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Advancing HIV-1 Vaccine Design: Understanding the Connection Between HIV-1 Viral
Envelope and the Induced Neutralizing Antibody Response, and the Development of an
Alphavirus-based HIV-1 Vaccine Vector

by

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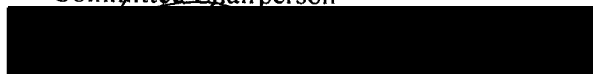
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DEDICATION

To my family

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July 30, 2014

ABSTRACT

Advancing HIV-1 Vaccine Design: Understanding the Connection Between HIV-1 Viral Envelope and the Induced Neutralizing Antibody Response, and the Development of an Alphavirus-based HIV-1 Vaccine Vector

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Induction of potent, broadly cross-reactive neutralizing antibodies (bcnAbs) remains a primary goal in HIV-1 vaccine development. Human clinical trials of HIV-1 vaccines have been largely unsuccessful until recently, when the RV144 Thai vaccine study demonstrated modest efficacy in preventing the acquisition of virus. These trials have highlighted the complexities of designing and delivering an immunogen capable of eliciting a humoral and/or cell mediated immune response. We investigated both of these aspects of vaccine design, beginning with a focus on screening for HIV-1 infected individuals who develop bcnAbs, and looking for connections between the envelope glycoprotein from early infection and the induced neutralizing antibody response. We also developed a potential replication competent HIV-1 vaccine vector based on the

alphavirus, Fort Morgan Virus, and tested its safety and immunogenicity in a small animal pilot study.

We screened sera from 157 individuals of the U.S. Military HIV Natural History Study Cohort for neutralization against a diverse panel of HIV-1 virus envelope clones. We found that bcnAbs were likely to be found across all HIV-1 disease progression groups, which included elite and viremic controllers, long term non-progressors, and rapid and typical progressors. Elite controllers, however, were found to be poor neutralizers. We compared six each of the best and worst neutralizers for differences in HIV-1 envelope neutralization sensitivity and sequence, and for differences in serum neutralization specificity. We found evidence of increased positive selection in envelope clones, and serum neutralization targeting specific epitopes in top neutralizers. Overall, envelope clones from top neutralizers were more sensitive to neutralization by monoclonal antibodies targeting CD4-induced epitopes. Additionally, we identified a patient (one of the top neutralizers) who possessed early envelope clones that were resistant to neutralization by monoclonal antibodies targeting the CD4 binding site and a glycan dependent epitope in V3, and also possessed serum antibodies directed towards the same two epitopes. We hypothesize that induction of bcnAbs may be related to the persistence of certain neutralization resistant viruses early in infection.

Alphaviruses have been shown to target dendritic cells and traffic to draining lymph nodes, where they produce high levels of antigen amidst resident antigen presenting cells. Fort Morgan Virus, carried by bedbugs that infest the nests of sparrows and swallows, is not known to be pathogenic in humans. We developed Fort Morgan Virus (FMV) as a replication competent vaccine vector that stably expresses HIV-1

envelope glycoproteins *in vitro*. We confirmed stable expression of in each successive round of plaque purification, a significant accomplishment in light of the combined size of the FMV RNA genome with HIV-1 transgene. Our novel FMV vector is one of only two non-attenuated, replication competent vectors that have been developed for HIV-1. In a small *in vivo* pilot study of safety and immunogenicity in rabbits, we found elevated, statistically significant serum anti-gp140 binding levels (measured by ELISA) in two immunized animals. Immunizations were well tolerated and no obvious abnormalities were observed in any of the animals. We completed a proof of concept FMV vaccine vector that merits further characterization and additional animal testing.

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CHAPTER 1: Background and Introduction

INTRODUCTION

Induction of potent, broadly cross-reactive neutralizing antibodies (bcnAbs) remains a primary goal in Human Immunodeficiency Virus-1 (HIV-1) vaccine development. Scientific difficulties in designing an efficacious HIV-1 prophylactic vaccine are split between the variable and complex nature of the envelope glycoprotein-based immunogen, and the vastly different delivery platforms that elicit humoral and/or cell mediated immune responses. For this dissertation research, we investigated both of these aspects of vaccine design in two parts. The focus of the first part is on broadly cross-reactive neutralization: the individuals who develop such a response, and connections between the envelope glycoprotein and the induced neutralization response. The second part describes the development of a potential replication-competent HIV-1 vaccine vector, and its safety and immunogenicity in a small animal pilot study.

Recent reverse vaccinology approaches, by which bcnAbs from HIV-1 infected patients who develop exceptionally potent and broad serum neutralizing ability are isolated and characterized, have greatly informed immunogen design. There have been conflicting reports on whether patients who possess bcnAbs are more likely to be of a particular disease progression group (classified as elite or viremic controllers, long term non-progressors, rapid progressors, or typical progressors). Clarifying this understanding can aid the search for an HIV-1 immunogen and vaccine design.

Since HIV-1 was first identified in the early 1980s, researchers have been optimistic about the development of a prophylactic vaccine. Early prototypes used soluble recombinant gp120 envelope glycoprotein (rgp120) to elicit neutralizing antibodies against surface antigens of the virus, an approach modeled after the hepatitis B vaccine. Vax004 and Vax003 were the first HIV human clinical efficacy trials to test

rgp120-based vaccines (81; 195). In both trials, the vaccines did not protect against HIV-1 infection nor did they delay disease progression.

By then, the importance of cell-mediated immunity was postulated in the control of viral replication and disease progression in long term non-progressive patients and in non-human primate challenge models. Primary virus strains were found to be more resistant to neutralization than laboratory adapted strains (58). More was understood about the tremendous viral diversity within infected individuals and additional subtype diversity among other HIV+ patients. A second group of experimental vaccines aimed at inducing cytotoxic T lymphocyte (CTL) responses was developed and studied in non-human primates. Human clinical trials were conducted utilizing a replication-deficient adenovirus Ad5 vector to express the three relatively conserved proteins: group-specific antigen (*gag*), polymerase (*pol*), and negative factor (*nef*) proteins (28; 103). During an interim analysis in the two trials concurrently testing this vaccine (Step and Phambili), the studies were halted due to concerns that the vaccine may have increased the risk of acquiring HIV.

The first demonstration of HIV vaccine efficacy was seen in the RV144 study conducted in Thailand, in which priming with a live recombinant canarypox vector (ALVAC-HIV) and boosting with recombinant gp120 (AIDSVAX B/E) yielded 31.2% efficacy at 42 months following first immunization. Immune correlates of protection were identified. The concentration of plasma IgG antibody specific for the V1V2 loop regions of gp120 was inversely correlated with reduced risk of infection, and high plasma concentration of IgA antibody to HIV envelope glycoprotein was directly correlated with acquisition of infection (196; 217). Additionally, serum IgG to V3 correlated with

decreased infection risk (101). Virus sieve analyses found that vaccine efficacy was higher against viruses matching the vaccine at residue 169 or mismatched at residue 181, both located within V2 of the envelope glycoprotein (221).

Lessons learned from these clinical trials of these vaccines, together with the identification of new, more potent and broadly cross-reactive neutralizing monoclonal antibodies (bcnMAbs) and the characterization of their neutralization epitopes, have redirected the field back towards an antibody-based vaccine (170; 232; 271). About 20% of HIV-1 infected individuals develop broadly cross-reactive neutralizing antibodies (bcnAbs) after 2-3 years of infection, but it is unclear whether these bcnAbs are more likely to be present within a certain disease progression group (262). Reverse vaccinology approaches have been taken to understand these bcnAbs and their targets in an effort to refine bcnAb-inducing immunogen design (264). Approaches to develop safer, CTL directed viral vaccine vectors that produce high levels of antigen expression are also underway (121).

HIV-1 Biology

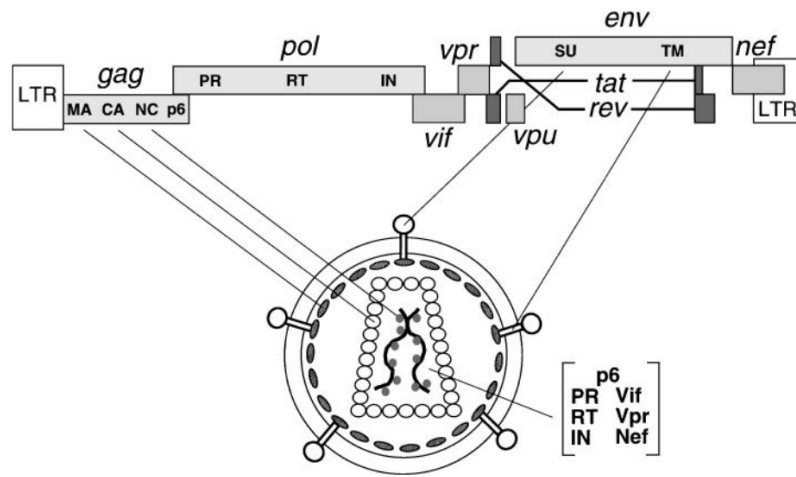
HIV-1 Replication and structure

HIV-1, an enveloped lentivirus of the *Retroviridae* family, possesses a cone shaped core that surrounds two positive sense strands of RNA genome. The virus acquires a lipid envelope from the plasma membrane of infected host cells during assembly and budding from its surface.

There are nine open reading frames (ORFs) in the ~9.8kb genome of HIV-1: *gag*, *pol*, *env*, *vif*, *vpr*, *nef*, *tat*, *rev*, and *vpu* (84). A schematic of the viral genome is shown in Figure 1. The viral Gag protein, a polyprotein containing the structural components matrix (MA), capsid (CA), nucleocapsid (NC) and p6, is necessary and sufficient for the formation of virus particles at the plasma membrane of an infected cell. The three proteins encoded by *pol*—protease (PR), reverse transcriptase (RT), and integrase (IN)—are contained within the virus core, where they are scaffolded on CA proteins. The *env* gene encodes gp160, an envelope glycoprotein precursor that is transported and heavily glycosylated via the endoplasmic reticulum (ER) and golgi (201). The two envelope glycoprotein subunit products produced from gp160 after furin cleavage, gp120 and gp41, are transported to the cellular plasma membrane. Heterodimers of gp120 and gp41 form noncovalently associated homotrimeric spikes on the outer membrane of the virus and mediate attachment and entry of the virus into target cells (134; 156; 220; 268). Each virus possesses approximately ten trimer spikes (304). The remaining six proteins are known as accessory proteins. Three of these, Vif, Vpr, and Nef, are also localized in the virus core. Tat and Rev modulate virus gene expression, and Vpu supports virus assembly (25; 78; 84; 149).

Figure 1: Organization of the HIV-1 genome

The HIV-1 genome encodes open reading frames for nine genes: *gag*, *pol*, *env*, *vif*, *vpr*, *nef*, *tat*, *rev*, and *vpu*. The cross-section of the HIV-1 virion shows an enveloped, cone shaped virus, with surface envelope glycoprotein spikes. Two copies of RNA genome are packed in the core of the virion, accompanied by nucleocapsid protein, and surrounded by capsid. Image used with permission from (84).

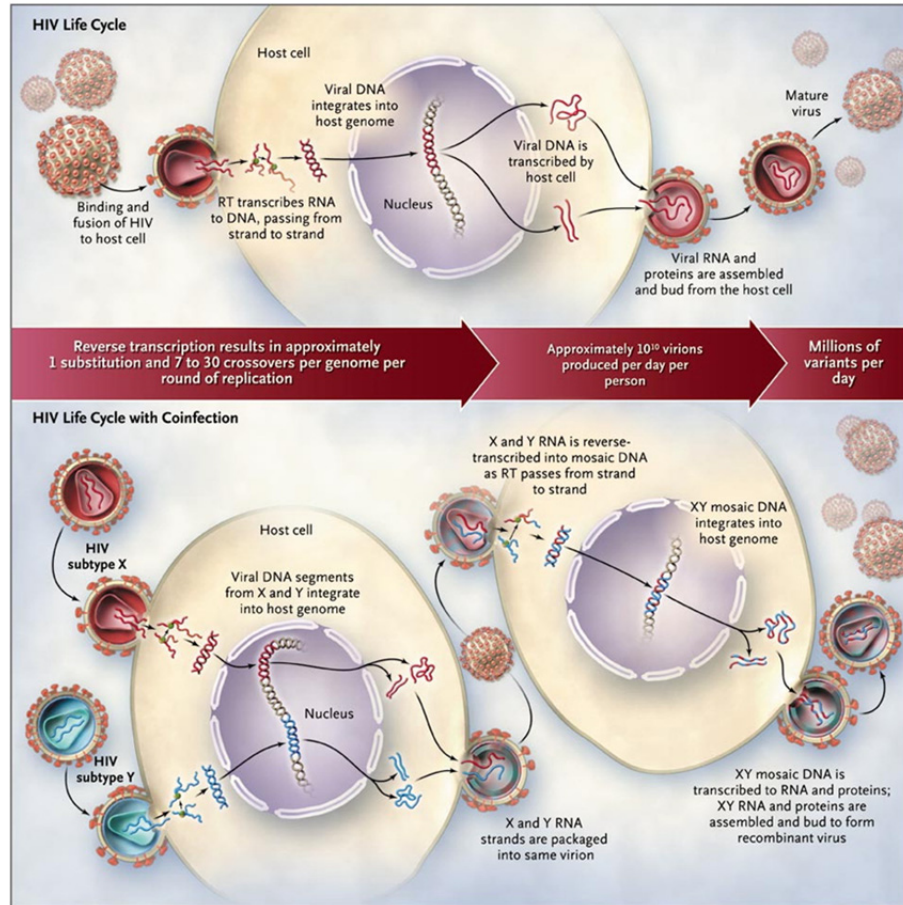


HIV-1 Life cycle

Viral entry into CD4+ T-cells proceeds stepwise as gp120 binds CD4, followed by coreceptor (CXCR4 or CCR5) binding and conformational changes in gp41 that facilitate membrane fusion. After the viral core enters the host cytoplasm, the viral reverse transcriptase enzyme synthesizes DNA copies of the viral RNA genome(s). HIV packages two single stranded copies of viral RNA genome. Either copy can be used as template for reverse transcription, and templates may switch during the process, generating diverse and novel recombinants. The mutation rate for reverse transcription *in vivo*, estimated to be 1.4×10^{-5} mutations per base per cycle, also contributes to form a highly heterogeneous quasispecies during replication (2; 85). A diagram of the HIV-1 viral life cycle is shown in Figure 2.

Figure 2: The HIV-1 Viral life cycle

HIV-1 binds CD4 and a coreceptor (CXCR4 or CCR5) to initiate fusion to the host cell. Reverse transcription and recombination events generate viral diversity with each round of replication. Image used with permission from (253).



DNA copies of the viral genome are actively transported to the nucleus, where they are inserted into the host DNA by the viral integrase protein (85). The integrated viral genome, known as provirus, provides a complete template for subsequent viral RNA production. The presence of the transcriptional transactivator Tat protein upregulates RNA synthesis. More than 30 viral RNAs are produced, some of which are multiply spliced before nuclear export (85; 206).

Virus assembly is directed by Pr55, the Gag precursor polyprotein. Pr55 associates with the plasma membrane and promotes Gag-Gag interactions. Pr55 also stimulates encapsidation of the two single-stranded copies of the viral RNA genome, association with envelope glycoprotein, and budding from the cell. The envelope glycoprotein gp160 is synthesized and oligomerizes in the ER. In the Golgi, gp160 is cleaved into gp120 and gp41 by Furin, and the gp120 domain is heavily glycosylated. The non-covalent association between gp120 and gp41 is only moderately strong, with the result that gp120 is shed from infected cells and virus particles. Finally, the p6 domain of Gag promotes budding and completes particle release (85; 113; 231).

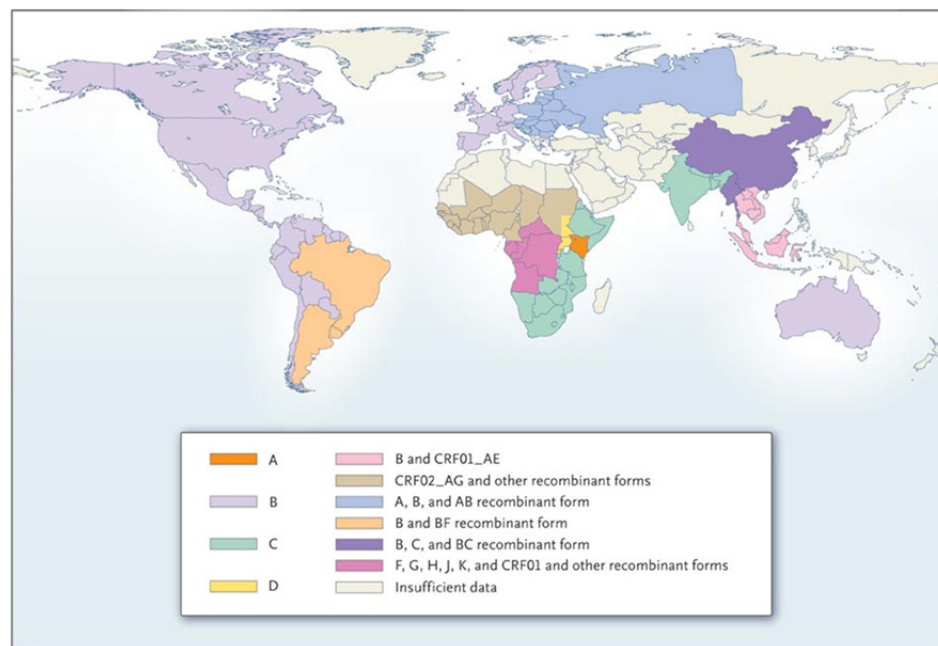
HIV-1 Origins and diversity

The major HIV-1 epidemic likely originated from a simian virus, SIVcpz, that infected populations of chimpanzees in southern Cameroon and was passed to human hunters via exposure to infected blood (253). This event and two other independent transmission events from great apes to humans have been deduced from phylogenetic analyses (127; 132). These events probably occurred early in the 20th century, and gave rise to the three HIV-1 groups: major (M), outlier (O), and nonmajor/nonoutlier (N). Strains similar to ones from groups M and N have been found in two geographically

separated populations of chimpanzees, and strains from group O are similar to ones found in gorillas and chimpanzees. In 2009, a new HIV-1 strain related to SIV that infects gorillas (SIVgor) was identified and classified in a new group, P (198). These HIV-1 groups are distinct from HIV-2, which was probably transmitted to humans in Africa from the Sooty mangabey monkey. Group M is the primary cause of the HIV-1 epidemic, and can be divided into subtypes (indicated by letters), and subsubtypes (denoted with numerals) (1; 9; 253). Subtypes can contain phylogenetically related strains that are approximately the same genetic distance from one another, or otherwise geographically or epidemiologically linked strains. Current group M subtypes include: A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K. Genetic variation between subtypes can be between 25 to 35%; within a subtype genetic variation can be between 15 to 20%. Presently, there are 58 HIV-1 circulating recombinant forms (CRFs), which result from the recombination of viruses from different subtypes (297). All subtypes are prevalent in the African continent. Worldwide, up to half of all HIV-1 infections are of subtype C. The prevalent subtype in Western Europe and the Americas is subtype B (109). A map of the global distribution of HIV-1 subtypes and recombinant forms is shown in Figure 3.

Figure 3: Global distribution of HIV-1 subtypes in 2008

This map shows the global distribution of HIV-1 subtypes and circulating recombinant forms in 2008. Presently, approximately half of all worldwide infections are subtype C. Infections in North America and Western Europe are predominately subtype B. Image used with permission from (253).



The earliest documented case of HIV-1 infection in humans was from a blood sample taken in 1959 from an individual in Kinshasa, Zaire (305). It has been proposed that the emergence of HIV-1 in the Americas occurred first in Haiti around the late 1960s before dispersing elsewhere (92). In 2012, there were 2.3 million new HIV-1 infections worldwide, the lowest number of annual new infections since the 1990s (260). Globally, approximately 35 million people are living with HIV (260).

HIV-1 Envelope glycoproteins gp120 & gp41

The three dimensional organization and function of the Env spike determine the target epitopes and mechanisms of neutralization. Infection of target cells by HIV-1 begins when the viral surface glycoprotein gp120 engages the cellular CD4 receptor and a chemokine co-receptor such as CCR5 or CXCR4 (51; 77). Some clones of HIV-1 envelope glycoprotein, such as the R2 clone, are capable of CD4-independent infection (53). The transmembrane component of the envelope spike, gp41, facilitates membrane fusion between the virus and the cell (43; 283).

Though there are five conserved regions (C1-C5) on gp120, trimeric Env is extremely heterogeneous (248). Differences in viruses within an individual stem from mutations that persist in the five variable regions (V1-V5) of gp120, inconsistent glycosylation, and the relatively flexible conformation of the trimer (173). The C1-C5 regions lie within the inner domain of the structure, which is shielded by a heavily glycosylated outer domain (140-142; 290; 291). Between the inner and outer domains, a flexible bridging sheet consisting of a four-stranded β -sheet formed by V1V2 and C4 is responsible for binding to both the CD4 and chemokine receptors (139; 141; 219). Structural disorder in V1-V5 is so prominent that even with the guidance of two dozen 3-

D crystal structures, the actual conformation of these loops in native trimers remains unknown (173).

The V3 loop of gp120 is a major component of the co-receptor binding region, and is essentially constant in length (34-35 amino acids) (37; 306). The amino acid sequence of V3 is highly variable (in all but subtype C viruses), but sequence variability is limited to only approximately 20% of amino acid positions, many of which are among the 14 amino acid “crown” in the middle of the V3 loop. The V3 loop is required for virus infectivity; deletion of V3 completely abrogates virus function (294). The amino acid residue N332 on the V3 loop is required for glycan dependent broad and potent neutralizing antibodies such as the PGT121 family of monoclonals (123; 273).

The V1V2 double loop region is highly variable in sequence and length, ranging from 50 to 99 amino acids, and increases in length during chronic infection (56). Quaternary epitopes in the V1V2 region are also the target of several broadly cross-reactive monoclonal antibodies such as PG9/PG16, CH01-04, and PGT141-145 (65). A net positive charge in the β -strand C region of V1V2 (which includes residues 168, 169, and 171) correlates with neutralization sensitivity to PG9/PG16 (65). Deletion of V1V2 abolishes virus entry (294). The V2 loop includes a motif that binds to $\alpha 4\beta 7$ integrin, which may direct the virus to gut mucosal cells.

The function of V4 and V5 are less well-defined. Both regions are involved in neutralization escape, and though V4-specific neutralizing antibodies have been found described in humans and immunized rabbits, no polyclonal or monoclonal V5-specific antibodies have been described (306).

When the Env trimer binds CD4, each of the three gp120 monomers transition from a 'closed' to 'open' conformation, shifting V1V2 loops from the apex of the trimer to the periphery, and permitting binding to a co-receptor such as CCR5 or CXCR4.

Prior to receptor binding, gp41 is masked by gp120 (282; 311). During the virus-cell membrane fusion process, which occurs within several minutes, gp41 undergoes structural changes to form a six-helix bundle in which the C-terminal helices of gp41 pack around the N-terminal helices in an anti-parallel manner (43; 251; 283). The membrane proximal external region (MPER) region of gp41 contains epitopes for numerous bnMAbs that may be accessible during fusion (16; 310).

Broadly neutralizing monoclonal antibodies to HIV-1

Prior to 2009, only five broadly neutralizing monoclonal antibodies to HIV-1 had been identified: 2F5, 4E10, 2G12, Z13e1, and 5H/11-BMV-D5. Since then, the implementation of high throughput neutralization screening and single B cell sorting and cloning has led to the isolation of more than 60 additional mAbs. These mAbs and their targeted epitopes are listed in Table 1.

Table 1: Broadly neutralizing monoclonal antibodies to HIV-1 and their targeted epitopes

gp41	V1V2	V2/V3	V3	V4	C1-C5	CD4bs	CD4i	gp120-gp41 interface
2F5	PG9	CH01-04	447-52D	1.7A	C11	VRC01-03	3BC176	8ANC195
4E10	PG16		PGT121-family	1.10C	2.12A	NIH45-46	48D	PGT151-family
10E8			PGT141-family	B23	2.3B	VRC-PG04, 04b	17b	
Z13e1			2G12	7.4H	8.2A	VRC-CH30-34	5.8H	
M66.6			10-1074			3BNC55, 60, 62, 117	2.5E	
7H6			HGN194			12A12, 21, 30	4.12D	
HK20						1B2530	ED11, 47	
5H/11-BMV-D5						1NC3, 7, 9	3.4F	
						CH103		
						b12		
						HJ16		
						F105		
						b6		

*Data adapted from (45; 117; 143)

Immune evasion

HIV-1 reverse transcriptase lacks proofreading activity, which contributes to the virus' relatively high mutation rate of approximately 3.4×10^{-5} mutations per base per replication cycle (253). Mutations accumulate rapidly as the virus replicates, collectively generating a swarm, or quasispecies of viruses within an individual. Immunodominant

epitopes are present on variable regions of the envelope glycoprotein, but induce neutralizing antibodies of limited breadth. Broadly cross reactive neutralizing antibodies, which develop in approximately 20% of HIV-infected individuals about 2-3 years after seroconversion, do not halt disease progression (131; 247). Consequently, an immune response that might clear infection is too little too late, if it even occurs at all (121).

In addition to harboring mutations in its variable regions, Env possesses a glycan shield that further supports immune evasion by cloaking immunogenic epitopes as ‘self’ (30; 281; 290). Other immune evasion features of Env include: a CD4 binding site (CD4bs) that is located within a crypt that sterically constrains antibody recognition (46; 141); a coreceptor binding surface that is inaccessible until CD4 binds (144); a quaternary structure of Env that is not rigid, and is therefore presented as a moving target (138; 295); and the accumulation of mutant, misfolded, truncated, or decayed Env that may also present irrelevant yet immunogenic epitopes for antibody induction (3; 54; 176; 190; 200).

Stages of infection and immune activation

During the initial stage of HIV-1 infection, a period of about 10 days following transmission known as the eclipse phase, most mucosal viral transmissions encounter a bottlenecking effect, such that a single virus genotype commonly initiates spreading infection from the site of exposure (126; 226). These primary founder viruses preferentially infect CD4⁺ T cells (rather than monocytes and macrophages) (226).

The next ~100 days of infection are known as the acute phase. The virus, which now can be detected as viral RNA in plasma, reaches lymphoid tissues and infects additional CD4⁺ T cells (76; 246; 301). Dendritic cells (DCs) bind HIV-1 via DC-SIGN

interactions and shuttle the virus to activated T cells (89). Rampant viral replication spreads the virus to other lymphoid tissues. In gut-associated lymphoid tissue (GALT), approximately 80% of CD4⁺ T cells can be depleted (20% by infection, 60% by apoptosis) within three weeks of infection (22-24; 168; 267). By the third to fourth week of infection, viremia peaks at more than a million RNA copies per ml of blood. B cell responses to new antigens are suppressed due to germinal center deficiency and loss (148).

Despite acute CD4⁺ T cell depletion, the innate immune response and CD8⁺ T cell responses are activated. Acute-phase proteins, which can be triggered by pro-inflammatory cytokines, are detectable in the plasma sometimes just before viral RNA can be detected. The production of these proteins can also be stimulated by lipopolysaccharide (LPS) from commensal bacteria translocating from the gut following HIV-1 infection of the GALT (22; 23; 215). Cytokines such as IL-15 and IL-18 are secreted and serve to enhance both innate and adaptive immune responses. Other cytokines and chemokines such as type I interferons, IFN γ , TNF, IL-10, IL-18, and CXCL10 are produced as viremia increases (245). Natural Killer (NK) cells can produce cytokines and can lyse virally infected cells, though in infected cells, HIV-1 downregulates expression of ligands for NK cells (277). Before the first antibodies are detectable, an initial HIV-1 specific CD8⁺ T cell response is mounted (18; 133; 188; 287). Following this short-lived CD8⁺ T cell response, virus mutations accelerate, selecting for escape mutants that replace the founder virus within ten days (13; 99; 226).

Over the next 12-20 weeks, CD4⁺ T cell levels partially recover, and viral load decreases, stabilizing at a viral set point which represents an equilibrium between virus

turnover and the immune response. Adaptive immune responses supply pressure resulting in innumerable escape mutants (177). The initial antibody response to Env is non-neutralizing. Neutralizing antibodies are produced, albeit slowly, to autologous virus at around 12 weeks post HIV-1 transmission, and to heterologous virus during a number of years after infection. Approximately 20% of patients develop neutralizing antibodies that cross-react substantially with heterologous viruses within about three years after onset of infection (262).

What follows is a period of chronic infection (8-10 years) during which selective pressure continues and escape mutants emerge, until cell mediated immunity is overwhelmed and progression to Acquired Immune Deficiency Syndrome (AIDS) begins.

The United States Centers for Disease Control and Prevention (CDC) defines AIDS (in adults and adolescents) as the stage at which one of the three following conditions is observed: $CD4^+$ T cells number fewer than 200 per microliter of blood, the percentage of $CD4^+$ T cells in total lymphocytes is less than 14% of total T cells, or the presence of an AIDS-defining condition (which includes various fungal, viral, parasitic and bacterial opportunistic infections, and certain cancers) (235).

Differing disease progression and virus control

Progression to AIDS is dependent on viral and host factors, but can be classified by clinical outcomes. In general terms, aside from typically progressing HIV-1 infected patients (TP) certain patients progress to AIDS very slowly, some taking over twenty years. These long term non progressors (LTNPs) include aviremic 'Elite Controllers' (EC) and Viremic Controllers (VC). On the other extreme, some patients (termed Rapid

Progressors, RP) progress rapidly, reaching AIDS in a few short years. These phenomena are naturally occurring and spontaneous.

Longitudinal viral load determinations are used to distinguish EC and VC, with the former having undetectable virus load and the latter having controlled viremia (compared to TP). Furthermore, a patient's disease progression group classification may change as infection persists (e.g. after a period of elite control, an EC patient may become viremic and progress to AIDS).

Specific criteria used to define these groups vary among different cohorts. For consistency, however, we will use the definitions used in the US Department of Defense HIV Natural History Study Cohort, the source of specimens in the present study (184).

Conflicting reports of broadly cross-reactive neutralization

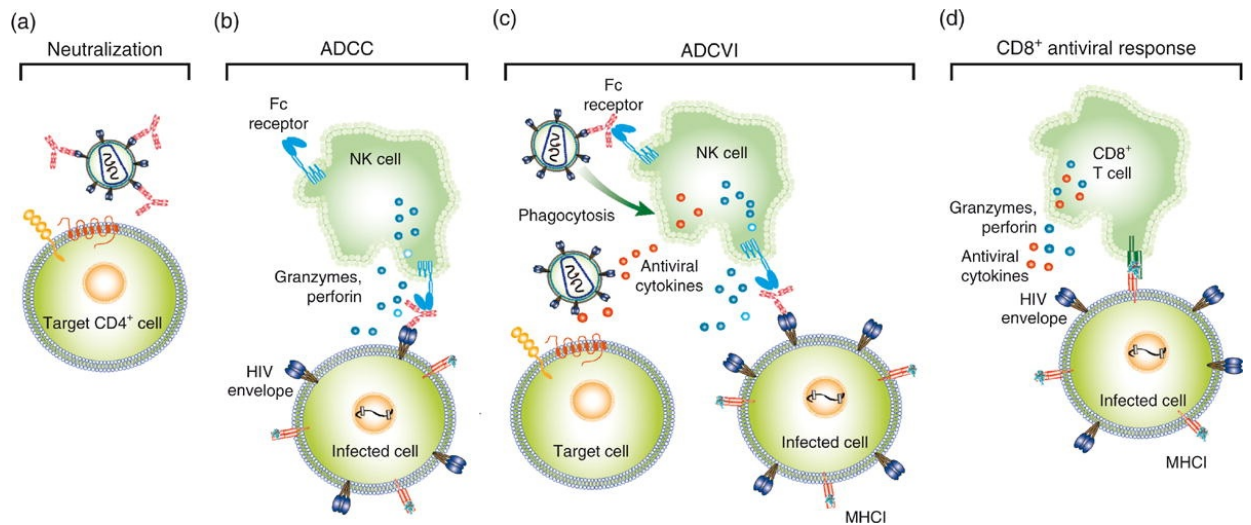
Broadly cross-reactive neutralizing antibodies (bcnAbs) are induced in approximate 20% of all HIV-infected individuals (262). However, conflicting reports about whether individuals in certain disease progression groups are more likely to possess bcnAbs than others (64; 110; 193). BcnAbs have been found in LTNPs in some (35; 175; 189; 194) but not all (67; 145; 171) studies. Viremic controllers were reported to induce better neutralization than elite controllers (162), who are unlikely to even develop bcnAbs (66; 73; 192). However, some elite controllers do indeed induce bcnAbs (145). Another study found similar neutralization patterns between LTNP and typical progressors (262).

HIV-1 Vaccine development

An effective HIV-1 vaccine will likely induce both antibody and cytotoxic T-lymphocyte (CTL) responses. Antibodies binding to the HIV-1 envelope can block entry into target cells, neutralizing the virus and preventing initiation of infection. Antibodies can also otherwise inhibit HIV-1 through antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular virus inhibition (ADCVI) (83). In ADCC, the Fc domain of antibodies bound to HIV-1 envelope on the surface of infected cells engages Fc receptors on certain innate immune cells (such as natural killer (NK) cells). Activation of Fc receptors on NK cells results in the secretion of perforin and granzymes, which causes lysis and apoptosis of infected cells. The functions of ADCVI include those of ADCC, Fc mediated phagocytosis of the virus, and secretion of antiviral cytokines (such as beta-chemokines) that interfere with virus activity. The CTL response does not directly inactivate virus, but rather it is mediated by short processed viral peptides in context with major histocompatibility complex class I (MHC-1) proteins that are presented on the surface of infected cells. Perforin, granzymes, and antiviral chemokines are produced upon CD8⁺ T cell recognition of the antigen/MHC-1 complex. Figure 4 illustrates these mechanisms of HIV-1 inhibition. The CTL response can suppress virus replication, reducing viremia. Activating both antibody and CTL responses together is hypothesized to reduce the probability of HIV-1 transmission (271).

Figure 4: Mechanisms of HIV-1 inhibition

Immune mechanisms of HIV-1 inhibition include neutralization, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular virus inhibition, and CD8⁺ cytotoxic T lymphocyte responses. Image used with permission from (229).



Several vaccine vectors developed for HIV-1 include DNA plasmids, and adenovirus and poxvirus vectors. Since DNA vaccines are nonreplicating, they are intrinsically safer than live attenuated viral vectors. They also do not elicit an immune response directed at the vector. These plasmids contain elements from human cytomegalovirus for eukaryotic gene expression. Two examples of DNA-based HIV vaccines include: PENNVAX-B, a mixture of three expression plasmids encoding subtype B *Env*, *Gag*, and *Pol* proteins, and HIVIS, which contains plasmids encoding subtype A, B, and C *Env*, and subtype B *Rev* proteins (124; 185).

Testing of DNA vaccines has not generated robust immune responses in humans, due to low rates of antigen expression and uptake by antigen presenting cells (40; 102; 160; 161). However, a recent study incorporating an additional DNA plasmid encoding human IL-12 and electroporation as a delivery method reported improved immunogenicity (HIV Vaccine Trials Network (HTVN) studies 070 and 080) (124). DNA prime followed by protein/viral vector boost vaccination generates better antibody responses than either DNA or protein alone, and increases CD8⁺ memory T cells (31; 115; 166; 256; 272; 276).

Recombinant adenovirus vectors were developed by Merck and the NIH Vaccine Research Center (VRC) after preclinical immunogenicity study results showed higher humoral and CTL responses with adenovirus serotype 5 (Ad5) vectors than with poxvirus vectors and DNA vaccines (8). Non-human primates immunized with Ad5 vector encoding *Gag* were found to control viral load following primary infection (241). The Merck vaccine (MRKAd5) consisted of equal parts of three recombinant Ad5 vectors expressing subtype B *Gag*, *Pol*, and *Nef*. The E1 region of the Ad5 vector was deleted,

rendering the virus replication incompetent, and was replaced with a promoter for human cytomegalovirus (44; 205; 293). MRKAd5 was tested in the Step and Phambili studies, both of which were terminated for futility (detailed in the following section).

Furthermore, the vaccine may have increased rates of HIV acquisition in uncircumcised men with pre-existing immunity to the Ad5 vector (28). Follow up studies have suggested that the effect of MRKAd5 attenuation is due to pre-existing Ad5 immunity, and may have been caused by a lack of innate immune activation (296). To bypass pre-existing immunity, next generation adenovirus vectors include rare human serotype vectors (rAd26, and rAd35), hexon-chimeric vectors (rAd5HVR48), and vectors derived from chimpanzees and other species (8). A replication competent recombinant adenovirus vector based on adenovirus serotype 4 (Ad4) has also been developed and tested in rabbits (5). Although the Ad4 vector construct replaces the E3 gene with the HIV-1 Env gene, the virus is not attenuated.

As viral vectors, poxviruses provide several advantages. Vaccinia, for example, can accept stable insertions of 25kb of DNA into its 200kb genome (243). It (and other poxviruses) remain in the cytoplasm of infected cells, and utilizes virally encoded polymerases for replication and transcription. Vaccinia is recognized as being relatively safe, and has been used in various gene therapy applications (167).

Modified Vaccinia Ankara (MVA), is a highly attenuated derivative of the chorioallantoid vaccinia virus Ankara (CVA). After more than 570 serial passages in primary chicken embryo fibroblasts (CEF), the MVA genome has lost about 30kb of genome and thus, possesses limited replication capacity in mammalian cells. Although

viral replication occurs, infection does not spread. As a vaccine, MVA can elicit strong immune responses to its expressed transgenes, but results are heterogeneous (98).

The NYVAC vaccinia strain was derived from a plaque-purified isolate of the Copenhagen vaccine strain (VACV-COP). Deletion of 18 open reading frames (ORFs) has rendered the virus highly attenuated, unable to produce infectious virus in humans (252). NYVAC provides high levels of gene expression and elicits antigen-specific, but limited immune responses in humans (97).

The Canarypox virus vector ALVAC was derived from a plaque-purified isolate of an attenuated canarypox virus subjected to 200 serial passages in chick embryo fibroblasts. ALVAC has been reported to be a potent CTL immunogen, and is well tolerated and safe in humans, (59; 80; 199). ALVAC was administered in conjunction with AIDSVAX (recombinant gp120) in the RV144 vaccine study, the first clinical trial to demonstrate efficacy (31.2%) for prevention of HIV acquisition (detailed in the following section) (217).

Human clinical trials of HIV-1 vaccines

To date, six human clinical trials of HIV-1 vaccines have been conducted. A summary of these six studies is shown in Table 2.

Table 2: Human clinical trials of HIV vaccines

Study	Pub. Year	Vaccines	Phase	Volunteers' risk	Location	Result	Ref.
Vax004	2005	AIDSVAX B/B gp120 in alum	III	MSM and women at high risk, (n=5,400)	USA, Europe	No efficacy	(81)
Vax003	2006	AIDSVAX B/E gp120 in alum	III	Injecting drug users, (n=2,100)	Thailand	No efficacy	(195)
HVTN502 Step trial	2008	MRKAd5 HIV-1 gag/pol/nef B	IIb	MSM and heterosexual women and men, (n=3,000)	USA	No efficacy; transient increased infection rate in vaccines	(28)
RV144	2009	ALVAC-HIV vCP1521 and AIDSVAX B/E rgp120 in alum	III	Community-risk, (n=16,400)	Thailand	31.2% efficacy at 42 mo, 60% at 12 mo against HIV acquisition. No effect on plasma viral load and CD4 count in infected individuals	(196; 217)
HVTN503 Phambili trial	2011	MRKAd5 HIV-1 gag/pol/nef B	IIb	Heterosexual men and women, (n=801)	Republic of South Africa	No efficacy; increase HIV infection rate in vaccines	(103)
HVTN505	2013	DNA and rAd5 (A, B, and C)	IIb	MSM with Ad5-specific antibody titers <1:18 (negative), (n=2,500)	USA	Stopped for futility; no efficacy on HIV acquisition, plasma viral load and CD4 count	(105)

Table adapted from Excler et al (74).

The first two trials (Vax004 and Vax003), antibody-inducing vaccines formulated with monomeric recombinant gp120 in alum, failed to prevent HIV-1 acquisition and did not affect HIV-1 disease progression (94; 195). The outcomes of these early trials shifted the focus of HIV-1 vaccine development to inducing cell-mediated immune responses, which were assessed in the Step (HVTN502) and Phambili (HVTN503) vaccine trials. Both studies tested the Merck vaccine (MRKAd5 HIV-1), a mixture of replication-defective Ad5 adenovirus vectors expressing subtype B genes for *gag*, *pol*, and *nef*. The Step trial, conducted in the US, was halted after an interim analysis showed no protection against HIV-1 acquisition. This led to the early termination of the Phambili trial, which had been proceeding concurrently in South Africa. Post-hoc analyses for both trials indicate an elevated infection rate in vaccinees. In the Step study, this risk was greatest in uncircumcised men with pre-existing neutralizing antibodies to Ad5 who received the Merck vaccine (55; 57). Additionally, no specific CD8+ cytotoxic T-lymphocyte (CTL) response recognizing vaccine-elicited epitopes was identified (222).

The HVTN505 trial tested a DNA prime (with DNA plasmids encoding genes for subtype B *Gag*, *Pol*, and *Nef*, and subtypes A, B, and C *Env*) followed by a replication-defective rAd5 boost (with a total of four prime-matched rAd5 vectors encoding the three subtypes of *Env* and subtype B *Gag-Pol*), in an attempt to induce both antibody and CTL responses. The trial was recently stopped due to findings showing absence of efficacy and an increase in HIV infection in vaccine recipients, among other reasons (105).

The only trial that has provided some evidence of vaccine efficacy is the RV144 study, which tested a recombinant canarypox vector (ALVAC-HIV) prime, followed by a bivalent monomeric gp120 boost (AIDSVAX gp120 B/E MN and CRF01_AE (A244)).

ALVAC-HIV (vCP1521) expresses *gag*, *protease* (subtype B strain LAI), gp120 (subtype CRF01_AE strain 92TH023) and gp41 (subtype B strain LAI). Vaccine efficacy at 42 months post first vaccination, the primary endpoint, was 31.2%. Post hoc analysis indicated 60% efficacy at 12 months post first vaccination (95; 217). Two initial correlates of risk were identified: the presence of plasma IgG antibody to gp70 V1V2 envelope proteins was inversely correlated with risk of infection, and plasma IgA to Env was directly correlated with an elevated rate of infection (108; 125; 307; 308). Subsequent analysis found that serum IgG antibody to V3 was directly correlated with decreased infection risk (101).

Studies examining breakthrough viruses can identify specific virus populations that are impervious to vaccine-induced immune responses. In these virus sieve analyses, sequence differences (such as those leading to escape mutations) in circulating viruses can be compared to identify critical targets to increase vaccine efficacy (93). For the RV144 study, virus sieve analysis identified increased vaccine efficacy if infecting strains of HIV-1 matched the RV144 vaccine at residue K169, or mismatched the vaccine at residue I181 (221). Both of these residues are located within the V2 region of the envelope glycoprotein.

Reverse vaccinology

In recent years, broadly cross reactive neutralizing antibodies (bcnAbs) isolated from “elite neutralizers,” HIV-infected patients whose serum is among the top 1% in neutralizing ability, have guided a different approach to vaccine design termed reverse vaccinology: using the structural information of a bcnAb and its epitope for immunogen design (263). Elite neutralizers are defined as patients whose serum is capable of

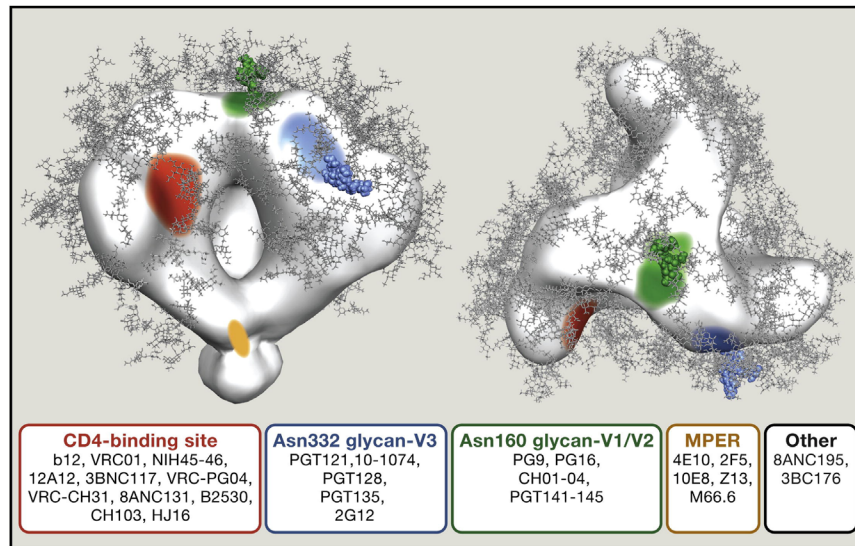
neutralizing more than one virus at an IC₅₀ dilution titer of 300 within a single subtype and across at least four subtypes (242). In this approach, single IgG⁺ memory B cells isolated from the elite neutralizer were plated in wells, and after activation, supernatants were screened for neutralizing ability against a panel of viruses (274). Neutralizing antibodies were cloned and expressed for further characterization.

Such bcnAbs isolated from elite neutralizers can elucidate conserved epitopes that serve as more focused targets for vaccine design. One recent study reported the identification of antibodies PGT151 and PGT152, and its recognition of a previously unknown complex glycan-dependent epitope in gp41 centered at residue N611 (and involving residues N637 and E647) (75). Previously known neutralizing epitopes on the HIV-1 envelope glycoprotein had been limited to the membrane proximal external region (MPER) of gp41, the CD4 binding site (CD4bs) of gp120, glycan dependent residues on V1V2 and V3, a glycan cluster on the outer domain of gp120. Approximate locations of these epitopes are presented in Figure 5.

The antibodies PGT151 and PGT152 selectively bound cleaved trimer (and did not bind uncleaved trimer). This finding highlights the possible importance of presenting more perfect, native-like trimers in Env immunogen design. Cleavage of the gp160 precursor into gp120 and gp41 is critical for native trimer assembly and fusion peptide exposure (218).

Figure 5: HIV-1 Epitopes targeted by broadly neutralizing monoclonal antibodies

Locations of epitopes on the HIV-1 envelope glycoprotein targeted by broadly neutralizing monoclonal antibodies. Image used with permission from (284).



Mechanism for induction of bcnAbs

The mechanism for induction of bcnAbs remains an important focus in HIV-1 research. Recent reports have shown that certain envelope glycoproteins from transmitted/founder viruses are more likely to bind to the germline or unmutated common ancestor (UCA) of the naïve B cell that ultimately produces bcnAbs (in a matter of months), and that the neutralization activity in polyclonal sera from a patient with bcnAbs may be a result of the combined activity of component monoclonal antibodies (68; 91; 154). Combined with the finding that most infections are initiated by a single

transmitted/founder virus, it is clear that the envelope glycoprotein plays an important role in the pathway for induction of bcnAbs (126).

Future directions

The results of the RV144 vaccine trial provide evidence that an efficacious HIV vaccine can be developed. Improving responses from both humoral and cell-mediated arms of the immune system should be a priority in the next vaccine tested. The identification of additional epitope targets by understanding bcnAbs from natural infection would greatly inform vaccine immunogen design and structural studies. However, equally important is the platform by which the immunogen is presented to the immune system.

USING ALPHAVIRUSES AS VECTORS

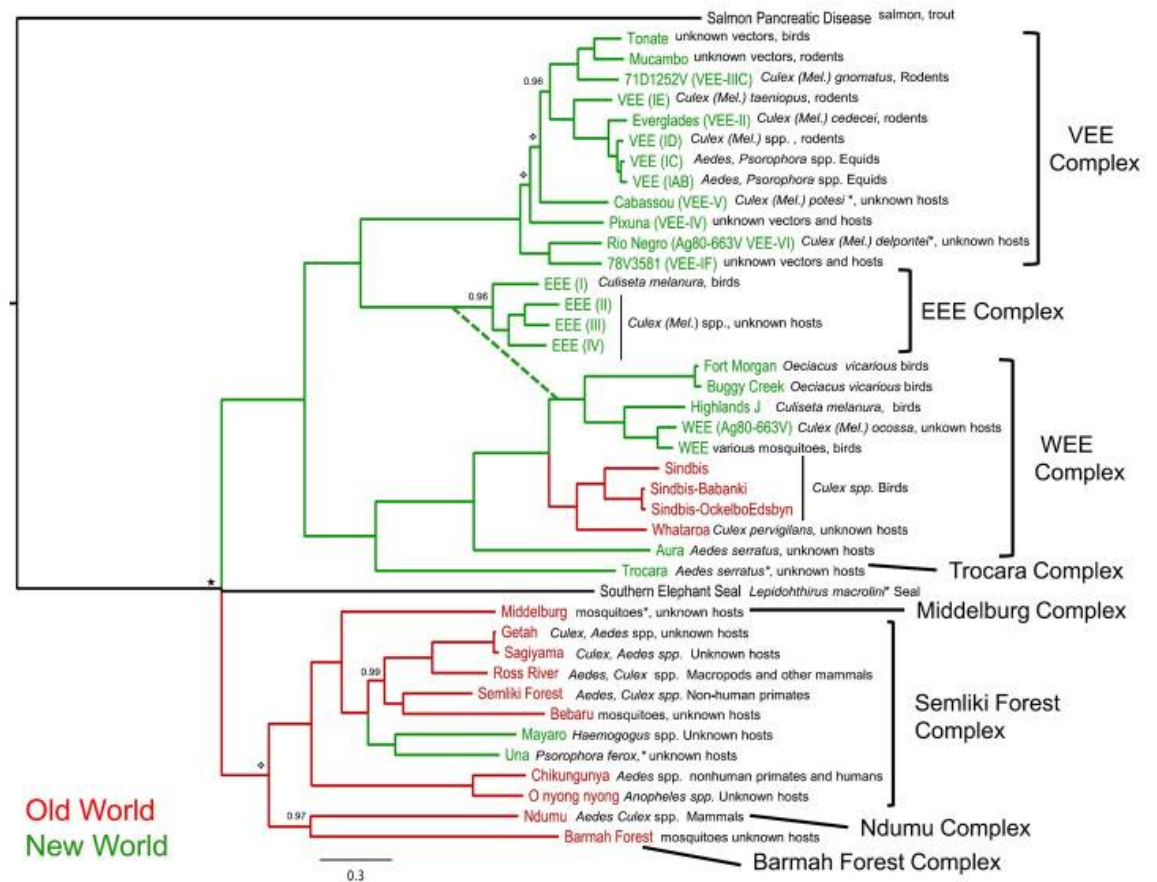
The use of alphaviruses as *in vivo* expression vectors in various applications, such as in immunizations and in cancer gene therapy, has been investigated in many studies (157; 211). Alphavirus expression systems offer many desirable features including broad host cell targets, high levels of transgene expression, relatively simple genome manipulation, positive sense RNA and its inherent infectivity, dendritic cell targeting, natural adjuvant effects, and replication competent or incompetent forms (211).

Alphavirus introduction: genome and replication

Alphaviruses are comprised of 29 enveloped virus species of the *Togaviridae* family. The initial taxonomic organization of the alphavirus genus was based on cross-reactivity to six antigenic complexes: eastern-, western-, and Venezuelan- equine encephalitis (EEE, WEE, and VEE, respectively), Semliki Forest, Barmah Forest, and Middelburg. More recently described alphaviruses have been assigned to complexes based on genetic analysis. The New World alphaviruses (EEE, VEE, and WEE complexes) tend to produce potentially fatal encephalitis disease as their most important manifestation, while the Old World alphaviruses (Semliki Forest, Barmah Forest, and Middelburg complexes) are generally associated with rheumatic disease (250). A phylogenic tree diagram of selected Old and New World alphaviruses is shown in Figure 6.

Figure 6: Phylogenetic trees of selected Old and New World alphaviruses

New World alphaviruses (green) tend to cause potentially fatal encephalitic disease. Old World alphaviruses (red) tend to cause rheumatic disease. Image used with permission from (278).



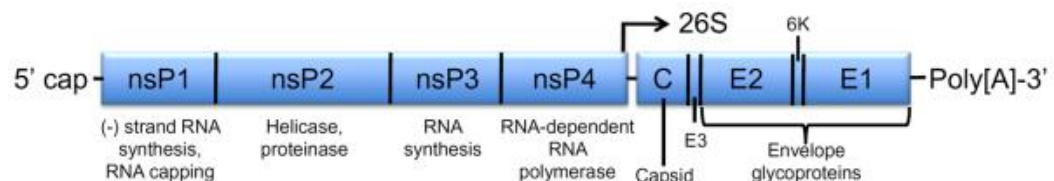
Alphaviruses vary in degree of pathogenicity; It is estimated that neurologic disease is caused by EEE in 1 of every 23 cases of human infection, and by WEE in 1 in 1000 infections (234). Fort Morgan Virus (FMV), which is in the WEE antigenic complex, is restricted to birds and their resident bed bugs, and is not known to infect humans.

Most individual alphavirus species are transmitted by a single (or small number of) species of mosquitos, lice, and/or bed bugs. Alphaviruses that infect humans always have at least one animal reservoir, from which its insect vector transmits it to other animals and humans. Some are not naturally transmitted to humans, generally the result of the restricted niches in which they exist in nature. Insect vectors maintain transmission among the target animal species.

The alphavirus genome is a single positive sense strand RNA of approximately 11kb that includes a 5'-end CAP and a 3'-end polyadenosine (polyA) tail, similar to typical messenger RNA (mRNA) (41; 211; 278). A diagram of the alphavirus genome is shown in Figure 7.

Figure 7: Alphavirus genome

The alphavirus genome is a single positive sense strand of RNA that encodes non-structural and structural polyproteins. Image used with permission from (278).



The viral RNA genome contains two open reading frames (ORFs) that encodes the non-structural (nSP) and structural polyproteins (SP). The ORF that encodes nSP is closer to the 5'-end and spans about two thirds of the genome. It encodes proteins for transcription of negative sense viral RNA, polyprotein cleavage, and RNA capping. The ORF that encodes SP (capsid, envelope glycoproteins) is the final third of the genome, located near the 3'-end.

Alphavirus replication takes place exclusively in the cytoplasm of infected cells. In the first 3-4 hours following viral entry, the four subunits of nSP (nSP1-4) are readily translated from the alphavirus genome (211). The yet unprocessed four subunit nSP1-4 complex forms the viral replicase (unprocessed Rep) and uses the alphavirus genome as a template to synthesize negative sense complementary RNA. As Rep is cleaved by proteases to form a tetramer of nSP1-4, its primary activity shifts to the synthesis of additional positive sense full length genomes from the newly produced negative sense template. From the negative sense template, Rep also recognizes a subgenomic promoter located upstream of the SP ORF, which yields the translated polyprotein product p62-6K-E1. This polyprotein is then processed by a cellular serine protease to yield the capsid protein and p62, from which three envelope glycoproteins (E1, E2, and 6K) will be produced and assembled in the Golgi (172). The viral genomic RNA is packaged in the capsid as a result of a packaging signal located in the nSP coding region. Upon transport to the plasma membrane, the nucleocapsid of the alphavirus assembles to contain 240 monomeric capsid proteins, 80 trimeric E1/E2 heterodimers, and one copy of genomic

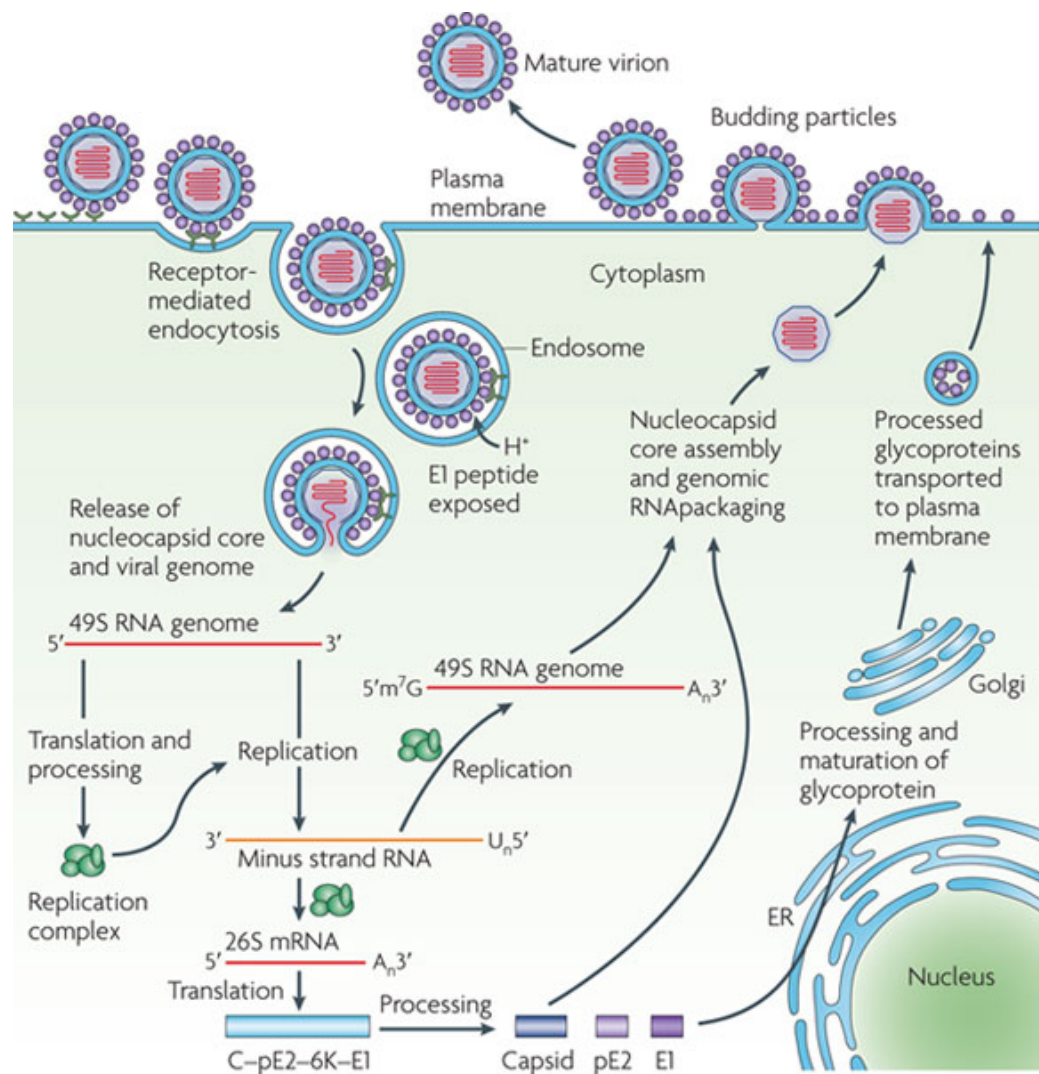
RNA. The capsid is icosahedral and coated with a lipid bilayer derived from the host plasma membrane (278).

Viral entry into the cytoplasm of cells is facilitated by receptor-mediated endocytosis initiated by attachment of the E2 glycoprotein to a host cell receptor (178; 266). Varied receptors have been identified, including a ubiquitously-expressed metal ion transporter natural resistance-associated macrophage protein (NRAMP2) that is used for entry into mammalian cells (223). Endosomal fusion and subsequent cytoplasmic entry is facilitated by the E1 glycoprotein, which contains a fusion peptide.

Several studies have suggested that the double-stranded RNA (dsRNA) intermediates that exist during alphavirus replication may trigger a “danger signal” in various intracellular pathogen-associated molecular patterns (PAMP) receptors (including Toll-like receptors TLR3, TLR7, and TLR8, and DExD/H-box helicases RIG-I and MDA5) leading to the induction of the innate anti-viral immune response via the autophosphorylation of dsRNA-dependent Protein Kinase R (PKR) (11; 147; 211). Subsequently, PKR phosphorylates translation factor eIF2 α , causing protein synthesis to be halted (41). Additionally, cytokines such as Type 1 interferons, IFN- α and β , are produced and pro-apoptotic pathways are activated. A diagram of the alphavirus life cycle is shown in Figure 8.

Figure 8: The alphavirus life cycle

Alphaviruses enter the host cell via receptor mediated endocytosis. The positive sense RNA genome is readily translated to polyproteins that form a replication complex that produces a minus sense RNA of genome. Viral replicase transcribes from the 26S subgenomic promoter from which structural proteins are translated and subsequently processed. Image used with permission from (237).



Alphavirus-based expression vectors

The pathobiology of and the host response to alphavirus-based expression vectors are attractive for vaccine development. These vectors can induce high levels of heterologous gene expression—greater than 20% of total cell protein—which is compartmentalized in the cytoplasm of the infected cell, thereby reducing or eliminating risk of integration in the host genome (209). Many lab strains of alphaviruses are non-pathogenic in humans. Additionally, the lack of pre-existing immunity against some alphaviruses in humans allows for a robust immune response to develop against the heterologous gene instead of only the virus vector (41).

Most alphavirus expression vectors are plasmid constructs that position a prokaryotic promoter such as the SP6 promoter upstream of a DNA copy of the RNA genome (211). After the plasmids are linearized, viral RNA can be transcribed *in vitro* using SP6 polymerase. Subsequently, cells can be efficiently transfected via electroporation with these transcripts, causing infection. When the viral genomic transcript encodes all viral genes, transfection of the virus genome will result in production of replicating virus. In order to avoid the use of potentially pathogenic viruses as *in vivo* vectors, non-propagating replicon expression vectors have been developed based on genomes of a number of alphaviruses. For these systems, viral genome is transfected into particle producing cells in two or three parts, one of which encodes the nSP and the other(s) encode the SP. The replicon genome encoding the nSP also encodes the downstream subgenomic promoter followed by the transgene which is intended to be expressed *in vivo* (41; 62; 211). Thus, virus packaging is accomplished when cotransfection of viral RNA (only nSP genes) and the helper RNA (only SP genes) into a single cell. Rep will produce products from both RNA sources. However, only

viral RNA (only nSP genes) will be packaged into virus particles. Helper RNA, lacking the packaging sequences within the Rep (nSP) gene, will not be incorporated. Thus, while virus particles produced from replicon systems can assemble and express heterologous proteins, they cannot cause a subsequent round of infection.

To prevent wild-type reversion due to recombination between a single helper RNA and viral RNA in some cases, two helper RNAs have been used, or a combination of mutations to attenuate activity have been introduced (211). Replicon-based vaccines developed from several different alphaviruses, including VEE, Sindbis Virus (SIN), and Semliki Forest Virus (SFV), have been tested to be safe and efficacious in many different species of animals and humans (21; 155; 209; 265).

Alphavirus-based vectors as vaccines

Alphavirus-based replicon systems or expression vectors have been applied as vaccines for a variety of viral and non-viral agents, and as adjuvants in conjunction with other vaccines. Alphavirus expression systems targeting over 27 different viruses have been developed and tested in animal models, resulting in the induction of neutralizing antibodies, cytotoxic T-lymphocyte (CTL) activity, and/or protection against challenge (157). The targeted viruses include (among others) Cytomegalovirus (14), Dengue (128), Ebola (208; 285; 286), Hepatitis B (70), Hepatitis C (26; 269), Hendra Virus (60), HIV-1 (20; 33; 41; 62; 82; 96; 153; 213; 279), Human Papillomavirus (49; 50; 114), Herpes Simplex Virus-1 (106; 233), Influenza (10; 19; 72; 163; 236; 270; 303), Marburg Virus (111; 112), Measles (186; 191), Nipah Virus (60), Rabies (230), Respiratory Syncytial Virus (47; 79), and Severe Acute Respiratory Syndrome Corona Virus (SARS-CoV) (240). Additionally, alphavirus-based systems have been developed for non-viral

infectious agents such as *B. anthracis* (255), *B. abortus* (32), *C. botulinum* (153), *M. tuberculosis* (129), *P. falciparum* (6; 259), Prions (136), and Staphylococcal enterotoxin (146). Furthermore, alphavirus replicon particles (containing no heterologous genes) were successfully used as an adjuvant in boosting specific T-cell and cytokine responses when administered with inactivated influenza vaccine in rhesus monkeys (38).

Alphavirus replicon vectors for VEE and Sindbis Virus (SIN) have been demonstrated to target dendritic cells (DC), professional antigen presenting cells that activate both memory and naïve T cells (88; 159). Upon initial subcutaneous infection with VEE (replicon) in mice, the virus spreads to Langerhans cells, DC of the skin (159). As early as 30 minutes later, the virus has spread to a deep cortex subsection of the draining lymph node typically associated with DC and T cells, seeding further infection. In a separate *in vitro* study, immature human DC were preferentially targeted for infection by SIN replicon particles (88). Increased affinity for human DC was shown to be dependent on a single amino acid substitution in the E2 glycoprotein of SIN (88).

Alphaviruses and HIV-1 are among a limited number of organisms that have been found to infect DC *in vivo*. Activation of DC by alphavirus vectors is in contrast to many other viral vaccine vectors, including those derived from adenovirus and vaccinia, which inhibit DC function and development (15; 71).

In most vertebrate cell lines, alphavirus vectors induce apoptosis in the infected cell (104; 152). The expression of the transmembrane regions of either of the E1 or E2 envelope glycoproteins is sufficient to induce apoptosis (119). Apoptosis occurs via both the intrinsic and extrinsic pathways within 24-48 hours of infection; there is no evidence that any alphavirus-encoded proteins possess anti-apoptotic activity (104; 137; 152; 181;

228; 261). For SIN replicons, apoptosis is triggered during cell entry but does not initiate prior to DC activation (118). It is hypothesized that apoptosis of DC cells at the lymph node may enhance presentation of antigen to other antigen presenting cells within the lymph node (4; 86).

Alphavirus-based expression vectors have also been developed to express HIV-1 proteins. In previous separate studies conducted by our laboratory, a VEEV replicon system was engineered to express different mutants of the HIV-1 envelope clone, R2.

The R2 envelope glycoprotein clone is an unusual clone isolated in 1989 from proviral DNA from a long-term non-progressive HIV-infected individual with broadly cross-reactive neutralizing antibodies. An unusual feature of the R2 clone includes a proline to methionine (PM) mutation in the V3 region (at residues 313-314), which confers capacity for CD4-independent infection (299). The R2 clone has been used in immunogenicity experiments to induce neutralizing antibodies in mice, rabbits, and non-human primates (62; 212; 213; 298; 300).

Moderately cross-reactive neutralizing responses were seen among all three animal models. Additionally, some of the immunized monkeys were protected from infection after challenge with the pathogenic simian-human immunodeficiency virus (SHIV) strain DH12R (Clone 7). However, induction of moderately potent neutralizing antibodies required multiple immunizations with VEEV replicon vectors, followed by administration of soluble gp140 in adjuvant. Despite the promise shown by VEEV replicon vectors in studies of immunization against other agents, the breadth and potency of the anti-HIV responses were less than desired.

There have been some efforts to improve immunogenicity of alphavirus replicon particle vaccine vectors. Increased expression of gp120 was achieved by modifying a SFV replicon expression vector to include a translation enhancer element upstream of an internal signal sequence to drive gp120 expression (82). Increased antibody titers were induced in mice when virus particles rather than soluble protein were used for booster immunizations (41).

Human clinical trials of an alphavirus replicon HIV-1 gag vaccine

Despite refinements in alphavirus-based vaccine design, including some that have yielded promising pre-clinical results, two recent human clinical trials of a replicon HIV-1 vaccine (AVX101) induced only low levels of specific cellular and humoral responses (279). The AVX101 vaccine is a VEEV replicon RNA encoding a modified *gag* gene from a subtype C HIV-1 isolate (DU422). Both clinical trials ended prematurely; the first study (HVTN 040) was halted due to vaccine instability, the second (HVTN 059) due to documentation issues at the contracted vaccine manufacturer. The clinical studies were able to demonstrate a dose-dependent antibody response to the VEEV virus vector. Furthermore, repeated immunizations with the vector do not appear to affect the ability to boost immune responses to other antigens.

Potential development of a replication competent alphavirus vaccine vector

Many viral infections result in immunity that lasts for many years or life. Live virus vaccines tend to replicate the immunity induced by natural infection, although not always to the same extent of durability. Non-infectious vaccines are commonly associated with more transient responses than live vaccines. The many *in vivo* expression vectors used in efforts to develop HIV-1 vaccines have either been non-replicating, or

highly attenuated. While VEEV replicon vectors transduce cells to produce very large amounts of protein, the protein expression lasts only for the duration of the single infection cycle. In cell culture, all susceptible cells are killed within a few days of inoculation with VEEV replicon particles. In order to study the possibility that use of a replication competent alphavirus expression vector might induce more potent and durable neutralizing antibody responses against HIV-1, we considered the pathobiology of all alphaviruses with respect to potential for use as a non-pathogenic vaccine vector.

Alphaviruses are insect-borne, and almost all infect and cause disease in more than one species of mammals. In many cases interspecies transmission is related to the insect vector being a mosquito species that bites multiple mammalian species. Western Equine Encephalitis Virus (WEEV) shares these properties.

At some point, virus of the WEEV lineage became established in a bird population in the Rocky Mountains. Fort Morgan Virus (FMV) was first isolated in 1973 from swallow bugs (*Oeciacus vicarius*) infesting the nests of Cliff Swallows (*Petrochelidon pyrrhonota*) and House Sparrows (*Passer domesticus*) in Fort Morgan, Colorado (34). FMV has since been detected in Colorado, South Dakota, Texas, and Washington. Swallow bugs, lacking the significant mobility to travel away from nesting sites, serve as a vector for FMV, transmitting the virus to nestling birds. After nesting is complete, the entire avian colony of a particular nest abruptly abandons the site. The swallow bugs, however, are able to survive overwintering until another group of avian hosts return. Viremia in Cliff Swallows and House Sparrows is estimated to be between 1-4 days (238). In this ecological niche, the virus has evolved such that there are now two distinctive clades of FMV that are clearly distinguishable by phylogeny from WEEV.

FMV is not known to infect humans and is non-pathogenic in laboratory animals (224). Previous experiments conducted by our laboratory have demonstrated susceptibility to wild type FMV infection in the following cell lines: Baby Hamster Kidney fibroblasts (BHK-21), African green monkey kidney epithelial (Vero), Human Embryonic Kidney 293 (HEK 293), mouse embryonic fibroblast (3T3), human cervical epithelial (HeLa), Chinese Hamster Ovary (CHO), human b-cell lymphoma (VC-1), and grivet kidney epithelia (BS-C-1).

In the absence of other lead candidate live virus vectors for use as HIV-1 vaccines, our laboratory pursued development of an FMV live virus vector. A full length, infectious molecular clone of FMV has been produced. Virus from this clone is infectious in, but not pathogenic for mice, and induces homologous neutralizing antibodies as a result of infection (all unpublished data). This molecular clone was used in this thesis project. (34; 224; 238).

SPECIFIC AIMS AND HYPOTHESES

Statement of Purpose

Stimulation of humoral and cell-mediated immunity is likely to be essential to the success of a HIV-1 vaccine. In recent years, tremendous efforts have been devoted to identifying those who develop bcnAbs in natural infection and cloning both antibody and envelope glycoprotein from these individuals to understand underlying mechanisms and relationships. The literature presents conflicting reports regarding the prevalence of bcnAbs among different disease progression groups. Though it is recognized that the envelope glycoprotein plays a role in binding germline naïve B cells that ultimately produce bcnAbs, it is unclear what features of the envelope glycoprotein are responsible for this process. Cell-mediated immunity may be achieved through the application of a replication competent viral vector, one that can mimic an authentic viral infection while expressing the envelope glycoprotein trimer. Two vectors (Ad5 and ALVAC) have been tested in human clinical trials but have yielded limited results.

The purpose of this research is four-fold: to compare the prevalence of bcnAbs among different HIV-1 disease progression groups, to investigate connections between the envelope glycoprotein and induced neutralization in patients with and without bcnAbs, to develop a replication-competent alphavirus-based vector that expresses HIV-1 envelope glycoprotein, and to conduct a pilot study using the vector in rabbits. Elucidating relationships between the envelope glycoprotein and induced neutralization, and the development of a replication-competent potential vaccine vector will advance HIV-1 vaccine design.

Specific Aim 1

To compare cross-reactivity and specificity of neutralizing antibody responses in HIV-1 infected patients who spontaneously control viremia or who fail to progress to immunodeficiency in the absence of therapy to those of patients with typically or rapidly progressive HIV-1 infection.

We hypothesize that long term non-progressors (LTNP) are more likely to have broadly cross-reactive neutralizing antibody (bcnAb) responses than HIV-1 infected patients who are viremic or non-viremic controllers, or who have typically or rapidly progressive HIV-1 infection.

Specific Aim 2

To compare the neutralization phenotypes and early envelope glycoprotein genes in patients with and without bcnAb.

We hypothesize that the neutralization phenotypes of envelope glycoproteins of patients with bcnAb responses differ from those without bcnAb responses. We predict that the specificity and induction of bcnAb responses correlates with the presence of certain envelope genotypes and/or phenotypes found early in infection.

Specific Aim 3

To clone and express green fluorescent protein (GFP) and HIV-1 envelope glycoprotein constructs in Fort Morgan Virus (FMV).

Based on the success of other alphavirus-based vectors that express HIV-1 proteins, we hypothesize that this is feasible.

Specific Aim 4

To evaluate safety and immunogenicity of Fort Morgan/HIV chimeric Virus in an animal model (rabbit).

We hypothesize that systemic infection of FMV and its expression of HIV-1 envelope glycoproteins is tolerated in an animal model, and that HIV-1 neutralizing antibody responses can be elicited by FMV/HIV chimeric virus infection.

CHAPTER 2: Materials and Methods

MATERIALS AND METHODS FOR AIMS 1 & 2

Cohort

All patient serum and plasma samples utilized in this study were taken from patients participating in the U.S. Department of Defense HIV Natural History Study (NHS). All protocols requiring the use of human specimens from the NHS cohort were reviewed and approved by the Infectious Disease Clinical Research Program (IDCRP) HIV Working Group, and the Institutional Review Board (IRB) at the Uniformed Services University of the Health Sciences (Protocol IDCRP-000-32).

The NHS is a prospective, observational, multicenter cohort study of HIV-infected military personnel and eligible beneficiaries that has enrolled over 5,400 patients since 1986. The cohort is ethnically balanced and geographically diverse, reflecting the general distribution of military personnel, and is similar to the general population in the United States. Patients enrolled in the NHS visit a military treatment facility approximately once every six months for follow up care and data collection (including demographic data, medical history, and laboratory measurements) (120; 164). Blood samples are collected and processed for storage of plasma, sera, and peripheral blood mononuclear cells (PBMC) aliquots.

Cohort participants were defined as seroconverters if they had a documented HIV-negative test date prior to the first HIV-positive date. The seroconversion date was estimated as the midpoint between these two dates. Cohort study participants were characterized with respect to nature of disease progression according to the following case definitions (39; 184):

Elite Controller (EC)

Individuals whose plasma HIV RNA levels were below the level of detection for at least 3 longitudinal HIV RNA determinations spanning at least a 12-month interval in the absence of anti-retroviral therapy (ART). Individuals may have had isolated occurrences of viremia up to 1000 copies/mL as long as such occurrences represent the minority of all available determinations.

Long Term Non-Progressors, 10-year (LTNP10)

Individuals (not EC) whose CD4 cell counts were at least 500 cells/ul for at least 10 years in the absence of ART or AIDS-defining events.

Viremic Controllers (VC)

Individuals (not EC or LTNP10) whose plasma HIV RNA levels were below 2000 copies/mL for at least 3 longitudinal HIV RNA determinations spanning at least a 12-month interval in the absence of ART. Individuals may have isolated occurrences of viremia greater than 2000 copies/mL as long as such occurrences represent the minority of all available determinations.

Long Term Non-Progressors, 7-year (LTNP7)

Individuals (not EC, LTNP10, or VC) whose CD4 cell counts were at least 500 cells/ul for at least 7 years in the absence of ART or AIDS-defining events.

Rapid Progressors (RP)

Individuals (not EC, LTNP10, VC, or LTNP7) whose HIV seroconversion window is less than 1 year and either: A) at least 2 CD4 T cell counts below 350 cells/ul within 3 years of seroconversion and no subsequent rise of CD4 T cell counts above 350/ul in the absence of ART, or B) ART-start within 3 years of seroconversion and CD4 T cell count within one month (30 days) of ART-start less than 350 cells/ul.

Typical Progressors (TP)

Individuals (not EC, LTNP10, VC, LTNP7, or RP) with at least 3 years of follow-up from seroconversion, and virus load counts always above 10,000 per mL and above 100,000 per mL on at least 3 occasions.

Hierarchy of disease progression groups

Since the criteria for each disease progression group is based on clinical measurements, it is possible for an individual patient to receive more than one disease progression group classification during follow-up. For example, an EC may develop viremia for a period of time during which he/she fits the criteria for VC. We set a hierarchy of disease progression groups based on the relative control of HIV-1 such that patients who received multiple classifications were assigned a single classification for the purposes of this study. The hierarchy used in the present study is as follows:

EC > LTNP10 > VC > LTNP7 > RP > TP.

Selection of patient samples

Patient samples were selected from the NHS sample repository based on serum or plasma availability and ART-naïve status at the time of sample collection. A total of 157 serum samples (taken 2-4 years post-seroconversion or first documented HIV-positive date) from individuals representing all disease progression groups (EC, LTNP10, VC, LTNP7, RP, and TP) were used in the present study to screen for HIV-1 neutralization activity. Additionally, early plasma samples (taken from the earliest available date within two years of seroconversion or first documented HIV-positive date) for each of the six best and six worst neutralizers were used for subsequent cloning and analysis.

Cell culture

All cell cultures were maintained in humidified incubators at 37°C and 5% CO₂ in air.

TZM-bl cells

The human cervical epithelial adenocarcinoma HeLa cell line TZM-bl (also known as JC53.bl), is engineered to stably express the CD4 and CCR5 co-receptors, and includes Tat-responsive reporter genes firefly luciferase (Luc) and *E. coli* β -galactosidase (280).

TZM-bl cells were maintained in Dulbecco's minimal essential medium (DMEM) (Lonza) supplemented with 10% Fetal Bovine Serum (FBS, Gibco), and L-glutamine, penicillin, streptomycin (Gibco). TZM-bl cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.

HOS-CD4-CCR5 cells

The Human Osteosarcoma HOS-CD4-CCR5 cell line is engineered to express the CD4 and CCR5 co-receptors via puromycin selection.

HOS-CD4-CCR5 cells were maintained in Dulbecco's minimal essential medium (DMEM) (Sigma) supplemented with 10% FBS (Gibco), L-glutamine, penicillin, streptomycin (Gibco), and puromycin (Sigma). HOS-CD4-CCR5 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau.

293T cells

The human embryonic kidney 293T cell line (HEK 293T/17) is a derivative of the HEK293 cell line that expresses the gene for Simian Virus SV40 T antigen. HEK 293T/17 cells are highly transfectable and are used for virus production.

HEK 293T/17 cells were maintained in Dulbecco's minimal essential medium (DMEM) (Lonza) supplemented with 10% FBS (Gibco), L-glutamine, penicillin, and streptomycin (Gibco). HEK 293T/17 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA).

Pseudovirus construction

Pseudotyped viruses were produced by cotransfection of two plasmids—one envelope and one virus backbone—in approximately 50% confluent 293T cells using the calcium phosphate precipitation method (Profection, Promega, Madison, WI) (214).

Six HIV-1 envelope clones representing a range of subtypes and neutralization sensitivities were used in neutralization assays to assess the neutralization breadth and potency of patient serum (239). The panel used in the present study (see Table 3) is similar to another that has been developed for high-throughput screening of serum for neutralization ability from HIV-infected individuals (242; 275). Aside from the envelope clone JRCSF, the clones are different from the panel used in those studies, however the composition of subtypes and neutralization sensitivity (tier) are identical. Heterologous envelope clones across different subtypes (rather than subtype B exclusively) were also chosen to avoid overestimating neutralization breadth and potency of a particular serum.

Table 3: Neutralization panel and envelope clone descriptions

Clone	Subtype	Neutralization Tier [*]	Origin	Reference
SS1196.1	B	1B	USA	(150)
JRCSF	B	2	USA	(135)
ZM197M.PB7	C	1B	Zambia	(151)
DU422.1	C	2	South Africa	(151)
Q23ENV17	A	1B	Kenya	(204)
93TH966.8	CRF01_AE	2	Thailand	(87)

^{*}Tiered sensitivity to neutralization: high (tier 1A), above average (tier 1B), moderate (tier 2), low (tier 3) (239).

Virus backbone plasmids pNL4-3.Luc.E-R- and pSG3ΔENV were used to produce pseudoviruses capable of infecting HOS-CD4-CCR5 or TZM-bl cells, respectively. For cotransfection in a 25cm² tissue culture flask, the following reagents were first combined in a sterile microfuge tube and allowed to precipitate at room temperature for 30 minutes: 2.5ug envelope plasmid, 7.5ug backbone plasmid, H₂O volume to 350ul, 50ul 2M CaCl₂, and 400ul 2X HEPES Buffered Saline (2X HBS). After incubation, the mixture is added to a 25cm² tissue culture flask of 293T cells (~50% confluent, seeded one day prior), and the flask was incubated in a humidified incubator at 37°C and 5% CO₂ in air. The ratio of envelope to backbone plasmid may be adjusted (to 1ug envelope + 10ug backbone, for example) to improve infectivity of produced virus.

For cotransfection in 24-well plates, the following reagents were first combined in wells of a 96-well plate with V-shaped wells: 1ug envelope plasmid, 0.333ug backbone

plasmid, H₂O volume to 22ul, 3ul 2M CaCl₂, and 25ul 2X HBS. After 30 minute room temperature incubation, the mixture is added to a single well of a 24-well plate containing 293T cells (~50% confluent, seeded one day prior), and the plate is incubated in a humidified incubator at 37°C and 5% CO₂ in air.

Approximately 18 hours after cotransfection, medium was replaced with fresh medium supplemented with 0.1mM sodium butyrate (Sigma) and cells were incubated for an additional 24 hours. After incubation, supernatant was harvested, centrifuged to remove cellular debris (10 minutes, 4,000 rpm, 4°C), and filtered through a 45µm (or smaller) pore syringe filter (EMD Millipore, Billerica, MA). Pseudovirus was used immediately, or supplemented with 10% FBS (Gibco) and stored at -80°C.

pNL4-3.Luc.E-R- was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau. pSG3ΔENV was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes and Dr. Xiaoyun Wu.

Pseudovirus neutralization assays

A robotic-assisted 384-well pseudovirus neutralization assay was developed to measure the ability of a reagent (serum, monoclonal antibody, etc.) to reduce infectivity (as reported by luciferase gene expression) of a pseudovirus in TZM-bl or HOS-CD4-CCR5 target cells. The 384-well assay was based on the 96-well assay method previously described by Montefiori et al., and by our laboratory (174; 212; 214). Briefly, 10µl of serum (or mAb, etc.) dilution was incubated with 10µl of pseudovirus for 1 hour at 4°C in 384-well white-walled, clear flat bottom, tissue culture plates (Greiner Bio-One, Monroe, NC). Normal human serum (Lonza, Walkersville, MD) was used as

negative control. After incubation, 10µl of target cell suspension (approximately 2,000-3,000 cells) was added to each well. Diethylaminoethyl-dextran (DEAE dextran, Sigma) or Polybrene (Santa Cruz Biotech) was added to TZM-bl or HOS-CD4-CCR5 target cell suspensions, respectively, to enhance pseudovirus infection. Plates were incubated for three days at 37°C and 5% CO₂ in air. After incubation, 30µl of reconstituted BrightGlo Luciferase Reagent (Promega, Madison, WI) was injected into each well using an automated luminometer (Berthold Centro XS3, Berthold Technologies, Bad Wildbad, Germany), and after at least five minutes, luminescence activity was measured for each well. Samples were tested in duplicate, and the 50% inhibitory concentration (IC₅₀) or inverse dilution (ID₅₀) were calculated as 50% reduction of relative light units (RLU), by method of Reed-Meunch or by Graphpad Prism regression analysis software (216). Each sample was tested in two independent experiments and geometric mean titers (GMT) were calculated.

Ranking of neutralization breadth and potency

The neutralization ability of patient sera was ranked by geometric mean titer (GMT) for each virus. The sum of the rankings for each individual patient serum was used to establish a final rank among patient sera. This generally corresponds with the breadth and, to some degree the potency, of neutralization.

Statistical analysis

Final rank results were analyzed by Kruskal Wallis test (Graphpad Prism) to determine whether statistically significant differences ($p \leq 0.05$) existed among groups. The final ranking was also stratified quartile groups for chi-square (χ^2) analysis

(Graphpad Prism) to test whether a significant difference ($p \leq 0.05$) existed between observed and expected results within a particular group of a quartile.

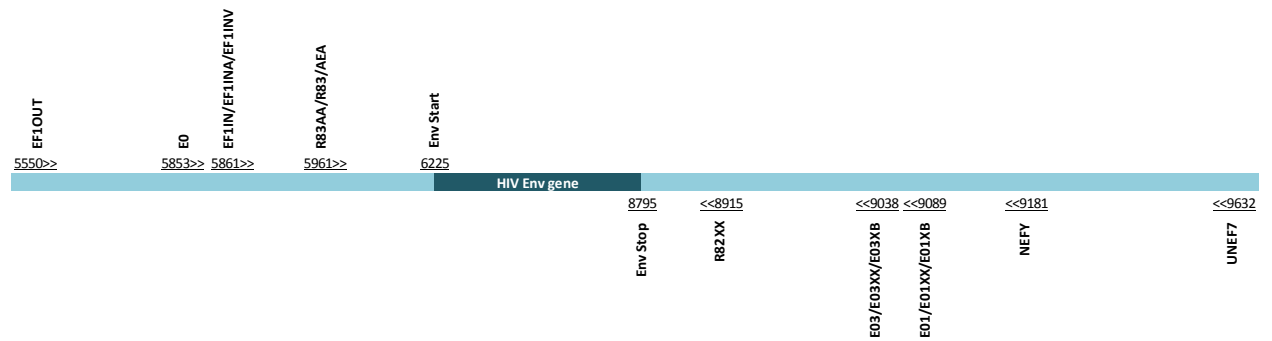
Viral RNA extraction and cDNA synthesis

Plasma samples taken from the earliest available date within two years of seroconversion (or documented HIV-positive date) were obtained for the top six and bottom six neutralizers. Viral RNA was extracted by spin column kit (QIAamp Viral RNA Kit, Qiagen, Germantown, MD), or by magnetic bead kit (MagMAX Viral RNA Isolation Kit, Ambion, Austin, TX) according to each manufacturer's protocol. cDNA was synthesized from RNA using the SuperScriptIII First Strand Synthesis System (Life Technologies, Grand Island, NY), using HIV-1 specific primers targeting anti-sense regions 3' of the envelope gene stop codon. Primers used for cDNA synthesis are shown in Table 4. Binding positions of these primers is shown in Figure 9.

Table 4: Primer sequences used for cDNA synthesis

Name	Primer Sequence
UNEF7	5' - GCACTCAAGGCAAGCTTTATTGAGGCTT
NEFY	5' - GTGTGTAGTTCTGCCAATCAGGGAA
E01	5' - TCCAGTCCCCCCTTTTCTTTTAAAAA

Figure 9: cDNA & Nested PCR Primer map (HXB2 Coordinates)



The thermocycler program for cDNA synthesis is as follows:

Incubate 65°C for 5 minutes (RNA, primer, dNTP, H₂O)
Place on ice for 1 minute
Add concentrated buffer, MgCl₂, DTT, RNaseOut, and SSIII enzyme
Incubate 50°C for 1 hour
Incubate 55°C for 1 hour
Incubate 70°C for 15 minutes
4°C hold
After addition of RNase H
Incubate 37°C for 20 minutes
Incubate 70°C for 15 minutes
4°C hold

Nested PCR and cloning of envelope genes

Nested PCR was performed using PlatinumTaq DNA Polymerase (Life Technologies, Grand Island, NY) using primers (developed by our laboratory and others) to amplify the envelope gene (179; 225; 227). Inner primers contained the following restriction endonuclease sites for cloning: *AflIII*, *EcoRI*, *EcoRV*, *XhoI*, and *XbaI*. Primers used for nested PCR are shown in Table 5.

Table 5: Primer sequences used for nested PCR of envelope genes

	Name	Primer Sequence
Outer 5' of Env gene	EF1OUT	5'- AGAGGATAGATGGAACAAGCCCCAG
	EF1IN	5'- TGGAAGCATCCAGGAAGTCAGCCT
	E0	5'- TAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTA
Outer 3' of Env gene	NEFY	5'- GTGTGTAGTTCTGCCAATCAGGGAA
	E01	5'- TCCAGTCCCCCCTTTTCTTTTAAAAA
	E03	5'- TAAGTCATTGGTCTTAAAGGTACCTG
Inner 5' of Env gene	EF1INA	5'- ATACGCCTTAAGGAAGCATCCAGGAAGTCAGCCT <i>AflIII</i>
	EF1INV	5'- ATACGCGATATCGAAGCATCCAGGAAGTCAGCC <i>EcoRV</i>
	R83AA	5'- ATACGCCTTAAGGCCTATGGCAGGAAGAAGCGG <i>AflIII</i>
	R83AEA	5'- ATATAGCCTTAAGGAATTCCCCTATGGCAGGAAGAAGCGG <i>AflIII</i> <i>EcoRI</i>
Inner 3' of Env gene	E01XX	5'- ATACGCCTCGAGGTCCCCCCTTTTCTTTTAAAAA <i>XhoI</i>
	E01XB	5'- ATACGCTCTAGAGCCCCCCTTTTCTTTTAAAAA <i>XbaI</i>
	E03XX	5'- ATACGCCTCGAGGTCGGTCTTAAAGGTACCTG <i>XhoI</i>
	E03XB	5'- ATACGCTCTAGAGCTCGGTCTTAAAGGTACCTG <i>XbaI</i>
	R82XX	5'- ATACCGCTCGAGCGGATACTGCTCCCACCCCATCTGC <i>XhoI</i>

The thermocycler program for nested PCR is as follows:

Incubate 94°C for 2 minutes
35 cycles of: 94°C for 15 seconds,
55°C for 30 seconds, and
68°C for 4 minutes
Incubate 68°C for 10 minutes
4°C hold

Positive second-round PCR products were purified in low melting point agarose gel, extracted by spin column kit (QIAQuick Gel Extraction Kit, Qiagen, Germantown, MD), digested by restriction endonuclease (New England Biolabs, NEB, Ipswich, MA), and ligated into an expression vector (pcDNA3.1(+) or pSV7d) using T4 DNA Ligase (NEB) (214). The vectors pcDNA3.1(+) and pSV7d are mammalian expression vectors, 5.4kb and 2.4kb in size, respectively. Chemically competent 5-alpha *E. coli* cells (NEB) were transformed with ligated product and plated on agarose petri dishes containing ampicillin for selection. After 24-36 hours incubation at 30°C, single colonies were picked from petri dishes, and expanded in additional LB bacterial growth media supplemented with ampicillin (Life Technologies, Grand Island, NY). Plasmids were extracted by spin column kit (QIAprep Spin Miniprep Kit, Qiagen, Germantown, MD), and positively-sized plasmids by agarose gel electrophoresis were subject to restriction endonuclease digestion for confirmation of the presence of insert (envelope gp160 gene).

Infectivity assays

Clones verified for the presence of insert (envelope gp160 gene) were tested for function in infectivity assays (214). A preliminary screen using pseudoviruses produced in 24-well plates identified infectious clones that were retested for infectivity after production in 25cm² tissue culture flasks.

A robotic-assisted 384-well infectivity assay was developed to measure the ability of a cloned envelope to cause infection (as reported by luciferase gene expression) in HOS-CD4-CCR5 target cells. The 384-well assay was based on the 96-well assay method previously described by Montefiori et al., and by our laboratory (174; 212; 214). Pseudoviruses were made by cotransfection of plasmids containing cloned envelope plasmid and NL4-3.Luc.E-R- virus backbone in 293T cells. Briefly, 20µl of pseudovirus dilution was incubated with 10µl of HOS-CD4-CCR5 cell suspension (approximately 2,000-3,000 cells) in 384-well white-walled, clear flat bottom, tissue culture plates (Greiner Bio-One, Monroe, NC). Polybrene (Santa Cruz Biotech) was added to HOS-CD4-CCR5 target cell suspensions to enhance pseudovirus infection. Plates were incubated for three days at 37°C and 5% CO₂ in air. After incubation, 30µl of reconstituted BrightGlo Luciferase Reagent (Promega, Madison, WI) was injected into each well using an automated luminometer (Berthold Centro XS3, Berthold Technologies, Bad Wildbad, Germany), and after at least five minutes, luminescence activity was measured for each well.

Major neutralization epitope presentation by functional envelopes

A panel of monoclonal antibodies (mAbs) was used in this study to assess the major neutralization epitope phenotype of functional cloned envelopes. The list of mAbs used in the present study are listed in Table 6.

Table 6: Monoclonal antibody information

mAb	Recognized Epitope	Reference
2F5	gp41 MPER	(207)
4E10	gp41 MPER	(249)
Z13e1	gp41 MPER	(310)
48D	CD4-induced region	(254)
17b	CD4-induced region	(254)
PG9	V1/V2 Glycan (N160)	(274)
PG16	V1/V2 Glycan (N160)	(274)
447-52D	V3 Loop	(100)
2G12	Oligo-mannose	(258)
PGT121	V3 Loop Glycan (N332)	(273)
PGT126	V3 Loop Glycan (N332)	(273)
VRC01	CD4 binding site	(289)
VRC03	CD4 binding site	(289)
NIH45-46 G54W	CD4 binding site	(61)
B12	CD4 binding site	(29)
F105	CD4 binding site	(203)
B6	CD4 binding site	(7)

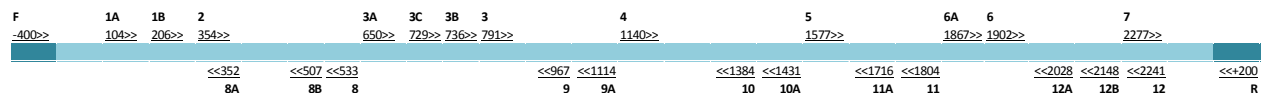
Nucleotide sequencing

Individual envelope gp160 clones were sequenced using a panel of forward and reverse primers developed by our laboratory and others (179; 227). Primers used for nucleotide sequencing are shown in Table 7. Approximate binding positions for these primers are shown in Figure 10. Bigdye 3.1 sequencing reactions were performed in 96-well plates, or individual PCR reaction tubes (Life Technologies, Grand Island, NY), and purified using dye terminator removal plates or cartridges (Edge Biosystems, Gaithersburg, MD). Sequences were obtained using an ABI-3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data was assembled using the Lasergene 8 Sequence Analysis Software Suite (DNASTAR, Madison, WI). Alignments were performed using online tools from the Los Alamos National Laboratory (<http://www.hiv.lanl.gov/content/index>) and the Mullins Laboratory of the University of Washington (<http://indra.mullins.microbiol.washington.edu/>). Dendrograms were created using MegAlign software from Lasergene 8.

Table 7: Primers used for sequencing

	Name	HXB2 Position	Primer Sequence
Forward Primers	1A	104-121	5'- TCACAGTCTATTATGGGG
	2	354-375	5'- TGTAATAATTAACCCCACTC
	3A	650-673	5'- CAATTCCCATACTATTGTGCCC
	3B	736-764	5'- AATGTCAGCACAGTACAATGTACACATGG
	3	791-807	5'- TGCTGTATAATGGCAGT
	4	1149-1166	5'- GGAATTTTCTACTGTAA
	5	1577-1597	5'- CAGCAGGAAGCACTATGG
	6A	1867-1887	5'- ATTTGGAATAACATGACCTGG
	6	1902-1919	5'- AGAAATTGACAATTACAC
	7	2277-2293	5'- CGACCTGCGGAGCCTGT
	F	Vector	5'- ATAATACGACTCACTATAGGG (T7 promoter)
	8A	332-352	5'- ATGGCTTTAGGCTTTGATCCC
Reverse Primers	8B	483-507	5'- GCTTGTGGTGATATTGAAAGAGCAG
	8	512-533	5'- TATCTTTCTGCACCTTATCTC
	9	944-967	5'- TTGTATAAAATGCTCTCCCTGGTC
	10	1364-1384	5'- TGTCTATACCACCATCTCTTG
	11	1785-1804	5'- TTCCAGAGCAACCCCAAAAC
	12A	2000-2028	5'- TATGTCAAACCAATTCCACAAACTTGCCC
	12B	2129-2148	5'- CAATGGTGAGTATCCCTGCC
	12	2222-2241	5'- ACCGGATGTGTCTCTGTCTC
	R	Vector	5'- TAGAAGGCACAGTCGAGG (BGH Poly(A) Signal)

Figure 10: Sequencing Primer Map (HXB2 Coordinates)



Serum neutralization specificity and blocking assays

SF162 mutants

The HIV-1 envelope clone SF162.LS (Subtype B, Tier 1A—high sensitivity to neutralization) contains a lysine residue at position 160 and an asparagine residue at position 332, thus it is naturally resistant to PG9/PG16-like mAbs, which recognize N-linked glycans associated with an asparagine at position 160 (48; 63; 239; 273; 274). Furthermore, it is sensitive to PGT121/PGT126-like mAbs, which recognize N-linked glycans associated with an asparagine at position 332. Two SF162 mutants were kindly provided by L. Stamatatos (Seattle Biomedical Research Institute): SF162_{K160N}, which introduces the PG9/PG16 mAb epitope, and SF162_{N332A}, which abolishes the PGT121/PGT126 mAb epitope. Increased neutralization of SF162_{K160N} compared to SF162 indicates the presence of PG9/PG16-like antibodies in serum. Additionally, decreased neutralization of SF162_{N332A} compared to SF162 indicates the presence of PGT121/PGT126-like antibodies in serum.

These SF162 envelope mutants were used in 384-well neutralization assays described in the previous pages of this chapter. Briefly, 10µl of serum dilution was incubated with 10µl of pseudovirus for 1 hour at 4°C in 384-well white-walled, clear flat bottom, tissue culture plates (Greiner Bio-One, Monroe, NC). Normal human serum (Lonza, Walkersville, MD) was used as negative control. Monoclonal antibodies were used as assay controls. After incubation, 10µl of target cell suspension (approximately 2,000-3,000 cells) was added to each well. Polybrene (Santa Cruz Biotech) was added to HOS-CD4-CCR5 target cell suspensions to enhance pseudovirus infection. Plates were incubated for three days at 37°C and 5% CO₂ in air. After incubation, 30µl of

reconstituted BrightGlo Luciferase Reagent (Promega, Madison, WI) was injected into each well using an automated luminometer (Berthold Centro XS3, Berthold Technologies, Bad Wildbad, Germany), and after at least five minutes, luminescence activity was measured for each well. Samples were tested in duplicate, and inhibitory concentration (IC₅₀) or the inverse dilution (ID₅₀) were calculated as 50% reduction of relative light units (RLU), by Graphpad Prism regression analysis software. Two independent experiments were conducted and geometric mean titers (GMT) were calculated.

Resurfaced stabilized core probes

The resurfaced stabilized core gp120 protein (RSC3) is based on the HXB2 Ds12F123 core and was designed to preserve the CD4 binding site (CD4bs) while altering or masking other immunogenic epitopes (302). Two additional RSC3 mutant probes were obtained: RSC3_{Δ371I}, which removes the isoleucine residue at position 371 and reduces binding of the gp120 CD4bs by b12 and VRC01, and RSC3_{Δ371I/P363N}, which also removes the isoleucine at residue 371 and includes a proline to asparagine mutation at residue 363 that completely abolishes the binding of CD4 to the gp120 protein, and therefore, cannot block viral entry (158).

The RSC3 mutant probes, used as blocking reagents, were preincubated with serum to assess the presence of CD4bs antibodies in a neutralization assay using SF162 pseudovirus. Greater neutralization after preincubation with RSC3_{Δ371I} or RSC3_{Δ371I/P363N} compared to preincubation with RSC3 indicates the presence of CD4bs directed antibodies.

Blocking experiments were conducted in 384-well neutralization assays described in the previous pages of this chapter. Briefly, 10 μ l of serum dilution was incubated with 10 μ l of RSC3 (or mutant) probe for 15 minutes at 37°C (as described by Lynch et al.) in 384-well white-walled, clear flat bottom, tissue culture plates (Greiner Bio-One, Monroe, NC) (158). After incubation, 10 μ l of SF162 pseudovirus was added to assay wells and plates were incubated for 1 hour at 4°C. Normal human serum (Lonza, Walkersville, MD) was used as negative control. Monoclonal antibodies were used as assay controls. After incubation, 10 μ l of target cell suspension (approximately 2,000-3,000 cells) was added to each well. Polybrene (Santa Cruz Biotech) was added to HOS-CD4-CCR5 target cell suspensions to enhance pseudovirus infection. Plates were incubated for three days at 37°C and 5% CO₂ in air. After incubation, 30 μ l of reconstituted BrightGlo Luciferase Reagent (Promega, Madison, WI) was injected into each well using an automated luminometer (Berthold Centro XS3, Berthold Technologies, Bad Wildbad, Germany), and after at least five minutes, luminescence activity was measured for each well. Samples were tested in duplicate, and inhibitory concentration (IC₅₀) or the inverse dilution (ID₅₀) were calculated as 50% reduction of relative light units (RLU), by Graphpad Prism regression analysis software. Two independent experiments were conducted and geometric mean titers (GMT) were calculated.

RSC3, RSC3 Δ 371I, and RSC3 Δ 371I/P363N proteins were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Drs. Zhi-Yong Yang, Peter Kwong, Gary Nabel.

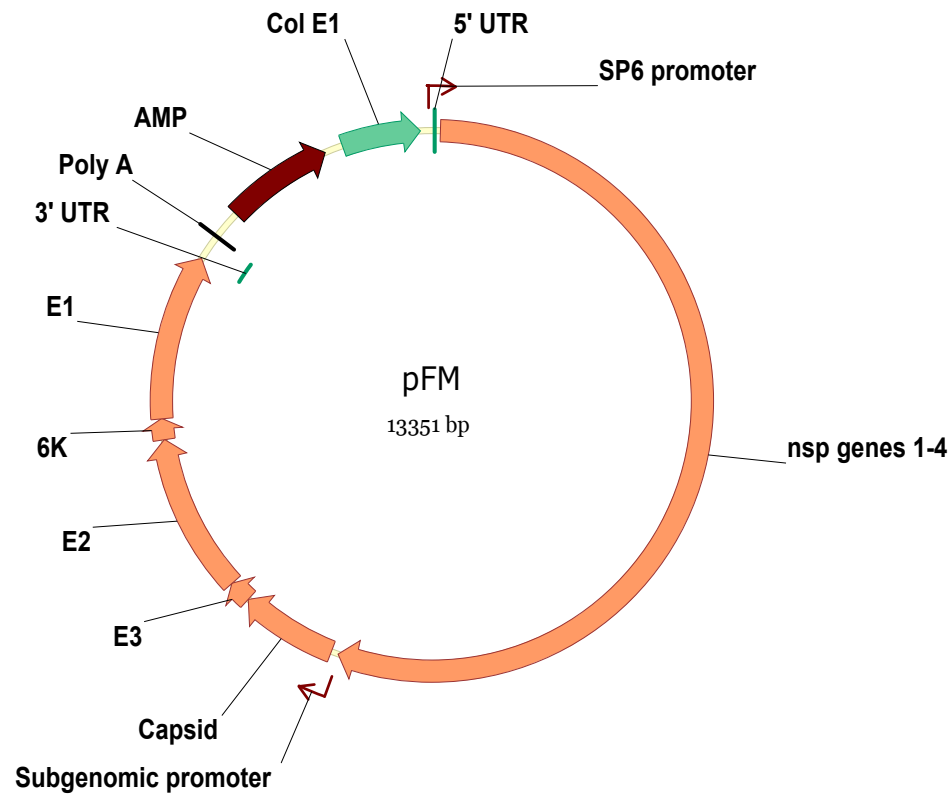
MATERIALS AND METHODS FOR AIMS 3 & 4

Fort Morgan Virus Plasmid pFM

Previous work in our laboratory has resulted in the construction of a full length infectious FMV cDNA clone (strain 77V-654, 11,381 bp) (unpublished data). The cDNA clone is contained in the plasmid pFM, which is 13,351 bp in size and contains the SP6 promoter sequence from the Sindbis Virus replicon vector pSinRep504. A plasmid map (provided by X. Liao) is shown in Figure 11. A *Bam*HI restriction endonuclease site was introduced previously within the 26S promoter sequence of pFM. The infectivity of virus produced from pFM (2.5×10^7 plaque forming units, pfu/ml) is of similar magnitude compared to wild-type FMV (7.5×10^7 pfu/ml) when measured by plaque assay (unpublished data).

Figure 11: Plasmid Map of pFM

Plasmid Map of pFM (provided by X. Liao) depicting the orientation of FMV genes and 26S subgenomic promoter.



Cloning of eGFP and HIV-1 Envelope into pFM

Three forms of the HIV-1 envelope glycoprotein clone, R2, were used to develop potential replication competent expression vectors. The first form, gp160, included a codon optimized full length sequence of the R2 gp160 Env (the part of the sequence encoding the cytoplasmic tail (CT) of gp41 was wild-type). The second form, gp160 Δ CT, was similar to the first, but contained a deletion in the cytoplasmic tail of gp160 which increases cell surface expression of the glycoprotein (12). The third form, gp140, was comprised of a codon optimized gp140 sequence that included a 15 amino acid flexible linker between the gp120 and gp41 sequences, and a sequence encoding a GCN4 trimerization motif (292). Each of these three forms of R2 Env were contained in the vector pcDNA3.1 (Life Technologies, Grand Island, NY) and had been previously developed in our laboratory. A control vector using the enhanced green fluorescent protein (eGFP) gene from pSinRep504 was developed in parallel.

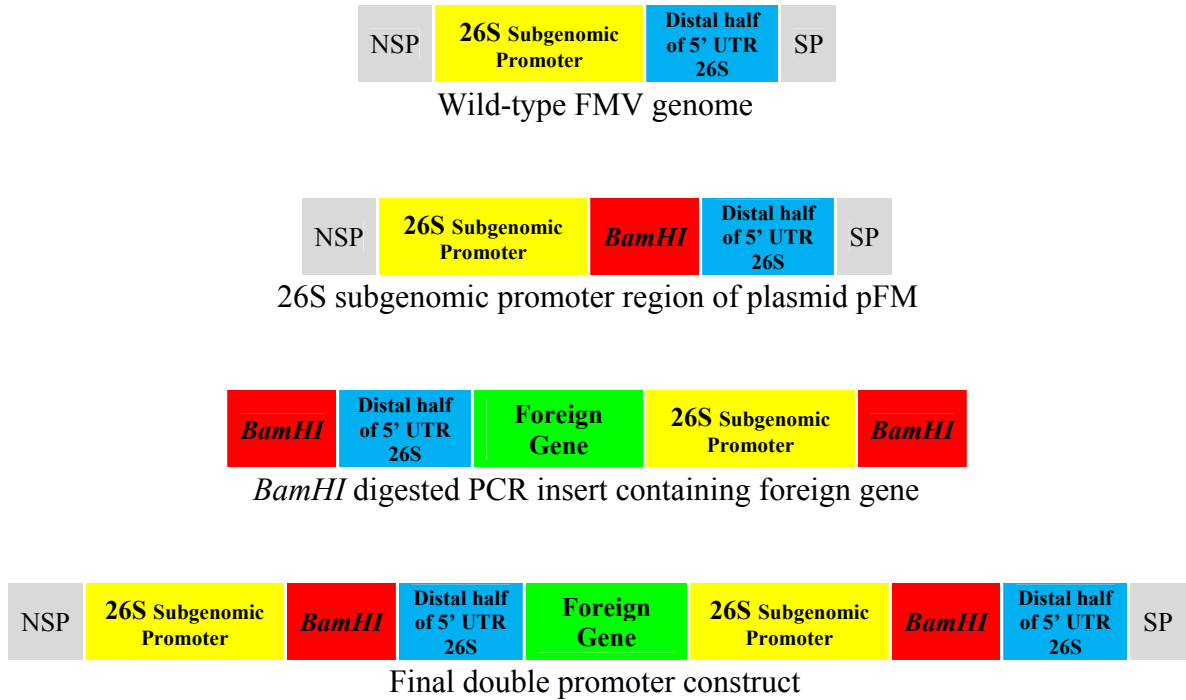
Upstream and downstream *Bam**HI* restriction endonuclease sites were incorporated in primer sequences for PCR amplification of the Env and eGFP genes. These primer sequences are listed in Table 8. An additional FMV 26S promoter sequence was included such that the mutant genome would contain two full length 26S promoters, one upstream of the introduced gene, and one in the wild type position upstream of the capsid coding sequence. A schematic of the cloning strategy is shown in Figure 12.

Table 8: Primer sequences used for PCR amplification of Env and eGFP genes

Name	Primer Sequence
gp160	FM160-1 5'- TAATAT <u>GGATCCT</u> GTGTTACGTTGCCTGCTCTTATGAGGGTGAAGGGG ATTAGAAGAAAC
	FM160-2 5'- TAATAT <u>GGATCCT</u> ATGTTGCGTCACCTATTTAGGTTACCCGTACAAAA TTATTGGTTGTTACAGCAGTGCCCTCTCCAGCCCCTGTCTGATTCTTGT TGGG
gp160ΔCT	FM160CT-1 5'- TAATAT <u>GGATCCT</u> GTGTTACGTTGCCTGCTCTTATGAGGGTGAAGGGG ATTAGAAGAAAC
	FM160CT-2 5'- TAATAT <u>GGATCCT</u> ATGTTGCGTCACCTATTTAGGTTACCCGTACAAAA TTATTGGTTGTTAGACTCGATTCACGATGGACAGGACCACGAACACG ATTCTC
gp140	FM140-1 5'- TAATAT <u>GGATCCT</u> GTGTTACGTTGCCTGCTCTTATGCGCGTGAAGGGG ATTCGCCGCAAC
	FM140-2 5'- TAATAT <u>GGATCCT</u> ATGTTGCGTCACCTATTTAGGTTACCCGTACAAAA TTATTGGTTGTTAAAGCTTCCTGCCCTCGATGCCGCCAGGGGCCTCGC CGATC
eGFP	FMGFP-1 5'- TAATAT <u>GGATCCT</u> GTGTTACGTTGCCTGCTCTTATGGTGAGCAAGGGC GAGGAGCTGTTC
	FMGFP-2 5'- TAATAT <u>GGATCCT</u> ATGTTGCGTCACCTATTTAGGTTACCCGTACAAAA TTATTGGTTGTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGG CGGCG

* *Bam*HI Restriction endonuclease site is underlined

Figure 12: Fort Morgan Virus Cloning strategy



The resulting amplicons were digested with *Bam*HI and ligated into pFM using T4 DNA Ligase (NEB). Antarctic Phosphatase (NEB) was used to prevent self-ligation of the digested pFM vector. The presence and orientation of the insert was confirmed by *Bam*HI digestion and nucleotide sequencing of the vector-insert junction.

Production of infectious virus

RNA transcripts were produced using the mMessage SP6 RNA transcription kit (Ambion, Austin, TX) from *Not*I linearized plasmids containing pFM, the R2 Env

mutants, and eGFP. RNA was transfected into Baby Hamster Kidney fibroblast (BHK-21, obtained from ATCC, Manassas, VA) cells by electroporation (280V, 30ms, GenepulserXL, Bio-Rad, Hercules, CA), after which cells were transferred to a 75cm² tissue culture flask containing DMEM medium, and incubated at 37°C, 5% CO₂. BHK-21 cells were maintained in DMEM (Sigma) supplemented with 10% FBS (Gibco), L-glutamine, penicillin, streptomycin (Gibco). Supernatant was collected 4-5 days post transfection and was used to infect a new flask of BHK-21 cells. When this new flask displayed moderate cytopathic effect (CPE), supernatant was harvested, supplemented with 10% fetal bovine serum (FBS), and stored at -80°C. Pelleted cell debris was also saved for Western blot analysis.

Assessment of transgene expression

Florescent microscopy

Cells infected with the FMV-eGFP mutant were visualized under florescent microscopy (Nikon Eclipse TS100 inverted microscope with Nikon Intensilight C-HGFI mercury fiber illuminator and Nikon B-2E/C florescence filter set, Nikon Instruments Inc., Melville, NY). An estimate of the percentage of GFP⁺ cells was recorded.

SDS-PAGE/Western blot

Proteins in supernatant and cell pellets from the R2 Env mutants of FMV were separated by SDS-PAGE electrophoresis and probed using a polyclonal rabbit anti-gp140 antiserum (R2143) in Western blot analysis for the presence of HIV-1 envelope glycoprotein expression.

When maximal CPE was observed in transfected/infected flasks, supernatant was harvested, and cell layers were then washed twice with PBS. Cell layers in flasks were

then scraped (using sterile cell scrapers) and harvested in 500 μ l PBS. For SDS-PAGE, samples were treated with cell lysis buffer (0.5% Triton X-100, 50mM Tris pH 7.4, 10mM Iodoacetamide, 10 μ g/ml Leupeptin) and separated in 8-12% polyacrylamide gel (30% Acrylamide, 0.75M Tris-HCl pH 8.8 or 0.25M Tris-HCl pH 6.8, 10% SDS, TEMED, 10% Ammonium Persulfate, H₂O) at 90-125V for approximately 1.5 hours. Samples were then transferred to nitrocellulose membranes at 30-100V for approximately 2 hours or overnight. Membranes were then blocked with Blotto (TBS/5% milk) and washed three times with TBS/T (TBS/2% Tween). The primary antibody used was polyclonal rabbit anti-gp140 antiserum (R2143). The secondary antibody used was Alkaline Phosphatase labeled goat-anti-rabbit IgG (Bio-Rad, Hercules, CA). Colorimetric development was performed using AP Color Development Reagent (Bio-Rad, Hercules, CA). Kodak BioMax XAR film were exposed to chemiluminescent membranes and developed using an automatic film processor.

Rabbit immunization preliminary study

All animal protocols used in these experiments were reviewed and approved by the Animal Care and Use Committee at Uniformed Services University of the Health Sciences (Protocol PMB09725). Eight female New Zealand White rabbits were immunized with FMV-gp160 Δ CT.269 (n=3), FMV-gp140.18 (n=3), or FMV-WT (n=2). Each immunized animal received two subcutaneous injections of 0.5ml each, at two injection sites (neck scruff, and lower hind leg). One rabbit was not immunized. A pre-immunization sample of whole blood (approximately 1ml each) was taken from the ear vein from eight (of nine) animals. On days 8-9 post-immunization, all animals were sedated (ketamine), exsanguinated by cardiac puncture, collecting 15ml of whole blood,

and animals were euthanized using xylazine. Dissection of spleen, liver and brain tissue was performed. Blood was processed aseptically and serum was frozen at -20°C.

Recovery of virus from dissected tissue

Dissected sections (measuring approximately 1 cm³) of spleen, liver, and brain tissue, were washed in sterile PBS before mechanical homogenization with ceramic spheres in 2 ml tubes (Lysing Matrix D, MP Biomedicals, Santa Ana, CA). Supernatant collected from centrifuged tissue homogenate was filtered using 0.45µm syringe filters, and subsequently applied to BHK-21 cells in 24-well tissue culture plates. Plates were incubated in a humidified incubator at 37°C and 5% CO₂ in air. Wells were visualized daily for the presence of CPE.

Plaque purification for transgene stability

R2 Env virus clones that yielded positive Western blot results were purified via repeated serial plaquing and Western blot testing. Plaque assays were performed in 6-well plates containing a monolayer of confluent BHK-21 cells (seeded one day prior). Plate wells were inoculated with virus dilution and incubated in a humidified incubator for 1 hour at 37°C, 5% CO₂ in air. After incubation, supernatant from wells was aspirated and replaced with supplemented agarose overlay. Agarose plugs above microscopically visualized plaques were picked (using sterile Pasteur pipets) from agarose overlays. Plaques from three rounds of repeated plaquing that maintained functional gene inserts, as demonstrated by positive ELISA or Western blot results in all progeny clones tested, were considered to primarily contain stable mutant viruses that express the R2 Env. The eGFP virus clone was similarly subjected to repeated plaque purification and confirmatory fluorescence microscopy.

Rabbit serum analysis

Serum was tested by Enzyme Linked Immunosorbent Assay (ELISA) for the presence of HIV-1 gp140-specific polyclonal antibodies. Diluted serum was tested in duplicate with four serial 5-fold dilutions starting at 1:100. ELISA plates were coated with gp140 antigen and stored at 4°C overnight. Plates were then washed six times with wash buffer and blocked with blocking buffer at room temperature for one hour. After blocking, plates were washed (six times), serum dilutions were added to each well, and plates were incubated at room temperature for one hour. After incubation, plates were washed (six times), Horseradish Peroxidase (HRP) labeled goat-anti-rabbit IgG was added to each well, and plates were incubated at room temperature for one hour. After incubation, plates were washed (six times), TMB substrate (KPL, Gaithersburg, MD) was added to each well, and plates were incubated at room temperature for 30 minutes. Colorimetric development was stopped using 2M H₂SO₄ and plates were read on a microplate reader (Bio-Rad Model 680, Hercules, CA) for absorbance at 450nm.

CHAPTER 3: Results and Discussion

RESULTS AND DISCUSSION FOR AIMS 1 &2

Evaluation of broadly cross-reactive neutralization and viral envelope glycoprotein phenotypes

The first phase of this project was to conduct a neutralization screen of individuals from different disease progression groups in the US Department of Defense HIV Natural History Study (NHS). After analyzing available specimens from the NHS sample repository database, we identified 97 patients to screen for broadly cross-reactive neutralizing antibodies (bcnAbs). These patients were selected based on sample availability at a date 2-4 years post seroconversion date or first documented positive, and prior to initiation of anti-retroviral therapy (ART). The number of individuals from each disease progression group is shown in Table 9.

Table 9: Patients for initial neutralization screening

Disease Progression Group	Number of Patients Identified (1st set, n=97)
Elite Controller (EC)	12
Long Term Non-Progressor, 10 years (LTNP10)	14
Viremic Controller (VC)	20
Long Term Non-Progressor, 7 years (LTNP7)	18
Rapid Progressor (RP)	13
Typical Progressor (TP)	20

We developed a 384-well pseudovirus neutralization assay based on the 96-well assay method previously described by Montefiori et al., and by our laboratory (174; 212; 214). A liquid handling pipetting robot (Precision XS, BioTek, Winooski, VT) was used to perform repetitive pipetting in 384-well plates. Initial assay development focused on the optimization of reagents for an assay length of 2-3 days, namely target cell concentration (for TZM-bl and HOS-CD4-CCR5) and volume, mAb/serum volume, DEAE/polybrene additive concentration, pseudovirus volume, and substrate type and volume. Once assay conditions were optimized, a series of 20 replicate assays were performed on multiple days to test for assay variability. Finally, neutralization assays in both 384- and 96-well plates were performed in parallel to correlate results. Overall, inter-assay NEUT50 titer variability in the 384-well assay was found to be approximately 2- to 10-fold, and NEUT50 titers were approximately 10-fold less in assays performed in 384-well plates than corresponding assays performed in 96-well plates.

To minimize neutralization assay variability caused by inconsistent preparations of pseudovirus, we assessed the feasibility of using frozen stocks of pseudovirus. A total of 23 envelope clones from standard neutralization panels were evaluated for stability after one freeze-thaw cycle. Eight of the envelope clones showed less than 10-fold drop in titer and were subject to further testing after several weeks of cold storage (-80°C). Six of these envelope clones were selected for our final virus neutralization panel, listed in Table 3 (in the previous chapter).

Serum samples from the 97 patients were tested against our six virus panel in 384-well TZM-bl neutralization assays. Preliminary results clearly distinguished sera that possessed bnAbs from sera that did not. However, results in midrange of neutralizers

were ambiguous, requiring a more sensitive neutralization assay. We selected the four viruses that were the most difficult to neutralize in the TZM-bl assay (ZM197M.PB7, DU422.1, Q23ENV17, and 93TH966.8) to use in the HOS-CD4-CCR5 assay (see Table 10 below). As others have reported, the HOS-CD4-CCR5 assay provides higher neutralization titers compared to the TZM-bl assay, possibly due the kinetics of viral entry with respect to the ratio of CD4:CCR5 on the target cell surface (90). On TZM-bl cells, this ratio is approximately 2, while the ratio is about 20 on stimulated peripheral blood mononuclear cells (PBMC) (202). Antibody-mediated neutralization has been shown to be more efficient on cells with lower surface concentrations of CCR5 (52).

Table 10: Pseudovirus clones and target cells used in neutralization assays

Clone	Subtype	Neutralization Tier [*]	Origin	TZM-bl Assay	HOS- CD4 ⁺ - CCR5 ⁺ Assay
SS1196.1	B	1B	USA	✓	
JRCSE	B	2	USA	✓	
ZM197M.PB7	C	1B	Zambia	✓	✓
DU422.1	C	2	South Africa	✓	✓
Q23ENV17	A	1B	Kenya	✓	✓
93TH966.8	CRF01_AE	2	Thailand	✓	✓

* Tiered sensitivity to neutralization: high (tier 1A), above average (tier 1B), moderate (tier 2), low (tier 3).

After the neutralization screen was completed for the 97 patients, geometric mean (GMT) NEUT50 titers were calculated for each serum/virus combination. The sum of the GMT NEUT50 titers for each serum was calculated and sorted by rank from highest to lowest. From this ranking, the top six neutralizers with available early plasma samples (earliest date within two years post seroconversion or documented positive date) were identified. However, as the top six neutralizers represented the top 27.8 percentile of all neutralizers, we were hesitant to proceed.

We identified and tested an additional 60 patient samples in the same selection and neutralization procedure performed for the initial 97 patient samples, with the addition of one more criterion: availability of early plasma. The cumulative number of individuals identified from each disease progression group is shown in Table 11.

Table 11: Additional patients for neutralization screening

Disease Progression Group	Number of Patients Screened (1st set, n=97)	Number of Patients Screened (2nd set, n=60)	Total Number of Patients Screened (1st and 2nd sets, n=157)
Elite Controller (EC)	12	4	16
Long Term Non-Progressor, 10 years (LTNP10)	14	0	14
Viremic Controller (VC)	20	48	68
Long Term Non-Progressor, 7 years (LTNP7)	18	1	19
Rapid Progressor (RP)	13	0	13
Typical Progressor (TP)	20	7	27

Results from the second set of serum neutralization assays were combined with data from the first set to complete neutralization ranking (see Table 12). To determine the percentage of individuals with bcnAbs, we identified the lowest ranked individual whose serum neutralized at least four viruses at a IC50 titer ≥ 100 . This individual was VC63, ranked 44 of 157. Since our ranking is based on cumulative neutralization titers across all viruses tested, we estimate that 44 of the 157 patients (28%) in our study possess bcnAbs.

Table 12: Ranking of serum based on neutralization ability

Geometric mean titer (GMT) of NEUT50 values. Results from two independent experiments. Final rank assigned by cumulative GMT for each sample, highest to lowest.

Table shading NEUT50 values: <20, red; 20-999, yellow; >1000, green.

		Target Cell	TZM	TZM	TZM	TZM	TZM	TZM	HOS	HOS	HOS	HOS	
		Envelope Clone	SS1196	JRC5F	ZM197M.PB7	DU422.1	Q23ENV17	93TH966.8	DU422.1	ZM197M.PB7	Q23ENV17	93TH966.8	
		Subtype	B	B	C	C	A	AE	C	C	A	AE	
		Neut. Tier	1B	2	1B	2	1B	2	2	1B	1B	2	Final Rank
Set	Sample	GMT	GMT	GMT	GMT	GMT	GMT	GMT	GMT	GMT	GMT	GMT	
1	LTNP7-3	180	1073	116	28	57	17	586	856	270	618		1
2	VC39	28	256	171	87	1145	70	481	497	1260	127		2
1	LTNP10-11	81	686	21	22	135	10	370	630	710	156		3
2	LTNP7-19	840	475	10	74	876	10	531	148	1534	168		3
2	VC22	41	54	119	28	25	15	575	391	277	121		5
2	EC14	31	53	57	24	10	27	797	367	812	139		6
2	VC33	35	42	41	23	10	45	140	685	335	674		7
2	VC30	505	280	15	10	307	20	118	476	567	33		8
2	VC32	236	73	46	10	10	10	461	744	106	126		9
1	LTNP7-13	56	105	10	15	10	15	541	415	140	501		10
2	VC53	395	350	32	10	10	30	108	336	116	135		11
1	EC1	210	2560	345	30	15	10	37	331	75	246		12
1	LTNP7-10	171	2560	20	41	17	28	247	98	244	50		12
2	VC43	192	373	32	41	10	151	957	183	17	67		12
1	TP10	137	1122	10	28	42	10	300	593	191	23		15
1	TP6	15	289	15	17	28	10	380	250	597	716		16
2	TP23	214	107	59	10	10	50	360	77	204	135		17
2	VC49	529	2560	14	19	199	17	269	10	493	43		18
2	VC31	163	31	28	23	10	61	111	114	562	54		19
2	VC58	148	92	15	10	25	17	233	69	598	57		19
2	VC60	84	47	10	10	10	10	532	112	490	171		21
2	TP27	107	22	10	10	10	118	211	706	10	605		22

1	RP2	32	66	36	26	21	40	49	196	58	189	23
1	VC15	41	569	10	10	30	10	169	135	17	788	23
2	VC38	244	29	10	10	10	59	81	168	64	385	23
2	VC29	136	59	29	15	302	10	594	10	134	59	26
1	LTNP7-4	102	27	10	10	10	10	495	454	25	80	27
2	VC35	99	117	254	19	29	10	106	25	180	45	27
2	VC46	439	84	35	10	10	66	56	86	200	29	29
1	TP2	27	23	24	10	16	10	298	264	763	28	30
2	VC54	114	116	33	10	10	84	119	82	66	22	31
1	VC4	42	65	15	10	10	10	296	142	132	60	32
1	LTNP7-15	18	502	10	14	24	10	61	267	92	215	33
1	TP14	10	218	10	10	37	10	255	452	21	198	34
1	LTNP7-2	90	424	21	10	10	10	170	51	123	48	35
2	VC48	103	32	52	10	10	138	1048	10	83	26	36
1	TP12	41	32	10	10	10	10	386	58	248	150	37
2	VC45	550	127	41	10	10	79	10	82	179	40	38
2	VC25	50	20	82	10	15	22	181	191	66	20	39
2	VC26	156	15	10	10	31	41	410	15	473	33	40
2	TP26	72	45	40	10	10	15	45	853	22	48	41
2	TP25	225	72	26	14	10	80	18	78	59	116	42
2	VC50	54	53	10	10	10	10	32	190	738	69	43
2	VC63	23	376	10	34	263	10	115	19	771	21	44
2	EC16	32	21	33	10	10	10	455	69	88	70	45
1	TP3	18	86	14	10	10	10	99	132	50	548	46
1	TP13	10	10	10	10	10	10	558	95	825	145	47
1	LTNP10-14	100	228	57	21	10	15	28	145	35	25	47
1	RP11	64	288	10	10	10	10	117	343	50	14	49
1	LTNP10-10	29	29	10	10	10	10	58	367	39	423	50
2	VC36	69	93	10	10	33	10	1299	15	68	20	51
2	VC66	84	295	133	10	10	68	19	26	10	226	52
1	VC16	15	110	10	16	42	10	67	177	454	20	53
2	VC67	28	28	46	10	10	10	46	277	10	550	53
1	TP5	16	98	14	10	14	10	115	63	60	301	55
1	TP18	50	45	24	10	10	10	215	131	10	31	56
2	VC23	60	18	58	10	43	14	37	46	27	330	57
1	VC1	10	10	16	10	10	25	128	128	50	402	58
2	VC41	60	54	19	10	10	61	84	10	376	20	58
1	LTNP7-14	18	16	10	10	10	10	194	126	170	51	60
1	TP11	10	10	10	10	10	10	173	309	566	29	61

1	VC17	36	50	10	10	10	10	18	243	168	71	61
1	VC12	17	14	10	10	10	10	157	150	507	30	63
1	LTNP7-5	19	1144	10	15	26	10	34	90	529	17	64
1	TP1	137	152	15	10	10	10	64	55	325	10	65
2	VC42	27	15	10	10	10	31	105	81	21	692	66
2	VC51	41	15	10	10	15	634	161	226	10	20	67
2	VC57	51	36	10	10	10	10	94	10	117	147	68
2	VC59	24	23	10	10	10	15	38	653	158	20	69
1	VC7	15	21	14	10	10	10	202	336	15	71	70
1	TP20	17	58	10	10	10	10	65	387	27	42	70
2	VC44	54	35	27	10	10	10	233	19	63	20	72
1	LTNP7-12	16	451	10	10	10	10	51	142	10	182	73
1	TP15	10	10	10	10	10	10	133	315	51	92	74
1	LTNP7-17	18	15	10	10	10	10	112	153	183	30	74
2	VC37	21	10	10	10	10	10	149	192	77	29	76
2	VC62	43	10	10	10	10	35	94	10	104	172	77
2	VC56	28	31	75	16	25	10	22	111	57	31	78
1	RP9	17	14	24	27	10	15	69	127	112	22	79
1	VC19	20	32	10	10	10	10	91	94	10	347	79
1	LTNP7-16	29	160	10	10	19	10	67	53	165	16	81
1	LTNP10-7	17	19	10	10	10	10	64	842	10	90	82
2	TP21	66	15	10	10	10	15	133	23	119	27	83
2	VC52	32	25	10	10	10	10	36	82	545	26	84
1	VC6	14	10	14	10	10	10	279	268	10	57	85
1	LTNP7-11	14	125	10	10	10	10	99	78	18	93	86
1	LTNP10-1	30	27	10	10	16	10	133	257	10	15	87
2	VC55	123	90	15	14	10	10	71	17	36	21	88
1	VC3	10	10	16	10	10	10	101	306	116	17	89
2	TP24	38	10	10	10	10	14	97	28	363	21	90
2	VC65	24	10	22	10	10	55	25	66	426	20	91
1	RP7	10	10	15	10	10	10	198	29	62	114	92
1	LTNP10-13	203	535	10	10	14	10	17	44	50	16	92
1	VC18	52	165	10	10	10	10	19	269	16	10	94
2	VC28	163	22	10	10	10	15	51	23	21	64	94
1	LTNP7-6	15	10	10	10	10	10	16	608	32	129	96
1	LTNP10-3	16	61	10	10	14	10	45	24	29	195	97
2	VC64	25	14	10	10	10	10	63	15	60	521	97
1	TP16	10	10	10	10	10	10	23	1194	54	63	99
1	TP17	10	10	10	10	10	10	68	107	128	32	100

1	VC13	19	10	10	10	10	10	347	136	14	17	101
1	LTNP10-8	52	1726	10	10	16	10	18	14	74	17	102
2	VC34	10	10	10	10	10	10	24	101	65	261	102
1	LTNP10-6	10	23	10	10	10	10	113	399	10	16	104
1	RP13	16	121	23	19	18	17	10	156	10	14	105
2	VC21	10	10	10	10	10	10	337	23	22	115	106
2	VC47	17	10	10	10	10	10	33	63	62	86	107
1	TP19	15	19	22	17	10	24	33	38	17	74	108
2	VC40	31	10	10	10	10	10	61	59	10	86	108
1	VC14	10	10	10	10	10	10	116	502	10	10	110
2	TP22	38	15	10	10	10	14	54	10	23	87	110
1	TP9	40	30	92	25	10	14	18	61	10	10	112
2	VC24	31	23	16	10	10	57	65	10	17	20	112
1	VC20	10	10	10	10	10	10	93	258	27	10	114
1	LTNP10-5	16	25	10	10	10	10	78	77	30	15	115
1	RP5	16	25	10	10	15	10	46	143	26	10	115
1	RP6	10	10	10	10	10	10	25	192	10	82	117
1	RP10	15	10	10	10	10	10	115	24	20	46	118
2	VC61	10	10	10	10	10	57	25	42	56	50	118
1	EC4	22	20	10	10	10	10	80	87	10	10	120
2	VC27	17	10	10	10	10	10	33	57	15	89	120
1	TP7	10	16	10	10	10	10	47	206	23	10	122
2	EC13	10	10	10	10	10	10	34	63	10	153	122
1	VC8	14	10	10	10	10	10	101	126	14	10	124
1	RP1	10	19	10	10	10	10	26	47	19	77	125
1	EC7	15	10	10	10	10	10	114	24	57	10	126
1	RP12	10	10	10	10	10	10	17	33	30	158	127
1	TP8	10	10	10	10	10	10	10	117	176	10	128
1	LTNP10-2	10	10	10	10	10	10	81	119	10	15	129
1	LTNP7-18	10	10	10	10	17	10	32	98	23	18	130
1	EC10	10	10	10	10	10	10	28	243	14	15	131
1	RP3	10	14	10	10	10	10	130	34	15	15	132
1	RP4	14	10	10	10	10	10	29	45	24	31	133
1	EC9	14	10	10	10	10	10	88	48	10	19	133
2	EC15	10	10	10	10	10	10	43	78	10	22	135
1	EC2	10	10	10	10	10	10	21	237	10	10	136
1	VC2	10	10	10	10	10	10	16	253	10	10	137
1	VC11	10	10	10	10	10	10	18	59	10	40	138
1	LTNP10-9	10	23	10	10	10	10	10	110	10	10	139

2	VC68	29	10	10	10	10	20	10	10	15	20	140
1	EC12	10	10	10	10	10	10	117	10	10	10	141
1	TP4	10	10	10	10	10	10	51	34	16	14	142
1	EC8	10	10	10	10	10	10	21	92	10	10	143
1	RP8	10	10	10	10	10	10	15	53	10	23	144
1	LTNP7-1	14	10	10	10	10	10	65	10	10	10	145
1	LTNP7-8	15	16	10	10	10	10	32	25	10	10	145
1	LTNP7-9	10	10	10	10	10	14	63	15	10	10	145
1	EC11	10	10	10	10	10	10	10	85	10	10	148
1	VC9	10	10	15	10	10	10	40	18	10	10	149
1	LTNP10-12	10	10	10	29	10	10	10	30	10	15	150
1	VC5	10	10	10	10	10	10	50	16	10	10	151
1	LTNP10-4	10	10	10	10	10	10	23	46	10	10	152
1	VC10	10	10	10	10	10	10	23	31	10	10	153
1	EC5	10	10	10	10	10	10	10	32	10	10	154
1	LTNP7-7	10	10	10	10	10	10	15	10	10	10	155
1	EC3	10	10	10	10	10	10	10	10	10	10	156
1	EC6	10	10	10	10	10	10	10	10	10	10	156

Table shading NEUT50 values: <20, red; 20-999, yellow; >1000, green

Figure 13: Neutralization ranking by disease progression group

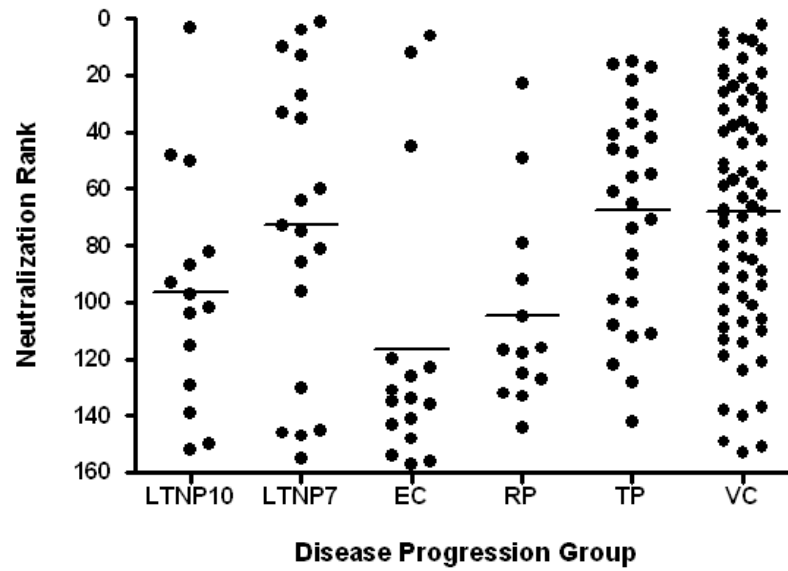
Each dot represents the neutralization ranking of serum from an individual belonging to a disease progression group. The vertical axis is displayed as rank order from highest (best neutralizer) to lowest (worst neutralizer). Horizontal lines indicate the median of each group. No significant difference (at $p \leq 0.05$) was observed when comparing the median ranks of LTNP10 and LTNP7 ($p=0.1353$, Kruskal-Wallis test, Graphpad Prism). Panel (a) displays the groups tested in each Kruskal-Wallis statistical analysis. Panel (b) displays LTNP10 and LTNP7 as separate groups. Panel (c) combines LTNP10 and LTNP7 patients into a single LTNP group.

(a) Groups tested by Kruskal-Wallis test, p-values

Groups Tested (Kruskal-Wallis)						p-value	
LTNP10	LTNP7	EC	RP	TP	VC	0.0003	***
LTNP10	LTNP7					0.1353	ns
ALL LTNP		EC	RP	TP	VC	0.0003	***
ALL LTNP				TP	VC	0.2933	ns
ALL LTNP		EC		TP	VC	0.0018	**
ALL LTNP			RP	TP	VC	0.0194	*

Figure 13 (continued): Neutralization ranking by disease progression group

(b) All disease progression groups



(c) LTNP10 and LTNP7 combined as LTNP

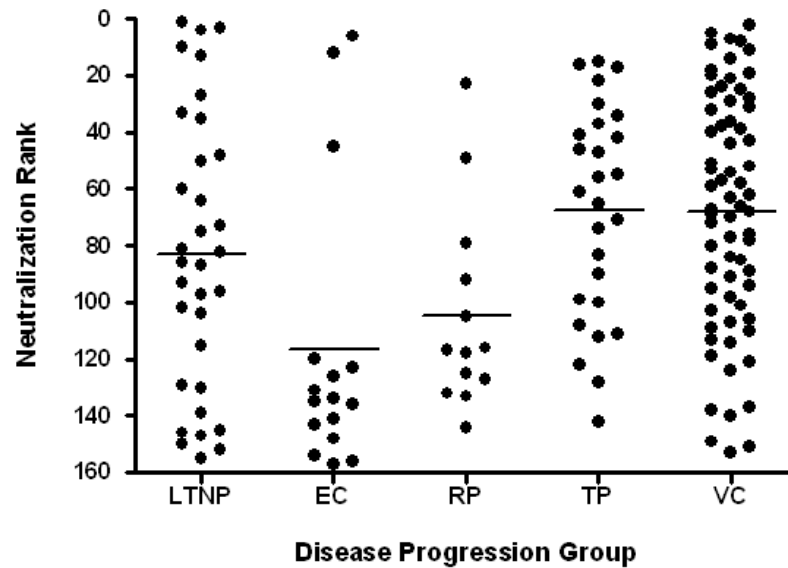


Figure 13 shows neutralization ranking by disease progression group. A highly statistically significant difference in median rank ($p=0.0003$) was observed when all six groups were compared using the Kruskal-Wallis test in Graphpad Prism. To investigate the underlying driver of the significant difference, we first compared the median rank of LTNP10 and LTNP7 groups using the same Kruskal-Wallis test. No significant difference ($p=0.1353$) was observed between the median rank of LTNP10 and LTNP7 groups. When LTNP10 and LTNP7 groups were combined, and EC and RP groups were excluded, no significant difference was observed ($p=0.2933$) between the median rank of LTNP, VC, and TP. When EC or RP were added to LTNP, VC, and TP groups, a significant difference was observed ($p=0.0018$ and $p=0.0194$, respectively) between median ranks. If rankings among all individuals were randomly distributed, it would be expected that the median rank of each group would be similar. However, these results indicate that EC and RP rankings are not randomly distributed.

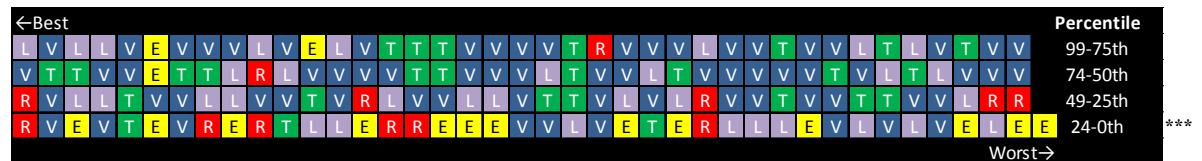
To further investigate trends in EC and RP rankings, we used a χ^2 test (Microsoft Excel) to compare observed versus expected frequencies of each disease progression group in quartiles of the neutralization ranking. In Figure 14, each colored square represents an individual and the letter in each square represents the disease progression group of the patient (L=LTNP, V=VC, E=EC, T=TP, R=RP). The groups LTNP10 and LTNP7 were combined, as they were not found to be statistically different. The first block displays individual rankings in order. The second block displays the rankings of each quartile, sorted by disease progression group. The third block displays the approximate expected frequency of each disease progression group per quartile, proportional to the total number of individuals in each group. No significant differences

were observed by χ^2 test for the first three quartiles. However, a significant difference was observed in the last quartile: when compared to expected frequencies, the number of EC was highly enriched ($p < 0.0001$), and the number of VC was slightly reduced ($p = 0.0126$). No significant differences were observed by χ^2 test for RP in any of the quartile groups.

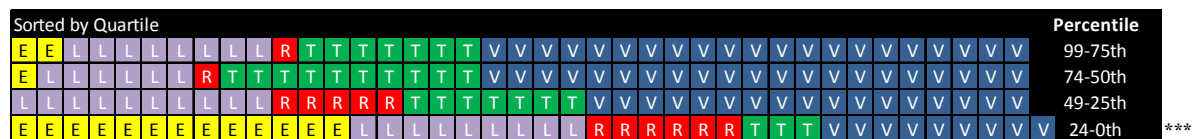
Figure 14: Neutralization ranking of disease progression groups by quartile

Each colored square represents an individual and the letter in each square represents the disease progression group of the patient (L=LTNP, V=VC, E=EC, T=TP, R=RP). The groups LTNP10 and LTNP7 were combined, as they were not found to be statistically different. Each row represents a quartile. The first block (a) displays individual rankings in order from best to worst (left to right, then top to bottom). The second block (b) displays the rankings of each quartile, sorted by disease progression group. The third block (c) displays the approximate expected frequency of each disease progression group per quartile, proportional to the total number of individuals in each group. No significant differences were observed by χ^2 test for the first three quartiles. However, a significant difference was observed in the last quartile (the bottom row of the four quartiles): when compared to expected frequencies, the number of EC was highly enriched ($p<0.0001$), and the number of VC was slightly reduced ($p=0.0126$).

(a) Individual rankings in order



(b) Individual rankings sorted by disease progression group within each quartile



(c) Approximate expected frequency of each disease progression group per quartile



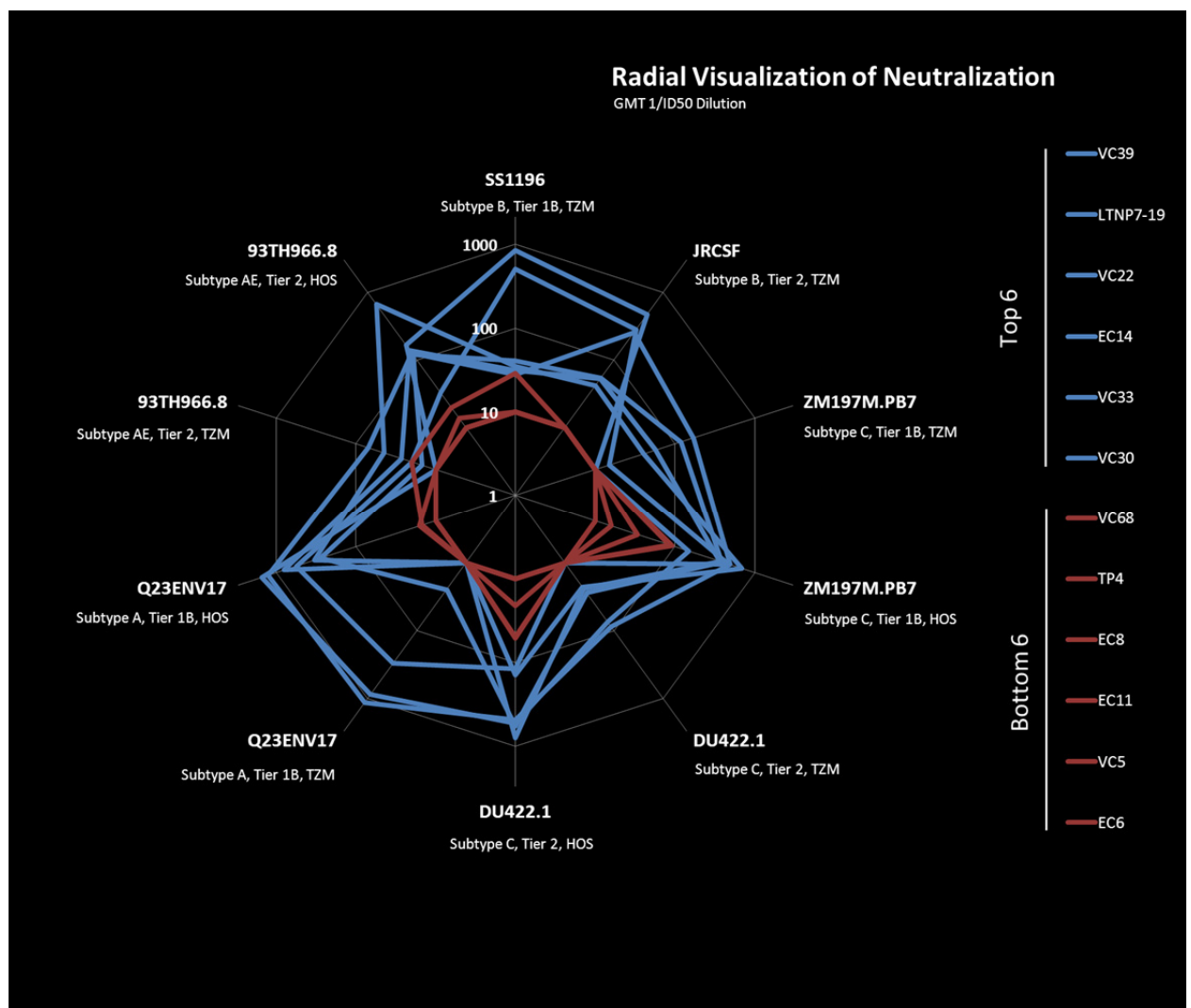
From the combined 157 patient sera tested, the top six and bottom six neutralizing individuals with available early plasma samples were identified for further investigation. The top six and bottom six represented the top 5 and bottom 10.8 percentile of neutralizers, respectively. The list of the top and bottom six neutralizers is shown in Table 13. A radial visualization of serum neutralization against our neutralization panel is shown in Figure 15.

Table 13: The top and bottom six neutralizers identified for further investigation

Neutralization Rank (out of 156)	Top 6 Neutralizers (Top 5 percentile)	Assigned Patient Code	Neutralization Rank (out of 156)	Bottom 6 Neutralizers (Bottom 10.8 percentile)	Assigned Patient Code
2	VC39	8	140	VC68	12
3	LTNP7-19	7	142	TP4	9
5	VC22	3	143	EC8	2
6	EC14	5	148	EC11	10
7	VC33	11	151	VC5	4
8	VC30	6	156	VC6	1

Figure 15: Radial visualization of neutralization for top and bottom six neutralizers

Geometric means of the 1/ID50 dilution for each patient serum against neutralization panel viruses are shown on a radial graph. Each blue or red line represents a single patient of the top or bottom six neutralizers, respectively. The center of the graph begins at 1 (titer), and each concentric ring outward increases by 10-fold.



With the top and bottom six neutralizers identified we proceeded with the next phase of this project – cloning and characterizing envelope genes. Plasma samples were obtained for each of the 12 individuals at a timepoint less than two years post seroconversion or documented positive date. Each of the 12 patients was given a new code (numbers 1-12) to reduce bias when processing samples. Initially, a spin column procedure was used to extract viral RNA from plasma samples. For samples with low RNA yield, a magnetic bead kit was used to recover additional viral RNA from plasma. Using a combination of these two methods, viral RNA was extracted from all 12 plasma samples, which was then used in reverse transcription PCR (RT-PCR) to generate cDNA.

We attempted to clone envelope genes via single genome amplification (SGA). Essentially PCR with limiting dilutions of (cDNA) template, SGA can yield with high (>95%) confidence, PCR products that originated from a single copy of template (225). This negates the chance of Taq-induced recombination events during PCR. To achieve such high confidence, however, approximately 80% of PCR reaction wells (or more) in a 96-well plate must be negative. Additionally, viral sequences obtained via SGA are proportionally representative of viral diversity, eliminating resampling bias. Anecdotal reports have suggested that virus clones obtained by single genome amplification (SGA) are more likely to be functional (F. McCutchan, personal communication). However, this was not our experience. We generated a total of 22 clones from three patients, none of which were functional. Facing limited time and resources, we opted to proceed with bulk PCR as an alternative to SGA. Unlike SGA, bulk PCR uses undiluted template as starting material.

In the early stages of nested PCR cloning of envelope genes, we used inner primers that contained the restriction endonuclease sites *EcoRI* and *XhoI*, as has been done previously by our laboratory (42). We encountered a number of envelope clones from one patient, that when digested with *EcoRI*, produced DNA fragments that were smaller than expected. Nucleotide sequencing confirmed the presence of an *EcoRI* recognition sequence within the envelope gene. A search of the online 2012 HIV-1 Sequence Alignment by the Los Alamos National Laboratory (LANL) Database (<http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html>) revealed that 19.2% of subtype B and 38.6% of all subtype envelope clones contain at least one *EcoRI* site within the envelope gene coding region. By eliminating the use of the *EcoRI* cloning site, only two other restriction endonuclease sites for cloning into pcDNA3.1 remained: *EcoRV*, which creates a blunt cut, or *AflIII*, which creates a four nucleotide overhang but ligates with <50% efficiency. We decided to proceed by using primers containing *AflIII* and *XhoI* sites.

Cloning into pcDNA3.1(+) vector

Clones that were confirmed to contain insert were tested for function in infectivity assays using pseudovirus produced in 24-well plates. Clones that appeared to be infectious were then subject to nucleotide sequencing. A total of 10-14 infectious clones and their sequences were generated for each patient. Pseudoviruses were then produced in 25cm² tissue culture flasks for further confirmation of infectivity prior to use in neutralization assays. Many of these pseudoviruses displayed low or no infectivity. Some issues were related to 293T cell viability and were traced to incorrect incubator

temperature calibration. However, after examining the translated sequence alignments, premature stop codons and/or frameshifts were found in the majority of envelope clones.

Cloning into pSV7d vector

In an effort to generate additional clones for screening, we changed cloning strategies and utilized pSV7d, a smaller expression vector (2.4kb versus the 5.4kb pcDNA3.1(+)). New PCR inserts were produced incorporating the restriction enzyme sites *EcoRI* and *XhoI* for cloning into pSV7d.

A total of approximately 10,000 bacterial colonies were processed through spin column purification and sized via agarose gel electrophoresis. Nearly 2,000 positively sized clones were screened for infectivity. Of these, 173 clones appeared to be functional. Although 57 of these clones possessed intact nucleotide sequences (sequences lacking frameshift mutations or premature stop codons), only 31 clones produced infectious titers suitable for testing in neutralization assays. At the deadline of this report, we were yet unable to obtain functional intact clones with usable infectious titers for two patients, VC22 (#3), and EC6 (#1), one each from the top and bottom groups. Additional cloning efforts to obtain multiple clones for all of the twelve patients are ongoing. Progress on testing infectious clones is shown in Table 14.

Table 14: Testing for infectious clones

	Code	Patient	First Screen Positive Clones (24-well)	Clones with Intact Sequences	Tested in Neutralization Sensitivity Assays*
Top 6	8	VC39	15	4	3
	7	LTNP7-19	11	3	2
	3	VC22	14	2	0
	5	EC14	17	4	1
	11	VC33	13	6	1
	6	VC30	16	12	9
Bottom 6	12	VC68	15	3	3
	9	TP4	14	11	7
	2	EC8	16	3	3
	10	EC11	13	1	1
	4	VC5	19	4	1
	1	EC6	10	4	0
Total			173	57	31

*Some clones with intact sequences produced infectious titers that were too low to use in neutralization assays.

Sequence analysis of cloned envelope genes

Analyses of sequence data was performed using consensus sequence alignments generated via the online LANL HIVAlign tool (<http://www.hiv.lanl.gov/content/sequence/VIRALIGN/viralalign.html>) for each patient.

Sequence analysis of the membrane-proximal external region (MPER)

The membrane-proximal external region (MPER) of gp41, residues 659-683 (HXB2 numbering), is targeted by several known monoclonal antibodies. The binding sites of mAbs 2F5, 4E10, Z13e1, and 10E8 are linear epitopes located within the MPER of gp41 (36; 116; 182; 183; 309). Mutations in residues D, K, or W of the core epitope of 2F5 (ELDKWA) are known to provide neutralization resistance (309). The E662A/K substitution is commonly observed and has been suggested as an escape mutation, but does not appear to contribute to neutralization resistance to 2F5 (36; 42; 257; 309). The S668N and N671S substitutions are also commonly observed, but lie outside of the recognition epitope of 2F5 (27; 183). Although the T676 residue is important for 4E10 binding, the commonly observed substitution to serine does not abrogate binding (36; 210). The residues N671 and D674 are required crucial for Z13e1 binding (182). The conserved residues W672, F673, W676, and K/R683 are required for 10E8 binding (116). The residue N677 contacts Z13e1 and 4E10 (244).

Table 15 shows a summary of amino acid mutations in the MPER region for all 12 patients. Seven amino acid substitutions were observed in the MPER region of the envelopes isolated from the 12 patients. Mutations were found in four of the top neutralizers, and in only one of the bottom neutralizers. Though many of these mutations do not affect neutralization sensitivity to known MPER mAbs, these mutations may be

due to positive selection, which appears to be more frequent in the top rather than the bottom neutralizers.

Four mutations observed, E662A/K, S668N, and T676S, have been reported to not affect neutralization sensitivity to MPER mAbs 2F5, 4E10, Z13e1, or 10E8. These mutations were found in three of the top neutralizers, LTNP7-19 (#7), EC14 (#5), and VC33 (#11), and in the only patient of the bottom neutralizers who possessed any MPER mutations, TP4 (#9).

The other two mutations observed were found in two of the top neutralizers. A N671S mutation in the MPER of clones from VC39 (#8) abrogates binding to Z13e1 and 10E8, and decreases binding to 4E10. A D674N mutation in clones from EC14 (#5) abrogates binding to Z13e1. A literature search for the mutations K665Q and N677Q, seen in clones from patient EC14 (#5), returned no results. The effect of the latter mutation, if any, is likely to be less significant because asparagine and glutamine share the same side chain amide functionality, but asparagine has one fewer side chain carbon atoms than glutamine.

Table 15: Analysis of amino acid mutations in the MPER region

		MPER Epitope			
		2F5	4E10	Z13e1	10E8
Top 6	8-VC39		N671S	N671S	N671S
	7-LTNP7-19	E662A	T676S	T676S	T676S
	3-VC22	-	-	-	-
	5-EC14	E662K K665Q		D674N N677Q	N677Q
	11-VC33	E662A	T676S	T676S	T676S
	6-VC30				
Bottom 6	12-VC68				
	9-TP4	E662A	T676S	S668N T676S	T676S
	2-EC8				
	10-EC11				
	4-VC5				
	1-EC6	-	-	-	-

Green – Not known to affect binding
Orange – Moderately decreases binding
Red – Abrogates binding
Black – Effect unknown

In the RV144 vaccine study, the presence of IgG antibodies directed to variable regions 1 and 2 (V1V2) of gp120 was inversely correlated with risk of HIV-1 infection (307). Neutralizing antibodies to the V1V2 region typically have long, protruding, anionic, heavy-chain complementarity-determining region 3 loops (CDR H3s) that recognize a quaternary glycopeptide epitope within the glycan shield (122; 169; 187).

The monoclonal antibodies CAP256 and VRC26.01-12 each recognize a V1V2 quaternary epitope, but are only partially dependent on glycans N160 and N156 (122). In the donor (CAP256) from which these antibodies were isolated, a series of longitudinal envelope sequences obtained by single genome amplification was examined to determine a mechanism for the development of these antibodies (68). Envelope sequences from this donor showed high levels of diversity between the superinfecting virus, first detected 15 weeks post-infection, and the primary infecting virus. Nearly all of the CAP256 monoclonal antibodies failed to neutralize the primary infecting virus, suggesting that the superinfecting virus was able to engage the naïve B cell that produced the antibody lineage. Engagement of the germline or unmutated common ancestor (UCA) of the naïve B cell and the subsequent gene rearrangement is hypothesized as the rate limiting step in the induction of bcnAbs (68).

In envelope sequences from the CAP256 donor, a rare K169I mutation was observed among superinfecting virus sequences, which dominated just before the development of neutralization breadth (68). Escape mutations at residues 166 and 169 were observed, resulting in a change in the net charge in the V2 epitope from +3 to 0 (68). At the same time, antibody CDR H3s became less acidic (-10 to -4). It is hypothesized that under positive selection by CAP256 antibodies, previously inaccessible

V1V2 conformations of the superinfecting strain were exposed, some of which contributed to the development of neutralization breadth. This hypothesis links one mechanism for the development of bcnAbs to the exposure of V1V2 conformations.

We examined V1V2 and V3 residues of consensus sequences of envelope clones obtained from each of the 12 patients in the present study. Figure 16 shows consensus and individual amino acid sequences of the V1V2 (residues 160-171), V3 (residues 332-334), and MPER (residues 659-683) regions, and results for neutralization sensitivity to monoclonal antibodies, and serum neutralization specificity findings.

Comparison of V1V2 and V3 regions between top and bottom neutralizers

None of the V1V2 sequences (160-171) were shared among the top neutralizers, and the net charge of the region ranged from +1 to +4 in clones from these individuals. Interestingly, two of the top six patients, VC39 (#8), and LTNP7-19 (#7), possessed the rare K169I mutation hypothesized to be the result of positive selection that exposed V1V2 conformations that preceded the development of neutralization breadth (68).

Consensus sequences for the bottom neutralizers were more homogeneous. Four of the bottom neutralizers shared identical V1V2 sequences and the net charge of the region was unvaryingly +3.

Taken together, these observations suggest that clones from the top neutralizers may have experienced more positive selection than clones from the bottom neutralizers, providing support for the hypothesis that conformational changes in V1V2 may induce bcnAbs.

Glycosylation at N160 is critical for recognition by PG9 and PG16 mAbs (63). Similarly, glycosylation at N332 is recognized by PGT121 and PGT126 mAbs (273).

Based on amino acid sequence alone, the R2 clone and clones from VC33 (#11), the former lacking the NxS/T glycosylation motif and the latter possessing a N160Y mutation, are unlikely to be sensitive to PG9 and/or PG16. All other patients possessed clones with N160, indicating neutralization sensitivity to PG9/PG16. Furthermore, each of the patients possessed clones with N332 (and the NxS/T motif across 332-334), indicating neutralization sensitivity to PGT121/PGT126.

Figure 16: Consensus and individual amino acid sequences of the V1V2, V3, and MPER regions, and results for neutralization sensitivity and serum neutralization specificity

Consensus (CON) and individual amino acid sequences of the V1V2, V3, and MPER region are presented. The net charge of the V1V2 region, and potential N-linked glycosylation sites at N160 and N332 are listed. Mutations in the MPER epitope are also included. Sensitivity to neutralizing monoclonal antibodies is displayed (see color legend for corresponding sensitivity). Serum neutralization sensitivity findings are also listed.

Nearly all clones were neutralized by at least one mAb for each epitope region. Nearly all clones were highly resistant to neutralization by Z13e1 and 2G12, and highly sensitive to PGT126.

The median number of mAbs that neutralized each clone was similar between top (7, range 5-9) and bottom (7.5, range 4-11) neutralizers. Overall, top neutralizers were more sensitive to neutralization by mAbs targeting CD4-induced epitopes. For top neutralizers: all clones from VC39 (#8) were highly resistant to V1V2/V3 mAbs, all clones from LTNP7-19 (#7) and EC14 (#5) were highly resistant to mAbs targeting CD4-induced epitopes. For bottom neutralizers: most clones from VC68 (#12) were highly resistant to CD4bs directed mAbs. Clones from bottom neutralizers were the most globally neutralization sensitive (9A36 and 2-105, 11/17 mAbs) and resistant (12-101, 9A29B, and 2-142, 4/17 mAbs).

[illegible]

Neutralization sensitivity of cloned envelopes

A total of 31 pseudovirus clones (from ten patients) displayed moderate infectivity when produced in 25cm² tissue culture flasks. These clones were tested in HOS-CD4-CCR5 neutralization sensitivity assays against a panel of mAbs described in Table 6 (in the previous chapter). These mAbs target known neutralization epitopes on the envelope glycoprotein. For each clone, the geometric mean of the IC₅₀ (ug/ml) was calculated from two independent neutralization assays (shown in Figure 16). The average geometric mean IC₅₀ of the clones for each patient is also shown. Additional assays will be performed pending additional completion of cloning and infectivity testing.

Comparison of neutralization sensitivity between clones from top and bottom neutralizers

In general, nearly all of the clones were neutralized by at least one mAb for each of the epitope regions tested (MPER, CD4-induced, V1V2, V3, and CD4 binding site). The 31 clones tested were mostly resistant to two mAbs, Z13e1 (MPER epitope) and 2G12 (oligomannose epitope), with 30 clones highly resistant to neutralization to each mAb.

The top and bottom six neutralizers were similarly sensitive to neutralization by the mAbs tested. The median number of mAbs that neutralized, to some extent, clones from the top and the bottom six neutralizers was 7 (ranging from 5-9) and 7.5 (ranging from 4-11), respectively.

Clones from two of the top neutralizers were highly resistant to neutralization of certain epitopes. All of the clones from VC39 (#8), were highly resistant to neutralization by the V1V2/V3 antibodies PG9, PG16, and 447-52D. All of the clones

from EC14 (#5) were highly resistant to neutralization by 48D and 17b, mAbs targeting CD4-induced epitopes.

Clones from three of the bottom neutralizers, VC68 (#12), TP4 (#9), and EC11 (#10), were also highly resistant to neutralization by 48D and 17b. Additionally, clones from VC68 (#12) were highly resistant to neutralization by all of the CD4bs mAbs.

The most neutralization sensitive and resistant clones were found in another two of the bottom neutralizers. Clones from patients TP4 (#9) and EC8 (#2) yielded the most globally neutralization sensitive clones 9A36 and 2-105, neutralized to some extent by 11 of the 17 mAbs tested, as well as the most globally neutralization resistant clones 9A124, 9A29B, and 2-142, neutralized by 4 of the 17 mAbs tested. Clone 12-101 from VC68 (#12), was also very resistant to neutralization, neutralized by only 4 of the 17 mAbs tested.

Overall, clones from the top neutralizers were more sensitive to mAbs targeting CD4-induced epitopes (6 of 16 clones sensitive to some extent), than were the bottom neutralizers (2 of 15 clones sensitive to some extent).

Analysis of envelope clone sequence and neutralization sensitivity

Two of the top six neutralizers possessed amino acid substitutions that conferred resistance to monoclonal antibodies targeting known epitopes. Our sequence analysis found that clones from VC33 (#11) possessed an N160Y mutation, abolishing the N-linked glycan for recognition by mAbs PG9/PG16. Indeed, the clone 11A140 obtained from VC33 was highly resistant to PG9 and moderately resistant to PG16 neutralization, concurring with previous reports that N160 is required for neutralization by PG9/PG16 (274). Analysis of the MPER region consensus sequence for clones obtained from

patient VC39 (#8) revealed a N671S mutation that affects one of two critical residues for recognition by Z13e1. Two of the three clones from VC39 were highly resistant, and the remaining clone was moderately resistant to neutralization by Z13e1. Clones from the aforementioned patients, VC33 (#11), and VC39 (#8), are likely to have undergone positive selection, developing resistance to neutralization by PG9/PG16- and Z13e1-like antibodies.

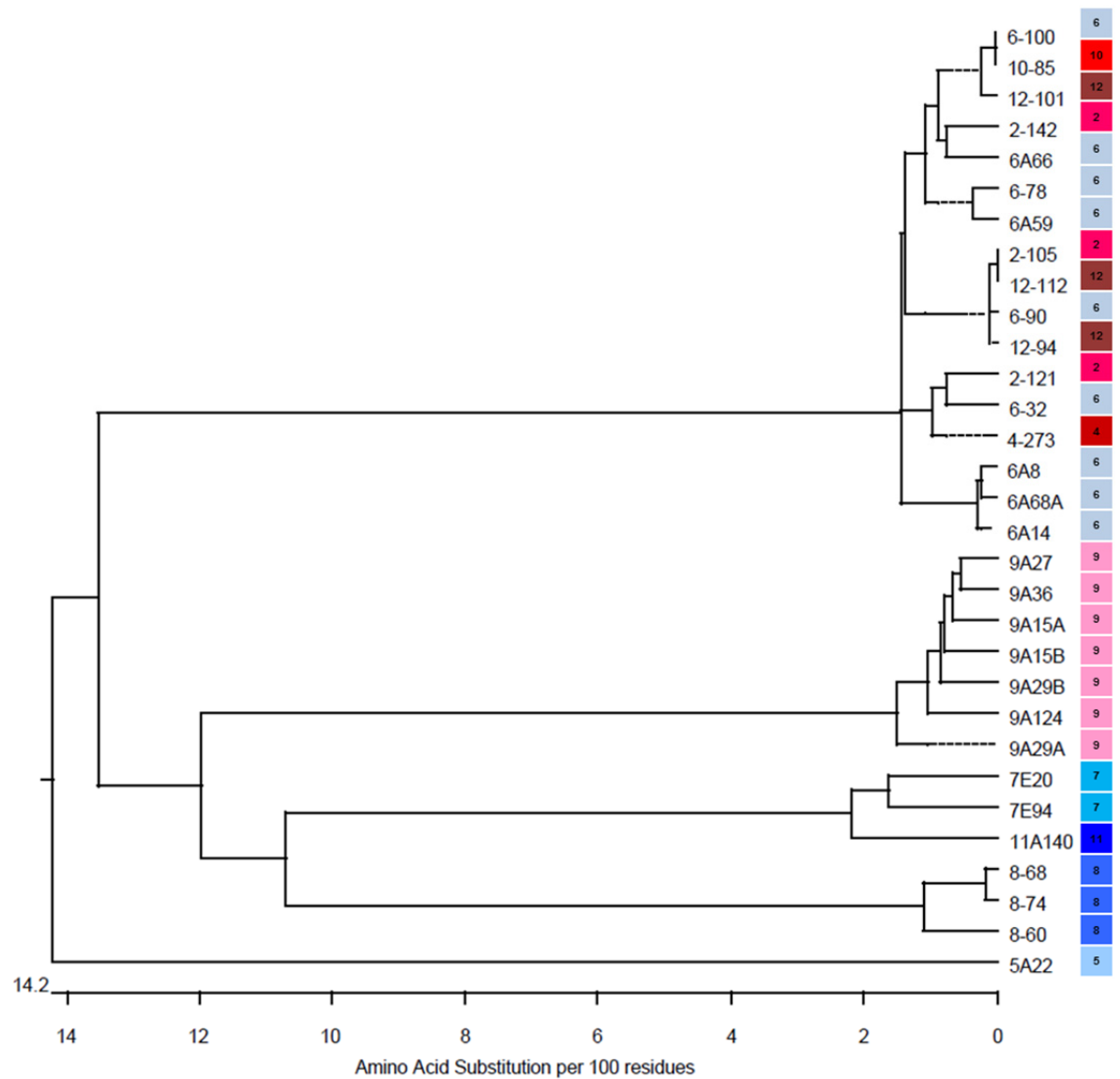
Nearly all clones were sensitive to neutralization by PGT126. Consensus sequences for clones obtained from each patient revealed the presence of N332, a glycan dependent PGT121/PGT126 epitope on V3. Out of 31 clones tested, 26 were highly sensitive to neutralization by PGT126. Two clones were moderately sensitive and the remaining three clones were highly resistant to neutralization by PGT126.

Phylogenic analysis of envelope clones

Amino acid sequences were aligned by ClustalW using DNASTar Lasergene MegAlign software to determine whether patients were infected with single or multiple strains of virus. The dendrogram is shown in Figure 17. Clones from individual patients cluster along a single major branch, suggesting that infection was initiated by a single virus strain.

Figure 17: Dendrogram of envelope clones

Amino acid sequences of envelope clones were aligned by ClustalW using DNASTar Lasergene MegAlign software. Color coding: reds (bottom neutralizers), blues (top neutralizers).



For the final phase of this project we employed serum specificity and blocking assays to evaluate the specificity of serum neutralization in each of the 12 patients. Patient serum used in our initial neutralization screen for bcnAbs (sample collected 2-4 years post seroconversion or documented HIV positive date) was tested in pseudovirus neutralization assays against the envelope clone SF162 and the SF162 mutants SF162_{K160N} and SF162_{N332A} for the presence of PG9/PG16-like and PGT121/PGT126-like antibodies. The clone SF162 contains a rare lysine substitution at residue 160 that confers resistance to neutralization by PG9 and PG16, monoclonal antibodies that recognize a N-linked glycan at the same residue (288). The mutant clones SF162_{K160N} and SF162_{N332A}, contain amino acid substitutions that confer sensitivity to neutralization by PG9/PG16 and resistance to neutralization by PGT121/PGT126, respectively.

We also tested the same patient serum for the presence of CD4 binding site (CD4bs) antibodies in a series of blocking neutralization assays using SF162 pseudotyped virus and the proteins RSC3, RSC3_{Δ391I}, and RSC3_{Δ371I/P363N}. Briefly, RSC3 is an engineered gp120 protein based on HXB2 that was designed to preserve the CD4bs while altering or masking other immunogenic epitopes (289; 302). Certain monoclonal antibodies that neutralize only neutralization sensitive Tier 1 strains bind strongly to RSC3 (158). Binding of such antibodies is abrogated in RSC3_{Δ371I}, which removes the isoleucine residue at position 371, and in RSC3_{Δ371I/P363N}, which in addition to the deleted isoleucine residue, includes a proline to asparagine mutation at residue 363 that completely abolishes the binding of CD4 to the gp120 protein, and therefore, does not block viral entry (158).

The envelope clone HXB2 was used in the blocking assay method described by Lynch et al., however we encountered difficulty producing HXB2 pseudovirus with high infectivity, and instead used SF162 in substitution (158). Table 16 shows IC50 and ID50 results from serum specificity and blocking assays.

Table 16: Results from serum specificity and blocking assays

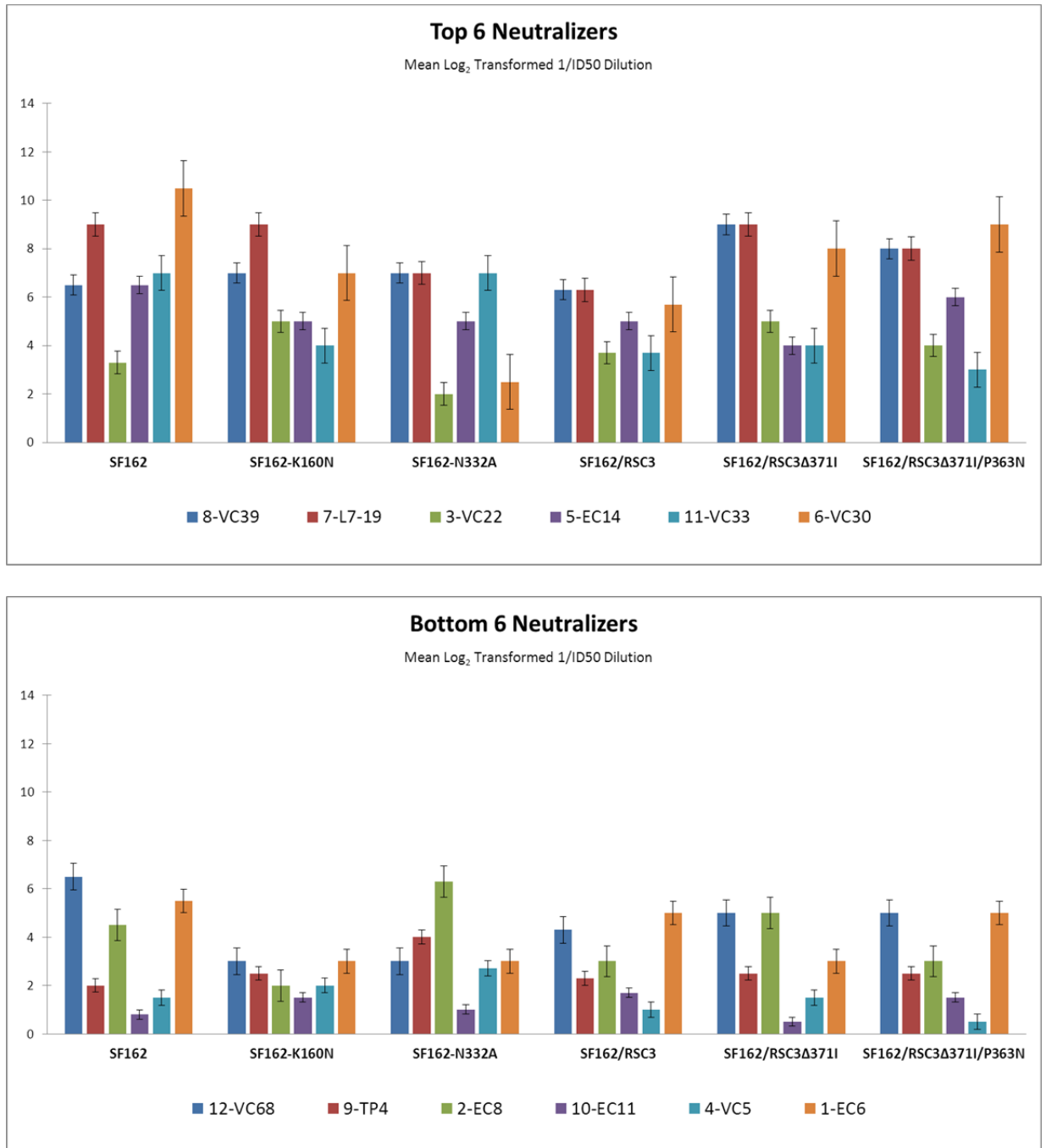
Monoclonal antibody control results are presented as mean IC50 concentration (ug/ml). Serum neutralization results are presented as mean Log₂ transformed 1/ID50 dilution. Each mAb or serum was tested in 2-4 independent experiments.

	mAb or Patient	SF162	SF162 _{K160N}	SF162 _{N332A}	SF162/RSC3	SF162/RSC3 _{A371I}	SF162/RSC3 _{A371I/P363N}
mAb Controls (Mean IC50, ug/ml)	PG9	>1	0.063	0.016	>1	>1	>1
	PG16	>1	>1	0.250	>1	>1	>1
	PGT121	0.098	0.040	0.016	0.110	0.532	0.625
	PGT126	0.086	0.040	0.250	0.047	0.625	0.625
	VRC01	0.438	0.625	0.250	>1	>1	>1
	VRC03	0.567	0.040	0.250	>1	1.000	0.625
	b12	0.172	0.625	0.250	>1	>1	0.625
Top 6 (Mean Log ₂ Transformation, 1/ID50 dilution)	8-VC39	6.5	7.0	7.0	6.3	9.0	8.0
	7-L7-19	9.0	9.0	7.0	6.3	9.0	8.0
	3-VC22	3.3	5.0	2.0	3.7	5.0	4.0
	5-EC14	6.5	5.0	5.0	5.0	4.0	6.0
	11-VC33	7.0	4.0	7.0	3.7	4.0	3.0
	6-VC30	10.5	7.0	2.5	5.7	8.0	9.0
Bottom 6 (Mean Log ₂ Transformation, 1/ID50 dilution)	12-VC68	6.5	3.0	3.0	4.3	5.0	5.0
	9-TP4	2.0	2.5	4.0	2.3	2.5	2.5
	2-EC8	4.5	2.0	6.3	3.0	5.0	3.0
	10-EC11	0.8	1.5	1.0	1.7	0.5	1.5
	4-VC5	1.5	2.0	2.7	1.0	1.5	0.5
	1-EC6	5.5	3.0	3.0	5.0	3.0	5.0

Our assay controls performed reasonably as expected. SF162 was resistant to neutralization by PG9 and PG16, SF162_{K160N} was sensitive to neutralization by PG9, and SF162_{N332A} was resistant to neutralization by PGT126. RSC3, designed to bind CD4 binding site antibodies, blocked neutralization of SF162 by VRC01, VRC03 and b12. In the presence of the RSC3 mutants, RSC3_{Δ391I}, and RSC3_{Δ371I/P363N}, neutralization of SF162 by VRC03 and b12 was greater than in the presence of RSC3, as expected.

In our serum specificity assays, the top six neutralizers possessed sera that, in general, neutralized SF162 at a higher 1/ID50 dilution than did the bottom six neutralizers. Figure 18 shows serum neutralization specificity differences between top and bottom neutralizers.

Figure 18: Comparison of serum neutralization specificities between top and bottom neutralizers



Comparison of serum neutralization specificities between top and bottom neutralizers

We found differences strongly indicating the presence of certain epitope targeted neutralization (shown in Table 14). Since statistical comparisons were not likely to be valid with such few replicates, our criteria for identifying differences in neutralization was a cut-off set at 4-fold or greater difference between SF162 and SF162 mutants or peptide controls.

Serum neutralization of SF162 was inhibited by RSC3 but not by RSC3_{Δ391I}, and RSC3_{Δ371I/P363N} in two of the top neutralizers, LTNP7-19 (#7), and VC30 (#6), strongly indicating the presence of CD4bs directed antibodies. Additionally, in VC30 (#6), the serum neutralization ID50 dilution for SF162 was 256-fold higher than for SF162_{N332A}, strongly indicating the presence of PGT121/PGT126-like antibodies. In another top neutralizer, VC33 (#11), serum neutralization of SF162 was inhibited by RSC3 and the RSC3 mutants, possibly due to affinity for a neutralizing epitope shared among the RSC3 and RSC3 mutant proteins.

Results for the bottom six neutralizers were varied. No consistent differences were observed in other patient sera that would indicate the presence of PG9/PG16-like, PGT121/PGT126-like, or CD4 binding site directed antibodies. Though this finding does not preclude the existence of such antibody specificities in these sera, it is likely that 12neutralization of SF162 was mediated by antibodies of other specificities.

RESULTS AND DISCUSSION FOR AIMS 3 & 4

Development of Fort Morgan Virus/HIV-1 Env Vaccine Vector

Four mutant pFM double promoter constructs were created: FMV-gp160, FMV-gp160 Δ CT, FMV-gp140, and FMV-GFP. We designed primers to extend the 3' end of the R2 Env and eGFP gene inserts to duplicate the 26S subgenomic promoter sequence such that the final construct would include two 26S promoters, one for the transgene, and one in the normal position upstream of the structural proteins.

Initial attempts at cloning R2 Env mutants into the single *Bam*HI restriction endonuclease site in pFM were frustrated by low ligation efficiency and poor transgene expression. The *Bam*HI cloning site was positioned downstream of the 26S promoter. The size of the insert (approximately 3 kb) in conjunction with the size of the vector (approximately 13 kb) likely hindered the success of ligation (see Figure 11 and 12 in the previous chapter). The application of Antarctic Phosphatase improved ligation efficiency (by preventing self-ligation of vector without insert). In vector that received insert, the orientation of the insert was paradoxically skewed such that the inverted orientation resulted in more than 70% of ligated products.

Viruses were produced by electroporation of in vitro transcribed RNA into BHK-21 cells. Peak CPE was observed in infected cells after 4-5 days. Expression of HIV-1 R2 Env glycoprotein was detected by Western blot in cell lysate and supernatant of BHK-21 cells infected with FMV-gp160, FMV-gp160 Δ CT, and FMV-gp140 mutants. Western blots were performed by L. Yan. GFP-positive cells were observed under fluorescence microscopy in BHK-21 cells that were infected with FMV-GFP.

Due to logistical reasons, the rabbit safety/immunogenicity pilot study was initiated before we were able to assess the stability of transgene expression over multiple virus passages. The nine New Zealand White rabbits used for the safety/immunogenicity experiment received immunizations as follows: FMV-gp160 Δ CT.269 (n=3), FMV-gp140.18 (n=3), FMV-WT (n=2), no injection (n=1). All animals appeared to tolerate blood draw and immunization procedures. No abnormalities were observed during immunization and dissection.

Several attempts at recovering virus from fresh or frozen dissected tissue were unsuccessful. Supernatant collected from filtered centrifuged homogenized tissue appeared to contain tissue debris and/or contaminating bacteria that clouded the growth media above the BHK-21 cell layer. No obvious CPE was observed.

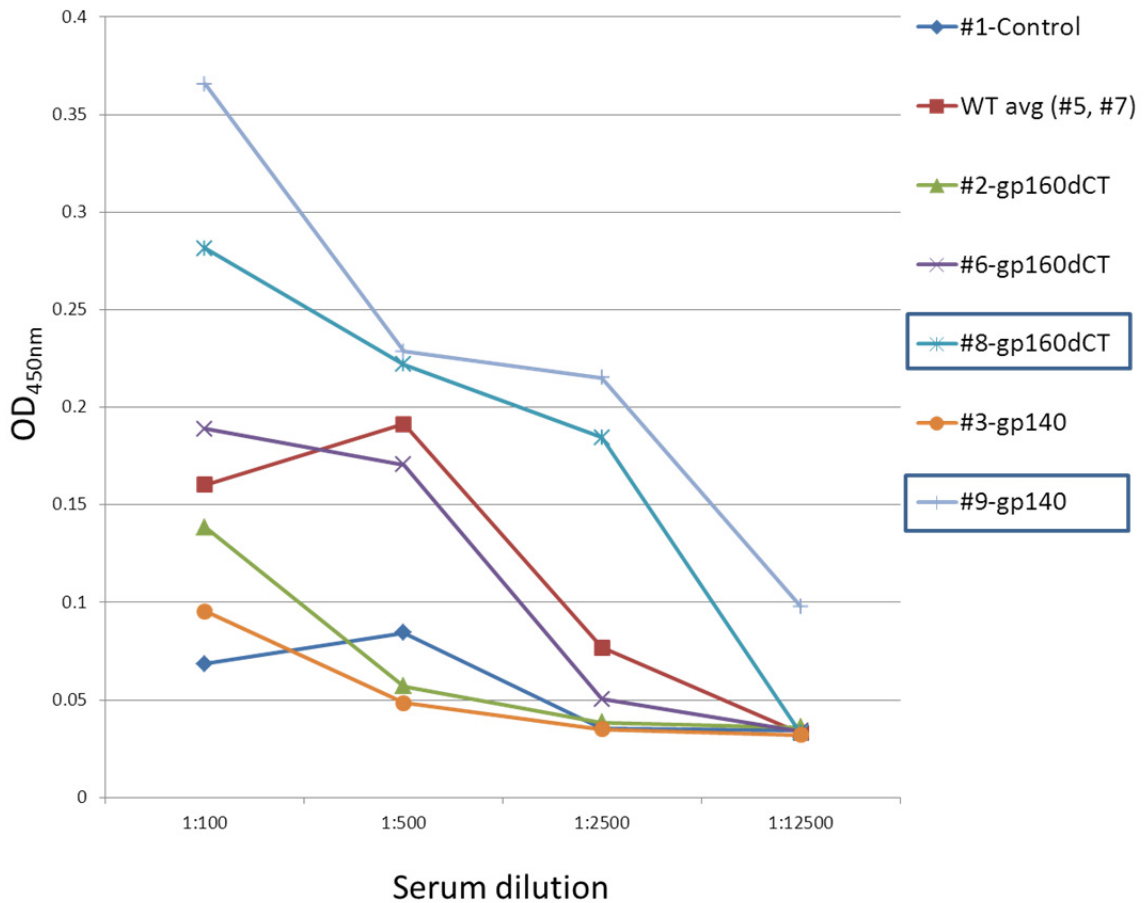
Rabbit serum collected from the pilot study was tested in pseudovirus neutralization assays against the same panel of viruses used to screen human serum from HIV-infected individuals in a different section of this dissertation research (refer to Chapter 2 for materials and methods). No significant neutralizing activity was detected.

Rabbit serum was also tested in our laboratory by ELISA for HIV-1 gp140-specific polyclonal antibodies. Diluted serum was tested in duplicate with four serial 5-fold dilutions starting at 1:100. Overall, antibody responses were poor. Two animals #8 and #9, (one each from FMV-gp160 Δ CT and FMV-gp140 groups, respectively) developed anti-gp140 antibodies that were higher than the average FMV-WT responses. ELISA results are summarized in Figure 19. When ELISA results for animals 8 and 9 were compared to the average of the animals receiving wild type FMV, a significant difference was observed (p=0.0308) using a two-way ANOVA model (Graphpad Prism).

Bonferroni post-tests (conducted following two-way ANOVA) found OD values for two of the dilutions were significantly higher for animals 8 and 9 compared to the average of the animals receiving wild type (for all post-tests, $p < 0.05$, Bonferroni posttests, Graphpad Prism).

Figure 19: Detection of HIV-1 gp140-specific antibodies in immunized rabbit serum

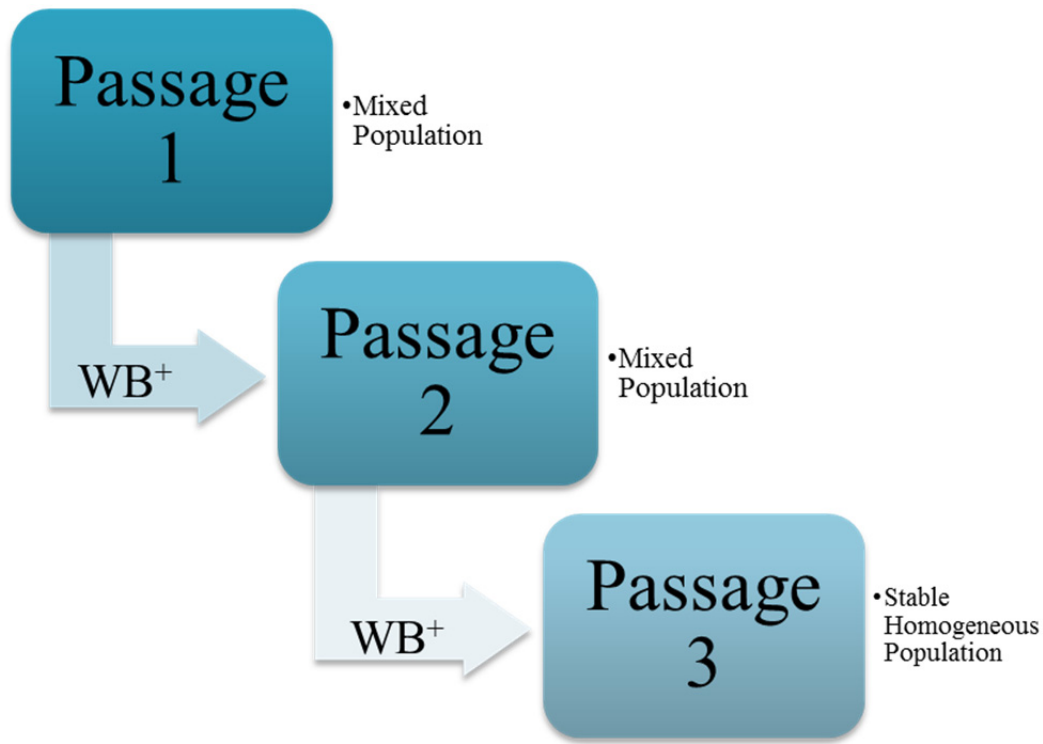
Enzyme Linked Immunosorbent Assay (ELISA) of binding of immunized rabbit serum to gp140 protein. Responses for animals #8 and #9 (FMV-gp160 Δ CT and FMV-gp140, respectively) were significantly higher than responses for animals treated with WT-FMV ($p=0.0308$, two-way ANOVA, Graphpad Prism).



We hypothesized that the weak antibody responses could be related to potential instability of the large recombinant genome, and/or some wild-type like virus (FMV infectious molecular clone without transgene) carried over from transcription could have contaminated the desired virus population. CPE was observed upon repeated passaging of virus, however, after the first passage, Western blot assays did not detect the presence of envelope glycoprotein. Our observations were similar to those of a group that had genetically modified Chikugunya virus (CHIKV), another alphavirus, to function as an attenuated vaccine candidate (197). In their report, they found that CHIKV/IRES (their modified attenuated vaccine candidate) consistently formed heterogeneous plaques in plaque assays after 10 passages, but virus was detected by RT-PCR only after the first passage.

We proceeded to subject virus passages to repeated plaque purification until R2 envelope glycoprotein or eGFP was detected for at least three passages. Plaque purification was performed with help from P. Zhang and Z. Zhang. Virus populations in the first two passages were often mixed; some, but not all plaques were Western blot positive for envelope glycoprotein. Figure 20 shows a schematic of this process.

Figure 20: Plaque purification stages



Western blot results for the detection of envelope glycoprotein in cell lysate and supernatant of cells transfected with FMV-gp160, FMV-gp160 Δ CT, and FMV-gp140 are shown in Figure 21. Additionally, passage by passage Western blot results for two FMV-gp160 Δ CT clones that were subject to three rounds of repeated plaque purification are shown in Figure 22. Florescence microscopy images of cells infected with plaque purified third passage FMV-eGFP virus are shown in Figure 23.

Figure 21: Western Blot of R2 Env Expression in Transfected BHK-21 Cells

Western blot was performed (by L. Yan) on infected BHK-21 cell lysate and supernatant to assess R2 envelope glycoprotein expression after transfection. Polyclonal rabbit anti-gp140 antiserum (R2143) was used for immunodetection. Blue boxes indicate the expected position of gp140.

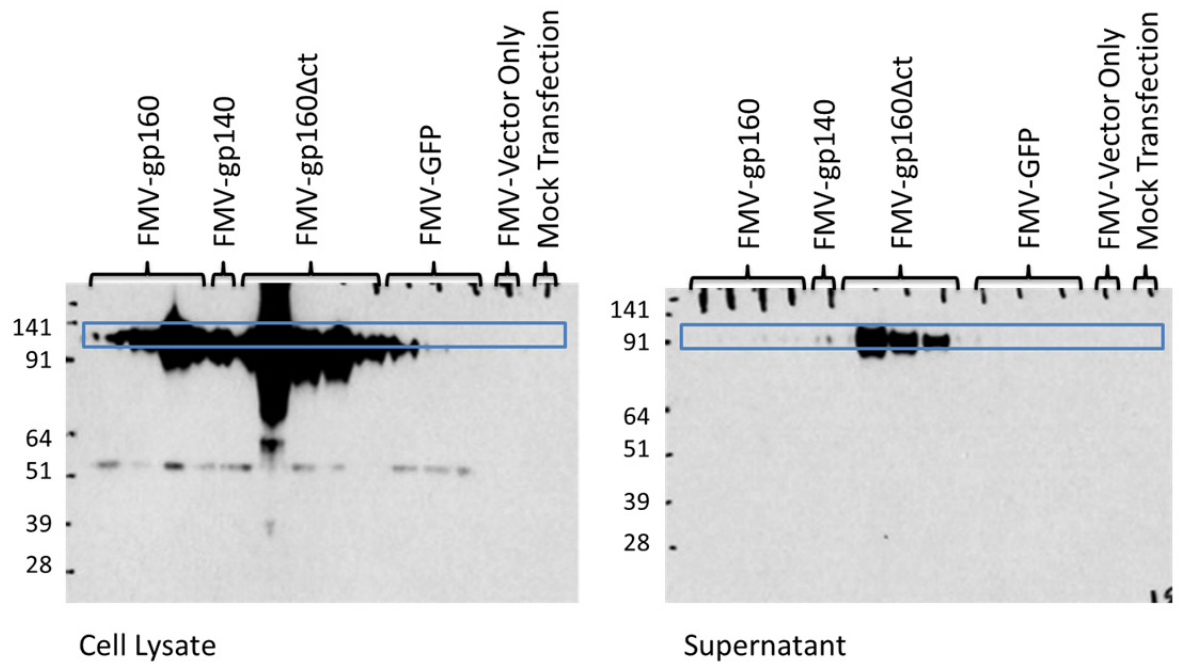


Figure 22: Western blot results by passage for two plaque purified FMV-gp160 Δ CT clones

For clones #7 and #14 of FMV-gp160 Δ CT, infected BHK-21 supernatant (S) and cell lysate (C) for each plaque purification passage (P1, P2, and P3) were probed for the expression of R2 gp160 Δ CT envelope glycoprotein by Western blot (performed by L. Yan). Polyclonal rabbit anti-gp140 antiserum (R2143) was used for immunodetection.

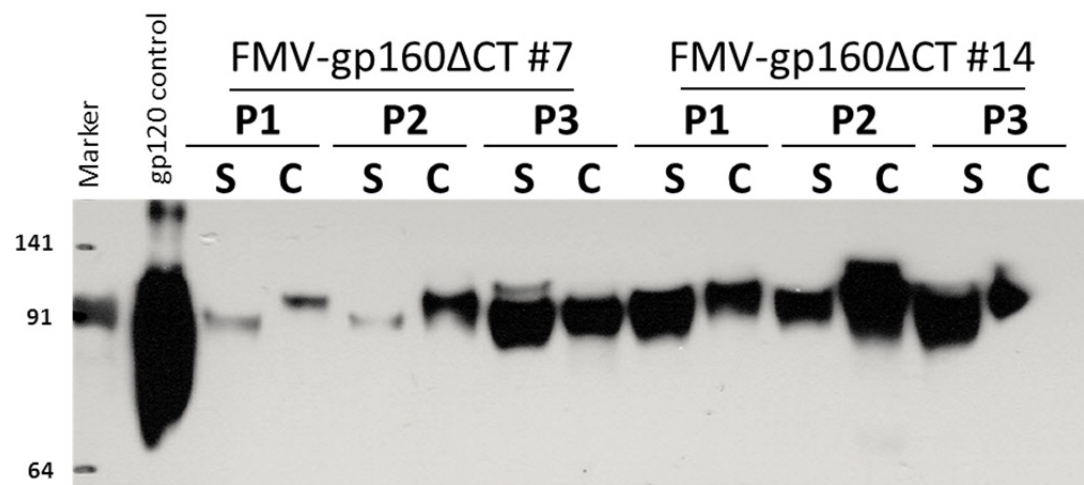
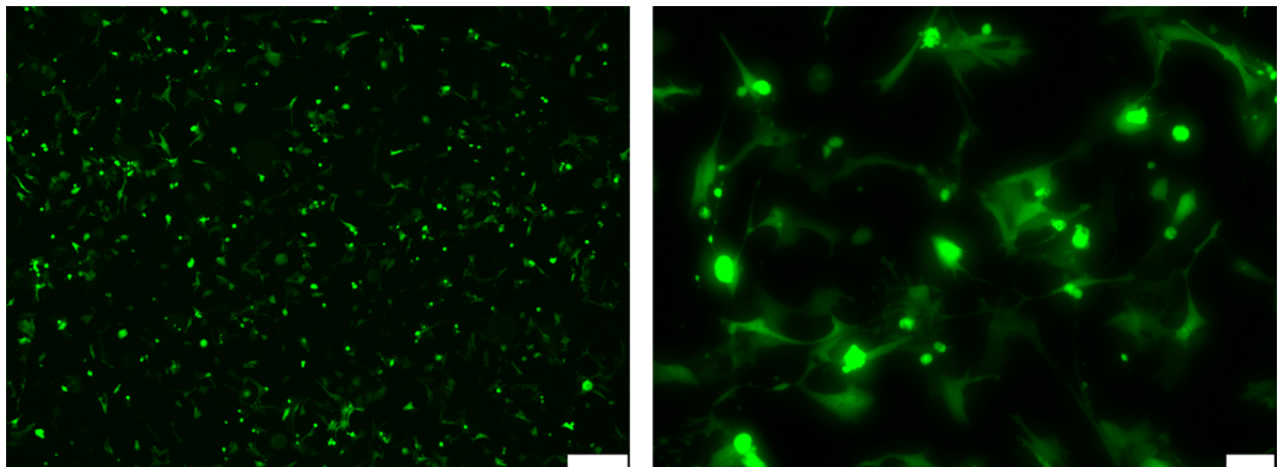


Figure 23: Fluorescence microscopy images of BHK-21 cells infected with FMV-GFP
5x magnification (left) and 20x magnification (right)



These results demonstrate a novel and significant accomplishment with respect to the stability and homogeneity of our plaque purified recombinant FMV clones, which are able to consistently express HIV-1 envelope glycoproteins and eGFP in successive passages *in vitro*.

CHAPTER 4: General Discussion

This project was conducted to compare the prevalence of broadly cross-reactive neutralization (BCN) among different HIV-1 disease progression groups, explore connections between envelope glycoprotein phenotypes and induced neutralization in those with and without BCN, develop an alphavirus-based replication-competent potential HIV-1 vaccine vector, and to evaluate the safety and immunogenicity of the vector in rabbits. Each of these elements is relevant in the pursuit of a suitable immunogen for a global HIV-1 vaccine.

Studies conducted over the past 20 years present conflicting findings regarding the presence of broadly cross-reactive neutralizing antibodies (bcnAbs) in different HIV-1 disease progression groups. Differences in methodologies involving assays and virus isolates, cohort size and composition, and definitions of breadth, potency, and disease progression group have indeed contributed to discrepant results.

Early assessments of neutralization tested patient sera against primary isolates of HIV-1 from other patients (175; 194). More recently, standardized luciferase-reporter based pseudovirus assays have been used to test the neutralization ability of patient sera against panels of recombinant viruses (165; 202). The selection of individuals to study was varied. In one study, 33 patients were selected from three sources: the Multicenter AIDS Cohort Study, the San Francisco City Clinic Cohort Study, and the lymph-node study of the National Institute of Allergy and Infectious Diseases (189). In another study, 113 patients from different disease progression groups were selected from a large multicenter cohort (67). And yet another study compared 22 controllers and 13 viremic patients without further stratification of disease progression groups (145). It is not

surprising that some (35; 175; 189; 194) but not all (67; 145; 171; 262) have found bcnAbs in LTNP.

In 2009, when we first started planning this study the number of HIV-1 cross-reactive monoclonal antibodies was small. These so-called first generation mAbs were mapped to a few epitopes which included the CD4bs, CD4-induced, glycans, MPER, and V3 (130). In the past five years, the employment of single cell antibody cloning from patients with broad neutralizing sera has accelerated the discovery of more potent and broad, second generation mAbs and their epitopes. Recently identified epitopes include glycan-dependent residues in V1V2 and V3, a non-autoreactive epitope on MPER, and a quaternary glycan-dependent epitope spanning both subunits of gp120 and gp41 (17; 75)

Understanding the mechanism by which bcnAbs develop in infection is a priority in HIV-1 vaccine development. There is a growing consensus, strengthened by the results of the RV144 vaccine study, that induction of bcnAbs will be necessary for a protective HIV-1 vaccine. Patients who possess bcnAbs may serve as a source for the isolation of broad and potent monoclonal antibodies, and virus envelope glycoproteins that may induce such antibodies.

Evaluation of NHS cohort for bcnAbs

We compared the cross-reactivity and specificity of neutralizing antibody responses in HIV-1 infected patients of different disease progression groups in the US Military HIV Natural History Study (NHS). To the best of our knowledge, our study is the first systematic comparison completed in a large, non-risk factor based cohort. Unlike cohorts that are composed of high risk individuals, the NHS cohort enrolls active duty military and eligible beneficiaries of the US Military health care system. Individuals

of the 5,400+ member NHS cohort, receives treatment at military health care facilities approximately once every six months. Further distinguishing this unique cohort is that treatment of enrolled patients occurs in parallel, facilitating more appropriate comparisons among different disease progression groups.

We first screened a selection of patients from all disease progression groups in the NHS cohort for bcnAbs. We found that long term non progressors (LTNP) were not more likely than any other group to possess bcnAbs. In our analysis, 7- and 10-year LTNP were not statistically different when median neutralization rank were compared. We did, however, find that elite controllers (EC) were more likely to be poor neutralizers, their prevalence enriched in the bottom quartile of all individuals tested. To a lesser extent, rapid progressors were also poor neutralizers. These latter results generally concur with those of other groups, reaffirming that EC are poor neutralizers and that bcnAbs are induced in approximately 20% of individuals 2-3 years after infection (66; 73; 192; 262). Furthermore, our results add to the hypothesis that some antigenic stimulation is required for the development of bcnAbs, though this proposed mechanism merits further investigation, as some EC have been found to possess bcnAbs (145).

Ultimately, the confirmation that EC are more likely to be poor neutralizers aids those involved with the reverse vaccinology approach to finding novel monoclonal antibodies or an immunogen for a HIV-1 vaccine, by reprioritizing the screening of HIV-1 infected patients for “elite neutralizers.”

Our sampling of one timepoint 2-4 years post seroconversion was appropriate given that neutralization breadth takes 2-3 years to develop. Neutralization screening of sera collected from selected individuals (from the top and bottom of the neutralization

ranking) at additional timepoints could potentially yield more information about the kinetics of the induction of bcnAbs, including when peak potency and breadth develop, and whether differences exist among different disease progression groups.

In our neutralization screen, we selected a virus panel that was similar to panels that have been used to assess serum neutralization ability in other studies (242; 275). Though we were unable to obtain identical virus strains, some of which are used exclusively by commercial laboratories, we matched the composition of subtypes and neutralization sensitivity tier as closely as possible. Therefore, we are confident that the six best and six worst neutralizers (with available samples) identified in our screen were indeed, among the best and worst neutralizers of the cohort.

HIV-1 envelope glycoprotein neutralization phenotypes and mutations

Though it has been reported that broadly cross-reactive neutralization antibodies (bcnAbs) are found in about 20% of HIV-1 infected individuals, the mechanism by which bcnAbs are induced remains unclear (262). It has been reported that a small number of viruses establish HIV-1 infection; 80% of HIV-1 infections are initiated by a single transmitted/founder virus (126). In a study of the co-evolution of broadly cross-reactive neutralization and a transmitted/founder virus, an accumulation of virus mutations were tracked prior to the development of neutralization breadth (154). Viral sieve analysis of the RV144 study—the first protective HIV-1 vaccine trial—reported increased vaccine efficacy if infecting strains of HIV-1 matched or mismatched the RV144 vaccine at certain residues within the V2 region (221). Taken together, these results suggest that the envelope glycoprotein from a single transmitted/founder virus can stimulate a protective antibody response in natural infection, and potentially, by vaccination. Finding

additional connections between the envelope glycoprotein and induced neutralizing antibody response may inform immunogen design.

Cloning of functional envelope genes from the best and worst neutralizers

For this part of the project, approximately 10,000 bacterial clones were processed through spin column purification and sized via agarose gel electrophoresis for the presence of envelope gene insert. Positively sized clones containing inserts were digested with restriction endonuclease for confirmation, resulting in nearly 2,000 clones to screen for infectivity. Of these, 173 clones appeared to be functional, albeit some low titer, after production in 24-well plates and screening in infectivity assays. We proceeded with nucleotide sequencing and neutralization sensitivity assays for the 173 clones in parallel, expediting the generation of data prior to confirming functionality of clones. (Our typical laboratory workflow is to confirm function of clones in 25cm² flasks before proceeding with nucleotide sequencing or neutralization assays.) Over 2,350 sequencing runs were completed. Analysis of the translated envelope sequences revealed the presence of frameshift mutations and premature stop codons in the majority of clones (116 out of 173, 67%).

The low number of genetically ‘normal’ (without frameshift or early stop codon) clones as well as functionality of clones obtained (after production in 25cm² flasks) was similar to our prior experience in our laboratory. Though 10-19 clones were obtained for each patient, a total of 31 clones (0-9 for each patient) were functional. Variation in the number of clones generated for each patient may be due to differences in the quality of the plasma sample after long-term storage.

We have identified four areas for further investigation: First, the number of false-positives when screening pseudovirus clones produced in 24-well plates was higher than desired. Taking this into account, a higher infectivity cutoff should be used in future screening of pseudovirus produced in 24-well plates. Screening should be repeated for confirmation of infectious clones. Second, based on our prior experience, it is likely that screening and testing additional clones will yield some high titer infectious clones. Third, perhaps there is a relationship between the presence of genetically defective virus (virus containing frameshifts and/or premature stop codons) and the ability to induce bcnAbs. Additional cloning efforts, and perhaps implementing SGA, may yield more evidence to support this observation. Finally, there is a chance that some genetically defective viruses were the result of Taq-induced errors or recombination events that occurred during bulk PCR.

When we designed this study, anecdotal reports suggested that virus clones obtained by single genome amplification (SGA) were more likely to be functional (F. McCutchan, personal communication). However, after we started using SGA to clone patient envelope genes, we found that none of the SGA clones were infectious.

Our difficulties in implementing SGA were likely related to the quality or quantity of viral RNA or cDNA used as starting template. RNA extraction from patient plasma, initially conducted using spin column purification, recovered very low concentrations of RNA. As a result, reduced efficiency impacted every subsequent step including reverse transcription to produce cDNA, and dilutions of nested PCR for SGA. Furthermore, none of the 22 clones we generated via SGA from three patients were

infectious. Facing limited time and resources, we opted to proceed with bulk PCR, which uses undiluted template, as an alternative to SGA.

Despite our experience with SGA, this more resource-intensive approach may have proven more beneficial in generating functional clones with intact genomes, as single genome clones could have been sequenced and verified before bacterial cloning. A recent anecdotal report suggested that transformed bacteria used for cloning may select for defective sequences, though this mechanism is unclear (B. Keele, personal communication). Using SGA would have also provided data on viral diversity.

From our (bulk) cloning and sequencing efforts, we obtained functional intact envelope clones for 10 of the 12 patients. We analyzed both the sequence data and neutralization phenotype of 31 clones, looking for unusual features between the top and bottom neutralizers that may be associated with the induction of bcnAbs.

Differences in neutralization epitopes between top and bottom neutralizers

Analysis of consensus sequences for the cloned envelopes reveals evidence of positive selection in the top but not the bottom neutralizers. More frequent mutations were observed in sequences of clones from the top six patients.

Two of the top six patients, VC39 (#8), and VC33 (#11), developed neutralization resistance mutations in the MPER region to Z13e1, and in V1V2 to PG9/PG16, respectively. Neutralizing epitopes in MPER are exposed during membrane fusion at the time of virus entry, however antibodies to MPER are rarely produced in natural infection, possibly due to autoreactivity with host phospholipids (16; 107; 310).

The V1V2 epitope has recently gained attention following molecular sieve analysis of sequences from the RV144 vaccine trial, and in the tracking of antibody and

envelope co-evolution in a patient (CAP256) with V1V2 neutralizing antibodies (68; 217; 221). For the RV144 study, virus sieve analysis identified increased vaccine efficacy in part, if infecting strains of HIV-1 matched the RV144 vaccine at residue K169 (221). In the CAP256 donor, superinfecting virus sequences were found to contain a rare K169I mutation preceding the development of neutralization breadth (68). The CAP256 donor also possessed envelope clones that were able to engage a germline or UCA naïve B cell line that ultimately produced bcnAbs. Changes in net charge of the V1V2 region can also affect sensitivity to the mAbs PG9/PG16, and can be indicative of positive selection (65; 68).

In our comparison of V1V2 epitope sequences between top and bottom neutralizers, we found that two of the top six neutralizers, VC39 (#8), and LTNP7-19 (#7) possessed the rare K169I mutation as described in the CAP256 donor (68). Additionally the net charge of the V1V2 region varied from +1 to +4 in the top six neutralizers, but was always +3 among the bottom six, providing evidence of positive selection in the top six.

The N332 residue in V3 was present in clones from all patients, indicating neutralization sensitivity to PGT121/PGT126 antibodies. Indeed, this result was confirmed in our neutralization sensitivity assays.

Early envelope clone phenotype and induced neutralization specificity

We probed neutralization phenotype of cloned envelopes from early plasma samples by testing neutralization sensitivity to a panel of monoclonal antibodies with known epitope specificities. Nearly all of the 31 clones tested were sensitive to neutralization by at least one mAb for each of the epitope specificities tested (MPER, CD4-induced, V1V2, V3, and CD4 binding site). Clones from the top and bottom six neutralizers were neutralized similarly; however, individual patients from each of the top and bottom groups possessed clones that were highly resistant to certain mAbs.

Overall, clones from top neutralizers were more sensitive to mAbs targeting CD4-induced epitopes than were clones from bottom neutralizers (6/16 vs 2/15 clones). Differences in neutralization sensitivity in these CD4-induced epitopes may contribute to differential induction of bcnAbs, though further studies are necessary to investigate this possibility.

On the envelope glycoprotein, the conserved CD4 binding site (CD4bs) and conserved N-linked glycans provide epitopes for broad and potent monoclonal antibodies. We tested sera taken 2-4 years post-seroconversion from the top six and bottom six neutralizers for the presence of antibodies directed towards the CD4bs, and epitopes recognized by the mAbs PG9/PG16 and PGT121/PGT126. Our results strongly suggested the presence of certain neutralizing antibody specificities in sera from two of the top six neutralizers. We were not able to determine serum specificities in sera from the other patients.

Juxtaposing neutralization sensitivity with serum specificity data, we examined whether certain early envelope neutralization phenotypes were associated with patterns of neutralization epitope specificity in sera collected 2-4 years post seroconversion.

Early envelope clones from one of the top six neutralizing patients, VC30 (#6), were highly resistant to most CD4bs mAbs and to PGT121 but highly sensitive to PGT126. Paradoxically, serum analyzed from the same patient, was found to possess both CD4bs-directed and PGT121/PGT126-like antibodies. Without further data, it is unknown whether the antibody specificity developed before or after the appearance of these particular envelope clones. Examining the temporal relationship between neutralization resistant infecting (or superinfecting) viruses and the subsequent induction of bcnAbs was outside the scope of this project, but certainly merits further investigation. Elucidating these relationships will inform vaccine design.

We have identified several interesting points in our analyses of early envelope phenotype and genotype, and induced neutralization specificity. Ongoing efforts to generate more functional intact clones will indeed provide relevant context for these observations, and allow us to further evaluate whether differences exist in the neutralization phenotypes and/or amino acid sequences of envelope glycoproteins from patients with and without bcnAbs.

Alphavirus vector development

We have developed a potential replication competent alphaviral vaccine vector based on Fort Morgan Virus (FMV) that stably expresses HIV-1 envelope glycoprotein or green fluorescent protein (GFP) *in vitro*. To the best of our knowledge, this FMV vector is one of only two non-attenuated, replication competent vectors that have been developed for HIV-1 (the other being adenovirus serotype 4, Ad4), and is a notable accomplishment in light of the combined size of the FMV RNA genome with HIV-1 transgene (~14kb) and the geometric requirements for alphavirus particle assembly (180).

A replication competent vector for HIV-1 provides many desirable advantages. Alphavirus replicon systems have been demonstrated as safe and efficacious. They produce high levels of protein expression and target dendritic cells (DC), important aspects of a potential vaccine. Alphavirus vectors have been shown to activate DC, contrasting with adenovirus and vaccinia vectors which inactivate DC by inhibiting DC maturation (15; 69; 71). Furthermore, alphaviruses are trafficked by DC to draining lymph nodes as early as 30 minutes following subcutaneous infection (88). Within 24-48 hours, apoptosis ensues, releasing additional antigen to other antigen presenting cells in the lymph node (152).

Possessing the advantages of other alphaviruses, Fort Morgan Virus (FMV), is also not known to infect humans and is non-pathogenic in laboratory animals. In general, humans are likely to lack pre-existing immunity to FMV. FMV was an ideal candidate for development as a potential replication competent vaccine vector, as subsequent rounds of infection could provide extended antigenic stimulation of humoral and CTL immune responses, potentially resulting in more durable immunity as seen in other live viral vaccines.

Pilot study of FMV vector in rabbits

We have shown that infectious molecular clones of FMV-WT and FMV-HIV envelope glycoprotein are tolerated in a rabbit model of infection. None of the animals displayed obvious abnormalities. Antibody binding to gp140 (as measured by ELISA), was slightly higher in two animals (one FMV-gp160 Δ CT and one FMV-gp140) than the average of two animals immunized with FMV-WT. Although this result was statistically significant, further confirmation is needed for several reasons: First, due to logistical reasons, the duration of our rabbit experiment (8-9 days) was likely too short to allow for a mature immune response to develop within the animals. It is possible that some of the animals may not have been non-responders if the experiment was continued for additional period of time, or if subsequent booster immunizations were administered. Second, we were able to investigate viral stability only after the rabbits were immunized. Since we found heterogeneous populations of virus in plaque assays, the experiment should be repeated with stable, repeatedly plaque purified virus.

We attempted to but were unsuccessful in recovering virus from dissected brain, spleen, or liver tissues from the rabbits. In a mouse pathogenicity experiment conducted previously by our laboratory, BALB/c mice were immunized either in the footpad (FP) or intraperitoneally (IP) with wild type FMV or molecular clones of FMV (produced from pFM). After 3, 5, 7, 10, or 14 days, the mice were sacrificed, and blood, brain, spleen, and liver were collected. Attempts at recovering virus from dissected brain, spleen, or liver tissues from these mice were also unsuccessful. These results are consistent with other results (A. Powers, personal communication).

For future development, quantifying levels of protein expression, and characterizing the extent of humoral and CTL immune activation are important priorities.

Additionally, building multivalent constructs, and testing expression of immunogens for other pathogens could expand the usability of the expression vector.

Overall conclusions

It is clear from a review of phase IIb and III HIV-1 vaccine efficacy trials that additional development of vaccine immunogens is needed. An ideal HIV-1 vaccine would protect against the acquisition of diverse strains of HIV-1 by inducing broadly cross reactive neutralizing antibodies (bcnAbs) as well as cell-mediated immunity. Broadly cross reactive monoclonal antibodies have been discovered as a result of natural infection, suggesting that such antibodies could be artificially induced. Recent research has applied a reverse vaccinology approach whereby bcnAbs from elite neutralizers have been used to determine corresponding recognition epitopes on the envelope glycoprotein.

We have shown that bcnAbs are equally likely to be found among all but one disease progression group in the US Military HIV Natural History Study; Elite controllers in our cohort were generally the worst neutralizers, consistent with many other reports. Paradoxically, however, some elite controllers have been reported to possess bcnAbs.

We have compared envelope neutralization phenotypes and serum neutralization specificity in patients with and without bcnAbs. We have found differences in neutralization sensitivity to mAbs targeting CD4-induced epitopes between top and bottom neutralizers. We have identified a patient with bcnAbs who possessed CD4bs directed and PGT121/PGT126-like serum neutralizing antibodies 2-4 years post-seroconversion, and early envelope clones that were highly resistant to neutralization by most CD4bs mAbs and PGT121. Additionally, in this patient and in other top neutralizers, we have described mutations affecting neutralization sensitive epitopes, providing evidence of positive selection in those who have bcnAbs.

We hypothesize that the induction of bcnAbs may be related to the presence of certain neutralization resistant envelopes early in infection, similar to another report

tracking virus and antibody co-evolution (68). This remains an important area for further investigation.

Ongoing efforts are being made to generate additional functional clones to further investigate connections between early envelope phenotype and genotype and induced neutralization. Our results should aid the search for an ideal immunogen by revealing some potential mechanism by which bcnAbs are induced.

We have developed an alphavirus based, replication competent vaccine vector that could be used to express HIV-1 envelope glycoproteins in humans. Since wild-type Fort Morgan Virus may not be pathogenic in humans, we expect the same to be true with our infectious molecular clone. This platform brings many desirable features of a vaccine vector, including broad host cell targets, high antigen expression, dendritic cell activation and trafficking, and natural adjuvant effects. We completed a proof of concept that needs to be further characterized with additional animal testing.

Both areas of investigation in this dissertation research improve on HIV-1 vaccine design. Findings from understanding the connection between the HIV-1 envelope and the induced antibody response may inform the design of immunogens for future vaccines. Additionally, the development of non-pathogenic, replication competent vaccine vectors can provide additional means to stimulate both humoral and cell-mediated arms of the immune system.

CHAPTER 5: References

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