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Antibody Responses Elicited by DNA Prime-Protein Boost HIV Vaccines: A Dissertation

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ANTIBODY RESPONSES ELICITED BY DNA PRIME-PROTEIN BOOST HIV-1 VACCINES

A Dissertation Presented

By

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Abstract

The best known correlate of protection provided by vaccines is the presence of pathogen specific antibodies after immunization. However, against the Human Immunodeficiency Virus-1 (HIV-1) the mere presence of antibodies specific for the viral Envelope (Env) protein is not sufficient to provide protection. This necessitates in depth study of the humoral responses elicited during infection and by vaccination. While a significant amount of effort has been invested in studying the evolution of antibody responses to viral infection, only limited progress in understanding antibody responses elicited through vaccination has been made. In the studies described here, I attempt to rectify this deficiency by investigating how the quality of a humoral response is altered with the use of different immunization regimens, in particular a DNA prime-protein boost regimen, or with the use of different model HIV-1 Env gp120 immunogens. In a New Zealand White (NZW) rabbit model, we demonstrate that the broader neutralizing activity elicited with the DNA prime-protein boost regimen may be the result of the elicitation of a higher avidity antibody response and a unique profile of antibody specificities. Specifically, use of a DNA prime-protein boost regimen elicits antibodies targeted to the CD4 binding domain of the HIV-1 Env, a specificity that was not frequently observed when only protein based immunizations were administered.

We extended this analysis to sera from healthy human volunteers who participated in early phase HIV vaccine trials utilizing either a protein alone immunization regimen, a canarypox prime-protein boost immunization regimen, or a DNA prime-protein boost immunization regimen. Evaluation of sera from these trials demonstrated that the use of a DNA prime-protein boost regimen results in an antibody

response with greater neutralization breadth characterized by an increased frequency and titer of antibodies targeted toward the CD4 binding site (CD4bs). In addition to this, the antibody response elicited by the DNA prime-protein boost regimen also exhibited the capability to mediate antibody dependent cell-mediated cytotoxicity (ADCC) activity as well as activation of the complement system.

Additionally, in an attempt to better understand the capabilities of antibodies elicited by a DNA prime-protein boost regimen, we generated gp120 specific monoclonal antibodies (mAbs) from a single DNA primed-protein boosted NZW rabbit. Analysis of mAbs produced from this animal revealed that use of this immunization regimen elicits an antibody repertoire with diverse epitope specificity and cross reactivity. Furthermore, these select mAbs are capable of neutralizing heterologous HIV isolates. Further application of mAb generation in rabbits may provide a valuable tool to study immunogenicity of different vaccines and immunization regimens.

Concurrently, while demonstrating that a DNA prime-protein boost regimen elicits a higher quality antibody response than that observed with other leading techniques, we also demonstrated that immunogen selection can play a vital role in the quality of the resulting antibody response. By immunizing with two closely related but phenotypically distinct model gp120 immunogens, known as B33 and LN40, we demonstrated that disparate gp120s have different intrinsic abilities to raise a heterologous neutralizing antibody response. Additionally, we showed that residues found within and flanking the b12 and CD4 binding sites play critical roles in modulating neutralizing activity of sera from animals immunized with LN40 gp120, indicating that

the broader neutralizing activity seen with this immunogen may be due to differential elicitation of antibodies to this domain.

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List of Abbreviations

ADCC	Antibody Dependent Cell mediated Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
Ab	Antibody
CD4bs	CD4 binding site
CD4i	CD4 inducible
CTL	Cytotoxic T Lymphocyte
ELISA	Enzyme Linked ImmunoSorbent Assay
EWB	ELISA Wash Buffer
Env	Envelope
HIV-1	Human Immunodeficiency Virus type I
IFA	Incomplete Freund's Adjuvant
mAb	Monoclonal Antibody
MLV	Murine Leukemia Virus
MPER	Membrane Proximal External Region
NAb	Neutralizing Antibody
NaSCN	Sodium Thiocynate
NZW	New Zealand White
PNGS	Potential N-Linked Glycosylation Site
SIV	Simian Immunodeficiency Virus
sCD4	Soluble CD4
TCLA	T Cell Line Adapted

Preface

Competition assays for Figure 3.7 were performed in the laboratory of Dr. James Binley.

Chapter I

Introduction

Acquired Immunodeficiency Syndrome (AIDS) remains one of the largest human epidemics affecting the world today. More than 33 million people are currently living with Human Immunodeficiency Virus Type 1 (HIV-1), and spread of this virus is continuing with an estimated 2.5 million new infections occurring in 2007. To date, 20 million deaths have been attributed to HIV-1 infection (2). The best hope of controlling this epidemic is through the development of a successful prophylactic vaccine. Recent setbacks from the STEP trial (17), which relied purely on the induction of cell-mediated immune responses, further highlights the need to develop vaccines that can elicit protective antibodies, such as neutralizing antibodies, against primary HIV-1 isolates. Unfortunately, eliciting a strong neutralizing antibody (NAb) response to the virus has proven to be an exceptionally difficult task.

Importance of antibodies for vaccines

The implementation of vaccination to protect a population against acquired disease has been one of the greatest successes of modern healthcare. Since Edward Jenner's discovery of vaccination to prevent smallpox, we have striven to utilize and manipulate immunological memory through vaccination in order to reduce severity, or in some cases, such as smallpox, the existence of a disease causing pathogen altogether. While understanding the exact protective mechanisms provided by vaccines remains an ongoing process, our best correlate of protection to date is the elicitation of pathogen specific antibody responses.

Using pox viruses as a model pathogen to study the necessity of antibody responses for protection, evidence indicates that virus specific antibodies are both absolutely necessary and sufficient for protection from viral acquisition (30). In this study, B and T cell populations were depleted from rhesus macaques before vaccination with the licensed smallpox vaccine, DryVax. Depletion of the $CD8^+$ cell population had no effect on the monkeys' ability to fight off a subsequent viral infection. Depletion of B cells before vaccination, however, resulted in rapid disease progression and death. Subsequently, both $CD4^+$ and $CD8^+$ were depleted 6 months after vaccination to assay their necessity in the memory response. Again, it was observed that T cell depleted macaques were resistant to viral infection, thus providing additional evidence that virus specific antibodies were the major protective component of the immune response.

The protective role of antibodies is not limited to pox viruses. Protective antibody responses have also been described against bacterial pathogens, toxins, and other viruses. These include but are not limited to *Haemophilius influenzae* (50), *Neisseria meningitides* (23), *Streptococcus pneumoniae* (14), diphtheria toxin (69), tetanus toxin (36, 98), hepatitis B virus (49), polio virus (35), and influenza virus (82). With such a strong historical precedent, it appears highly probable that any protective immune response against HIV will require virus specific antibodies as a major component. Despite this precedent however, until recently, much of the work on HIV vaccines had moved away from inducing a protective antibody response.

T cell HIV vaccines

In recent years, the focus of the HIV vaccine field has largely been on the induction of strong cell mediated immune responses against the virus. This is especially

true for the large effort put forth in inducing strong cytotoxic T lymphocyte (CTL) responses directed against the virus.

Focus on the induction of CTL responses was driven by a number of discoveries implicating $CD8^+$ T cells as vitally important in the prevention and control of viral infection. Early work on the role of CTL responses in viral infection determined that the induction of CTLs is the primary correlate for the control of viremia in early infection (15, 53). These findings were corroborated with the discovery that $CD8^+$ T cells were absolutely required to control SIV infection (95). Additional evidence in human patients capable of controlling viral replication without therapy, so called “elite controllers”, supported this notion further when strong and effective CTL responses correlated with viremic control in these individuals (11, 81).

The theory behind the design of a T cell vaccine is that the presence of a strong and immediate CTL response present at the time of viral exposure would, at a minimum, reduce chronic viral loads in infected individuals by reducing acute viremia. This theory was supported by data indicating that strong CTL responses were shown capable of protecting against viral infection in SHIV protection models (4, 7, 97). Because of the success in raising strong T cell responses and the protection seen in SHIV challenge models, the T cell vaccine appeared to be an attractive platform for vaccine development. However, despite this success in raising strong T cell responses and the protective capabilities of the vaccines when facing SHIV challenges, the effect of these vaccines on more highly pathogenic SIV challenges was much less substantial (20, 59). Therefore, it is unfortunate, but not entirely surprising, that T cell based vaccines, as shown in the

STEP trial, despite being safe and immunogenic in humans, ultimately proved ineffective in the best of cases and possibly detrimental in the worst (1, 17, 54, 74, 104).

Antibodies against the HIV Envelope

While there will be continued effort to improve the magnitude and breadth of T cell responses in future HIV-1 vaccine development, the failure of the STEP trial has resulted in a renewed focus on the induction of humoral responses to HIV-1 as a means to provide an early, and possibly even sterilizing immune response. The induction of a strong functional antibody response, such as in the form of broadly NAbs, is currently one of the most sought after goals in the field of HIV-1 vaccine development.

Unfortunately, HIV-1 contains an array of protective mechanisms that makes the elicitation of a broad and potent NAb response an exceptionally difficult task. Much of the difficulty in raising functional antibody responses can be attributed to the high degree of diversity found in the envelope (Env) glycoprotein, the major target of NAbs to the virus (52). In addition to the difficulty in overcoming high levels of sequence diversity, functional NAbs must also be able to overcome a series of intrinsic defenses present in the HIV-1 Env. This includes high levels of glycosylation, epitope masking by variable loops, cryptic binding domains, the high degree of entropy present in the Env protein, and masking of functionally important domains by quaternary interactions resulting from trimerization of the Env complex (75). Additionally, because HIV, as a retrovirus, integrates into the host cell's genome, there exists only a very narrow window for NAbs to act before the establishment of a persistent infection.

Despite all of the protective mechanisms the virus utilizes, a number of monoclonal antibodies (mAb) have been identified that are capable of neutralizing a

relatively wide number of primary isolates (13, 18, 124). Study of these antibodies is especially important because it reveals weaknesses in the viral Env that can be exploited by the immune system to prevent viral infection. Five of these antibodies, 2G12, b12, PG9, PG16, and 447-52D recognize gp120, the surface subunit of Env. The first of these, 2G12, targets a cluster of carbohydrate moieties on the heavily glycosylated "silent face" of gp120 (92, 93). The mAb b12 binds to a region that overlaps the CD4 binding site (CD4bs) (122). PG9 and PG16 are two recently discovered antibodies that most efficiently recognize an epitope present on the trimeric form of Env (110). These two antibodies recognize a conformational epitope consisting of residues in the V2 and V3 regions of gp120. The fifth antibody, 447-52D, binds to the tip of the V3 loop of gp120 and has been shown to have some neutralization breadth against clade B viruses (39). Unfortunately, this region is frequently masked upon trimerization of the Env, often times limiting both the breadth and potency of other antibodies targeted to this region (79). Other broadly neutralizing mAbs, 2F5, Z13, and 4E10, target the membrane proximal external region (MPER) of gp41 (72, 126).

To date, the only means of providing sterilizing immunity has been the passive transfusion of these mAbs before or shortly after viral challenge (24, 31, 45-47, 64, 66). The success of these studies demonstrates that sterilizing immunity based on an antibody mediated mechanism is indeed feasible, and strengthens the argument for the generation of an antibody based HIV vaccine.

Novel immunization modalities for raising HIV specific humoral responses

Unfortunately, until very recently, there was little progress made in the pursuit of an antibody based vaccine that prevented HIV infection. The most prominent setbacks

were the failed phase III Vaxgen trials (33, 80). These trials employed a traditional subunit based vaccine consisting of gp120 adjuvanted in alum. While these trials were very successful in raising binding antibodies, and even NAbs against autologous T Cell Line Adapted (TCLA) strains of HIV, they did not succeed in generating a heterologous NAb response against relevant primary isolates and, ultimately, did not prevent HIV infection. Since these results became public, it became apparent that a large effort needed to be put forth to raise the quality of the antibody response generated through immunization. One of the primary means by which researchers have attempted to do this is to experiment with novel methods of immunization, including DNA vaccines, HIV proteins delivered in a viral vector, and heterologous immunization regimens that deliver the same immunogen, but deliver it via two different methods. This last method, a heterologous prime-boost regimen, is arguably the most interesting given that its use has been reported to provide partial protection against HIV acquisition in a phase III trial, RV144, the details of which will be discussed shortly.

Viral vector based vaccines

One novel approach that has been tested in the HIV vaccine field is to use viral vectors to deliver HIV-1 antigens. One such application of this approach has been evaluated in a phase I human trial using an adenovirus vector to deliver HIV-1 Env antigens (21). This study used an adenovirus delivery system that was made replication incompetent through the deletion of the E1, E4 and part of the E3 regions of the viral genome. Genes encoding a gag-pol fusion protein were inserted into the virus, intended for elicitation of T cell responses, as well as three gp140 Env genes, each derived from a single clade A, clade B, and clade C isolate. Four weeks after immunization, 93% of

individuals were capable of recognizing the homologous clade B Env by IP-Western blot. When antibody responses were measured by ELISA however, only 50% of the individuals were capable of recognizing one of the three antigens used in the vaccine formulation. Despite the positive binding titers induced in some individuals, no neutralizing activity was detected against the highly sensitive isolate SF162, or the TCLA isolate HXB2. Therefore, while this vaccine proved to be relatively safe, it failed to generate a highly immunogenic humoral response against even highly sensitive HIV-1 viruses.

The use of a canarypox virus to deliver HIV antigens has also been evaluated (91). In this phase II human trial, uninfected individuals were immunized with the canarypox vector vCP1452, encoding the gp120 protein of MN fused to the gp41 region of the HIV isolate LAI, plus the entire gag gene and CTL epitopes derived from the nef and pol proteins. The canarypox immunization was either administered alone, or boosted with a subunit protein consisting boost of a bivalent formulation of gp120s derived from the HIV-1 isolates MN and GNE8 for a total of four immunizations. Positive binding antibodies were raised against the gag protein in 23-36% of individuals depending on the immunization group. More relevantly however, between 70% and 83% of individuals raised NAb responses against the homologous isolate MN. Notably, individuals that received only canarypox based immunizations elicited lower titers of NAbs against MN. Neutralization of the heterologous isolate IIIB was also evaluated in a limited number of samples. When heterologous neutralization was taken into account, those individuals who received only the canarypox based immunization fared significantly worse than those who received a subunit protein booster. Specifically, the individuals who received only

canarypox immunizations never successfully neutralized IIIB, while individuals who received a subunit protein boost were able to neutralize the virus in 70% of cases. The generation of antibody responses capable of neutralizing TCLA strains of virus using a similar canarypox prime-protein boost immunization regimen has also been mirrored in a number of other studies, demonstrating the utility of a heterologous prime-boost regimen (10, 32, 41, 105).

DNA vaccines

The use of DNA vaccines to raise humoral responses against HIV-1 was first seen in the early nineties where it was shown that a DNA plasmid encoding HIV-1 Env derived from a TCLA isolate was capable of raising HIV-1 specific antibody responses in small animals (62, 111). The antibodies raised by this approach were capable of binding recombinant Env protein as well as neutralizing the HIV-1 isolate IIIB. The utility of this approach was further demonstrated in a SHIV challenge model in cynolomogous macaques (16). In this study, animals that received DNA immunizations generated a strong immune response that, upon viral challenge, resulted in a decreased viral load compared to unimmunized animals. Furthermore, in this study, one of four immunized animals was protected from viral challenge upon completion of the DNA immunization regimen.

Other than a DNA vaccine's obvious ability to generate an immune response, there are a number of positive aspects that make DNA based immunizations an attractive option for use as a platform for an HIV-1 vaccine. The first of these is the endogenous production and processing of a chosen antigen. When a DNA immunization is given, antigen encoding plasmids are taken up directly by cells at the injection site of the host,

thereby making antigen production similar to that of a live attenuated vaccine. This allows the protein to undergo well regulated translation processes, allowing for native folding, as well as normal post translational modifications, such as glycosylation, of the antigen of interest. Additionally, because of the endogenous production of the antigen, the produced protein can be efficiently presented to the immune system through class I and class II MHC complexes, allowing for an efficient T cell response to the antigen. In addition to native antigen production and processing, the DNA vaccine has also proven to be a very safe alternative to subunit and live attenuated vaccines (19, 63, 90, 107, 112). Because DNA vaccines are normally non-replicative, non-integrative, and can only encode the protein(s) of interest, DNA vaccines allow the researcher to elicit an antibody response with the specificity of a subunit vaccine and the native antigen processing of a live attenuated vaccine, all without the safety risk of reversion of an attenuated viral strain into a more pathogenic one.

In addition to its relative safety, DNA based immunizations provide an excellent platform for studying different properties of a particular antigen, screening of different immunogens (71, 88), identifying immunogenic and neutralizing domains of a target (114), as well as identifying effective immunization regimens (89).

Unfortunately a number of caveats still exist that prevents the widespread application of this technique. One of these caveats is the relatively low *in vivo* transfection efficiency. The inefficient uptake of the DNA plasmid by host cells leads to low levels of *in vivo* antigen production. Because of this, a significant effort has been applied to increase the potency of DNA vaccines. This includes studying different delivery mechanisms for the DNA itself. These methods include electroporation (44,

119), needle free jet systems (3, 42, 106), gene gun (106, 119), and microneedle injections (83), all of which are intended to increase the efficiency of DNA delivery over a traditional intramuscular injection (119).

Increasing the efficiency of DNA delivery is only one aspect in the effort to increase the potency of DNA immunizations. Work has also focused on the design of the DNA constructs themselves in order to enhance antigen production on the level of the individual transfected cell. One of the primary improvements that has been made is the advent and implementation of codon optimization to maximize the efficiency of tRNA usage in the cell (27, 115, 125). Optimizing each codon to utilize the most prevalent tRNA present in the cell allows for more efficient protein translation, resulting in a higher quantity of antigen being produced. Other work to increase the amount of antigen produced has focused on manipulating the leader sequences and promoters of these constructs (115). Here, it was demonstrated that simultaneous manipulation of the immunogen leader sequence, promoter, and codon usage resulted in improved immunogenicity of a gp120 protein in a mouse model (115).

Despite some of these limitations, the ease of use and safety of DNA vaccines has lead to the evaluation of these vaccines in human clinical trials in attempts to raise HIV-1 specific antibody responses. In one trial, three DNA immunizations encoding three Env antigens from clades A, B, and C, as well as the T cell antigens, gag, pol, and nef, were given to healthy human volunteers using a needle free injection system (22). Antibody responses from this trial were then evaluated by ELISA and neutralizing antibody assays. Humoral responses recognizing the clade A and C Envs were generated in 71% of individuals with the clade B being recognized in 64% of individuals in the trial. Despite

the presence of binding antibodies, none of the vaccinated individuals generated NAbs against even the sensitive HIV-1 isolate, MN. This indicates that use of an immunization regimen consisting of a DNA vaccine alone may not be immunogenic enough to provide a protective response.

This pattern of raising a binding antibody response but not a Nab response has been observed in other DNA only vaccine trials as well. One such trial delivered DNA encoding a gag-pol-nef fusion protein plus modified Env constructs derived from clades A, B and C via a needle free injection system (40). Similar to the previous DNA only trials, binding antibodies, as determined by ELISA, were raised in 60% of individuals. Again, however, a total lack of NAb responses were raised against the sensitive HIV-1 isolate, MN. A third trial, utilizing only DNA-based immunizations encoding gag, pol, env, rev, tat, and vpu, delivered by a needle based intramuscular injection, also failed to produce any detectable NAb responses against the HIV-1 isolates ADA or MN (70). As a whole, these trials have highlighted the fact that, in humans, neither DNA based nor viral vector based immunization platforms are immunogenic enough on their own to become a viable vaccine.

Heterologous prime-boost vaccine regimens

In summary, the use of a single modality immunization has produced less than stellar humoral responses in HIV clinical trials in humans. Although several traditional and novel vaccine types have been tested, each appears to have their shortcomings. While traditional subunit protein was shown to be immunogenic, it was not protective. DNA and viral vectored vaccines, on the other hand, were overall not immunogenic enough to generate a robust response. The question that now remains is whether or not higher

quality antibody responses can be generated through the combination of different immunization regimens relative to their use alone.

DNA vaccine prime-viral vector boost

Based upon the limited ability of vaccines utilizing a single modality to raise an effective antibody response against HIV-1, combinations of heterologous immunization approaches have also been attempted. One such study used a DNA prime and adenovirus boost to elicit cellular and humoral immunity in rhesus macaques (65). In this study immunization of rhesus macaques with a chimeric HxBc2/BaL gp145 construct delivered either by a DNA prime-adenovirus boost or strictly repeated immunizations with recombinant adenovirus type 5 (rAd5) was evaluated. Immunization with a single Ad5 vector generated higher binding titers against the gp140 protein compared to immunization with only DNA vaccine. However, repeated boosting of the rAd5 immunized animals with additional rAd5 virus did not enhance the antibody response. In contrast, if a DNA prime was given to the animals prior to a rAd5 boost, a rapid rise in Env antibody binding titers were observed. Neutralizing activity raised by the two immunization approaches was also evaluated. Neutralization of the 89.6 isolate was found to be significantly greater in animals that first received a DNA prime prior to adenovirus boosting, indicating the superiority of this combination immunization approach relative to immunization with only rAd5. However, the breadth of neutralization using the DNA prime rAd5 boost format was still somewhat limited, with only about a third of tested clade B isolates being neutralized by sera generated from immunization with either an 89.6 or chimeric HxBc2/BaL construct.

Canarypox prime-protein boost

In the phase II canarypox prime-protein boost trial discussed above (91), the authors demonstrated that using the heterologous prime-boost regimen elicited a significantly higher and broader NAb response than immunization with only the canarypox vector. This work was continued further by evaluating a canarypox prime-protein boost regimen in a large phase III trial known as RV144 (85). This trial is interesting for several reasons, with the primary reason being that it is the first HIV vaccine trial ever to provide partial protection from HIV acquisition. In a modified intent-to-treat group, the authors report a statistically significant, 31% drop in HIV acquisition compared to a placebo controlled group. The second interesting point is that this trial used two pre-existing products that alone were either not very immunogenic, as was the case for the canarypox vaccine (91), or did not provide any protective capacity as was observed with the AIDSVAX B/E protein vaccine (80). However, the observation that, when combined, this vaccine can provide partial protection is a remarkable result. While the exact mechanism of this protection remains unclear, it provides additional evidence that suggests that the combination of two different immunization regimens can provide a higher quality immune response than either immunization regimen can alone. The relative success of this trial may provide a glimpse of the power of heterologous prime-boost regimens, and justifies further investigation into, not only canarypox plus protein combinations, but other heterologous prime-boost immunizations as well.

DNA prime-protein boost

Our group has been working with a heterologous prime boost regimen, the DNA prime-protein boost regimen, for nearly fifteen years. We and others have shown, in historical data covered here, in original data presented later, and in agreement with the

recent RV144 trial, that a heterologous prime-boost regimen elicits a higher quality humoral response than immunization with either modality alone.

Despite improvements in the design of the DNA construct and the increase in efficiency in the delivery, DNA immunizations are still only capable of producing limited quantities of antigen at levels much lower than those produced by inactivated or subunit vaccines. Because of this, as witnessed in DNA vaccine alone human HIV-1 trials, this vaccination modality is not immunogenic enough on its own to generate an effective antibody response against the virus when delivered by a traditional needle injection. Despite this, DNA immunizations are highly effective in priming the body's immune system and work best when used in combination with another immunization approach, usually with DNA administered as a priming immunization followed by a boost of other modality. One of the simplest and most effective of these combination approaches for the elicitation of humoral immunity is a DNA prime followed by a subunit protein boost (60).

Early studies using the DNA prime-protein boost approach utilized TCLA derived Env proteins in the vaccine formulation (87). Rabbits in this study were immunized with DNA based immunizations encoding gp120, gp140, or a replication incompetent form of HXB-2 and subsequently boosted with rgp160 derived from the HIV-1 isolate IIIB in Incomplete Freund's adjuvant (IFA). While only limited binding antibodies were generated following DNA immunization, boosting with recombinant protein greatly increased binding titers in immunized animals. Analysis of serum avidity elicited by each immunization regimen indicated that use of a combination approach elicited a higher avidity antibody response than that which was observed with the use of DNA

immunizations alone. In addition, the combination DNA prime-protein boost approach generated significantly greater homologous NAb titers than that observed with immunization of naïve animals with only recombinant protein. Heterologous NAb responses against MN and SF2 were also generated using this prime boost immunization regimen. Titers in immunized animals varied from 1:148 to exceeding 1:3000 against MN and 1:37 to 1:269 against SF2.

Initial work demonstrated that a DNA prime-protein boost strategy is an effective means by which to raise antibody responses in both small animals and non-human primates (6, 55). However, many of these studies suffered from the inability to neutralize the more relevant primary isolates of HIV-1. One breakthrough study, in an attempt to overcome the limitations of TCLA derived immunogens, used gp120 derived from the primary isolate JR-FL as a model immunogen (113). In this study, rabbits were immunized in either a DNA prime-protein boost format, or with only recombinant gp120 protein derived from the primary isolate JR-FL. Sera generated by both immunization approaches contained high levels of binding antibody to homologous envelope, but the NAb response generated by each immunization regimen differed dramatically. One example of this was observed with the neutralization of the sensitive isolate SF162. Both immunization regimens were capable of generating a Nab response against this sensitive isolate, however, the DNA primed animals did so with a much higher titer. Additionally, animals that received a DNA prime were capable of neutralizing the homologous strain of HIV-1, JR-FL, in a PBMC based neutralization assay. Inhibition of this neutralization resistant primary isolate was not observed in animals that were immunized with only protein. Sera raised by the DNA prime-protein boost approach were also frequently

capable of neutralizing other heterologous clade B isolates, including 1196 and 0692, a phenomenon that was absent in animals that received only protein based immunizations.

The superiority of the DNA prime-protein boost approach was proven again concurrently in a separate study looking at the effect of oligomerization state on the elicitation of NAbs (8). Here, the use of a DNA prime-protein boost approach elicited a 12-fold higher binding antibody response when compared to immunization with only protein, clearly demonstrating the potential of this platform for eliciting an antibody response to the virus. This increase in binding titer was accompanied by a slight increase in the frequency of neutralization of the autologous JR-FL virus between vector primed and trimer primed animals.

While the DNA prime-protein boost approach was able to enhance the binding and neutralizing antibody response elicited when compared to immunization with subunit protein alone, the overall breadth of neutralizing activity was still somewhat limited. The most likely explanation for this was the use of only a single, subtype B Env in the vaccine formulation. One attempt to increase the breadth of the neutralizing activity was to include multiple, genetically distinct Envs into a single polyvalent formulation (118). In this study, rabbits were immunized in a DNA prime-protein boost format consisting of either monovalent or polyvalent formulations of gp120 derived from clades A, B, C, D, E, F, and G. Sera generated by immunization with these constructs were then tested in a pseudovirus based neutralization assay against a panel of 14 viruses from clades A-E. Data from this study revealed that immunization with a polyvalent Env formulation significantly increased the breadth of neutralization against this multiclade panel of

pseudoviruses, as evidenced by an almost doubling in the number of isolates neutralized compared to the monovalent immunization groups (118).

The success of the DNA prime-protein boost immunization regimen further demonstrated its promise in non-human primate studies. To this extent, a modification of the polyvalent formulation above, consisting of two clade B gp120s, one clade C gp120, and one clade E gp120, plus gag derived from NL4-3 was tested for its protective efficacy in rhesus macaques (73). Animals received a combination of DNA and protein based immunizations and were subsequently rectally challenged with the R5 SHIV BaL. At the time of challenge, immunized animals had generated an antibody response capable of neutralizing the sensitive isolates of HIV, MN and SF162, as well as the BaL challenge strain. Immunization with the above regimen protected four out of six macaques from infection with the SHIV, based upon detection of viral RNA in the blood (a sterilizing immunity). Relative to a control group of seven naïve animals who all became infected and demonstrated high viral loads, the remaining two immunized macaques that became infected demonstrated lower levels of viral RNA in the blood. Because the gag antigen is from an HIV-1 isolate, while the macaques were challenged with a SHIV whose gag antigen is derived from SIV, the protection is likely mediated by Env-induced immunity, most likely due to anti-Env antibodies.

Other studies have also confirmed the utility of the DNA prime-protein boost approach in non-human primates. One of these studies used this prime-boost approach in neonatal macaques (84). Immunization of animals in this study with DNA encoding vpu and the IIIB Env, followed by boosting with recombinant IIIB derived gp160, protected out of 15 animals from a homologous IIIB intravenous SHIV challenge. Another study

utilizing the DNA prime-protein boost approach immunized rhesus macaques with gp120 or gp160 forms of envelope derived from HXBc2 (55). Homologous NAb titers exceeding 1:1000 were generated as a result of immunization with these constructs. Following an intravenous challenge of SHIV HXBc2, none of the immunized monkeys became infected based as evidenced by an inability to re-isolate the challenge HXBc2 virus at every bleed after challenge.

As a result of data demonstrating that a DNA prime-protein boost approach could provide sterilizing immunity in a SHIV protection model, the DNA prime-protein boost approach was tested in a Phase I clinical trial (117). Again, a polyvalent Env formulation was used, this time consisting of five Envs from clades A, B, C, and E. After three DNA immunizations and two protein boosts, humoral responses were evaluated using solid phase antibody binding and NAb assays. Immunization with this polyvalent Env vaccine formulation and regimen elicited broad and high titer binding antibody responses against gp120 antigens from clades A through H of HIV-1 in all individuals enrolled in the trial when evaluated by ELISA and Western blot analysis. Additionally, NAb responses were detected in 100% of individuals against the sensitive HIV-1 isolates MN, NL4-3, and SF162 at titers as high as 1:2000. Neutralization of the homologous primary isolates included in the vaccine was also frequently observed. Specifically, neutralizing activity was detected in more than 60% of individuals against the subtype C vaccine strain 96ZM652. In addition to eliciting NAbs against sensitive and homologous isolates, a result not always observed in previous trials, the new polyvalent DNA prime-protein boost regimen also generated neutralizing activity against difficult to neutralize heterologous primary isolates from clades A, B, C, D, and E in the high throughput

pseudovirus based PhenoSense neutralization system (117). Positive NAb responses were identified in each of the 22 vaccines included in the analysis with about 60% of vaccinees having positive NAb responses against 80-100% of pseudotyped viruses included in the assay. This represents a significant improvement over the NAb responses reported with immunization via protein, DNA, or viral vectors alone as well as combinations of viral vector prime-protein boost and DNA prime-viral vector boost vaccines. In addition to strong NAb responses, this DNA prime-protein boost regimen also elicited a polyfunctional T cell response (5). This is important to note because, despite the setback from STEP, it is still highly probable that a balanced B and T cell response will be required to provide the best possible protection against HIV. Additionally, while this vaccine was successful in generating a cross reactive antibody response, it also proved to be safe and well tolerated (51).

Research Framework and Objectives

The previous data described above laid the foundation for the original body of work described herein. While it has become increasingly accepted that use of the DNA prime-protein boost regimen elicits a higher quality antibody response than immunization with only protein, the underlying mechanism of this phenomenon remains unclear. The work described in the following chapters attempts to identify specific attributes of the humoral response resulting from a DNA prime-protein boost immunization regimen that may account for the higher quality NAb activity observed when this regimen is used. We attempt to identify the role of the DNA and protein components in the priming and boosting phases of an immunization regimen in contributing to the final antibody response. We expand this analysis using a second antigen formulation and identify a

unique profile of antibody specificities resulting from immunization with a DNA prime-protein boost regimen in small animals. We extend this characterization to human serum samples resulting from immunization with a protein alone regimen, a canarypox prime-protein boost regimen, and a DNA prime-protein boost regimen, thus allowing us to determine if the patterns we identify in small animals are also seen in healthy, immunized humans.

Also, using the DNA prime-protein boost regimen as a tool, we attempt to demonstrate that individual gp120 immunogens have different capabilities of raising a heterologous NAb response and that the broader NAb response elicited by select gp120s is due to recognition of conserved domains on the HIV Env.

Finally, in order to allow us to directly compare the capabilities of antibodies elicited through immunization to those elicited through infection, we generate HIV specific mAbs from a single DNA primed-protein boosted rabbit.

Chapter II

Analysis of the quality of antibody responses elicited by immunization with DNA, protein, or DNA plus protein

Introduction

Previous data from our lab has indicated that gp120 formulations delivered by a DNA prime-protein boost approach are capable of eliciting an antibody response with the ability to neutralize isolates from many of the major subtypes of HIV (117, 118). Additional data in the NZW rabbit model has indicated that the combination DNA prime-protein boost regimen elicits a broader NAb response than immunization with only protein (Fig 2.1). However, the underlying mechanism driving this phenomenon remained unclear.

The previous studies that first noted this phenomenon lacked several controls that would have helped elucidate this phenomenon further. In this chapter, we attempt to address these shortcomings by making a more stringent comparison between humoral responses elicited by different immunization regimens. In order to accomplish this, rabbits were immunized in one of five different ways using JR-FL gp120 as a model HIV antigen. The first two methods of immunization repeat the regimen used in previous studies (117, 118). The first of these is the delivery of three DNA empty vector immunizations followed by two protein immunizations in IFA. The second employed three DNA primes with a JR-FL gp120 construct followed by two protein boosts with recombinant JR-FL gp120 protein. A direct comparison between these groups allows us to control for any nonspecific effects of the DNA priming. However, there remains a

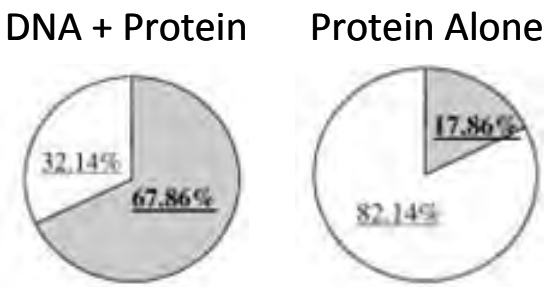

Fig 2.1. Neutralization breadth of sera resulting from immunization with either a DNA plus protein or a protein alone regimen. Pie charts demonstrating the capability of serum from animals immunized with either a DNA plus protein regimen or a protein alone regimen to neutralize a panel of HIV isolates. Grey shaded regions indicate the percentage of viruses from a 14 virus panel capable of being neutralized by sera from each immunization regimen. Figure adapted from (118).

large caveat with this comparison, this being that the vector prime-protein boost group only receives two immunizations that contain antigen, while the JR-FL DNA prime-protein boost received five immunizations containing antigen. In order to address this, in the current study, we included an additional control consisting of a group of rabbits that received five JR-FL protein immunizations. This allowed us to directly answer the question of whether three additional protein immunizations could increase the breadth of the NAb response.

The next control that was lacking in the previous study is the presence of a five DNA immunization group. Inclusion of this control would have allowed us to evaluate whether a DNA immunization is the primary determinant of the specificity of the final immune response, whether it elicits a unique profile of antibody specificities itself, or whether a combination of the DNA and protein plays a unique role in determining the final antibody profile.

A third control that would have been interesting for inclusion in the previous study is one where the priming and boosting antigens were not precisely matched. This mismatched antigen regimen could result in a broader recognition of HIV antigens compared to a matched antigen regimen due to the exposure of B cells to greater sequence diversity.

In the current study, with the inclusion of these additional controls to address missing elements of the previous work, we monitored the effects of different immunization regimens on the resulting antibody response. We evaluated differences in antibody binding titer, specificity, neutralizing activity, and avidity elicited by each of

these regimens and identified a unique profile of antibody characteristics elicited when a DNA component is included in the immunization regimen.

Results

In this study, rabbits were immunized in one of five schedules in order to provide a direct comparison as to the relative immunogenicities of homologous vs. heterologous prime-boost vaccinations. These approaches (summarized in Fig 2.2) utilize HIV-1 JR-FL gp120 as a model antigen delivered as either a DNA vaccine or a recombinant protein vaccine.

Groups of NZW rabbits were immunized with one of the following regimens: 1) five JR-FL gp120 DNA immunizations; 2) five JR-FL gp120 protein immunizations; 3) three JR-FL gp120 DNA immunizations followed by two JR-FL gp120 protein immunizations. The first two schedules are homologous prime-boost using the same type of vaccine. The third schedule is a heterologous prime-boost regimen which administered DNA and protein immunizations to the same animals at different time points. Consistent with previous studies, three DNA-based immunizations were given in the priming phase, followed by two protein-based immunizations in the boosting phase (108, 117). Two additional immunization schedules were included as controls. In one schedule, rabbits received three empty DNA vaccine vector immunizations followed by two JR-FL gp120 protein boosts. The purpose of this control is to exclude the non-specific effect by a DNA plasmid as the prime. Another control group received the JR-FL DNA priming immunizations, but was boosted with a 5-valent gp120 protein vaccine, which included the JR-FL gp120 and four other gp120 proteins from clades A, B, C and E. Inclusion of this group allowed for the comparison between the polyvalent and

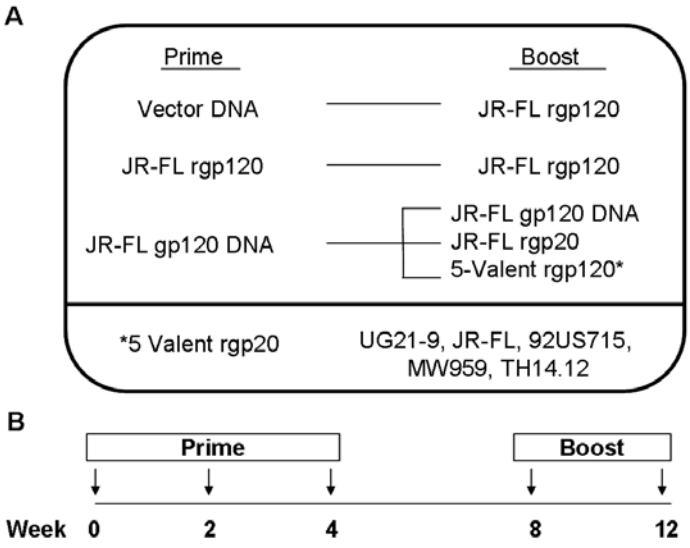

Fig 2.2. Study design and immunization schedule for rabbits receiving JR-FL gp120based immunizations. Rabbits were immunized with one of five prime-boost regimens: 1) three DNA vector immunizations plus two JR-FL gp120 protein boosts; 2) five JR-FL gp120 protein immunizations; 3) five JR-FL gp120 DNA immunizations; 4) three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein boosts; 5) three JR-FL gp120 DNA immunizations plus two 5-valent gp120 protein boosts. The 5-valent protein mixture consisted of gp120s from clades A (UG21-9), clade B (JR-FL + US715), clade C (MW959), and clade E (TH14.12). “Priming” immunizations were administered at Weeks 0, 2, and 4. “Boosting” immunizations were given at Weeks 8 and 12.

monovalent boosts. In all of the above studies, the "priming" immunizations were given at Weeks 0, 2, and 4 with "boosting" immunizations given at Weeks 8 and 12 (Fig 2.2).

Generation of gp120 specific antibodies as measured by ELISA

The overall immunogenicity for each immunization regimen was first determined by measuring the binding titers of serum IgG for each individual rabbit against the JR-FL gp120 antigen by ELISA (Fig 2.3). All rabbits, regardless of immunization regimen, generated a significant gp120-specific antibody response. Despite the observation that rabbits receiving five protein immunizations tended to generate a slightly lower binding antibody response, endpoint serum dilution titers among these groups were statistically indistinguishable from each other. Additionally, variation of individual animals within a single group was also minimal, with no animals deviating more than a single dilution step from the group geometric mean titer.

Analysis of antibody specificity elicited by each immunization regimen

We chose to use a pseudoviral based competitive binding assay to examine antibodies of a particular specificity capable of binding to an HIV-1 viral Env spike as previously reported (25, 68, 108). Knowing that the V3 loop is an immunodominant epitope of gp120, whose recognition is sometimes responsible for the neutralization of select viruses, we began by assaying sera for the presence of V3 directed antibodies using a known V3 directed monoclonal antibody, 447-52D, in a competitive binding assay (Fig 2.4A). Consistent with the immunodominant nature of the V3 loop, all immunization regimens elicited high titer antibody responses capable of outcompeting binding of 447 to this domain. In many cases, titers approached or exceeded 1:1000. However, animals that received only protein immunizations or immunizations with only the empty DNA

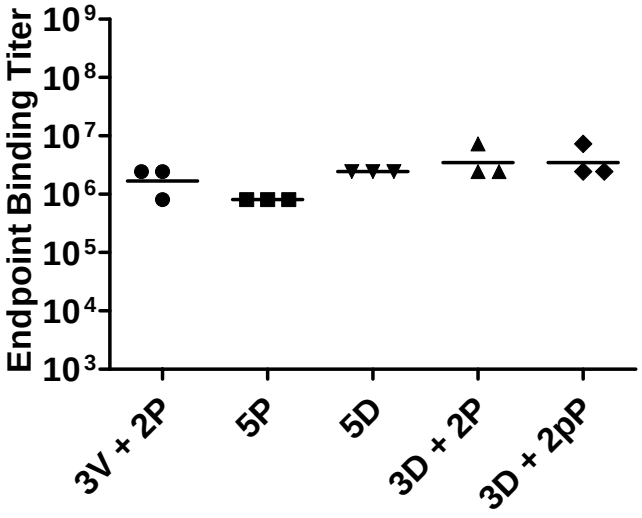

Fig 2.3 Endpoint binding titers of sera from rabbits receiving JR-FL based immunizations. Endpoint binding titers from samples collected two weeks after the final boosting immunization were determined against the autologous JR-FL gp120 protein by ELISA. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP) . Symbols denote individual rabbits. Bars denote geometric mean within a group.

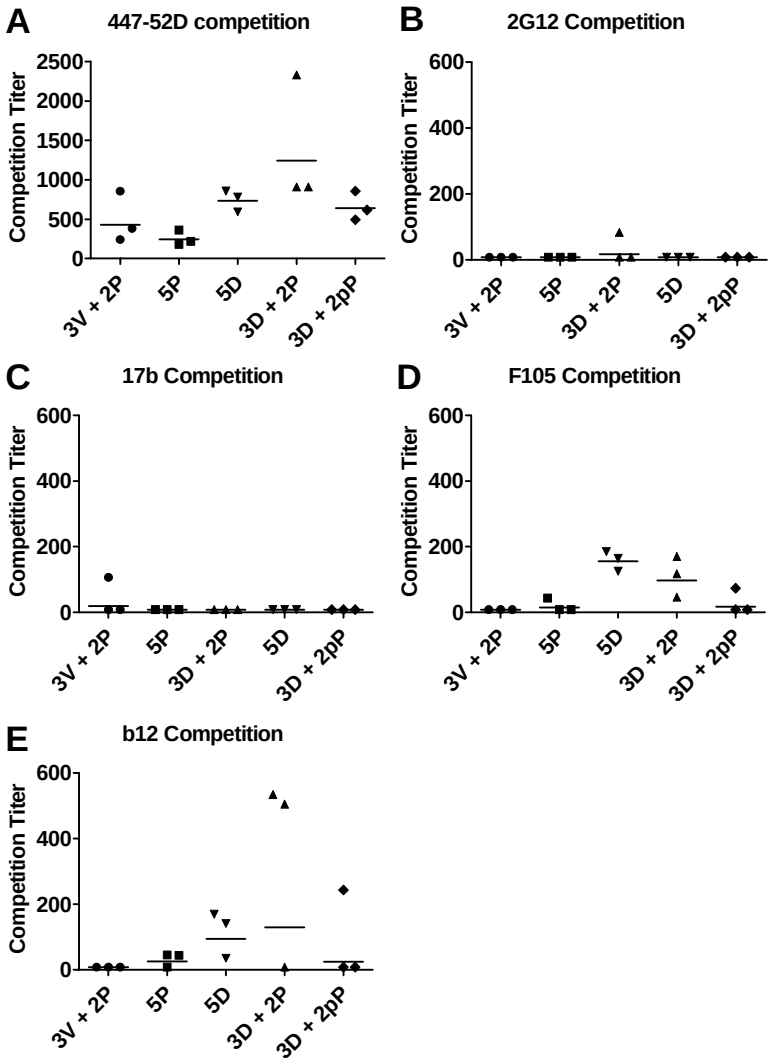

Fig 2.4. Specificity of JR-FL based vaccine induced antibody responses as determined by mAb competition. The ability of serially diluted polyclonal serum to outcompete binding of mAbs to a JR-FL & VSV-G pseudotyped virus was measured. Competition titers indicate the serum dilution preventing 50% of pseudoviral binding to the ELISA plate. % competition was calculated according to the following equation: % competition = [(RLUs in absence of sera – RLU in presence of sera)/RLUs in absences of sera]*100. A. Competition against the V3 directed mAb 447-52D. B. Competition against the glycan directed mAb 2G12. C. Competition against the co-receptor binding site directed mAb 17b. D. Competition against the narrowly neutralizing CD4bs mAb F105. E. Competition against the broadly neutralizing CD4bs mAb b12. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP).

vaccine vector prime had the lowest V3 loop-targeted antibody responses. The group with the highest antibody responses to this region included rabbits that received the DNA prime-protein boost regimen with the matched JR-FL boost. Rabbits in this group elicited a significantly higher V3-directed antibody response than rabbits that received five protein-based injections ($p=0.02$). Interestingly, the group that received a polyvalent gp120 boost had a lower 447-52D-like antibody response than the monovalent (JR-FL gp120) boost, suggesting that the polyvalent boost, which included gp120s from different subtypes, may dilute the focus of V3 specific antibodies away from subtype B V3 epitope.

Next, we expanded our analysis to antibodies against other important gp120 epitopes. This was conducted by determining any differences in the fine specificity of the antibodies elicited by each prime-boost immunization regimen. First, we looked for the presence of antibodies targeted to a cluster of carbohydrates recognized by the human mAb, 2G12 (Fig 2.4B). Of the 15 rabbits tested in this study, only one that received the homologous DNA prime-protein boost was capable of outcompeting binding to the mAb 2G12, indicating that antibodies of this specificity are rare with any of the immunization schemes used in the current study. This observation is also consistent with data in HIV infected humans, indicating that antibodies of this specificity are rarely elicited (94).

Next, we evaluated the rabbit immune sera for the presence of antibodies targeted to the co-receptor binding site by testing competition with the human mAb, 17b (Fig 2.4C). Again, with only a single rabbit capable of outcompeting binding, and at a titer barely reaching our cutoff of 50% reduction in binding at a 1:40 serum dilution, we determined that antibodies targeted to this domain were also largely absent. This result is

also consistent with a previous report showing that the failure to elicit co-receptor-targeted antibodies in rabbits was not unusual (34).

The presence of CD4bs specific antibodies in sera from immunized animals was also evaluated using either the narrowly neutralizing monoclonal antibody, F105 (Fig 2.4D), or the broadly neutralizing antibody, b12 (Fig 2.4E). When the mAb F105 was used as a competitive binding target, five of the six animals that received only protein immunizations demonstrated no capability of outcompeting binding to this monoclonal antibody. In contrast, all animals that received five DNA immunizations generated antibodies capable of outcompeting binding to F105, and did so with the highest competition titer among all groups with an average reciprocal dilution of approximately 150. Similarly, all three animals in the matched JR-FL DNA prime-protein boost group generated an antibody response capable of outcompeting binding to F105. Interestingly, when the protein boost formulation was changed from a single JR-FL gp120 to five recombinant gp120 proteins from different HIV-1 subtypes, antibodies capable of competing binding to F105 were largely absent, with only one of the three animals capable of doing so at a lower serum dilution.

This general trend continued when we tested the ability of the rabbit sera to outcompete binding to a second CD4bs mAb, b12, which can neutralize a wide range of primary HIV-1 isolates (Fig 2.4E). Again, the animals that received only two JR-FL gp120 protein-based immunizations with the empty DNA vector prime could not outcompete binding to b12 in any instance. Consistent with the F105 competition results, animals that received immunizations with only protein only sporadically elicited antibodies targeted to the CD4bs. In those sera where competition was detected against

b12, it was of very low titer, barely making our cutoff of a 1:40 dilution. Again, in the animals that received five DNA immunizations, we detected CD4bs directed antibodies at titers very similar to those observed against F105, at approximately a 1:150 dilution. Two out of the three rabbits that received a monovalent DNA prime-protein boost were capable of outcompeting binding to b12. This is one rabbit less than was capable of outcompeting binding to F105 within the same group. However, the two animals that did generate antibodies capable of outcompeting binding to b12 did so with high 50% competition values approaching serum dilution of 1:600. In rabbits that received the polyvalent protein boost, we again noticed that only one out of the three animals was capable of outcompeting binding to b12. This change in the boost formulation may result in the generation of antibodies that recognize the CD4bs of Envs from other clades. If this is the case, these non clade B CD4bs Abs may be unable to outcompete binding of the largely subtype B-specific antibodies, F105 and b12, to the viral envelope.

Neutralization of Tier 1 HIV-1 viruses

The competition analysis performed above indicated that every immunization regimen elicited antibodies to the V3 loop. However, since the ability to outcompete binding to V3-directed antibodies only confirms the presence of this type of antibody in the immune sera but does not reveal any information about their functionality, we evaluated how well these V3-directed antibodies are capable of neutralizing sensitive isolates of HIV-1. In order to do this, we utilized two viruses with a known sensitivity to V3-mediated neutralization. The first virus was SF162 (Fig 2.5A). Consistent with the presence of high titer V3-directed antibodies in rabbit sera, this virus was neutralized by serum from every animal in the study. However, the potency at which this was achieved

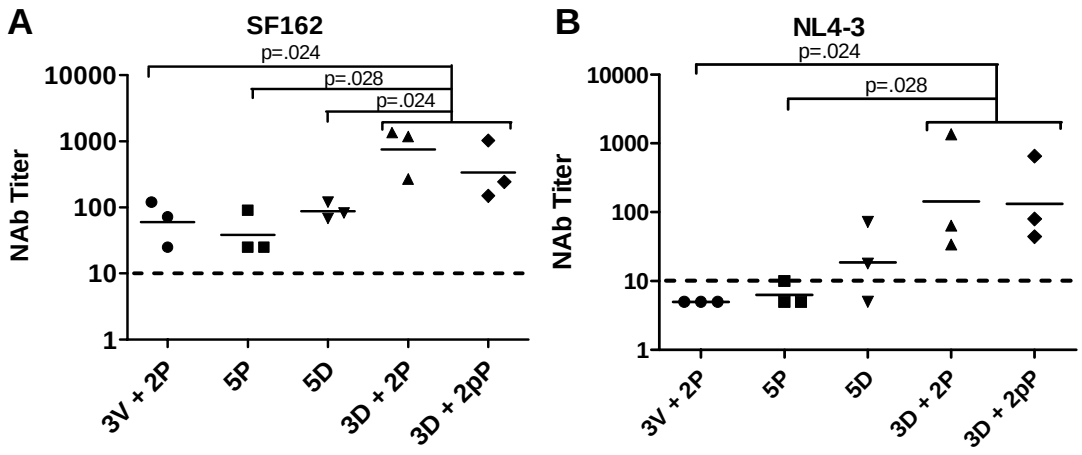

Fig 2.5 Neutralizing activity of sera elicited by different JR-FL based vaccine regimens against sensitive HIV isolates. Rabbit sera collected two weeks after the final boost immunization were tested for their ability to neutralize Tier 1 sensitive HIV isolates in the TZM-bl assay system A. Serum NAb titers against SF162. B. Serum NAb titers against NL4-3. Neutralization was calculated according to the following formula: % neutralization = [(Preimmune RLUs – Immune RLUs)/(Preimmune RLUs)]*100. NAb titer is defined as the serum dilution capable of inhibiting 50% of viral infection. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP).

differed between immunization groups. Geometric mean NAb titers in groups whose immunizations consisted of only a single vaccine modality were all below 1:100. Specifically, geometric mean NAb titers for animals that received two protein, five protein, or five DNA immunizations were 1:60, 1:38, and 1:88, respectively. In contrast, animals that received three DNA prime immunizations and were boosted with two protein immunization of either matched JR-FL protein or a polyvalent gp120 protein mix, achieved geometric mean NAb titers of 1:754 and 1:334, respectively. The increases in potency seen against SF162 when the heterologous DNA prime-protein boost regimen was used were statistically higher than those seen with the use of two protein immunizations ($p=.024$), five protein immunizations ($p=.028$), and the use of five DNA immunizations ($p=.024$). Rabbit sera were next tested against NL4-3, an HIV-1 isolate slightly more resistant to neutralization (Fig 2.5B). Again, consistent with the SF162 neutralization data, the antibody responses generated through a heterologous immunization regimen were significantly more potent than those generated through immunization with any single vaccination modality. Animals that received vector primes followed by two protein immunizations were completely incapable of neutralizing this virus. This trend only improved slightly in animals that received five protein immunizations. Within this group, only serum from a single animal was capable of neutralizing the virus, and only at a NAb titer of 1:10. Rabbits that received five DNA immunizations neutralized this isolate with slightly more frequency and potency. Two of the three animals in the group neutralized the virus with a geometric mean NAb titer of 1:19. In contrast to the sporadic neutralization seen with the single modality immunizations, all six rabbits that received the JR-FL DNA prime and either monovalent

or polyvalent protein boost were capable of neutralizing NL4-3. Geometric mean NAb titers of 1:143 and 1:132 were achieved for the two DNA prime-protein boost groups, respectively. This demonstrates another 7-fold increase in potency over the next best immune sera from animals that received five DNA immunizations. Again, the potency of neutralization observed when a combination DNA prime-protein boost regimen was administered was significantly higher than the potency of neutralization seen when two protein ($p=0.024$) or five protein ($p=0.028$) immunizations were given.

Further investigation of this V3-mediated neutralization led us to investigate its role in neutralizing the autologous HIV-1 JR-FL isolate. Initial screening of all sera against a JR-FL pseudovirus showed that greater than 50% neutralization was achieved in only a single animal in the JR-FL DNA prime-JR-FL protein boost group (Fig 2.6A). Previous studies have demonstrated that exposure to soluble CD4 (sCD4) sensitizes envelopes to V3-mediated neutralization (120). We utilized this phenomenon to further study the functionality of the V3-directed antibodies being generated by each immunization regimen. In these assays, JR-FL pseudovirus was exposed to sCD4 at 5 μg/mL and sera was then added to the virus to determine if any increases in neutralization were observed (Fig 2.6B). This produced even more striking differences in neutralization between immunization groups than those observed with SF162 or NL4-3. Against the sCD4 exposed homologous JR-FL virus, rabbits that received either two or five JR-FL protein immunizations were completely incapable of neutralizing the virus. Rabbits that received five DNA-based immunizations fared better with two of three animals capable of neutralizing the virus. When animals that received the JR-FL DNA prime and monovalent protein boost regimen were evaluated, sera from all three animals were

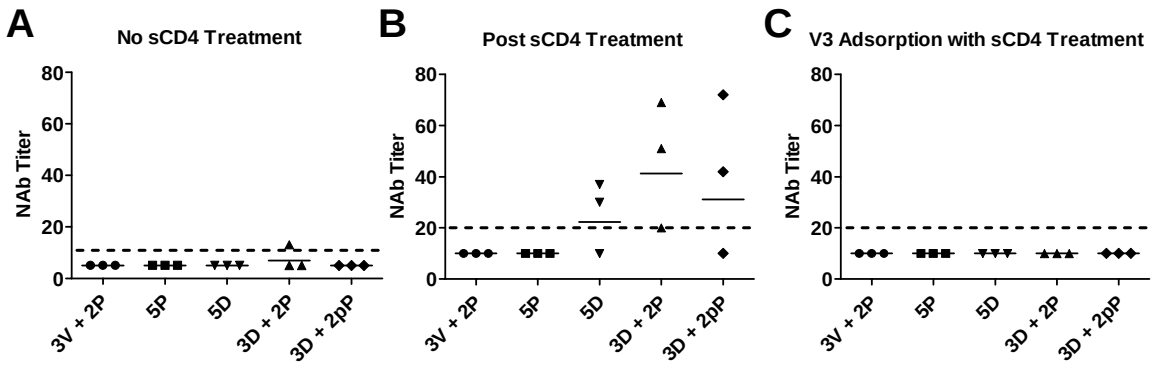

Fig 2.6. Ability of serum from rabbits who received JR-FL based immunizations to neutralize sCD4 exposed JR-FL. The functionality of V3-directed antibodies elicited by each immunization group was evaluated by their ability to neutralize a sCD4 exposed JR-FL pseudovirus. A. Neutralization of JR-FL prior to exposure to sCD4. B. Neutralization of JR-FL after exposure to sCD4. C. Neutralization of JR-FL after exposure to sCD4 when sera are preincubated with a matched V3 peptide. Neutralization in the absence of sCD4 was calculated according to the following formula: % neutralization = [(Preimmune RLUs – Immune RLUs)/(Preimmune RLUs)]*100. When virus was treated with sCD4, neutralization was calculated as follows: % neutralization = {[(Preimmune + sCD4 RLUs) – (Immune + sCD4 RLUs)]/(Preimmune + sCD4 RLUs)}*100. NAb titer is defined as the serum dilution capable of inhibiting 50% of virus infection. Dotted line indicates the lowest serum dilution tested. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP).

capable of neutralizing the sCD4 treated virus. In rabbits that received the JR-FL DNA prime and polyvalent protein boost, two out of three could neutralize the virus. Therefore, the inclusion of the DNA prime in the immunization regimen increased the quality of the V3-directed antibody response, making it more capable of neutralizing not only the V3 sensitive viruses, such as NL4-3, but also the more resistant JR-FL virus upon sCD4 treatment.

Despite evidence that wild type rabbits are not capable of generating antibodies to CD4 inducible sites, such as the co-receptor binding site (34), we wanted to confirm that the observed neutralization against JR-FL was in fact mediated by recognition of the V3 loop. Sera were first incubated with a JR-FL V3-matched peptide (CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC) at 25 μg/mL prior to the addition to sCD4 exposed JR-FL. As expected, this step eliminated all observed neutralization of the JR-FL virus (Fig 2.6C).

Neutralization of Tier 2 primary HIV-1 isolates

While we have already demonstrated some differences in the capacity of sera from each immunization regimen to neutralize sensitive or Tier 1 isolates, we also wanted to determine if there were any differences in the ability of these sera to neutralize other, more resistant primary isolates. To do this we chose to evaluate sera from each immunization group against viruses from the standard NIH Tier 2 clade B primary isolate panel (56). While, overall, these viruses are much more resistant to neutralization, we observed the superiority of the DNA prime-protein boost approach in eliciting sera capable of neutralizing these viruses (Table 2.1). When only two protein immunizations were given, none of the animals elicited a NAb response against any of the Tier 2 viruses.

Table 2.1: Neutralization of heterologous primary isolates by sera from rabbits who received JR-FL based immunizations.

	3 Vector 2 JR-FL Protein			5 JR-FL Protein			5 JR-FL DNA			3 JR-FL DNA 2 JR-FL Protein			3 JR-FL DNA 2 5-Valent Protein		
	617	618	619	620	621	622	626	627	628	623	624	625	629	630	631
6535.3	30	29	29	53	45	31	48	26	47	39	60	67	47	57	59
AC10.0.29	4	23	10	28	33	25	19	6	16	9	6	48	48	55	65
CAAN5342.A2	29	35	37	48	40	36	22	32	42	14	17	59	47	39	74
PVO.4	0	8	0	22	12	22	7	0	16	7	0	37	14	7	36
QH0692.42	24	22	29	25	34	33	39	46	61	24	43	61	25	50	50
REJO4541.67	0	0	25	37	23	29	12	8	30	0	11	35	0	4	24
RHPA4259.7	0	0	12	16	10	6	18	37	51	0	7	36	27	21	27
SC422661.8	10	29	8	33	27	32	21	29	14	17	25	55	44	52	66
THRO4156.18	0	0	0	0	1	0	0	0	0	0	0	22	0	0	28
TRJO4551.58	3	27	33	42	23	26	27	32	41	2	2	29	32	32	51
TRO.11	0	7	31	23	31	40	39	23	26	8	24	32	4	10	25
WITO4160.33	0	1	8	27	0	9	14	2	23	0	0	18	0	0	10
MLV	0	11	1	6	8	0	3	0	15	0	4	14	6	0	13

Numbers indicate % neutralization at a 1:10 serum dilution

When five protein immunizations were given, neutralization of these Tier 2 viruses was also absent with the exception of one animal (Rabbit #620) that was capable of neutralizing the 6535.1 isolate. Furthermore, administration of five DNA immunizations did not improve neutralization of these viruses; as Rabbits #626 and #627 were not capable of neutralizing any of the Tier 2 viruses while Rabbit #628 neutralized two out of the 12 isolates (QH0692.42 and RHPA4259.7) at a 1:10 dilution. The neutralizing activity of sera from animals immunized with a DNA prime-protein boost immunization regimen improved slightly, but not dramatically. Within this immunization group, one of the animals (Rabbit #625) was capable of neutralizing four of the 12 isolates. However, this was not typical as Rabbit #624 could only neutralize one of the 12 isolates and Rabbit #626 could not neutralize any. When animals were given the JR-FL DNA prime followed by a polyvalent protein boost, the consistency and breadth of neutralization were further increased. The best neutralizer we encountered, Rabbit #631, neutralized six isolates (6535.3, AC10.0.29, CAAN5342.A2, QH0692.42, SC422661.8, and THRO4156) at a 1:10 dilution. Rabbit #630 neutralized four isolates in the same panel as Rabbit #631. Rabbit #629 however, could not achieve 50% neutralization against any of the viruses tested. The observed neutralization was HIV-1-specific as none of the sera neutralized a control Murine Leukemia Virus (MLV) pseudotyped virus (Table 2.1).

Evaluating the avidity of elicited antibody responses

Previous studies have suggested that a heterologous DNA prime-protein boost approach was able to elicit antibody responses with higher avidity than the homologous DNA or protein alone approaches (87, 113). Importantly, recent evidence has indicated that antibody avidity may correlate to better protection against SHIV challenge even in

the absence of a NAb response in a non-human primate SHIV challenge study (121). In the current study, the avidity of rabbit immune sera being elicited by each immunization group was evaluated by measuring how well the serum remained bound to gp120 in the presence of increasing concentrations of sodium thiocynate (NaSCN) in an ELISA based assay. Evaluating the sera elicited by each regimen in this manner revealed that serum avidity differed greatly between different immunization regimens (Fig 2.7). By comparing bound IgG from our test animals against an IgG standard, we were able to calculate at which concentration of NaSCN 50% of the original bound IgG becomes displaced. Sera from animals who received only two protein injections were most easily displaced by NaSCN. Half of all bound IgG was displaced with an average of a 1.9 M solution of NaSCN. When five protein immunizations were given instead of two, 50% of IgG remained bound to the plate in a 2.8 M solution of NaSCN. Serum avidity increased further with the use of five DNA immunizations. Sera from rabbits that received this regimen maintained 50% of IgG binding at a concentration of 3.4 M NaSCN. The use of a DNA prime-protein boost approach improved this further to an average 50% displacement at 3.6 and 3.9 M NaSCN for the monovalent and polyvalent gp120 boosted groups, respectively. These observed increases in binding avidity with the DNA prime-protein boost regimen were found to be statistically higher than those seen when only protein-based immunizations were used ($p = 0.024$). Because only relatively small gains in serum avidity were seen with the inclusion of a protein boost, it is likely that the use of a DNA immunization induces an initial antibody response with a higher avidity than is observed with an initial protein immunization. The increase in binding avidity may be

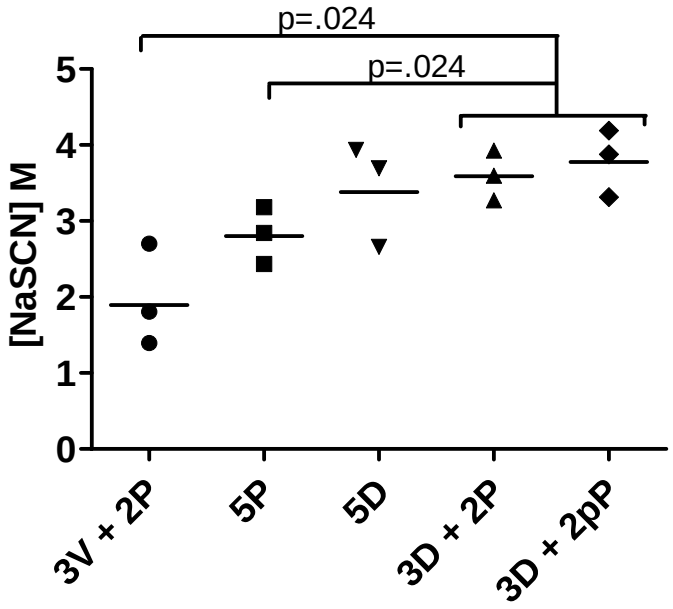

Fig 2.7 Measurement of serum avidity elicited by different JR-FL based immunization regimens. Sera were evaluated for their ability to be displaced from autologous JR-FL gp120 by increasing molar concentrations of sodium thiocynate (NaSCN). Dots indicate concentration of NaSCN required to displace 50% of bound IgG. Bars indicate group geometric mean. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP).

one of the reasons why the DNA prime-protein boost regimen appears to be superior to either DNA or protein alone in eliciting a functional antibody response.

Discussion

Recent studies have suggested that a heterologous prime-boost vaccination approach in which the same antigen is delivered sequentially by different types of vaccines is more effective in eliciting humoral immune responses than a homologous prime-boost regimen which utilizes only a single vaccine modality (61). In the current study we have built upon previous work (108, 113) in evaluating humoral responses generated using HIV-1 gp120 antigen as a model antigen delivered by different prime-boost regimens. In this chapter, a rigorous comparison of immunizations was conducted with two or five protein vaccinations, five DNA vaccinations, or a combination of DNA and protein vaccinations in a rabbit model. We demonstrated that all regimens studied were capable of eliciting an equivalent binding antibody response. However, sera generated by each of these immunization regimens proved to differ greatly in more important characteristics including specificity, neutralizing activity, and avidity. This finding may have a significant impact on the future development of vaccines.

Different vaccine delivery approaches have been developed based on the available technology at any given time in history. To date, efficacy and safety have been the final parameters driving the development of different vaccine delivery approaches. However, little work has been done to compare the detailed parameters of humoral responses resulting from these different vaccine delivery approaches. Unfortunately, immune correlates of protection are not well understood, even for licensed human vaccines. While antibodies are well recognized for playing a major role in protection for many

successful vaccines, it is frequently not clear what specific mechanism contributes to such protection. This situation became even more complicated with the discovery of a newer generation of vaccination approaches, such as DNA vaccines, viral vector-based vaccines, and heterologous prime-boost strategies, because there have been very few studies examining how these newer immunization regimens affect the quality of the final antibody response.

Due to the challenge of developing an HIV vaccine, many novel approaches have been developed and tested with the goal of raising an optimal antibody response to the HIV-1 Env glycoprotein. The current study utilized some of these novel approaches in order to conduct a detailed analysis on the quality of antibody responses elicited by different prime-boost vaccination strategies.

We hypothesized that the measurement of binding antibodies against a protein antigen by a polyclonal animal serum may not reflect the difference in detailed antibody profiles of such immune sera. It is well-known that the gp120 form of HIV-1 Env protein-based immunizations typically leads to antibodies targeted to the immunodominant V3 loop. We used this region as the first model antigen determinant to identify a number of differences among different prime-boost immunization regimens. As expected, all immunization regimens used in our study generated antibodies to this domain, based upon their ability to outcompeting binding to the V3-directed mAb, 447-52D. However, it became apparent that immunization regimens that included a DNA-based immunization elicited antibodies that were more capable of outcompeting binding to 447-52D. This manifested itself at a functional level in DNA-immunized animals as it was observed that these animals were more capable of neutralizing the V3 sensitive

isolate, NL4-3. Similarly, only sera from animals that received a DNA immunization demonstrated neutralizing activities against the CD4-sensitized primary isolate, JR-FL. Through the use of V3 peptide adsorptions, we further demonstrated that this neutralizing activity was mediated by antibodies recognizing the exposed V3 loop after CD4 treatment. This indicates that the DNA priming step either provides a more relevant antigen conformation or generates antibodies with some biophysical quality that is superior than those generated through the protein alone vaccination approach.

Further investigation into epitopes outside of the V3 loop also yielded interesting results. While little to no antibodies targeted to CD4-induced (17b-like) or glycan (2G12-like) epitopes were seen through immunization with any regimen, a significant number of antibodies capable of outcompeting the CD4bs mAbs, F105 and b12, were observed. However, these CD4bs directed antibodies were only seen in animals that had received some form of DNA based immunization. Also interesting to note is data suggesting that the fine specificities of these antibodies can be shifted with different types of protein boosts. Vaccination regimens that included the JR-FL monovalent gp120 formulation as either DNA alone or in a DNA prime-protein boost format, elicited detectable levels of F105 or b12 competition in almost every instance. However, when the JR-FL DNA prime was followed by a polyvalent gp120 protein boost, only a single rabbit was capable of outcompeting binding to F105 and b12. This may reveal a shift in specificity to CD4 binding sites of subtypes other than clade B, or potentially, but less likely, a loss of CD4bs directed antibodies altogether. The fact that antibodies to this domain are being generated at all is potentially important based upon evidence that broadly neutralizing activity in some individuals is mediated by antibody recognition of

this domain (57). This observation makes the use of DNA vaccines a potentially attractive platform for HIV vaccine development.

While using competition assays to dissect antibody specificities revealed interesting trends, likely the most relevant comparison is that of the neutralizing activity elicited by each regimen. Consistent with previous reports (108, 113), animals that received a heterologous DNA prime-protein boost were better capable of neutralizing relevant primary isolates. Neutralization of primary isolates was almost completely lacking in rabbits that received antigen by only a single vaccine modality. In addition to the issue of the method of immunization, and in concurrence with previous data (118), a polyvalent formulation of Env antigens appears to play an important role in eliciting a broader NAb response than immunization with only a single Env antigen. This may be the result of too much focus on a single Env leading to a more potent, but less broadly Nab response when only a monovalent formulation is used.

Additional data demonstrating that immunization with some form of DNA based vaccine increases serum avidity to the vaccine antigen is also enlightening. It may be possible that the smaller amount of antigen being produced from the initial DNA immunizations results in a higher avidity antibody response. This may prove to be an important facet of a potential vaccine in light of recent data indicating that serum avidity inversely correlates with viral load after challenge (121). This result points to the need for future studies to understand how DNA immunization may affect the generation of B cells that produce high avidity antibodies.

In summary, the studies detailed in this chapter expanded on preliminary findings that a combination prime-boost regimen elicits a different profile of antibody responses

than immunization with only protein. One interesting point however, is found in the comparison between the 5 DNA immunization regimen, and the DNA prime-protein boost regimen. Overall, despite similar avidity levels and antibody specificity, as measured by competition assay, the 5 DNA immunization group was largely unable to neutralize any heterologous primary isolates. This indicates that a protein boost given after a DNA immunization may refine the specificity of antibodies originally elicited during the DNA priming phase. However, without fine mapping data, this hypothesis is difficult to prove.

Chapter III

Unique elicitation of CD4 binding site antibodies by a DNA prime-protein boost regimen in rabbits

Introduction

In our pre-clinical and clinical immunogenicity studies, we reported that polyvalent gp120 formulations, delivered by the DNA prime-protein boost approach, elicited NAbs effective against viruses belonging to several different major subtypes of HIV-1 (117, 118). In chapter II we began to elucidate the mechanism underlying this phenomenon further by identifying unique components of the antibody response that are elicited when a DNA component is included in the immunization regimen. We next wanted to further this analysis in a preclinical study which laid groundwork for the eventual DNA prime-protein boost trial in humans (117).

Utilizing a polyvalent formulation of gp120s identical to that used in the DNA prime-protein boost clinical study (117), NZW rabbits were immunized with either a DNA prime-protein boost regimen or a protein alone regimen. The resulting antibody responses were evaluated for their fine specificity using peptide mapping and competitive binding assays in order to determine if unique regions of gp120 were being recognized by sera from animals given a DNA prime-protein boost regimen.

Results

Eliciting neutralizing antibodies in rabbit sera using a polyvalent DNA prime-protein boost Env vaccine formulation DP6-001

In order to begin to investigate the mechanism behind the broader neutralization seen when a DNA prime-protein boost regimen is used, we immunized rabbits with the

same 5-valent gp120 vaccine formulation that induced low titer but positive NAbs effective against a wide range of primary Env antigens in a recently completed phase I clinical study (117). NZW rabbits were immunized with a 5-valent gp120 formulation in either a DNA prime-protein boost or protein alone regimen as described in Figure 3.1. Sera collected from rabbits two weeks after the 2nd protein boost in both groups were used to evaluate peak level gp120 specific IgG and NAb responses. By evaluating sera in this manner, we determined that the peak level gp120-specific binding titers were very similar between the two immunization groups (Fig. 3.2). However, similar binding antibody titers did not lead to a similar functional neutralizing antibody response. We determined that sera from rabbits that received the polyvalent DNA prime-protein boost possessed higher neutralizing activities against a panel of pseudotyped viruses in the PhenoSense neutralization system (Table 3.1). Here, we examined three sensitive viruses (MN, NL4-3 and SF162) and 12 viruses relatively resistant to neutralization with Env antigens derived from primary isolates of clades A, B, C, D, and E. In this assay, only the neutralizing activity present at a 1:30 dilution is reported to provide a more stringent cutoff for positivity. All sera, regardless of immunization regimen, neutralized the Tier 1 sensitive isolates MN, NL4-3, and SF162, at this serum dilution. In contrast to this, only sera from the DNA prime-protein boost group were capable of frequent neutralization of the more resistant primary isolates. We determined that the DNA prime-protein boost group neutralized 8 out of the 12 resistant viruses tested. Neutralization of primary isolates from clades A, C, and D was observed in almost every case in the DNA primed rabbit sera. Three viruses (AC10.0.29, PVO.4 and QH0692.42) from the NIH's Tier 2 clade B standardized panel (56) and JR-CSF (another neutralization resistant

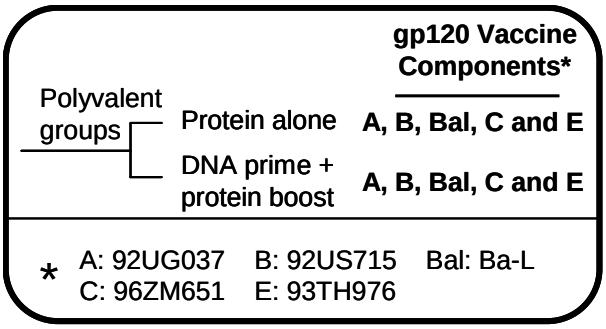

Fig 3.1: Immunization groups and 5-valent gp120 vaccine formulation. Rabbits received either a DNA prime-protein boost or protein alone immunization regimen.

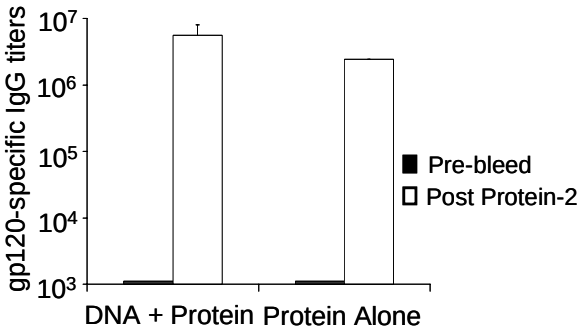

Fig 3.2. gp120 specific IgG titers in rabbits immunized with either a DNA prime-protein boost regimen or a protein alone regimen against autologous antigens. Data are shown as geometric mean titers with bars indicating standard deviation within a group.

Table 3.1. Neutralizing antibody responses against a multi-clade panel of HIV isolates elicited by polyvalent envelope formulations

HIV-1 isolate (subtype)	% Neutralization for vaccine group:					
	DNA prime + protein boost				Protein alone	
	29-1	29-2	30-1	30-2	33-1	33-2
MN (B)	99.9	99.9	99.9	99.9	99.1	92.7
NL4-3 (B)	97.6	98.6	99.7	96	94.3	93.9
SF162 (B)	99.9	99.9	99.9	99.9	99.8	98.4
92RW020 (A)	54	57.5	55.6	50.4	—	—
94UG103 (A)	—	51	51.6	55.4	—	—
SC422661.8 (B)	—	58.6	—	56.4	—	—
93IN905 (C)	62.1	66.6	66.7	63.3	—	59.2
98CN006 (C)	53	56.1	—	50	—	—
92UG046 (D)	53.4	56.1	54.7	54.1	—	—
94UG114 (D)	57.2	57.7	55.2	60.3	—	—
92TH021 (E)	—	—	51.9	53.9	—	50.2

Numbers indicate the percent neutralization of indicated virus at a 1:30 serum dilution. "–" indicates less than 50% neutralization at a 1:30 serum dilution.

isolate) were not neutralized by any of the rabbit sera (data not shown). Two rabbit sera had neutralizing activity against SC422661.8, from the NIH's Tier 2 clade B standardized panel. The clade E isolate, 92TH021, was neutralized by only two out of four DNA primed sera. In contrast, sera from rabbits immunized with only protein did not neutralize most of the primary viruses. Additional negative controls were also assayed in the PhenoSense assay. Rabbit immune sera were tested against Murine Leukemia Virus (MLV). Against this irrelevant isolate no neutralization was seen. Additionally, preimmune rabbit sera were tested against all HIV isolates in this panel, again no neutralization was observed, indicating that all neutralization seen here is HIV specific.

Epitope mapping of the polyvalent sera using linear overlapping peptides

To determine differences in antibody specificity between DNA primed versus protein alone polyvalent rabbit sera, we first measured serum binding to linear 15-mer peptides with an 11 residue overlap derived from the group M consensus gp120 sequence. In order to fairly evaluate reactivity irrespective of endpoint titer, all rabbit sera were normalized to equivalent gp120 specific binding levels of 200 ng/mL. Sera from both groups had strong reactivity to peptides derived from the C1, C2, V3, and C5 segments of gp120 (Fig 3.3), including two prominent regions within the V3 loop. Very little reactivity was observed against peptides derived from the V1/V2 and V4 loops of gp120 for any of the sera tested.

Interestingly, rabbit sera from the DNA primed group exhibited unique positive binding against six regions that were poorly recognized by the protein only sera (see arrows in Fig. 3.3). The size of these six regions vary: peptides 11 (p11) and 30 (p30) were recognized as single peptides, peptides 48-49 (p48-p49) and 56-67 (p56-p57) as two

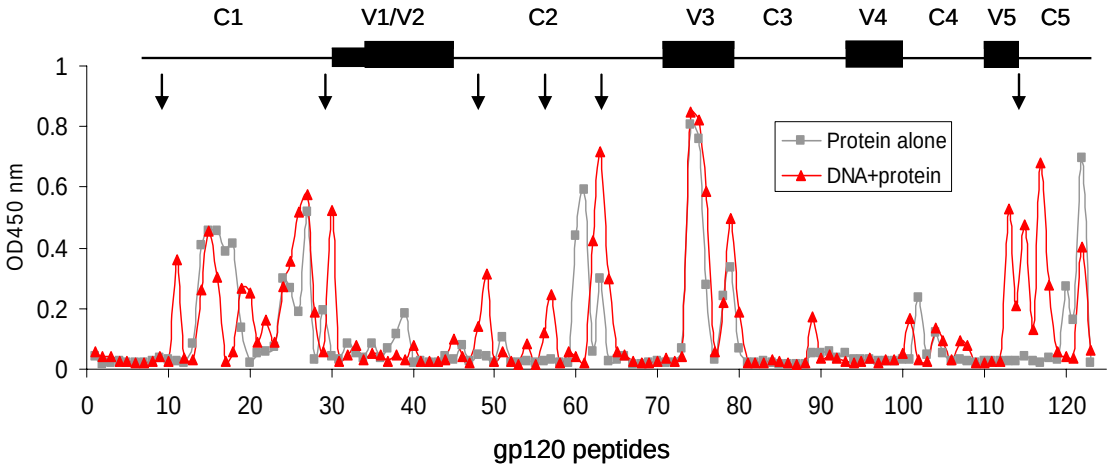

Fig 3.3. Recognition of Consensus M linear overlapping peptides by normalized rabbit sera. Sera from rabbits immunized with either a DNA prime-protein boost immunization regimen (red curves) or a protein alone immunization regimen (black curves) were screened for recognition of overlapping peptides from the gp120 subunit by ELISA. Each data point represents average OD values from each animal per immunization regimen. Increases in optical density (OD) indicate stronger recognition of the linear peptide. Arrows indicate individual or clusters of peptides that were uniquely recognized by animals who received a DNA prime-protein boost regimen.

adjacent peptides, and peptides 61-64 (p61-p64) and peptides 113-117 (p113-p117) as a cluster of several neighboring peptides. Further analysis of these six highly reactive regions indicate that three of them, p30, p61-p64 and p113-p117 contain amino acid residues that are part of either the CD4bs or are involved in the binding of the neutralizing mAb b12 based on a previously published Env structure (48, 122). The sequences of these peptides are listed in Fig. 3.4. Average binding to these peptides was compared between sera from the two immunized groups. The DNA prime-protein boost group sera had significantly greater recognition of these three regions when compared to the protein alone group sera ($p < 0.01$) (Fig. 3.4). Overlay of these sequences onto the crystal structure of gp120 demonstrates that the locations of these peptides (in blue) are in the known CD4 binding region on gp120, overlapping with either previously reported CD4 binding residues (Fig. 3.4B, in red) or b12 binding residues (Fig 3.4C, in orange).

Because a polyvalent Env formulation was used in this rabbit immunization study and the sequence differences of these Env proteins, particularly in the variable loops, may have precluded recognition of the consensus M peptides, we generated overlapping peptides (20mers with 10 aa overlapping) to each of the variable loops from four primary gp120 antigens that were used in our study: 92UG037 (clade A), 92US715 (clade B), 96ZM651 (clade C) and 93TH976 (clade E). These peptides were then tested for antibody recognition with the polyvalent rabbit sera by ELISA (Fig 3.5). Some sequence-specific variable loop recognition was observed by sera from immunized rabbits against peptides derived from the V1/V2 loop of clades A and E Env antigens, in contrast to peptides derived from the V1/V2 loop of clades B and C Env antigens. However, there was no differential recognition in this region between the DNA primed

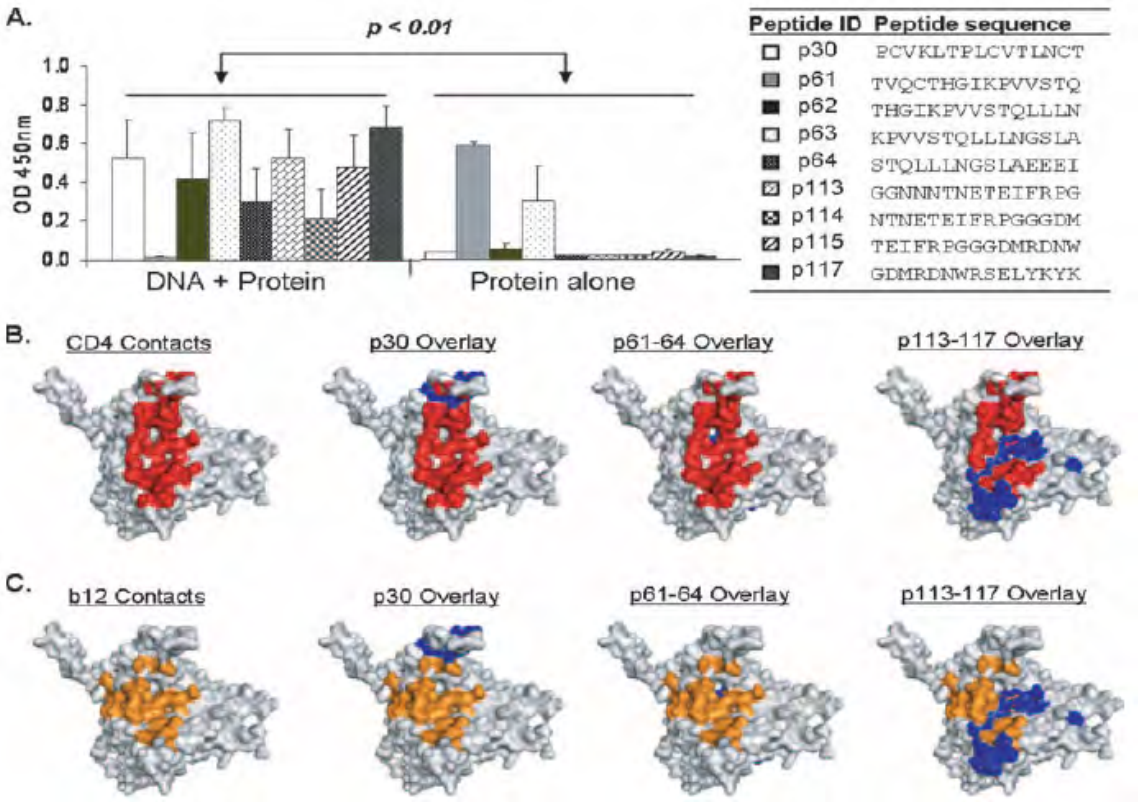

Fig 3.4 Additional analysis of three regions of gp120 uniquely recognized by DNA primed-protein boosted rabbits. A) Recognition of linear peptides that contain CD4 or b12 contact residues by sera from DNA plus protein or protein alone groups by ELISA. Data is represented as the mean OD values within a group with error bars denoting standard deviation. Sequences of recognized peptides are listed on the right. B) Location of the peptides (blue) uniquely recognized by sera from DNA prime-protein boosted animals mapped onto the crystal structure of JR-FL gp120 liganded with sCD4 and mAb X5. CD4 contact residues are highlighted in red. C) Location of the peptides (blue) uniquely recognized by sera from DNA prime-protein boosted animals mapped onto the crystal structure of JR-FL gp120 liganded with sCD4 and mAb X5. b12 contact residues are highlighted in orange.

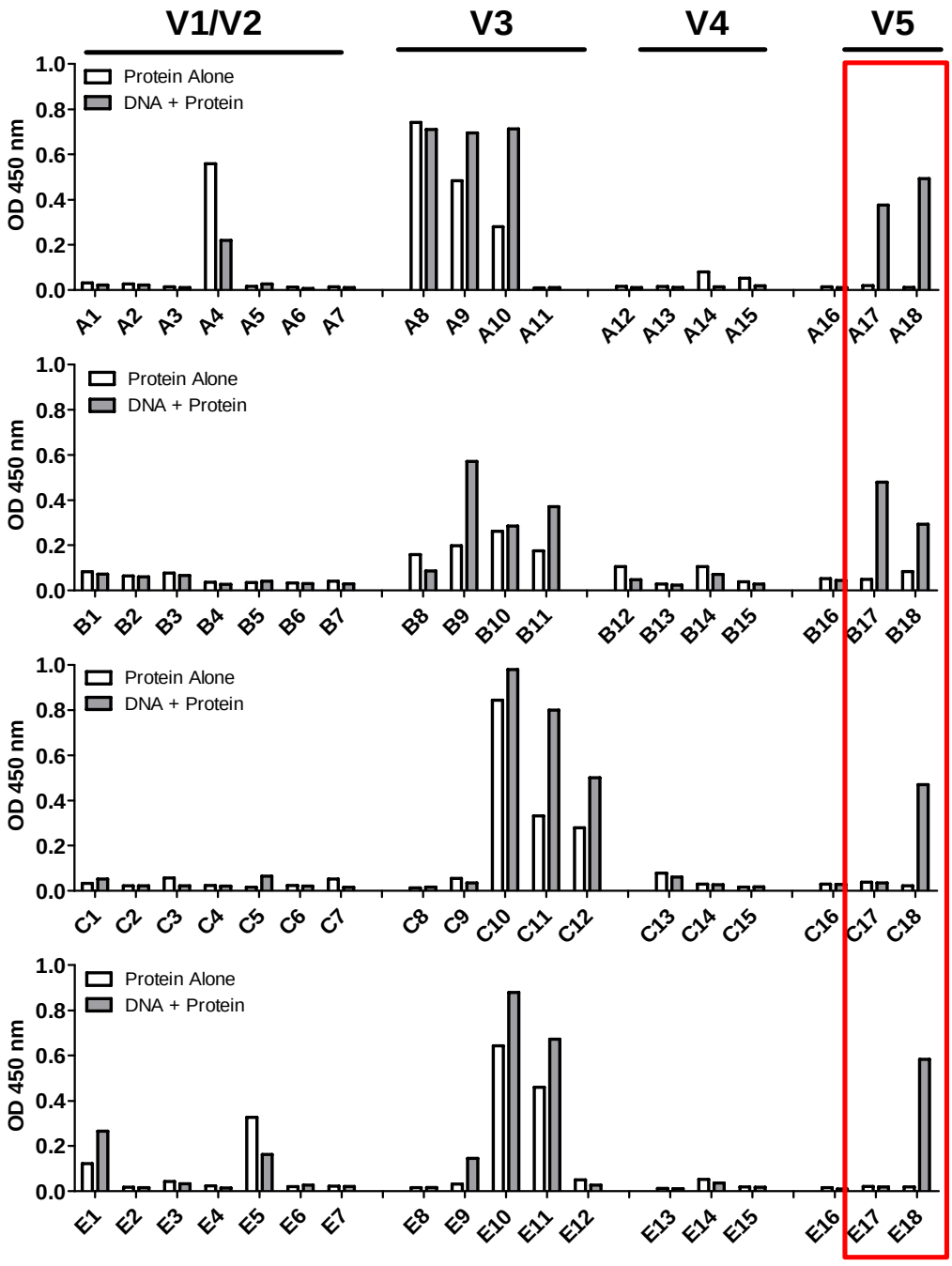

Fig 3.5. Polyvalent rabbit sera recognition of linear peptides with sequences derived from the variable loops of the vaccine antigens. Peptides derived from the variable loops of gp120s from clades A, B C, and E were used in graphs A, B, C, and D respectively. Gray bars indicate recognition of peptides from sera of DNA prime-protein boosted animals while white bars indicate recognition of peptides from sera of protein alone immunized animals. The red box corresponds to peptides spanning regions that were also seen to be uniquely recognized by DNA primed-protein boosted animals against consensus M peptides.

and protein alone groups. Reactivity to the V3 loop was observed for both the DNA prime-protein boost and protein alone immunization groups across all gp120 derived peptides tested here (Fig. 3.5). Once again, very little reactivity was generated against peptides in V4 region (Fig. 3.5). For the V5 region, it is striking to find that there are 1-2 peptides, located at the junction of the V5 and C5 regions, that were recognized by the sera elicited with the DNA prime-protein boost approach but not the protein alone sera (Fig. 3.5). Peptides 17 and 18 in Fig. 3.5 correspond to the amino acid sequences included in peptides 115 to 117 as shown in Fig. 3.3, and are therefore, in the region involved in CD4 binding (96).

Mapping the specificity of polyvalent sera using virus capture competition

Competitive virus capture was used to further map antibody specificities. Here, polyclonal sera were monitored for their ability to outcompete binding of mAbs with known specificity to an HIV pseudovirus. We began by looking at antibodies targeted to the immunodominant V3 loop of the HIV Env. Using the V3 specific mAbs 39F and LE311 we demonstrated that antibodies with similar V3 directed specificities in the polyclonal sera were found in significantly higher titers in animals that received a DNA prime (Fig. 3.6). Next, we tested our sera against the glycan specific antibody, 2G12. Against this particular mAb, no significant competition was detected in rabbits immunized with either immunization regimen. Due to its conserved nature across multiple clades, we also tested for the presence of antibodies targeted to the CD4bs. To do this we used two antibodies targeted to this domain, the broadly NAb, b12, and the narrowly NAb, 15e. The use of these antibodies as competitive targets revealed that higher titers of CD4bs specific antibodies were being elicited in animals that received a

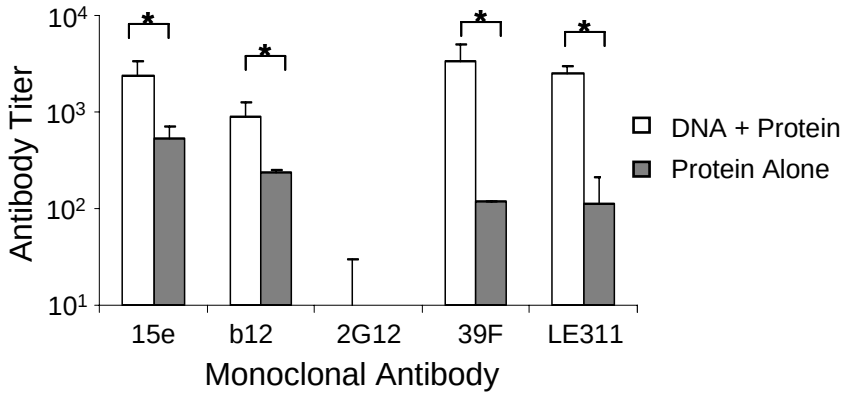

Fig 3.6. Detection of antibody specificities in polyvalent rabbit sera by competition assay. Monoclonal antibodies were used to probe the specificities of antibodies present in polyclonal sera elicited by a DNA prime-protein boost or protein alone immunization regimen. Antibody titer indicates the serum dilution capable of preventing 50% of virus binding to a mAb coated plate. % competition was calculated according to the following equation: % competition = [(RLUs in absence of sera – RLU in presence of sera)/RLUs in absences of sera]*100. White bars indicate group mean titers from sera resulting from a DNA prime-protein boost regimen. Gray bars indicate antibody titers from protein alone immunized animals.

DNA prime compared to those that received only protein. The data described here demonstrate that the DNA prime-protein boost approach is more effective in eliciting V3 and CD4bs antibodies compared to the protein alone approach. However, these data do not prove that these antibodies are responsible for the improved NAb activities observed in the DNA primed rabbit sera (Table 3.1).

To elucidate any potential role of the elevated levels of V3 directed antibodies in the neutralization of HIV isolates, we attempted to adsorb the V3-specific antibodies by incubating the sera with 15-mer clade B consensus peptides spanning the N-terminal strand of the V3 loop (TRPNNNTRKSIHIGPGRAF) as studies have shown this area to be the target of several neutralizing V3 mAbs (9, 101-103). A pilot experiment was conducted to confirm that these peptides were capable of adsorbing V3 mediated neutralizing activity in rabbit sera by using serum from a control rabbit that was immunized with only a V3 peptide fused with a carrier protein (123). V3 peptide adsorption of this serum resulted in a greater than 95% decrease in the neutralizing activity against pseudotyped virus expressing Env from HIV-1 isolate SF162, an isolate very sensitive to V3 mediated neutralization (Fig 3.7A).

When sera from the polyvalent DNA prime-protein boost rabbits were first incubated with these V3 peptides and then tested for their neutralizing activities against pseudotyped virus expressing Env from the HIV-1 isolate SF162, we observed that the sera isolated from DNA prime-protein boosted animals were more sensitive to a depletion in neutralization (Fig. 3.7B). Sera from rabbits that received a DNA prime had an average of 79% reduction in NAb titers as compared to a 50% reduction in animals that received only protein-based immunizations. The greater sensitivity of the DNA primed

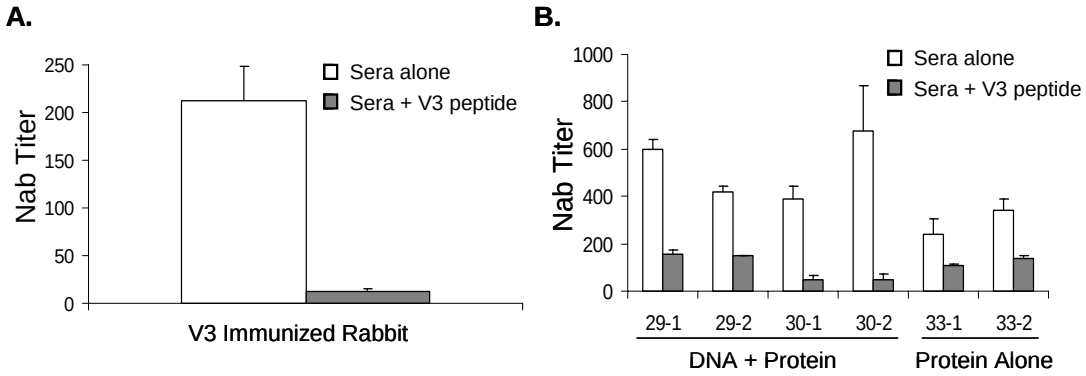

Fig 3.7. Effect of V3 peptide adsorption on the neutralizing activity of polyvalent rabbit sera against SF162. Clade B consensus V3 peptides were incubated with rabbit sera at 30 ug/mL prior to exposure to the SF162 pseudotyped virus. Neutralization was calculated according to the following formula: % neutralization = [(Preimmune RLUs – Immune RLUs)/(Preimmune RLUs)]*100. NAb titer indicates the serum dilution that prevents 50% of virus infection. A) Effect of peptide adsorption on the neutralizing activity of sera from a rabbit that was immunized with only a V3 fusion protein. B) Effect of V3 adsorption on the neutralizing activity of sera from rabbits who received either a DNA plus protein regimen, or a protein alone regimen. Error bars indicate standard deviation from replicate experiments.

sera to V3 peptide adsorptions supports the previous data that V3-directed antibodies are present at higher levels in DNA primed animals.

We next addressed the role of V3-directed antibodies in the neutralization of more resistant primary isolates. We repeated the V3 peptide adsorptions in an attempt to neutralize two clade B primary isolates, SS1196 (Fig. 3.8A) and SC422661.8 (Fig. 3.8B). We observed that V3 peptide adsorption had very little effect on the neutralization of SS1196 with only an average of a 9% reduction in neutralization. Against the second primary isolate, no reduction in neutralization was observed in the presence of the V3 peptide. This indicates that while V3 antibody levels may be elevated in the DNA primed animals, they play a minimal role in the neutralization of the more neutralization resistant isolates tested here. This suggests that the increased levels of CD4bs antibodies or other unknown conformational antibodies may contribute to the enhanced neutralizing activities of the DNA primed sera against more resistant primary viral isolates.

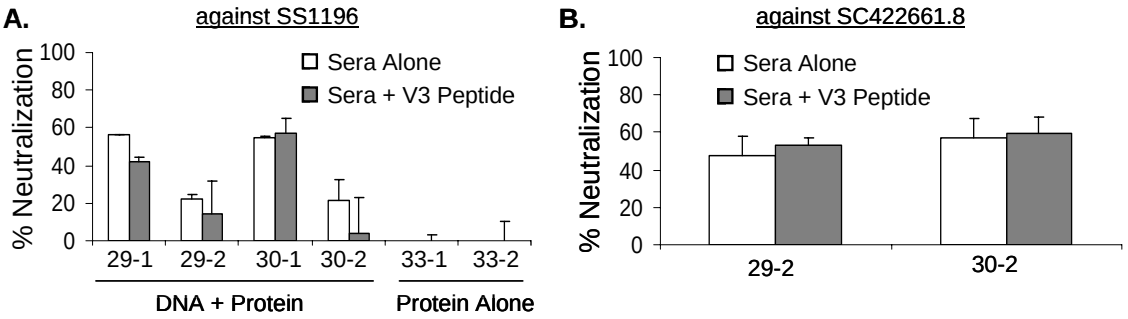

Fig 3.8. Effect of V3 peptide adsorption on the neutralizing activity of polyvalent rabbit sera against primary HIV-1 isolates. Clade B consensus V3 peptides were incubated with rabbit sera at 30 ug/mL prior to exposure to the SF162 pseudotyped virus. Neutralization was calculated according to the following formula: % neutralization = [(Preimmune RLUs – Immune RLUs)/(Preimmune RLUs)]*100. NAb titer indicates the serum dilution that prevents 50% of virus infection. A) Effect of V3 peptide adsorption on neutralization of the clade B isolate SS1196. B) Effect of V3 peptide adsorption on the neutralization of the clade B isolate SC422661.8.

Discussion

The phenomenon of a DNA prime-protein boost regimen eliciting a broader NAb response when compared to immunization with only protein was demonstrated previously (109, 118). However, the mechanism underlying the phenomenon was unclear. Our previous study implicated the use of a DNA component in the immunization regimen in altering the antibody specificity and avidity of the resulting antibody response. In particular, we observed an increase in the presence of antibodies capable of outcompeting binding to V3 and CD4bs specific mAbs in a competitive binding assay.

In this study, we confirmed previous data indicating that immunization with a DNA prime-protein boost regimen elicits a superior NAb response. Through the use of peptide mapping we provide additional evidence that a unique profile of antibody responses were being elicited when a combination DNA plus protein regimen was administered. We observed a unique pattern of linear epitope recognition in the sera of animals that received a DNA prime. These uniquely recognized areas were largely focused within the conserved regions of gp120, in particular the C1, C2 and C5 regions. Many of these uniquely recognized peptides, in particular the ones derived from the C2 and C5 regions, contained known contact residues for either sCD4 or the CD4bs directed mAb, b12. This peptide binding data was further supported by additional competitive binding data which indicated that animals that received a combination DNA plus protein regimen produced sera that was more capable of outcompeting binding to V3 and CD4bsspecific antibodies. When taken together, these data implicate that the combination of DNA and protein immunizations are better able to elicit antibodies to functionally conserved domains such as the V3 loop and CD4bs. Because antibodies to the CD4bs

have been implicated in broad neutralization in HIV infected individuals (57), it is possible that the unique elicitation of antibodies to this domain are responsible for the broader neutralization observed when a combination prime-boost immunization is administered. Unfortunately, because CD4bs specific antibodies are difficult to selectively deplete, we were unable to definitively prove that antibodies with these specificities are responsible for the broader neutralization seen when a combination immunization is used. Despite this, we were able to demonstrate that the increased levels of V3 directed antibodies are likely not entirely responsible for the increased breadth of neutralization, as very little neutralizing activity was adsorbed using V3 peptides against select clade B isolates.

Chapter IV

Unique elicitation of CD4 binding site antibodies by a DNA prime-protein boost regimen in humans

Introduction

After establishing in small animals that a different profile of antibody responses are being elicited when a combination DNA prime-protein boost regimen was used compared to only protein, we next wanted to determine if this effect was also seen in the sera of immunized humans. In order to do this, we obtained samples from three different HIV vaccine trials. In these trials, healthy human volunteers received one of three different immunization regimens. Volunteers from the first of these trials, HVTN 041, received a protein alone regimen. Volunteers from the second trial, DP6-001, received a DNA prime-protein boost regimen and volunteers from the third trial, HVTN 203, received a canarypox prime-protein boost regimen. This trial is of particular interest as it was one of the phase II trials leading up the phase III RV144, which showed partial protection from HIV acquisition. Using sera from these trials, we evaluated several characteristics that could potentially be important in providing protection. The parameters that we evaluated include binding titers, neutralizing activity, specificity, ability to mediate antibody-dependent cell-mediated cytotoxicity, and activation of the complement pathway.

Results

All three of the clinical trials included in the current analysis were designed to elicit HIV-1 Env-specific antibody responses (Table 4.1). HVTN 203, was an early phase clinical study designed to test the immunogenicity of the canarypox prime-protein

Table 4.1: Summary of Vaccine Trials

Trial	Prime Immunizations			Boost Immunizations			HIV-1 strains	Adjuvant
	Type	Dose	Weeks	Type	Dose	Weeks		
HVTN 041	N/A	N/A	N/A	gp120 protein	5, 20, or 100 ug	0, 4, 12	W61D	AS02A (QS-21 + 3D-MPL)
HVTN 203	Canarypox	$10^{7.26}$ TCID50	0, 4, 12, 24	gp120 protein	600 ug	12, 24	MN, GNE8	Alum
DP6-001	DNA	1.2 mg	0, 4, 12	gp120 protein	375 ug	20, 28	A, B, Bal, C, E *	QS21

* A: 92UG037 B: 92US715 Bal: Ba-L C:96ZM651 E: 93TH976

boost regimen prior to the full scale RV144 efficacy trial in Thailand. While these two studies are similar in the application of the canarypox prime-protein boost concept, they differ in the exact antigens delivered to participants in the trial. HVTN 203 expressed a clade B Env by canarypox vector, and was boosted with a bivalent clade B/B Env protein formulation from HIV-1 isolates MN and GNE8 (91). RV144 on the other hand expressed a clade E Env by canarypox vector, and was boosted with bivalent clade B/E Env proteins, where the two clade E Env antigens in the prime and boost were not matched (85). Volunteers in the HVTN 203 trial received a total of four canarypox vector immunizations in addition to protein boosts adjuvanted with alum that were given twice and overlapped with the last two canarypox immunizations. Protein boosts consisted of the same recombinant Env protein vaccine that failed to show any protective efficacy in a Phase III clinical trial when used alone (33). HVTN 041 tested the immunogenicity of an Env protein derived from the HIV-1 isolate, W61D, without any prior immunizations using other forms of vaccines. A unique feature of HVTN 041 is the use of adjuvant, AS02A, a combination of MPL and QS-21 in the oil-in water emulsion SB62, to generate strong Env-specific antibody responses in healthy human volunteers (37). The DP6-001 trial used a DNA prime-recombinant protein boost immunization approach delivering a 5-valent Env formulation from HIV-1 isolates of clades A, B, C, and E (117). Human volunteers were first immunized three times with Env expressing DNA vaccines, followed with two boosts using matched recombinant Env proteins in QS-21 adjuvant. All of the Env proteins used in three studies were in the form of gp120.

Evaluating neutralizing antibody activity elicited by each trial

Neutralizing antibody activity has been a key parameter in HIV vaccine research to measure the protective potential of immune sera specific for HIV-1 Env antigens (43, 100). Neutralizing antibody activities in sera included in the current report were previously tested and showed diverse profiles (37, 91, 117). Both the HVTN 203 and 041 trials elicited positive human Env specific antibody responses that were only capable of neutralizing T cell line adapted (TCLA) or highly sensitive HIV-1 isolates (37, 91) . Data from the HVTN 203 trial indicated that most individuals elicited a serum neutralizing antibody response against MN, however, less frequent neutralization toward the TCLA strain IIIB was observed. Serum antibodies from recipients of the HVTN 041 vaccine were only capable of neutralizing the autologous W61D TCLA strain. In contrast, individuals participating in the DP6-001 trial elicited antibody responses that were capable of neutralizing a number of primary isolates from a diverse subset of clades (A, B, C, D, and E) albeit at low titers (117). The main objective of the current study is to understand if there is any difference in the quality of these sera that may account for the difference of their neutralizing activities. Because previous neutralizing activity analyses for sera from each trial were done in different systems, making direct comparisons difficult, a new set of neutralization assays were conducted in the TZM-bl assay system. In this analysis, three model HIV-1 primary Env antigens with varying degrees of sensitivity to neutralization were used to confirm the previously reported neutralizing profiles for these three sets of sera.

The vast majority of all sera tested, including 10 of 12 sera (83%) from the HVTN 203 study, 11 of 12 sera (92%) from the HVTN 041 study, and 20 of 21 samples (95%) from the DP6-001 study, were capable of neutralizing SF162, a primary isolate highly

sensitive to neutralization (Fig 4.1A). Geometric mean ID50 titers were 1:62 for the HVTN 203 trial sera, 1:164 for the HVTN 041 trial sera, and 1:104 for the DP6-001 trial. Statistically, sera from the HVTN 041 trial were significantly more potent neutralizers than those from the HVTN 203 study against the sensitive isolate SF162 (p=0.027), but not significantly different from DP6-001.

Neutralizing activities against SS1196, a primary isolate that is moderately sensitive to neutralization, allowed for some differentiation of the potential of each trial sera (Fig 4.1B). Only 4 of the 12 sera (33%) from the HVTN 203 trial were capable of neutralizing SS1196 at a 1:10 dilution. In contrast, 8 of the 12 sera (67%) from the HVTN 041 trial and 18 of the 21 sera (86%) from the DP6-001 trial were capable of neutralizing SS1196. No significant difference in titers were observed among the three sets of sera (p=0.09). However, geometric mean NAb titers for both the HVTN 041 and DP6-001 trials were 14 and 13, respectively, much lower than that seen against the highly sensitive SF162 isolate.

The third pseudotyped virus tested in the current analysis expressed Env from the HIV-1 isolate, SC422661.8, a Tier 2 virus representative of those found shortly after the establishment of HIV-1 infection in a new patient and known to be highly resistant to neutralization (56). A significant drop in neutralizing activities was observed with sera from all three vaccine trials against this virus (Fig 4.1C). None of the sera from the HVTN 203 trial were capable of reaching 50% neutralization at the lowest dilution tested (1:10). Similarly, neutralizing activity against this isolate was only observed in two sera (17%) from the HVTN 041 trial. However, 10 of the 21 sera (48%) from the DP6-001 trial were capable of neutralizing SC422661.8 at a 1:10 dilution. The lack of neutralizing

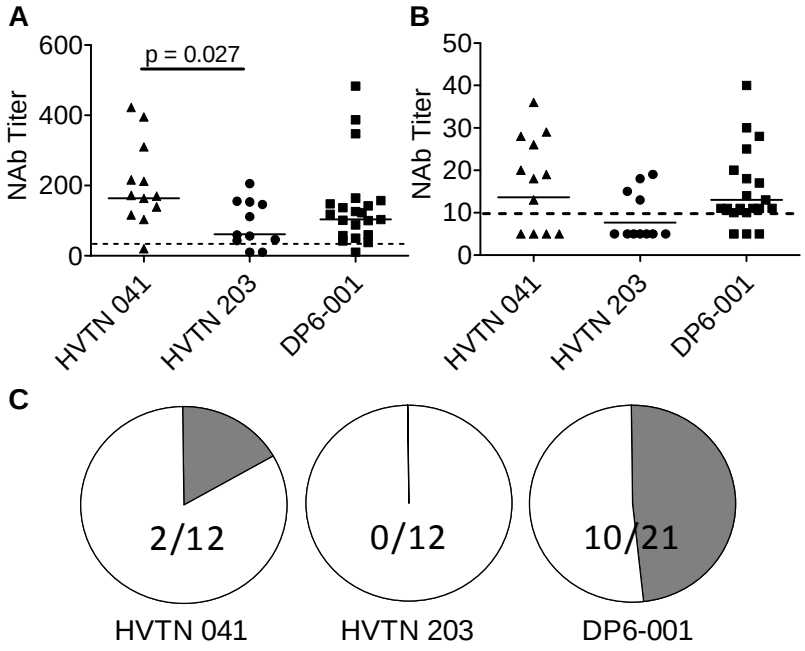

Fig 4.1: Confirmation of neutralizing activities against representative HIV isolates. Neutralization was calculated according to the following formula: % neutralization = [(NHS RLUs – Immune RLUs)/(NHS RLUs)]*100. Neutralizing antibody titers at 50% inhibition for each serum are shown against either SF162 (A) or SS1196.1 (B). Neutralizing activities against SC422661.8 (**C)** is shown as the fractions of individual sera from each trial either capable of achieving at least 50% inhibition of infection at a 1:10 serum dilution (shaded portion) or unable to achieve 50% inhibition (open portion).

activity from the HVTN 203 and 041 trials against more resistant isolates, and the low titer, but broader neutralization seen in the samples from the DP6-001 trial are all consistent with previously reported neutralization profiles (37, 91, 117). It is interesting to note that the broader neutralization seen in the DP6-001 samples is present despite the fact that, on average, individuals in the DP6-001 had either lower or equivalent titers of Env-specific binding antibodies when compared to other two trial sera.

In order to understand what features of the antibody responses elicited by each of these sera may be responsible for the difference in their neutralization profiles, a wide spectrum of analyses were conducted to understand the quality of different sera. The first was an evaluation of Env specific binding antibodies. Because each trial tested here was formulated with at least one clade B component, we chose JR-FL gp120 as the model detecting antigen to examine binding titers (Fig 4.2). In addition to being derived from a clade B isolate, JR-FL Env antigen is both well characterized and completely heterologous to all gp120s included in these studies. When sera was tested for the ability to bind JR-FL gp120, we determined that binding antibody titers generated in the HVTN 041 trial were significantly higher than those generated in either the HVTN 203 ($p=0.04$) or DP6-001 ($p=0.0003$) clinical trials, suggesting that gp120 adjuvanted with AS02A is an exceptionally immunogenic formulation. The geometric mean binding titer of the HVTN 041 trial was 3.3 fold higher than that of the HVTN 203 trial and 4.5 fold higher than that observed for the DP6-001 trial. The levels of binding antibodies elicited in the HVTN 203 and the DP6-001 trials were not statistically different.

Antibodies directed to CD4 inducible (CD4i) epitopes are frequently elicited in HIV infected individuals (26). However, their role, if any, in controlling viral infection

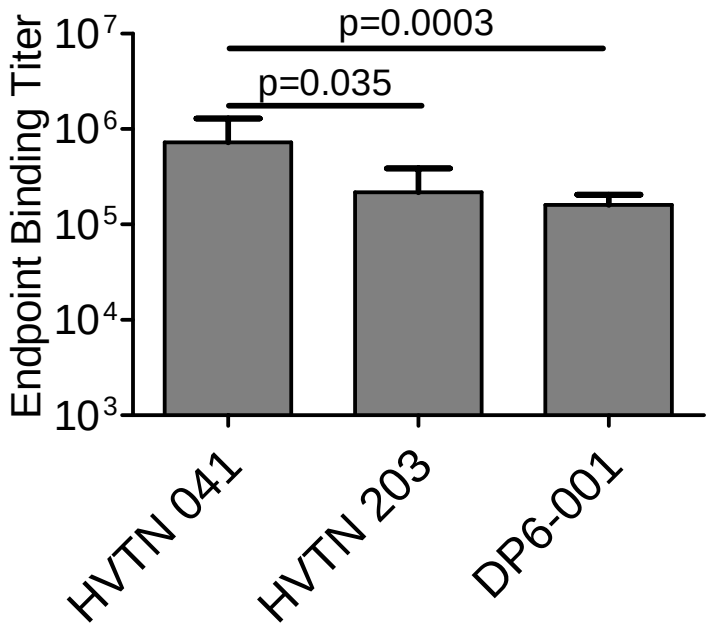

Fig 4.2: Geometric mean endpoint binding titers of sera from each of three human vaccine trials against a recombinant gp120 protein of the heterologous clade B JR-FL. Error bars indicate standard error.

remains unclear. Prior exposure of pseudovirus to sCD4 can expose CD4i epitopes, such as the co-receptor binding site, on the viral envelope (34). In an attempt to determine if sera from any of the HIV vaccine trials being tested in this study elicited CD4i directed antibodies, sera from each trial were assayed for their ability to outcompete binding to 17b, a mAb that targets the co-receptor binding site. We found that 17b-like antibodies were elicited in high frequency and in high titer in all three vaccine trials (Fig 4.3A): 7 of 12 sera (58%) from the HVTN 203 trial, 9 of 12 sera (75%) from the HVTN 041 trial, and 17 of 21 sera (81%) from the DP6-001 trial were able to outcompete binding to 17b at a 1:40 serum dilution. In addition to being elicited frequently, these antibodies were all found at relatively high titer, with most sera able to outcompete binding to 17b at dilutions in the hundreds. No statistically significant differences were observed in either the frequency or titer of the 17b-competing antibodies elicited among three trials.

Next, we evaluated if the CD4i antibodies found in the sera from each trial are functional in a modified neutralization assay. Pseudotyped viruses expressing Env from the JR-FL isolate were treated with sCD4 prior to incubation with serum in order to expose CD4i epitopes, such as the co-receptor binding site, on the viral envelope. We found that while prior sCD4 treatment, JR-FL was difficult to neutralize (Fig. 4.3B), all three immunization regimens elicited significant neutralizing activities against JR-FL Env pseudotyped viruses upon exposure to sCD4: 7 of 12 (58%) individuals from HVTN 203, 10 of 12 individuals (83%) from HVTN 041, and 20 of 21 individuals (95%) from DP6-001 showed positive neutralizing activities in this assay (Fig 4.3C). Geometric mean neutralizing titers for HVTN 203, HVTN 041, and DP6-001 were 1:28, 1:44 and 1:49, respectively, without any statistical differences among the three trials. This data

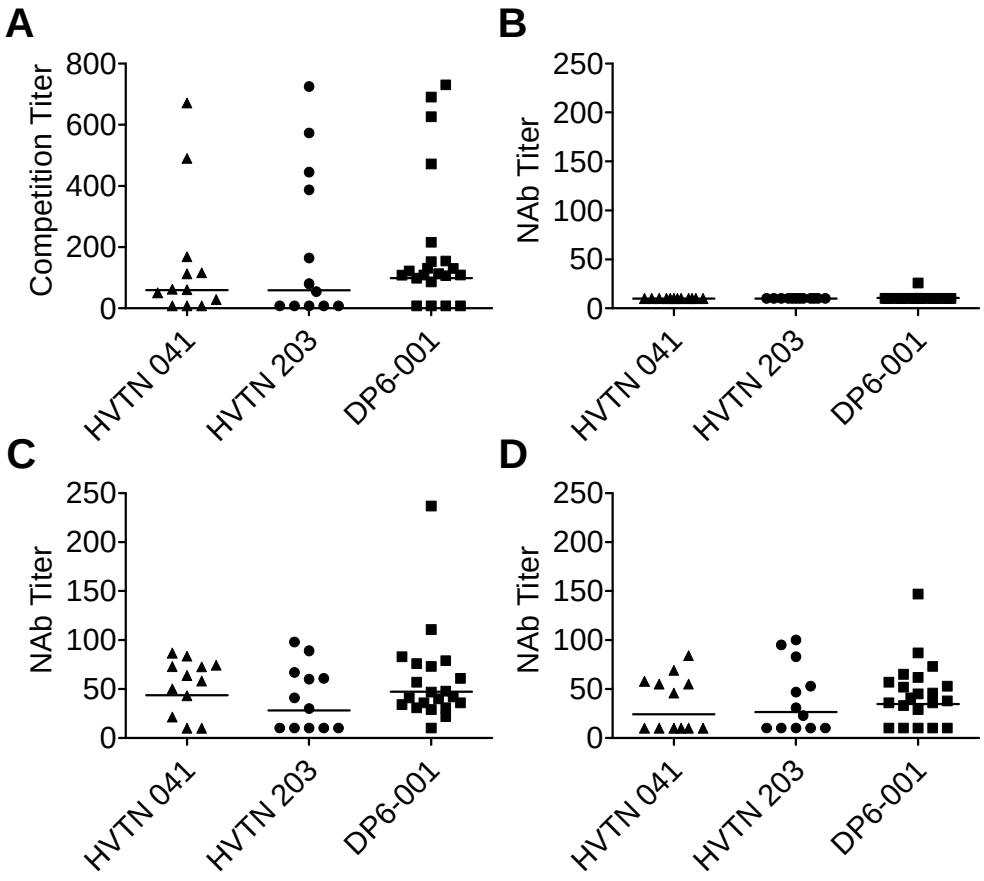

Fig 4.3: Analysis of antibodies against CD4 inducible (CD4i) epitopes. A) The presence of co-receptor binding site-directed antibodies was assayed by competition with the mAb, 17b. Competition titer indicates the serum dilution capable of outcompeting 50% of pseudoviral binding to 17b. % competition was calculated according to the following equation: % competition = [(RLUs in absence of sera – RLU in presence of sera)/RLUs in absences of sera]*100. B) Neutralizing antibody titers against HIV-1 JR-FL isolate without sCD4 treatment. C) Neutralizing antibody titers against HIV-1 JR-FL isolate with sCD4 treatment. D) Effect of V3 peptide treatment on neutralizing activity against sCD4 treated JR-FL. With no sCD4 treatment % neutralization was calculated according to the following formula: % neutralization = [(NHS RLUs – Immune RLUs)/(NHS RLUs)]*100. When virus was treated with sCD4 neutralization was calculated as follows: % neutralization = {[(NHS + sCD4 RLUs) – (Immune Sera + sCD4 RLUs)]/(NHS + sCD4 RLUs)}*100. NAb titer indicates the serum dilution preventing 50% of pseudovirus infection.

suggests that under the proper conditions, CD4i antibodies present in vaccinee sera would be capable of neutralizing heterologous isolates of HIV-1.

Because it has been reported previously that sCD4 treatment better exposes the V3 loop to antibodies specific for this domain (120), we attempted to determine if the neutralizing activity observed after sCD4 treatment was due to recognition of the V3 loop or due to recognition of the co-receptor binding site by the 17b-like antibodies detected through competition. To answer this question, vaccinee sera were incubated with a synthetic peptide matched to the V3 loop sequence of the JR-FL Env prior to the exposure of sCD4-treated JR-FL. This resulted in a slight drop in the geometric mean NAb titer of HVTN 203 sera to 26, of HVTN 041 sera to 25, and of DP6-001 sera to 34 (Fig 4.3D). This drop in potency was also accompanied by a drop in the frequency of positive neutralizing sera to 6 of 12 individuals (50%) in the HVTN 041 trial and to 16 of 21 individuals (76%) in the DP6-001 trial (Fig 4.3D). The observation that a V3 peptide was able to block some, but not all of the neutralizing activity indicates that both V3-directed and co-receptor binding site-directed antibodies may play a role in the neutralization of JR-FL after exposure to sCD4.

In order to further evaluate the presence of antibodies that target other potentially neutralizable epitopes without sCD4 treatment, additional competitive binding assays were conducted to determine if any of the human immune sera were capable of outcompeting binding to known neutralizing mAbs. Overall, human immune sera included in the current study only outcompeted binding to the mAb 2G12 at very low frequency. None of the 12 sera from the HVTN 041 trial, 2 of the 12 sera (17%) from the

HVTN 203 trial, and 5 of the 21 sera (24%) from DP6-001 trial outcompeted binding to 2G12 (Fig 4.4A).

Antibodies capable of outcompeting binding to the neutralizing mAb, 447-52D, which is specific for the crown of the V3 loop, were elicited nearly ubiquitously in all of the vaccinee sera tested (Fig 4.4B). The geometric mean competitive binding titers against 447-52D were 1:108 for the HVTN 203 sera, 1:409 for the HVTN 041 sera, and 1:187 for the DP6-001 sera. Statistically significant differences in the titers of V3-directed antibodies were observed in the HVTN 041 sera relative to the HVTN 203 sera ($p=0.008$) and the DP6-001 sera ($p=0.046$).

A unique profile of CD4bs directed antibodies was observed upon examination of the ability of the immune sera to outcompete binding to the neutralizing mAb, IgG1 b12 (Fig 4.4C). Only 4 out of 12 sera (33%) from either the HVTN 203 trial or the HVTN 041 trial generated an antibody response capable of outcompeting binding to b12. However, 20 out of 21 sera (95%) from participants in the DP6-001 trial were capable of outcompeting binding to b12 and did so with high titers, sometimes exceeding a 1:500 dilution.

The roles of antibodies in a viral infection are not limited to binding and neutralization of cell-free virus. Antibodies can bind to cells coated with HIV-1 Env and mediate their killing through an interaction with Fc receptor bearing effector cells. In order to evaluate sera's ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC), a fluorometric ADCC killing assay was used. When sera from each trial were evaluated in this system, we determined that antibody responses elicited by all three immunization regimens were capable of mediating ADCC activity in an equivalent

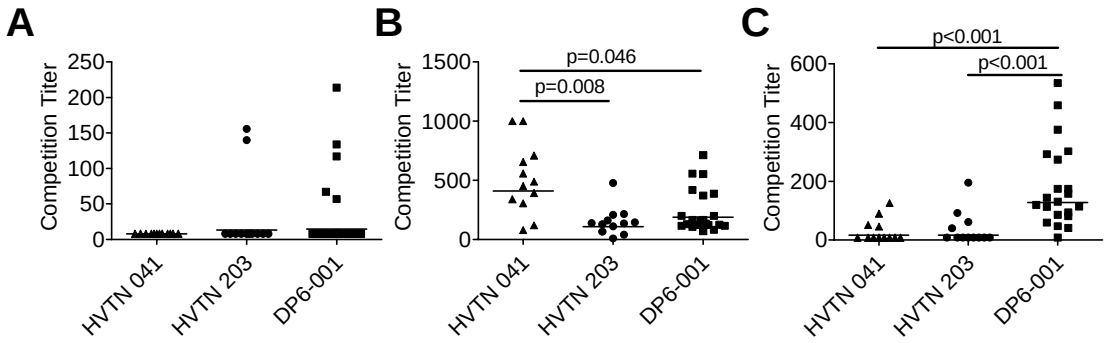

Fig 4.4: Specificity of vaccine-induced antibody responses as determined through mAb competition. The ability of serially diluted human immune serum to outcompete binding of mAb to a JR-FL & VSV-G pseudotyped virus was measured. % competition was calculated according to the following equation: % competition = [(RLUs in absence of sera – RLU in presence of sera)/RLUs in absences of sera]*100. Competition titers indicate the serum dilution preventing 50% of pseudoviral binding to the mAb. A) Competition with carbohydrate-specific mAb, 2G12. B) Competition with V3 loop-specific mAb, 447-52D. C) Competition with CD4bs-specific mAb, b12.

fashion. Mean lysis of the CEMNK^{r} target cells mediated by sera from each trial was found to be approximately 20%. (Fig. 4.5).

A second intrinsic characteristic of antigen specific antibody is the ability to mediate activation of the complement pathway. In order to evaluate human sera's ability to mediate activation of the complement cascade, we conducted an ELISA based assay to monitor deposition of C4, a downstream product of complement activation, by gp120 specific antibody found in vaccinee sera. When this was done, we found that gp120 specific antibodies from the sera from all three trials were capable of activating the complement pathway in a concentration dependent manner. Again however, we observed no differences in the abilities of sera from each trial to activate the complement cascade. A representative assay result is shown in Fig. 4.6.

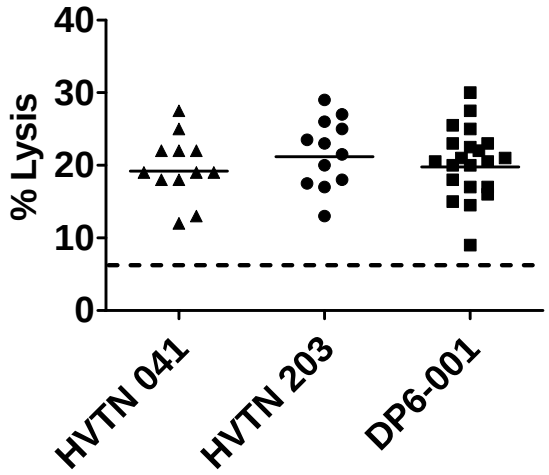

Fig 4.5: Ability of vaccinee sera to mediate ADCC activity. CEMNK^{r} target cells were pulsed with gp120 prior to exposure of vaccine serum at a 1:100 dilution. Target cell lysis indicates the ability of vaccinee serum to mediate cell killing by PBMC from a normal human donor as measured by the percentage of CFSE^{-} target cells within the PKH26^{hi} population. Dotted line indicates background cell lysis observed with a normal human sera control.

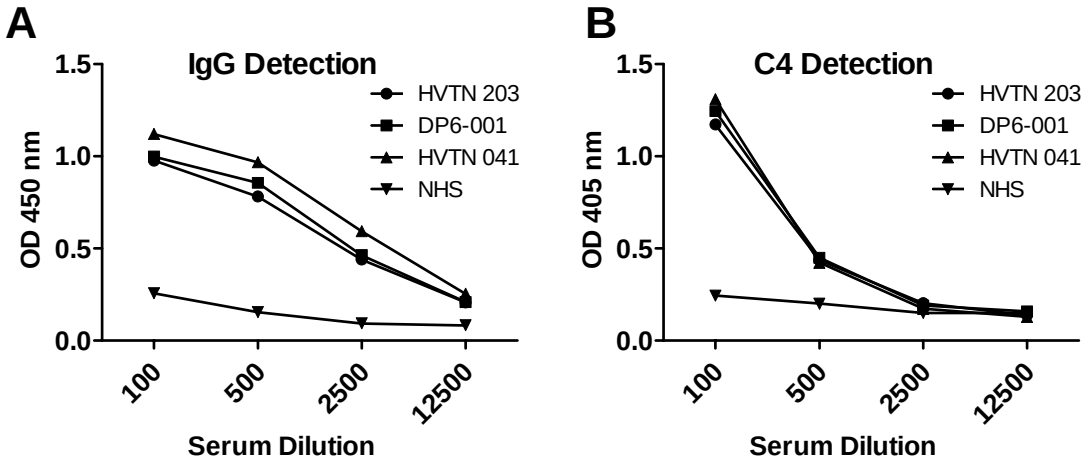

Fig 4.6: The ability of Env specific antibodies to activate the complement cascade present in complement intact normal human sera was determined using deposition of C4 as a marker for complement activation. A representative plot with data from a single individual from each trial is shown. A) gp120 specific IgG measurement and B) C4 detection in the same testing sera.

Discussion

In the current chapter, a side-by-side comparison was conducted on the quality of human antibody responses elicited by three candidate AIDS vaccines focusing on HIV-1 Env specific antibodies. Vaccines from all three studies included a gp120 protein vaccine component but only two of the studies included priming immunizations using either a viral vector- or DNA-based vaccine. Although the sample sizes are relatively small, our results suggest that the antibody profiles elicited by each vaccination regimen are different. This information is valuable for the development of AIDS vaccines with potential to elicit protective antibody responses.

Volunteers from the HVTN 041 trial had the highest Env specific serum IgG titers among the three trials evaluated here. This was achieved without receiving a priming immunization from any gene based vaccine. Because previous studies using recombinant gp120 proteins alone adjuvanted in alum did not generate high binding antibodies (33), it is very likely that the strong adjuvant (a mixture of QS-21 and MPL in the oil in water emulsion SB62) used as part of the HVTN 041 trial vaccine formulation played an important role in the high immunogenicity observed for this recombinant gp120 protein vaccine. A potential caveat to this binding analysis is that the differences in binding antibody responses could be due to different degrees of mismatch between the vaccine immunogens and JRFL gp120. Even taking this in account however, all three trials elicited a significant binding antibody response above 10^5 against this model antigen.

Consistent with the binding antibody results and the immunodominant nature of the V3 loop, competition assays revealed the presence of the highest levels of antibodies

specific for the V3 loop among participants in the HVTN 041 trial. Participants in the HVTN 041 trials also showed high neutralizing activities against highly sensitive and moderately sensitive pseudoviruses, but poor neutralizing activity against more resistant viruses, supporting the hypothesis that recombinant protein-based HIV vaccines are highly capable of eliciting antibodies against stable structures that may be masked in viruses more resistant to neutralization.

Sera from the DP6-001 trial, which employed a DNA prime-protein boost approach, presented a different antibody profile than that seen in HVTN 041. This regimen was effective in eliciting Env specific binding antibody responses with similar characteristics compared to the highly immunogenic HVTN 041 formulation, albeit at a slightly lower quantitative level. Most significantly, however, DP6-001 sera demonstrated greater potential to neutralize more resistant HIV-1 isolates, as previously reported (117). Furthermore, sera from the DP6-001 trial exhibited a greater preference to elicit antibodies to conformational epitopes, such as the CD4bs, a finding initially observed in rabbit immune sera following administration of a similar DNA prime-protein boost regimen (108). While it is difficult to completely attribute the better neutralizing activities of the DP6-001 sera to the presence of CD4bs antibodies, conformational antibodies such as those specific for the CD4bs and other conformational epitopes have been found responsible for the broad neutralizing activities in some HIV infected patients (57, 110).

Sera from HVTN 203 had the least impressive antibody responses in many of the parameters measured in the current report. While the canarypox prime-recombinant Env protein boost approach was more effective in eliciting higher binding antibody responses

than the previous studies using this same Env alone (33), HVTN 203 trial sera were only similar to or less effective than the other two trials in eliciting Env-specific antibody responses. Most importantly, HVTN 203 trial sera were less effective than HVTN 041 trial sera in eliciting binding antibody titers, and less effective than DP6-001 trial sera in eliciting antibodies specific for conformationally sensitive epitopes. Furthermore, HVTN 203 participants elicited antibodies with the least potent neutralizing activity. It is not clear whether these differences between the canarypox vector prime and the DNA vaccine prime can be attributed to the fact that the canarypox vector expresses multiple unrelated viral vector proteins in addition to the HIV-1 Env and the DNA vaccine only focuses on the expression of Env.

Because the same canarypox prime-recombinant Env protein boost approach was used in the recent RV144 trial, which showed a low level of protection against HIV-1 for the first time in a field trial, the results presented in the current report raise several interesting questions. If a canarypox prime-recombinant Env protein boost approach offers any unique protective benefit over the other two approaches, it is then necessary to identify new biomarkers other than those included in the current study since none stood out as a unique marker for the success of the HVTN 203 trial vaccine. Alternatively, either of the other two approaches evaluated in the current study may have a better protective potential over the canarypox prime-recombinant Env protein boost approach if the higher responses in binding and neutralizing assays observed in sera obtained from only the HVTN 041 or DP6-001 trials are any indication. However, more advanced studies are needed to answer these questions. Interestingly, since each vaccination approach has a relatively specific antibody response profile, as discovered in this report,

it may be possible to link the efficacy of any future vaccine formulation to the antibody profile it exhibits.

The current report also indicates a great need to expand the scope of research to include diverse types of antibody responses when a candidate HIV vaccine is evaluated. The presence of NAbs has been used almost exclusively to judge the protective potential of vaccine-induced antibody responses. Our data suggests that other parameters, for example, possibly the induction of conformation dependent antibodies, can also provide unique insight to differentiate the quality of antibodies elicited by vaccines. In recent studies of HIV infected individuals with broadly neutralizing activity, the neutralizing fraction of sera has often been mapped to those antibodies directed towards the CD4bs (12, 57, 58, 94). Because of this, it is exciting to observe the elicitation of antibodies with similar specificities as those seen in HIV infected individuals through the use of a DNA prime-protein boost regimen. On the other hand, no differences were observed in preliminary analyses of ADCC or complement-mediated antibody effects among three sera included in the current study.

In summary, studies described in this chapter directly compared serum antibody responses from three different HIV vaccine clinical trials. Antibody profiles elicited by the three different immunization regimens are similar with regards to the high titer binding antibodies that are capable of neutralizing sensitive isolates, mediating killing of antigen-coated cells by Fc bearing effector cells, and activating the complement cascade. Differences between the vaccine trials became apparent when the neutralizing capabilities of sera from each trial were evaluated. We found that participants in the DNA prime-protein boost trial were capable of neutralizing more resistant primary isolates. In

contrast, participants from the canarypox prime-protein boost and the protein alone trials demonstrated a much more limited neutralization capacity. Additionally, profiles of antibody specificities differed between the three trials. We found that participants from neither the HVTN 203 nor the HVTN 041 trials were capable of efficiently eliciting antibodies directed to the CD4bs of the HIV-1 Env antigen. Because of the unique antibody profile and the ability to better neutralize primary isolates, the DNA prime-protein boost regimen offers another heterolgous prime-boost platform for further HIV vaccine development in addition to the recent promising RV144 canarypox prime-protein boost regimen.

Chapter V

Mapping the structural basis for antibody responses elicited by DNA prime-protein boost vaccines

Introduction

After establishing that a combination DNA prime-protein boost regimen is capable of eliciting a higher quality antibody response, as defined by the ability to elicit antibodies to functionally conserved domains, a higher avidity antibody response, and enhanced neutralizing activity against heterologous isolates, we wanted to apply this technique to answer a fundamental question in the field of HIV vaccinology: whether or not any gp120 immunogen is superior to any other in eliciting a heterologous NAb response. The high degree of sequence diversity in the HIV Env results in a protein that has a huge diversity of phenotypes. Polymorphisms within Env are responsible for changes in structure which can alter viral tropism, antigenicity of Env, and potentially even the ability to be transmitted to a new host. However, thus far, no one has been able to demonstrate that two naturally occurring isolates differ in their ability to raise a heterologous NAb response. In this study, we answer this question with the use of two naturally occurring isolates, LN40 and B33.

Results

Model envelopes and immunization regimen.

The LN40 and B33 gp120s were selected for this study due to their high degree of sequence similarity but opposing phenotypic properties. A sequence alignment of the gp120 subunits from these two Envs are shown in Fig 5.1. To summarize, there are a total of 51 amino acid polymorphisms within gp120, which also account for a total of five

```
HXB2    1 MRVKEKYQHL WRWGWRWGTM LLGMLMICSA TEKLWVTVYY GVPVWKEATT  50
B33       ---------- ---------- ---------- ---.....  ..........
LN40      ---------- ---------- ---------- ---.....  .......T..


HXB2   51 TLFCASDAKA YDTEVHNVWA THACVPTDPN PQEVVLVNVT ENFNMWKNDM 100
B33       ........E. .......... .......... ......K... ......R.N.
LN40      .......... .......... .........S ....P..... .D......N.


HXB2  101 VEQMHEDIIS LWDQSLKPCV KLTPLCVSLK CTDLKNDTNT NSSSGRMIME 150
B33       .......... .......... .......T.N ...FR.A... .......-..
LN40      .......... .......... .......T.N ....R.A... ....E.K-..

                                    V1/V2
          ____________________________________________________
HXB2  151 KGEIKNCSFN ISTSIRGKVQ KEYAFFYKLD IIPIDNDTTS YKLTSCNTSV 200
B33       G......... ..--..D... .......... V...E..... .R.I......
LN40      E........Y VTPTL.D.K. ....T..... VM...K.N.. .R.I......


HXB2  201 ITQACPKVSF EPIPIHYCAP AGFAILKCNN KTFNGTGPCT NVSTVQCTHG 250
B33       .......I.. .......... .........D .K...K.... ..........
LN40      .......I.. .......... .........D .K...K.... ..........


HXB2  251 IRPVVSTQLL LNGSLAEEEV VIRSVNFTDN AKTIIVQLNT SVEINCTRPN 300
B33       .K........ .......... ....E...N. ..N......E A.........
LN40      .K........ .......... ....E...N. .........E ..........

                          V3
          ____________________________________
HXB2  301 NNTRKRIRIQ RGPGRAFVTI GKI-GNMRQA HCNISRAKWN NTLKQIASKL 349
B33       .....S.NL- -......LY.T .E.T.DI... ...L.S...E ....K.VI..
LN40      .....S.HL- -......Y.T .E.T.DI... ...L.E...E ....K.VI..


HXB2  350 REQFGNNKTI IFKQSSGGDP EIVTHSFNCG GEFFYCNSTQ LFNSTWFNST 399
B33       G....K.... V..P...... ...K...... ......D... ......N---
LN40      .......... ..N.P..... ...R...... .........K ......N---

               V4
          _________
HXB2  400 WSTEGSNNTE GSDTITLPCR IKQIINMWQK VGKAMYAPPI SGQIRCSSNI 449
B33       -V.K.L.--. .NG....... .........E .......... ..........
LN40      -G..RLD..K .NG....... .........E .......... ..........

                    V5
          ___________
HXB2  450 TGLLLTRDGG NSNNESE--I FRPGGGDMRD NWRSELYKYK VVKIEPLGVA 497
B33       ...I...... .NKS...PE. .......... .......... ..R.......
LN40      ...I...... GD..G.KPE. .......... .......... ..........


HXB2  498 PTKAKRRVVQ REKR 511
B33       .......... ....
LN40      .......... ....
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Fig 5.1. Sequence alignment of B33 and LN40 gp120s used for DNA immunizations. HXB2 gp120 sequence is included as a reference. "." indicates a sequence identity. "-" indicates a sequence gap. The numbering system used in the alignment, is the standard HXB2 numbering system.

changes in potential N-linked glycosylation sites (PNGS). These polymorphisms result in two Envs with very different phenotypic characteristics (Table 5.1). Briefly, B33 has been shown to be highly macrophage tropic, sensitive to neutralization by mAb b12 and resistant to neutralization by the mAb 2G12. LN40 however, is not macrophage tropic, is resistant to b12, and sensitive to 2G12 (29, 76-78).

In order to evaluate their immunogenic potentials, rabbits were primed with either B33 gp120 DNA or LN40 gp120 DNA at Weeks 0, 2, and 4. Every animal was then given the same 5-valent protein boost, consisting of gp120s from clades A, B, C, and E. Half of the animals from each group were boosted on Weeks 8 and 12 while the other half were boosted on Weeks 38 and 42 (Fig 5.2). Because all animals received identical protein boosts, any differences seen in the resulting antibody responses should be due to effects of the initial priming immunizations with either B33 gp120 or LN40 gp120.

Evaluating binding titers generated through immunization

Two weeks after the final protein boost, animals were bled and the resulting binding antibody response was evaluated against the homologous B33 and LN40 gp120s as well as a heterologous clade B gp120, JR-FL. Through these assays, we determined that high titer binding antibody responses were generated against both autologous and heterologous clade B Envs by priming with either LN40 or B33 gp120 constructs (Fig 5.3). Against B33 gp120, no differences in the binding titers were observed regardless of the priming immunization given (Fig 5.3A). However, a slight difference in binding was seen against the LN40 gp120. Sera from animals primed with the LN40 gp120 recognized the homologous LN40 gp120 protein with approximately a three-fold higher titer than animals primed with the heterologous B33 gp120 (Fig 5.3B). Despite this,

Table 5.1 Summary of B33 and LN40 Envelope Phenotypes

Envelope	Characteristics
NA420 B33	Highly macrophage tropic, high affinity for CD4, b12 sensitive, 2G12 resistant
NA420 LN40	Poorly macrophage tropic, low affinity for CD4, b12 resistant, 2G12 sensitive

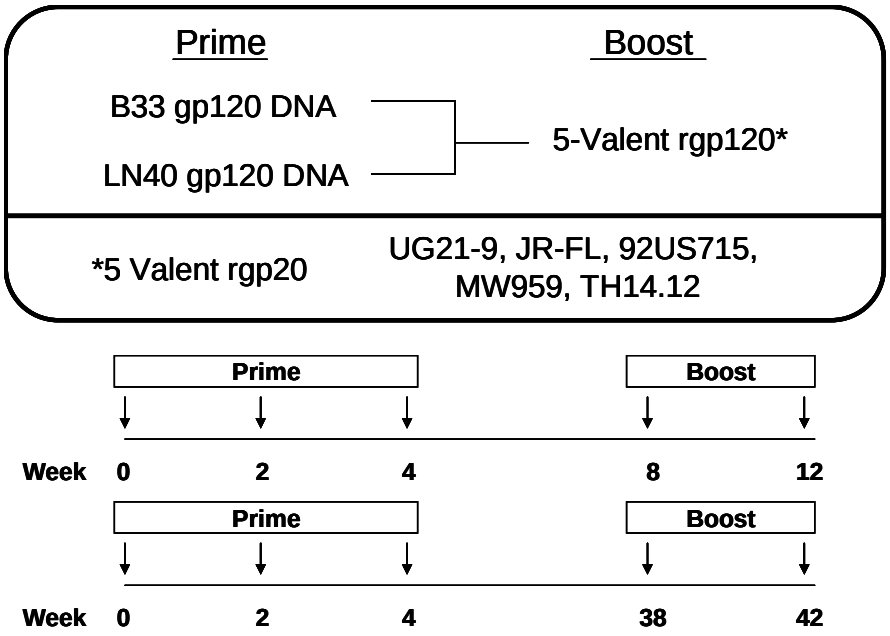


Fig 5.2: Study design and immunization regimen to study B33 and LN40 immunogenicity. Rabbits received three DNA primes of either LN40 gp120 or B33 gp120 at Weeks 0, 2, and 4. Protein boosts consisting of a 5-valent mixture of gp120s were given as an early boost at Weeks 8 and 12, or as a late boost at Weeks 38 and 42.

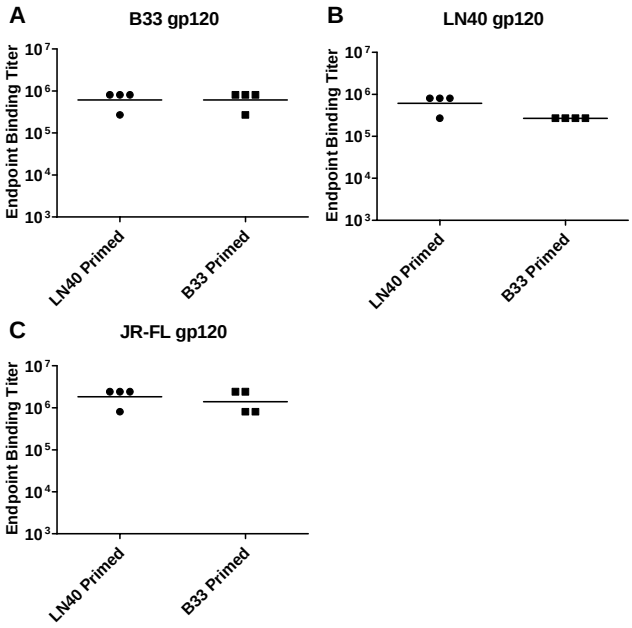


Fig 5.3: Endpoint binding titers of sera from rabbits primed with B33 and LN40 gp120s. Serially diluted rabbit sera collected 2 weeks after the final protein boost were assayed for binding against homologous and heterologous clade B gp120s. **A.** Endpoint titer against B33 gp120. **B.** Endpoint titer against LN40 gp120. **C.** Endpoint titer against JR-FL gp120. Endpoint titer is defined as the last serum dilution giving at least double the OD of a preimmune control.

endpoint binding titers against the LN40 gp120 still exceeded 10^5 in both immunization groups. Against the completely heterologous JR-FL gp120, no differences in the endpoint binding titers were observed between immunization groups (Fig 5.3C).

Evaluating neutralizing activity in sera from B33 and LN40 immunized rabbits

In spite of the observation that immunization with B33 and LN40 generated similar, high titer binding antibody responses, we found that the neutralizing activity in sera from LN40 primed rabbits was much more broadly reactive (Table 5.2). All animals regardless of whether they were primed with B33 or LN40, were capable of neutralizing the sensitive isolates SF162 and NL4-3, indicating that a functional NAb response can be elicited through priming with both B33 and LN40 gp120s. However, when the ability to neutralize more relevant primary isolates is taken into account, only animals primed with LN40 gp120 were capable of neutralizing these viruses. Differences in the neutralizing activity of sera elicited by each gp120 began to become apparent against the moderately sensitive clade B isolate SS1196.1. Three of the four animals primed with LN40 gp120 were capable of neutralizing this virus at a 1:10 dilution. However, none of the animals primed with B33 gp120 were capable of neutralizing this isolate. When evaluating the neutralizing activity of the elicited sera against more prototypical primary isolates, specifically those tier 2 isolates representative of viruses seen shortly after establishment of infection (56), the differences in the neutralizing activity between the two immunization groups becomes more dramatic. While the overall breadth and potency remains limited, even within the LN40 immunized animals, it is notable that only rabbits primed with the LN40 constructs are capable of neutralizing any of the primary isolates tested. All four of the LN40 primed animals were capable of achieving 50%

Table 5.2: Neutralization of heterologous clade B isolates by sera from LN40 and B33 primed animals

	LN40 Primed				B33 Primed			
Virus	491	652	653	654	492	655	656	657
SF162	96	94	90	89	93	94	89	89
NL4-3	71	71	98	87	80	72	69	73
SS1196.1	66	56	86	22	28	36	22	37
6535.3	80	57	88	51	29	3	11	73
AC10.0.29	16	19	25	13	20	11	10	13
CAAN5342.A2	54	60	63	49	49	20	42	40
PVO.4	15	15	28	22	23	33	31	23
QH0692.42	52	51	57	19	42	38	13	32
REJO4541.67	6	0	21	6	0	20	12	16
RHPA4259.7	31	53	52	33	23	30	29	29
SC422661.8	24	17	11	9	9	11	9	31
THRO4156.18	24	46	29	35	21	15	7	10
TRJO4551.58	0	19	24	10	10	9	20	9
TRO.11	23	29	32	24	22	0	17	37
WITO4160.33	45	13	0	0	28	0	0	34
MLV	8	4	14	0	0	25	0	5

Numbers indicate percent neutralization at a 1:10 serum dilution

neutralization of the 6535.3 isolate at a 1:10 dilution. Three of the four animals elicited an antibody response capable of neutralizing CAAN5342.A2 and QH0692.42 at the same dilution. Additionally, half of the LN40 primed animals were capable of neutralizing REJO4541.67. In contrast, the only positive neutralization hit we observed in animals primed with B33 was against the 6535.3 isolate, where Rabbit #657 achieved greater than 50% neutralization at a 1:10 dilution.

To further verify this phenomenon, we purified the rabbit IgG over a protein A column and re-evaluated the neutralizing activity against the tier 2 clade B panel (Table 5.3). These results mirror results seen when only serum was used. Animals primed with LN40 gp120 elicited a more potent NAb response than animals primed with B33. In some instances, such as seen against neutralization of 6535, LN40 priming resulted in a NAb response that was 50 times more potent than the neutralization seen in animals primed with B33. While this particular result is exceptional, against the QH0692.42, REJO4541.67, and TRJO4551.58 isolates individual rabbit NAb potencies are frequently between 2- and 10-fold higher in LN40 primed animals than in B33 primed animals.

Evaluating the specificity of neutralizing activity using chimeric viruses

Because the LN40 primed animals appeared to elicit a unique NAb response capable of neutralizing a greater breadth of viruses with increased potency, we wanted to map the specificity of the observed neutralizing activity. Preliminary neutralization of the homologous B33 and LN40 viruses indicated that neutralizing activity elicited by each gp120 was strain specific, with respect to these particular viruses. In every case, sera elicited through priming with the LN40 gp120 was capable of neutralizing the

Table 5.3: IC50s against heterologous clade B isolates represented as the concentration of gp120 specific IgG from LN40 and B33 primed animals.

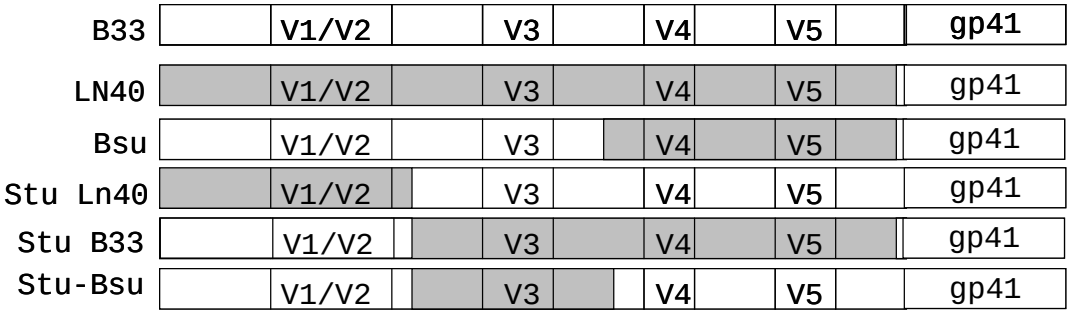
	LN40 Primed				B33 Primed			
Virus	491	652	653	654	492	655	656	657
6535.3	0.63	1.67	0.16	4.30	5.80	3.81	3.48	2.04
AC10.0.29	NA	4.09	6.66	NA	NA	4.12	4.30	4.44
CAAN5342.A2	7.57	4.57	5.35	4.04	7.84	4.36	NA	NA
PVO.4	5.85	2.38	4.70	4.80	7.81	2.64	3.62	3.78
QH0692.42	1.24	1.10	2.34	4.19	4.13	2.45	3.10	3.73
REJO4541.67	2.64	0.62	0.52	2.62	2.72	1.75	2.51	2.48
RHPA4259.7	4.10	4.73	7.41	NA	5.47	NA	NA	NA
SC422661.8	3.71	3.00	6.47	NA	4.28	NA	NA	NA
THRO4156.18	2.61	NA	NA	NA	4.55	NA	NA	NA
TRJO4551.58	4.27	0.47	0.59	1.55	4.42	3.10	3.39	3.23
TRO.11	3.05	1.38	4.26	2.78	3.62	2.30	1.93	2.42
WITO4160.33	1.24	3.30	3.34	5.20	3.76	4.22	NA	NA
MLV	NA	NA	NA	NA	NA	NA	NA	NA

ug/mL gp120 specific IgG
10-5
5-2.5
2.5-1.25
1.25-.625
.625-.312

Numbers indicate IC50 against each virus in ug/mL gp120 specific IgG
NA: 50% neutralization Not Achieved

homologous LN40 pseudovirus while sera elicited by priming with the B33 gp120 was capable of neutralizing the autologous B33 pseudovirus. However, sera from LN40 primed animals could not neutralize B33 and sera from B33 primed animals could not neutralize LN40 (Fig 5.4). This allowed us to further dissect the specificity of the neutralizing activity using a series of chimeric viruses. In total, four chimeric viruses, all of which have been previously described (29), were used for this purpose (Fig 5.4A). Similar to previous neutralization results with heterologous clade B isolates, none of the B33 primed animals achieved 50% neutralization of any of the four chimeric viruses tested (Fig 5.4B). The LN40 immunized animals, however, were able to neutralize two of the four chimeric viruses tested (Fig 5.4B). The first of these, Stu-B33, contains the C1, V1/V2, and N-terminal of C2 of the B33 virus and the remaining C-terminal of the LN40 virus. At a 1:10 serum dilution, 50% neutralization of this virus was achieved by sera from three of the four LN40 primed animals. The fourth animal, while not achieving 50% neutralization at a 1:10 dilution, still exhibited a 32% increase in the neutralization potency of Stu B33 over the parental B33 virus. The second chimeric virus that was sensitive to neutralization by sera from the LN40 primed animals, Stu-Bsu, contains the C-terminal portion of C2, the V3 loop, and the N-terminal portion of C3 from the LN40 gp120. The remainder of the gp120 from this Env is derived from the B33 isolate. Again, sera from three of the four animals neutralized this chimeric virus. Similar to what was seen with the Stu B33 chimera, the fourth LN40 primed animal, while not achieving 50% neutralization, was still more capable of neutralizing this Stu-Bsu chimeric isolate than the B33 parent isolate.

A



B

	LN40 Primed				B33 Primed			
Virus	491	652	653	654	492	655	656	657
B33	5	16	17	14	52	65	56	73
LN40	63	69	67	60	11	10	21	20
Bsu	0	20	15	14	44	8	9	41
Stu LN40	16	0	0	0	13	0	20	19
Stu B33	68	61	64	46	8	21	40	32
Stu Bsu	68	55	54	46	4	20	25	30

Numbers indicate percent neutralization at a 1:10 serum dilution

Fig 5.4: Neutralization of chimeric viruses by rabbits primed with B33 or LN40 gp120. **A.** Schematic of chimeric viruses. White regions indicate Env portions derived from B33. Grey regions indicate Env portions derived from LN40. **B.** Neutralization of chimeric viruses. Neutralization was calculated according to the following formula: % Neutralization = [(Preimmune RLUs – Immune RLUs)/(Preimmune RLUs)]*100.

V3 peptide adsorption of sera from LN40 immunized animals

After narrowing down the region of neutralizing specificity to between the sequence spanning the C-terminal of C2 to the N-terminal of C3 from the parental LN40 Env, we wanted to confirm that the activity we are observing was not simply due to recognition of the V3 loop. To account for this possibility we absorbed the immune sera with 25 ug/mL of consensus B peptide (CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC) prior to the exposure of virus. When this V3 adsorption was done against the highly V3 sensitive SF162 isolate, a greater than 97% reduction in the NAb titer was observed with sera from all the LN40 primed animals (Fig 5.5A). However, V3 adsorption had very little effect on the neutralization of the fully homologous LN40 virus. In all four cases, less than a 10% reduction in neutralization was observed (Fig 5.5B). We then subjected the Stu-Bsu chimera to the same analysis. When this was tested, a larger proportion of the neutralizing activity was adsorbed with the V3 peptide. Between 14% and 26% reduction in neutralization was observed with Rabbits #491, #652, and #653. Only a 4% reduction was observed with Rabbit #654 (Fig 5.5C). This bigger drop in neutralization may be due to a less efficient masking of the V3 loop by the V1/V2 loop derived from the B33 isolate. The V1/V2 loop of B33 is both shorter and less highly glycosylated than that of the V1/V2 loop from LN40, which may result in V3 being more exposed in the Stu-Bsu chimera. Despite the bigger reduction in neutralization with V3 adsorption at this dilution step however, the majority of the neutralizing activity directed against this chimera remained intact, indicating that not all neutralizing specificity was targeted to the V3 loop. We also wanted to test one of the primary isolates that was capable of being

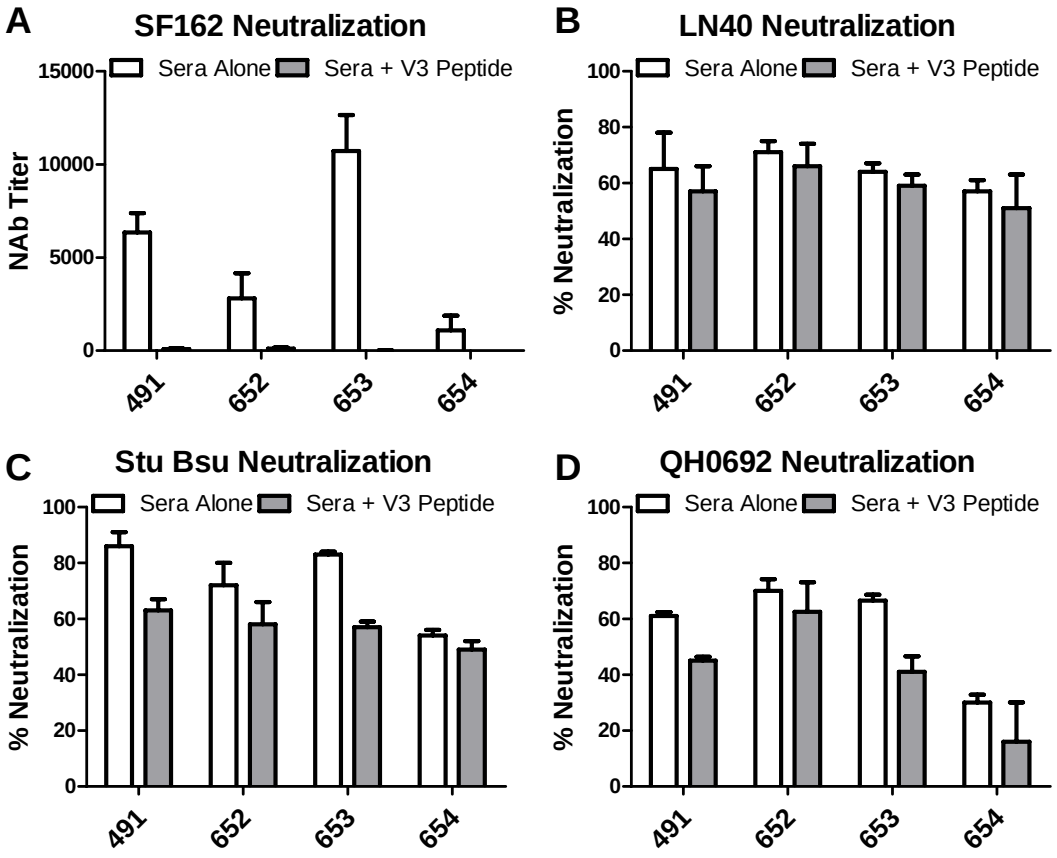


Fig 5.5. Effect of V3 adsorption on the neutralizing activity of sera from LN40 primed rabbits against homologous and heterologous viruses. Prior to incubation with virus, sera was incubated with a clade B consensus V3 peptide at 25ug/mL to determine the role of V3 directed antibodies in neutralization activity of sera from LN40 primed animals **A.** Effect of V3 adsorption on the neutralization of SF162. Y axis indicates neutralizing antibody titer (ID50). **B**. Effect of V3 adsorption on homologous LN40. Y axis indicates the percent neutralization at a 1:10 dilution. **C.** Effect of V3 adsorption on chimeric Stu-Bsu virus. Y axis indicates the percent neutralization at a 1:10 dilution. **D.** Effect of V3 adsorption on the neutralization of QH0692at a 1:10 serum dilution. Neutralization was calculated according to the following formula: % Neutralization = [(Preimmune RLUs – Immune RLUs)/(Preimmune RLUs)]*100. NAb titer indicates the serum dilution preventing 50% of pseudoviral infection.

neutralized by sera from the LN40 primed animals. To this end, we evaluated the effect of V3 adsorption on the neutralizing activity of LN40 primed sera against the QH0692 isolate (Fig 5.5D). Similar to what was seen with the V3 adsorption of Stu-Bsu we observed a slight drop in the capability of our sera to neutralize this virus, however, the majority of the neutralizing activity remained intact. This is further evidence that neutralizing specificities outside of the V3 loop are responsible for the broader neutralization we are observing from sera of LN40 primed animals.

Identification of residues important for maintaining the neutralizing activity of LN40 primed sera

After eliminating the V3 loop as the likely cause of the broader neutralization observed with the LN40 primed sera, we wanted to perform a fine mapping analysis using point mutations to determine exactly where the NAbs were targeted. Because we already have evidence that the neutralizing specificity is targeted to the Stu-Bsu region of Env, we began mutagenesis studies to determine the effects of point mutations within and surrounding this region. Interestingly, closer inspection of this region revealed that it contained contact residues for CD4 as well as residues that flank the CD4 binding loop at the C-terminal end. Additionally, this area had previously been found to confer sensitivity to mAb b12 (29). Because of this, we evaluated the effects of mutations in and around this region for their effect on neutralization of the parental LN40 virus (Table 5.4).

We began by evaluating the effect of a polymorphism found at position 283 which is known to be part of the CD4bs. The threonine at this position in LN40 was mutated to the asparagine found in B33. When this mutation was tested against sera from LN40 primed animals, we found that relative to the parental LN40 virus, the LN40N

Table 5.4 Point mutations affecting the neutralizing activity of sera from LN40 primed animals

Virus	Mutation	LN40 Primed			
		491	652	653	654
LN40		71	79	66	66
B33		14	20	14	11
LN40 N	T283N	8	17	20	19
B33 T	N283T	50	51	21	23
LN40 NL	H310N, F317L	58	51	41	22
LN40 K	R373K	71	61	52	42
LN40 D	N386D	55	71	57	46
LN40 386 ΔGlycan	T388V	65	76	59	54
LN40 KD	R373K, N386D	21	21	14	0
LN40 KV	R373K, T388V	22	27	20	8
LN40 VKPS	I360V, N362K, Q363P, P364S	44	64	43	41

Numbers indicate percent neutralization at a 1:10 serum dilution

mutation eliminated almost all neutralizing activity. Interestingly, when the reverse mutation was made in the parental B33 isolate, a N283T mutation, the sera from two of the LN40 primed animals regained some of the neutralizing activity against this B33T mutant relative to parental B33, thus indicating that this position is important for the targeting of NAbs in sera from LN40 primed animals.

Having some evidence that the neutralizing activity in sera from LN40 primed animals was modulated by residues within the CD4bs lead us to evaluate other regions known to be important in CD4 and b12 binding. Previous work has implicated two residues at positions 373 and 386 in mediating resistance to b12 in the parental LN40 virus (29). Because of this, we next evaluated the role of residues at these positions in modulating the neutralizing activity of sera from LN40 primed rabbits. In the parental LN40 virus, we mutated the arginine at position 373 to the lysine that is found in this position in B33 to produce the LN40 K mutant. In LN40, at position 386, there is an asparagine which is the site of a PNGS. Because of this, we made two mutations that would affect this position. The first was to directly eliminate the asparagine at position 386, and thus the glycosylation, by making an asparagine to aspartic acid mutation to create the LN40 D mutant. The second mutant we generated that affects this residue, LN40 386Δglycan, eliminates the glycosylation site by mutating the threonine at position 388 to a valine, thereby leaving the asparagine residue intact but unable to be glycosylated due to the elimination of the NxS/T glycosylation signal sequence. When we evaluated these mutants for their affect on neutralization we found that all three mutants, LN40 K, LN40 D, and LN40 386Δglycan, only resulted in marginal reductions

in neutralization compared to the wildtype LN40. In most instances, we observed less than a 10% reduction in neutralization against each of these mutants.

However, when we made these mutations in combination with each other we saw very dramatic effects. When we tested the LN40 KD and LN40 KV mutants, both of which affect residues 373 and the glycosylation site at position 386, we saw almost a complete loss of neutralizing activity from the sera of LN40 primed animals. These residues, which were critical in modulating resistance to b12, also appear to be critical in modulating resistance to the antibodies generated through immunization with LN40.

As further evidence suggested that antibodies targeted to the CD4bs were the cause of the observed neutralizing activity, we wanted to evaluate additional mutations that flank the CD4 binding loop. Because this region is highly polymorphic, we made four mutations, in concert, in the LN40 backbone. These mutations, an I360V mutation, an N362K mutation, a Q363P mutation, and a P364S mutation combined to form the LN40VKPS mutant. When tested for its effect on neutralization, these mutations appear to play a less critical, but still modulatory role, in the neutralization sensitivity of the virus. These mutations, in concert, resulted in an average drop of 22% neutralization relative to the parental LN40.

Additional mutations were also made in the crown of the V3 loop of LN40 to make it identical to that of B33. We determined that these two mutations, H310N and F317L, also play a role in modulating sensitivity of the LN40 virus to its autologous sera. In this case, we observed a 15-44% drop in neutralization of the LN40 NL mutant relative to the parental LN40 virus. Interestingly, this also provides evidence that multiple

antibodies targeted to distinct epitopes may act in conjunction to neutralize a single HIV isolate.

Identification of residues important for neutralizing activity of B33 primed sera

While we demonstrated that mutations in and surrounding the CD4bs had the largest effect on the neutralizing activity of sera from LN40 primed animals, the reason for the narrow neutralization seen in sera from B33 primed animals was less clear. Historically, antibodies targeted to the V3 loop of the HIV Env have been implicated in narrow, type-specific neutralization. For this reason, we began investigating if B33 was only being neutralized because of the presence of V3-specific antibodies. To test this, we introduced two sets of mutations into the B33 virus. The first set altered the crown of the B33 V3 loop to look like that of LN40 by introducing an N310H and a L317F mutation to generate the B33 HF mutant. These mutations caused an approximately 20% drop in neutralization in two of our sera, and almost no change at all in the other two sera (Table 5.5).

However, two additional polymorphisms between LN40 and B33 that flanked the V3 loop also existed. We introduced these mutations, A291S and N310H, in conjunction with the N310H and L317F mutations in the B33 backbone to generate the B33 SHFE mutant. When we did this, almost all neutralization of the mutant virus was lost against the sera from B33 primed animals. This result indicates that the narrow neutralization phenotype induced by priming with B33 is likely due to the elicitation type specific V3 directed NAbs by this particular Env.

Table 5.5 Point mutations affecting the neutralizing activity of sera from B33 primed animals

		B33 Primed			
Virus	Mutation	492	655	656	657
LN40		11	10	21	20
B33		52	65	56	73
B33 HF	N310H, L317F	55	42	53	48
B33 SHFE	A291S, N310H, L317F, S353E	25	26	19	23

Numbers indicate percent neutralization at a 1:10 serum dilution

Discussion

In the previous chapters, we established that a DNA prime-protein boost regimen elicits a unique profile of antibody responses, which appears capable of eliciting antibodies to the CD4bs, and correlates with increased breadth of neutralization. Knowing this, we wanted to determine if we could utilize the DNA prime-protein boost regimen as a tool to answer another fundamental question of whether or not all HIV antigens have equivalent abilities to raise a heterologous NAb response.

One common criticism of the previously failed phase III Vaxgen trials (33, 80) was the use of gp120s only distantly related to those found in circulation. In this set of studies, we attempted to determine whether the antigen selection used in a potential vaccine is important within the context of generating a binding and neutralizing antibody response against HIV. This study utilized two gp120s , LN40 and B33, as model antigens to address this question. These two Envs were ideal for this purpose because they were isolated from the same individual at the same time point, making them much more similar to each other than to any other gp120 from heterologous isolates, while still maintaining distinct antigenic phenotypes.

Dissection of the binding antibody response revealed very little difference between the LN40 and B33 gp120s when used in the priming phase of a DNA prime-protein boost regimen. High titers of cross reactive binding antibodies were seen regardless of what gp120 was used as a priming immunization. In contrast to this similarity, the quality of the NAb response differed greatly. This result alone warrants re-evaluation of antibody responses generated in future vaccine trials. No longer should the

generation of a binding antibody response be considered sufficient for furthering the development of a potential vaccine.

In this study, we demonstrated that LN40, when used as a priming immunization, generated a broader heterologous NAb response than priming with the B33 gp120. Epitope mapping analysis revealed that the enhanced neutralization observed in the sera from LN40 primed animals is likely due recognition of the CD4bs of gp120 (Fig 5.6). Again, this data is in agreement with numerous other studies which have indicated that recognition of the CD4bs may be a critical domain recognized by sera from HIV infected individuals with broadly neutralizing activity (12, 57, 58, 94). To date, however, no group has been able to demonstrate that immunization with two different naturally occurring gp120s can result in differential elicitation of antibodies to a conserved domain. This is especially remarkable given the overall sequence similarity present in the LN40 and B33 epitopes.

In summary, using B33 and LN40 gp120s as model immunogens in a DNA prime-protein boost format we have demonstrated that even very similar proteins can elicit a different NAb response. This NAb response cannot be predicted solely on the presence of binding antibodies against multiple antigens but instead lies in the fine specificity of antibodies being elicited by each immunogen.

Mapping of the neutralizing activity from LN40 primed animals revealed that mutations within and surrounding the CD4bs had the greatest detrimental effect on neutralization of the autologous LN40 isolate. This evidence suggests that the broader neutralizing activity elicited by priming with LN40 may be due to the elicitation of CD4bs antibodies. With regards to the highly type specific neutralization seen in sera

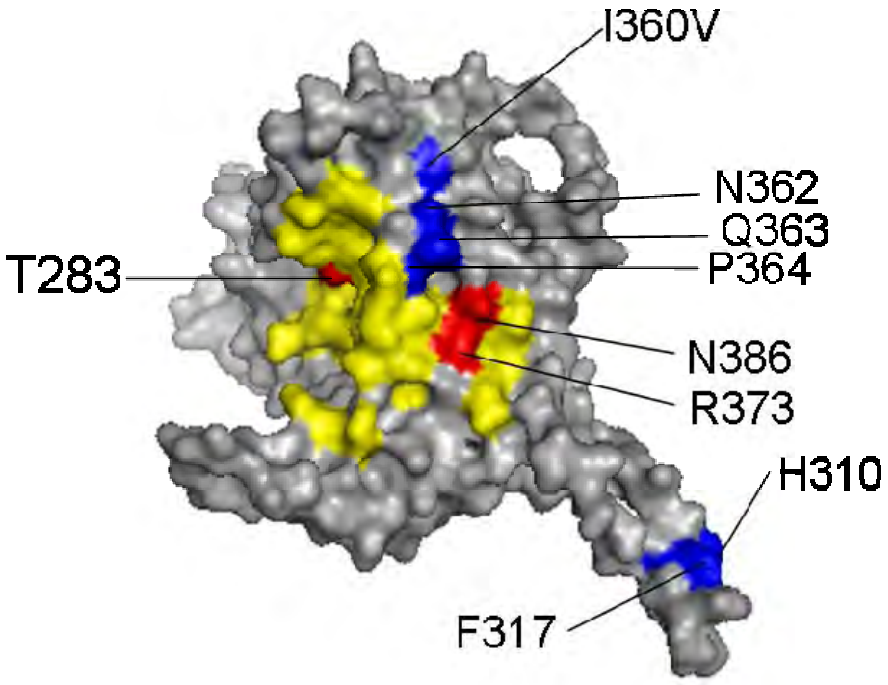


Fig 5.6. Schematic of amino acid residues important for maintenance of neutralizing activity in sera from LN40 primed rabbits. Crystal structure of gp120 liganded with sCD4 and X5. Yellow highlighted residues indicate the binding footprint of mAb b12. Red highlighted positions indicate residues critical for neutralization of LN40 by autologous sera. Positions highlighted in blue indicate residues which modulate, but not ablate, neutralization of LN40 by autologous sera. Letters indicate the residue found at that position in LN40. Numbers indicate the position of that residue according to the HxBc2 numbering standard.

from B33 primed animals, mutations to the V3 loop had the greatest detrimental effect on the neutralizing activity of autologous sera. This suggests that the B33 Env may be eliciting V3 directed antibodies targeted to an abnormally type specific epitope. These findings warrant further investigation of other Env proteins that may further enhance the NAb response against HIV isolates or potentially mimic the antibody specificities seen in those individuals capable of mediating broad neutralization of HIV.

Chapter VI

Generation of HIV Specific Monoclonal Antibodies in Rabbits

Introduction

Much of our knowledge of the potential of the HIV Env protein as a vaccine antigen, in particular its exploitable weaknesses, comes from the study of Env specific mAbs. Hundreds of Env specific mAbs have been generated from B cells of HIV infected individuals. Of these mAbs, only a handful has been studied in any great detail. This subset that has been well studied is largely due to their ability to cross react with or neutralize a wide variety of HIV isolates. The most well studied of these antibodies are the CD4bs specific antibody, b12, the glycan specific antibody, 2G12, the V3 directed antibody, 447-52D, the V2/V3 specific antibodies, PG9 and PG16, and the MPER specific antibodies, 2F5 and 4E10.

To date, the cross reactivity and neutralizing capabilities of these select mAbs are the standard by which all other humoral responses are judged. Despite the influence that these antibodies have in the HIV vaccine field, there is still an alarming lack of information about how well antibodies elicited through immunization compare to these well defined mAbs. Part of the reason for this, is that direct comparison of polyclonal sera and mAbs is not a fair comparison. For most of these mAbs, the concentration that they were present at in the original donors is not known. Therefore, we have no way of knowing if the concentrations that these antibodies neutralize at are in fact relevant. Additionally, a direct comparison of known mAbs to antibodies generated through immunization necessitates the production of monoclonals from immunized animals or individuals. Despite the huge number of antibodies generated from infected individuals,

very few have been studied from immunized animals. To the best of our knowledge, in one of the most useful animal models, NZW rabbits, no HIV specific mAbs have ever been isolated.

All previous analyses in this body of work have studied humoral responses elicited through immunization in the context of antibodies present in a polyclonal population. However, working with polyclonal sera presents a set of challenges which makes definition of the capabilities of a particular subset of antibodies a difficult task. To begin, within a polyclonal population the actual number of antibodies targeted to a particular epitope and the total number of epitopes being recognized is unknown. As a corollary to this, it is theoretically possible that different antibodies specific for the Env protein could have interfering or synergistic effects when evaluated in binding or neutralization assays. Furthermore defining the epitopes themselves, or capabilities of antibodies targeted to a specific epitope, through mutagenic analysis runs the risk of inadvertently altering a distal region of the epitope being studied, confounding any resulting analysis.

In this study, we attempt to overcome these challenges and identify the capabilities of antibodies elicited through immunization by generating mAbs from a single immunized animal. In the current chapter, we report the isolation of thirty-six gp120 specific hybridomas from a single DNA primed-protein boosted rabbit. Analysis of these antibodies reveals that a wide range of specificities are represented in the hybridoma population. These antibodies recognize both linear and conformational epitopes and are targeted to multiple distinct regions of gp120. In addition, these

antibodies demonstrate different levels of binding cross reactivity as well as neutralization of heterologous isolates.

Results

Immunization Schedule

A single NZW rabbit was immunized using the model clade B gp120 antigen JR-FL in a modified DNA prime-protein boost schedule in order to examine the gp120specific B cell responses at the time of splenocyte isolation (Fig 6.1). Following our standard immunization regimen of three DNA primes and two protein boosts, the rabbit was given one additional DNA immunization and a final 400 µg protein boost delivered intravenously in PBS. Four days after the final protein boost the spleen was isolated and shipped to Epitomics for hybridoma production.

Defining mAb binding characteristics

Screening of cell free supernatants from lines surviving fusion and selection with the E240 fusion partner from 4,000 individual wells revealed the generation of 36 gp120 specific hybridoma lines. Recognition of the autologous JR-FL gp120 by antibody secreted from these cell lines are shown by ELISA in Fig 6.2. As demonstrated by the data here, all 36 lines secreted antibody that recognized JR-FL gp120 in its native form.

Because some of the most highly conserved regions of gp120, such as the CD4bs, are conformational in nature, we were also interested in determining if these mAbs recognized conformationally sensitive epitopes on the JR-FL Env. To test this, we evaluated each mAb for recognition of JR-FL gp120 by Western blot under denaturing conditions. This data revealed that antibodies specific for both conformational and linear epitopes were being elicited by our DNA prime-protein boost regimen. We

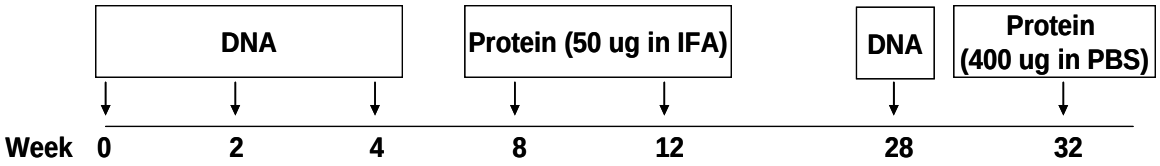


Fig 6.1. Immunization schedule for production of rabbit mAbs. A single NZW rabbit received three JR-FL gp120 DNA immunizations by gene gun at Weeks 0, 2, and 4. This was followed by two JR-FL protein immunizations delivered intramuscularly in IFA at Weeks 8 and 12. A final DNA immunization was given by gene gun at Week 28. The final protein boost was given as a 400 µg dose in PBS intravenously at Week 32. The spleen was isolated four days after the final protein immunization.

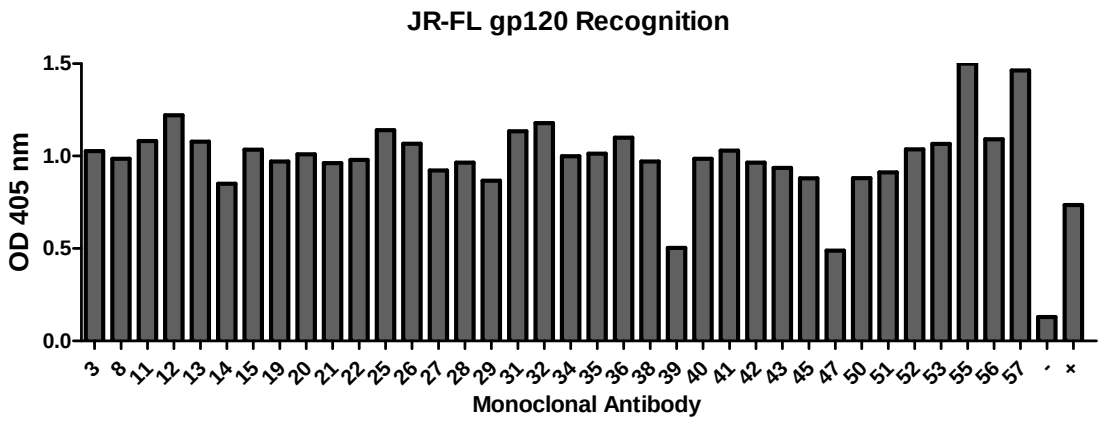


Fig 6.2 Recognition of JR-FL gp120 by mAbs. Supernatants from hybridoma lines were screened for recognition of the autologous antigen JR-FL gp120. Numbers on the X axis indicate mAb ID number. "-" indicates recognition of JR-FL from a polyclonal pre-bleed sample at a 1:10,000 dilution. "+" indicates recognition of JR-FL of a polyclonal sera sample isolated in week 30 at a 1:10,000 dilution.

found that 19 of the isolated antibodies do not recognize linearized JR-FL based upon the absence of a JR-FL gp120 band on the Western blot (Fig 6.3). These antibodies were identified as mAb numbers, 8, 11, 12, 19, 21, 22, 25, 26, 27, 29, 31, 32, 36, 38, 39, 42, 47, 52, and 57. The remaining 17 isolated antibodies, mAb numbers, 3, 13, 14, 15, 20, 28, 34, 35, 40, 41, 43, 45, 50, 51, 53, 55, and 56, recognized the denatured form of JR-FL. However, even among these antibodies there was a high degree of variability as to how well they recognized the denatured form of the antigen. An example of this occurred with mAbs #53 and #55. Antibody #53 recognized the denatured form of JR-FL very efficiently, whereas #55 barely recognized it at all. This occurred despite the fact that #55 recognized JR-FL gp120 by ELISA more efficiently than #53 (Fig 6.3).

While identifying the type of epitope being recognized by each antibody is valuable, we also wanted to determine if it was possible at this stage to identify individual epitopes being targeted by each monoclonal. In order to do this, we utilized the same competitive binding assay used in the previous studies. We chose the V3 directed mAb 3074, the CD4bs directed mAb b12, and the glycan specific antibody 2G12 as competitive targets for our monoclonals with unknown specificity. While many of our antibodies were not capable of outcompeting binding to any of our known monoclonals, we did observe several marginal and two strong competitive hits against 3074, b12, and 2G12 (Table 6.1). Of particular interest were the mAbs #52, #53 and #56. Antibody numbers 52 and 53 both prevented greater than 50% of viral binding to mAb b12, potentially indicating that their binding footprints overlap with that of the CD4bs. Antibody number 56, on the other hand, exhibited the strongest competition seen in the assay against 3074, providing evidence that it may be targeted to the V3 loop of gp120.

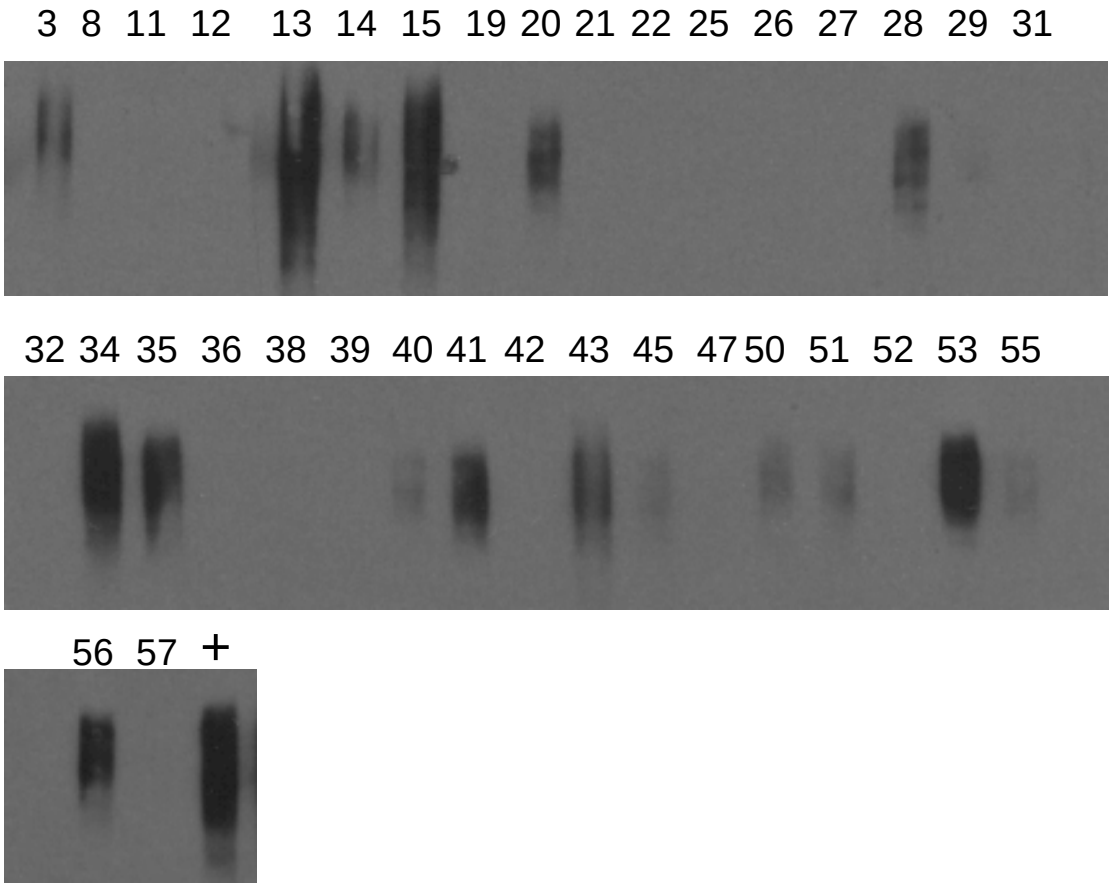


Fig 6.3 mAb recognition of denatured JR-FL gp120. Numbers indicate mAb ID number. "+" indicates recognition of denatured JR-FL gp120 by polyclonal sera collected at Week 30 at a 1:500 dilution.

Table 6.1 Ability of unknown mAbs to outcompete binding to mAbs of known specificity

	Competing mAb		
mAb	**3074**	**b12**	**2G12**
3	-12	22	14
8	-17	-44	13
11	-29	39	-1
12	-35	46	-16
13	2	2	7
14	20	11	17
15	17	24	11
19	17	27	20
20	-1	32	22
21	-11	26	22
22	1	3	19
25	-21	40	18
26	2	8	23
27	20	2	13
28	11	18	10
29	16	44	17
31	37	44	51
32	11	41	39
34	13	30	27
35	-6	29	14
36	0	7	22
38	19	33	20
39	22	4	14
40	32	38	17
41	31	45	40
42	8	20	30
43	22	21	28
45	-3	4	-2
47	-8	-11	-5
50	21	42	27
51	17	-2	11
52	27	72	10
53	21	56	39
55	-7	28	-2
56	83	34	22
57	19	5	6

Numbers indicate % reduction in luciferase activity upon competition with unknown mAbs at a 1:2 dilution.

The final analysis performed at this stage was to monitor the antibodies' ability to neutralize different HIV isolates (Table 6.2). Unfortunately, due to the low concentration of antibodies present in the culture supernatants at this stage, almost always below 1 μg/mL, very little neutralization was seen. The exceptions to this were seen in neutralizing assays with mAbs #28 and #56. Antibody #28 demonstrated marginal neutralization against the SS1196 isolate while antibody #56 demonstrated relatively potent neutralization against SF162.

Based upon the binding and neutralizing data at this stage we selected twelve antibodies for further examination. These antibodies, #13, #15, #20, #27, #28, #31, #35, #41, #43, #52, #53, and #56, were all subjected to further binding analysis. In an attempt to determine the exact specificities of these antibodies, we mapped recognition of linear overlapping peptides derived from the clade B consensus sequence.

Of the twelve antibodies selected for further analysis, four antibodies did not react strongly with any of the peptides tested (Fig 6.4). Of these four antibodies, three did not react with denatured JR-FL in a Western blot analysis. The fourth antibody, #28, did react well with denatured JR-FL gp120 by Western blot but was still negative by peptide mapping. This lack of recognition may be due to an epitope that is longer than the 15 residues encompassed by our peptides, or more likely sequence polymorphisms between the peptides and the JR-FL immunogen which could be precluding recognition. If sequence polymorphisms between JR-FL and the peptides are in fact precluding recognition, it may be possible that mAb #28 is targeted to the Env variable loops, however, this is pure conjecture at this stage.

Table 6.2 Ability of mAbs to neutralize HIV pseudoviruses

	Pseudovirus				
mAb ID	NL4-3	SS1196	SF162	JR-FL	MLV
3	22	12	17	12	15
8	21	24	25	25	18
11	27	28	21	25	36
12	21	29	25	16	37
13	17	28	24	21	40
14	21	29	25	16	9
15	17	28	24	21	11
19	20	17	13	18	4
20	17	22	-1	26	4
21	21	21	9	19	3
22	19	22	17	19	11
25	15	16	17	8	21
26	11	18	22	15	6
27	14	26	29	7	15
28	7	58	39	7	28
29	14	31	8	10	22
31	13	27	12	28	3
32	13	19	-4	17	-5
34	0	9	-3	5	-9
35	1	4	13	9	0
36	12	15	19	16	0
38	3	16	2	8	4
39	9	20	13	6	17
40	10	31	-8	22	-8
41	8	20	-21	34	10
42	15	16	8	25	8
43	16	18	-8	10	-4
45	1	4	-29	-7	-13
47	8	15	6	-5	24
50	22	26	17	33	23
51	18	32	20	14	27
52	9	32	11	23	3
53	20	22	14	33	13
55	9	3	-21	12	-17
56	12	10	88	5	-3
57	26	24	11	17	7

Numbers indicate % neutralization of indicated pseudovirus by culture supernatants at a 1:2 dilution

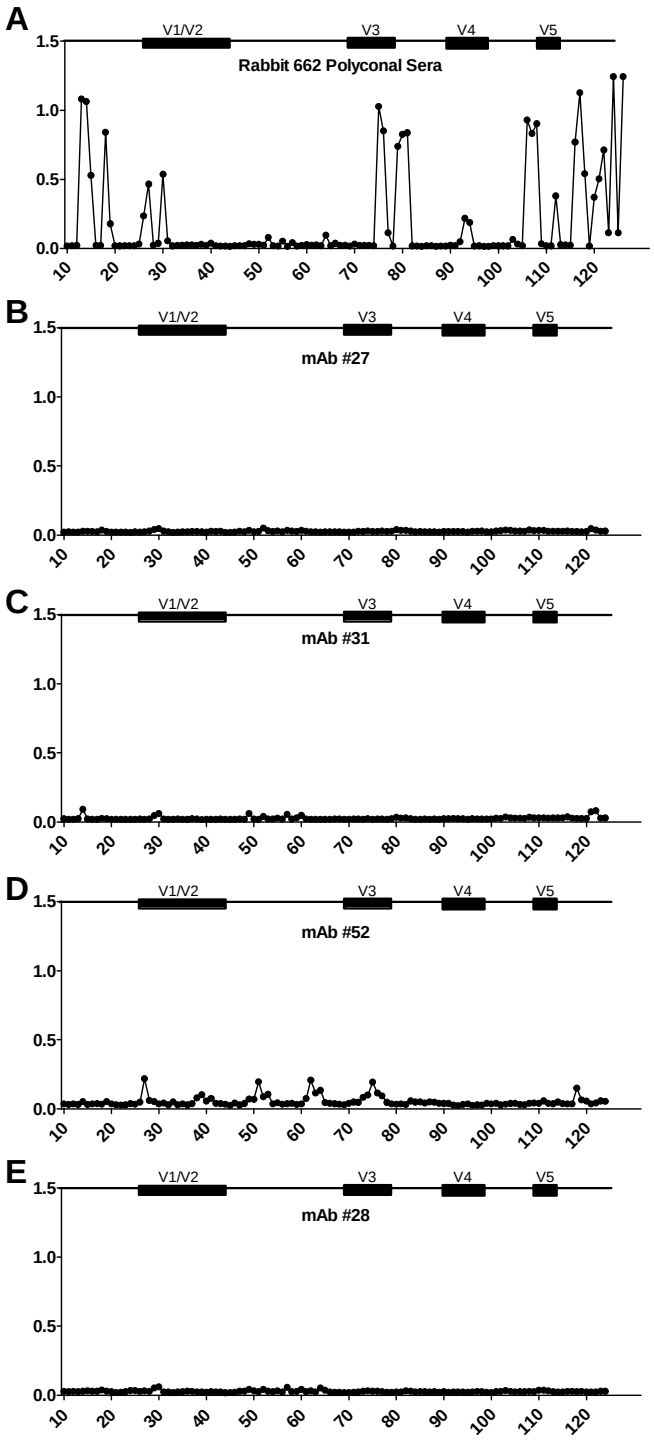


Fig 6.4 Monoclonal antibodies with no recognition of consensus B linear peptides **A.** Peptide recognition of polyclonal sera from R662 collected at Week 30 at a 1:1000 dilution. **B.** Peptide recognition of mAb #27. **C.** Peptide recognition of mAb #31. **D.** Peptide recognition of mAb #52. **E.** Peptide recognition of mAb #28.

Of the remaining eight mAbs tested, three recognized a linear epitope in the C1 region of Env (Fig 6.5). These three mAbs, #15, #35, and #41, all recognized peptides encompassing the sequence TTLFCASDAKAYDTEVHNVWATH. Such similar recognition suggests that these three antibodies are either identical or clonally related to each other.

The next grouping of antibodies recognized two distinct epitopes within the V3 loop of Env. Two of these antibodies, #20 & #43, recognized an epitope on the C-terminal strand of V3 (Fig 6.6A & B). This epitope encompassed the sequence RAFYTTGEIIGDIRQAHCNISRA. Interestingly, these two C-terminal V3 antibodies also reacted weakly with a peptide derived from the N-terminal region of C5. This peptide spanned the sequence RPGGGDMRDNWRSEL. If confirmed, this may provide insight as to the position of the V3 loop in an unliganded gp120 structure. The remaining V3 specific antibody, mAb #56, recognized peptides derived from the N-terminal strand and beta turn of the V3 loop. Specifically the mAb recognized peptides spanning the sequence TRPNNNTRKSIHIGPGRAF (Fig 6.6D). This sequence specificity correlates well with the competition data for this antibody because it strongly outcompeted binding to the V3 directed mAb 3074, which has a similar epitope specificity. Additionally, this antibody also potently neutralized the isolate SF162, which is known to be highly sensitive to V3 mediated neutralization. Furthermore, the N-terminal strand/crown of the V3 loop is the target of several neutralizing V3 directed antibodies. Recognition of this epitope may explain the relatively potent neutralization of SF162 by this particular mAb as well.

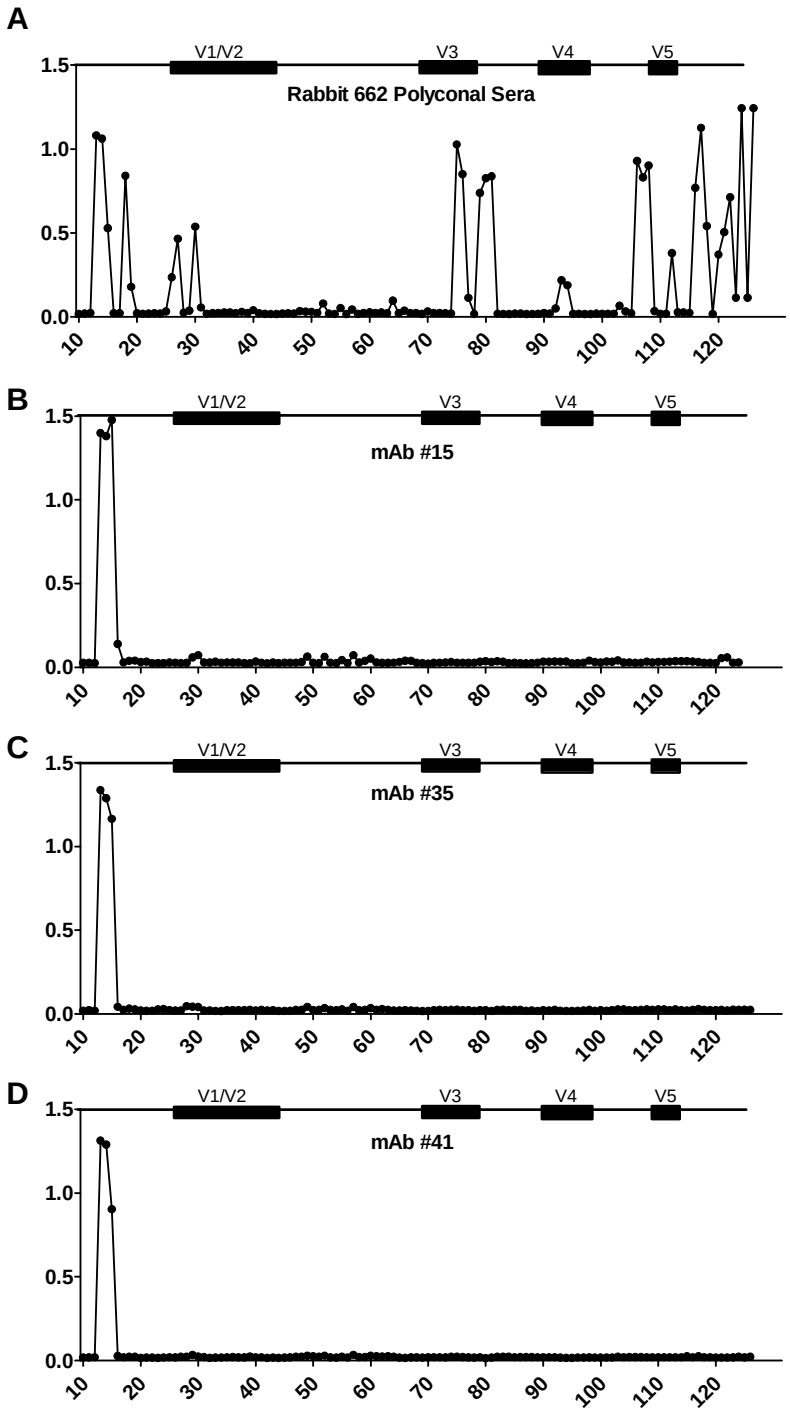


Fig 6.5 Recognition of consensus B peptides by mAbs with specificity for the C1 region of Env **A.** Peptide recognition of polyclonal sera from R662 collected at Week 30 at a 1:1000 dilution. **B.** Peptide recognition of mAb #15. **C.** Peptide recognition of mAb #35. **D.** Peptide recognition of mAb #41.

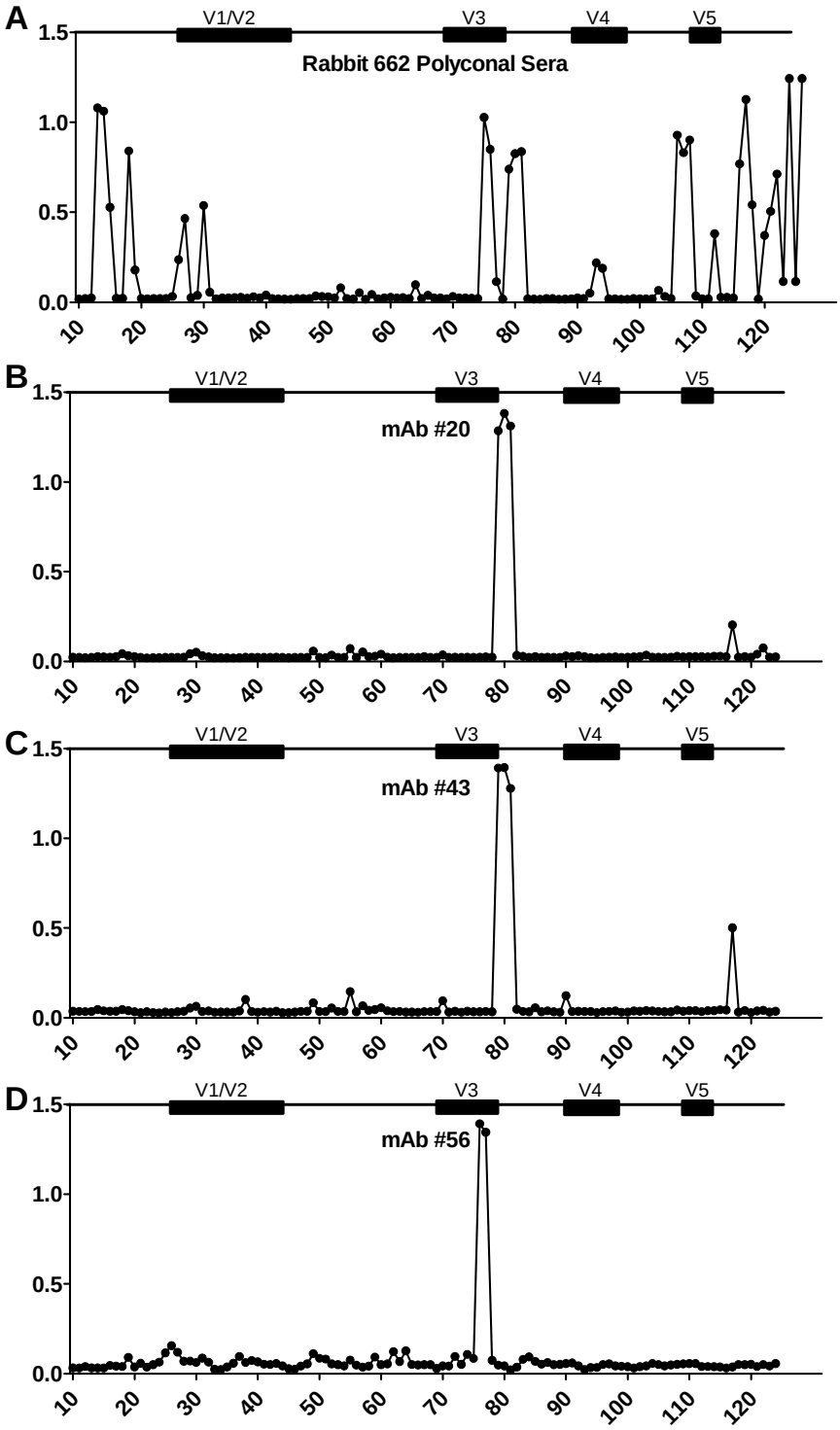


Fig 6.6 Recognition of consensus B peptides by mAbs with specificity for the V3 loop of Env **A.** Peptide recognition of polyclonal sera from R662 collected at Week 30 at a 1:1000 dilution. **B.** Peptide recognition of mAb #20. **C.** Peptide recognition of mAb #43. **D.** Peptide recognition of mAb #56.

The next epitope recognized by a mAb produced in this study was found in the C5 region of Env. This monoclonal, #13, recognized a set of two peptides from C5 with the sequence KYKVVKIEPLGVAPTKAKR (Fig 6.7B).

The final antibody isolated in this study, #53, is particularly interesting. This single antibody appears to recognize peptides from two different regions of gp120 (Fig 6.8B). The first of these regions is found in the C1 region and spans the sequence ATHACVPTDPNPQEV. The second region, found in C4, spans the sequence MWQEVGKMYAPPIRGQIRCSSN. This is an interesting collection of peptide recognition due to the fact that residues that are recognized in the C4 region are known CD4 contact residues. This coupled with the fact that #53 weakly outcompeted binding to b12 suggests that this antibody may be targeted to the CD4bs.

After identifying regions of gp120 that some of these mAbs are targeted to, we next wanted to determine how cross reactive these antibodies are with gp120s from other clades. We evaluated this cross reactivity in an ELISA based assay where we monitored binding of the mAbs to gp120s derived from clades A (92UG037), B (92US715 & BaL), C (96ZM651), and E (93TH976) (Fig 6.9). When this was tested we observed that each mAb had a unique pattern of cross reactivity. We first evaluated our antibodies with unknown specificity against this panel of gp120s (Fig 6.9A). Of these, mAbs #27, #31, and #52 did not cross react with any of the tested gp120s other than the homologous JR-FL. Antibody #28 fared slightly better with weak cross reactivity with 92US715, 96ZM651, and 93TH976. We next evaluated the C1 specific mAbs #15, #35, and #41. We observed recognition of the clade A and the 92US715 clade B gp120 by mAbs #35

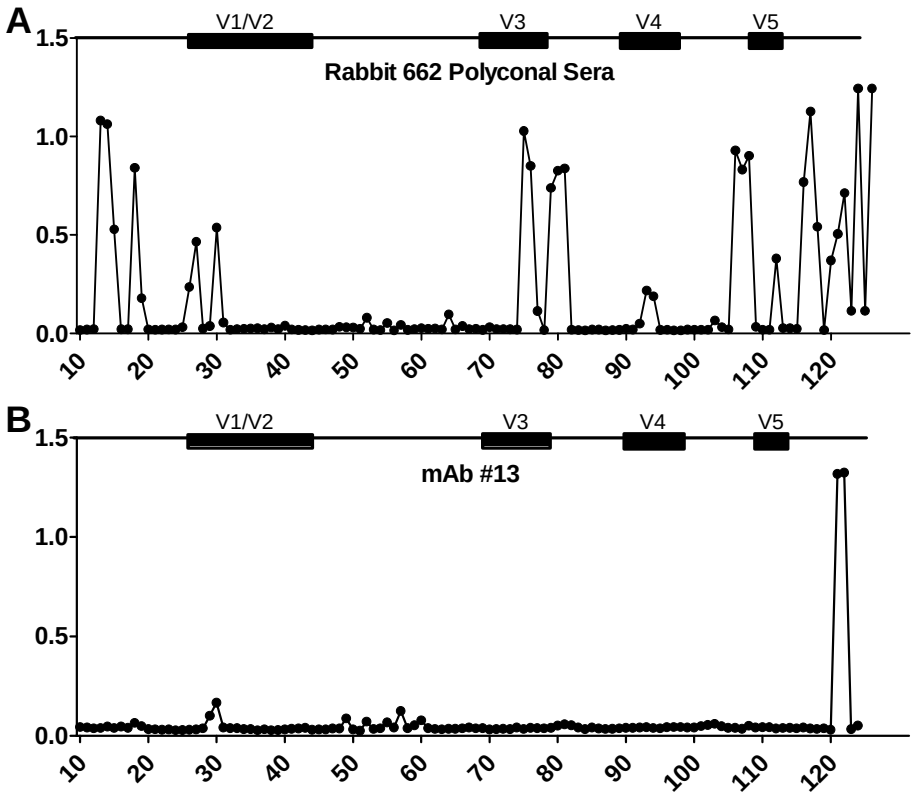


Fig 6.7 Recognition of consensus B peptides by mAbs with specificity for the C5 region of Env **A.** Peptide recognition of polyclonal sera from R662 collected at Week 30 at a 1:1000 dilution. **B.** Peptide recognition of mAb #13.

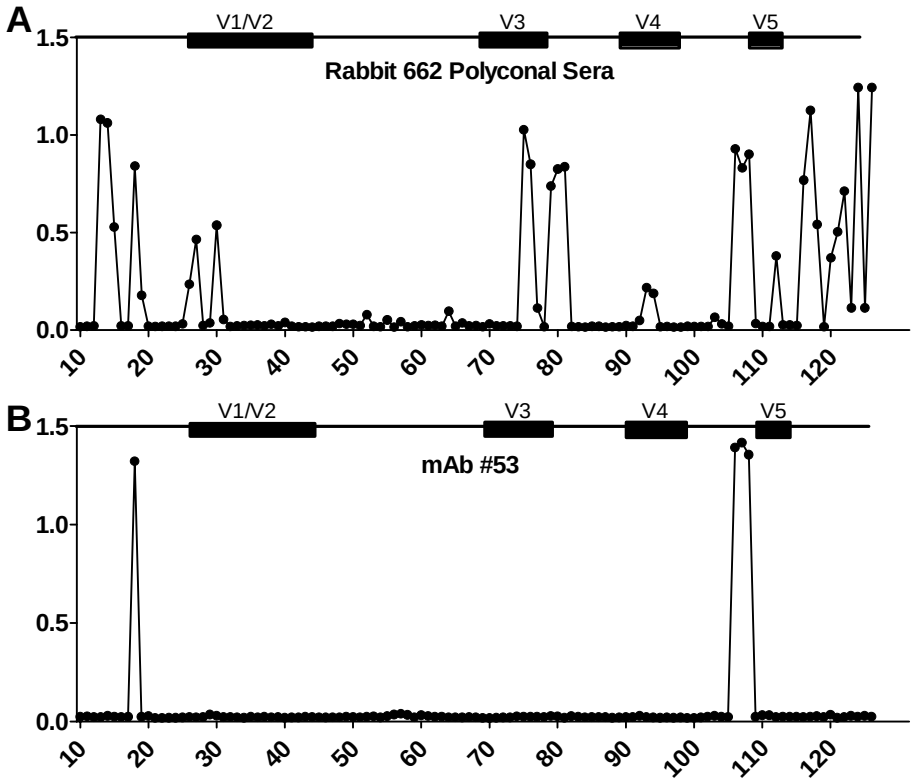


Fig 6.8 Recognition of consensus B peptides by mAbs with specificity for multiple regions of Env **A.** Peptide recognition of polyclonal sera from R662 collected at Week 30 at a 1:1000 dilution. **B.** Peptide recognition of mAb #53.

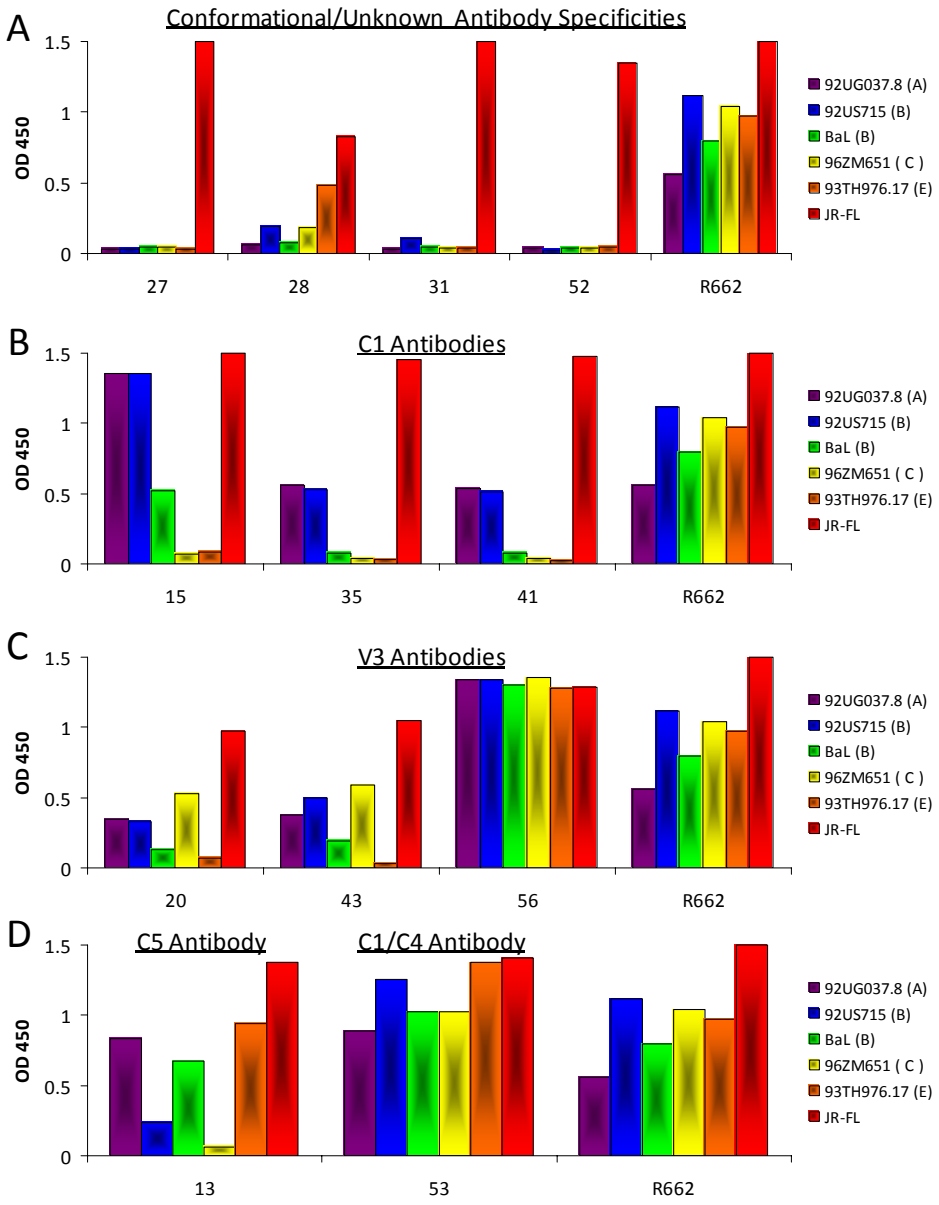


Fig 6.9. Cross reactivity of mAbs to gp120s of different clades. Hybridoma supernatants were screened for recognition of gp120s derived from isolates from clade A (92UG037.8), clade B (92US715, BaL, & JR-FL), clade C (96ZM651), and clade E (93TH976.17). "R662" indicates recognition of the various gp120s by polyclonal sera from the parental rabbit at a 1:1000 dilution. **A.** Recognition of gp120s by conformational or unknown antibody specificities **B.** Recognition of gp120s by C1 specific antibodies **C.** Recognition of gp120s by V3 specific antibodies **D.** Recognition of gp120s by a C5 specific antibody (#13) or a C1/C4 specific antibody (#53)

and #41. However, no recognition of the clade C or clade E gp120 in this study was seen. Recognition of this multiclade panel by mAb #15 was similar to that of #35 and #41, with the exception that mAb #15 also recognized the clade C gp120.

The V3 directed antibodies were also tested for recognition of this panel of gp120s (Fig 6.9C). Monoclonals #20 and #43, the two C-terminal strand V3 specific antibodies, were able to weakly cross react with all the gp120s in this panel except for the clade E gp120. The V3 directed antibody specific for the N-terminal strand, #56, demonstrated the broadest cross reactivity of all the mAbs tested in this study. This mAb was able to recognize every single gp120 it was tested against.

Finally, we tested the C5 specific mAb #13 and the C1/C4 specific mAb #53 (Fig 6.9D). We found that mAb #13 recognized 5 out of the 6 gp120s it was tested against. The one exception to this was the lack of reactivity to the clade C gp120. When mAb #53 was tested against these gp120s, we determined that it is as cross reactive as the V3 mAb #56. Monoclonal #53 was capable of recognizing all six of the gp120s in this panel.

Discussion

The type of mutagenic analysis used in the previous chapter is an extremely valuable means by which to dissect the neutralizing activity and specificity of polyclonal sera. However, due to the diverse number of epitopes being targeted by antibodies in polyclonal sera it is difficult to define exact antibody specificities and activities. This difficulty stems from the risk of one mutation having global effects on the target Env and affecting a region distal to the experimental mutation. Because we wanted to study the capabilities of individual antibodies generated with our DNA prime-protein boost approach, we generated mAbs from a single DNA primed-protein boosted rabbit. This allowed us to directly compare capabilities of antibodies elicited through immunization to those broadly NAbs that were elicited through natural infection.

After hybridoma fusion and selection, we identified 36 lines which all secreted antibody that recognized the autologous JR-FL gp120. Initial characterization of these lines revealed that antibodies recognizing both linear and conformational epitopes were being represented approximately equally. Additionally, some of these antibodies demonstrated the capability to neutralize heterologous virus and outcompete binding to known neutralizing mAbs.

After this initial screening, we selected 12 antibodies for further testing. Additional epitope mapping revealed that antibodies recognizing linear epitopes were found to target the C1, V3, C4, and C5 regions of Env. When we tested these antibodies for binding cross reactivity against multiple gp120s from different clades, we observed a highly variable pattern of cross reactivity. While some of these antibodies, such as #27, #31, and #52, only react with the autologous JR-FL gp120, others, such as #53 and #56,

cross react with every gp120 they have been tested against. This data suggests that some of the antibodies being elicited through this vaccination regimen have greater abilities to cross react with heterologous gp120s than sera from some HIV infected individuals (117). Additionally, we provide evidence that these antibodies are targeted to functionally important domains, such as the CD4bs and the V3 loop. Also, some of these antibodies are capable of mediating neutralization of heterologous HIV isolates.

In this study, we have proven that it is viable to generate mAbs from DNA primed-protein boosted rabbits. From the antibodies that were generated, we were able to demonstrate that immunization with gp120 elicits antibodies to both linear and conformational epitopes, that these antibodies have diverse specificities for different regions of gp120, and these antibodies vary in their cross reactivity to Envs of different clades. Importantly, mAbs that were generated appear to be targeted to functionally important regions such as the CD4bs and the V3 loop. While thus far, only limited neutralization has been performed, these assays are ongoing. We hope to demonstrate that mAbs generated through this immunization approach have the capability to neutralize diverse isolates of HIV.

Chapter VII

Materials and Methods

HIV-1 gp120 DNA vaccines

All constructs used for DNA immunizations were HIV-1 gp120s cloned into the pJW4303 vector, as previously reported (113). DNA was produced in HB101 bacterial cells then isolated and purified using the Qiagen Plasmid Maxi or Mega Kits. Env gp120 constructs derived from the JR-FL, 92UG037, 92US715, Ba-L, 96ZM651, & 93TH976 were all codon optimized. Env gp120s from LN40 and B33 were of wild type codon usage.

HIV-1 gp120 protein vaccines

Recombinant HIV-1 gp120 proteins were produced from Chinese Hamster Ovary (CHO) cells. The JR-FL gp120 protein was produced by Progenics and provided by Dr. John Warren at Division of AIDS, NIAID, NIH. The gp120s derived from 92UG037.8, Ba-L, 96ZM651, and 93TH976.17 were produced in CHO cells at Advanced BioScience Laboratories. Other gp120 Env glycoproteins from subtypes A (UG21-9), B (92US715), C (MW959), and E (TH14.12) were all produced by Dr. James Arthos. Secreted proteins from stably transfected CHO cell lines were harvested and purified over a lectin affinity column.

Antibodies

The CD4bs directed mAbs, b12 and b6 were obtained as a gift from Dr. Dennis Burton or purchased from Polymun. The V3-directed mAb, 447-52D and 3074, were provided as a gift from Dr. Susan Zolla-Pazner. The co-receptor binding site antibody 17b and the V3 directed antibodies 39F and LE311 were provided by James Robinson.

The mAbs 2G12, and F105 were obtained through the NIH AIDS Research & Reference Reagent Program.

Rabbit Immunizations

NZW rabbits at 6-8 weeks of age were purchased from Millbrook Farm (Amherst, MA) and housed in the animal facility managed by the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS) in accordance of the protocol approved by UMMS' Institutional Animal Care and Use Committee (IACUC).

When DNA immunizations were given, DNA encoding the appropriate immunogen or the pJW4303 vector control were coated onto 1 micron gold beads at a ratio of 2 μg of DNA per milligram of gold beads and delivered to animals via a Bio-Rad Helios gene gun onto shaved abdominal skin. Each animal received 36 μg of DNA per immunization. Where appropriate, protein immunizations were administered consisting of either 50 μg of a single gp120 protein or 10 μg each of a 5-valent formulation of gp120s. Prior to injection, 50 μg of gp120 protein was diluted in 500 μL PBS and mixed with 500 μL IFA. The 1 mL adjuvanted protein solution was then injected intramuscularly into the lumbar region of rabbits. Serum was collected for antibody studies two weeks prior to the first immunization and two weeks after each animal immunization.

Endpoint Binding ELISA

All coating antigens with the exception of LN40 and B33 gp120 used in Chapter VI were purified gp120s identical to those used for the immunization of rabbits. In chapter VI, LN40 and B33 antigen used for coating plates was produced by transient transfection of 293T cells. Recombinant gp120 protein was coated onto 96 well

microtiter plates (Costar) at 1 μg/mL in 100 μL of PBS for 1 hr at room temperature. When LN40 and B33 protein was coated on ELISA plates, 100 μL of serum free cell culture supernatants was used. Plates were then washed 5 times in PBS containing 0.1% Triton-X (EWB) and blocked overnight at 4°C in PBS containing 4% whey by weight (whey dilution buffer) and 5% powdered milk. The following morning, plates were washed 5 times in EWB and serially diluted rabbit sera, collected at 2 weeks following the final protein immunization, was added to the wells in a volume of 100 μL. Plates were washed 5 times in EWB and 100 μL of biotinylated anti-rabbit secondary antibody (Vector Labs) at 1.5 μg/mL was incubated on the plate for 1 hr at room temperature. Plates were washed 5 times with EWB and incubated with 100 μL of a streptavidin horseradish peroxidase construct (Vector Labs) at 500 ng/mL. Plates were washed 5 times with EWB and developed for 3 min in 100 μL of a 3,3'5,5'-tetramethylbenzidine substrate solution (Sigma). The reaction was stopped with addition of 25 μL of 2N H_2SO_4. Endpoint titers as reported are defined as the last dilution of a serially diluted serum sample with greater than double the background optical density of a preimmune serum sample.

Peptide Binding ELISA

Microtiter plates (96 wells) were coated with 100 μl of individual overlapping 15 mer peptides (4 μg/ml) derived from the consensus HIV-1 M group gp120 sequence, obtained from the NIH HIV Research and Reference Reagent program. Plates were incubated at room temperature for 1 hour, then washed 5 times with 200 μl wash buffer (0.1% Triton-X in PBS) and blocked overnight in 4% whey dilution buffer containing 1% by weight powdered milk. Plates were washed again and incubated with 100 μl rabbit

sera normalized to 200 ng/ml gp120-specific IgG for 1 hour at room temperature. Plates were washed again and incubated with 100 µl of 1 µg/ml biotinylated anti-rabbit Ab (BA-1000, Vector Labs) for 1 hour at room temperature. After additional washes, 100 µl of 500 ng/ml HRP conjugated streptavidin (Vector Labs) was added for 1 hour at room temperature. Plates were washed 5 times with EWB and developed for 3 min in 100 µL of a 3,3'5,5'-tetramethylbenzidine substrate solution (Sigma). The reaction was stopped by the addition of 25 µl 2N H_2SO_4. Plates were then read using a Dynex® OpSys MR plate reader.

In chapter III, the polyclonal rabbit sera were normalized using a semi-quantitative ELISA to determine the concentration of gp120-specific IgG in each. The gp120 used to coat plates was the matched 5-valent mixture (1 µg/mL of each component). The concentration of gp120-specific IgG in each serum was determined against a standard curve generated using a known rabbit IgG (Southern Biotechnology Associates), as previously described (116). Rabbit sera were prepared to normalized concentrations of 200 ng/mL of gp120-specific IgG for screening of overlapping peptides. In chapter V, the concentration of gp120 specific purified IgG was calculated in a similar fashion.

HIV-1 Neutralization Assays

Neutralization assays reported here were done in one of two ways. In most assays neutralization was performed using the TZM-bl reporter cell line as previously described (67). In some indicated cases, the Phenosense neutralization system was utilized by Monogram Biosciences (86).

Phenosense Neutraliation Assay

The PhenoSense neutralization assay was conducted through a subcontract using the PhenoSense Assay system at Monogram Biosciences. In this system, pseudovirus was harvested 48 hours after co-transfection of HEK293 cells with pCXAS-Env libraries plus an HIV genomic vector that contains a firefly luciferase indicator gene. Pseudoviruses were the incubated for 1 hour at 37°C with heat-inactivated rabbit sera at graded dilutions. U87 cells expressing CD4 and the CCR5 and CXCR4 co-receptors were inoculated with virus-Ab mixtures in the absence of added cations. Virus infectivity was determined 72 hours post-inoculation by measuring the amount of luciferase activity expressed in infected cells. Neutralizing activity was calculated as the percent inhibition of viral replication (luciferase activity) at each antibody dilution compared with a rabbit serum free control: % inhibition = [1 − (luciferase + immune sera)/(luciferase − immune sera)] × 100. Pre-bleed rabbit sera were also included as negative controls. MuLV was also included in each assay to rule out non-specific neutralizing activities.

TZM-bl Neutralization Assay.

HIV-1 pseudovirions were produced through cotransfection of the $pSG3^{\Delta env}$ backbone (NIH AIDS Research Reference and Reagent Program) and an Env gp160 bearing plasmid in HEK 293T cells. Pseudovirus containing supernatants were cleared of cell debris by low speed centrifugation. Pseudoviruses were then titered out on the TZM-bl cell line before use. For a typical neutralization assay, 200 $TCID_{50}$ of pseudovirus was incubated with rabbit sera for 1 hr at 37°C. The pseudovirus/sera mix was then added to 10^4 TZM-bl cells in a final concentration of 20 µg/mL DEAE Dextran. Plates were incubated at 37°C for 48 hours and developed with luciferase assay reagent according to the manufacturer's instruction (Promega). Neutralization was calculated as the percent

change in luciferase activity in the presence of preimmune sera versus that of luciferase activity in the presence of immune sera [(Preimmune RLUs – Immune RLUs)/(Preimmune RLUs)]*100.

In peptide adsorption experiments the same neutralization protocol was applied as described above except, prior to the exposure of sera to the pseudovirus, the sera were incubated with an equal volume of a V3 peptide at 25-30 µg/mL for 30 minutes at 37°C.

Additionally, in some assays, pseudovirus was exposed to sCD4 prior to the addition of sera. In these assays, JR-FL pseudovirus was preincubated with 5 µg/mL sCD4 (NIH AIDS Research and Reference Reagent Program) prior to the addition of serum. In these assays, percent neutralization was calculated with the light signal of JR-FL in the presence of sCD4 serving as our baseline light signal.

Competitive Binding Assays

Competitive binding assays were performed as previously described (25, 28) with minor modifications. Pseudovirions bearing the JR-FL Env and Vesicular Stomatitis Virus (VSV) glycoprotein were produced with the pSG3$^{\Delta Env}$ backbone in 293T cells. Microtiter plates were coated with 50 µL of mAb at 5 µg/mL for 1 hr at room temperature. Plates were then blocked in PBS with 3% BSA overnight at 4°C. Rabbit sera was heat inactivated at 56°C for 30 min, serially diluted, and incubated with pseudovirus correlating to 2.5 ng of p24/well for 1 hr prior to the addition to the virus/sera mixture to the ELISA wells. Pseudovirus/sera mixture was then incubated on the ELISA wells for 3 hrs at room temperature. Plates were washed 5 times with sterile PBS and overlayed with 10,000 TZM-bl cells per well. Plates were then incubated for 48 hrs at 37°C. Luciferase activity was determined per the manufacturer's instruction

(Promega). Competition titer is reported as the serum dilution at which the luciferase signal is reduced by 50% compared to a serum negative control. When competition with the co-receptor binding site antibody 17b was tested, the pseudovirus was incubated with sCD4 at 5 µg/mL for 30 min at 37°C prior to the exposure of sera.

NaSCN Displacement

JR-FL gp120 was coated onto 96 well microtiter plates (Costar) at 1 µg/mL in 100 µL of PBS for 1 hr at room temperature. Plates were then washed 5 times in PBS containing 0.1% Triton-X (EWB) and blocked overnight at 4°C in PBS containing 4% by weight whey (whey dilution buffer) and 5% powdered milk. Rabbit sera were then added to the plate at either a 1:30,000 or 1:100,000 dilution and incubated at room temperature for 1 hr. Plates were then washed 5 times in EWB. NaSCN was then added at various (0, 1, 2, 3, 4, 5 M) concentrations in PBS for 15 min followed by 5 washes in EWB. Bound IgG was detected as described above. Determination of IgG quantity remaining on the plates was done using linear regression analysis of a standard IgG curve (Southern Biotech). Data is reported as the NaSCN concentration required to displace 50% of IgG initially bound on the plate.

Antibody Dependent Cell mediated Cytoxicity (ADCC)

The ability of serum from immunized individuals to mediate ADCC activity was performed as previously described with minor modifications (38). Briefly 1 x 10^6 $CEMNK^r$ cells were dual stained with 2.5 x 10^{-6} M PKH-26 (Sigma) and 5 x 10^{-8} M CFSE (Molecular Probes, Invitrogen). Cells were then washed in PBS and incubated with 5 µg of JR-FL gp120 for 1 hr at 25°C. Following another wash in PBS, $CEMNK^r$ cells were plated at 5000 cells/well in 50 µL RPMI with 10% FBS in a 96 well tissue culture

dish. 100 µL of sera diluted in RPMI with 10% FBS were then added to the cells at a final dilution of 1:100. After a 15 minute incubation, 50 µL of effector cells were added to the plate at a 50:1 E:T ratio. Plates were centrifuged at 400g for 3 min to promote cell-cell contact and then incubated at 37°C for 4 hours. Following the 4 hour incubation, cells were subjected to flow cytometric analysis on a LSRII cytometer for loss of CFSE fluorescence within the PKH-26^{hi} target cell population. Cell lysis is defined as the percentage of $CEMNK^{r}$ cells in the PKH-26^{hi} population that lost CFSE fluorescence. Negative controls included normal human serum as well as $CEMNK^{r}$ cells that were not pulsed with gp120.

Detection of complement activation

The downstream product of complement activation, C4, was detected in an ELISA based assay. 50 uL of JR-FL gp120 was coated on a microtiter plate at 1 µg/mL overnight at 4°C. Plates were then washed five times in PBS with .025% Tween-20 (EWB). Plates were blocked in 200 µL of PBS containing .025% Tween-20 and 3% BSA. After washing, serial dilutions of human sera that had been heat inactivated for 30 min at 56°C was then added to the gp120 coated plate and incubated for 1 hr at room temperature (RT). After washing, intact normal human sera was incubated on the plate at a 1:100 dilution for 1 hr at RT. Plates were then washed and a goat anti-C4 antibody (Immunology Consultants Laboratories) was added to the plate at a 1:1000 dilution for 1 hr at RT. After washing an AP conjugated anti-goat secondary was added to the plate at a 1:1000 dilution and incubated for 1 hour at RT. After washing the plate was then developed with 50 µL of AP development solution consisting of diethanol amine buffer plus phosphatase substrate (Sigma).

Site Directed Mutagenesis

All site directed mutagenesis was perform using the Stratagene Quikchange II site directed mutagenesis kit according to the manufacturers instructions. Mutagenesis was verified by sequencing the gp120 Env.

Production of rabbit hybribdoma lines

Rabbit hybridoma lines were produced as previously described by contract with Epitomics (99). Briefly, splenocytes harvested from immunized rabbits were fused with the 240E 1-1-2 fusion partner at a ratio of 2:1 with 50% PEG 4000 at 37°C in serum free media. Cells were then plated out in microtiter plates in culture media with 15% FBS. After 72 hours, HAT was added and media was changed every 5 days. Wells containing antigen specific cell lines were screened by testing cell free culture supernatant for recognition of JR-FL gp120 antigen by ELISA.

Chapter VIII

Final Comments and Conclusions

The primary goal of the HIV vaccine field is to develop a vaccine that is able to elicit an immune response capable of providing protection from viral acquisition. Unfortunately, there is still no known natural correlate of protection from HIV infection. To date, the only means of providing sterilizing immunity in an experimental system is the passive transfer of NAbs (24, 31, 45-47, 64, 66). This evidence makes it a strong likelihood that any successful vaccine will require a strong, high quality antibody response as an essential component.

A large portion of our knowledge of HIV specific antibody responses comes from the study of HIV infected individuals, especially those whose sera possess broadly neutralizing activity. Through examination of these individuals' sera or mAbs produced from these patients, we have identified numerous weaknesses in the viral Env protein that can be exploited by the host antibody response to prevent viral infection. These regions include the CD4bs, the V3 loop, unique patterns of glycosylation, the coreceptor binding site, conformational epitopes involving the V2 and V3 loops, and the MPER region of gp41. While the field is beginning to gain an understanding of how the host antibody response evolves in an attempt to counter the virus, data is still sorely lacking on the extent of our capabilities to mimic this response by exploiting viral weaknesses through vaccination. In the work presented in this dissertation, I attempted to address this gap in knowledge by using one of the few tools available that has been shown to induce a high quality NAb response to HIV, a heterologous prime-boost regimen consisting of a DNA prime followed by protein boosting.

In this body of work we have studied the humoral responses elicited by the DNA prime-protein boost immunization regimen. Using this as a baseline, we have compared antibody responses elicited by DNA alone and protein alone regimens in small animals and protein alone and canarypox prime-protein boost regimens in humans. Based on these comparisons, we determined that a DNA prime-protein boost regimen elicits a higher quality antibody response than those elicited by other regimens. The parameters for this assessment include the elicitation of a broader heterologous NAb response, the unique elicitation of antibodies to the CD4bs, and the elicitation of a higher avidity binding antibody response. These aspects are enhanced in sera from DNA prime-protein boosted individuals while still maintaining the ability to mediate other potentially relevant protective mechanisms, such as ADCC and complement activation. All of these parameters make the DNA prime-protein boost regimen a strong candidate for further vaccine development.

Using the DNA plus protein regimen we were also able to demonstrate that even closely related gp120s will elicit a different heterologous NAb response. Using LN40 and B33 gp120s as model immunogens, we demonstrated that priming with LN40 gp120 elicits a broader heterologous NAb response. Through the use of mutagenic epitope mapping, we implicated the elicitation of antibodies to the CD4bs in LN40 primed animals as the likely mechanism for this broader neutralization. The evidence presented here that not all gp120s are created immunogenically equal may have implications for the selection of antigen formulations in future vaccines.

Additionally, we have also demonstrated that generation of mAbs from DNA primed-protein boosted rabbits is both feasible and provides a valuable tool for studying

the characteristics of antibody responses elicited through immunization. The mAbs generated here were targeted to a diverse set of epitopes within gp120, including functionally conserved ones such as the CD4bs and the V3 loop. While some these mAbs proved to have only a very narrow specificity, others cross reacted to every gp120 tested in this study. While only limited neutralization assays have been completed, we have observed and expect to continue to see that some mAbs generated in this study have the ability to neutralize heterologous isolates of HIV. Of the neutralizing antibodies that have been identified thus far, we found that they are targeted to multiple epitopes, which in the context of a polyclonal sera, could play either additive or synergistic roles in neutralizing heterologous isolates of HIV. Collectively, these results indicate that a DNA prime-protein boost regimen elicits a high quality antibody response targeted to a diverse set of epitopes which, in some cases, is capable of mediating neutralization of diverse heterologous isolates.

Chapter IX

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