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Antibody response of mice and rabbits targeting HIV-1 using global panel DNA and SOSIP gp140 protein

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Antibody response of mice and rabbits targeting HIV-1 using global panel DNA and SOSIP gp140 protein

by

Kari Loni Rohl

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

MASTER OF SCIENCE

Major: Immunobiology

Program of Study Committee:
Michael W. Cho, Major Professor
  Joan Cunnick
  Cathy Miller
  James Roth
  Mark Ackermann

Iowa State University

Ames, Iowa

2016

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APPENDIX B

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I would first like to thank Dr. Cho for allowing me into his lab and giving me the opportunity to work on this project. I would also like to thank Dr. Joan Cunnick, Dr. Cathy Miller, Dr. James Roth, and Dr. Mark Ackermann for all of the advice and assistance they provided me as my committee members.

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Finally, yet most importantly, I would like to thank my family. Mom, Dad, Gena and Luke, you have all been so supportive and encouraging to me throughout my studies, and especially during the writing of this thesis. I could not have done this without you guys!
Human Immunodeficiency Virus type 1 (HIV-1) is a highly variable virus that continues to cause a worldwide pandemic. The search for a vaccine has focused on the elicitation of broadly neutralizing antibodies (bnAbs), which can prevent the attachment and/or entry of the majority of HIV strains. In this thesis, we attempt to elicit more common neutralizing antibodies (nAbs), which are type-specific in their ability to block HIV infection. However, we propose to elicit a wide range of these type-specific nAbs to mimic the breadth of protection found in true bnAbs to protect the host from multiple HIV strains. We immunized both mice and rabbits with a twelve virus panel, termed global panel, that represents seven different HIV-1 clades. The immunizations were performed using particle mediated epidermal delivery (PMED) loaded with plasmids that could express the desired env proteins in vivo. The mice did not elicit any neutralizing activity against tier 1 or tier 2 viruses. The rabbits had neutralization against one of the tier 1 viruses tested, but not against any of the tier 2 global panel viruses.
CHAPTER 1: GENERAL INTRODUCTION

Thesis Organization

This thesis is divided into three chapters. Chapter 1 is a literature review of HIV. This contains a general overview of HIV including the history, the variability, the proteins and structure, with a special focus on the envelope glycoprotein. Chapter 1 is followed by a review of DNA vaccines, including how they produce proteins in vivo, the relative safety of DNA vaccines, and the different administration techniques, focusing on gene gun.

Chapter 2 is a manuscript in preparation entitled “Antibody response of mice and rabbits primed with global panel DNA and boosted with BG505 SOSIP gp140 protein.” This manuscript describes an attempt to induce neutralizing antibodies against HIV using twelve different strains of HIV DNA delivered using a gene gun. The contributions of each author are as follows: Kari Rohl performed all of the DNA preparations, animal immunizations, and all the characterization assays. Feng Jiao made the pseudoviruses for the neutralization assays, and assisted with the animal immunizations. Kari Rohl and Michael W. Cho wrote and revised the manuscript.

Chapter 3 summarizes the conclusions and includes suggestions for future work. This thesis also includes an appendix section containing other unpublished projects where Kari Rohl was the primary investigator.
**Literature Review**

Human Immunodeficiency Virus (HIV) was discovered in 1983 [1-3]. HIV type 1 (HIV-1) causes Acquired Immune Deficiency Syndrome (AIDS), which reduces the immune system to negligible levels. HIV type 2 (HIV-2) is morphologically similar, but is antigenically distinct and more closely related to Simian Immunodeficiency Virus (SIV) [4,5]. HIV-2 is less virulent and limited to West Africa. For the remainder of this document, HIV refers to HIV-1. The most recent statistics on HIV from UNAIDS [6,7] indicate that there were 1.2 million AIDS related deaths in 2014; 150,000 of those deaths were children under the age of 15. Two million people were infected with HIV, and nearly 37 million people are currently living with HIV as of 2014.

HIV is split into four main groups: M (major), O (outlier), N (non-M or O), and P (Fig 1.1). Group M is the pandemic group and is divided into ten clades (A-K) (reviewed in [8]). HIV is highly variable, and in group M alone there is a 25-35% divergence in sequence between the clades, and up to 20% sequence divergence within any clade. Infections from clade C represent nearly half (48%) of those worldwide, and clade C is the prevalent strain in Africa and India. Clade B has the highest number of complete sequences as it is more prevalent in developed countries with easier access to patient samples, but this clade only accounts for about 12% of infections worldwide [9,10]. Infection by a second strain of HIV is possible and results in numerous HIV interclade recombinant viral strains (Circulating Recombinant Forms (CRF)) [11-13].
HIV proteins

HIV is a member of the Lentivirus genus and the Retroviridae family. HIV is an enveloped virus containing two copies of a single-stranded, positive sense RNA that is 9.2k nucleotides long [14,15]. It encodes typical retrovirus structural proteins: Gag, Pol, and Env. Additionally, HIV expresses regulatory proteins Tat and Rev, as well as several accessory proteins: Nef, Vif, Vpr, and Vpu (Fig 1.2).

Gag is expressed from an unspliced viral mRNA. This membrane-associated Gag polyprotein results in the virion budding from the infected cell. The Gag polyprotein is cleaved [16,17], yielding the matrix (MA), capsid (CA), and nucleocapsid (NC) and p6 proteins. MA proteins increase stability by attaching to the inner portion of the lipid bilayer. A small portion of the MA proteins attaches to the complex that shuttles the viral DNA into the host nucleus [18-22]. CA forms the conical shaped core of viral particles (Fig 1.3). The NC protein ensures that viral RNA is incorporated into the new virions [23,24]. NC also plays a role in reverse transcription [25-27]. The p6 region of Gag is important for the inclusion of viral protein R (Vpr) into the new virion and for budding [28,29].

The Pol polyprotein coding region overlaps with the Gag coding region, though they use different reading frames. During the maturation process, the protease cleaves the Pol polypeptide into the protease (PR), reverse transcriptase (RT), and integrase (IN) proteins. HIV RT makes a double-stranded DNA copy from the single-stranded viral RNA. As RT does not contain a proof-reading mechanism, there is a high error rate, resulting
in several point mutations in each newly made genome copy [30-34]. The IN is responsible for inserting the proviral DNA into the host cell DNA [35,36].

Tat (transactivator of transcription) is the regulatory protein that is required for HIV replication [37,38]. Tat binds RNA and promotes the production of full-length transcripts [39-41]. Rev regulates the transition from early to the late phase gene expression [42,43]. Rev also exports unspliced or incompletely spliced viral RNA from the nucleus [44].

Negative regulation factor (Nef) is the initial viral protein that accumulates in an infected cell. Nef has many different functions, one of which is the down-regulation of CD4, as cell-surface localized CD4 inhibits the budding and Env expression of new virions [1,3]. Vpr assists in the infection of non-dividing cells by helping it to localize the preintegration complex (PIC) to the nucleus [45]. Viral protein U (Vpu) enhances viral budding. Viral infectivity factor (Vif) prevents HIV inactivation by interfering with APOBEC3G [1,4], which is an innate antiviral protein that will terminate the viral life cycle [6].

**HIV envelope**

The Env glycoprotein (gp) is originally expressed as a 160kD protein from a single mRNA. It is made in the endoplasmic reticulum and transports through the Golgi apparatus, where glycosylation occurs. Here, 25-30 complex N-linked carbohydrate side chains are added to Asp residues. This process is required for newly formed virions to be infectious [46]. The cellular protease furin cleaves the original gp160 protein into two
subunits: gp41 and gp120. These subunits are non-covalently bound, and present as a trimer that is embedded in the virion surface as well as infected cells. gp41 contains the transmembrane domain and the fusion peptide, while gp120 engages the cellular receptor and co-receptor [9].

HIV binds to its major receptor, CD4, with a specific structural, but non-linear domain called the CD4 binding site (CD4bs) [11]. CD4 is an immunoglobulin (Ig)-like protein that is expressed on helper T cells and primary macrophages. The binding of the CD4 is not enough for viral fusion and entry. HIV also requires co-receptor binding to either CCR5 or CXCR4 [14]. The binding of CD4 causes extreme conformational changes in the Env that allow co-receptor binding and subsequent viral entry into the host cell [16]. gp120 contains five variable loops, labeled V1-V5. The V3 loop is not involved in binding to CD4, but it does interact with the co-receptors CCR5 and CXCR4 and is partially responsible for the susceptibility of different cells to specific strains of HIV [18,20].

The primary function of gp41 is to facilitate the fusion of the viral and cellular membranes. Before gp120-CD4 attachment, gp41 is hidden inside of the viral envelope. However, once CD4 binding has occurred, the gp41 fusion peptide is exposed which, due to its hydrophobic nature, inserts into the host cell membrane [23]. This insertion fastens the virion to the host cell and allows for the fusion peptides to change into a six-helix bundle [24,25,27]. Because the fusion peptide is buried in the host cell membrane, and the transmembrane region of gp41 is lodged in the virion membrane, the formation
of the six-helix bundle brings the two membranes together and a fusion pore forms [25-28], allowing the viral genome to enter the host cell.

**DNA vaccines**

It was first demonstrated in the 1990s that direct inoculation of an antigen-expressing DNA plasmid induced humoral and cellular immunity [28-30,32-34]. The general strategy for DNA immunizations is the same, no matter the immunization route (Fig 1.4). The plasmid vector has a high-efficiency promoter that guides the transcription of the desired antigen in a cell’s nucleus. This DNA plasmid is injected into the skin or muscle, where it will enter a cell and synthesize the antigen. The plasmid antigen is translated and expressed by the transfected cells, and induces an immune response against the antigen.

The intracellular transcription and translation of plasmid DNA mimic the replication of a virus during infection. Both systems require cellular machinery to cross the plasma membrane and to translate their proteins. Since the processing of the plasmid protein is the same as the viral proteins, it results in antigen that is likely folded in its native conformation. Since this imitates both the glycosylation and the post-translational modifications, it gives rise to products that resemble a natural infection more closely than protein immunization, and it should favor the production of neutralizing antibodies.

DNA immunizations overcome the safety risks that are associated with live (attenuated) vaccines. Live attenuated vaccines have the ability to revert to their
virulent wild type status, and they also have the potential to spread to unintended individuals [30-35]. DNA vaccinations do not have the genetic material to revert to a virulent virus. They only contain the limited genetic material encoding a specific antigen that is inserted into the immunized plasmid.

There are many different ways of vaccinating with DNA. The most common are intra-muscular DNA injection (IM) or by particle mediated epidermal delivery (PMED) using a “gene gun.” Gene gun inoculation requires far less DNA than needle injection because it is very effective at propelling the plasmid into the cells of the epidermis. Gene gun bombardment primarily elicits a humoral response [35-37], whereas injection into muscle results in a strong cellular-mediated response without much Ab response [37-39].

There have been several gene gun DNA vaccines that have been successful in eliciting protective immunity against viral infections such as rabies [39-42], influenza [42,43,47,48] and foot-and-mouth disease [44,49]. There have also been a few studies that focus on developing a strong Ab response against HIV with most targeting gp120 or gp41 protein [1,3,50-52]. Studies show the antibody production is lower than protein alone, but when boosted with protein, the neutralization activity is higher than with either alone [53-55].

**HIV vaccines**

To combat infections one can either resort to treatment following infection or prevention to block infection at its outset. HIV is historically difficult to treat due to the
error prone replication (reviewed in [56]), which quickly yields HIV strains resistant to certain treatments [57]. However there has been some success with antiretroviral therapy (ART) and the more recent highly active ART (HAART). ART suppresses the number of HIV virions circulating, giving the immune system a chance to fight off infections on its own and reducing the risk of transmitting the virus to others. Unfortunately, current ART cannot get rid of all of the HIV as some remain hidden in reservoirs that ART and the immune system cannot access [58-60]. This results in a lifetime of consistent use of expensive medications.

A subset of individuals who have been chronically infected with HIV will develop broadly neutralizing antibodies (bnAbs). These Abs are able to bind to the majority of HIV strains and prevent them entering the host cell, thus neutralizing the virus [61,62]. Individuals who elicit bnAbs tend to have consistently low levels of viremia [63]. HIV infection could be prevented altogether if a vaccine induced bnAbs. For HIV vaccine development, we focus on the env glycoprotein because it is the only portion exposed before infection.

There has been only a handful of vaccine trials that progressed to Phase IIb and Phase III clinical trials. Of those, only one clinical trial successfully induced some protection against HIV. The RV144 vaccine used a combination of two previously failed HIV immunogens [64]. They primed with a recombinant canarypox viral vector containing the env, gag, and pro genes (ALVAC-HIV) then boosted with a gp120 protein vaccine (AIDSVAX-B/E). This vaccination schedule lowered the rate of infection by 31.2%
as compared with the group given the placebo. However, it did not affect the viral load of those who did become infected during the trial.

A vaccine is considered by many scientists to be the best way to conquer the HIV pandemic. ART is becoming more efficient, but preventative measures are a more cost-effective way of managing the disease and will allow for a better quality of life.

**Figures**

![HIV phylogenetic tree depicting groups and clades.](image)

*Fig 1.1*: HIV phylogenetic tree depicting groups and clades.
**Fig 1.2**: HIV genome to scale, including reading frames and long terminal repeats.
Fig 1.3: Structure of the HIV virion.

Fig 1.4: Schematic overview of DNA immunization.
References


CHAPTER 2: ANTIBODY RESPONSE OF MICE AND RABBITS PRIMED WITH

GLOBAL PANEL DNA AND BOOSTED WITH SOSIP GP140 PROTEIN

A manuscript in preparation

Kari Rohl, Feng Jiao, and Michael W. Cho

Abstract

The search for an HIV-1 vaccine that induces broadly neutralizing antibodies (bnAbs) capable of targeting multiple HIV strains has been unsuccessful. Based on research by deCamp et al. [1], we proposed an immunization strategy with a diverse group of HIV env genes that are statistically shown to be a representative sample of the global epidemic. We hypothesized that even though this strategy may not elicit true bnAbs, it could generate type-specific neutralizing Abs targeting many different HIV clades, therefore protecting the host against infection. Mice and rabbits were immunized with 12 different HIV envs from several different clades representing the potential breadth of infection. Immunizations were primed with submixtures of env DNA using a gene gun, and a subsequent SOSIP gp140 protein boosts. There was no neutralizing activity from the mouse serum against tier 1 viruses, or against the tier 2 global panel viruses. The rabbits showed neutralization against a single tier 1 virus, and no noticeable neutralization against the global panel viruses.
Introduction

A significant problem for vaccine development against Human Immunodeficiency Virus type 1 (HIV-1) is the difficulty in eliciting broadly neutralizing antibodies (bnAb) against the many circulating viral strains. The only target for vaccines against HIV is the envelope protein (env) as this is the only portion of the virus exposed and available for antibodies (Abs) to bind. HIV env consists of two main proteins arranged in non-covalent heterotrimer: gp120 and gp41. gp120 is outer portion of the env and contains the binding site for the CD4 receptor. After gp120 binds CD4, the env undergoes drastic conformational changes to reveal gp41, which can now insert itself into the host cell membrane and begin the fusion process to permit HIV virion entry.

DNA immunization has been investigated since the 1990s [2-5]. A plasmid encoding for a protein is injected into the skin or muscle. Once the plasmid enters a cell, it is trafficked into the nucleus and directs the synthesis of the specified antigen. Resulting protein production is similar to viral protein replication during a natural infection. Both rely on host machinery to translate and transport the products. However, the utilization of a DNA plasmid encoding a protein immunogen is significantly safer to deliver a protein antigen than a live attenuated vaccine, as there is no potential for virulence reversion [6]. There are several different ways to deliver a DNA vaccine; the two most common ways are through intra-muscular DNA injection (IM) and by particle mediated epidermal delivery (PMED) using a gene gun. The gene gun is extremely effective at injecting the plasmid DNA into the cells of the epidermis without using needles, and requires far less DNA than needle injection [7].
Eliciting bnAbs is a major goal for HIV vaccination studies. Studies have shown that the passive transfer of bnAbs taken from chronically infected HIV patients can provide protection against most routes of infection [8-12]. Characterization of these bnAbs revealed they target highly conserved regions, such as the base of the V3 loop [13], the CD4 binding site [14], and the membrane-proximal external region (MPER) of gp41 [15]. The development of bnAbs correlates with a high viral load and chronic infection, which allows the B cells sufficient time to undergo extensive somatic hypermutation [16].

A vaccine strategy is needed that can mimic infection with diverse antigens over an extended period of time. Polyvalent vaccines composed of multiple different env proteins produce a broader nAb response than a monovalent env protein in rabbits and macaques [17-19]. The variability in the multiple env proteins could assist in selecting for a consensus epitope, such as the conserved regions in the env, as well as with B cell cross-linking.

In a study by deCamp et al. [1], the authors determined that there were twelve virus strains that accurately represent the global epidemic of currently circulating strains, which they termed the global panel. They tested 219 env-pseudotyped viruses against the serum of 205 chronically infected individuals, collected from around the globe. The twelve strains that they determined statistically represented the spectrum of HIV are highly sensitive to the known bnAbs. Immunization with these twelve strains could potentially produce nAbs that cover the spectrum of HIV strains.
Based on these studies, we proposed that a polyvalent immunization would induce nAbs that cover the possible breadth of HIV infection. We hypothesized that DNA immunizations using the gene gun would effectively deliver multiple HIV env plasmids, thus potentially inducing broader neutralization activity than a single env immunogen. Using the global panel DNA to elicit nAbs against a broad spectrum of HIV strains would be able to protect an individual from infection of most if not all HIV strains.

Results

Mouse immunizations

We divided the global panel envs into six groups based on their clade containing between one and three strains (Table 2.1). The gp160 plasmid DNA of each of the groups was mixed and loaded together onto the gene gun bullets.

For the first immunization, we immunized with one bullet each from group 1, group 3, and group 6 (Fig 2.1). The second immunization was performed with one bullet each from group 2, group 4 and group 5. The antibody titers against gp120 after the first and second immunization reached $10^3$ (Fig 2.2a). Unfortunately, these did not increase after the third (groups 1, 3, and 6) or the fourth (groups 2, 4, and 5) immunizations as anticipated. Although the titers did not increase, neither did they decrease after a two-week waiting period. We followed the gene gun DNA immunizations with two protein boosts using 30ug of SOSIP gp140 adjuvanted with zinc chitosan (ZnCh). After the second protein boost, corresponding to the sixth immunization and collected at the
terminal bleed, the antibody titer against gp140 reached above $10^6$ and $10^5$ against gp120 (Fig 2.2 a and b).

Next, we analyzed whether any neutralizing activity was present in the sera against laboratory-adapted (tier 1) pseudoviruses, which are considered easy to neutralize, and later against tier 2, global panel pseudoviruses, which are primary isolates and harder to neutralize [20]. We tested pooled sera from after the fourth immunization against several tier 1 pseudoviruses: SS1196, MN.3, MW965, DH12, Q23, and Bal.26. No neutralization activity was observed (Fig 2.3). In fact, we saw enhancement of viral infectivity, which is common for non-neutralizing serum (YQ, KLR, and MWC unpublished observation). The tier 1 viruses were not tested with the after the sixth immunization serum due to a lack of serum from the mice.

Serum was tested for neutralization against the global panel pseudoviruses after the sixth collection. There was no neutralizing activity (Fig 2.4). BJOX002000 had some weak neutralization (slightly above 50%) at the initial 1:20 dilution, but quickly dropped off at the lower dilutions. Most of the others showed enhancement of the serum at the initial dilution.

**Rabbit immunizations**

The rabbits were immunized similarly to the mice. Even though the mice immunization data was generally negative, neutralization from the rabbit serum may be more likely due to their longer CDR3 regions as compared to the CDR3 region mice [21], which is a required hallmark of some anti-HIV antibodies. The dosage of DNA and
protein was increased given the larger animal size, and the timeline was extended (Fig 5:). The rabbits were immunized with 6ug of DNA every two weeks, and serum was collected before the subsequent immunization, for a total of five DNA immunizations. The first four were global panel groups, as described for mice. The fifth was SOSIP gp140 DNA. The rabbits were boosted with 100ug of gp140 protein on ZnCh on week 24 and on week 26.

The antibody titer from the DNA immunizations alone was unexpected as there was no observed gp120 or gp140 specific response (Fig 2.6 a and b). After the first protein boost (sixth immunization), the antibody titer rose to at least $10^4$ for all the rabbits against gp120, and $10^5$ against gp140. The second boost with gp140 increased the titer to $10^6$ against both gp120 and gp140.

All neutralization assays were performed with serum collected after the seventh immunization (second gp140 ZnCh boost). First, we tested the serum against three tier 1 viruses: SF162, DH12, and Bal.26 (Fig 2.7). All three rabbits could neutralize SF162. None of the rabbit sera could neutralize DH12, and rabbit 2 (Rb 2) was able to weakly neutralize Bal.26. We next tested pseudoviruses produced from the global panel (Fig 2.8). None of the twelve viruses were neutralized. Five of the twelve assays showed the same viral infectivity enhancement observed in the mouse serum, while the other seven did not.
Discussion

Studies have shown that a polyvalent DNA vaccine is more effective at creating neutralizing antibodies than their monovalent counterparts [17,18,22]. We grouped the strains by clade, then immunized with three different clades per immunization (Table 1). By doing this, we attempted to activate a wider variety of antibodies, thus perhaps increasing the likelihood of B cell cross-reactivity [23,24]. The sequential immunization of different HIV env (Figs 2.1 and 2.5) should be more advantageous than simultaneous immunization of all the same env strains [24]. This will help to guide the antibodies toward conserved regions of the env, and thus be more likely to yield nAbs.

The Ab titer (Fig 2.2) in mice greatly increased in response to the DNA immunizations and increased more so after protein boosts. The titer against gp140 after the second protein boost is similar to what we have observed after two protein immunizations with gp140 on ZnCh alone. DNA vaccines often produce lower titers of Abs than protein vaccines. However, vaccinations that are primed with DNA then followed by protein boost have higher neutralization activity than a protein only, or a vaccination primed with protein and followed by a DNA boost [25,26].

Even though the neutralization assay against the tier 1 viruses did not show any neutralizing activity (Fig 2.3), the assay was only performed prior to the protein boost. The mouse sera neutralization assays against the tier 2 viruses in the global panel (Fig 2.4) were tested after the terminal bleed. Even though the majority of the pseudoviruses analyzed were not neutralized, BJOX002000 showed slight neutralization. This is a promising result because it could indicate that if the serum IgG were further
purified and concentrated, it may have additional neutralizing activity below the level of
detection for the sera assay.

Titers of rabbit serum following the DNA vaccination were undetectable (Fig 2.6),
which was a highly unexpected result. However, after the protein boost, the titer
increased substantially. The titer after the first protein boost was ten fold higher than
the initial titer from a single protein immunization with SOSIP gp140 ZnCh. The second
protein boost in this study produced a titer that was on par with a second gp140 protein
only immunization (H. Shi, unpublished data). The rabbit neutralization assays against
the tier 1 viruses demonstrated some neutralization (Fig 2.7); however, they were
performed after the second protein boost, so a direct comparison cannot be made
between mouse and rabbit assays. All three rabbits neutralized SF162. Only R2
neutralized Bal.26, and none of the rabbit sera were able to neutralize DH12. One
possibility for the low neutralization activity was that the serum nAb concentration was
below detection standards for this assay, and thus purified IgG could yield higher
neutralizing activity. This assay was not performed at this time because tier 1
neutralization was not the overall goal of this study. We are more interested in the
neutralization ability against tier 2 viruses.

The tier 2 neutralization data (Fig 2.8) did not demonstrate neutralization like
the tier 1 assays; however, due to the background from the serum components, it is
difficult to tell whether a higher concentration will yield better neutralization or not. The
rabbit serum could potentially have neutralizing activity at a higher concentration but
Abs would need to be purified from the serum to assess this claim. While we did not see
any neutralization of the tier 2 viruses of the global panel viruses, we did see some against the tier 1 viruses. This is a starting point for further studies into global panel gene gun immunizations.

Further experiments should include a detailed peptide mapping of the V3 region of multiple different gp120 constructs, ideally based on sequences native to the global panel viruses, due to the variability in the immunized antigens. These epitope binding sites should be compared to maps from rabbits immunized with SOSIP gp140 ZnCh alone to see how the DNA prime altered the binding repertoire of the Abs.

**Materials and Methods**

**Enzyme-linked immunosorbent assay (ELISA)**

ELISAs were performed as previously described [27]. The gp120 or gp140 was coated onto 96-well Nunc-Immuno plates and incubated at 4°C overnight at a concentration of 30ng/well in coating buffer (15mM Na2CO3, 35mM NaHCO3, 3mM NaN3, pH 9.6). Plates were blocked with 200μl of blocking buffer (PBS, pH 7.4, 2.5% skim milk and 5% calf serum) for 1hr at 37°C. Plates were washed five times with wash buffer (PBS containing 0.1% Tween 20).

Serum was added 1:300 in blocking buffer for 2hrs at 37°C. Wells were subsequently washed ten times with wash buffer. The secondary antibody (anti-mouse-HRP or anti-rabbit-HRP) was added 1:3000 in blocking buffer, and incubated for 1hr at 37°C. Wells were washed ten times again, then 100μL of TMB substrate (BioRad) was added. The plate was incubated for 10min at room temperature (RT) and stopped using
50μL of 2N H2SO4. The absorbance was read at 450nm on a microplate reader (Versamax by Molecular Devices). All experiments were performed in duplicates.

**Gene gun bullets**

Initially, 20-25mg of 1.5micron gold particles were measured, then were quick washed twice with 500μL spermidine solution. Gold particles were resuspended in 150μL spermidine and transferred to a new 1.5mL tube. 2ug of plasmid DNA/ mg of gold was added to the tube while vortexing at a low speed. An equal amount (150μL) of 10% CaCl2 was added while vortexing. The mixture was incubated for 10min to allow the gold to precipitate.

The polyvinylpyrrolidone (PVP) mixture (5% 20x PVP in Ethanol (EtOH)) is made by first adding 1mL of PVP/EtOH mixture to flat bottom vials per 1mg gold. The mixture is spun down at top speed for 10 seconds. The supernatant is removed, and the DNA/gold mixture was washed 3 times with 800μL EtOH while vortexing. The pellet was resuspended in a small portion of the PVP/EtOH mixture (~500μL). This mixture was transferred into the flat bottom vial. A 2.5in section of tubing (0.125in x 0.250in silicone tubing) was cut and attached to a 3mL syringe. A 25in segment of the small tubing (0.079in x 0.098in Tefzel tubing) was cut and inserted in the tubing apparatus. The N2 tank was attached to the tubing apparatus and the flow was set to 20PSI and the flowmeter (Dywer VFA) to 8 liters/ min (LPM) air. After pressing the load button, the vial was mixed by vortexing and sonication, then the slurry was pulled into a 3mL syringe. The slurry was pushed into the end of the tubing apparatus and the spin cycle activated.
Excess EtOH was collected as it was forced out of the apparatus. The tubing was allowed to dry for at least 1hr (N2 flow through at 20PSI). Finally, the tubing was cut into 0.5in pieces (bullets). Each bullet contains 1ug of DNA.

**Animal immunizations**

Female BALB/CJ mice were purchased from the Jackson Lab. Three non-overlapping gene gun shots were given to each animal on shaved abdominal skin. Blood was collected from the lateral tail vein two weeks after each immunization.

Three New Zealand white female rabbits were purchased from Charles River (USA). Six non-overlapping shots were delivered to each animal on shaved abdominal skin. Blood was collected from the central ear artery two weeks after each immunization. All of the studies conducted were approved by IACUC.

**Neutralization assay**

Virus neutralization assays were performed using single cycle HIV pseudovirus infections of TZM-bl cells as described elsewhere [27-29]. Heat inactivated rabbit sera (56°C for 1hr) was diluted in 100µl of cell culture media (DMEM plus 10% heat inactivated FBS and 1% penicillin/streptomycin). Samples were serially diluted from 1:20 to 1:43740 in cell culture medium and pre-incubated for 1hr at 37°C before adding cells. After a 48hr incubation, cells were lysed and luciferase activity determined using a microtiter plate luminometer and Bright-Glo Luciferase Assay System (Promega). Neutralization titers are the sample dilution at which relative luminescence units (RLU)
were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in cell control wells.

Acknowledgments

We would like to thank Dr. Fuller for allowing us to use their gene gun and for the training that you gave us with the equipment.

Panel of Global HIV-1 Env Clones (cat# 12670) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. David Montefiori.

Tables

Table 2.1: Immunization groups showing individual strains and clades.

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**Fig 2.1:** Mouse immunization timeline showing the immunization groups and the serum collection.
Fig 2.2: Mouse serum titer by week, measured by ELISA (a) against MCON6 gp120 protein, and (b) against SOSIP gp140 protein.
Fig 2.3: Mouse tier 1 pseudovirus percent neutralization by serum dilution
Fig 2.4: Mouse tier 2 global panel percent neutralization by serum dilution
Fig 2.5: Rabbit immunization timeline showing the immunization groups and the serum collection.
Fig 2.6: Rabbit serum titer by week, measured by ELISA (a) against MCON6 gp120 protein, and (b) against SOSIP gp140 protein.
Fig 2.7: Rabbit tier 1 pseudovirus percent neutralization by serum dilution (a) SF162, (b) Bal.26, and (c) DH12.
Fig 2.8: Rabbit tier 2 global panel percent neutralization by serum dilution
References


CHAPTER 3: CONCLUSION AND FUTURE DIRECTIONS

Conclusion

As discussed in Chapter 2, global panel DNA immunizations followed with gp140 protein boosts did not elicit strong neutralization activity in mice or rabbit sera. The inability of the sera to neutralize may be due to low levels of nAb present. We could improve upon this study in several potential ways. One possibility is that the membrane bound env is not as immunogenic as the secreted form. As reported from this laboratory, when gp120 protein alone was immunized, antigen specific titers of up to $10^7$ were observed [1]. Thus, one possibility is to use plasmids that express secreted versions of the global panel gp120 subunit to induce a broad range of nAbs. Alternatively, it is currently hypothesized that gp120 alone may not be as ideal of an immunogen, as the tendency to favor type-specific nAbs and non-nAb elicitation may distract the immune system from potentially eliciting true bnAbs [1]. Therefore, a construct based on the gp160 DNA plasmids, but modified to express gp140 by deleting the cytoplasmic tail and transmembrane region of the protein could also be used. This may lead to a stronger immune response and therefore more antibodies, which could induce greater numbers of nAbs. Another possibility would be to increase the amount of DNA administered at each immunization. Other studies have immunized with 36-50ug of DNA per immunization [2,3]. Increasing the amount of DNA administered could increase the Ab titers. The more DNA that can be introduced to the animal, the more
cells that can uptake and express the plasmid protein. This increased expression should lead to more Ab exposure and a better Ab response.

**Future Directions**

Many different experiments could further clarify the results of this project in the near future. The first step would be to purify the IgG from the rabbit serum and test the neutralization activity against the global panel viruses using higher concentrations of antibodies. This should also reduce the viral infectivity enhancement that seems to be caused by some of the serum components. The purified IgG should also be tested against a wider variety of tier 1 viruses, and compared to the neutralization activity of gp140 protein immunizations alone (H. Shi unpublished data).

Additionally, it is important to understand the exact binding locations of the gp120/ gp140 specific antibodies. A peptide ELISA would show the binding location of Abs that have a linear epitope. A standard peptide ELISA contains overlapping peptides from a consensus sequence [4,5]. Each well is coated with a different peptide and the serum or purified IgG is added to each to test the ability to bind to that linear epitope. Since this study used a wide variety of HIV strains, in addition to the consensus sequence peptides, V3 loop peptides specific to the global panel strains should be tested. The results of the consensus sequence peptide mapping should be compared to the results from our laboratory when rabbits were immunized with SOSIP gp140 ZnCh alone. This information may let us conclude whether or not the global panel DNA immunization enhanced the breadth of Abs produced.
References


APPENDIX A: PRELIMINARY GENE GUN DATA

Conclusions

We first needed to establish that DNA immunization via gene gun would successfully induce antibodies targeting HIV-1 immunogens in our lab, using this system. The mice were immunized with DNA coding for an immunogen with demonstrated efficacy in mice and rabbits. The first immunogen used was the engineered outer domain GT6 (eOD-GT6) derived from gp120, followed by a larger immunogen, BG505 SOSIP-gp140. This allowed a comparison of the immune response to previous studies that we have done in our lab using these antigen [1].

The mice were immunized every two weeks for 10 weeks using 2ug of DNA per immunization (Fig A1). We immunized with eOD-GT6 for the first three immunizations, and for the following three immunizations, we used SOSIP gp140. After the third immunization, the antibody (Ab) titer reached to 6x10^4 (Fig A2). Additional immunizations with gp140 DNA did not further increase the Ab titer. The titer after the terminal bleed (week 12) dropped slightly to 3x10^4. Thus, we confirmed that we could successfully perform gene gun immunizations.

Materials and Methods

All assays were performed as described in the Chapter 2 Materials and Methods (page 26).
### Immunization

<table>
<thead>
<tr>
<th>Week</th>
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<th>eOD 2</th>
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**Fig A1:** Mouse immunization timeline showing the immunization groups and the serum collection.
Fig A2: Mouse serum titer by week, measured by ELISA against eOD-GT6 protein.

References

APPENDIX B: HR1-ID-HR2

Conclusions

The purpose of this experiment was to construct a protein that would be useful as a treatment of HIV. We hypothesized that if we could create a construct that mimicked the structure of HIV after it has entered a cell, we could target CD8 T cells toward infected cells and lyse them before too many new HIV virions were made. We cloned the immunodominant (ID) region of an M group consensus (MCON6) region and ligated it in between the heptad repeat 1 and 2 (HR1, HR2). The ID region is highly immunogenic, and there should be high titers against this region of the protein when used as an antigen. To that end, we would immunize either mice or rabbits and collect their serum antibodies. We would then use these antibodies in an Antibody Dependent Cellular Cytotoxicity (ADCC) assay to test the ability of the serum antibodies at activating CD8 T cell activity.

Following construction of HR1-ID-HR2, we tested binding of the two antibodies known to bind to the six helix bundle, NC-1 and our 2C2 [1] (Fig B1), against HR1-ID-HR2 and HR1-54Q. It is not surprising that 2C2 did not bind to HR1-ID-HR2, because three quarters of its binding region was not included in the protein construct because is not considered part of the ID region (Fig B2). In contrast, 2C2 was readily able to bind the HR1-54Q (unpublished data) construct, as the epitope was fully intact. NC-1 binds to HR1-ID-HR2, but less so than it does to HR1-54Q.
The plans for this construct include immunizing either mice or rabbits, and evaluating the antibodies produced on their ability to activate ADCC. The protein will be loaded onto ZnCh and immunized subcutaneously.

**Materials and Methods**

**HR1-ID-HR2 cloning**

The immune-dominant region of HIV-1 MCON6 was PCR amplified using primers (forward: aaaggaTCCGGCATCGCAGCAGCAGTCCAACCTGCTGCGG reverse: aaaGAATTttaatggatgtgatgtgatgCTCGTTCTTCTCTGCTGG) with a BamHI and an EcoRI site (respectively) inserted for digestions. It was then ligated into the pET vector containing the HR1-HR2 domains (pET 54Q trimer vector) using standard molecular biology techniques.

**Protein expression**

The ligated plasmids were transformed into T7 competent cells (New England Biolabs) for *E. coli* expression. A small volume of LB was inoculated with a single colony from the transformed *E. coli* cells. The next day, the cultures were used to inoculate a large volume of LB. Once the optical density value (OD) reached an OD$_{600}$ level of 1, the cultures were induced with 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG). After 4hrs, the culture was spun down at 5000RPM for 20min.
Cell lysis

The pellet was resuspended in phosphate buffered saline (PBS) and vortexed. The suspension was transferred into a glass beaker for sonication (10min at 63% Amp, 40sec on, 20sec off). The cells were lysed using the French Press, one time at 20000PSI. The lysed cells were spun down at 10000RPM for 10min then the pellet was resuspended in 8M urea. The supernatant was then spun down again.

Protein purification

To purify the HR1-ID-HR2 protein, the supernatant of the cell lysis was mixed end-to-end with Ni-NTA beads overnight. The mixture was allowed to flow through a column two times. The beads were then washed with a decreasing concentration of urea (8M, 6M, 4M, 2M, 1M) 100mL each, then with100mL of 40mM imidazole in PBS. The protein was eluted with 250mM in 10mL PBS.

Protein dialysis

The protein was dialyzed to remove the imidazole. To dialyze, the solution was loaded into Spectra/ Por molecularporous membrane tubing (size 3, MWCO: 3500, spectrumlabs) with 10% glycerol. The buffer was 20mM Heps, 25mM NaCl, and 10% glycerol. They were allowed to dialyze at room temperature, and the buffer was changed every 3hr, at least 3 times. The solution was then removed from the dialysis tubing and measured using a spectrophotometer at 280nm.
**NC-1:** binds 6 helix bundle

*Fig B1:* Sequence of MCON6 ID region with alignment of 2C2 antibody
Fig B2: ELISA with equal molar coated with HRI-ID-HR2 or HR1-54Q against (a) 2C2 and (b) NC-1
References