part (gp41 residues 656 to 668; HXB2 numbering) of MPER while three others; 4E10, Z13e1, and 10E8; bind the C-terminus half (gp41 residues 668 to 683; HXB2 numbering). The exact epitope, neutralizing breadth and neutralizing potency varies among these antibodies. While both m66.6 and 2F5 binding involves ^{664}DKW^{668} residues, 2F5 is more broad and potent of the two antibodies[122,124]. The less potent and broad of the N-terminal bnAbs, Z13e1, binds the epitope ^{668}SLW<u>N</u>WF<u>DIT</u>N^{667} (critical residues underlined)[125]. However, 4E10 (binding epitope ^{672}<u>WF</u>D<u>IT</u>NW<u>LW</u>^{680}) and the recently discovered 10E8 (binding epitope 671<u>NWF</u>DITNWLWYI<u>R/K</u>^{683}) show significant breadth by neutralizing about 98% of

the HIV-1 strains[123]. Additionally, the potency of 10E8 is much higher than 4E10, thereby making it one of the broadest and most potent bnAb isolated to date[123]. The epitopes targeted by these bnAbs are continuous as opposed to conformational epitopes on gp120. Furthermore, as revealed by the recent crystal structures of the native envelope, MPER might not be involved in interprotomer interactions and fold independently in the vicinity of a viral membrane[126,127]. Hence, a significant amount of resources have been invested in MPER-based vaccine design.

Several different approaches have been tried to induce anti-MPER bnAbs in animals. Early studies testing the efficacy of MPER peptide-based vaccines failed to induce neutralizing antibodies, probably due to improper MPER structure in absence of other regions, as well as lacking T cell epitopes required to induce robust CD4+ T cell immunity. Most of these approaches tested the immunogenicity of the 2F5 epitope containing peptide alone[128,129] or coupled to carrier proteins[130-132]. One study tested a slightly larger peptide containing the partial HR2 and complete MPER domains in conjugation with subunit B of cholera toxin[133]. Some studies have used chimeric viruses to display MPER epitopes[134-141], but most of these studies report low anti-MPER antibody titers, probably due to the presence of other immunogenic epitopes outside the MPER epitope. MPER peptides have also been delivered on engineered scaffolds optimized to mimic antibody bound peptide conformations[142-144]. However, these approaches have largely failed because antibody-bound conformations, which might result from induced fit, might not represent the native MPER structure bound by naïve B cells. Different recombinant fusion proteins have also been tested to better expose MPER domain by grafting it onto the gp120 variable loops[145,146] or by replacing the gp41 HR2

domain with HA2 to mimic a fusion-intermediate[147]. Finally, to present MPER in a more native conformation, multiple studies have delivered MPER using virus-like particles and liposomes, but overall, these studies too have failed in eliciting anti-MPER bnAbs.

A handful of studies have demonstrated weak to modest cross-clade neutralizing activity[138,140,141,148-150]. Guinea pigs immunized with rhinoviruses displaying the 2F5 epitope (ELDKWA) and boosting with the similar MPER epitope coupled to carrier peptides were reported to induce modest serum neutralizing activity[140]. A second study also showed similar results in guinea pigs using chimeric rhinovirus displaying the 4E10 epitope[141]. Guinea pigs also elicited weak MPER-specific neutralizing antibodies using a mutated heamagglutinin-gp41 chimeric DNA vaccine or protein-containing virus like particles[138]. Kreb et al generated a multimeric MPER antigen by fusing it to the self-assembling E2 protein of *Geobacillus stearothermophilus*[149]. Rabbits immunized with the MPER 60-mer antigens and boosted with gp160 DNA elicited antibodies that showed weak neutralization against the HIV-2/HIV-1 chimeric viruses. One study reported the immunization of llamas with gp41-based proteoliposomes[148]. While no serum neutralization was detected, following hybridoma generation, a variable domain of a single heavy chain (VHH), named 2H10, was isolated and its binding was mapped to ^{657}EQELLELDK^{665} (critical residues underlined). This "nanobody" further demonstrated modest cross-clade neutralization in its bivalent form. Finally, Lai et al described the immunization of guinea pigs with a gp41- fusion intermediate with liposomal delivery that has demonstrated the best, albeit still modest, serum based cross-clade neutralization reported to date[150]. Regardless, none of these studies induced neutralizing antibodies that can match the breadth and potency of bnAbs isolated from patients.

Two different hypotheses have been proposed to explain these failures. The first hypothesis is based on cross-reactivity and knock-in mice experiments performed using 2F5 and 4E10[77,78,151,152]. These studies showed that anti-MPER bnAbs are self-reactive, and hence such B cells are eliminated during development in a healthy individual. The development of these antibodies is probably possible due immune dysfunction and loss of tolerance mechanisms in a small population of HIV-1 infected patients. However, the recently discovered and more potent 10E8 bnAb does not demonstrate similar cross-reactivity to lipids or self antigens[123]. Hence, lipid cross-reactivity or recognition of self-antigens per se is not a necessary property of MPER targeting bnAbs. Instead, the limited understanding of viral and host factors involved in the generation of these bnAbs has been suggested as a more likely reason for failure of past attempts[153].

While the MPER domain is only accessible post receptor binding[88-92], it is likely that MPER exhibits multiple structures due to the conformational plasticity of gp41. This, along with high hydrophobicity, has complicated efforts to elucidate native MPER structure(s) that can engage naive B cells and mediate bnAb development. In the absence of this crucial information, MPER-based vaccine development must rely on logical, yet somewhat empirical design and testing of different vaccine antigen and adjuvant formulations as undertaken in this dissertation.

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CHAPTER 2

EFFECT OF POLYETHYLENE GLYCOL-BASED SILENCING OF GP41 ENVELOPE CLUSTER II IMMUNODOMINANT EPITOPE ON MPER IMMUNOGENICITY

A manuscript in preparation

Saikat Banerjee, Habtom H. Habte, Michael W. Cho

Abstract

The membrane proximal external region (MPER) is highly conserved and capable of eliciting broadly neutralizing antibodies (bnAbs) including 4E10, in patients. However, attempts to elicit similar bnAbs using different MPER based immunogens have been unsuccessful. We had previously designed and characterized a gp41 antigen consisting of the heptad repeat region 2 (HR2) and MPER. This antigen, named gp41-54Q, elicited strong antibody titers in rabbits, but the sera failed to neutralize pseudovirus. Linear epitope mapping revealed strong responses against the non-neutralizing cluster II immunodominant epitope. Attempts to characterize this response at the monoclonal level resulted in identification of the non-neutralizing 2C2 antibody, which binds the C terminus half of this epitope. In this study, we attempted to mask this immunodominant epitope using amine reactive polyethylene glycol (PEG). PEGylation of the lysine residues in this region decreased 2C2 binding severely but retained 4E10 binding, suggesting that the MPER was accessible. Immunogenic characterization of the PEGylated gp41-54Q in rabbits revealed lower overall antibody titers. Linear epitope mapping showed strongly decreased antibody responses toward the cluster II region. However, the primary antibody response was diverted towards the HR2 domain, and the MPER bound little antibody. Our results suggest that silencing the cluster II region alone

is not sufficient to enhance MPER immunogenicity and highlights how neighboring domains might influence the immune response toward the MPER.

Introduction

The isolation and characterization of multiple bnAbs from infected patients in the last fifteen years has enhanced our understanding of the host response to HIV-1(van Gils and Sanders, 2013). While these findings have highlighted the vulnerable epitopes on the virus envelope, translating this knowledge into designing effective vaccines capable of generating similar bnAbs has been difficult. Of the different epitopes that elicit bnAbs, the MPER domain of the gp41 subunit remains an attractive candidate for two major reasons. First, this short region is a hotspot for multiple bnAbs including 10E8, which remains one of the broadest and most potent neutralizing antibody isolated (Huang et al., 2012; Purtscher et al., 1994; Stiegler et al., 2001; Zwick et al., 2001). Second, the neutralizing epitopes on MPER are contiguous as opposed to complex structural epitopes present on the gp120 subunit. However, MPER immunogen design has been difficult due to its hydrophobic nature and the inability to characterize MPER structure in context of the native virion (Montero et al., 2008). Furthermore, the gp41 subunit undergoes significant conformational changes during the fusion process, thereby making it a challenging target (Mao et al., 2013; Melikyan, 2008). Hence, there is a need for systematic, immunogenic characterization of MPER in the context of different gp41 conformations.

Towards this goal, we have previously characterized a gp41 ectodomain antigen (unpublished data). This antigen, named gp41-54Q, contained 54 amino acids spanning

both HR2 and MPER domains. Following immunization, rabbits elicited strong antibody responses that were predominantly directed against the cluster II region, located in the HR2 domain, along with low levels of MPER-targeting antibodies (Supplementary Fig. 1). The cluster II epitope has been shown to elicit non-neutralizing antibodies mostly (Frey et al., 2010; Hioe et al., 1997). One of the immunized rabbits was used for hybridoma generation, resulting in the identification of a non-neutralizing antibody (2C2) that coincidentally targeted the cluster II region. Interestingly, 2C2 was able to prevent 2F5 binding to the MPER in competition assays (data not shown). Other have also reported similar 2F5 competing, cluster II antibodies in patients (Alam et al., 2008).

Based on these findings, it was hypothesized that the induction of non-neutralizing antibodies against the cluster II region might interfere with the generation of MPER-targeting bnAbs in immunized rabbits. Upon "silencing" of this dominant epitope, antibody response might be redirected to other subdominant epitopes (including the MPER) on gp41-54Q. In this study, the cluster II immunodominant epitope on gp41-54Q was modified by PEGylation of the two lysine residues: K655 and K665. The effectiveness of this epitope masking was evaluated by monoclonal antibody binding and rabbit immunization experiments.

Results

Generation and characterization of PEGylated gp41-54Q antigens

To mask the non-neutralizing immunodominant epitope on gp41-54Q using a mild method, *N*- Hydroxylsuccinimide (NHS) esters of PEG molecules were selected as they specifically bind primary amines on the protein. Two of these reactive sites were

present on the lysine residues (K655 and K665) that flank the C-terminus end of the immunodominant cluster II region, as indicated in Fig. 1A. Apart from these, the N-terminus end of the trypsinized gp41-54Q was also PEGylated. Since the efficiency of masking might depend on the size of the PEG molecule, two PEG polymers of different chain lengths, PEG4 and PEG8, were tested. Following labeling, quantification of unmodified primary amines using 2,4,6-trinitrobenzene sulfonic acid (TNBSA) revealed that 75-85% of the primary amines were PEGylated (data not shown).

The effect of the modification on epitope accessibility was tested using ELISA binding to different antibodies. We had previously isolated a non-neutralizing antibody, named 2C2, from a gp41-54Q immunized rabbit (unpublished data). The binding specificity for 2C2 was mapped to the immunodominant region, (^{655}KNEQELLALDK^{665}) with the underlined residues being critical as per binding analyses with alanine scanning mutant peptides. As shown in Fig. 1B, there was a significant loss in recognition of both PEG4-54Q (~250 fold) and PEG8-54Q (~1000 fold) in comparison to the unmodified protein, suggesting that K655 was successfully modified to block access to 2C2. This remarkable decrease in binding might also be due to modification of the K665 residue. While K665 is not critical for 2C2 binding, PEGylation of this residue might cause steric interference with antibody binding. Binding was also reduced (~20 fold) to the MPER binding neutralizing antibody 2F5 that recognizes the core epitopes ^{662}A/ELDKWA^{667}, suggesting that K665 residue was also successfully PEGylated. However, the lack of completely inhibition of 2F5 binding suggests that this residue might not be uniformly PEGylated in agreement with the total percentage of primary amine modification mentioned earlier. This modification also mildly affected Z13e1 binding to its epitope

^{666}WASLWNWFDITN^{677}, probably because of the steric hindrance caused by the flexible PEG molecule bound to adjacent K665 residue. Importantly, binding to 4E10 (core binding epitope ^{672}WFDITNWLW^{680}) remained unaffected, suggesting that PEGylation did not block access to the C-terminus end of MPER. Overall, the ELISA binding data to different antibodies revealed that accessibility of the cluster II region can be blocked without affecting the antibody binding to the C terminus end of gp41-54Q.

Immunogenic characterization of PEGylated gp41-54Q antigens

The immunogenicity of PEG4-54Q and PEG8-54Q was tested in rabbits using the zinc-chitosan adjuvant. Three rabbits were immunized three times for each of these modified antigens. Rabbits were bled prior to and two weeks after each immunization. The isolated sera were used for determining antibody titers using ELISA against the corresponding antigen. In case of PEG4-54Q immunized animals (Fig. 2; top panels), serum antibody titers reached between 10^2 to 10^3 post first immunization. Titers increased significantly upon second immunization, ranging from 10^4 to 10^5. Following the third immunization, there was only a slight increase in titer for rabbit R1, but an almost 10-fold increase was observed for rabbits R2 and R3. For animals immunized with PEG8-54Q (Fig. 2; bottom panels), serum antibody titers after each immunization were found to be lower than that seen PEG4-54Q rabbits, suggesting that the larger PEG molecule was more efficient in immunosilencing. Regardless, following three immunizations the titers ranged between 10^4 and 10^5 in all animals, which is at least 10-100 fold less than antibody titers for rabbits immunized with the unmodified protein (unpublished data).

To determine the epitopes against which the antibody response was directed, peptide ELISAs were performed after the third immunization using a mixture of N-and C-terminus biotinylated overlapping 10-mer peptides (Fig. 3) as previously described (Habte et al., 2015). The linear peptides recognized by rabbits immunized with PEGylated antigens were strikingly different from those for animals immunized with unmodified gp41-54Q (Fig. S1). For rabbits immunized with PEG4-54Q (Fig. 3, top panel), the response towards the cluster II region was much lower, suggesting that PEGylation of this region was successful in blocking response to this otherwise immunodominant epitope. However, two of the three rabbits (R2 and R3) showed no response towards the C-terminus end of MPER. Surprisingly, strong response was seen against peptides ^{629}MEWEREISNY^{638}, ^{632}EREISNYTDI^{641}, ^{635}ISNYTDIIYR^{644} and ^{638}YTDIIYRLIE^{647} located at the N-terminus end of the HR1 domain. For R1, the epitope recognition was different and included weak response towards peptides ^{668}SLWNWFDITN^{677}, ^{671}NWFDITNWLW^{680} and ^{674}DITNWLWYIK^{683} in the MPER C-terminus end.

Rabbits immunized PEG8-54Q recognized fewer linear epitopes (bottom panel). While R1 did not bind any linear peptides, R2 recognized only peptide ^{638}YTDIIYRLIE^{647}. R3 recognized few N-terminus end peptides in the HR1 domain but also bound peptides ^{647}EESQNQQEKN^{656} in the cluster II region and ^{659}ELLALDKWAS^{668} overlapping between cluster II and MPER. The response elicited against these two peptides was unexpected since they both contained lysine residues that were PEGylated.

To further understand serum binding to these peptides, ELISA was performed against N-terminus and C-terminus biotinylated peptides separately. As shown in Fig. 4, for both peptide 647 and 659, serum from PEG8-54Q R3 bound C-terminus end biotinylated peptides well but failed to recognize N-terminus end biotinylated peptides. One possible explanation for this difference in binding to the peptides with the same sequence but different orientation is the accessibility of the binding residues after coating on the plate. In the model described in Fig. 4, regions closer to the plate are somewhat inaccessible due to their proximity to the coating surface where as regions away from the plate can be readily bound by antibodies. Since antibodies recognized the peptides that were attached to the plate via their C-terminus, it is likely that epitopes bound were located at the N-terminus ends of these peptides. Consistent with this theory, these epitopes were away from the lysine residue that was PEGylated in the protein, thereby explaining the possibility of antibody response to these regions. It is important to note that such binding was observed only in one of the three rabbits immunized with PEG8-54Q, suggesting that it might not be a common phenomenon. In comparison to PEG4, PEG8 has a longer and more flexible chain, which might have allowed antibody access to the nearby regions in the cluster II region as shown in case of R3.

Finally, in agreement with the lack of strong response towards the MPER domain, sera from all immunized rabbits failed to display any neutralizing activity (data not shown).

Discussion

To elicit anti-MPER bnAbs through immunization, MPER containing antigens need to meet at least two criteria. First, the antigen should generate a strong antibody response against the MPER domain. Second, the antibodies elicited should bind the MPER domain on the surface of the native virion. However, achieving both of these criteria has been difficult. One possible hypothesis for this failure is that inducing non-neutralizing antibodies could directly interfere with neutralizing antibody elicitation to neighboring domains (Alam et al., 2008; Cleveland et al., 2000). Hence, reducing the immunogenicity of non-neutralizing epitopes might be a possible solution (Garrity et al., 1997).

In this study, the immunogenicity of the cluster II non-neutralizing epitope was silenced using a simple but effective method of blocking via PEGylation. As revealed by ELISA, this modification also reduced 2F5 binding, and thus is not recommended for the generation of 2F5-like antibodies. This masking did not affect the downstream 4E10 binding and is equally unlikely to restrict access to the 10E8 epitope. Hence, PEGylation of primary amines can be performed for antigens designed to elicit 4E10 or 10E8-like antibodies, albeit after replacing the K683 residue with the alternate R683 (Huang et al., 2012).

Both PEG4 and PEG8 were successful in preventing immune responses against the cluster II region. The extent of immunosilencing was greater for PEG8, probably due to its longer chain length. Strangely, the longer chain length also allowed the elicitation of antibodies in the vicinity of the modified residues in one rabbit. Since PEG8 did not provide any additional benefits in terms of site specific silencing in vivo, it is fair to

conclude that use of shorter PEG4 might be sufficient in blocking immunodominant epitopes. Unfortunately, effective masking of the cluster II region did not result in enhancement of antibody response towards neutralizing epitopes in the MPER. Instead, antibodies were now elicited against the N-terminus end of the HR2 domain with little to no response against MPER. These findings suggest that the N-terminus end of the HR2 domain might be more immunogenic than the MPER upon masking of the cluster II region. Our study serves as a cautionary note that silencing the cluster II region might not be sufficient to enhance MPER immunogenicity for some gp41-based antigens. However, we cannot conclude whether this is true in case of all other antigens since MPER immunogenicity depends heavily on the context in which it is presented (Habte et al., 2015). It is possible that other gp41 antigens containing an immunodominant cluster II region might benefit from this approach. Therefore, we recommend that PEGylation should be considered as an effective means of silencing immunodominant epitopes. Furthermore, PEG based epitope masking might be pursued for other proteins in general.

Materials and Methods

PEGylation of gp41-54Q

The gp41-54Q gene segment, which encodes the C-terminal 54 amino acids of the gp41 ectodomain from an M group gp160 consensus sequence (MCON6), was PCR-amplified from pcDNA-MCON6gp160 (kindly provided by Dr. Beatrice Hahn, University of Alabama; (Gao et al., 2005)). PCR reaction was carried out using a sense primer (5'-CGCGGATCCGAGTGGGAGCGCGAGATC-3'; underline denotes*Bam*HI site) and an antisense primer (5'-

GATGAATTCTTAATGGTGATGATGGTGATGCT**G**GATGTACCACA-GCCA-3’; underline denotes *Eco*RI site). A point mutation was introduced (indicated in bold) to mutate K683 into Q683 to eliminate a trypsin cleavage site. The PCR-amplified DNA fragment was digested with *Bam*HI and *Eco*RI and ligated into corresponding sites in pGEX-2T (GE Healthcare Life Sciences) or pET-21(a) (Novagen), to generate pET-gp41-54Q, respectively. Final constructs were sequenced confirmed.

The gp41-54Q protein was expressed and purified as previously described (Shi et al., 2010) and cleaved using trypsin to remove the T7 expression tag. The cleaved, purified protein was modified using Methyl-PEG-NHS esters- MS(PEG)4 (Thermo Scientific; Cat# 22341) and MS(PEG)8 (Thermo Scientific; Cat# 22509) as per the manufacturer’s instructions. Both modified and unmodified proteins were treated with 2,4,6-trinitrobenzene sulfonic acid (TNBSA) solution (Thermo Scientific; Cat# 28997) to determine the amount of free primary amines and the extent of successful PEGylation using the manufacturer’s protocol.

Rabbit immunization

Six New Zealand white female rabbits (2.5 to 3 kg) were purchased from Charles River (USA) and housed under specific pathogen free environments in compliance with the animal care guidelines at Iowa State University. To evaluate the immunogenic properties of the PEGylated antigens, three rabbits each were immunized subcutaneously with PEG4-54Q and PEG8-54Q on weeks 0, 4 and 9 with the adjuvant zinc chitosan, which was prepared and used as previously reported (Habte et al., 2015; Qin et al., 2014). 200 μg of the antigen was loaded onto 200 mg of Zn-chitosan in phosphate-buffered

saline (PBS, pH 8.0) by continuous agitation for three hours at room temperature. For every immunization, each rabbit was immunized with 200 µg of PEGylated antigens.

Enzyme-linked immunosorbent assay (ELISA)

All antigens were coated onto 96-well Nunc-Immuno Plates (Nunc; # 439454) at 30 ng/well using antigen coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) overnight at 4 ºC. The plates were blocked for 1 hr at 37 ºC using 200 µl/well of PBS (pH 7.5) containing 2.5% skim milk and 5% Calf Serum (CS). The plates were washed 5× with 0.1% Tween 20 in PBS.

For ELISA with cluster II- and MPER-binding antibodies, all antibodies (2F5(Buchacher et al., 1994; Purtscher et al., 1994; 1996), 2C2, 4E10(Stiegler et al., 2001) and Z13e1(Nelson et al., 2007; Zwick et al., 2001)) were diluted in blocking buffer at a concentration of 1 µg/ml and further serially diluted three folds. 100 µl of the diluted antibodies were added to each well and incubated for 2 hr at 37 ºC. The plates were washed 10×, and 100 µl of horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-human, 1:3000 dilution; Thermo Scientific; Cat# 31410) was added to each well and incubated for 1 hr at 37 ºC. Wells were washed 10× and developed by adding 100 µl TMB HRP-substrate (Bio-Rad) for 10 min. After stopping reactions using 50 µl of 2 N H_2SO_4, plates were read at 450 nm on a microplate reader (Versamax by Molecular Devices). All experiments were performed in duplicates.

For antibody end point titers, rabbit sera were initially diluted 1:10 in blocking buffer, and further subjected to three fold serial dilutions. 100 µl of the diluted sera were added to each well and incubated for 2 hr at 37 ºC. The plates were washed 10×, and 100

µl of horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit, 1:3000 dilution; Thermo Scientific; Cat# 31430) was added to each well and incubated for 1 hr at 37 ºC. The rest of the steps were same as described above.

For peptide ELISA with overlapping 10-mer peptides, individual peptides were biotinylated and coated as previously described (Habte et al 2015).

Neutralization assays

TZM-bl cell-based HIV-1 pseudovirus neutralization assays were performed as previously described (Li et al., 2005; Qin et al., 2014; Wei et al., 2002). Viruses tested were SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B). Murine leukemia virus Env-pseudotyped virus was used as a negative control.

Acknowledgments

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Consensus group M Env peptides (Cat# 9478); HIV-1 anti-gp41 2F5 from Dr. Hermann Katinger (Cat# 130220), 4E10 from Dr. Herman Katinger (Cat# 10091), and Z13e1 from Dr. Michael Zwick (Cat# 11557). This work was supported by a grant from the NIH, NIAID (P01 AI074286) grant. MWC has an equity interest in NeoVaxSyn Inc., and serves as the CEO/President. NeoVaxSyn Inc. did not contribute to this work or the interpretation of the data.

Figures

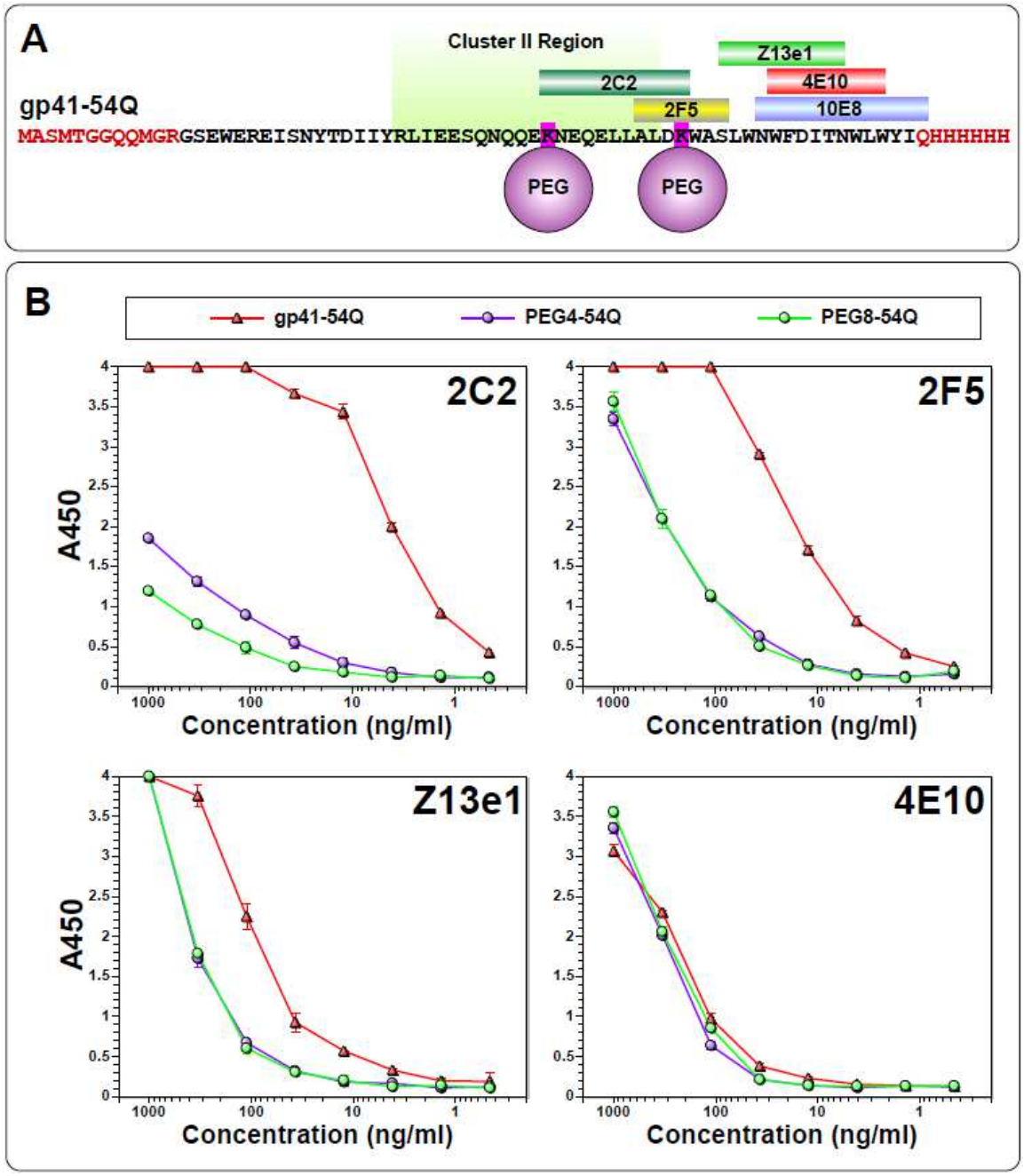


Fig 1: Design and characterization of PEGylated gp41-54Q. (A) PEGylated gp41-54Q. The sequence of gp41-54Q is shown along with binding sites for the non-neutralizing antibody 2C2 and bnAbs 2F5, 4E10 and Z13e1. Even though gp41-54Q does not contain the K683 residue essential for 10E8 binding, its epitope is shown. The two potential sites for PEGylation within the cluster II immunodominant region are also shown. (B) ELISA with 2C2, 2F5, Z13e1 and 4E10 reveal that while the cluster II region

is masked in both PEG4-54Q and PEG8-54Q, the C terminus end of the MPER is readily accessible.

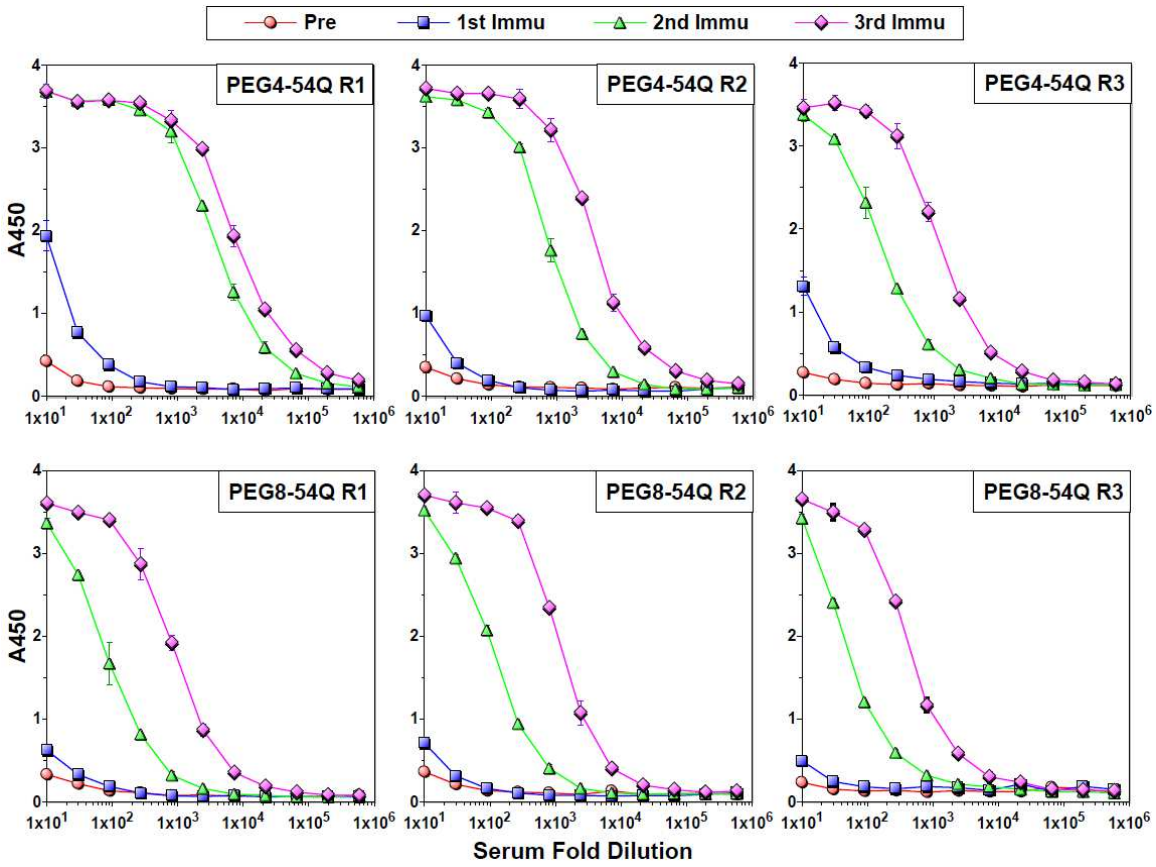


Fig 2: Immunogenicity of PEGylated gp41-54Q. ELISA was performed with sera collected post first, second and third immunization to determine endpoint titers for rabbits immunized with either PEG4-54Q or PEG8-54Q.

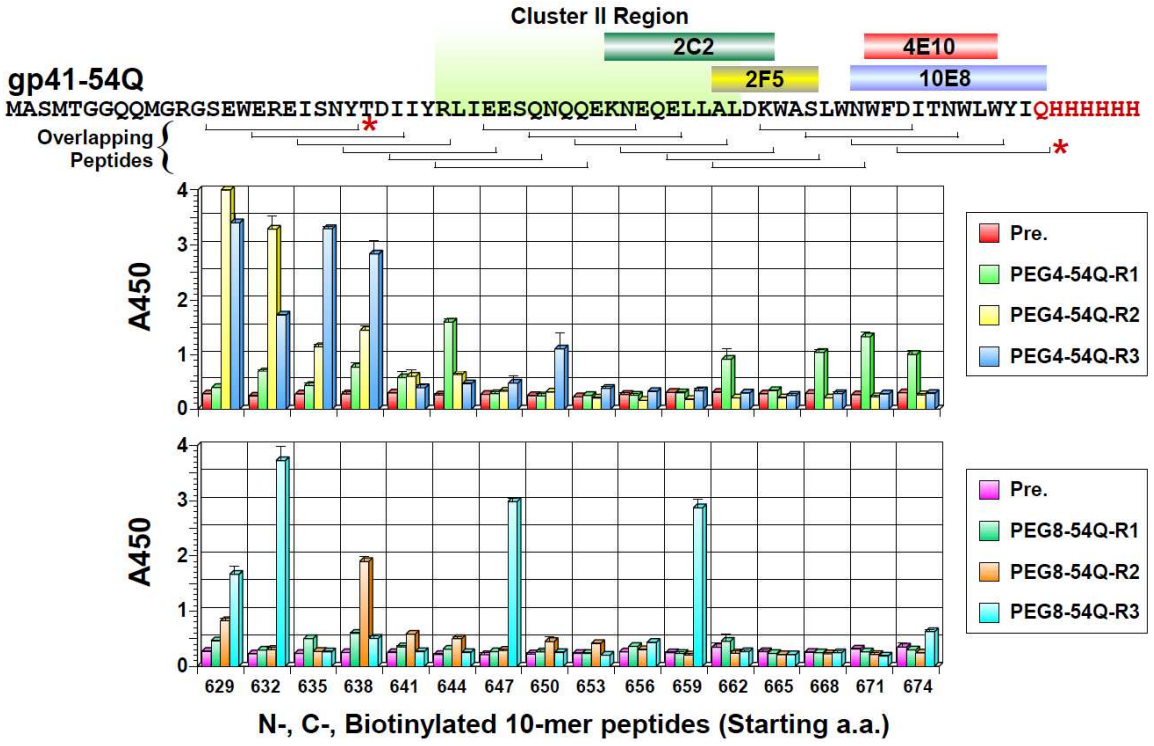


Fig 3: Mapping of linear immunogenic epitopes. Sera post third immunization was tested for ELISA binding to biotinylated overlapping peptides spanning the HR2 and MPER domains. Pre-immune serum was used as negative control. The sequence of the gp41-54Q is aligned with the peptide numbering and individual peptides are marked using horizontal brackets. The cluster II region and core binding epitopes for 2C2, 2F5, 4E10 and 10E8 are indicated.

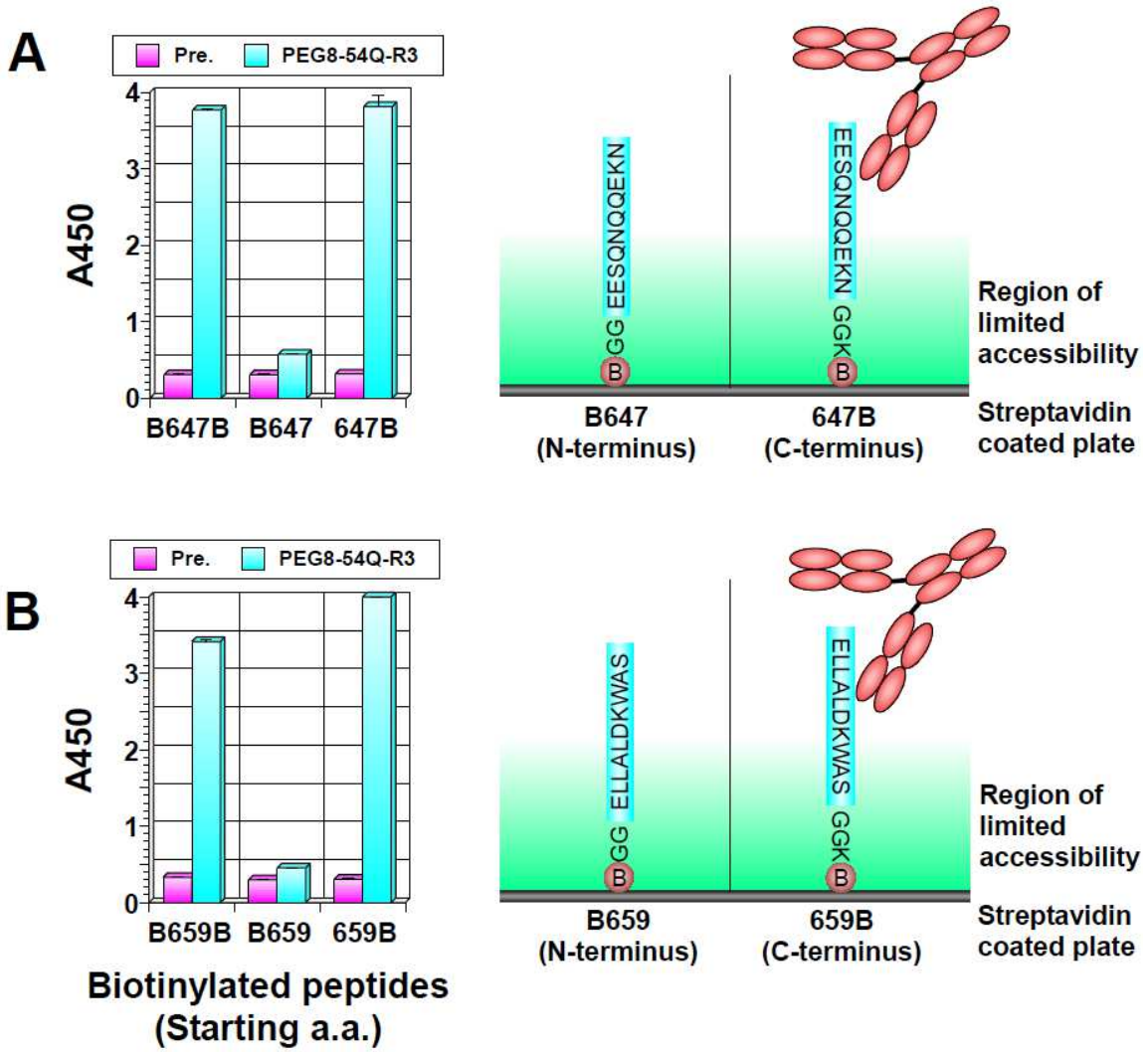
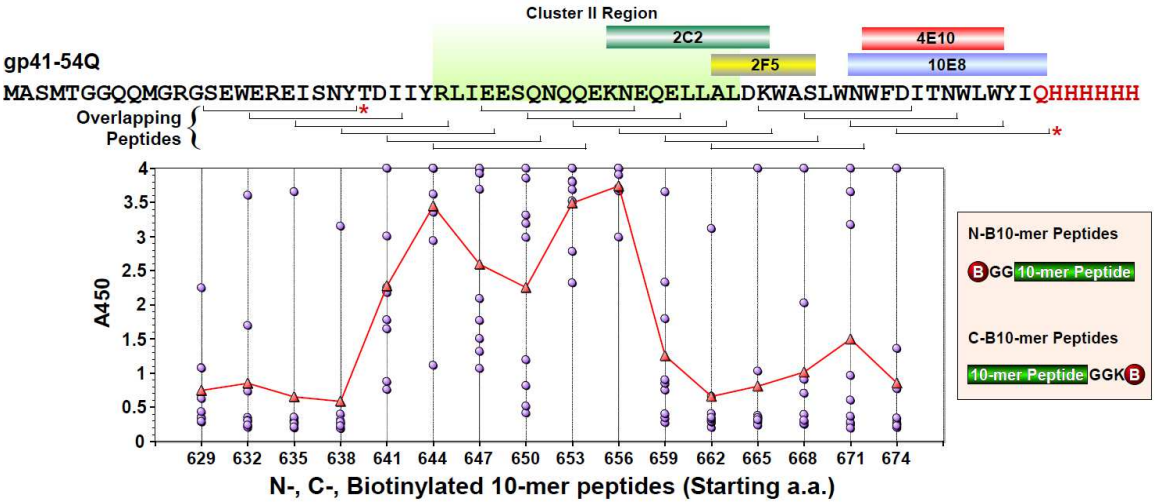


Fig 4: Binding analysis of PEG8-54Q R3. Serum from R3 was tested separately against N- and C-terminus biotinylated peptides (A) 647 and (B) 659. Binding to the mixture of both sets of peptides was used as a positive control. Serum showed strong binding only to the C-terminus biotinylated peptides. This difference in binding to N- and C-terminus biotinylated peptides can be explained by the adjoining model where regions close to the plate are less accessible to antibody binding. Hence, binding to C-terminus biotinylated peptides means that the binding epitope lies at the N-terminus end of the peptide which also happens to be away from the lysine residues (and are modified on the PEGylated antigens).



Supplementary Fig 1: Linear epitope mapping of antibody response against gp41-54Q. Sera from nine rabbits from three different experiments were used for epitope mapping using overlapping 10-mer peptides spanning the entire gp41-54Q. A mixture of N-terminus and C-terminus biotinylated peptides were used for the assay. Horizontal brackets represent the sequence of each peptide. The first peptide (MEWEREISNY) and the last peptide (DITNWLWYIK) are marked with an asterisk to indicate slight sequence differences from original antigen. A450 values for individual rabbits are represented with purple spheres where as average values are indicated with red triangles. The most immunodominant epitope overlaps with the cluster II region. The binding epitope (KNEQELLALDK) for the non-neutralizing antibody 2C2 (isolated from one gp41-54Q immunized rabbit) is indicated along with core binding epitopes for anti-MPER bnAbs 2F5 (ALDKWAS), 4E10 (WFDITNWLW) and 10E8 (NWFDITNWLWYIK).

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CHAPTER 3

IMMUNOLOGICAL CHARACTERIZATION OF PUTATIVE GP41 FUSION INTERMEDIATES OF HIV-1

A manuscript in preparation

Saikat Banerjee, Heliang Shi, Habtom H. Habte, Yali Qin, Michael W. Cho

Abstract

The gp41 envelope subunit plays an important role in HIV-1 fusion to host cells. The membrane-proximal external region (MPER) domain of this protein is a target for several broad neutralizing antibodies (bnAbs) including 4E10 and 10E8. Elicitation of similar antibodies through immunization has been difficult, partly due to the complex structural properties of gp41. In our attempt to better understand the immunogenicity of MPER in different structural contexts, we had previously reported its characterization using an antigen containing a stable six-helix bundle comprising of HR1 and HR2 domains. In this study, we introduced multiple mutations or deletions to destabilize the six-helix bundle, thereby generating antigens that mimic the soluble fusion intermediate state. All antigens induced strong humoral response in rabbits. Elicited antibodies showed strong binding to MPER peptide containing the 4E10 and 10E8 epitopes. Peptide scanning in this region using alanine mutants revealed that immunized rabbits targeted several of the critical residues involved in 4E10 binding. Most importantly, the HR1-Δ10-54K construct was successful in eliciting antibodies that target the W672 residue critical for 4E10 binding. Our results demonstrate that changes outside the MPER domain can influence the presentation of neutralizing epitopes and significantly alter the binding face targeted by vaccine-induced antibodies.

Introduction

It is widely hypothesized that a successful vaccine should induce neutralizing antibodies against multiple clades of HIV-1 (Excler et al., 2015; Walker and Burton, 2008). Such broadly neutralizing antibodies (bnAbs) are elicited in a small population of HIV-1 infected patients, suggesting that the generation of these antibodies is a complex process. However, isolation of these antibodies has helped the field of vaccine development immensely by understanding both the epitopes targeted and also the unique features that contribute to their broad neutralizing ability. Most of the bnAbs identified to date target multiple conformational epitopes on the gp120 subunit of the virus envelope protein and act by blocking CD4 receptor or CCR5/CXCR4 co-receptor binding (van Gils and Sanders, 2013). Unfortunately for vaccine development, attempts to elicit similar antibodies have largely failed due to the high sequence variability, extensive glycosylation of gp120 and the presence of decoy immunodominant epitopes that give rise to largely strain specific neutralizing antibodies (Pantophlet and Burton, 2006; Sodroski et al., 1998; Wei et al., 2003).

In contrast to gp120 bnAbs, several bnAbs target linear epitopes on the gp41 subunit and block essential conformational changes that are required for virus fusion to the host cell. Further, these linear epitopes reside in a highly conserved, ~22 amino acid long domain called the membrane proximal external region (MPER) (Montero et al., 2008). The discovery of the highly potent and broad neutralizing antibody 10E8 (Huang et al., 2012), along with previously characterized bnAbs 2F5, 4E10 and Z13e1 (Purtscher et al., 1994; Stiegler et al., 2001; Zwick et al., 2001), has renewed interests in designing MPER-based vaccines.

So far, eliciting anti-MPER bnAbs through vaccination has been largely elusive. Direct immunization with MPER peptides alone or coupled to carrier proteins (Decroix et al., 2001; Joyce, 2002; Liao et al., 2000; Matoba et al., 2006; McGaughey et al., 2003; Ni et al., 2004), scaffold-based MPER epitope presentation (Correia et al., 2010; Guenaga et al., 2011; Ofek et al., 2010), MPER liposomal delivery (Dennison et al., 2011; Hanson et al., 2015; Hulsik et al., 2013; Lai et al., 2014; Matyas et al., 2009; Mohan et al., 2014; Serrano et al., 2014; Venditto et al., 2013; 2014), MPER containing hybrid/fusion proteins (Coëffier et al., 2000; Hinz et al., 2009; Krebs et al., 2014; Law et al., 2007; Liang et al., 1999; Mantis et al., 2001; Strasz et al., 2014) and chimeric viruses or virus like particles (Arnold et al., 2009; Benen et al., 2014; Bomsel et al., 2011; Eckhart et al., 1996; Jain et al., 2010; Kamdem Toukam et al., 2012; E. Kim et al., 2014; Luo et al., 2006; Marusic et al., 2001; Muster et al., 1995; Ye et al., 2011; Yi et al., 2013; Zhang et al., 2004) are some of the different approaches attempted for MPER-based HIV-1 vaccines but only a handful have demonstrated modest cross-clade neutralizing activity (Hulsik et al., 2013; Krebs et al., 2014; Lai et al., 2014; Ye et al., 2011; Yi et al., 2013).

The gp41 protein undergoes large conformational changes during virus fusion, and it is likely that the MPER domain also assumes several structural conformations. To better understand MPER conformation, multiple crystal structures of short MPER peptides in complex with bnAbs have been generated (Cardoso et al., 2007; Julien et al., 2008; Ofek et al., 2004). Since attempts to induce bnAbs using constructs based on these structures have failed so far (Correia et al., 2010; Guenaga et al., 2011; Ofek et al., 2010), it is possible that these antibody bound structures do not represent the native MPER conformation that engages naïve B cells and triggers the development of known bnAbs.

Thus, further studies that characterize the structural and immunological properties of MPER in context of larger gp41-based proteins are required.

In a previous study, we generated a soluble gp41 construct named gp41-HR1-54Q consisting of HR1, HR2 and MPER domains (Shi et al., 2010). While the HR1 and HR2 domains of this protein formed a post-fusion six-helix bundle, the MPER domain remained solvent accessible and highly flexible (Shi et al., 2010). Immunological characterization revealed strong immune response against the C terminus end of the MPER domain, overlapping 4E10 and 10E8 epitopes(Habte et al., 2015). Despite competition against these bnAbs, sera from immunized rabbits failed to neutralize pseudoviruses. Further analysis revealed that sera antibodies bound the face of the alpha helix opposite to the face bound by 4E10 and 10E8. It is possible that the failure to elicit antibodies against this binding face was due to presentation of the highly flexible MPER in the context of "near post-fusion" conformation. Studies on gp41 conformational changes during fusion suggest that MPER presentation might be more optimal in the fusion intermediate state (Chakrabarti et al., 2011; de Rosny et al., 2004; Dimitrov et al., 2007; Finnegan et al., 2002; Frey et al., 2008; M. Kim et al., 2011). However, reports evaluating the immunogenicity of gp41 fusion intermediate have typically characterized antigens consisting of trimerized HR1 domains that lack the MPER (Bianchi et al., 2010; Qi et al., 2010). Only one other study has attempted to generate an MPER-containing fusion intermediate by replacing the HR1 domain with HA2 region of influenza (Hinz et al., 2009). While this study failed to elicit neutralizing antibodies, it is unclear whether these results were due to the presence of non-HIV-1 fragments (like the HA2 or the artificial trimerization domain) or due to use of Fruend's adjuvant that might interfere

with the presentation of the highly hydrophobic MPER domain. Thus, detailed characterization of MPER immunogenicity should be performed in the context of the gp41 fusion intermediate using different antigens and adjuvants to answer valuable questions pertaining to vaccine design.

To generate putative fusion intermediate variants of gp41-HR1-54Q, we primarily disrupted the intramolecular interactions between HR1 and HR2 domains by introducing mutations or deletions in the HR1 domain. The generated putative fusion intermediates (pFIs) possessed different structural and antigenic properties from gp41-HR1-54Q. All pFIs induced strong antibody responses during rabbit immunizations with the adjuvant zinc-chitosan. Linear epitope mapping further revealed that the pFIs generated distinct patterns of peptide recognition across HR2 and MPER domains. Detailed mapping of the antibody response against the 4E10 epitope revealed that antibodies elicited by the pFIs targeted the peptide face opposite to that bound by gp41-HR1-54Q induced antibodies. Furthermore, antibodies induced by one of the constructs, HR1-Δ10-54K, targeted several residues critical for 4E10 binding, including W672. These results suggest that structural changes outside the MPER can significantly alter its presentation permitting elicitation of antibodies towards the neutralizing face.

Results

Design of gp41-HR1-54Q variants

The trimeric structure of gp41-HR1-54Q is stabilized by both inter- and intramolecular interactions (Shi et al., 2010). During protein folding, intramolecular interactions form the post-fusion hairpin between HR1 and HR2 domains of individual

protein molecules. Intermolecular interactions between individual HR1 molecules cause the formation of the final six-helix bundle by trimer assembly in solution, post protein folding. The trimeric interface between HR1 molecules is lined with I548, Q552, L555 and I559 residues and highlights the importance of hydrophobic interactions to hold the core together (Shi et al., 2010). To generate pFIs of gp41 from its post-fusion conformation, different point mutations or deletions were introduced to disturb the intramolecular interactions between HR1 and HR2 domains (Fig. 1A). It was hypothesized that such mutations or deletions would open up the hairpin loop thereby simulating conformations prior to the formation of the six-helix bundle. All mutations or deletions were restricted to the HR1 domain only so as to avoid changing the conformation of the HR2 or MPER domains. While designing the variants, we also experimented with the trimeric nature of gp41-HR1-54Q. The residues lining the trimeric interface (I548, Q552, L555 and I559) were unchanged in variants with point mutations but were partially or completely removed in deletion variants.

First, to reduce the hydrophobic interactions within the hairpin loop, two point mutations- L565A and L568A were introduced in the HR1 domain (Fig 1B, named HR1-AA-54Q). These two residues were hypothesized to be critical due to their close proximity to HR2 domain residues I635 and Y638. The next construct was designed to contain two point mutations, L568E and K574E, which were hypothesized to introduce repulsive forces and disrupt the stable six-helix bundle in two ways (Fig 1C, named HR1-EE-54Q). The L568 residue on the HR1 domain was in close proximity to HR2 domain residue E634 within the same gp41-HR1-54Q molecule. The K574 residue on the HR1 domain of one molecule was in close proximity to E632 located on the HR2 domain of a

neighboring molecule. Thus, HR1-EE-54Q contained point mutations that were designed to destabilize both inter- and intramolecular forces in the trimeric gp41-HR1-54Q. Interestingly, in a recent study, L565R and L568E mutations were also used for generating prehairpin intermediates by disrupting the intramoelcular interactions between HR1 and HR2 domains (Gao et al., 2013). However, the study did not provide any immunogenic characterization of these proteins.

Finally, to reduce the interactive face between the HR1 and HR2 domains, ten and seventeen residues were deleted from the N terminus end of HR1. These two variants were named HR1-Δ10-54K and HR1-Δ17-54K, respectively. The terminal residue on these constructs was also reverted to K as it was later reported to be critical for 10E8 binding (Huang et al., 2012).

Structural characterization of pFIs

Due to its stable, six-helix bundle conformation, gp41-HR1-54Q is surprisingly resistant to cleavage by the enzyme trypsin. To test if the introduced mutations and or deletions were able to destabilize this structure, the pFIs were treated with trypsin. As shown in Fig. 2A, while gp41-HR1-54Q remained mostly undigested after one hour trypsin digestion, the pFIs showed different degrees of trypsin sensitivity. HR1-EE-54Q was the most sensitive to trypsin digestion followed by HR1-Δ10-54K, HR1-Δ17-54K and HR1-AA-54Q. The difference in accessibility of trypsin cleavage sites on gp41-HR1-54Q and the pFIs suggested that structure of the pFIs was significantly different from gp41-HR1-54Q. Strangely, the HR1-Δ10-54K was more susceptible to trypsin digestion compared to the HR1-Δ17-54K, which has a shorter HR1 domain.

Next, ELISA was performed using NC-1, a mouse mAb capable of recognizing the post-fusion six-helix bundle structure (Fig. 2B) (Jiang et al., 1998). This binding requires HR2 domain region ranging from residues 642-657 (Yuan et al., 2009) which is present among all the antigens. Hence, any changes in NC-1 binding would suggest conformational loss in the variants and not simply due to the induced mutations or deletions in HR1 domain. Three of the variants, HR1-EE-54Q, HR1-Δ10-54K and HR1-Δ17-54K showed no NC-1 binding. Interestingly, HR1-AA-54Q could be recognized, but the binding affinity was much weaker than that of gp41-HR1-54Q. These results further supported the idea that introduced point mutations and deletions were successful in disrupting the post-fusion conformation, albeit to different extents.

In the gp41-HR1-54Q crystal structure, the N terminus end of the HR1 domain contains residues I548, Q552, L555 and I559; which line the trimeric interface between the HR1 cores of the six-helix bundle. Thus, loss of most or all of these residues will likely disrupt the trimeric conformation observed in gp41-HR1-54Q. The oligomeric status of all pFIs was tested using ELISA with 126-7 (Gorny et al., 1989; Robinson et al., 1991; Tyler et al., 1990; Xu et al., 1991; Yuan et al., 2009), a human mAb antibody that can specifically recognize a native trimeric form of the HIV-1 envelope shared between both prefusion and post-fusion conformations of gp41 (Yuan et al., 2009). As shown in Fig. 2C, gp41-HR1-54Q, HR1-AA-54Q and HR1-EE-54Q were bound equally well by 126-7 suggesting that the trimeric conformations of these proteins were similar. However, 126-7 failed to recognize both HR1-Δ10-54K and HR1-Δ17-54K along with another protein lacking the HR1 domain (names gp41-54Q). It is important to note that the trimer specific binding epitope for 126-7 has been described as ranging between

residues 641-648 in the HR2 domain (Yuan et al., 2009). Since the only difference between all the constructs tested for 126-7 binding is the presence or absence of the complete or partial HR1 domains, it is likely that this region is also involved in formation of a 126-7 reactive trimer.

Antigenic characterization of pFIs

These results suggested that the introduced mutations or deletions had changed the structure of the variants significantly from the post-fusion conformation of gp41-HR1-54Q. To test how these changes had affected the presentation of MPER, ELISA was performed with four bnAbs (Fig. 3). While all pFIs showed overall strong binding to 2F5, there were slight differences. 2F5 bound HR1-AA-54Q and gp41-HR1-54Q with virtually same affinity, while binding to HR1-Δ17-54K was slightly enhanced. Both HR1-EE-54Q and HR1-Δ10-54K showed slightly reduced binding. The differences in binding to 4E10 were more obvious. While HR1-Δ17-54K showed similar recognition as gp41-HR1-54Q, HR1-Δ10-54K showed slightly lower recognition. Interestingly, HR1-AA-54Q and HR1-EE-54Q showed an even greater reduction binding to 4E10. The binding to Z13e1 also followed a similar pattern to that of 4E10 except that the binding of HR1-AA-54Q was less reduced. The two pFIs containing the terminal K residue, HR1-Δ10-54K and HR1-Δ17-54K, were recognized well by 10E8, while all other pFIs showed poor binding, which is not unexpected given how critical the terminal K is for 10E8 binding. Taken together, these results suggested that despite individual differences in recognition by different bnAbs, the MPER was fairly accessible in our pFIs. These

findings are also interesting because they demonstrate that changes in the distant HR1 domain can also influence the exact MPER conformation.

Immunogenic characterization of pFIs

Next, the immunogenicity of pFIs was evaluated in rabbits using the zinc-chitosan adjuvant that elicited strong antibody response against gp41-HR1-54Q (Habte et al., 2015). A total of eight rabbits were divided in four groups of two rabbits each. Each group was immunized with one pFI at weeks 0, 4, 9 and 15. Serum was collected prior to immunization and two weeks post immunization, and antigen specific antibody titers were determined using ELISA (Fig. 4). Based on the antibody responses after a single round of immunization, the immunogenicity of pFIs appeared to be similar or lower than that of gp41-HR1-54Q (Habte et al., 2015). For rabbits immunized with HR1-AA-54Q, titers more than $1x10^5$ after single immunization. The antibody titers increased more than ten folds upon subsequent immunization. While the titers remained almost the same for R2 after two more immunizations, titers reached above $1x10^7$ for R1 after the fourth immunization. Rabbit immunization with a single dose of HR1-EE-54Q elicited lower titers ranging approximately between $1x10^4$ to $1x10^5$. Titers were boosted following subsequent immunization and remained at $1x10^6$ or more after the fourth immunization. HR1-Δ10-54K and HR1-Δ17-54K showed strikingly low immunogenicity based on titers elicited after a single round of immunization. However, titers were successfully boosted to $1x10^6$ or higher for all rabbits following subsequent immunizations. Titers even reached higher than $1x10^7$ for the HR1-Δ17-54K R1 following third immunization with but decreased more than ten fold after the fourth immunization.

Detailed characterization of the antibody responses against pFIs

Since the 54 amino acids spanning the HR2 and MPER domains of the pFIs were identical to that of gp41-HR1-54Q except the terminal K683 in HR1-Δ10-54K and HR1-Δ17-54K, the linear immunogenic epitopes in this region were further mapped using a mixture of N- and C-terminus biotinylated, overlapping 10-mer peptides (Fig. 5). The linear peptides recognized by rabbits immunized with HR1-AA-54Q were somewhat similar to the response seen against gp41-HR1-54Q, especially towards the C-terminus end. While the rabbit sera also bound some peptides spanning the HR2 domain, MPER domain peptide 671 (^{671}NWFDITNWLW^{680}) was recognized equally well by both rabbits. For HR1-EE-54Q immunized rabbits exhibited a similar pattern with most of the linear immunogenic peptides located in the N terminus of the HR2 domain and the C terminus end of MPER. Little to no antibody recognition was seen for peptides spanning the cluster II region (^{644}RLIEESQNQQEKNEQELLAL^{663}) that typically elicits non-neutralizing antibodies (Alam et al., 2008; Frey et al., 2010; Hioe et al., 1997). Rabbits immunized with HR1-Δ10-54K strongly recognized peptides 632 (^{632}EREISNYTDI^{641}), and 635 (^{635}ISNYTDIIYR^{634}) in addition to 629 (^{629}MEWEREISNY^{638}), suggesting a shift in the response despite the recognition of peptide 671. The most striking change in linear epitope recognition was seen in rabbits immunized with HR1-Δ17-54K. While peptide 671 showed strong signal in both rabbits, the majority of the peptides spanning the HR2 domain including the cluster II region also bound strongly.

Since all rabbits elicited immune response towards the C terminus end of the MPER, the sera was titered using a 13-mer 671 peptide (^{671}NWFDITNWLWYIK^{683}) that contained the complete epitopes for both 4E10 and 10E8 (Cardoso et al., 2005; Huang et

al., 2012). As shown in Fig. 6, all sera recognized this longer peptide, albeit to different extents. While binding to 13-mer peptide agreed well with binding to shorter peptides (Fig. 5), there was a major difference in binding affinity of HR1-Δ17-54K R2. This serum displayed rather weak binding to the 13-mer despite showing very strong binding to the 10-mer 671 peptide at the same dilution. It is important to note that while the 10-mer binding assay was performed using a mixture of both N- and C-terminus biotinylated peptides, the 13-mer peptide was only biotinylated at its C-terminus K683 residue. Furthermore, this serum exhibited good binding against the 10-mer, C-terminus biotinylated 671 peptide (data not shown). Hence, the difference in binding to the 13-mer peptide might be due to other factors like peptide length, coating efficiency, and peptide conformations.

Finally, all rabbit sera were tested for neutralization using a luciferase-based assay in TZM-bl cells against pseudoviruses SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B). Unfortunately, none of the sera possessed any neutralization activity (data not shown).

Fine mapping analyses of sera targeting near 4E10/10E8 epitope

To understand why antibodies elicited by FIs failed to neutralize despite targeting near the 4E10 and 10E8 epitopes, a fine epitope binding analyses was performed using alanine scanning mutants of the 13-mer 671 peptide. As shown in Fig. 7A, all sera (except the poorly binding sera from the previously mentioned HR1-Δ17-54K R2) were normalized to give comparable binding signals to the wild type peptide before performing the alanine scan analysis. First, for all rabbits tested, binding to peptide containing the

D674A mutation was poor, probably in part due to the loss of helical conformation of this peptide as discussed elsewhere (Brunel et al., 2006). For R1 in both HR1-AA-54Q and HR1-EE-54Q immunized groups (Fig. 7B and 7C; top panel), the binding pattern was similar with little decrease in binding upon alanine replacements of residues at the N-terminus end. This suggests that these rabbits elicit a somewhat diverse polyclonal antibody response towards the 4E10 and/or 10E8 epitope. Surprisingly, R2 from both HR1-AA-54Q and HR1-EE-54Q groups (Fig. 7B and 7C; bottom panel) appeared to target residues W678 and L679, suggesting the epitope binding specificities of these rabbits were not as broad as those discussed above. A similarly focused response targeting residues W678 and L679, but also including I675, was observed in HR1-Δ17-54K R1. It is important to note that animal-to-animal variation as seen in HR1-AA-54Q and HR1-EE-54Q immunized rabbits is common, and this type of variation has been reported in our previous reported gp41-HR1-54Q study. The alanine scan pattern for HR1-Δ10-54K immunized rabbits (Fig. 7D) appeared slightly different due to the recognition of W672 residue, in addition to residues I675, W678 and L679. Overall these binding specificities are quite different from gp41-HR1-54Q immunized rabbits that recognized residues F673, I675, T676, N677, W678 and Y681 to different degrees(Habte et al., 2015). In comparison, antibodies elicited by pFIs lacked N677 binding but recognize a new L679 residue, suggesting a shift in MPER binding.

While alanine scan patterns showed slight differences between the rabbits immunized with different pFIs, the real meaning of these binding patterns were revealed upon mapping the critical binding residues onto the structure of the peptide co-crystalized with 4E10 (Fig. 8) (Cardoso et al., 2007). Since critical binding residues for HR1-Δ10-

54K immunized rabbits included residues for rabbits immunized with other pFIs, these were plotted onto the 4E10 peptide under the assumption that our 13-mer peptide also is a similar alpha helix. As shown in Fig. 8A, gp41-HR1-54Q immunized rabbits recognized faces opposite to or perpendicular to the 4E10 binding face while overlapping residues F673 and T676. Surprisingly, the binding epitope for HR1-Δ10-54K immunized rabbits was rotated so that the residues overlapping with 4E10 binding face now involved L679 and W672 (Fig. 8B, C and D) but lacked residues T676 and F673. As mentioned earlier, antibodies against all of the other pFIs also targeted the L679 residue that lies opposite to the N677 residue recognized by gp41-HR1-54Q. Due to the additional recognition of the W672 residue, we conclude that HR1-Δ10-54K came the closest to eliciting antibodies against the 4E10 epitope. The lack of sera neutralizing activity in these animals is most likely due to the inaccessibility of this epitope on the native virion.

Discussion

In a previous study, we described the structural and immunological characterization of gp41-HR1-54Q, which represented a near post-fusion form of gp41 with an exposed MPER (Habte et al., 2015; Shi et al., 2010). While this antigen was unable to elicit neutralizing activity in rabbits, it elicited antibody response overlapping the 4E10 and 10E8 epitope. Since it has been previously speculated that anti-MPER bnAbs primarily target the gp41 fusion intermediate form (Chen et al., 2014; Dimitrov et al., 2007; Frey et al., 2008), we asked two important questions. First, is it possible to convert the post-fusion structure of gp41-HR1-54Q to a fusion intermediate form?

Second, is such an intermediate form capable of eliciting bnAbs in rabbits via immunization?

To answer these questions, we generated four pFIs by introducing mutations or deletions in the HR1 region of gp41-HR1-54Q. These changes were able to destabilize the structure by different degrees as demonstrated by differences in trypsin sensitivity and recognition by mAbs NC-1 and 126-7. All of the pFIs elicited strong immune response in rabbits with titers reaching $1x10^7$ or higher after multiple immunization. However, upon closer examination of earlier antibody titers, it is clear that these variants are not as immunogenic as gp41-HR1-54Q, which was able to elicit titers as high as $1x10^6$ after a single dose of immunization (Habte et al., 2015). Based on this subtle difference it is tempting to speculate that the gp41 post-fusion form might be inherently more immunogenic due to its stable conformation. Interestingly, antibody titers against HR1-AA-54Q, which has comparatively milder disruptions in the six-helix bundle, were similar to gp41-HR1-54Q. Despite having identical sequences for HR2 and MPER domains, different pFIs elicited remarkably different antibody responses towards the linear peptides in this region. Again, these results suggest that conformations and immunogenicity of HR2 and MPER domains are highly dependent on the context in which they are presented. Despite the differences in recognition of other regions, all pFIs showed strong response against the C terminus end of MPER represented by peptides 671 (^{671}NWFDITNWLW^{680}) and 674 (^{674}DITNWLWYIK^{683}).

Even though the pFIs elicited strong immune responses, sera from the immunized rabbits failed to neutralize the virus. Antibodies that bind to the HR1 domains can prevent the six-helix bundle formation and hence, prevent virus fusion (Gustchina et al.,

2009; 2007; Louis et al., 2005; 2003; Miller et al., 2005; Nelson et al., 2008). D5 and HK20 represent two such human antibodies with modest neutralization (Corti et al., 2010; Miller et al., 2005). To elicit similar antibodies through immunization, a few studies have generated HR1 based constructs fused to artificial trimerization domains (Bianchi et al., 2010; Qi et al., 2010). While these studies reported weak neutralization, the lack of similar activity in our study is probably due to the HR1 mutations or deletions introduced in our construct. In fact, residues L568 and K574 are directly involved in binding to both D5 and HK20 (Luftig et al., 2006; Sabin et al., 2010). In addition, the accessibility of this hydrophobic pocket might be limited due to steric constraints (Eckert et al., 2008; Hamburger et al., 2005; Sabin et al., 2010). At the same time, it is important to highlight that our study is significantly different from the studies discussed above because it focuses on the characterization of the MPER domain in context of a soluble fusion intermediate. A recently reported study (Vassell et al., 2015) discussed the generation of similar gp41 constructs containing HR1, HR2 and MPER domains. While the authors claimed that their immunogens presented the MPER in a fusion intermediate conformation due to the truncated HR1 domain, all of them showed strong binding to NC-1, suggesting the formation of the six-helix bundle. In contrast, all of our antigens, including HR1-Δ10-54K and HR1-Δ17-54K represented distinct conformations as they failed to bind NC-1. Despite these differences, both studies failed to elicit anti-MPER neutralizing antibodies, suggesting that the generation of a soluble gp41 fusion intermediate by disrupting the post-fusion form is not sufficient to elicit bnAbs.

A detailed binding analysis of this polyclonal response using the alanine scan mutants of the13-mer peptide harboring the 4E10 and 10E8 epitopes revealed some

important findings. First, all pFIs elicited antibodies that targeted the L679 residue instead of N677. This highlights a remarkable shift from the antibody binding pattern elicited by gp41-HR1-54Q. Additionally, the HR1-Δ10-54K variant was able to elicit antibodies that bind W672 residue. Alanine replacement at this residue has been reported to reduce 4E10 binding by over 1000-fold highlighting its importance (Brunel et al., 2006). Both I675 and L679 also contribute significantly to 4E10 binding (Brunel et al., 2006; Cardoso et al., 2005). However, the failure to recognize F673 and T676 suggest differences in binding patterns between our vaccine-induced antibodies and 4E10. In the absence of a crystal structure of HR1-Δ10-54K, it is hard to speculate how the ten-residue deletion from the N terminus end influenced the overall MPER conformation to facilitate this major shift in antibody binding to the 4E10 epitope. Nevertheless, these results clearly demonstrate that changes outside the MPER domain can significantly influence the binding face targeted by anti-MPER antibodies elicited by immunization. These results also highlight a major hurdle in MPER-based vaccine design. Since gp41-based antigens are difficult to crystallize, we cannot predict the exact MPER conformation despite characterizing antigens using SPR or ELISA-based bnAb binding assays. In the absence of definitive methods, MPER-based vaccine design continues to heavily rely on empirical design and characterization. However, analyzing the success of a vaccine candidate based merely on the ability to neutralize provides insufficient information for improvement. Performing a detailed evaluation of the immune response elicited in animal models, especially in terms of binding to critical epitopes, might prove to be more fruitful in terms of future vaccine design.

It is also important to acknowledge that the failure to elicit anti-MPER neutralizing antibodies might be due to the absence of a lipid membrane. This idea is support by a recent report where a fusion intermediate form of gp41 was covalently linked onto liposomes to deliver MPER in a membrane context(Lai et al., 2014). This fusion intermediate form was also constructed by replacing the N terminus part of the HR1 domain with a GCN4 trimerization domain. However, the authors did not replace the immunodominant C-C loop with a flexible linker. Unlike our study, this construct elicited modest cross clade neutralization against Tier 1 viruses belonging to subtypes A, B and C. Future studies that combine our putative fusion intermediates with a lipid-based delivery might be able to elicit similar or better responses, especially since they do not contain the non-neutralizing immunodominant C-C loop. Overall, the results presented in this study further our understanding of the complex structure and immunogenicity of gp41 envelope and provide new insights into development of MPER based vaccines.

Materials and Methods:

Cloning, Expression and Purification of pFIs

In order to generate pFI constructs with point mutations, the QuikChange® XL Site directed mutagenesis kit was used as per the manufacturer's instructions using the original gp41-HR1-54Q plasmid as the template(Shi et al., 2010). For HR1-AA-54Q, the mutations L565A and L568A were introduced using the sense primer 5'-GAGGCCCAGCAGCAC**GCC**CTGCAG**GCC**ACCGTGTGGGGCATC-3' and the antisense primer 5'-GATGCCCCACACGGT**GGC**CTGCAG**GGC**GTGCTGCTGGGCCTC-3'. For HR1-

EE-54Q, the mutations L568E and K574E were introduced using the sense primer 5'-GCACCTGCTGCAG**GA**GACCGTGTGGGGCATC**G**AGCAGGGAGGAGG-3' and the antisense primer 5'-CCTCCTCCCTGCT**C**GATGCCCCACACGGTC**TC**CTGCAGCAGGTGC-3'.

For the deletion variants, 10 and 17 residues were deleted from the N terminus end of the HR1 domain as shown in Fig 1A. Both constructs were synthesized from IDT (Integrated DNA Technology) in the pUC57 backbone with flanking restriction sites for BamHI and EcoRI at the 5' and 3' ends of the constructs respectively. The sequence was also altered to code for the terminal 683K residue instead of the 683Q as in gp41-HR1-54Q. These constructs were cloned into the pET-21a vector (Novagen; cat#69740-3) using BamHI and EcoRI. All constructs were expressed and purified similar to gp41-HR1-54Q (Shi et al., 2010). The final proteins were dialyzed into 1x PBS (pH 8.0) and stored at -80 degrees.

Trypsin sensitivity assay

All pFIs were incubated with trypsin at 1:100 (enzyme: protein) mass ratio for one hour at 37 degrees. 3 μg of untreated and trypsin treated samples were then run on a Novex® 10-20% tricine gel (Thermo Fisher Scientific; cat# EC6625BOX)

Rabbit immunization

Eight New Zealand white female rabbits (2.5 to 3 kg) were purchased from Charles River (USA), housed under specific pathogen free environments and used in compliance with the animal protocol approved by IACUC of Iowa State University. To

evaluate the immunogenicity of the pFIs, rabbits were divided into four groups of two and each group was immunized with a different pFI in complex with the Zn-chitosan adjuvant. The immunization protocol including the adjuvant preparation, antigen/adjuvant dosage, the immunization and bleeding schedule were all exactly the same as that previously described for gp41-HR1-54Q (Habte et al., 2015).

Enzyme-linked immunosorbent assay (ELISA)

All ELISAs were performed using the standard protocol described for gp41-HR1-54Q (Habte et al., 2015) except for the use of an alternate blocking buffer consisting of PBS (pH 7.5) with 2.5% skim milk and 5% calf sera. For ELISAs testing the binding of antibodies NC-1, 126-7, 2F5, 4E10, Z13e1 and 10E8, coating antigen amounts for all other antigens equimolar to 30 ng/well of gp41-HR1-54Q using the same coating conditions as described for gp41-HR1-54Q. In order to determine end point titers, all antigens were coated at 30 ng/well. The end-point ELISA titers were defined as serum dilution factor that gave readings of average + 2x SD of the background as described previously(Qin et al., 2014). Coating for linear epitope mapping using 10-mer biotinylated peptides and 13-mer alanine scanning was also performed as previously described (Habte et al., 2015).

Neutralization assays

Neutralization assays were performed in TZM-bl cells as previously described (Li et al., 2005; Qin et al., 2014; Wei et al., 2002). Viruses tested included SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B). Murine leukemia

virus Env-pseudotyped virus was used as a negative control.

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Figures

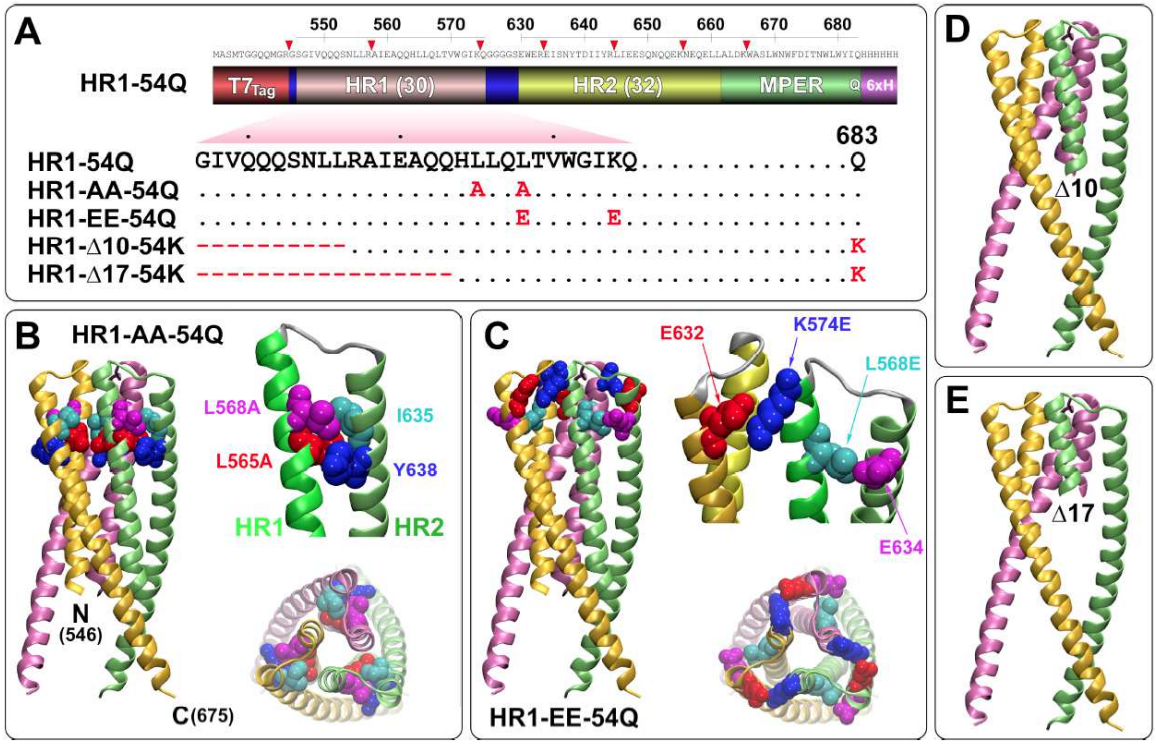

Fig 1: Design of putative fusion intermediates of gp41-HR1-54Q. (A) A domain structure of gp41-HR1-54Q consisting of the T7 tag FP, heptad repeat 1 (HR1), GGGGS linker, heptad repeat 2 (HR2), membrane proximal external region (MPER) and the 6x His tag is shown. The HR1 domain sequence, along with the terminal 683Q residue, is indicated for gp41-HR1-54Q. Point mutations and deletions introduced in the HR1 domain to generate variants HR1-AA-54Q, HR1-EE-54Q, HR1-Δ10-54K and HR1-Δ17-54K are indicated. The terminal 683Q residue was also reverted back to 683K in HR1-Δ10-54K and HR1-Δ17-54K. (B) The mutations introduced in HR1-AA-54Q- L565A and L568A are plotted on the gp41-HR1-54Q crystal structure (pdb: 3K9A) (Shi et al., 2010) to highlight the proximity of these residues to the neighboring I635 and Y638 residues located on the HR2 domain. (C) The mutations introduced in HR1-EE-54Q- L568E and K574E are plotted on the gp41-HR1-54Q crystal structure to display the effect of these mutations on the neighboring E632 and E634 residues. The truncations introduced at the N terminus end of the HR1 domain are plotted onto the gp41-HR1-54Q trimer to show the possible effect of these changes on the exposure of the HR2 and MPER domains in variants (D) HR1-Δ10-54K and (E) HR1-Δ17-54K.

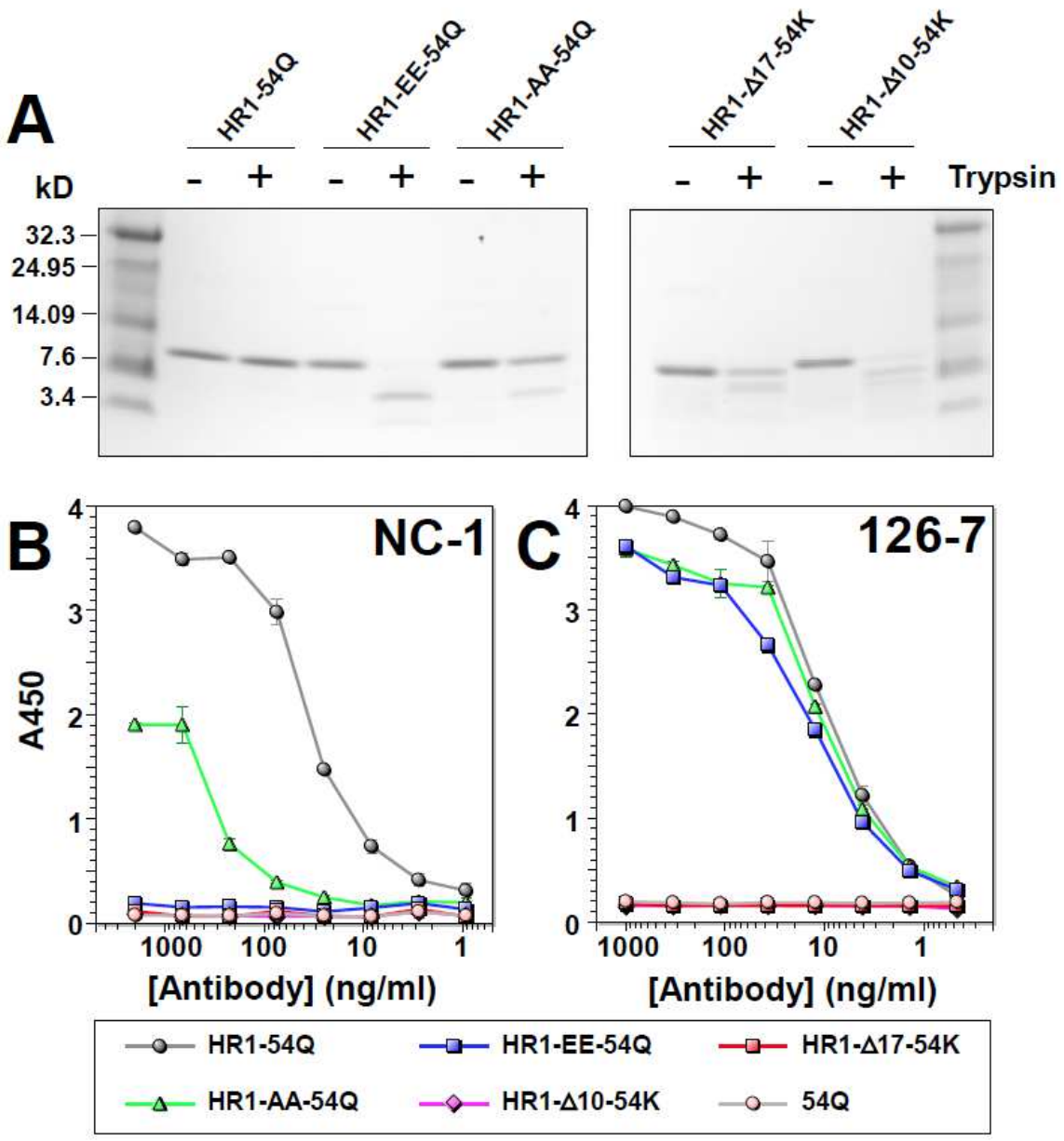

Fig 2: Structural characterization of putative fusion intermediates. (A) Differences in sensitivity to trypsin digestion of pFIs in comparison to gp41-HR1-54Q. (B) ELISA with mAb NC-1 to detect the presence of the six-helix bundle in the variants. gp41-HR1-54Q was used as a positive control while another protein (gp41-54Q), that lacks the HR1 domain, was used as a negative control. (C) ELISA with mAb 126-7 was used to detect the presence of a trimer in the variants. gp41-HR1-54Q and gp41-54Q were used as positive and negative controls respectively.

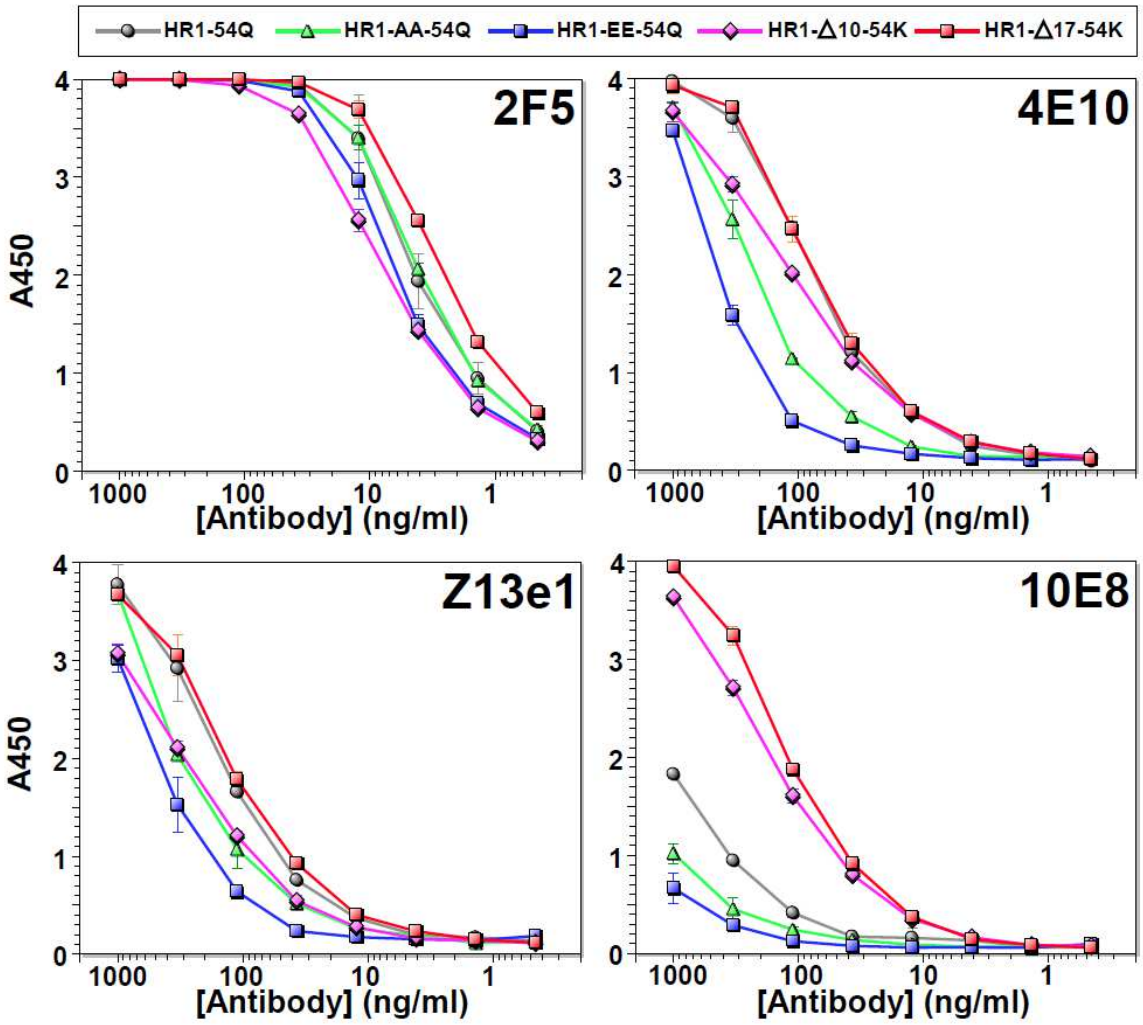

Fig 3: Antigenic characterization of putative fusion intermediates. The antigenic integrity of the variants was tested by performing ELISA with bnAbs 2F5, 4E10, Z13e1 and 10E8.

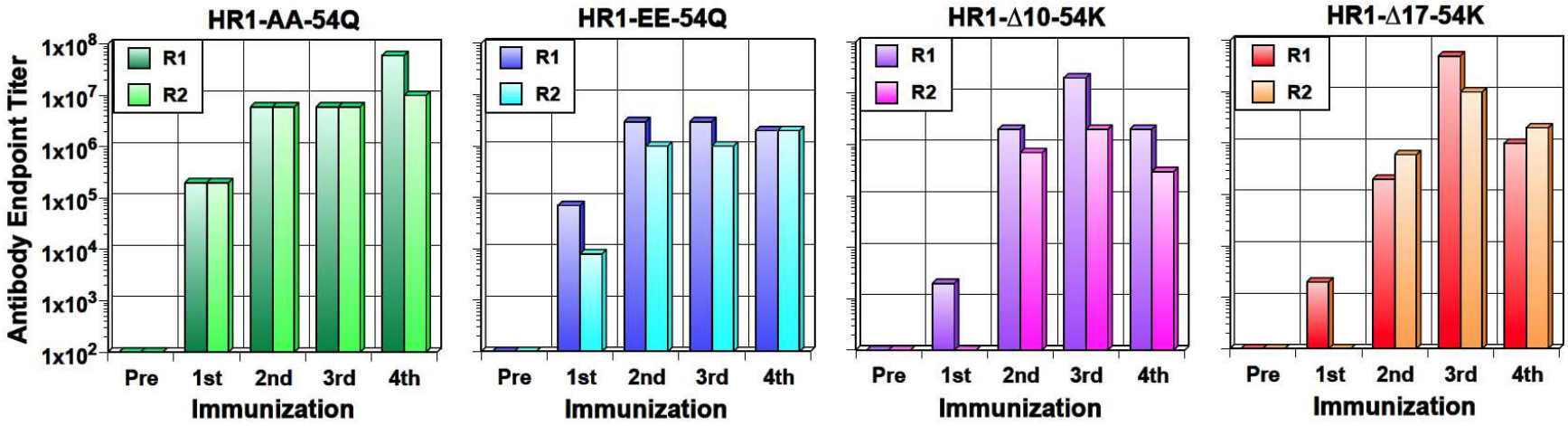

Fig 4: Antibody titers of putative fusion intermediates. Sera post every immunization were tested using ELISA to determine the end-point antibody titer against the corresponding antigen. Preimmune serum was used as a negative control.

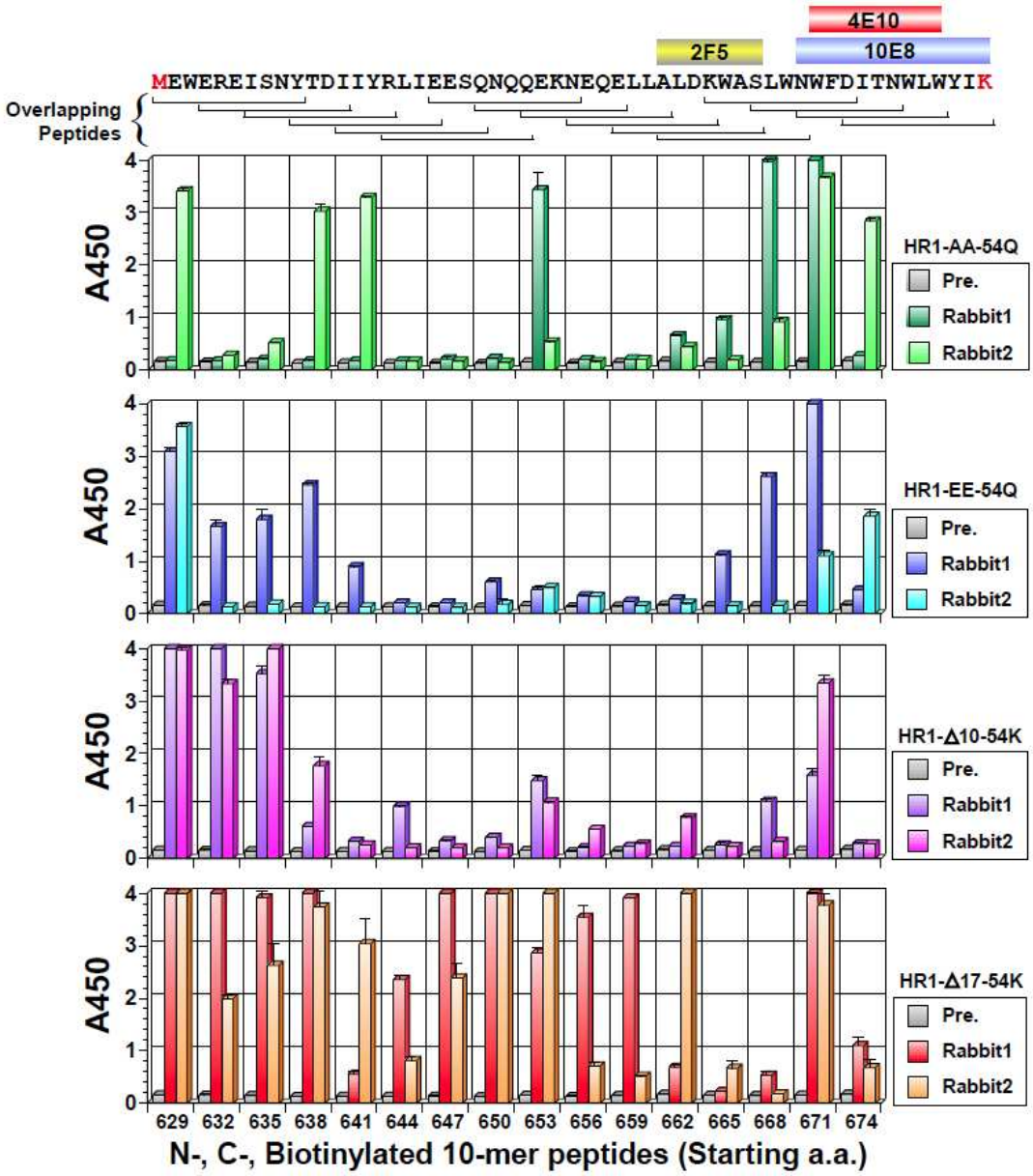

Fig 5: Mapping of linear immunogenic epitopes. Sera post fourth immunization was tested for binding against biotinylated 10-mer peptides (mixture of both N-terminus and C-terminus biotinylated peptides) spanning both HR2 and MPER domains. The amino acid sequence of each peptide is indicated by horizontal brackets. The first and last residue in the entire peptide set is indicated in red as they can differ in the four variants. The core binding epitopes for 2F5, 4E10, and 10E8 bnAbs are also indicated. Preimmune serum was used as a negative control.

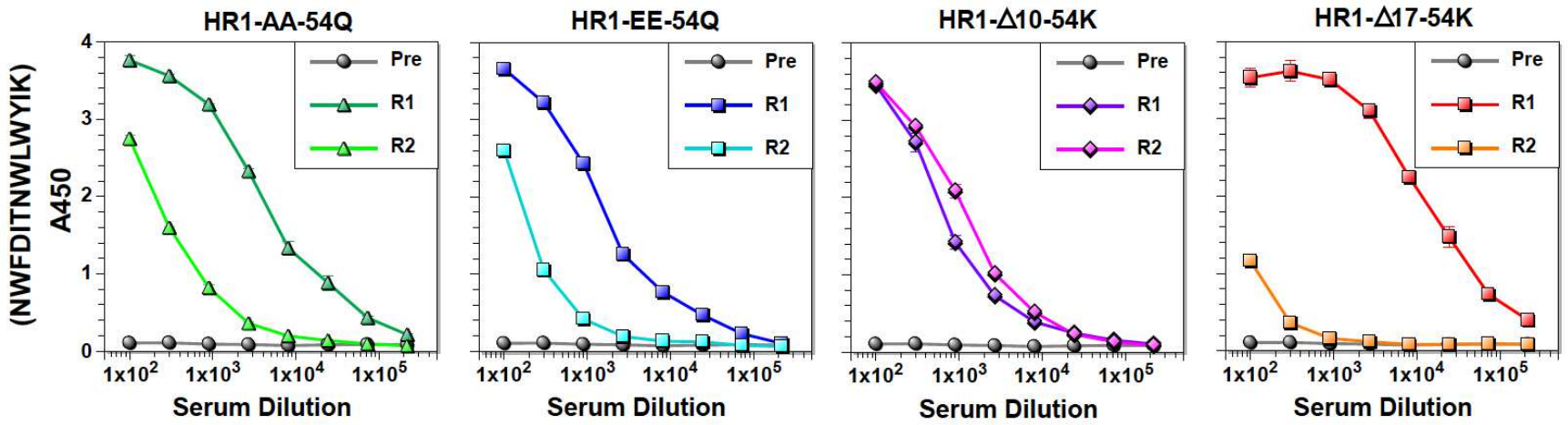

Fig 6: Antibody titers against MPER peptide. Sera post fourth immunization was tested for binding against biotinylated 13-mer peptide (^{671}NWFDITNWLWYIK^{683}) containing the complete binding epitope for both 4E10 and 10E8. Preimmune serum was used as negative control.

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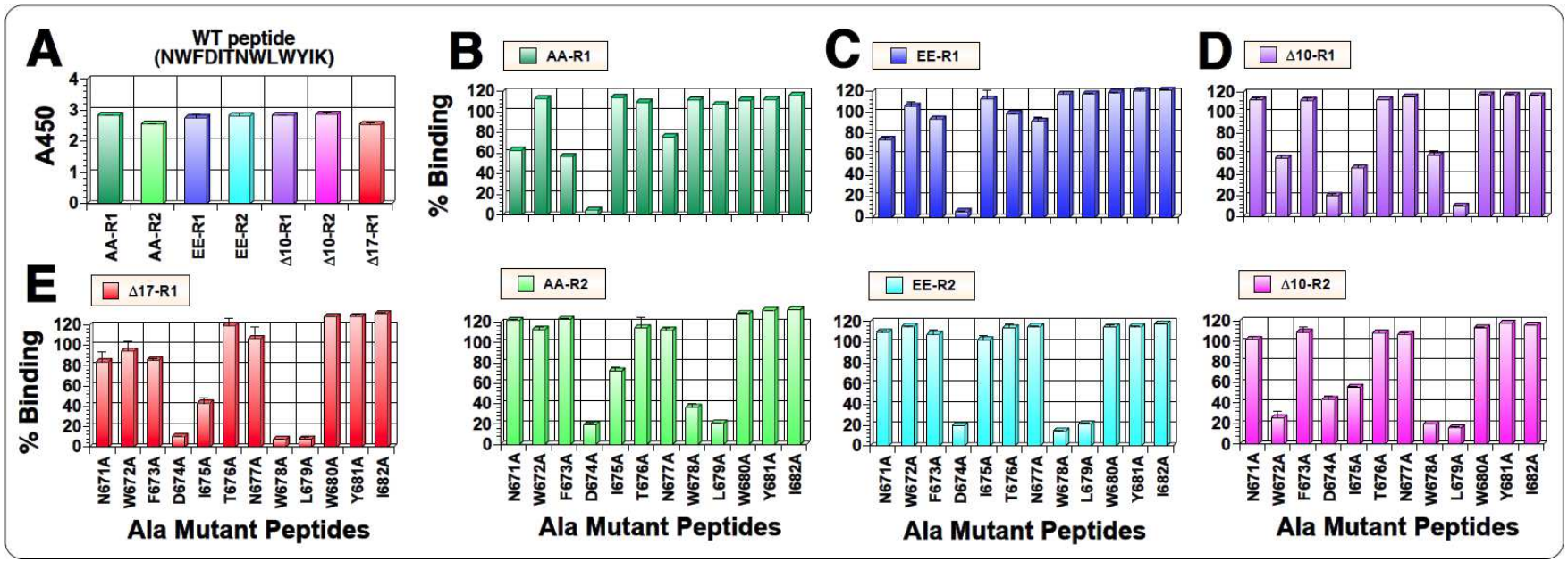

Fig 7: PepScan analysis of the C-terminus end of MPER. (A) Sera post fourth immunization was tested for binding against biotinylated 13-mer peptide (^{671}NWFDITNWLWYIK^{683}). The sera dilutions were normalized to give comparable binding signal (AA-R1 at 2000-fold dilution; AA-R2 and EE-R2 at 100-fold dilution; EE-R1 at 600-fold dilution; Δ10-R1 at 300-fold dilution; Δ10-R2 at 400-fold dilution; Δ17-R1 at 5000-fold dilution). (B-E) The same sera dilutions were tested for binding to mutant peptides. Results are shown as the percentage of binding to the wild type peptide showed in panel (A).

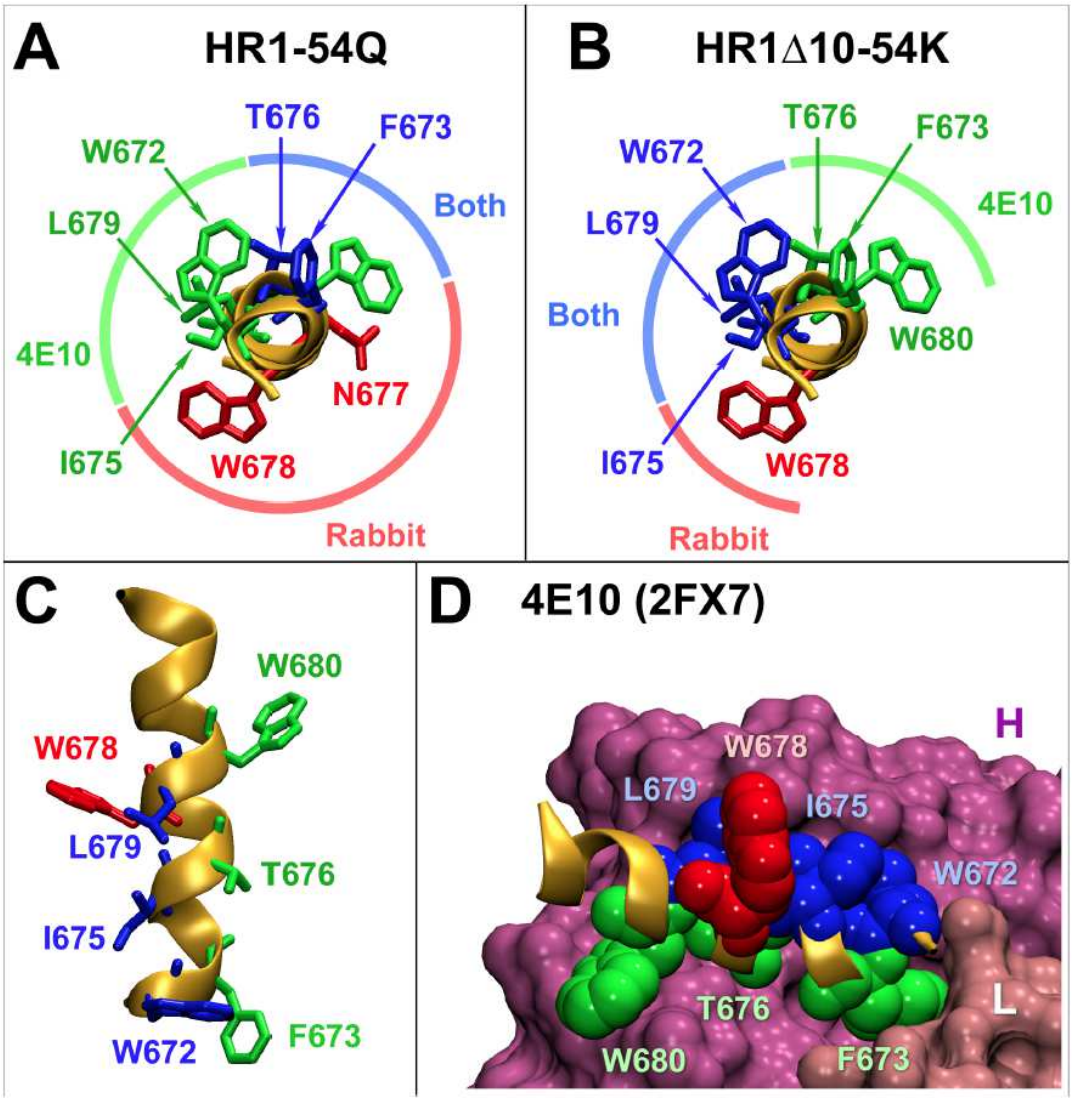

Fig 8: Structural comparison of critical binding residues for HR1-Δ10-54K immunized sera with those of HR1-54Q immunized sera and 4E10. (A) Critical binding residues for sera from gp41-HR1-54Q immunized rabbits are plotted (in red) onto the peptide co-crystalized with 4E10 (pdb: 2FX7) (Cardoso et al., 2007). Residues critical for 4E10 binding are shown in green. Residues common to both are shown in blue. (B) Critical binding residues for sera from HR1-Δ10-54K immunized rabbits were also plotted on the same peptide revealing significant difference form the binding pattern seen in gp41-HR1-54Q. (C) Lateral view of the peptide displaying critical binding residues for HR1-Δ10-54K rabbit sera and 4E10. (D) Position of all the HR1-Δ10-54K critical residues in context of the 4E10 bound peptide. The heavy and light chains for the antibody are indicated as H and L.

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CHAPTER 4

IMMUNOLOGICAL CHARACTERIZATION OF A GP41-MPER ANTIGEN CONTAINING THE NATIVE TRANSMEMBRANE DOMAIN OF HIV-1

A manuscript in preparation

Saikat Banerjee, Yali Qin, Michael W. Cho

Abstract

Eliciting broadly neutralizing antibodies through immunization is an important goal in the field of HIV-1 vaccine development. Several patient isolated antibodies targeting the membrane-proximal external region (MPER) of the gp41 envelope subunit demonstrate cross-clade neutralization with high potency. However, it has been difficult to induce such antibodies through vaccination. In the absence of definitive information about native MPER conformations capable of generating broad neutralizing antibodies (bnAbs), vaccine development efforts must focus on a systematic immunological characterization of MPER in different structural contexts. We have previously described the immunogenicity of MPER in the context of multiple gp41 ectodomain antigens. In this study, a gp41-54TM antigen consisting of the heptad repeat 2 (HR2), MPER and transmembrane (TM) domains was generated and was successfully delivered using liposomes. The antigen-loaded liposomes were bound well by MPER bnAbs, suggesting that the accessibility of the MPER domain remained unhindered. Immunization with loaded liposomes mounted strong antibody response in rabbits. Detailed epitope analysis revealed that antibodies were elicited against two major linear peptides including one harboring the 4E10 epitope. Our result demonstrates that the MPER domain can be successfully targeted using a liposomal delivery system and serves as a template for designing future anti-MPER vaccines.

Introduction

Despite more than 30 years of research, efforts to develop a clinically successful HIV-1 vaccine have largely failed. While there might be multiple correlates of protection, it is widely hypothesized that antibodies capable of neutralizing multiple clades of HIV-1 are an important component of a successful vaccine (Excler et al., 2015; B. D. Walker and Burton, 2008). Indeed, multiple studies have demonstrated that these broadly neutralizing antibodies (bnAbs) can confer protection through passive immunization (Balazs et al., 2012; Ferrantelli et al., 2003; Hessell et al., 2009; Mascola et al., 2000; Shibata et al., 1999). These broadly neutralizing antibodies (bnAbs) are elicited in a fraction of the virus-infected population and are generated against a few well-defined, conserved epitopes on the HIV-1 envelope protein. Recent advances in isolation of bnAbs (Bonsignori et al., 2011; Gaebler et al., 2013; Gray et al., 2011; Scheid et al., 2009; L. M. Walker et al., 2009; Wardemann et al., 2003), coupled with their structural characterization at high resolution (Huang et al., 2012; Julien et al., 2013b; Pejchal et al., 2011; Scharf et al., 2014; Zhou et al., 2010), have intensified efforts to design newer immunogens and immunization strategies that can induce bnAbs against these vulnerable sites.

The gp120 envelope subunit contains multiple sites; namely the receptor-binding site, the glycan-V3 loop, and the V1/V2 loop region; which can generate bnAbs (van Gils and Sanders, 2013). However, the highly conformational and discontinuous nature of these epitopes, the extensive glycosylation and the presence of other immunodominant decoys provides substantial challenges towards designing a gp120-based vaccine (Pantophlet and Burton, 2006; Sodroski et al., 1998; Wei et al., 2003). In comparison,

the gp41 subunit contains only one major epitope, named the membrane proximal external region (MPER), that is capable of eliciting bnAbs including 4E10 and 10E8 that neutralize about 98% of the HIV-1 isolates (Huang et al., 2012; Montero et al., 2008; Stiegler et al., 2001). Furthermore, as revealed by the recent crystal structures of the native envelope, this continuous, linear epitope might not be involved in interprotomer interactions and might fold independently in the vicinity of a viral membrane (Julien et al., 2013a; Pancera et al., 2014). These properties favor the use of MPER as a vaccine candidate against HIV-1.

Unfortunately, most attempts to elicit bnAbs using MPER-based vaccines has failed (discussed in (Habte et al., 2015)). While a few of these have elicited modest cross-clade neutralizing antibodies (Hulsik et al., 2013; Krebs et al., 2014; Lai et al., 2014; Ye et al., 2011; Yi et al., 2013), none have matched the breadth and potency of bnAbs elicited in infected patients. At least two major explanations have been suggested for these failures. First, it has been argued that anti-MPER bnAbs are self-reactive and hence prevented from generation by elimination of these self-reactive B cells during differentiation. This idea is largely based on the cross-reactivity and knock-in mice experiments performed using two anti-MPER bnAbs: 2F5 and 4E10 (Alam et al., 2008; Doyle-Cooper et al., 2013; Haynes et al., 2005; Verkoczy et al., 2013). However, the recent discovery of the more potent 10E8 bnAb that lacks such cross-reactivity (Huang et al., 2012) suggests that such generalization about anti-MPER bnAbs might not be true. The second and more likely reason for the failure of MPER-based vaccines is the limited understanding of viral and host factors involved in the generation of these bnAbs (Stamatatos et al., 2009). While the MPER domain is accessible to bnAbs only after

receptor engagement, the lack of structural information about specific MPER conformation(s) that can trigger the development of these bnAbs is a major hurdle in MPER-based vaccines. Hence, studies that evaluate MPER immunogenicity in different gp41 contexts are necessary.

In our attempt to design gp41-based vaccines, we have previously characterized constructs that present the MPER in a near post-fusion conformation (Habte et al., 2015), putative fusion-intermediate conformations (unpublished, Chapter 3) and a putative pre-fusion conformation (unpublished, gp41-54Q). While none of these induced detectable neutralizing activity in sera from immunized rabbits, their characterization revealed important differences in immunogenicity and the epitopes targeted. MPER presentation in both the context of a near post-fusion conformation and a fusion-intermediate conformation elicited antibodies against the C-terminus end of MPER that contains the 4E10 and 10E8 epitopes. However, one of the fusion-intermediate constructs generated antibodies targeting closest to and partially overlapping the neutralizing face of MPER. In comparison, animals immunized with the gp41 pre-fusion state mounted responses predominantly towards the cluster II epitope. Regardless, all of these constructs lacked the neighboring transmembrane (TM) domain following the C-terminus end of MPER. The lack of the TM domain might have allowed higher MPER flexibility and elicitation of antibodies to the non-neutralizing face of MPER.

In this study, using the gp41-54TM antigen, we characterized MPER immunogenicity in the presence of both of its neighboring domains, namely the heptad repeat 2 (HR2) domain and the TM domain. It was hypothesized that incorporation of the transmembrane domain might provide more structural rigidity to the otherwise free

MPER as observed in our previous studies characterizing soluble gp41 antigens(Habte et al., 2015) (Chapter 3 and unpublished, gp41-54Q). Second, the transmembrane domain would anchor the MPER on a membrane, thereby mimicking the MPER presentation on the native virion. The antigen was delivered on liposomes with the TLR4 agonist monophosphoryl lipid A (MPLA) adjuvant as it can be directly incorporated in the liposomes.

Results

Design and characterization of gp41-54TM

The gp41-54TM antigen contained 54 amino acids spanning HR2 and MPER along with the native TM domain of HIV-1 (Fig. 1A). The protein was expressed in *E.coli*, purified using a C-terminus histidine tag and refolded in the presence of detergent (Fig. 1B). As shown in Fig. 1C, the antigenicity of gp41-54TM was evaluated using ELISA binding to different antibodies. The protein was recognized well by all anti-MPER bnAbs including 2F5, 4E10, Z13e1 and 10E8; suggesting that the MPER domain was readily accessible. Of all the anti-MPER bnAbs, 2F5 showed the strongest binding to gp41-54TM suggesting better exposure of this region. In line with this observation, strong binding signal was also detected using another antibody, 2C2, which recognizes the non-neutralizing epitope upstream of the 2F5 epitope.

Characterization of gp41-54TM on the surface of liposomes

To design a suitable platform for the delivery of gp41-54TM, simple liposomes comprising of phosphatidyl choline were generated and loaded with the antigen by

extensive dialysis. The quality of the antigen-loaded liposomes was evaluated by immunogold staining with antibodies. Initially, small, homogenous, liposomes were generated by extrusion through 100 nm polycarbonate filters. However, each liposome showed little immunogold labeling when using 2F5 as the primary antibody (Supplementary Fig. 1). These results might suggest that each liposome was only able to hold a small amount of antigen, or that loaded antigens were not accessible due to the rigid structure with sharper lipid curvature. Since the ultimate goal was to develop a platform that can induce a strong antibody response, it was important to generate liposomes that displayed multiple antigen copies so as to crosslink the B-cell receptors upon immunization. Therefore, larger liposomes were generated using 1 μm polycarbonate filters. This generated a more polydisperse liposomal population consisting of smaller and larger liposomes. Interestingly, the larger liposomes demonstrated much better labeling as shown in Fig. 2. The antigen was recognized well by bnAbs 2F5, 4E10, Z13e1 and 10E8; suggesting that these MPER epitopes were accessible on the surface of the liposome. Labeling was also seen against the non-neutralizing 2C2 antibody in agreement with the ELISA data. The presence of several gold beads on the surface of these large liposomes suggested that multiple antigens were loaded onto each liposome. Unloaded liposomes were used as negative controls and did not show any labeling with the 10E8 antibody.

Immunogenic characterization of gp41-54TM

For rabbit immunizations, the adjuvant MPLA was used since it is easily incorporated into the liposomes during their formulation. Following antigen loading, the

total solution containing both loaded and unloaded liposomes were used for immunization. While the unloaded liposomes did not contain any antigen, they would still possessed adjuvant properties due to the MPLA. As shown in Fig. 3, antibody titers reached about 10^3 to 10^4 after a single dose of immunization. Following the second immunization, titers increased to above 10^5 for rabbits R1 and R2 and above 10^6 for rabbit R3. Titers were boosted slightly following subsequent immunization, reaching as high as 10^7 for rabbit R3. Overall, the gp41-54TM protein was fairly immunogenic when delivered on liposomes along with the MPLA adjuvant.

Detailed characterization of the antibody responses against gp41-54TM

The ectodomain of gp41-54TM is identical to that of the soluble gp41-54Q antigen (unpublished study) except for the terminal 683 residue (K in gp41-54TM versus Q in gp41-54Q). To evaluate how the incorporation of a transmembrane domain affected the immunogenicity of linear epitopes in the HR2 and MPER domains, peptide ELISA was performed using overlapping 10-mer peptides spanning these domains(Habte et al., 2015). Multiple linear peptides were bound strongly by the rabbit sera after third immunization (Fig. 4). The two major peptides that were recognized by all three rabbits were the HR2 domain peptide 638 (^{638}YTDIIYRLIE^{647}) and the MPER peptide 671 (^{671}NWFDITNWLW^{680}), which also contains the core binding epitope for the bnAb 4E10. Several other peptides were recognized somewhat sporadically for different rabbits. Rabbit R1 bound peptide 662 (^{662}ALDKWASLWN^{671}) that contains the complete binding epitope for 2F5. Rabbit R1 also recognized peptides 629 (^{629}MEWEREISNY^{638}), 632 (^{632}EREISNYTDI^{641}), 650 (^{650}QNQQEKNEQE^{659}), while

rabbit R3 recognized peptides 644 (^{644}RLIEESQNQQ^{653}) and 647 (^{647}EESQNQQEKN^{656}). In comparison, animals immunized with the soluble gp41-54Q recognized a different pattern after third immunization (Supplementary Fig. 2). First, most of the gp41-54Q immunized animals did not show strong binding to either peptide 638 or 671 as recognized by gp41-54TM immunized animals. Second, peptides spanning ^{641}IIYRLIEESQNQQEKNEQELLQLDK^{665} appeared to be highly immunogenic in majority of the animals immunized with soluble gp41-54Q. While peptides 644, 647 and 650 were also sporadically recognized in gp41-54TM immunized animals, the response to this cluster II non-neutralizing region was less robust compared to the gp41-54Q animals.

Despite eliciting antibodies against the peptide that contains the 4E10 epitope, none of the rabbit sera were able to neutralize pseudoviruses SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B) (data not shown). To understand how the vaccine-elicited antibodies bind the 671 peptide differently as compared to 4E10 bnAb, we attempted to perform sera binding analysis using alanine scanning mutant peptides as previously described (Habte et al., 2015). Oddly, rabbit sera did not show good binding to the C-terminus biotinylated peptide used for this analysis (data not shown). This suggests that antibodies elicited by gp41-54TM are significantly different from those elicited by ectodomain only antigens gp41-HR1-54Q and derivative fusion intermediates. Future evaluation of sera using N-terminus biotinylated, 13-mer 671 peptide and its alanine scan mutants might provide better understanding of the exact binding face that gp41-54TM-induced antibodies target. Results from these experiments will be critical in understanding how the addition of a transmembrane domain influences

the exposure of the 4E10 binding and thus neutralizing face of MPER, thereby improving the design of next generation of gp41-based immunogens.

Discussion

Epitope binding studies of 4E10 and 10E8 bnAbs suggest that the MPER peptide contains a neutralizing face and a non-neutralizing face (Brunel et al., 2006; Huang et al., 2012). To elicit 4E10- and 10E8-like bnAbs, MPER vaccines must induce antibodies that bind this neutralizing face. We have previously evaluated the immunogenicity of a wide variety of MPER antigens that contained only the gp41 ectodomain ((Habte et al., 2015) and Chapter 3). While these antigens had significant differences at their N-terminus end, the C-terminus end of MPER was always free. It is possible that such antigen design inherently contributed to a flexible MPER capable of eliciting antibodies against the faces opposite or perpendicular to the neutralizing antibody binding face.

To restrict MPER presentation so that antibodies are mounted against the neutralizing face, a new antigen named gp41-54TM was designed to include the neighboring transmembrane (TM) domain. The TM domain allowed anchoring of the MPER to the surface of phosphatidyl choline liposomes thereby mimicking a membrane environment. As shown in this study, membrane presentation of MPER did not restrict access by bnAbs suggesting that these epitopes remained accessible. Interestingly, the use of larger liposomes allowed us to load multiple antigens on the same platform, which might be important for eliciting strong B-cell responses. In line with this, our gp41-54TM delivery on liposomes containing the MPLA adjuvant induced robust antibody response in rabbits. Furthermore, all three rabbit elicited antibodies against the linear

peptide containing the 4E10 epitope. These results suggest that it is possible to elicit antibodies against the 4E10 epitope using a larger gp41 antigen that is delivered in the context of the membrane. It is important to note that the linear epitopes recognized were somewhat different from those elicited by the gp41-54Q antigen. Overall, in comparison to the membrane bound gp41-54TM, the soluble gp41-54Q antigen elicited a more robust response against the non-neutralizing cluster II region. It is possible that the ectodomain might fold differently in these two immunogens, especially since the hydrophobic MPER in gp41-54Q does not contain any C-terminus transmembrane domain. Surprisingly, despite strong binding of 2F5 to gp41-54TM, rabbits failed to elicit strong antibody response to this region., highlighting that epitope antigenicity does not always correlate with its immunogenicity.

Multiple studies have described the use of liposomal delivery systems in attempts to induce MPER-targeting bnAbs. While the liposome composition, immunization protocol and immunized host vary among these studies, these approaches can be categorized based on the antigen and its presentation on the delivery platform. First, several groups have delivered MPER peptides that lack any lipid anchors or transmembrane domains (Dennison et al., 2011; Devito et al., 2004; Karasavvas et al., 2008; Matyas et al., 2009; Mohan et al., 2014; Serrano et al., 2014; Yang et al., 2013). It is likely that these peptides associated with the liposomes based on their hydrophobic nature. Of these, only one reported the isolation of a weakly neutralizing monoclonal antibody, W320, which binds overlapping the 2F5 epitope (Matyas et al., 2009). Alternatively, short MPER peptides have also been chemically conjugated to lipid anchors for direct liposomal incorporation (Hanson et al., 2015; Kim et al., 2013;

Venditto et al., 2013; Watson et al., 2011; Watson and Szoka, 2009). Unfortunately, these approaches have also failed to generate any neutralizing antibodies. This suggests that short MPER peptides might not be effective despite liposomal delivery.

Multiple studies have also tested the immunogenicity of MPER in the context of the transmembrane domain (Benen et al., 2014; Hulsik et al., 2013; Kamdem Toukam et al., 2012; Kim et al., 2013; Lenz et al., 2005). While some have used antigens that contain MPER and TM domains with or without the cytoplasmic tail (CT) (Kamdem Toukam et al., 2012; Kim et al., 2013), others have characterized antigens containing HR2, MPER and TM domains similar to gp41-54TM but using different delivery systems (VLPs or liposomes of different composition) (Benen et al., 2014; Hulsik et al., 2013; Lenz et al., 2005). Of the later studies, Benen et al reported that following a DNA-prime VLP-boost strategy, sera from immunized rabbits showed weak neutralizing activity against SF162 and MW965.26 (Benen et al., 2014). Interestingly, another study that immunized llamas with gp41-proteoliposomes, reported the generation of a virable domain only-single chain antibody that bound overlapping the 2F5 epitope and neutralized both Tier1 and Tier 2 viruses (Hulsik et al., 2013). It is important to note that prior to isolation of the single chain antibody by hybridoma generation and extensive screening in this study, no neutralizing activity was detected in the whole sera. Whether or not gp41-54TM induced any low levels of neutralizing antibodies remains to be determined using similar methods. However, compared to the studies mentioned above, our immunization scheme generated stronger overall antibody response.

Most importantly, while some of the previous studies elicited antibodies targeting the region bound by 2F5 (Dennison et al., 2011; Devito et al., 2004; Huarte et al., 2012;

Kamdem Toukam et al., 2012; Matyas et al., 2009; Serrano et al., 2014; Watson et al., 2011; Watson and Szoka, 2009; Yang et al., 2013; Zhang et al., 2014), our immunogen was successful in eliciting immune response against the peptide harboring the 4E10 epitope. This is significant since 4E10 has much broader neutralizing ability than 2F5 (Binley et al., 2004; Huang et al., 2012). Hence, our results suggest that gp41-54TM might be a good starting point for designing the next generation of MPER-based vaccines to specifically target the 4E10 epitope. One potential strategy might be to use gp41-54TM as a priming antigen along with different boosting antigens that present more native structures to better direct antibody maturation to facilitate recognition of the native virion. The effect of factors like liposome size and composition, especially through the incorporation of cholesterol to mimic the native membrane (Brügger et al., 2006), can also be further explored. Finally, since gp41-54TM contains only the HR2, MPER and TM domains, the ectodomain might mimic a prefusion state. It is speculated that the neutralizing face of the MPER might be better accessible in a fusion intermediate form (Chakrabarti et al., 2011; de Rosny et al., 2004; Dimitrov et al., 2007; Finnegan et al., 2002; Frey et al., 2008; Kim et al., 2011). In line with this theory, constructs mimicking the gp41 fusion intermediate have been reported to induce modest cross-clade neutralizing antibodies when delivered on liposomes after covalent linking (Lai et al., 2014). This antigen also contained the immunodominant C-C loop. Hence, future constructs that present some of our previously described fusion intermediates (that lack the immunodominant loop) along with a transmembrane domain might further enhance the effectiveness of such vaccine approaches.

Materials and Methods:

Cloning, Expression and Purification of gp41-54TM

The gp41-54TM fragment was amplified by PCR from Mcon6gp160 (46) using sense 5'- GCGCGGATCCGAGTGGGAGCG-3' and antisense 5'-GCGCGAATTCTTAATGGTGATGATGGTGATGCTGGCGCACGCGGTTCAC-3' primers. The amplified product was digested at indicated BamHI and EcoRI sites and cloned into pET-21a vector (Novagen; cat#69740-3) using the same sites. The resulting plasmid, following sequence confirmation was designated as pET-gp41-54TM. For expression, *E.coli* T7 Express IysY/I^q (New England Biolabs; cat#C3013I) was transformed with pET-gp41-54TM and cultured overnight at 37 °C in superbroth containing ampicillin (50 μg/ml). Cells were diluted 1:100 in fresh superbroth containing ampicillin (100 μg/ml) and grown to 5.0 A_{600} at 37 °C. Protein expression was then induced using 1mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested 5 hour post-induction by centrifugation at 15000g rpm for 15 min. The cell pellet was resuspended in 1X PBS (pH 7.4) and lysed by passing once through French Press at 20000 psi. Lysed cells were collected by centrifugation at 15000g for 20 min. The pellet was completely solubilized in 1X PBS (pH 7.4) containing 2% OG (n-octyl-β-D-glucoside) by sonication. Following centrifugation at 15000g for 30 min, the supernatant was collected, and the detergent solubilized protein was bound to Ni^{2+}-NTA (nitrilotriacetic acid) Superflow (Qiagen; cat#30450) by mixing on an end to end shaker overnight at 4 °C. The mixture was loaded onto a column, and resin bound protein was denatured by passing at least 10 bed volumes of 6M urea in 1X PBS (pH 8) containing 1% OG and 20 mM imidazole. The protein was renatured by passing 10 bed volumes of

1X PBS (pH 8) containing 0.5% OG, 20 mM imidazole and a decreasing step gradient of urea at 4, 2, and 0 M. The protein was eluted with 1X PBS (pH 8) containing 1% OG and 300 mM of imidazole. The protein was finally dialyzed against a buffer containing 20 mM HEPES, 20 mM NaCl (pH 8.0), 1% OG and 15% (v/v) glycerol.

Liposome production and characterization

For initial characterization, simple liposomes were prepared by dissolving phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) (Avanti Polar Lipids; cat# 850355P) in chloroform at a final concentration of 10 mg/ml. After solvent evaporation under a stream of nitrogen gas, the sample was lyophilized to remove any residual chloroform. Multilamellar vesicles were generated by resuspending the lipid film in 1x PBS (pH 8) at a final concentration of 10 mg/ml. The lipid suspension was then extruded through either 100-nm or 1-μm polycarbonate membrane to generate liposomes. For protein loading, the gp41-54TM was added to the liposomes at a mass ratio of 1:50 (protein: lipid) and incubated for 30 min at room temperature with end-to-end mixing. The sample was then dialyzed against 1x PBS (pH 8) for 48-72 hours with a final dialysis performed using 1x PBS (pH 7.4).

Both unloaded and loaded liposomes were analyzed using antibody-based immunogold labeling and visualized with a JOEL 2100 transmission electron microscope. 3 μl of the diluted liposome sample (1:20 dilution) was applied on a carbon-coated copper grid. After 3 min incubation, excess liquid was carefully blotted away. Blocking was performed for 30 min by floating the grid on top of 50 μl of blocking buffer (0.1% AURION BSA-c^{TM} in 1X PBS, pH 7.4). The grid was then floated on top

of 50 µl of primary antibody solution (made in blocking buffer at 10 µg/ml concentration) for 1 hour. This was followed by 10 min incubation in blocking buffer to remove unbound antibodies and blotting away the excess liquid. Following two more washes, the grid was floated on the secondary antibody solution (1:40 dilution in blocking buffer) for 1 hour. The grid was washed twice by floating on the blocking buffer for 5 min. Three more similar washes were performed using distilled water. Following a 30 sec staining with 20 µl of 1% uranyl acetate solution, grids were blotted carefully and allowed to dry completely before visualization at 20000x magnification. Different primary antibodies used for staining were 2F5(Buchacher et al., 1994; Purtscher et al., 1996; 1994), 4E10(Stiegler et al., 2001), Z13e1(Nelson et al., 2007; Zwick et al., 2001), 10E8 (Huang et al., 2012) and 2C2 (unpublished data). Anti-human antibody conjugated to 10 nm gold was used as a secondary antibody for 2F5, 4E10, Z13e1 and 10E8 while anti-rabbit antibody conjugated to 10 nm gold was used as secondary antibody for 2C2.

Rabbit immunization

Three New Zealand white female rabbits (2.5 to 3 kg) were purchased from Charles River (USA), housed under specific pathogen free environments and used in compliance with the animal protocol approved by IACUC of Iowa State University. Liposomes were prepared as described earlier but with the addition of the adjuvant monophosphoryl lipid A (MPLA) to the phosphatidylcholine (PC) solution in choloroform. The amount of MPLA was 100 µg for every 10 mg of PC. The gp41-54TM protein was loaded onto pre-formed liposomes as described earlier. Rabbits were immunized subcutaneously on weeks 0, 4 and 11 with 200 µg of protein (along with 10

mg of PC and 100 μg of MPLA) per immunization. Blood was collected pre-immunization and 2 weeks post-immunization. Processed sera were stored at -80 °C.

Enzyme-linked immunosorbent assay (ELISA)

All ELISAs were performed using the standard protocol described for gp41-HR1-54Q (Habte et al., 2015) except for the use of an alternate blocking buffer consisting of PBS (pH 7.5) with 2.5% skim milk and 5% calf sera. Briefly, for assays testing binding to monoclonal antibodies as well as for serum antibody titers, gp41-54TM was coated at 30 ng/well. All antibodies inlcuding 2F5, 4E10, Z13e1, 10E8 and 2C2, were used at an initial concentration of 1 μg/ml followed by three-fold serial dilution. Secondary antibody used for 2F5, 4E10, Z13e1 and 10E8 was a goat anti-human, horseradish peroxidase (HRP)-conjugated (Thermo Scientific; Cat# 31410) while a goat anti-rabbit, horseradish peroxidase (HRP)-conjugated secondary antibody was used for 2C2 (Thermo Scientific; Cat# 31430). All secondary antibodies were used at 1:3000 dilution in the blocking buffer as previously described. Coating for linear epitope mapping using 10-mer biotinylated peptides and 13-mer alanine scanning was also performed as previously described (Habte et al., 2015).

Neutralization assays

Neutralization assays were performed in TZM-bl cells as previously described(Li et al., 2005; Qin et al., 2014; Wei et al., 2002). Viruses tested included SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B). Murine leukemia virus Env-pseudotyped virus was used as a negative control.

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The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 anti-gp41 mAb 2F5 from Dr. Hermann Katinger (Cat# 130220), 4E10 from Dr. Herman Katinger (Cat# 10091), Z13e1 from Dr. Michael Zwick (Cat# 11557) and 10E8 from Dr. Mark Connors (Cat# 12294). We would like to thank Tracey M. Stewart and Dr. Harry T. Horner at the Microscopy and Nanoimaging Facility at Iowa State University for helping with immunogold labeling and liposome visualization using TEM. This work was supported by a grant from the NIH, NIAID (P01 AI074286) grant. MWC has an equity interest in NeoVaxSyn Inc., and serves as the CEO/President. NeoVaxSyn Inc. did not contribute to this work or the interpretation of the data.

Figures

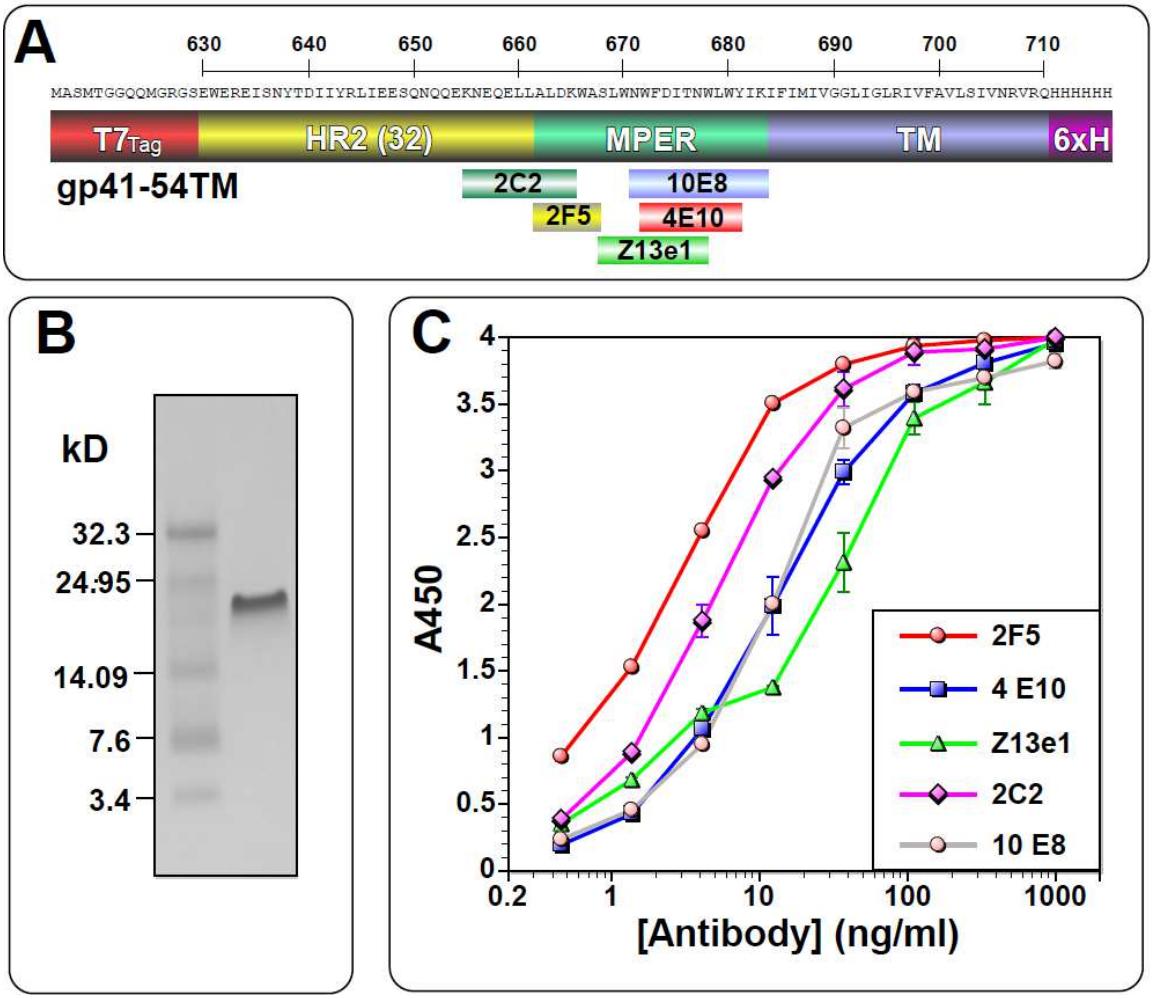


Fig 1: Design and antigenic characterization of gp41-54TM. (A) A domain structure of gp41-54TM consisting of the T7 tag, heptad repeat 2 (HR2), membrane proximal external region (MPER), transmembrane (TM) and the 6x His tag is shown along with the aligned sequence on top. (B) The purified protein was run on a tricine-SDS gel to check its purity. (C) ELISA binding of monoclonal antibodies (mAbs) 2F5, 4E10, Z13e1, 2C2 and 10E8.

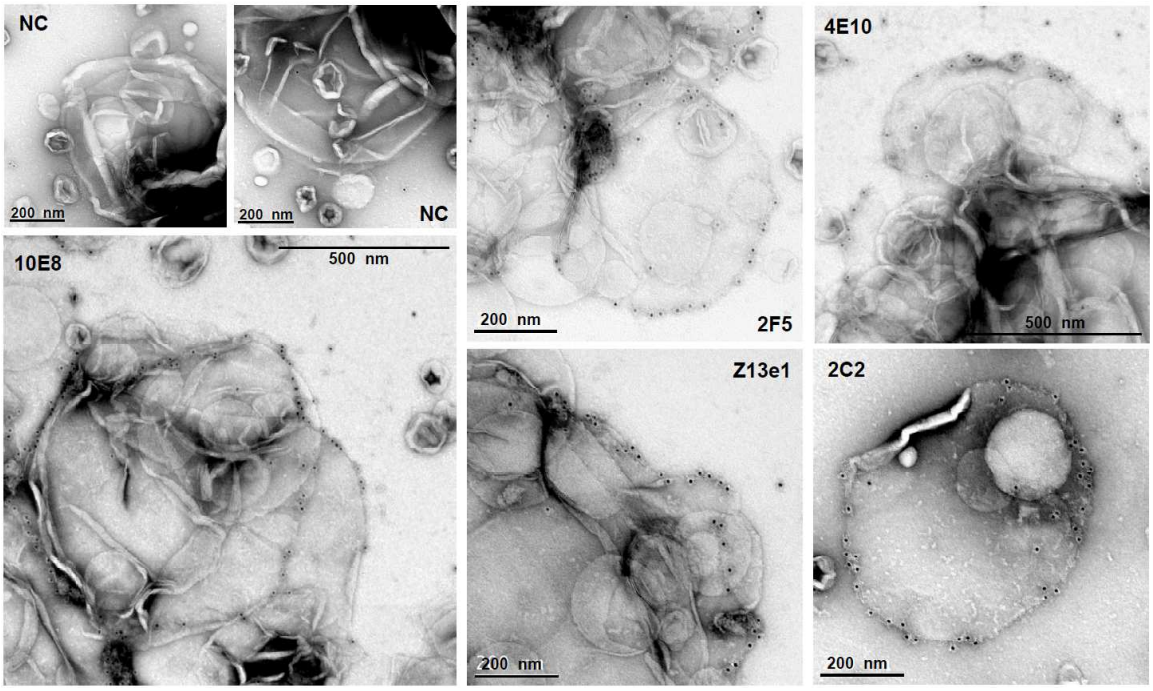


Fig 2: Immunogold labeling of gp41-54TM proteoliposomes. MPER accessibility on liposomes was tested by binding to bnAbs 2F5, 4E10, Z13e1 and 10E8 . All anti-MPER bnAbs were able to bind the gp41-54TM loaded liposomes. Strong binding was also observed forand cluster II-binding rabbit mAb 2C2. Unloaded liposomes (showed no binding to 10E8 as indicted in the panels labeled NC) are also shown.

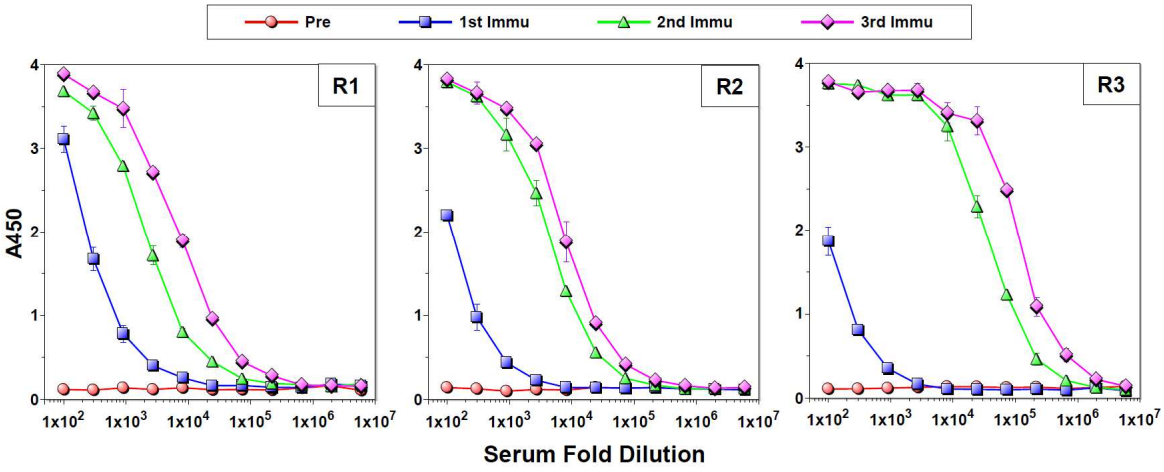


Fig 3: Antibody titers. Sera after each immunization were used to test for binding to gp41-54TM. Pre-immune serum was used as negative control.

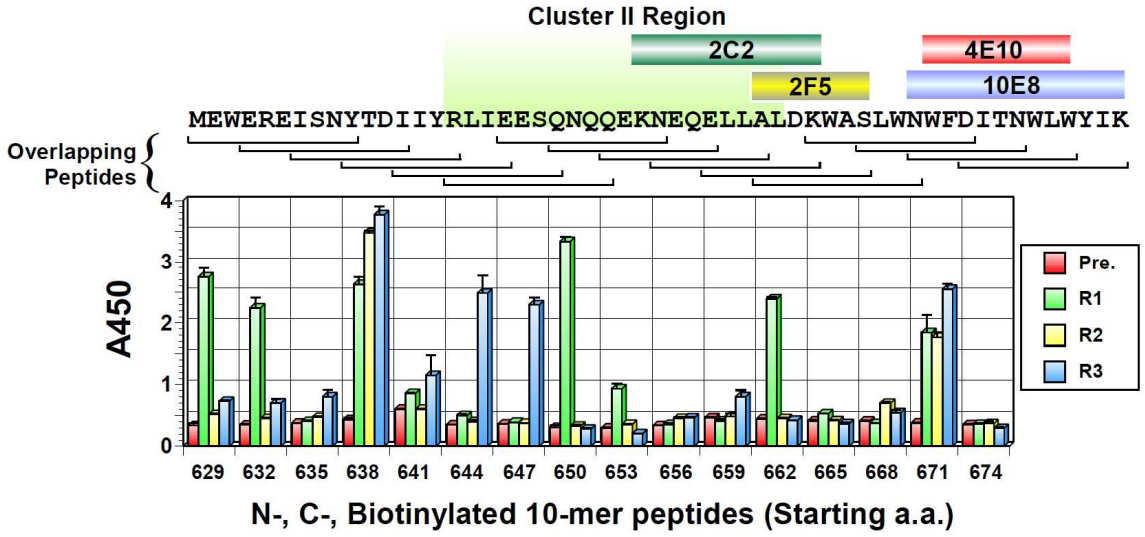
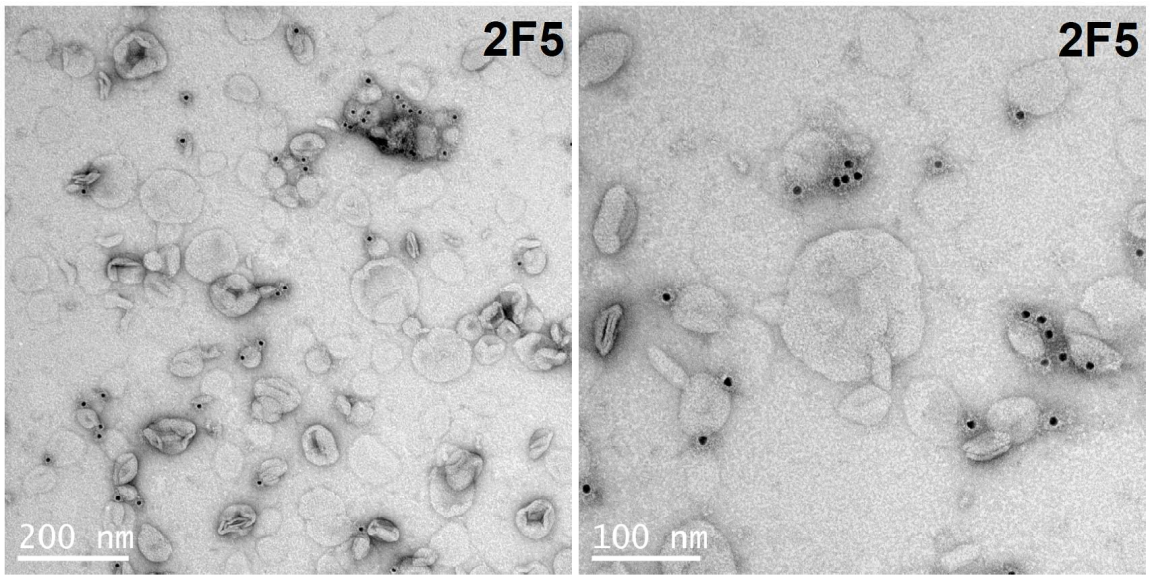
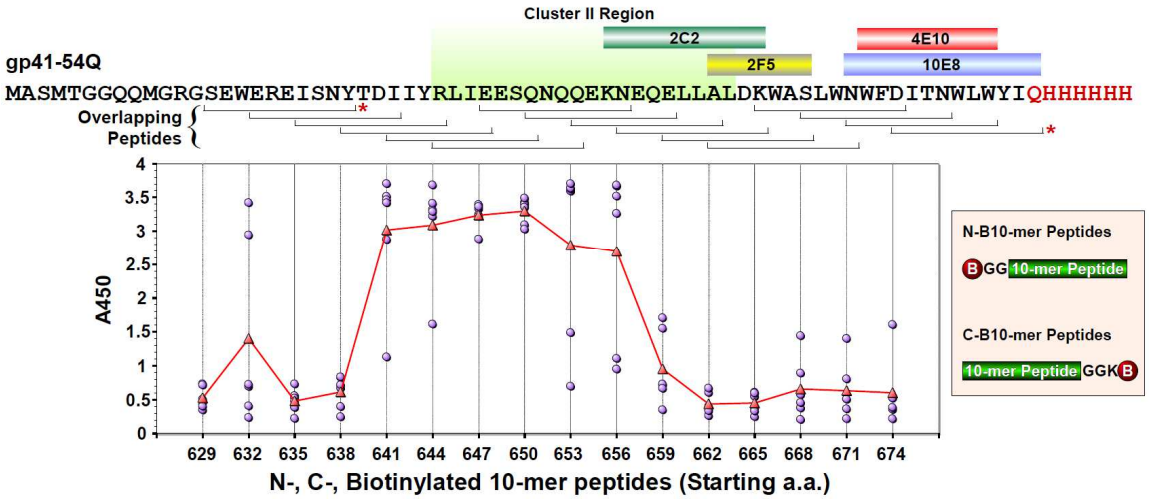


Fig 4: Mapping of linear immuniogenic epitopes for gp41-54TM immunized rabbits. Sera post third immunization was tested for binding against biotinylated 10-mer peptides (mixture of both N-terminus and C-terminus biotinylated peptides) spanning both HR2 and MPER domains. The amino acid sequence of each peptide is marked by horizontal brackets. The core binding epitopes for 2F5, 4E10, and 10E8 bnAbs and 2C2 are indicated. The cluster II region is also highlighted. Pre-immune serum was used as a negative control.



Supplementary Fig 1: Immunogold labeling of smaller liposomes. Smaller liposomes generated using a 100 nm polycarbonate filters were loaded with gp41-54TM by extensive dialysis and tested for binding to 2F5. As shown in both panels, little gold labeling was observed on each liposome suggesting poor antibody binding.



Supplementary Fig 2: Mapping of linear immuniogenic epitopes for gp41-54Q immunized rabbits. Sera (post-third immunization) from 6 rabbits from two different experiments were used for epitope mapping using overlapping 10-mer peptides spanning the entire gp41-54Q. A mixture of N-terminus and C-terminus biotinylated peptides were used for the assay. Horizontal brackets represent the sequence of each peptide. The first peptide (MEWEREISNY) and the last peptide (DITNWLWYIK) are marked with an asterisk to indicate slight sequence differences from original antigen. A450 values for individual rabbits are represented with purple spheres where as average values are indicated with red triangles. The most immunodominant epitope overlaps with the cluster II region. The binding epitope (KNEQELLALDK) for the non-neutralizing antibody 2C2 (isolated from one gp41-54Q immunized rabbit) is indicated along with core binding epitopes for anti-MPER bnAbs 2F5 (ALDKWAS), 4E10 (WFDITNWLW) and 10E8 (NWFDITNWLWYIK).

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CHAPTER 5

CHARACTERIZATION OF MPER-TARGETING HYBRIDOMAS GENERATED FROM RABBITS USING A NOVEL PRIME-BOOST IMMUNIZATION

Saikat Banerjee, Hojin Moon, Yali Qin, Heliang Shi, Michael W. Cho

Abstract

Broadly neutralizing antibodies elicited against the membrane proximal external region (MPER), including 4E10 and 10E8, isolated from infected individuals can neutralize up to 98% of all HIV-1 isolates. Attempts to elicit similar antibodies using different MPER-based vaccine strategies have not been successful. In our continued efforts to fully characterize MPER immunogenicity, we have tested novel prime-boost immunization strategies. In a comparative study, rabbits were primed with either soluble or membrane bound MPER antigens. Both groups were then progressively boosted using larger immunogens to attempt to direct antibody maturation and facilitate recognition of native MPER structure on the virion. Rabbits primed with the soluble antigen elicited strong antibody response against peptides containing the 4E10/10E8 epitopes. Although none of the rabbits demonstrated serum-neutralizing activity, one rabbit was selected for hybridoma generation to understand why the elicited response was non-neutralizing. Following extensive screening using MPER peptides containing epitopes for 2F5 and 4E10/10E8, three novel hybridomas named 6C10, 9F6 and 21B5 were identified. Detailed epitope mapping of these hybridomas revealed that while 9F6 recognizes the peptide overlapping the 4E10 eptiope, 21B5 binding to the MPER requires the ^{680}YIK^{683} residues in addition to the upstream 4E10 epitope, similar to the epitope binding requirement for 10E8. The 6C10 hybridoma bound an epitope overlapping but slightly

upstream of the 2F5 epitope. Further characterization of these hybridomas will provide valuable information for MPER-based vaccine design.

Introduction

The human immunodeficiency virus 1 (HIV-1) has evolved multiple strategies to evade the immune system. The virus uses a mixture of low spike density (Klein et al., 2009; Zhu et al., 2006), variable glycosylation (Binley et al., 2010; Moore et al., 2012), transient epitope exposure (Frey et al., 2008), conformational masking (Kwong et al., 2002), steric occlusion (Labrijn et al., 2003), and non-neutralizing immunodominant decoys including non-functional envelope monomer, stumps and uncleaved precursors (Moore et al., 2006; Poignard et al., 2003) as means to counter the generation of functional antibodies.

The primary antibody response in infected individuals first comprises of largely non-neutralizing antibodies (Tomaras et al., 2008) followed by neutralizing antibodies specific against the autologous infecting virus (Deeks et al., 2006; Gray et al., 2007; Moog et al., 1997; Richman et al., 2003; Wei et al., 2003). However, following 2-4 years of infection, about 20% of the infected individuals elicit high levels of antibodies that can neutralize multiple strains of HIV-1 (Binley et al., 2008; Dhillon et al., 2007; Gray et al., 2007; 2011; Hraber et al., 2014; Y. Li et al., 2009), and about 1% of the infected patients can neutralize hundreds of viral quasi-species from multiple HIV-1 clades (Simek et al., 2009). These broadly neutralizing antibodies (bnAbs) target a few select conserved sites of vulnerability on the virus, either by blocking receptor/coreceptor binding or by

preventing the envelope protein from undergoing conformational changes critical for virus fusion to the host cells.

The ability of these antibodies to confer protection following passive immunization (Balazs et al., 2012; Ferrantelli et al., 2003; Hessell et al., 2009; Mascola et al., 2000; Shibata et al., 1999) has triggered the search for a vaccine capable of inducing similar antibodies in animal models. These vaccine efforts typically focus on two major epitopes targeted by some of the broadest bnAbs isolated so far. Several gp120-based vaccine strategies have been evaluated to target the CD4 receptor-binding site (reviewed in (Georgiev et al., 2013)). These efforts have been complicated due to the highly conformational nature of this epitope along with the presence of immunodominant epitopes and heavy glycan shield on the gp120 subunit (Pantophlet and Burton, 2006; Sodroski et al., 1998; Wei et al., 2003). Another epitope of interest has been the membrane proximal external region (MPER), which represents a stretch of ~22 amino acid long, highly conserved domain in the gp41 subunit. This region is targeted by multiple patient-isolated bnAbs including 2F5, Z13e1, 4E10 and 10E8 (Huang et al., 2012; Kwong et al., 2013; Purtscher et al., 1994; Stiegler et al., 2001; van Gils and Sanders, 2013; Zwick et al., 2001). The last two bnAbs demonstrate neutralization against about 98% of the HIV-1 isolates (Huang et al., 2012).

The elicitation of similar MPER targeting bnAbs through vaccination is one of the major goals in the field of HIV-1 vaccine development. However, identifying the optimal MPER conformation that can bind germline B-cells and trigger bnAbs generation has been difficult due to multiple reasons. First, the MPER resides in the highly dynamic gp41 subunit that undergoes multiple structural changes to mediate virus fusion to the

host cell (Melikyan, 2008). It is likely that MPER accessibility varies to a great extent during the pre-fusion, the fusion intermediate and the post-fusion states of gp41. Furthermore, the MPER conformation might also be influenced by the availability and conformation of neighboring domains. These issues might be responsible for the failure of small MPER peptide-based vaccines (Decroix et al., 2001; Joyce, 2002; Liao et al., 2000; Matoba et al., 2006; McGaughey et al., 2003; Ni et al., 2004). While multiple structural studies defining MPER structure bound to bnAbs reveal critical information about the binding face that needs to be targeted (Cardoso et al., 2007; Julien et al., 2008; Ofek et al., 2004), these conformations might not represent the native structure which interacts with the germline or precursor antibodies involved in the maturation process. This is evident by the failure of scaffold-based MPER epitope presentation to generate bnAbs (Correia et al., 2010; Guenaga et al., 2011; Ofek et al., 2010a). Chimeric virus based MPER approaches have typically induced poor anti-MPER antibody responses, probably due to presence of other immunodominant epitopes outside the MPER domain (Arnold et al., 2009; Eckhart et al., 1996; Kusov et al., 2007; Luo et al., 2006; Marusic et al., 2001; Ye et al., 2011; Zhang et al., 2004). Other approaches involving presentation of MPER in hybrid/fusion proteins (Coëffier et al., 2000; Hinz et al., 2009; Krebs et al., 2014; Law et al., 2007; Liang et al., 1999; Mantis et al., 2001; Strasz et al., 2014), virus like particles (Benen et al., 2014; Bomsel et al., 2011; Jain et al., 2010; Kamdem Toukam et al., 2012; Kim et al., 2014) and MPER liposomal delivery (Dennison et al., 2011; Hanson et al., 2015; Hulsik et al., 2013; Lai et al., 2014; Matyas et al., 2009; Mohan et al., 2014; Serrano et al., 2014; Venditto et al., 2013; 2014) have also failed, likely from the inability to present the MPER in the presence of other poorly defined structural

elements necessary to mimic the native conformation. Finally, an additional hurdle in generation of anti-HIV-1 bnAbs is that the germline B-cell has to undergo extensive somatic hypermutation, typically in an environment that has high antigenic heterogeneity. The necessity to replicate this extended period of antibody maturation in a vaccination setting needs to be determined. Regardless, a handful of animal studies reported the elicitation of weak to modest neutralizing antibodies using MPER-based vaccines (Hulsik et al., 2013; Krebs et al., 2014; Lai et al., 2014; Ye et al., 2011; Yi et al., 2013). Hence, there is a need to improve the neutralizing breadth and potency of vaccine induced antibodies as well as to characterize MPER immunogenicity in context of different gp41 structures.

In this study we have tested and compared two novel prime boost strategies for inducing MPER-targeting neutralizing antibodies. It was hypothesized that while smaller priming antigens might be better to focus the antibody response towards MPER, the use of progressively larger antigens for boosting might allow antibodies to mature in a manner so that they can recognized MPER on the surface of the native virion. Two different antigens, one soluble (group 1) and one membrane bound (group 2), were used for priming. All animals were then subjected to subsequent immunizations with different combinations of protein, DNA and recombinant vaccinia virus boost. The detailed antibody response was characterized at the polyclonal level following each immunization. One of the animals was further used for hybridoma generation and characterization of the antibody response at the monoclonal level.

Results

Prime-boost immunization using a membrane-bound priming antigen

We had previously demonstrated that gp41-54TM proteoliposomes induced antibodies against the peptide containing the 4E10 epitope (Chapter 3). In this study, three rabbits were primed using similar gp41-54TM proteoliposomes (Fig. 1A). The rabbits were then boosted four weeks later using a combination of proteoliposomes and a 54CT DNA vaccine that codes for a highly similar but larger protein containing the same HR2 and MPER domains, but also containing the additional cytoplasmic tail (CT) as in the native virus gp41 protein. For the third boost, rabbits were immunized using a combination of 54CT DNA and recombinant vaccinia virus expressing gp160 (rVV-gp160). A final fourth boost was performed using gp41-54TM and rVV-gp160. Serum was collected two weeks after each immunization, and antibody titers against gp41-54TM were analyzed using ELISA (Fig. 1B).

All three rabbits showed slightly different antibody response following each immunization. Antibody titer for rabbit R1 (in green) reached between 10^3 and 10^4 after the first immunization and was continuously boosted following subsequent immunizations, reaching more than 10^6 after the fourth immmunization. For rabbit R2 (in yellow), the response after the first immunization was the strongest among all these rabbits, and titers reached as high as 10^5 after the second immunization. However, titers dropped following the third immunization and remained at slightly above 10^4 even after the fourth immunization. Antibody titers for rabbit R3 (in blue) reached between 10^5 and 10^6 after the second immunization and did not show further increase after the third and fourth immunizations.

To map the linear epitopes targeted across the gp41 ectodomain, sera after each immunization was also analyzed using overlapping, 10-mer biotinylated peptides that spanned HR2 and MPER domains. As shown in Fig. 2, following priming with gp41-54TM, antibodies were raised against multiple peptides in the HR2 domain, but little response was detected against peptides spanning the MPER domain. For all three rabbits, while there was slight difference in the exact peptides targeted, antibodies were raised against the N-terminus (peptides 629, 632, 635, 638, 641) and C-terminus (peptides 653, 656, 659) ends of HR2. Rabbit R2 (in yellow) elicited some antibodies against the ^{659}ELLALDKWAS^{668} peptide containing the 2F5 epitope.

Following the first boost with gp41-54TM proteoliposomes and 54CT DNA, the response against the HR2 domain was further enhanced for rabbit R3 (in blue) and included additional peptides in the HR2 central region (peptides 641, 644, 647, 650, 653). Rabbit R3 also showed weak response against all MPER spanning peptides (peptides 662, 665, 668, 671, 674). Antibody response to different linear epitopes remained unchanged for rabbit R2. Interestingly, little to no linear epitopes were bound by sera from rabbit R1. Since this rabbit still showed increase in overall antibody response against gp41-54TM, it is possible that antibodies were elicited against conformational epitopes that could not be detected using overlapping peptide ELISA. After the third immunization with 54CT DNA and rVV-gp160, there was an overall decrease in linear epitopes targeted across HR2 and MPER domains. Once again, it is possible that the antibodies elicited by the rabbits at this point bound conformational epitopes or epitopes on gp160 that are outside the HR2 and MPER. Alternately, epitopes presented by gp41-54TM might not be accessible on rVV-gp160. Following the fourth immunization using

gp41-54TM and rVV-gp160, the antibody binding was enhanced considerably towards cluster II region peptides 653 and 659 for rabbit R3. Rabbit R1 also showed increased binding for peptide 653, while there was no significant binding increase for any peptides in case of rabbit R2. Overall, these results demonstrate that this prime boost strategy (group 1) was unable to induce antibodies against the linear peptides that span the MPER domain. Additionally, all rabbits failed to demonstrate serum neutralizing activity (data not shown).

Prime-boost immunization using a soluble priming antigen.

Next, three new rabbits were immunized and bled similar to the protocol used for the gp41-54TM based prime-boost strategy described above but with slight changes (Fig. 3). Most importantly, the gp41-54TM antigen was replaced with a soluble antigen, named MPER28x3, which consists of three tandem repeats of MPER28 (representing 6 residues from the HR2 domain and the 22 MPER residues). As shown in Fig. 3, following the first immunization with MPER28x3, antibody titers reached 10^3 or slightly above for all three rabbits. Titers were increased by about 100-fold for all rabbits after the second immunization with MPER28x3 and 54CT DNA. The subsequent boost with 54CT DNA and rVV-gp160 did not enhance the antibody response any further for rabbits R1 (in green) and R2 (in yellow), while rabbit R3 (in blue) showed slight increase. Overall titers remained between 10^5 and 10^6 for all three rabbits. Following the fourth boost, antibody titers reached almost 10^6 for rabbits R1 and R3 while titers for rabbit R2 remained the same.

Linear epitope mapping using overlapping, 10-mer biotinylated peptides were then performed with sera following each immunization (Fig. 4). Strangely, little to no response was observed against any linear peptides after the first immunization. However, strong antibody response was detected against the cluster II immunodominant region (peptides 650, 653, 656) following the second immunization with MPER28x3 and 54CT DNA. Little response was also seen against the MPER peptide ^{668}SLWNWFDITN^{677} for rabbit R3. After the third immunization with 54CT DNA and rVV-gp160, while the cluster II region still remained immunodominant, some antibody response could be detected against other linear epitopes. Rabbit R2 showed good binding to peptide ^{662}ALDKWASLWN^{671} that contains the 2F5 epitope. In addition to peptide ^{668}SLWNWFDITN^{677}, rabbit R3 also bound peptide ^{671}NWFDITNWLW^{680} that contains the 4E10 epitope. Rabbit R1 and R2 also recognized additional HR2 domain peptides upstream of the cluster II region. THeourth immunization with MPER28x3 and rVV-gp160 further enhanced anti-MPER antibodies in rabbits R2 and R3. Binding was seen against peptides ^{668}SLWNWFDITN^{677} and ^{671}NWFDITNWLW^{680} in rabbit R3 and peptides ^{671}NWFDITNWLW^{680} and ^{674}DITNWLWYIK^{683} in rabbit R2.

Overall, despite the strong response against the cluster II immunodominant region, this prime boost strategy (group 2) was successful in inducing antibodies against the linear peptides covering the C-terminus end of MPER that contains the 4E10 and 10E8 epitopes. Unfortunately, sera from these rabbits did not exhibit neutralizing activity (data not shown). It is possible that MPER-binding antibodies failed to neutralize due to their inability to access the MPER on the native virus. Alternately, it is also possible that the quantity of neutralizing antibodies was too low in the serum to be detected by our

neutralization assay. To explore these possibilities at a monoclonal level, rabbits R2 and R3 were further used for hybridoma generation.

Generation of hybridoma and epitope mapping using ELISA

Rabbits R2 and R3 from group 2 were injected intravenously with 200 μg of MPER28x3 antigen in PBS without any adjuvant at week 35 (Fig. 3A), and sacrificed four days later to harvest the spleen. Spleen from rabbit R2 was frozen for future use, while spleen from rabbit R3 was used for hybridoma generation. Fusion was performed for fourteen 96-well plates, and viable hybridomas post-HAT selection (about over 70% of the total) were screened against the MPER28x3 immunogen. Since the vast majority of the hybridoma supernatants showed detectable signal (over 95%), they were further tested for binding to 15-mer peptides containing epitopes for 2F5 (^{657}EQELLALDKWASLWN^{671}) and 4E10/10E8 (^{669}LWNWFDITNWLWYIK^{683}). 15-mer peptides were chosen preferentially over 10-mer peptides because they could contain longer epitopes, especially the entire 10E8 epitope. Based on strong binding to either of these peptides, three hybridomas (6C10, 9F6 and 21B5) were selected for further characterization.

To map the binding epitope for all three hybridomas, peptide ELISAs were performed using overlapping 15-mer peptides. As shown in Fig. 5A, the 6C10 hybridoma showed strong binding to peptides ^{649}SQNQQEKN<u>EQELLAL</u>^{663}, ^{653}QEKN<u>EQELLAL</u>DKWA^{667} and ^{657}<u>EQELLAL</u>DKWASLWN^{671} suggesting that the common residues between these peptides (underlined) was the core binding epitope. In agreement with this, peptides ^{645}LIEESQNQQEKN<u>EQE</u>^{659} (upstream) and

^{661}LALDKWASLWNWFDI^{675} (downstream) that lacked the complete ^{657}EQELLAL^{663} epitope could not be bound by 6C10. Hence, while 6C10 was identified after screening with ^{657}EQELLALDKWASLWN^{671} peptide, these results indicated that it binds slightly upstream of and partially overlaps with 2F5 epitope (^{662}ALDKWAS^{668}) also present in the same peptide. However, 6C10 does not bind any of the critical residues (^{664}DKW^{666}) reported for 2F5 binding (Ofek et al., 2004). Thus, 6C10 binds the junction between MPER and cluster II region. Interestingly, we have previously isolated another anti-cluster II, non-neutralizing monoclonal antibody from rabbits immunized with gp41-54Q. This antibody, named 2C2, recognizes the ^{655}KNEQELLALDK^{665} epitope with the critical residues underlined (unpublished data). Since 6C10 does not require the 665K residue for binding, its binding mechanism to this region might be different from 2C2.

Next, binding analyses were performed for both 9F6 and 21B5 hybridomas that were originally screened using the ^{669}LWNWFDITNWLWYIK^{683} peptide containing the complete epitope for both 4E10 and 10E8 bnAbs. Interestingly, both 9F6 and 21B5 failed to bind overlapping peptides upstream (^{665}KWASLWNWFDITNWL^{679}) and downstream (^{673}FDITNWLWYIKFIM^{686}) of the peptide used for the original screening experiment. This suggested that the binding epitope of these hybridomas is completely contained within the ^{669}LWNWFDITNWLWYIK^{683} peptide. Interestingly, binding analysis with a mixture of the N-terminus and C-terminus biotinylated 10-mer peptide ^{671}NWFDITNWLW^{680} revealed a major difference between 9F6 and 21B5 binding. 9F6 showed strong recognition of this 10-mer peptide, thus indicating that the binding epitope was completely contained in the peptide and overlapped completely with the 4E10 epitope sequence. In contrast, 21B5 failed to bind the 10-mer peptide. These results

suggested that 21B5 requires the additional residues ^{681}YIK^{683} for binding, similar to that of 10E8 binding (Huang et al., 2012).

Detailed epitope mapping of 9F6 hybridoma

Both 4E10 and 10E8 bind the neutralizing face of MPER. To further define the MPER peptide face bound by 9F6 and 21B5, alanine scanning analysis was performed using the C-terminus biotinylated, 13-mer peptide (^{671}NWFDITNWLWYIK^{683}) as described in our previous study (Habte et al., 2015). Interestingly, while 21B5 could bind the unbiotinylated 13-mer 671 peptide, it failed to bind the C-terminus biotinylated form of this peptide (data not shown). This difference might be due to two reasons. First, since the terminal 683K was biotinylated, it might affect binding directly, suggesting that this residue is critical. Second, even if the 683K residue is not critical for 21B5 binding, the 13-mer peptide would be bound to streptavidin plates using terminal K683, thereby limiting the accessibility of other binding residues (within ^{680}YIK^{683}) at the C-terminus end. In our experience, the use of biotinylated peptides offers higher sensitivity than unbiotinylated peptides in alanine scanning assays. Hence, alanine scanning analysis for 21B5 must be performed using longer biotinylated peptides or N-terminus biotinylated peptides.

Unlike 21B5, 9F6 showed good binding to the 13-mer 671 peptide biotinylated at the C-terminus end (Fig. 6A) and hence was used for further binding analysis using alanine mutant peptides (Fig, 6B). Previously, our 4E10 binding analyses (Habte et al., 2015) revealed critical binding including residues W672, F673, I675, T676 and L679, similar to that reported by others (Brunel et al., 2006). The effect of N671A and D674A

has been suggested to be primarily due to disruption of peptide structure (Brunel et al., 2006). In comparison, 9F6 hybridoma binding was severely affected by W672A, F673A, N677A and to a lesser extent by T676A and W680A. Thus, 9F6 binding to the ^{671}NWFDITNWLW^{680} epitope involves three residues (W672, F673, and T676) that are also critical for 4E10 binding. Alanine mutations at these residues decrease 4E10 binding by over a 1000-fold (Brunel et al., 2006). Interestingly, unlike 4E10, alanine mutations at I675 and L679 did not affect 9F6 binding at all. Also, 9F6 binding involved other residues including N677 and W680 that are not necessary for 4E10 binding. However, the W680 residue has been shown to be important for neutralization by 4E10 (Brunel et al., 2006; Zwick et al., 2005) and hence considered part of the 4E10 epitope. Thus, as revealed by our assay, the binding epitopes for 9F6 (^{671}N<u>WF</u>DI<u>TN</u>WL<u>W</u>^{680}) and 4E10 (^{672}<u>WF</u>D<u>IT</u>NW<u>LW</u>^{680}) contain remarkable similarities. To further visualize and compare the binding of 9F6 and 4E10, the critical binding residues were mapped onto the MPER peptide that was co-crystallized with 4E10 (Cardoso et al., 2005). As shown in Fig. 7A and 7B, 9F6 shared a significant overlap with the 4E10 binding face. However, 9F6 binding was slightly offset to include residue N677, whereas 4E10 binding involved residues I675 and L679.

Discussion

The antigenic environment in HIV-1 infected patients is quite complex. Besides the variability in envelope sequence and glycosylation patterns, the virus also presents non-functional spikes, and critical neutralizing epitopes on functional spikes are only exposed transiently following receptor and co-receptor binding. It is hard to identify

which component(s) of this antigenic cocktail are important for the development of bnAbs. In comparison, vaccines traditionally contain a single, well-defined immunogen. For HIV-1 vaccines, while small subunit immunogens induce strong antibody response, they might not be able to mimic the correct conformation in the absence of other domains. Furthermore, antibodies elicited towards such subunit immunogens might not be able to access the same epitopes on the whole virus where epitope accessibility might be obscured due to other structural components. Alternately, larger, conformationally correct envelope spike-based vaccines like virus like particles might provide too many epitopes, which might be immunodominant and distract antibodies from being elicited towards subdominant neutralizing epitopes. Hence in this study, a more complex vaccination approach combining both smaller and larger antigens was employed. Rabbits were primed with smaller antigens to first focus the antibody response against the MPER. Two different priming immunogens were used: one membrane bound and one soluble. Rabbits were then immunized with sequentially larger immunogens in subsequent immunizations, but in combination with the smaller immunogen used for the previous immunization.

For the first group using the membrane bound gp41-54TM as the priming antigen, the prime-boost strategy failed to elicit antibodies against the MPER domain. As shown in our previous study (Chapter 3), gp41-54TM alone elicited antibodies against the peptide containing the 4E10 epitope, albeit after three immunizations. It is possible that the combination of this antigen with other boosting antigens diverted the antibody response away from the MPER domain. In comparison, two of three rabbits primed with the soluble MPER28x3 generated MPER-targeting antibodies after the prime-boost

strategy. However, the bulk of the immune response was still directed primarily against the cluster II non-neutralizing epitope, which might be a drawback of this immunization strategy. Furthermore, anti-MPER antibodies were truly boosted only after the fourth immunization. Immunization with MPER28x3 alone can also induce similar antibodies against the MPER as well as the cluster II region (unpublished data). However, the quality of MPER targeting antibodies might differ between the two experiments in terms of exact binding epitopes and affinities for both the vaccine immunogen and the native virion. Regardless, immunization with MPER28x3 alone (unpublished data) or with the prime-boost strategy tested in this study failed to exhibit any detectable neutralizing activity in the sera.

To understand why gp41-based antigens fail to elicit bnAbs, we previously evaluated sera binding to the MPER using alanine-scanning analysis of the 4E10 and 10E8 peptide. While this approach has provided valuable insights into the overall humoral response against MPER, individual antibody binding patterns to certain residues can remain hidden because of the presence of complementary binding patterns exhibited by other abundant antibodies. To overcome this issue, we analyzed the antibody response at the monoclonal level by generating hybridomas from rabbit R3 from the MPER28x3 group (group 2). Following fusion, hybridomas were screened using specific peptides of interest rather than the entire immunogen. This approach allowed us to directly identify hybridomas specific to peptides containing the 2F5 and 4E10/10E8 epitopes.

First, using ELISA-based binding analysis, the core binding epitope for the 6C10 hybridoma was defined as ^{657}EQELLAL^{663}. This epitope lies slightly upstream and

partially overlaps with the 2F5 binding epitope (^{662}AL<u>DKW</u>AS^{668}). Interestingly, our previously isolated, non-neutralizing 2C2 rabbit monoclonal antibody also overlaps this epitope (^{655}<u>K</u>N<u>EQ</u>ELLALDK^{665}). However, neither 6C10 nor 2C2 recognize the critical residues for 2F5 binding. One study described the isolation of a modestly neutralizing rabbit monoclonal antibody, named WR320, that binds a slightly larger epitope (^{661}LELDKWASL^{669}) overlapping the cluster II and MPER regions (Matyas et al., 2009). In another report, a variable domain of a single heavy chain (VHH), named 2H10, was isolated from llamas and its binding mapped to ^{657}<u>E</u>QEL<u>LE</u>LD<u>K</u>^{665} (critical residues underlined) (Hulsik et al., 2013). 2H10 further demonstrated modest cross clade neutralization in its bivalent form. Both of these studies reported neutralizing activity only after isolation of monoclonal antibodies following hybridoma generation, suggesting that the level of such antibodies in whole serum was probably too low. Future experiments performed with the cloned 6C10 antibody will be necessary to map the fine binding specificities and evaluate its neutralizing ability.

Next, two other hybridomas, named 9F6 and 21B5, were isolated using the 15-mer peptide containing both the 4E10 and 10E8 epitopes. Binding analysis revealed that the 21B5 hybridoma binding epitope included the complete ^{671}NWFDITNWLWYIK^{683} epitope, and the presence of ^{681}YIK^{683} is critical for binding, similar to the broad and highly potent human monoclonal antibody 10E8. Failure to bind the C-terminus biotinylated 13-mer peptide further confirmed the importance of this region to 21B5 binding. Unfortunately, this also prevented the fine mapping of the residues using our current alanine scanning peptides. Performing future binding studies using longer peptides or N-terminus biotinylated peptides might circumvent this. In comparison, the

core binding epitope for the 9F6 hybridoma was defined as ^{671}NWFDITNWLW^{680}, which also contains the complete 4E10 epitope sequence. In comparison, only one other study has reported the isolation of a non-neutralizing antibody WR316 (^{668}SLWNWF^{673}) that partially overlaps the 4E10 epitope (Matyas et al., 2009). Fine mapping of the 9F6 binding epitope revealed a substantial overlap with the 4E10 binding face, involving residues W672, F673, T676, and W680. However, 9F6 binding is slightly offset due to the inclusion of N677 residue instead of I675 and L679 that are involved in 4E10 binding. Whether or not this difference is enough to prevent virus neutralization needs to be further evaluated using the cloned 9F6 antibody. Since 4E10 exhibits some cross-reactivity (Alam et al., 2008; Haynes et al., 2005), lipid-binding tests should also be performed to determine whether 9F6 demonstrates similar properties.

Finally, it is important to acknowledge that while these hybridomas show good overlap with binding epitopes for well-characterized bnAbs, they might not neutralize the virus due to steric occlusion by the bulky gp120 subunit that sits atop the gp41 subunit. To overcome this challenge, anti-MPER bnAbs have evolved with special features like long heavy chain CDR3s along with hydrophobic patches at the tip necessary for lipid interactions (Huang et al., 2012; Ofek et al., 2010b; Sun et al., 2008). These antibodies undergo extensive somatic hypermutation to develop such special characteristics. Whether or not our hybridomas contain antibodies with similar characteristics and neutralizing ability requires to be tested in future. Regardless, to our knowledge, the results discussed in this study are the closest reports about eliciting vaccine-induced antibodies that share similar binding characteristics as two of the broadest, patient-isolated, neutralizing antibodies: 4E10 and 10E8. Future structural and functional

characterization of these hybridomas will provide valuable insights for HIV-1 vaccine development.

Materials and Methods:

Rabbit immunization

Six New Zealand white female rabbits (2.5 to 3 kg) were purchased from Charles River (USA), housed under specific pathogen free, BSL-1 (pre-vaccinia immunization) and BSL-3 (post-vaccinia immunization) environments. All animals were tested in compliance with the animal protocol approved by IACUC of Iowa State University. The rabbits were divided into two groups of three rabbits each. Rabbits were in both groups were immunized on weeks 0, 4, 11 and 29.

For group 1 (Fig. 1A), rabbits were primed by subcutaneously injecting 200 μg of gp41-54TM on DPPC liposome with MPLA adjuvant as described (Chapter 3). For the second immunization, rabbits were injected subcutaneously with 50 μg of gp41-54TM/liposome. Rabbits were also injected intradermally with 200 μg of gp41-54CT DNA, followed by electroporation using the AgilePulse In Vivo System (BTX, Harvard Apparatus). The gp41-54CT DNA was derived from pcDNA-MCON6gp160 (kindly provided by Dr. Beatrice Hahn (Gao et al., 2005)) and designed to code for the HR2, MPER, TM and CT domains (details to be described elsewhere). For the third immunization, rabbits were injected with 200 μg of gp41-54CT DNA as described above and with rVV-gp160 ($1x10^8$ PFUs) through intradermal injection. The recombinant vaccinia virus expressing gp160 from the DH12 HIV-1 isolate was propagated and purified as described previously (Cho et al., 2001; 1998). For the fourth immunization,

both 50 µg of gp41-54TM and rVV-gp160 were administered as described above. Blood was collected pre-immunization and two weeks post-immunizations. Processed sera were stored at -80 °C.

For group 2 (Fig. 3A), rabbits were primed by subcutaneously injecting 200 µg of MPER28x3 with the zinc-chitosan adjuvant as previously described for other antigens (Habte et al., 2015; Qin et al., 2014). The MPER28x3 construct coded for three tandem repeats of the 28 amino acids from the gp41 ectodomain (details to be described elsewhere). Each 28 amino acid long repeat consists of 6 residues from the HR2 domain and 22 MPER residues. The protein was expressed in *E.coli*, refolded and purified as previously described (Habte et al., 2015). All subsequent immunizations were performed as that of group 1 rabbits except that the second gp41-54TM immunization was replaced with MPER28x3. Blood was collected, processed and stored as above.

Hybridoma generation

Rabbits R2 and R3 from group 2 were injected intravenously using 200 µg of soluble MPER28x3 in 1x PBS without any adjuvant on week 35. Four days later, the spleen was harvested. Spleen for Rabbit R3 was used for fusion as described previously with minor modifications (Qin et al., 2015; Spieker-Polet et al., 1995). Briefly, rabbit splenocytes were fused with fusion partner cell line 240E-1 (kindly provided by Dr. Katherine L. Knight (Spieker-polet 1995)) at a ratio of 2:1 with 50% PEG 1500 (Sigma-aldrich P7181) and selected by growing in HAT (hypoxanthine, aminopterin and thymidine) media (Sigma-aldrich H0262). Supernatants collected from the hybridomas

were then tested initially for specific binding to MPER28x3, and subsequently screened by testing binding against MPER peptides of interest as described below.

Enzyme-linked immunosorbent assay (ELISA)

All ELISAs were performed using the standard protocol described earlier (Habte 2015), except for the use of an alternate blocking buffer containing 2.5% milk and 5% calf sera in 1X PBS (pH 7.5). For ELISAs testing sera antibody titers, the coating antigens (30 ng/well) used for group 1 and group 2 animals were gp41-54TM and MPER28x3. The end-point ELISA titers were defined as serum dilution factor that gave readings of average + 2x SD of the background as described previously (Qin et al., 2014). Linear epitope mapping for sera was performed using the mixture of N- and C-terminus biotinylated 10-mer peptides (Habte et al., 2015).

For screening hybridomas, HIV-1 consensus group M Env (15-mer) peptides from the NIH AIDS Reagent Program (Cat# 9487) were used as binding antigens. Peptide 9136 (^{657}EQELLALDKWASLWN^{671}) containing the 2F5 epitope and peptide 9139 (^{669}LWNWFDITNWLWYIK^{683}) were coated at 100 ng/well using standard ELISA protocol. 100 µl of the hybridoma supernatant was directly added to each well and incubated for 2 hrs prior to continuing. Similar binding analysis was performed for hybridoma epitope mapping using upstream and downstream peptides (Fig. 5) from the same peptide set. Alanine scanning analysis of the 9F6 binding epitope was also performed using the 13-mer 671 peptide (^{671}NWFDITNWLWYIK^{683}) as previously described (Habte et al., 2015), but the hybridoma supernatant was diluted 1:2 with blocking buffer to prevent signal saturation. For all ELISAs testing hybridoma binding,

goat anti-rabbit, horseradish peroxidase (HRP)-conjugated antibody (Thermo Scientific; Cat# 31430) was used as secondary antibody.

Neutralization assays

Neutralization assays were performed in TZM-bl cells as previously described (M. Li et al., 2005; Qin et al., 2014; Wei et al., 2002). Viruses tested included SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B). Murine leukemia virus Env-pseudotyped virus was used as a negative control.

Acknowledgments

We are grateful to Dr. Beatrice Hahn for providing pcDNA-MCON6gp160. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Consensus group M Env peptides (Cat# 9487) and 4E10 from Dr. Herman Katinger (Cat# 10091). This work was supported by a grant from the NIH, NIAID (P01 AI074286) grant. MWC has an equity interest in NeoVaxSyn Inc., and serves as the CEO/President. NeoVaxSyn Inc. did not contribute to this work or the interpretation of the data.

Figures

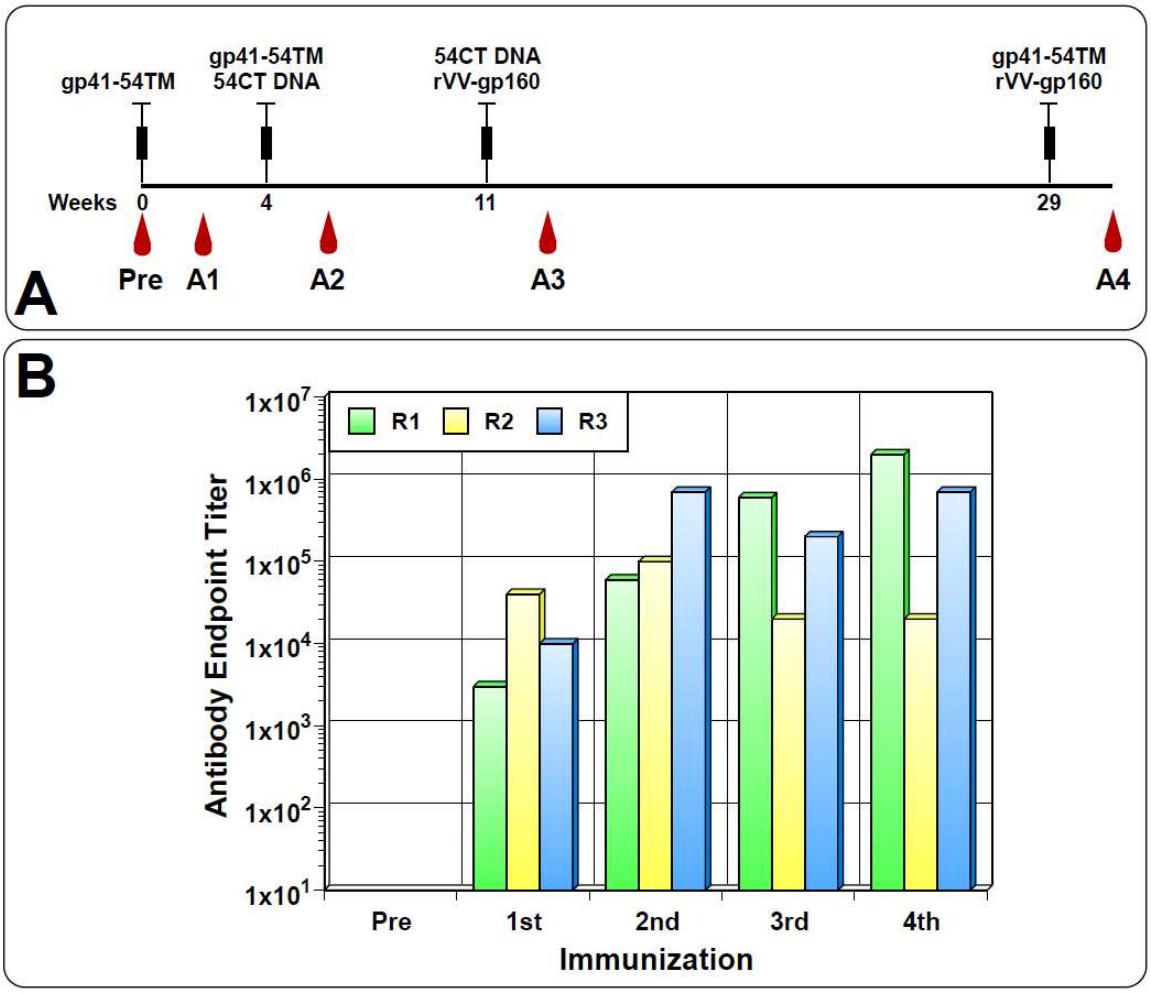

Fig 1: Prime-boost strategy using the membrane bound gp41-54TM primogen. (A) Immunization protocol for group 1 rabbits that were immunized on weeks 0, 4, 11 and 29 and bled pre-immunization and two weeks post every immunization. (B) Antibody endpoint titers were determined after each immunization by performing ELISA against gp41-54TM. Pre-immune sera were used as negative controls.

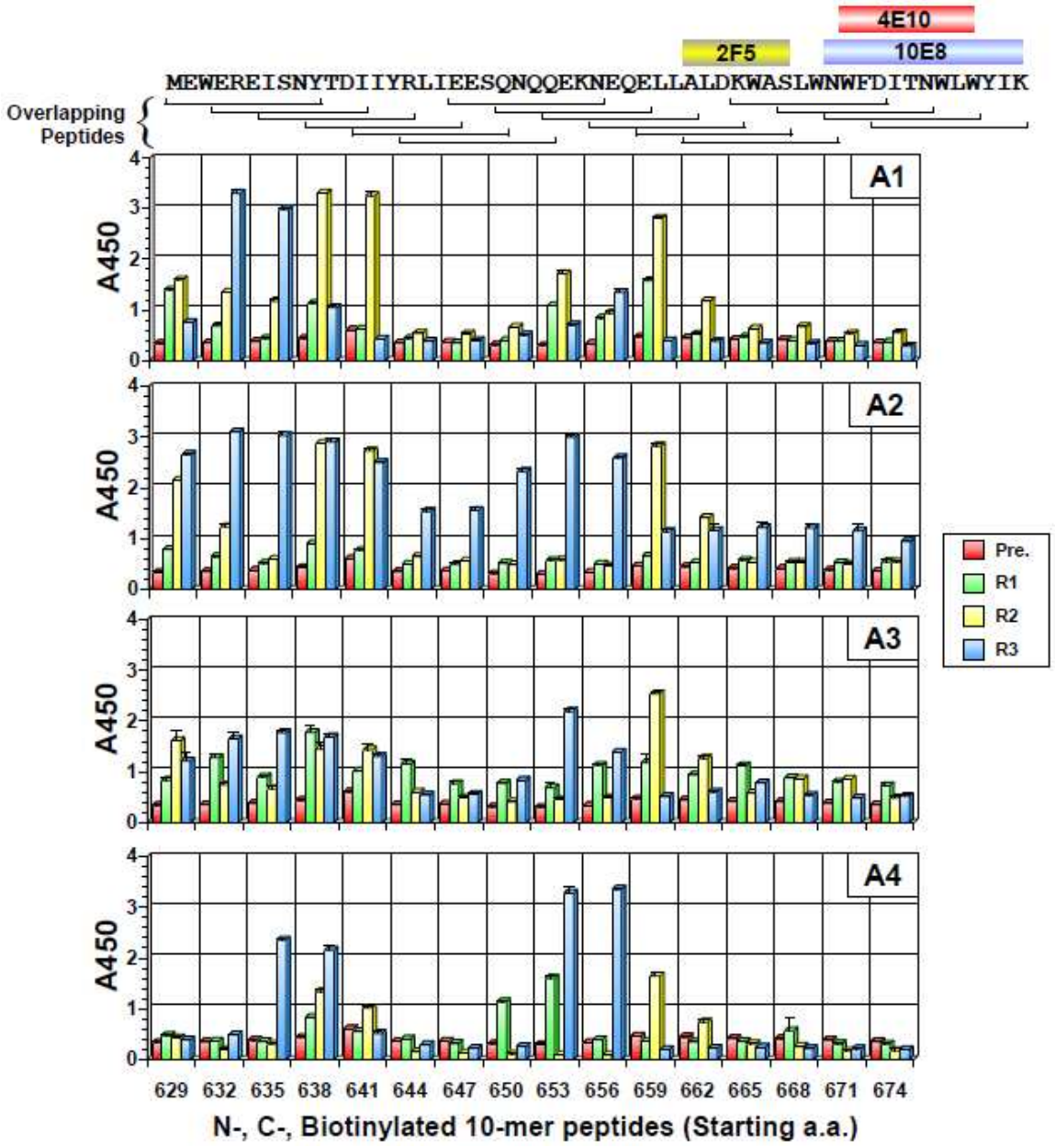

Fig 2: Linear epitope mapping for rabbits primed with gp41-54TM. Sera collected two weeks after first (A1), second (A2), third (A3) and fourth (A4) immunization was tested for binding to a mixture of N- and C-terminus biotinylated peptides spanning both HR2 and MPER domains of gp41. Pre-immune serum was used as negative control. Horizontal brackets on top indicate the sequence for each peptide and the core epitopes for bnAbs 2F5, 4E10 and 10E8 is shown.

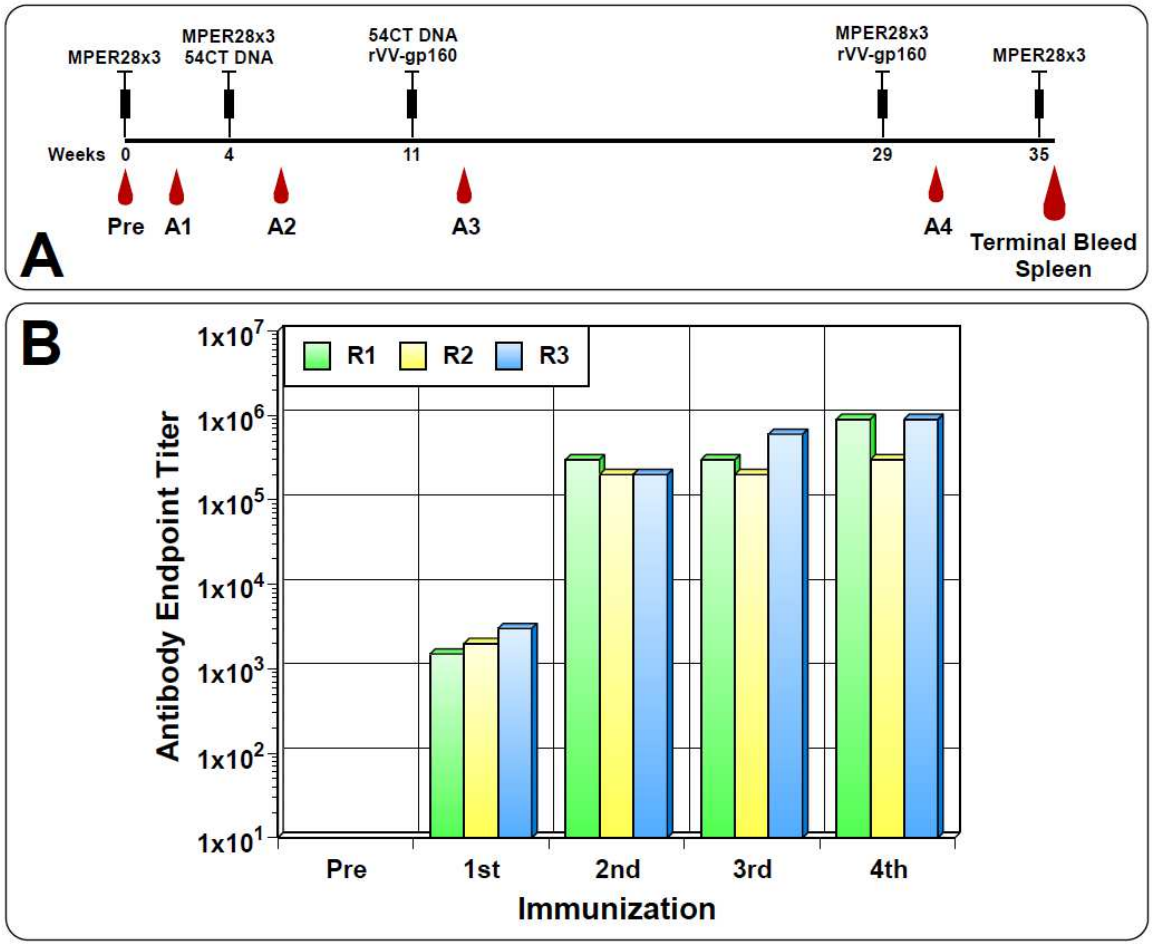

Fig 3: Prime-boost strategy using soluble MPER28x3 primogen. (A) Immunization protocol for group 2 rabbits that were immunized on weeks 0, 4, 11 and 29 and bled pre-immunization and two weeks post every immunization. Rabbit R2 and R3 were also boosted intravenously with MPER28x3 and sacrificed 4 days later to harvest the spleen for hybridoma generation. (B) Antibody endpoint titers were determined after each immunization by performing ELISA against MPER28x3. Pre-immune sera were used as negative controls.

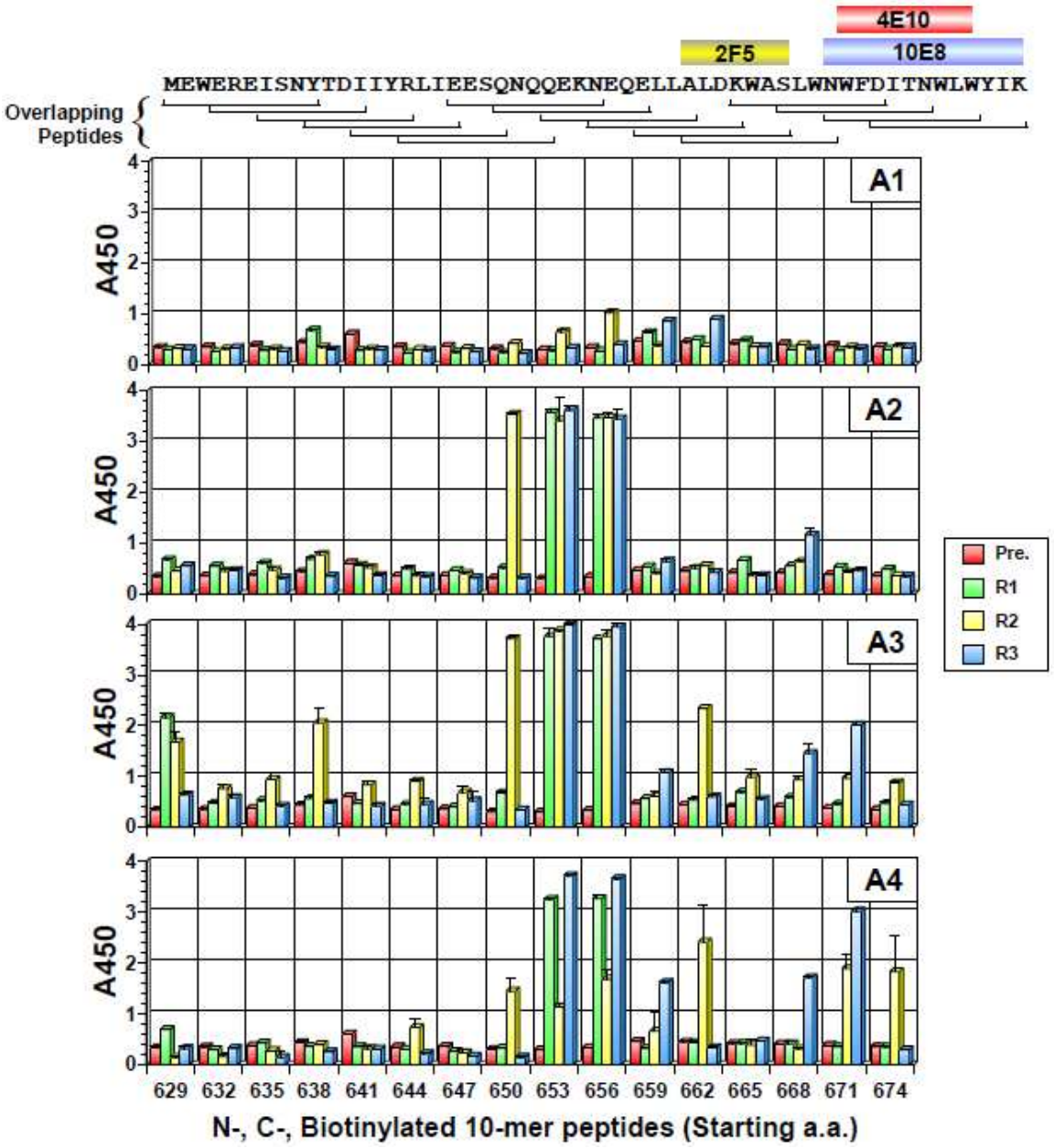

Fig 4: Linear epitope mapping for rabbits primed with MPER28x3. Sera collected two weeks after first (A1), second (A2), third (A3) and fourth (A4) immunization was tested for binding to a mixture of N- and C-terminus biotinylated peptides spanning both HR2 and MPER domains of gp41. Pre-immune serum was used as negative control. Horizontal brackets on top indicate the sequence for each peptide and core-binding epitopes for bnAbs 2F5, 4E10 and 10E8 is shown.

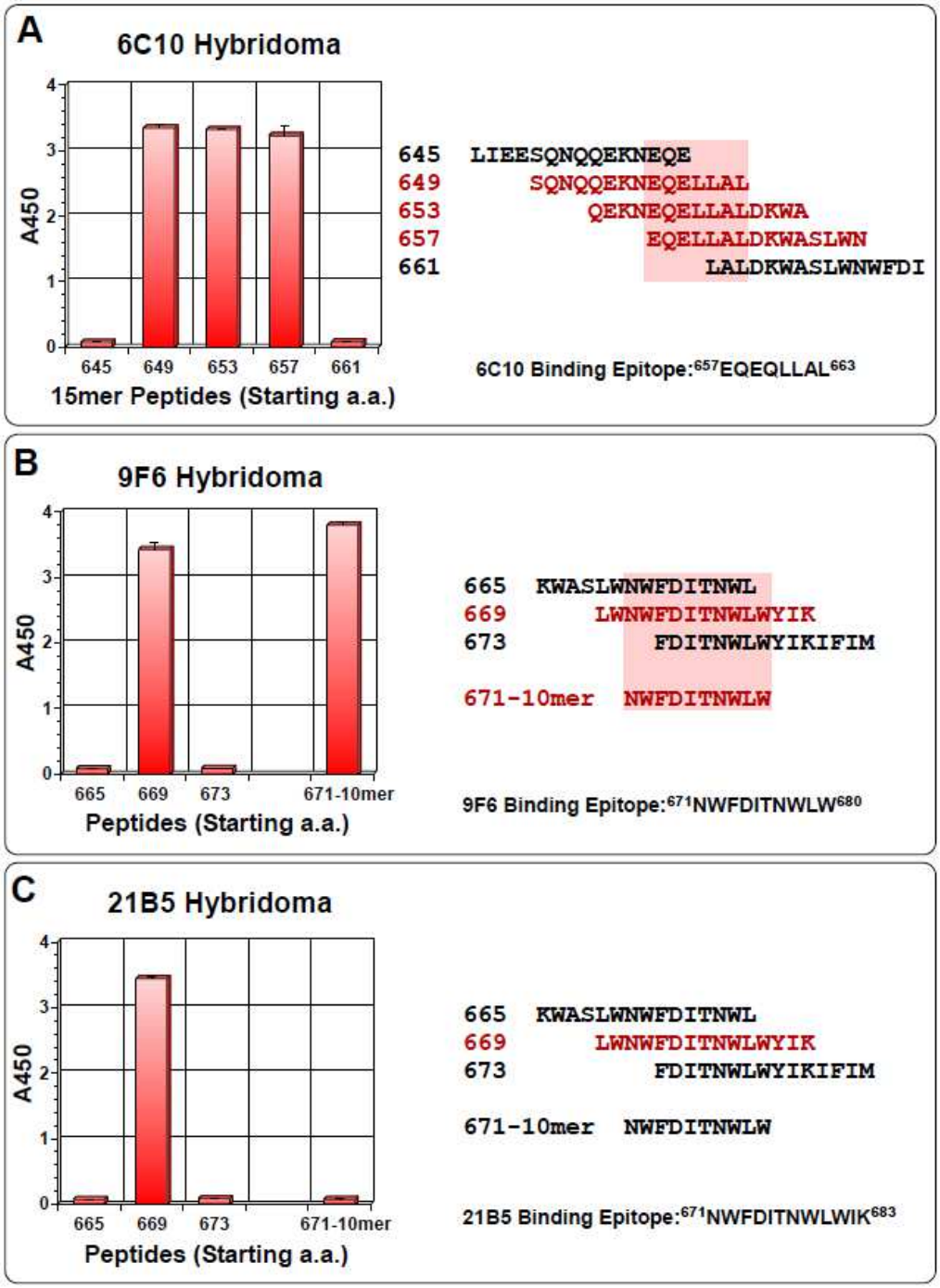

Fig 5: Hybridoma epitope mapping. Three different hybridomas, (A) 6C10, (B) 9F6 and (C) 21B5 were tested for binding with overlapping peptides to determine their binding epitope. Binding was also tested against shorter 10-mer peptide (mixture of both N- and C-terminus biotinylated peptides) for both 9F6 and 21B5 hybridomas. Peptide sequences for all peptides tested are aligned. Peptides showing positive binding signals are represented in red, and common epitopes between positive peptides are highlighted in red.

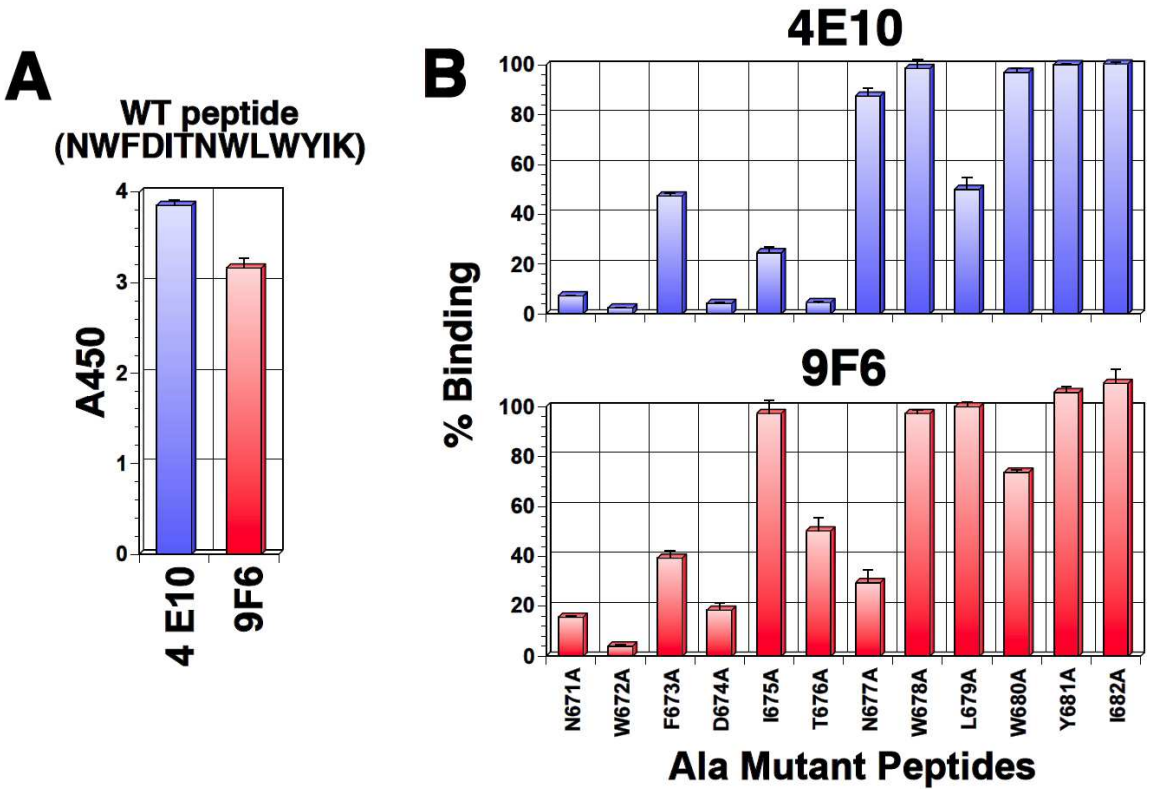

Fig 6: PepScan analysis of 9F6 epitope. Binding of the 9F6 hybridoma was tested against (A) wild type, C-terminus biotinylated 13-mer peptide (sequence indicated) and (B) mutant peptides containing alanine residues at different positions. The percentage binding relative to wild type peptide is plotted. As a positive control, 4E10 binding was also tested previously (Habte et al., 2015) and has been re-plotted here for reference.

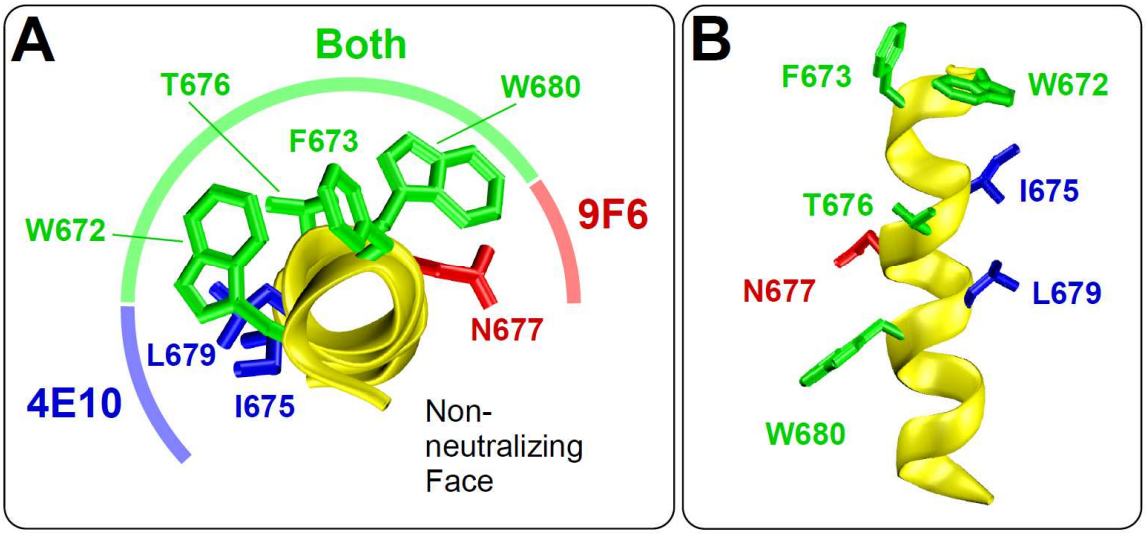

Fig 7: Structural comparison of critical residues targeted by 9F6 and 4E10. The critical residues identified from PepScan analysis for both 9F6 and 4E10 were plotted onto the co-crystal structure of a peptide bound to 4E10 (pdb: 2FX7)(Cardoso et al., 2007). (A) Top view showing the arrangement of different binding residues and the relative position of the non-neutralizing face of MPER. Critical binding residues shared by 9F6 and 4E10 are shown in green while unique binding residues for 9F6 and 4E10 are shown in red and green respectively. Significant overlap is observed in the binding of 9F6 and 4E10. (B) Lateral view of the peptide displaying critical binding residues for 9F6 and 4E10.

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CHAPTER 6

CONCLUSION AND FUTURE DIRECTION

Despite more than three decades of HIV-1 research, the goal of designing the perfect vaccine that can prevent against the large variety of circulating strains has yet to be achieved. However, the quest to meet this challenge has provided us with great appreciation for the complex mechanisms evolved by the virus to counteract the host immune system. While these past failures highlight how little is truly understood about the functioning of the immune system in response to a pathogen, they have also challenged us to develop newer methods that push the boundaries of immunology, virology, structural biology, molecular biology and vaccinology. From a vaccine perspective, the induction of bnAbs remains one of the major goals of an HIV-1 vaccine. This dissertation presents an evaluation of different vaccine approaches to target the highly conserved gp41 MPER domain. These approaches include (a) silencing of a non-neutralizing immunodominant epitope, (b) use of soluble, putative fusion intermediate forms of gp41, (c) use of a membrane-bound gp41 antigen, and (d) use of novel prime-boost immunization strategies. Several interesting conclusions were made as discussed in the different chapters.

The immunogenicity of a particular epitope can be influenced by the presence of other neighboring epitopes. In fact, HIV-1 uses immunodominant epitopes (e.g. V3 loop) that elicit non-neutralizing or strain-specific neutralizing antibodies as a decoy to prevent the host immune system from focusing on subdominant epitopes capable of eliciting bnAbs[1-3]. Interestingly, evaluation of MPER immunogenicity in context of a different antigen, gp41-54Q, revealed that the antibody response was directed predominantly

against the cluster II region in the HR2 domain (unpublished data). Additionally, the cluster II immunodominant region generally gives rise to non-neutralizing antibodies capable of competing with anti-MPER bnAbs like 2F5[4-6]. Hence, in Chapter 2, the cluster II epitope was masked on gp41-54Q, and the ability of this approach to redirect antibody response to other subdominant epitopes, especially the MPER, was evaluated. Results showed that the MPER domain is quite accessible despite masking of the cluster II epitope. However, while this modification was successful in preventing responses against the cluster II region, induced antibodies were redirected towards the N-terminus end of HR2 instead of the MPER domain. It is important to emphasize that epitope immunogenicity is context dependent. Hence, these results might be specific to gp41-54Q and similar antigens that contain only HR2 and MPER domains. Whether this strategy can generate MPER targeting antibodies in context of a larger gp41 antigen remains to be determined.

The MPER domain was found to be highly immunogenic in a different antigen, named gp41-HR1-54Q[7]. However, antibodies were directed against the non-neutralizing face of MPER and hence failed to neutralize the virus. Since the crystal structure of this antigen revealed that it was in a near post-fusion state, it is likely that this conformation was less than optimal to target the MPER on native virions prior to fusion. Furthermore, others have shown that the putative gp41 fusion intermediate form can present MPER in a more optimal conformation for binding to bnAbs[8-13]. Chapter 3 described attempts to generate putative fusion intermediates (pFIs) of gp41 by disrupting the near post-fusion conformation of gp41-HR1-54Q. Detailed antigenic evaluation revealed that sequence changes outside of the MPER, even as far as the HR1 domain, induced overall

conformational changes that influenced MPER immunogenicity. While both near post-fusion and pFIs generated strong antibodies against the MPER domains, there was a surprising difference between how these antibodies bound MPER. Such differences would remain un-noticed using traditional methods that only employ overlapping peptides to determine targeted epitopes. However, as shown in this chapter and our previous study, polyclonal sera binding analysis using alanine scanning peptides of a known epitope might reveal whether a vaccine can target the correct binding face. This is especially important for MPER-based vaccines because the MPER contains a neutralizing face that is bound by bnAbs and a non-neutralizing face that might be hidden at the viral membrane interface. While one of the pFIs came close to targeting the complete neutralizing face bound by the bnAb 4E10, it was evident that antibodies generated also targeted part of the non-neutralizing face. It is likely that since MPER in gp41 ectodomain constructs lacked any structural constrains (e.g. transmembrane domain) at the C-terminus end, it was flexible and accessible on both the neutralizing and non-neutralizing face. These results also suggest other gp41 ectodomain-only antigens that contain an MPER domain with a free C-terminus end might have similar problems in restricting antibody response to the neutralizing face. As discussed in this chapter, the lack of neutralization seen in two other similar pFIs by other groups[14,15] suggest that soluble pFIs of gp41 might not be sufficient.

To limit antibody responses towards the neutralizing face of MPER, a gp41 antigen consisting of the HR2, MPER and TM domains was designed. As described in Chapter 4, the gp41-54TM proteliposomes were successful in displaying the MPER in a way that could be accessed by different bnAbs. The antigen elicited strong antibody

responses and targeted the MPER C-terminus that contained the 4E10 epitope. Interestingly, sera from immunized animals failed to bind the C-terminus biotinylated 13-mer peptide that was used for alanine scanning analysis described previously. This difference in binding suggested that antibodies elicited by the transmembrane domain containing gp41 antigen were significantly different from those elicited by ectodomain only containing antigens. Future binding analyses need to be performed, either using longer peptides or using N-terminus biotinylated 13-mer alanine scanning peptides to understand this difference in binding. These experiments will also reveal whether gp41-54TM was successful in inducing antibodies against the neutralizing face of MPER. However, it is important to remember that sera from gp41-54TM immunized rabbits failed to neutralize pseudoviruses. Based on this, induced antibodies might target part of the non-neutralizing MPER face. Alternately, it might be possible that while these antibodies can indeed bind the neutralizing face of MPER, they might not access the MPER domain on the virion. For example, the CDR3 regions of these antibodies might not be long enough, and their ability to reach MPER might be hindered by the presence of the bulky gp120 head. The other potential reason for the failure of gp41-54TM might be that it lacks the right conformation. With regards to this, it might be interesting to present some of the previously described pFIs (Chapter 3) in the context of the TM domain and deliver them on liposomes. The liposome composition could also be altered to include cholesterol since it is in abundance in the native HIV-1 membrane[16].

All of the previous attempts involved multiple immunizations using a single antigen. While these subunit vaccines elicited strong antibody responses, they may not have contained all the structural elements required to mimic the native virion.

Unfortunately, use of larger, more native virion-like antigens might have their own drawback due to the presence of immunodominant non-neutralizing epitopes. Hence, a strategy was designed to prime rabbits with smaller antigens to first direct antibody response towards desired epitopes and then successively boost with larger antigens to direct maturation of these antibodies to bind the native virion. In Chapter 5, this strategy was tested in two groups using different priming antigens. The membrane bound priming antigen followed by boosts with other antigens failed to elicit response against MPER. Interestingly, three successive immunizations with gp41-54TM induced some MPER-targeting antibodies (Chapter 4). Hence, one possibility is that the combination with other antigens diverted the immune response to regions outside the MPER. In comparison, the prime-boost approach using the soluble MPER28x3 induced some MPER targeting antibody response. However, this response appeared to be triggered at least in part due to boosting with the priming antigen. Unfortunately, rabbits still did not demonstrate serum neutralization activity.

To evaluate whether any antibodies were raised against the neutralizing face of MPER, hybridomas were generated from one rabbit. While this is a time consuming and labor intensive process, the analysis of humoral immune response at the monoclonal level has the potential to identify rare antibodies that might not be detected in assays utilizing whole sera due to different clonal quantities and specificities. For example, if antibodies binding the non-neutralizing face are in abundance, MPER peptide based alanine scanning analysis of the total sera will only detect the predominant response. Three different hybridomas were generated and their epitopes were characterized. While the 6C10 hybridoma bound the junction between the cluster II and MPER regions, two other

hybridomas revealed interesting binding patterns. First, binding analysis of 21B5 revealed that residues ^{681}YIK^{683} were required for binding in addition to the 4E10 epitope sequence. This requirement is similar to that of 10E8. Since this hybridoma failed to bind the C-terminus biotinylated 13-mer 671 peptide, future binding analysis must be performed using either longer or N-terminus biotinylated peptides to determine which other residues are critical. This result will further reveal whether the binding epitope is the same as 10E8. Next, 9F6 binding analysis revealed a striking overlap between its epitope and the 4E10 epitope. To our knowledge, this is the first report to demonstrate that antibodies can be targeted so close to the 4E10 epitope.

Isolation and characterization of all three antibodies, but especially 9F6, will be critical for future progress. First, the binding affinities of the purified antibody should be tested and compared to 4E10 and 10E8 bnAbs. While 9F6 shows striking overlap with the 4E10 epitope, binding interactions and affinities to individual residues may or may not be the same as 4E10. Next, whether 9F6 can bind the same epitope in context of the native virion will important to address. Successful binding of the native virion is expected to result in neutralization, and will merit further testing to determine neutralization breadth and potency. If initially negative, the neutralization assay should be repeated using Fab (fragment antigen-binding) fragments as they are less bulky than whole antibodies and hence might be able to access the MPER epitope, before concluding that they are non-neutralizing.

The inability to neutralize is equally likely because of several factors. First, unlike 4E10, 9F6 does not bind residues I675 and L679. Interestingly, 10E8 does not bind I675 residue either, but its binding involves N671 and Y/K683 residues[17]. While the

role of N671 in 9F6 binding must be confirmed using crystallography, alanine scanning demonstrated that Y683 is not required. Hence, future neutralization assays will be important to analyze the effect of these differences. Second, MPER binding bnAbs isolated from patients have unique properties like long HCDR3s[18] and/or polyreactivity. 9F6 may not replicate these qualities, especially since in patients these properties might have been generated after extensive somatic hypermutation and maturation. Future antibody sequencing and lipid binding assays must be performed to answer these questions. 9F6 was generated from rabbits, an animal model that might inherently lack the required immune components to mimic the antibody maturation observed in infected humans. In fact, comparative analysis of HCDR3s suggests that rabbits lack long D and J segments[18,19]. Furthermore, of the few reported studies that resulted in the elicitation of MPER targeting antibodies with modest neutralizing abilities[20-25], four were performed in guinea pigs[20,23-25] and one in llamas[21]. With regards to this, it might be interesting to test whether other animal models, including non-human primates, can generate similar 9F6-like or better antibodies following the same immunization protocol. Finally, while the immunogenicity of the MPER28x3 antigen alone has been tested before (unpublished data), lack of antibody characterization at the monoclonal level prevents us from concluding whether similar 9F6-like antibodies can be generated by immunizing rabbits with MPER28x3 only.

Overall, the MPER domain is an attractive candidate for HIV-1 vaccine design. However, as demonstrated in this dissertation and by many others before, the lack of structural information about MPER conformation is a difficult challenge to overcome, especially for designing future vaccines. While the approaches tested here are somewhat

empirical, the results presented will hopefully contribute to future efforts in the fight against the global HIV-1 pandemic.

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APPENDIX

IMMUNOGENIC PROPERTIES OF A TRIMERIC GP41-BASED IMMUNOGEN CONTAINING AN EXPOSED MEMBRANE-PROXIMAL EXTERNAL REGION.

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Habtom H. Habte, Saikat Banerjee, Heliang Shi, Yali Qin, Michael W. Cho

Abstract

The membrane-proximal external region (MPER) of HIV-1 gp41 is an attractive target for vaccine development. Thus, better understanding of its immunogenic properties in various structural contexts is important. We previously described the crystal structure of a trimeric protein complex named gp41-HR1-54Q, which consists of the heptad repeat regions 1 and 2 and the MPER. The protein was efficiently recognized by broadly neutralizing antibodies. Here, we describe its immunogenic properties in rabbits. The protein was highly immunogenic, especially the C-terminal end of the MPER containing 4E10 and 10E8 epitopes (^{671}NWFDITNWLWYIK^{683}). Although antibodies exhibited strong competition activity against 4E10 and 10E8, neutralizing activity was not detected. Detailed mapping analyses indicated that amino acid residues critical for recognition resided on faces of the alpha helix that are either opposite of or perpendicular to the epitopes recognized by 4E10 and 10E8. These results provide critical information for designing the next generation of MPER-based immunogens.

Introduction

The envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) serves a critical role of mediating virus entry into host cells. This protein is also

immunologically important because it is the sole target against which neutralizing antibodies are elicited in infected individuals. The two subunits of the envelope glycoprotein, gp120 and gp41, pose different sets of challenges for HIV-1 vaccine design. Besides being heavily glycosylated and highly variable, the gp120 subunit contains many immunodominant epitopes that act as decoys, which provide limited, if any, protection (Pantophlet and Burton, 2006; Sodroski et al., 1998; Wei et al., 2003). While several anti-gp120 broadly neutralizing antibodies (bnAbs) have been isolated from infected individuals (Blattner et al., 2014; Buchacher et al., 1994; Burton et al., 1991; Diskin et al., 2011; Falkowska et al., 2014; Klein et al., 2012; Scharf et al., 2014; Scheid et al., 2011; Walker et al., 2011; 2009; Wu et al., 2010), most of the epitopes targeted by these antibodies are non-linear and highly conformational. Hence, designing gp120 antigens that can present the neutralizing epitopes in the correct conformation, while limiting response to other non-protective immunodominant epitopes, has been a difficult task.

In comparison, gp41 is smaller, less variable and less glycosylated. It contains a highly conserved domain (~22 amino acid residues) called the membrane-proximal external region (MPER) that lies between heptad repeat region 2 (HR2) and the transmembrane (TM) domain. This MPER contains linear epitopes targeted by a number of bnAbs, including 2F5, Z13e1, 4E10 and 10E8 (Huang et al., 2012; Kwong et al., 2013; and reviewed in Montero et al., 2008; Purtscher et al., 1994; Stiegler et al., 2001; van Gils and Sanders, 2013; Zwick et al., 2001). Unfortunately, the structure of gp41 is thought to be highly dynamic, undergoing significant conformational changes upon receptor binding and during the fusion process(Mao et al., 2013; Melikyan, 2008). In the native, pre-

fusion state, gp41 presumably exists in a metastable conformation that stores the free energy needed for membrane fusion. Following gp120 binding to CD4 and to a co-receptor, gp41 transforms into a fusion-active intermediate in which the N-terminal fusion peptide (FP) inserts into the host-cell membrane. Subsequently, the two heptad repeat regions, HR1 and HR2, are brought together to form a highly stable six-helix bundle, which concomitantly leads to the formation of a hairpin structure that completes the fusion of the viral and cellular membranes (Melikyan, 2008). This metastable and transient nature of the gp41 structure has made it difficult to design vaccine antigens that can present epitopes in their native form so as to generate potent bnAbs.

Significant efforts have been made for developing MPER-based vaccines (Montero et al., 2008). Some of the vaccine candidates evaluated so far include immunogens based on short MPER peptides, either alone or coupled to carrier proteins (Decroix et al., 2001; Joyce, 2002; Liao et al., 2000; Matoba et al., 2006; McGaughey et al., 2003; Ni et al., 2004); the use of artificial scaffolds containing stabilized MPER epitopes (Correia et al., 2010; Guenaga et al., 2011; Ofek et al., 2010); hybrid/fusion proteins (Coëffier et al., 2000; Hinz et al., 2009; Krebs et al., 2014; Law et al., 2007; Liang et al., 1999; Mantis et al., 2001; Strasz et al., 2014); chimeric viruses or virus-like particles displaying MPER epitopes (Arnold et al., 2009; Benen et al., 2014; Bomsel et al., 2011; Eckhart et al., 1996; Jain et al., 2010; Kamdem Toukam et al., 2012; Kim et al., 2007; Luo et al., 2006; Marusic et al., 2001; Muster et al., 1995; Ye et al., 2011; Yi et al., 2013; Zhang et al., 2004); and presentation of MPER peptides on liposomes (Dennison et al., 2011; Hanson et al., 2015; Hulsik et al., 2013; Lai et al., 2014; Matyas et al., 2009; Mohan et al., 2014; Serrano et al., 2014; Venditto et al., 2013; 2014). Despite these

efforts, none of them succeeded in inducing bnAbs against the MPER, albeit a few recent studies reported induction of modest levels of cross-clade neutralizing activity (Hulsik et al., 2013; Krebs et al., 2014; Lai et al., 2014; Ye et al., 2011; Yi et al., 2013). These results highlight the difficulty in eliciting anti-MPER bnAbs through vaccination.

There likely are multiple reasons for these unsuccessful attempts. Short peptide-based immunogens might be lacking helper T cell epitopes that are needed to induce robust $CD4^+$ T cell immunity. In addition, peptides, in the absence of neighboring domains, might not fold into the conformation that may exist in the native trimeric envelope spikes on virus particles. However, merely mimicking bnAb-bound conformations might not be sufficient to elicit such antibodies since MPER epitopes constrained in artificial scaffolds also failed to elicit bnAbs (Correia et al., 2010; Guenaga et al., 2011; McGaughey et al., 2003; Ofek et al., 2010). Chimeric viruses with MPER grafts have shown to induce poor anti-MPER antibody titers (Eckhart et al., 1996; Kusov et al., 2007; Luo et al., 2006; Zhang et al., 2004). This could be due to the presence of other epitopes that might be more immunogenic than the MPER and distract immune responses away from it. Thus, the relative immunogenicity of the target epitope is important when in the context of a large protein. Finally, choosing appropriate adjuvants could also be an important factor, not only for enhancing immune responses *per se*, but also for making sure that the adjuvant being used is able to preserve the correct conformation of critical neutralizing epitopes.

As a part of our efforts to develop gp41-based HIV-1 vaccine candidates, one of our goals has been to better understand the relationship between antigenic structures and their immunogenic properties. Towards this goal, we generated various gp41 constructs

containing the MPER. One of these constructs, gp41-HR1-54Q, contains a portion of HR1 connected to HR2 by a short linker, followed by the MPER and a 6xHis tag. Not surprisingly, structural analyses of this construct indicated that it forms a stable six-helix bundle, which represents a post-fusion state (Shi et al., 2010). However, considering that the MPER was extended away from the six-helix bundle and that it was efficiently recognized by bnAbs 2F5, Z13e1 and 4E10 (Shi et al., 2010), we evaluated its immunogenic properties in rabbits. Although our antigen elicited strong antibody responses against the C-terminal end of the MPER that harbors 4E10 and 10E8 epitopes, no neutralizing activity was detected. Despite this failure, the results of our study demonstrate that the region targeted by 4E10 and 10E8 can be made highly immunogenic, even in the context of a large protein.

Results

Rationales for gp41-HR1-54Q design and its structure.

For designing an immunogen based on gp41, we wanted to (1) incorporate as much of gp41 as possible in order to provide sufficient helper T cell epitopes; (2) make sure that critical neutralizing epitopes on the MPER are accessible (*viz.* 2F5, Z13e1 and 4E10; 10E8 was not discovered at the time this study began); (3) ensure that the antigen is expressed efficiently, rendered soluble and easy to purify; and (4) minimize the immunodominant epitopes that induce non-neutralizing antibodies. One of the constructs we generated, gp41-HR1-54Q, is shown in Fig. 1A. The immunodominant C-C loop between the HR1 and HR2 was replaced with a GGGGS linker. Concomitantly, the C- and N-terminal ends of HR1 and HR2 were also trimmed by six and two amino acids,

respectively. While this flexible linker allowed the HR1 and HR2 domains to freely interact with each other, we hypothesized that replacement of the C-C Loop with the linker would avoid diverting immune responses away from the MPER domain. Secondly, the fusion peptide (FP) was removed to enhance solubility. Furthermore, the fusion peptide-proximal region (FPPR) between FP and HR1 was removed to eliminate any possible interactions between FPPR and MPER, which could interfere with recognition by bnAbs.

As shown in Fig. 1B, gp41-HR1-54Q was expressed at high levels (>120 mg/l of purified protein). Although the protein fractionated in insoluble inclusion bodies, the protein could be readily solubilized with urea, refolded by step-wise removal of urea, and purified to homogeneity (Shi et al., 2010). Although our original intent was to remove the $T7_{Tag}$ by cleaving it with trypsin, as we previously observed that other potential digestion sites were resistant (data not shown), the tag also could not be cleaved, suggesting inaccessibility of the site. As shown by the crystal structure of the protein (Fig. 1C; (Shi et al., 2010)), HR1 and HR2 domains formed a highly stable six-helix bundle structure. The N-terminal eight amino acids of MPER were also highly ordered (^{662}ALDKWASL^{669}). The N-terminal 12 residues containing the $T7_{Tag}$, as well as the last eight residues (^{676}TNWLWYIQ^{683}) and the 6xHis tag were not ordered and their structures could not be defined. In addition, the side chains of six residues at the end (^{670}WNWFDI^{675}) could not be resolved, suggesting some flexibility. In contrast to the structure of our gp41-HR1-54Q, a crystal structure of two peptides encompassing FPPR-HR1 (a.a. 528-581) and HR2-MPER (a.a. 628-683) regions (Fig. 1D; (Buzon et al., 2010)), which was reported nearly at the same time of our structural study, indicated that

FPPR interacts with MPER to enhance stability of the six-helix bundle. As a result, the MPER region became highly ordered and its structure could be resolved further downstream to Y^{681}. Thus, the structural state of our immunogen might represent a "near post-fusion", rather than the "post-fusion", in regards to the MPER.

Antigenicity and immunogenicity of gp41-HR1-54Q

We have previously shown that gp41-HR1-54Q could be efficiently recognized by three bnAbs against MPER (2F5, Z13e1 and 4E10; (Shi et al., 2010)). 10E8, which was more recently isolated, also binds the protein, albeit with lower affinity (data not shown; Fig. 5). This is likely due to the fact that our immunogen contains K683Q substitution and that K or R683 is one of the amino acid residues recognized by 10E8 (Huang et al., 2012). Since these results indicated that the epitopes targeted by the bnAbs were accessible and could fold into correct conformations, we proceeded to evaluate the immunogenicity of gp41-HR1-54Q.

Six rabbits were immunized with gp41-HR1-54Q. Zn-chitosan was used as an adjuvant/delivery platform, which we have recently shown to induce strong antibody responses against gp120-based antigens (Qin et al., 2014a). Zn-chitosan was particularly well suited for our immunogen compared to many adjuvants that are oil/lipid-based considering that the MPER regions is highly hydrophobic. Rabbits were immunized four times subcutaneously on weeks 0, 4, 9 and 15. Pre- and post-immune sera (2 weeks post-immunization) were collected and antibody titers were determined by ELISA against the immunogen (Fig. 2). Strong antibody responses were observed in all of the animals. In particular, we were quite surprised to see end-point antibody titers approaching nearly

$1x10^7$ even after only a single immunization. Antibody titers increased substantially after the second immunization in most of the animals resulting in end-point titers between $1x10^7$ and $1x10^8$; however, titers did not increase further after the third or the fourth immunizations, indicating that antibody responses reached the maximum level after two immunizations. Despite having induced high levels of antibodies against gp41-HR1-54Q, none of the sera exhibited neutralizing activity against HIV-1 pseudoviruses in a standard TZM-bl cell based neutralization assay (data not shown).

Detailed characterization of antibody responses.

Despite failing to exhibit neutralizing activity, understanding the properties of antibodies elicited is important as they may provide hints as to why they failed to neutralize, and facilitate designing better immunogens. Towards this goal, immunogenic epitope mapping analyses were conducted by ELISA using various protein fragments and peptides spanning different segments of gp41-HR1-54Q (Fig. 3).

First, ELISAs were done with three long peptides available from the NIH repository, that cover the entire length of the immunogen: HR1 (N36), HR2 (C34) and MPER (^{661}LELDKWASLWNWFDITNWLWYIK^{683}). Despite some sequence differences in the N-terminal half of the C34 peptide, it was used since the cluster II region was quite conserved. Although not unexpected, antibodies against N36 were not detected. Considering that HR1 forms the inner core of the six-helix bundle, it is possible that they are simply not exposed enough to elicit antibody responses. In this regard, it was surprising to see little to no reactivity against C34 or MPER peptides since they are well exposed. This lack of reactivity could be due to a possibility that the vast majority

of the antibodies are against non-linear epitopes and that these peptides do not contain the full structural elements necessary to form the epitopes. Alternatively, these peptides simply might not be able to fold into conformations that mimic the structure of the whole protein. Yet, another possibility is that the way in which they are coated onto the surface of ELISA plates hides the epitopes or sterically hinder efficient antibody binding. Some differences in the amino acid sequences in the N-terminal half of the C34 peptide with our immunogen could also contribute.

To further characterize antibodies, two larger protein fragments were used: gp41-HR1-HR2 and gp41-54Q, which are similar to gp41-HR1-54Q but lack either MPER or HR1, respectively (Fig. 3). Not surprisingly, HR1-HR2, which would form a stable six-helix bundle, was efficiently recognized, indicating that a large proportion of antibodies recognize non-linear, or highly conformational epitopes on the six-helix bundle. But, what was interesting was that gp41-54Q, which is unable to form a six-helix bundle, was also well recognized. This suggested that gp41-54Q folded into a structure that is different from C34 or MPER peptides individually. Alternatively, although not exclusively, the two segments joined together may have allowed the protein to expose epitopes when coated onto the ELISA plate.

To identify epitopes recognized by antibodies that bind gp41-54Q, we conducted ELISA with overlapping “10-mer” peptides (Fig. 3). However, rather than coating plates with peptides directly using the traditional method, peptides were biotinylated and layered onto streptavidin-coated plates. Considering that the peptides are very short, we suspected that direct coating of the peptides onto plates could potentially mask epitopes. Since antibodies could bind at either N- or C-terminal ends of the peptides, peptides were

biotinylated at either ends of the peptides, thereby generating two sets of biotinylated peptides. We rationalized that using two different sets of the peptides would enhance our ability to detect antibody binding. Furthermore, two glycine residues were inserted as a spacer to avoid steric clashes between antibodies and the plate. To minimize the amount of work, wells were coated with both types of peptides simultaneously. Surprisingly, high levels of antibodies were detected against a number of peptides (Fig. 3). Although there were some animal-to-animal variations, overall, the MPER was more immunogenic than HR2. The three most immunogenic peptides were ^{671}**NWFD**ITNWLW^{680}, followed by ^{668}SLW**NWFD**ITN^{677} and ^{665}KWASLW**NWFD**^{674}. The common amino acid residues on these peptides are ^{671}NWFD^{674}, suggesting they might play a critical role. Consistent with this interpretation, the reactivity of adjacent peptides that lack NWFD (^{662}ALDKWASLWN^{671} and ^{674}DITNWLWYIK^{683}) decreased precipitously.

Quantification of antibodies against 671 peptide.

The 671 peptide (^{671}**NWFD**ITNWLW^{680}) encompasses the entire 4E10 epitope and most of the 10E8 epitope, which extends further out to K/R^{683} (Cardoso et al., 2005; Huang et al., 2012). Since it was the most immunogenic peptide in the region that encompasses HR2 and MPER, we were curious about the amount of antibodies directed at this peptide. Antibody levels were compared with those directed against HR1-HR2 six-helix bundle. As shown in Fig. 4 (left panel), all six animals mounted strong antibody responses against the six-helix bundle with end-point titers reaching $2x10^5$. While this is high, it was at least 100-fold less than the titer against the whole immunogen (Fig. 2), indicating that there are significant levels of antibodies directed against other epitopes. In

contrast, antibody levels against the 671 peptide varied from animal-to-animal, with end-point titers ranging from about $1x10^4$ to greater than $2x10^5$ (Fig. 4, right panel). Considering that the 671 peptide is significantly smaller than HR1-HR2 six-helix bundle (~7-fold), this result indicates that the peptide is highly immunogenic in the context our gp41-HR1-54Q.

Competition analyses with bnAbs 4E10 and 10E8.

Although antibodies bound biotinylated 671 peptides, they did not bind the full length, unbiotinylated MPER peptide. To determine whether antibodies that target the 671 peptide could indeed bind the epitopes recognized by 4E10 or 10E8 in the context of gp41-HR1-54Q, we conducted antibody competition analyses with the two mAbs. As shown in Fig. 5, both 4E10 and 10E8 could be competed away with antisera in a dose-dependent manner. 10E8 was more easily competed, which is likely due to the fact that gp41-HR1-54Q has Q at position 683, instead of K or R, which is one of the residues important for 10E8 binding. Although the assay might not prove that antibodies bind exactly at the same epitope, it does confirm that antibodies do indeed bind at or near the 4E10 and 10E8 binding site close enough to compete.

The antibody titers against the 671 peptide and their ability to compete with 4E10 or 10E8 did not seem to have clear correlation. For example, rabbit #3, which showed the highest antibody titer, was best able to compete with 4E10 or 10E8. However, there were a few notable exceptions. For example, although rabbit #5 had lower antibody titer against the 671 peptide than rabbits #1, #4 and #6, antibodies from the animal were better able to compete with 4E10 and 10E8. Another example is rabbit #2, which showed the

lowest antibody titer against the peptide. While it competed poorly against 4E10, it competed better than rabbit #4 and competed equally with rabbits #1 and #6 against 10E8. These results reveal complexity in evaluating antibody responses and that multiple parameters must be considered, including quantity, affinity, epitope targets and antigens being used for analyses.

Fine mapping analyses of antibodies targeting near 4E10/10E8 epitopes.

To further define amino acid residues critical for antibody recognition, ELISA was conducted using a panel of 13-mer (^{671}NWFDITNWLWYIK^{683}) alanine scanning mutant peptides. First, ELISA was done with the wild type peptide (Fig. 6A). Due to significantly higher levels of antibodies against the peptide for rabbits #3, #5 and #6, higher dilution of antisera was used for the three rabbits (1:2700 compared to 1:100 for the other rabbits) to avoid oversaturation. As a positive control, effects of mutations on 4E10 binding were evaluated. As shown in Fig. 6B, mutations at N671, W672, D674, and T676 severely affected 4E10 binding. Mutations at F673, I675 and L679 also affected binding to a lesser extent. It has been shown that mutations at N671 and D674, both of which lie on the non-neutralizing face, affect 4E10 binding because these residues are critical for maintaining the alpha helical conformation of C-terminal MPER peptides(Brunel et al., 2006). Although W680 is important for neutralization, it is not critical for binding(Brunel et al., 2006; Zwick et al., 2005). Thus, these results are consistent with previously published reports(Brunel et al., 2006) and validate our assay.

ELISA results from the six rabbits varied significantly from animal-to-animal. In general, three patterns were observed: (1) rabbits #1 and #2, (2) rabbits #3 and #4, and (3)

rabbits #5 and #6. For rabbits #1 and #2, mutations at D674 and N671 affected binding the most, followed by mutations at F673, N677, W678 and L679 (Fig. 6C). Mutations at I675 and T676 also affected, albeit weakly. For rabbits #3 and #4, mutations at D674 and N671 also affected binding (Fig. 6D). However, none of the mutations at other sites (with the exception of F673) significantly affected binding. It should be noted that these assays were conducted with polyclonal sera. Thus, one possible explanation is that a large diversity of antibodies was induced in these animals such that a mutation at a single site would not result in significantly reduced binding. In contrast, the affects of mutations on antibody binding were quite severe for rabbits #5 and #6 (Fig. 6E); virtually all mutations, except for W672 and I682, had affected binding. As with all other rabbits, the mutation at D674 affected binding most severely, possibly due to the importance of this residue for folding into a stable alpha helix. Other critical residues were N671, F673, T676, N677 and W678. Mutations at L679 and W680 also affected, albeit weakly. Y681 was also critical, but only for rabbit #5. The fact that mutations strongly affected antibody binding for rabbits #5 and #6, in contrast to rabbits #3 and #4, suggested that a limited number of highly dominant antibodies might have been generated in rabbits #5 and #6.

The epitope recognized by 4E10 is ^{672}**<u>WF</u>**D**<u>IT</u>**NW**<u>LW</u>**^{680} (Cardoso et al., 2005). 10E8 has a slightly larger footprint, ^{671}**<u>NWF</u>**DI**<u>T</u>**NWLWYI**<u>R</u>**^{683} (Huang et al., 2012). The results from the ELISA with alanine scanning mutant peptides clearly showed that residues important for recognition by the rabbit sera overlap with those critical for 4E10 and 10E8 binding (bold/underlined). Despite this, we did not detect any neutralizing activity in our rabbit sera. To better understand possible reason(s) for the lack of

neutralizing activity, the amino acid residues critical for binding were plotted onto a peptide that was co-crystallized with 10E8 (Fig. 7). The analyses were done based on an assumption that the C-terminal 13-mer peptide used for the ELISA, and the corresponding residues on gp41-HR1-54Q, also existed in an alpha helix. The analyses revealed that the critical residues for 4E10/10E8 and rabbit antibodies were on different faces of the alpha helix. For rabbits #5 and #6, they were separated by about 90° with overlap at F673 and T676 (Fig. 7C). For rabbits #1 and #2, they were completely on the opposite side, with overlap at I675 and L679 on one side and F673 on the other. Thus, the likely reason why rabbit antibodies failed to neutralize HIV-1 is because the faces of the alpha helix recognized by them might not be fully accessible on the trimeric envelope structure on the virion surface.

Discussion

Despite many failures to induce potent bnAbs against gp41 MPER during the past decades, it remains an important goal towards developing a protective AIDS vaccine. Towards this goal, we have been designing various MPER-based immunogens, one of them being gp41-HR1-54Q. We had previously reported its crystal structure (Shi et al., 2010) and its immunogenicity was examined in this study. Although we failed to induce bnAbs using this construct, we believed it was important to characterize its immunogenic properties in detail to learn why it may have failed. Indeed, we have made a number of important observations, which we believe would facilitate future vaccine development efforts.

First, strong immune responses were induced against gp41-HR1-54Q in rabbits. The antibody titers elicited seemed to be much stronger than previously characterized gp41-based immunogens, reaching nearly $1x10^7$ end-point titers even after a single immunization. This could be attributed to a potent adjuvant effect of Zn-chitosan (Qin et al., 2014a; Seferian and Martinez, 2000). It could also be attributed to a stable structure of the six-helix bundle formed by HR1 and HR2. Strong antibody responses against the six-helix bundle, especially against the cluster II region within HR2, have also been observed in HIV-1 infected patients (Alam et al., 2008; Frey et al., 2010). It should be noted, however, that the end-point antibody titers against HR1/HR2 six-helix bundle were only $2x10^5$ (Fig. 4), about 100-fold less than the titers against the whole antigen. This suggests that other regions/conformations of the antigen were also immunogenic. Indeed, significant levels of antibodies were also detected against gp41-54Q (Figure 3), a construct that contains just HR2 and MPER and would not be able to form the six-helix bundle. Substantial antibody levels were also detected against MPER using biotinylated 10-mer peptides, although not when the 23 amino acid MPER peptide was used. In this regard, it should be pointed out that antibody detection by ELISA depends significantly on what protein or peptide fragments are used and how they are attached to plates (*i.e.* direct coating vs. using biotinylated peptides).

Although we were able to induce high titers of antibodies against MPER using gp41-HR1-54Q, they failed to exhibit neutralizing activity. Detailed mapping analyses indicated that the antibodies targeted epitopes that overlap with those of 4E10 and 10E8. However, the critical residues of the epitopes seemed to lie on the face of the MPER alpha helix perpendicular to, or opposite side of, the residues recognized by 4E10 and

10E8 (Figs 6 and 7). The crystal structure revealed that the MPER region of our gp41-HR1-54Q is highly flexible and disordered; the very C-terminal eight residues of MPER as well as the 6xHis tag could not be observed and the last eight residues that could be seen could be resolved only at the level of the backbone atoms (Shi et al., 2010). Being in a "near post-fusion" state without FPPR, it appears that MPER on our immunogen is flexible enough to be recognized by 4E10 and 10E8 as well as the antibodies induced in rabbits. However, on the native trimeric envelope structure on virus particles, flexibility of MPER is likely more limited being not only bound to the membrane, but also connected to a large cytoplasmic domain. In such a rigid state, it is possible that the epitopes being recognized by the rabbit antibodies are not fully exposed on either the pre-fusion structure or on fusion intermediates that may exist during the fusion process. Alternatively, these epitopes might be exposed, but the angle of approach required for binding might not be possible in the context of the protein situated on the viral membrane.

Given that 4E10 and 10E8 epitopes are accessible on our immunogen, we are unsure as to why antibody responses were not induced against these epitopes. One possibility is that the epitopes that induced antibodies is inherently and overwhelmingly more immunogenic such that faster antibody responses against these epitopes prevented any immune responses being mounted against the 4E10 or 10E8 epitopes (due to steric competition). If this was the case, perhaps reducing immunogenicity of the epitopes by amino acid substitutions or by masking (*e.g.* by glycosylation, PEGylation or immune complexing) could render 4E10/10E8 epitopes more immunogenic. Another possibility is that the epitopes on our HR1-54Q that induced antibodies are more favorably targeted

than 4E10/10E8 epitopes when presented in the context of a stable six-helix bundle. In this case, immunogens with less completely formed (or less stable) six-helix bundle structures that might mimic fusion intermediates could be better immunogens. Alternatively, although not exclusively, immunogens that include the transmembrane domain (with or without the cytoplasmic tail) might be necessary to provide proper rigidity of, or spacing between, the three MPER on a trimeric structure that would hide the non-neutralizing face of the 4E10/10E8 peptide.

Recently, there have been a couple of reports describing immunogenic properties of antigen constructs very similar to ours, which also contained HR1, HR2 and MPER domains. In a report by Vassell *et al.* (Vassell et al., 2015), authors evaluated immunogenicity of several constructs comprised of MPER with different lengths of HR1 and HR2 in rabbits. Constructs were made with or without two different trimerization domains (GCN4 or foldon). The immunogens were based on HIV-1_{HXB2} strain, in contrast to ours, which was based on M group consensus sequence (MCON6). Compared to our study, antibody responses were significantly weaker with end-pointers reaching only 4-8x10^4. More importantly, antibody titers directed against MPER ranged only between about 100 and 5,000, which are 100- to 1000-fold less than what we observed. Two notable differences between the two studies are (1) we used 200 μg of antigen per immunization while they used 50 μg, and (2) we used Zn-chitosan as an adjuvant in contrast to their study, which used complete/incomplete Freund's adjuvant. Thus, our study demonstrates that it is possible to overcome poor immunogenicity of MPER by using a suitable antigen with appropriate dosage and an adjuvant. In this regard, one interesting observation from their study is that of all the immunogens they evaluated, the

construct that induced best antibody responses against MPER was FDA26, which lacked a C-terminal trimerization domain, suggesting that rigidity of the region makes it less immunogenic.

In contrast, Wang *et al.* (Wang et al., 2011) reported the elicitation of neutralizing antibodies, albeit with limited breadth and potency, in rabbits upon immunization with a similar gp41 antigen named NCM(TAIV). This construct, based on HXB2 strain, contains N36 HR1 connected to C34 HR2 *via* a GGGKLGGG liner followed by MPER. It also carries two point mutations: T569A and I675V, which have been reported to increase the exposure of the neutralizing epitopes in the MPER region (Blish et al., 2008). Interestingly, the same construct without the mutations or with a single mutation individually, induced much weaker antibody responses, especially against MPER region. The exact mechanism of enhanced immune responses rendered by these mutations, or the nature of neutralizing activity, currently remains unknown. Furthermore, the absence of detailed epitope mapping data in the report and the lack of further follow up studies limit our ability to fully compare immunogenic properties of NCM(TAIV) and gp41-HR1-54Q.

In recent years, significant advances have been made in discovering potent bnAbs against HIV-1 (Bonsignori et al., 2011; Gaebler et al., 2013; Gray et al., 2011; Scheid et al., 2009; Walker et al., 2009; Wardemann et al., 2003) and determining high-resolution structures of the bnAbs (Huang et al., 2012; Julien et al., 2013b; Pejchal et al., 2011; Scharf et al., 2014; Zhou et al., 2010) as well as novel envelope antigens (*e.g.* germline targeting eOD-GT6 and stable trimeric SOSIP gp140; (Bartesaghi et al., 2013; Jardine et al., 2013; Julien et al., 2013a; Lyumkis et al., 2013)). While structure-based, rational

immunogen design can facilitate vaccine development efforts, much of vaccine research still remains an empirical process because immunology still is a "black box" and we are unable to predict immunological responses to a given immunogen with any precision. As such, vaccine development efforts remain a reiterative process for which understanding why a vaccine candidate failed to induce desired immune response is important. Our study reveals detailed information on immunogenic properties of gp41-HR1-54Q. The availability of its crystal structure allows us to have better understanding of the relationship between antigenic structures and their immunogenic properties. We hope to use this information to design next generation of MPER-based immunogens.

Materials and Methods:

Rabbit immunization

Female New Zealand white rabbits (2.5 to 3 kg) were purchased from Charles River or Myrtle's Rabbitry and housed under specific pathogen free environments. Rabbits were cared for and used following animal protocols approved by IACUC at Case Western Reserve University or Iowa State University. To evaluate immunogenic properties of gp41-HR1-54Q, which was expressed and purified as previously described (Shi et al., 2010), rabbits were immunized subcutaneously with the protein four times (weeks 0, 4, 9 and 15) using Zn-chitosan as an adjuvant. Zn-chitosan was prepared and used as previously reported (Qin et al., 2014a). The protein was loaded onto Zn-chitosan at a ratio of 200 μg to 200 mg, respectively, in phosphate-buffered saline (PBS, pH 8.0) by continuous agitation for three hours at room temperature. Rabbits were immunized with 200 μg of gp41-HR1-54Q per each immunization.

Enzyme-linked immunosorbent assay (ELISA)

To determine the end point titers, gp41-HR1-54Q was coated onto 96-well Nunc-Immuno Plates (Nunc; # 439454) at 30 ng/well using antigen coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) overnight at 4 ^oC. Uncoated surface was blocked using 200 µl of PBS (pH 7.5) containing 2.5% skim milk and 25% Fetal Bovine Serum (FBS) for 1 hr at 37 ^oC. The plates were subsequently washed 10× with 0.1% Tween 20 in PBS. Rabbit sera were serially diluted (three folds) in the blocking buffer, and 100 µl was added to each well and incubated for 2 hr at 37 ^oC. The plates were washed 10×, and horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit, 1:3000 dilution; Thermo Scientific; Cat #31430) was added to each well and incubated (100 µl, for 1 hr at 37 ^oC). Wells were washed 10× and developed by adding 100 µl TMB HRP-substrate (Bio-Rad) for 10 min. Reactions were stopped with 50 µl of 2 N H_2SO_4. Plates were read on a microplate reader (Versamax by Molecular Devices) at 450 nm. All experiments were performed in duplicates.

For ELISA with other proteins (gp41-HR1-HR2, gp41-54Q) and peptides (N36, C34), coating antigen amounts used were molar equivalents to that used for gp41-HR1-54Q (30 ng/well). The details of the expression and purification of gp41-HR1-HR2 and gp41-54Q will be described elsewhere. As described in the results section and Fig. 3, constructs for these proteins were the same as gp41-HR1-54Q, except for the lack of MPER or HR1 domain respectively. The gp41-HR1-HR2 protein ended at L661 with RSELVPR thrombin cleavage site at the C-terminus. For ELISA with overlapping peptides, 10-mer peptides were biotinylated with EZ-Link Sulfo-NHS-LC Biotin as per the manufacturer's instructions (Thermo Scientific, Cat #21327). A mixture of both N-

and C-terminally biotinylated peptides (100 ng each) were used for coating onto streptavidin-coated plates (Thermo Scientific, Cat #15500). For alanine scan analysis, 13-mer ^{671}NWFDITNWLWYIK^{683} peptides were also biotinylated similarly at the C terminus end using the K683 residue. All other steps for ELISA were the same as described above.

Competition assays

For competition assays, plates were coated overnight with 30 ng/well of gp41-HR1-54Q. Antibodies used for competition included 4E10 (Stiegler et al., 2001) and 10E8 (Huang et al., 2012) at a final concentration of 1 μg/ml. The rest of the assay was performed as previously described (Qin et al., 2014b).

Neutralization assays

TZM-bl cell-based HIV-1 pseudovirus neutralization assays were done as previously described (Li et al., 2005; Qin et al., 2014a; Wei et al., 2002). Viruses tested were SF162 (tier 1A, clade B), MW965.26 (tier 1A, clade C), and MN.3 (tier 1A, clade B). Murine leukemia virus Env-pseudotyped virus was used as a negative control.

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(P01 AI074286). MWC has an equity interest in NeoVaxSyn Inc., and serves as CEO/President. NeoVaxSyn Inc. did not contribute to this work or the interpretation of the data.

Figures

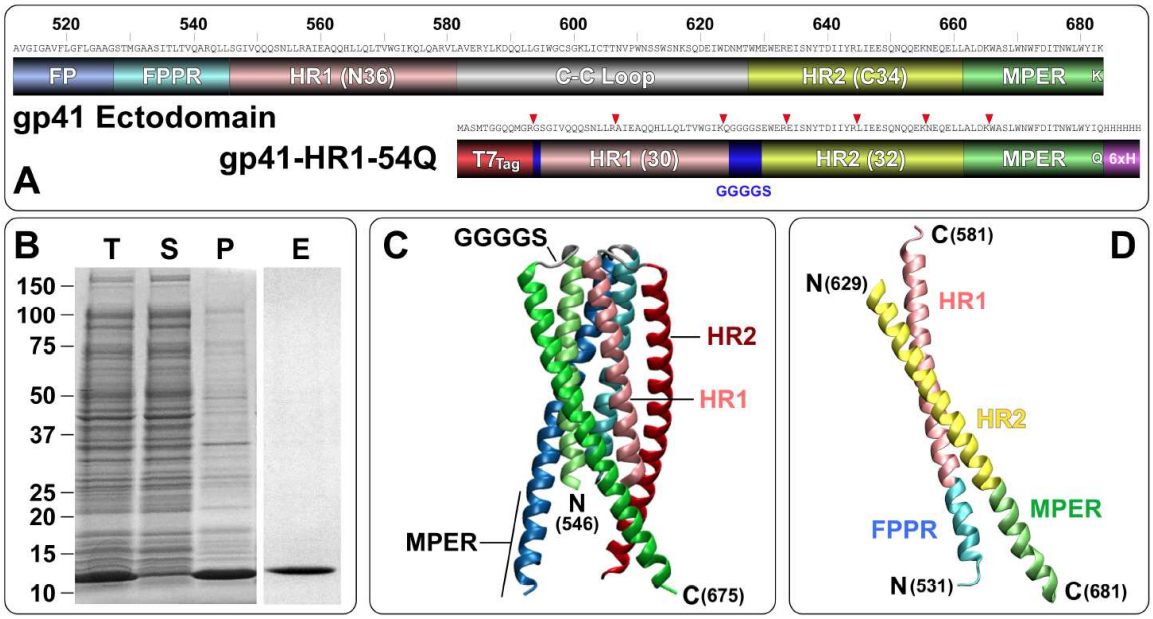

Fig 1: Generation of gp41-HR1-54Q. (A) A domain structure of gp41 ectodomain is shown at the top consisting of FP (fusion peptide), FPPR (fusion peptide proximal region), HR1 (heptad repeat region 1), immunodominant C-C loop, HR2 (heptad repeat region 2) and MPER (membrane-proximal external region). In comparison, gp41-HR1-54Q consists of shortened HR1 and HR2 domains linked together by a GGGGS linker in place of the C-C loop. The construct also has an N-terminal T7 expression tag and a C-terminal 6xHis tag. (B) SDS-PAGE of the expressed and purified protein stained with Coomassie blue showing total (T), supernatant (S), pellet (P) and elution (E) fractions. (C) A crystal structure of the gp41-HR1-54Q (pdb: 3K9A) (Shi et al., 2010) indicating individual domains. (D) A crystal structure of the post fusion complex (pdb: 2X7R) formed by two peptides containing the FPPR-HR1 and HR2-MPER domain (Buzon et al., 2010).

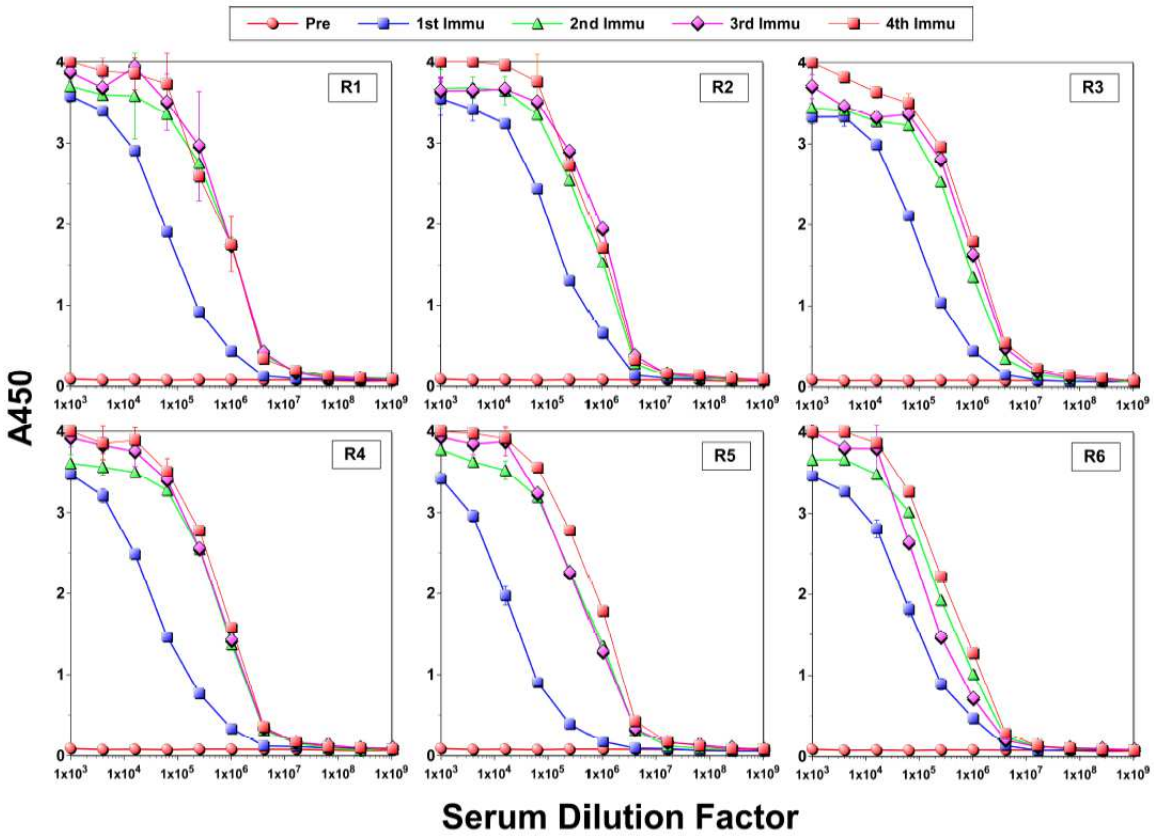

Fig 2: Antibody Titers. Sera from six immunized rabbits (R1-R6) were tested for binding to gp41-HR1-54Q after each of the four immunizations. Pre-immune serum was used as a negative control.

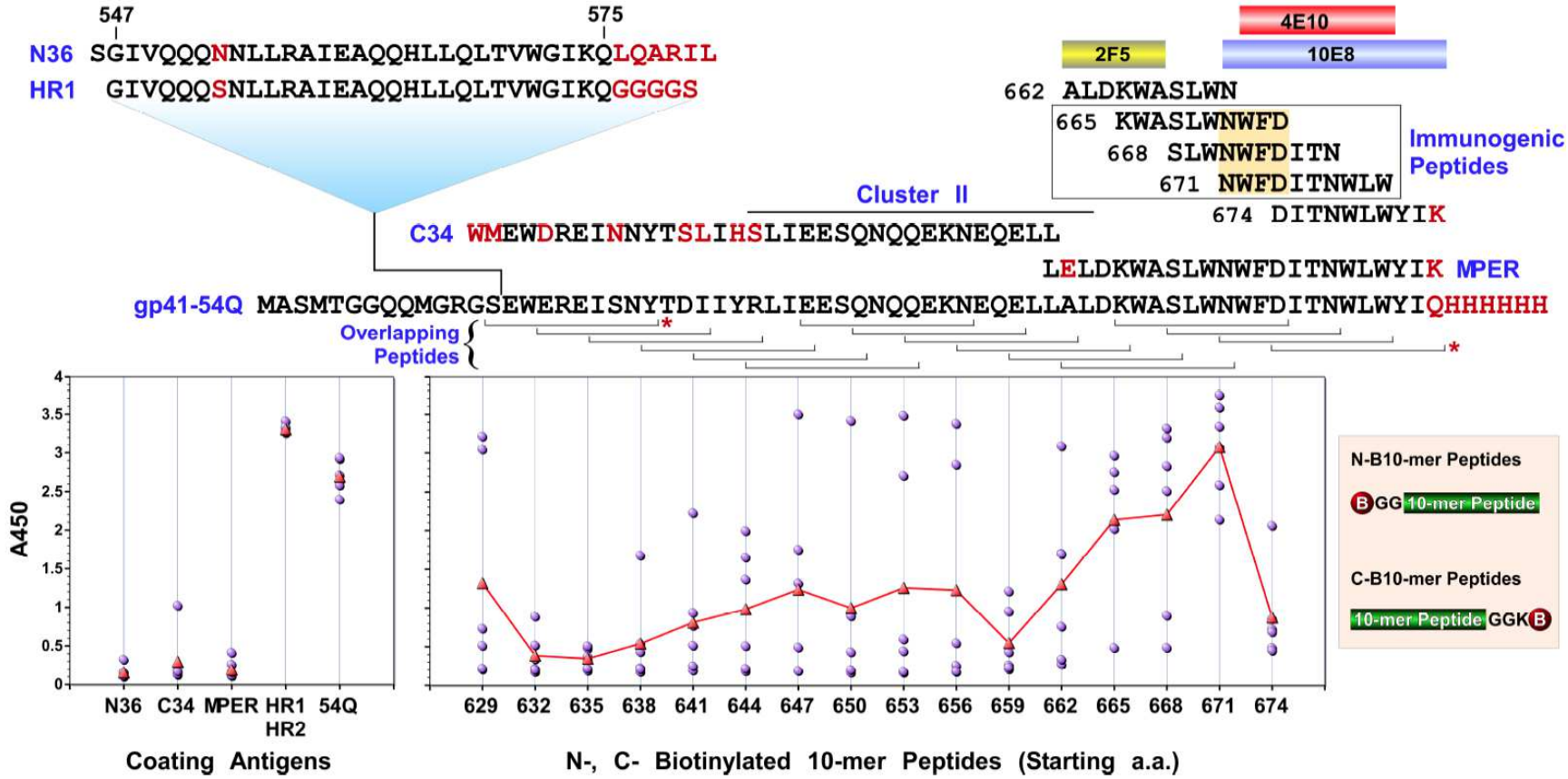

Fig 3: Mapping of Immunogenic Epitopes. Sera after fourth immunization were tested for binding against N36 peptide (HR1), C34 peptide (HR2), MPER peptide, gp41-HR1-HR2 (comprised of HR1 and HR2 domains) and gp41-54Q (comprised of HR2 and MPER domains). Purple spheres indicate ELISA A450 values for individual rabbits while the average values are plotted with red triangles. The amino acid sequences of N36, C34 and MPER, are compared with our antigen above the graph (conserved residues in black;

differences in red). ELISA was also performed against biotinylated 10-mer peptides spanning both HR2 and MPER domains. For each 10-mer, a mixture of N-terminus biotinylated (N-B10-mer) and C-terminus biotinylated (C-B10-mer) peptides were used. The amino acid sequence each 10-mer peptide is indicated by horizontal brackets. The first peptide (MEWEREISNY) and terminal peptides (DITNWLWYIK) are marked with an asterisk to indicate slight sequence differences from the original antigen. The three most immunogenic peptides, along with two adjacent peptides, are indicated separately and the important binding residues are highlighted. The core binding epitopes for 2F5, 4E10 and 10E8 bnAbs are also indicated.

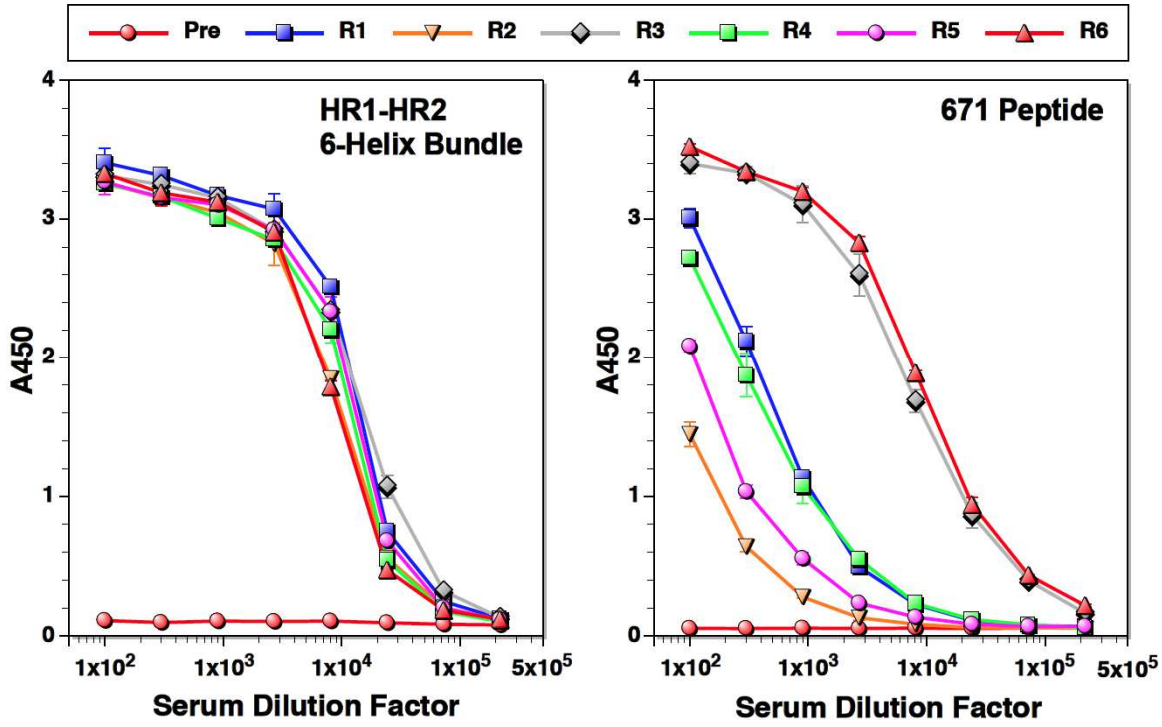

Fig 4: Antibody titers against six helix bundle and MPER peptide. Sera after fourth immunization showed strong binding antibody titers against gp41-HR1-HR2. Binding antibody titers were also high against the biotinylated, 10-mer 671 peptide that harbors the complete 4E10 epitope and the partial 10E8 epitope suggesting strong response against MPER.

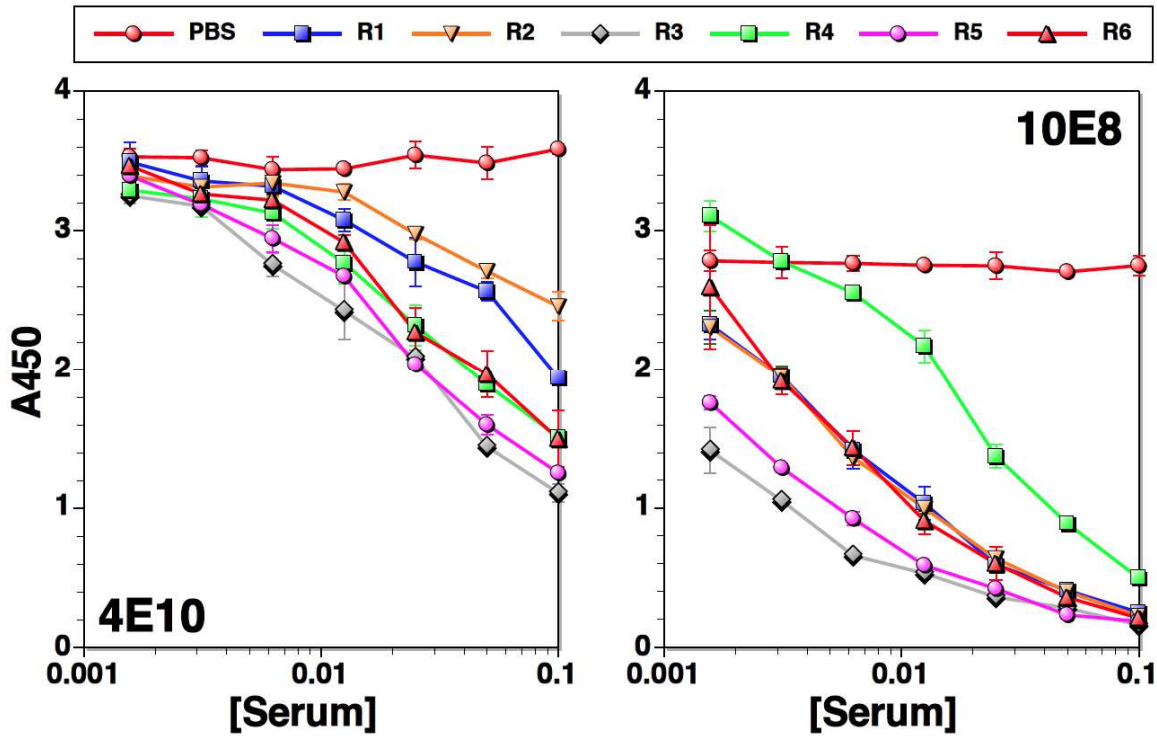

Fig 5: Competition assay against bnAbs. Sera after fourth immunization could compete against both 4E10 and 10E8 for gp41-HR1-54Q binding.

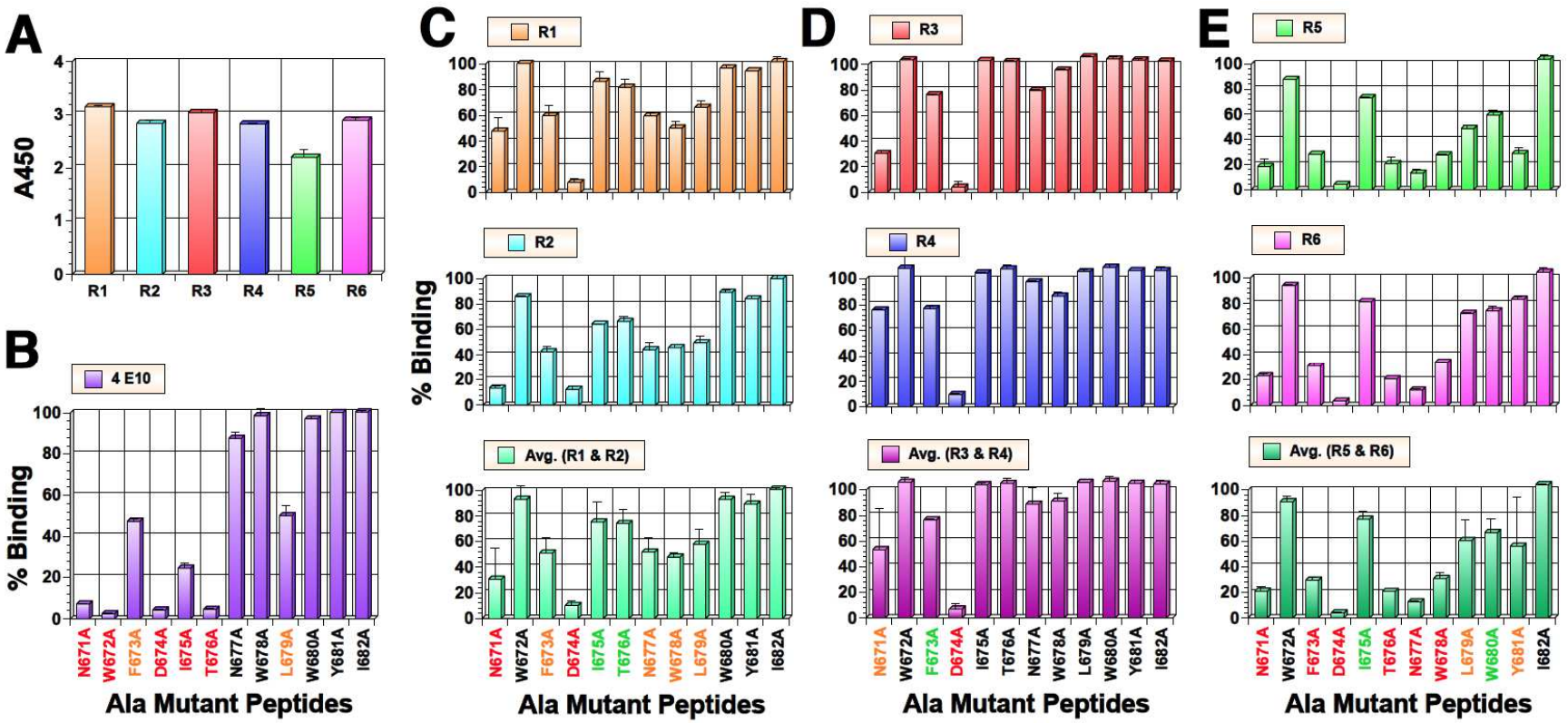

Fig 6: PepScan analysis of the C-terminus end of MPER. (A) Sera after fourth immunization were tested for binding to a biotinylated wild type 13-mer peptide (^{671}NWFDITNWLWYIK^{683}). The sera dilutions were normalized to give comparable binding signal (R1, R2 and R4 were tested at 100-fold dilution while R3, R5, and R6 were tested at 2700-fold dilution). (B) Binding of 4E10 (1 µg/ml) to mutant peptides was evaluated as a positive control. (C-E) The same dilutions of rabbit serum samples were tested for binding mutant peptides. Results are shown as the percentage of binding to the wild type peptide shown in panel (A). Three different patterns of antibody responses are shown on different columns with the average calculated at the bottom. The labeling of the mutant peptides are color coded based on the extent of reduction in binding as follows: red: <31%; orange: 31-61%; green 61-80%.

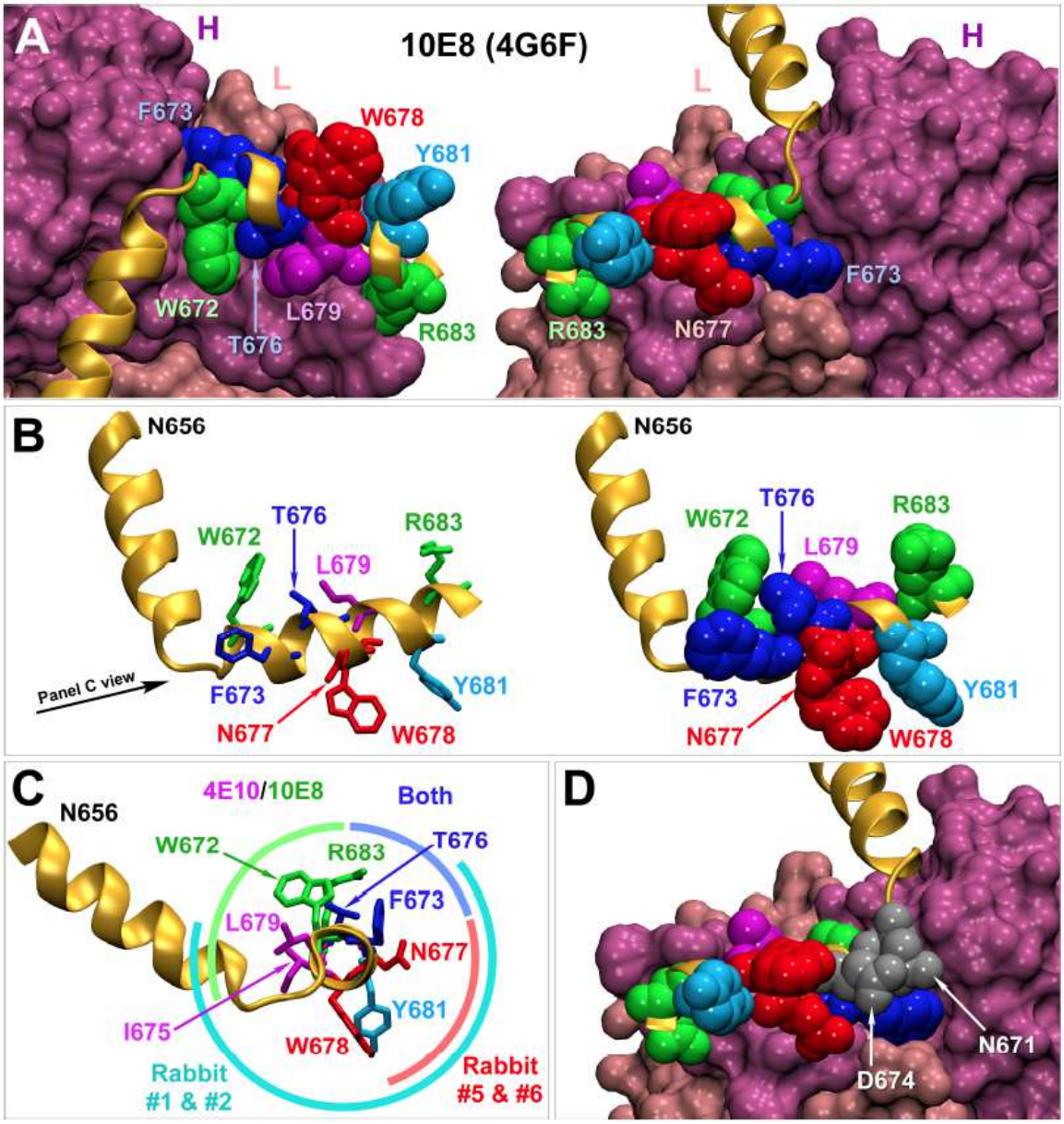

Fig. 7. Structural comparison of critical residues targeted by antibodies induced in rabbits with those of 10E8 and 4E10. (A) A co-crystal structure of a peptide bound to 10E8 is shown (pdb: 4G6F). Amino acid residues critical for binding by 10E8 (W672 and R683) and antibodies from rabbits #5 and #6 (N677 and W678) are shown in green and red, respectively. The residues important for binding by both antibodies (F673 and T676) are shown in blue. Only the most critical residues are shown. N671 and D674 are not shown, as their affect on binding may be indirect. L679, which moderately affects binding of 4E10, as well as rabbits #1, #2, #5, and #6, is shown in magenta. Y681 (cyan), which affected rabbit #5 quite significantly is also shown. Heavy (H) and Light (L) chains are indicated. (B, C) Views of the peptide bound to 10E8 from different

angles. Panel C shows a view through the axis of the alpha helix at the C-terminus (from N671 to R683), which reveals that residues recognized by 4E10/10E8 and rabbit antibodies are situated on different faces of the helix. (D) A crystal structure of a peptide bound to 10E8 illustrating the locations of N671 and D674, which shows that these two residues lie on the binding face of the helix for rabbit antibodies. Thus they could be directly involved in binding antibodies in addition to being important for maintaining alpha helix conformation of the peptide.

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