Modulating the Antigenicity and Immunogenicity of HIV-1 Envelope by a Conserved N-Linked Glycan

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Abstract

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HIV-1 establishes persistent infection in part due to its ability to evade host immune responses. Occlusion by glycans contributes to masking conserved sites that are targets for some broadly neutralizing antibodies (bNAbs). Previous work has shown that removal of a highly conserved potential N-linked glycan (PNLG) site at amino acid residue 197 (N7) on the surface antigen gp120 of HIV-1 envelope glycoprotein (Env) increases neutralization sensitivity of the mutant virus to CD4 binding site (CD4bs)-directed antibodies and enhanced the ability of Env to generate cross-reactive neutralizing responses as compared to its WT counterpart. However, it is not clear if the role of the N7 glycan is conserved among diverse HIV-1 isolates and if other glycans in the conserved regions of HIV-1 envelope glycoprotein (Env) display similar functions. In this work, I examined the role of conserved PNLGs on the antigenicity of HIV-1

Env, particularly the role of the N7 glycan, in a panel of HIV-1 representing different clades, tissue origins, coreceptor usage, and neutralization sensitivity. I demonstrate that the absence of the N7 glycan increases the sensitivity of diverse HIV-1 isolates to CD4bs- and V3 loop-directed antibodies, indicating that the N7 glycan plays an important and conserved role modulating the structure, stability or accessibility of bNAb epitopes in the CD4bs and coreceptor-binding region, thus representing a potential target for immunogen design. I also examined if Tier 2 neutralizing antibodies and V1/V2 directed non-neutralizing antibodies previously correlated with protection in a clinical HIV-1 vaccine could be elicited by a poxvirus prime-gp120 boost strategy in a rabbit model using Env with or without the N7 glycan. I demonstrate that immunized rabbits generated cross-reactive neutralizing activities against >50% of Tier 2 global HIV-1 isolates tested in addition to V1/V2 binding antibodies. These findings demonstrate that antibody responses that have been correlated with protection against HIV-1 acquisition in humans can be elicited in a preclinical model by a poxvirus prime-gp120 boost strategy. However, the N7 glycan had little or no impact on Env immunogenicity in the context of the poxvirus prime-gp120 boost used, indicating the need for further improvements in immunization strategy by optimizing variables such as the nature of the priming and boosting immunogens. This work helps to define some of the parameters that may affect the antigenicity of Env and inform HIV-1 vaccine design.

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Chapter 1

Introduction

Human Immunodeficiency Virus (HIV)

HIV, the etiologic agent of acquired immunodeficiency syndrome (AIDS), is a member of the *Retroviridae* family, *Lentivirus* genus (1-3). HIV-1 emerged in humans through cross-species transmission on at least seven occasions from apes and monkeys infected with simian immunodeficiency virus (SIV), a retrovirus that can infect non-human primates. SIV infections in natural hosts are generally non-pathogenic. However, SIV infection in unnatural non-human primate hosts can lead to simian AIDS. Virus strains from chimpanzees infected with SIV_{cpz} are believed to have crossed the species barrier into humans, resulting in HIV-1, the major pandemic form of HIV. HIV-2, a lesser pandemic version of HIV, is believed to have been transmitted from sooty mangabeys infected with SIV_{sm} (4-8). At the end of 2014, an estimated 78 million people have become infected with HIV and 39 million people have died due to AIDS-related illnesses (www.unaids.org, 2014 Global Report). In addition to preventative interventions such as male circumcision and vaginal microbicides (9-12), the development of antiretroviral therapy (ART) programs averted an estimated 7.6 million deaths between 1995 and 2013. However, only 41% of all adults living with HIV had access to treatment in 2014 (www.unaids.org, 2014 Global Report). Because of the inaccessibility of therapy and the emergence of drug resistant HIV-1 due to poor adherence, ART treatment remains a suboptimal and expensive intervention. Given these limitations, and the fact that an estimated 2 million people became newly infected with HIV in 2014, an effective vaccine would be a practical and cost effective strategy to halt the spread of the HIV/AIDS pandemic. Of the vaccines that made it to human clinical trials, only one was moderately efficacious (~31%, RV144 Trial) (13) and, to date, no vaccine regimen has generated broadly neutralizing antibodies (bNAbs) capable of preventing infection in humans. Generating a

vaccine capable of eliciting a protective immune response against HIV-1 infection has been difficult in part due to the genetic and biological diversity of HIV-1. Because of HIV-1's diversity, it is generally believed that an effective prophylactic HIV-1 vaccine must elicit bNAbs against diverse primary isolates of HIV-1. HIV-1 has evolved many protective mechanisms to evade host immune responses, including occlusion of potential targets by glycans (14-20), rendering it difficult to elicit antibodies targeting bNAb epitopes by vaccination or natural infection. The work presented in this dissertation defines a single N-linked glycan on Env that is highly conserved among diverse HIV-1 isolates that can modulate the antigenicity of Env to known bNAbs. Additionally, we attempt to generate antibody responses that have been associated with protection in the RV144 trial in a small animal model. In an attempt to better inform HIV-1 vaccine design, we examine how antibody responses may be influenced by the specific Env immunogen used and how the presence or absence of the N-linked glycan modulates the immunogenicity of Env.

HIV-1 Infection

The majority of HIV infection in adults results from a single virus transmitted via mucosal exposure (21, 22). While the risk of infection associated with different mucosal exposure routes varies (23), the early events resulting in a productive HIV-1 infection begin when the virus crosses the epithelial barrier of mucosal surfaces. $CD4^+$ T cells and Langerhans cells are thought to be the first cell types infected by HIV-1, but dendritic cells may help facilitate an active infection (24-26). After initial infection, the virus cannot be detected in plasma for 7 to 21 days even though HIV-1 is replicating in the mucosa, submucosa, and draining lymphoreticular tissues (21, 27-29). At the end of this time frame known as the eclipse phase, virus reaches the draining lymph node where they infect $CD4^+/CCR5^+$ T cells (**Figure**

1.1). The virus replicates and spreads rapidly throughout the body to other lymphoid tissues, particularly the gut-associated lymphoid tissue (GALT) where there are high numbers of activated $CD4^+/CCR5^+$ memory T cells (30-33). During the acute phase of infection, characterized by the sequential appearance of viral markers and antibodies in the blood (34), ~80% of $CD4^+$ T cells in the GALT are depleted within the first three weeks of HIV-1 infection. Once virus is detected, viremia can be suppressed, but not eliminated, by administration of antiretroviral treatment (ART). ART can restore normal $CD4^+$ T cell numbers in blood, but not in the GALT. Consequently, B cell responses are also impaired because the development of

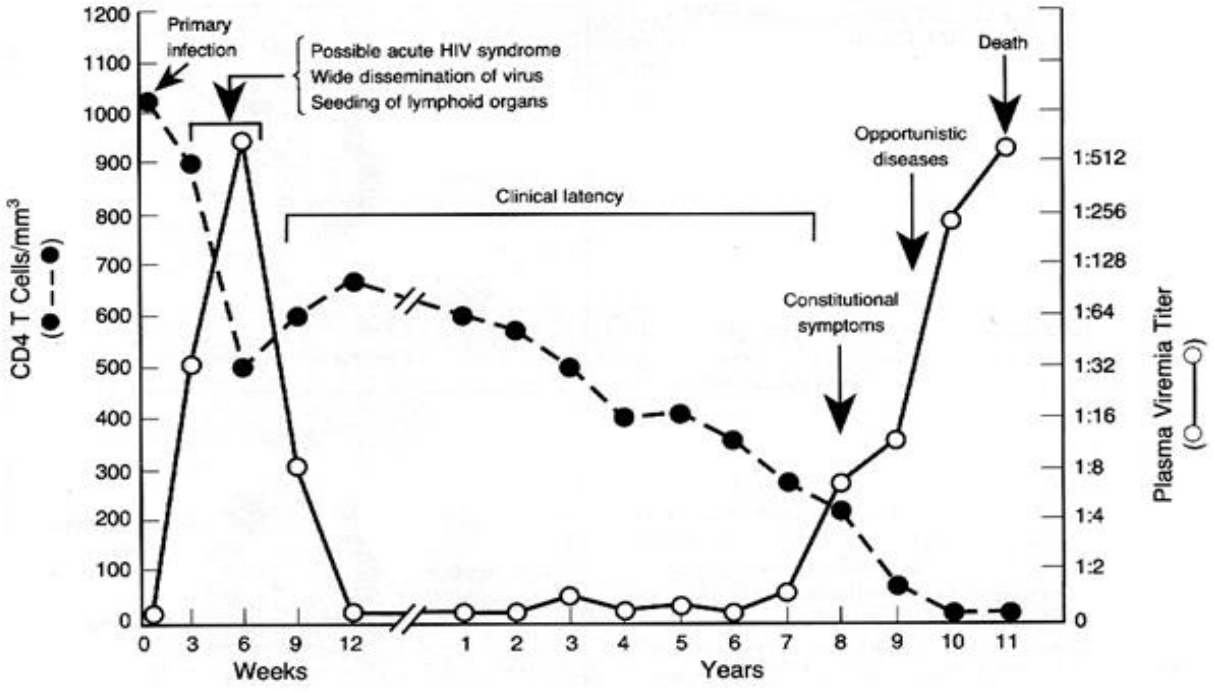

Figure 1.1: Typical course of HIV infection. After HIV-1 initiates infection at the mucosal sites, there is a widespread dissemination of virus and a concomitant burst in plasma viral load, which often coincides with a sharp decrease in the number of $CD4^+$ T cells in peripheral blood. High viral replication induces HIV-1 specific immune responses, subsequently reducing the plasma viral load. A prolonged period of clinical latency where reduced viremia is sustained for several years ensues. However during latency, the $CD4^+$ T cell counts continue to decrease to a critical level below which opportunistic infections arise, and death may occur. Reproduced with permission from reference (35).

germinal centers relies on $CD4^+$ T cell responses (36). While virus replicates in lymphoid tissues, plasma viremia peaks 21-28 days after initial HIV-1 infection (30, 32).

This burst in HIV-1 replication during the acute infection phase induces the adaptive immune responses, leading to a more stable viral load known as the viral set point during the clinical latency stage. This set point is thought to result from host replenishment of target cells and immune clearance of virus and infected cells, generating a balance between virus turnover and the immune responses (37, 38). This viral set point stage can be maintained for years without any clinical symptoms during the chronic infection phase. Eventually, immune exhaustion, disrupted T-cell homeostasis, and chronic immune activation and inflammation contribute to the development of AIDS and death occurs if no treatment is administered (39).

HIV-1 Genome and Lifecycle

HIV-1 has a 9.8kb genome consisting of two copies of single stranded positive-sense RNA that encode nine genes. The genes encoded include transcriptional transactivator (*tat*), regulator of virion (*rev*), viral infectivity factor (*vif*), viral protein R (*vpr*), viral protein U (*vpu),* negative factor (*nef),* group specific antigens (*gag*), polymerase (*pol),* and envelope glycoprotein (*env*). After the viral genome enters the host cell cytosol, viral RNA is reverse transcribed to double-stranded DNA by *Pol* encoded reverse transcriptase (RT) (40). The newly synthesized viral double stranded DNA is then translocated across the nuclear pore in a pre-integration complex catalyzed by Vpr (41). Once in the nucleus, the viral DNA is integrated into the host cell chromosomal DNA by *Pol* encoded integrase (40). Tat activates the transcription initiation and elongation of viral DNA (42), and Rev promotes the nuclear export, stabilization, and utilization of viral mRNAs in cytoplasm (43). These mRNAs are translated into viral proteins

using host cell translation machinery. Viral proteins, including capsid proteins encoded by *gag* that enclose the viral genome, along with two single-stranded copies of viral RNA assemble to form new immature virus particles (44, 45). Once immature virus buds from the infected cell, *pol* encoded protease cleaves the Gag and GagPol polyprotein precursors, triggering the conversion of the immature virus particle into the mature virion (40). *Vif* encodes a protein that promotes the infectivity of cell-free virus particles and triggers the degradation of antiviral proteins (46). *Vpu* encodes a protein that degrades CD4 in the endoplasmic reticulum and enhances virion release from the plasma membrane (47). *Nef* encodes a protein that downregulates CD4 and major histocompatibility complex (MHC) class I molecules (48). Most of these proteins and genes are important for viral replication, however, envelope glycoprotein (Env) initiates infection by interacting with host cell receptors, thus playing an important role in virus infection and cell-cell transmission. Env is the only viral protein exposed on the virus surface, and therefore, the lone target of protective antibodies that potentially block infection of target cells (49).

HIV-1 Envelope Protein Function and Dynamic Properties

The *env* gene encodes the viral envelope glycoprotein (Env) gp160, a polyprotein precursor that is translated from a singly spliced mRNA (50, 51). Concomitant with translation, gp160 is glycosylated with N- and O-linked oligosaccharide side chains in the rough endoplasmic reticulum (ER) (52, 53). N-linked glycans are covalently attached to Asn residues in the Asn-X-Ser/Thr glycan sequon, where X is any amino acid except Pro, while O-linked glycans can attach to an oxygen atom in Ser, Thr, and Cys residues. These glycosylated gp160s oligomerize in the ER before being trafficked to the Golgi complex. High-mannose oligosaccharide side chains on the glycoproteins undergo further complex modifications along the secretory path in the trans-Goldi network (TGN) (54-57). The addition of sugar residues to

the core glycan structure gives rise to three major types of N-linked glycans on Env: high mannose, complex, or hybrid glycans (58). In the Golgi, gp160 is proteolytically cleaved into the mature subunit glycoprotein gp120 and transmembrane glycoprotein gp41 by host cellular furin or furin-like proteases at the Lys/Arg-X-Lys/Arg-Arg motif (59, 60). The gp120 and gp41 subunits associate through noncovalent interactions. Three molecules each of gp120 and gp41 form a functional heterotrimeric HIV-1 envelope spike on the viral surface (**Figure 1.2**). Due to the relatively weak noncovalent gp120/gp41 bonds, gp120 can shed from the cell surface, generating a heterogeneous population of surface Env in the form of gp41 stumps in addition to uncleaved gp160 (61, 62). Additionally, the abundance of Env protein on the cell surface is highly regulated by an endocytosis signal in the gp41 region. This, in combination with the weak

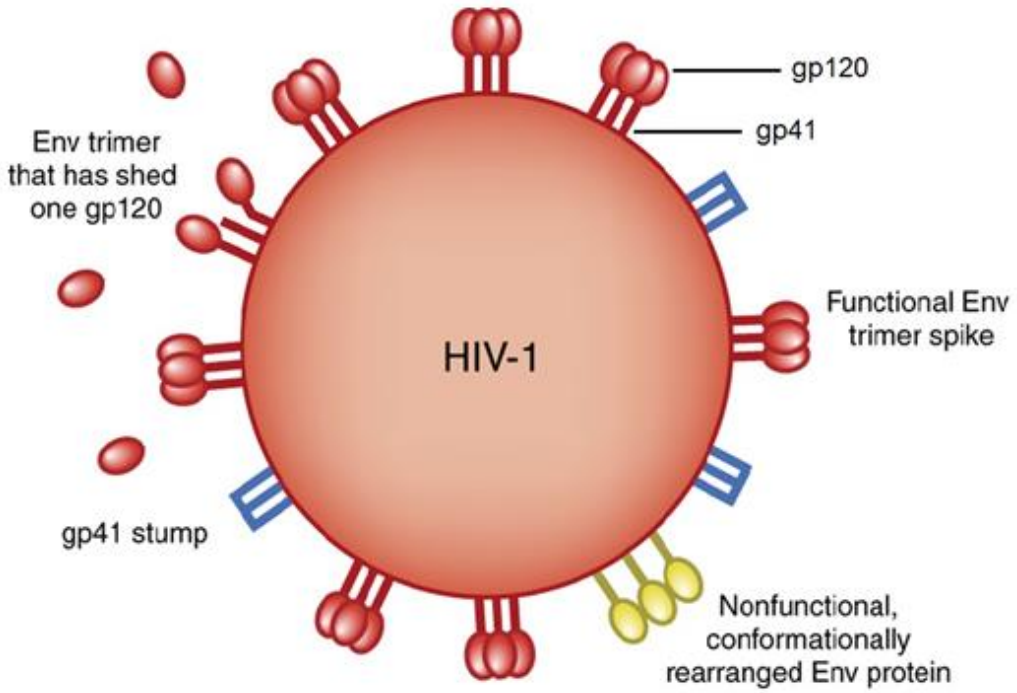

Figure 1.2: A representation of different forms of Env on the surface of the viral membrane. Three gp120 and gp41 domains linked through non-covalent interactions form the functional Env trimer. The gp41 transmembrane unit hooks the Env into the host-derived viral membrane. Other forms of Env are expressed on the virus including Env that has shed one or more gp120, gp41 stumps, and conformationally rearranged Env. These forms could also be expressed on infected cells. Other forms of Env protein may be expressed on HIV-1 virions. Adapted with permission from reference (63).

noncovalent gp120/gp41 bonds, contributes to the relatively low levels (~14 spikes, on average) of mature Env spikes on virus particles (59, 64).

The gp120 protein has five relatively constant domains (C1-C5) separated by five variable domains (V1-V5) (**Figure 1.3A**). A number of highly conserved Cys residues form intramolecular disulfide bonds crucial to the formation of Env. Some of these disulfide bonds border the variable regions, folding the V1-V5 domains into loops (**Figure 1.3B**) (53, 65, 66). Recombination, point mutations, insertions, and deletions contribute to the sequence variability in the V1-V5 regions. These domains, particularly the V1/V2 loop, can vary in loop length and the number of glycosylation sites (17, 67-71). In contrast, the C1-C5 domains show relatively little length variation. These conserved regions of gp120 help define the virus' CD4-tropism because the conserved domains interact with the virus receptor, CD4. The CD4 receptor binding site is formed from distal conserved domains, particularly C1, C3, and C4, folded in the Env tertiary structure (72-74). HIV-1 infects cells that express CD4 including $CD4^+$ T lymphocytes, macrophages & dendritic cells because the virus' entry process is initiated by the binding of surface gp120 to CD4, the primary receptor**.** Once gp120 binds CD4, the protein undergoes a conformational change that exposes the coreceptor binding site (75). The major coreceptors for HIV-1 are C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4). Viral tropism is, in large part, determined by gp120 binding affinity to these coreceptors (76). HIV-1 that primarily utilizes CCR5, referred to as "R5" viruses, predominantly infects monocytes/macrophages while virus that primarily uses CXCR4, referred to as "X4" viruses, infects T cell lines and primary T cells (77-80). Dual tropic (R5X4) strains can use either CCR5 or CXCR4 coreceptors to enter host cells. Most transmitter/founder (T/F) HIV-1 isolates are R5 viruses, but X4 isolates can arise during the late stage of infection. The switch to X4

tropism is typically associated with a decline in peripheral blood $CD4^+$ T cells and the onset of AIDS symptoms **(Figure 1.1)** (81, 82). The V3 loop may be critical for membrane fusion and coreceptor binding to CCR5 or CDXCR4 (83-85), but it is not directly involved in CD4 binding (86, 87). Mutations in the V3 loop can drive the switch from R5 to X4 tropism (88). The coreceptor binding site, including parts of the V3 loop, and CD4 receptor binding sites are highly conserved regions that are targeted by some bNAbs (89-92).

After gp120 binds the coreceptor, further conformational changes trigger a membrane fusion reaction mediated by gp41 that delivers the viral core into the host cell's cytoplasm. The gp41 transmembrane subunit of Env attaches to the gp120 subunits through non-covalent bonds (**Figure 1.2**) and mediates fusion between the virus and host cell plasma membrane (93). The gp41 subunit has three domains: an extracellular domain, a highly conserved transmembrane domain that anchors the Env in the lipid (94), and a C-terminal cytoplasmic domain **(Figure 1.3A)**. The C-terminal cytosolic domain influences Env endocytosis, virus infectivity, cell-surface Env expression, gp120 shedding, and Env-induced fusion (95-105). The extracellular N-terminal domain consists of a fusogenic peptide region, a polar region, a highly conserved Trp-rich domain that is targeted by several neutralizing antibodies (Nabs) referred to as the membrane-proximal external region (MPER) (106-108), and two hydrophobic regions that form α-helical coiled-coil structured regions referred to as the heptad-repeat regions HR1 and HR2 (109-118). After gp120 binds to the CD4 and the coreceptor, gp41 undergoes conformation changes that expose the fusion peptide. The fusion peptide penetrates into the target cell membrane, causing membrane destabilization (119). The HR1 and HR2 motifs form a stable six-helix bundle that brings the viral and cell membranes into close contact for fusion to occur (110, 113).

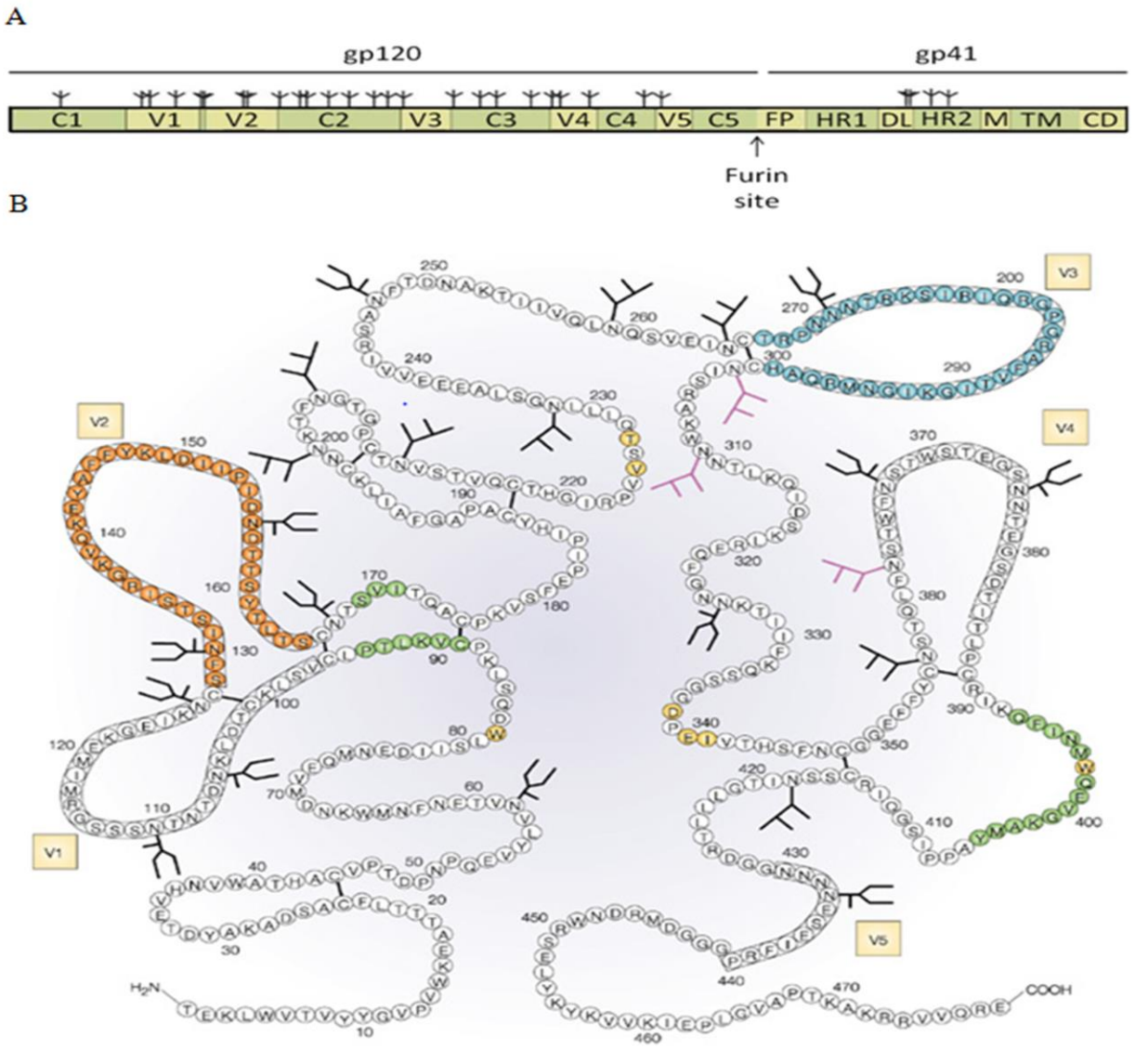

Figure 1.3: Primary structure of HIV-1 Env indicating the disulfide bonds and glycosylation sites: (**A**) Linear segments of Env are designated as follows: C1-C5, conserved regions 1-5; and V1-V5, variable regions 1-5; FP, fusion peptide; HR1, heptad repeat 1; HR2, heptad repeat 2; M, membrane proximal ectodomain region (MPER); TM, transmembrane domain; CD, cytoplasmic domain. Glycans are represented by tree-like symbols. Adapted with permission from reference (120). (**B**) The gp120 showing predicted disulfide bonds and the location of variable regions marked in boxes (V1-V5) is shown. The glycosylation sites containing high mannose-type and/or hybrid-type oligosaccharide structures are indicated by the branched structures, and glycosylation sites containing complex-type oligosaccharide structures are indicated by the U-shaped branches. Epitopes targeted by bNAbs are highlighted as follows: CD4-binding domain (yellow highlight), the CD4-induced epitope (green highlight), an epitope composed of high mannose residues (purple highlight), the V2 loop (orange highlight), and the V3 loop (blue highlight). Reproduced with permission from reference (121).

Antibody Response to HIV-1 Infection

The initial antibody response to Env usually appears 1 week after infection and typically consists of non-neutralizing antibodies (non-Nabs) that target sites on the gp41 stalk that do not select for viral escape (122) (**Figure 1.4**). These non-Nabs normally target nonnative forms of Env, thus limiting virus spread and infection through the action of its fragment crystallizable (Fc) region initiating destruction of the virus or infected cell by the innate immune system. Non-neutralizing antibody Fc effector activities include antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cell-mediated virus inhibition (ADCVI), or antibody-dependent cellular cytotoxicity (ADCC) (123). It is not known why the initial HIV-1 antibody response is directed against non-neutralizing epitopes, but it may be related in part to the immunodominance of denatured or non-functional Env on the virus surface compared to the relatively rare frequency of native trimers (62). These non-Nabs are insufficient to suppress viremia (122). By the time antibodies that neutralize the transmitted founder (T/F) virus are detected three months after initial infection, the virus has established latency in the host (15). The Nabs that target functional trimers prevent infection through the fragment antigen-binding (Fab) region recognizing Env and blocking functions that mediate virus entry into cells. The early Nabs typically neutralize the infecting virus strain (autologous virus) only and cannot neutralize more divergent viruses (heterologous viruses). These Nabs can be highly potent against the autologous virus, exerting selective pressure on the virus to generate escape mutants that are less sensitive to host-generated Nabs (15, 124). These escape variants arise rapidly and easily because the majority of autologous Nabs target epitopes on the variable loops of Env (15, 125). This results in an arms race between antibodies generated against new HIV-1 mutants and HIV-1 rapidly mutating, generating viral quasi-species to evade these antibodies. Mutations that arise in HIV-1 Env may result in the

exposure of more conserved regions, which may stimulate the production of Nabs that target these conserved regions of Env. Antibodies that target conserved epitopes on Env can neutralize heterologous viruses.

Neutralization breadth may arise by a gradual maturation or focusing of the Env-specific B cell response to a conserved epitope, or it may be due to the accumulation of Nabs directed against several distinct epitopes (126-128). The development of cross-reactive Nabs capable of neutralizing multiple heterologous viruses generally takes 2-4 years after serconversion (129). Approximately 20% of HIV-1 infected individuals develop cross-reactive sera antibodies capable of neutralizing different isolates. Among HIV-1 infected individuals, approximately 1% known as "elite neutralizers" develop bNAbs capable of neutralizing many different HIV-1 isolates (128). The characterized bNAbs recognize one of five conserved epitopes on the HIV-1 Env

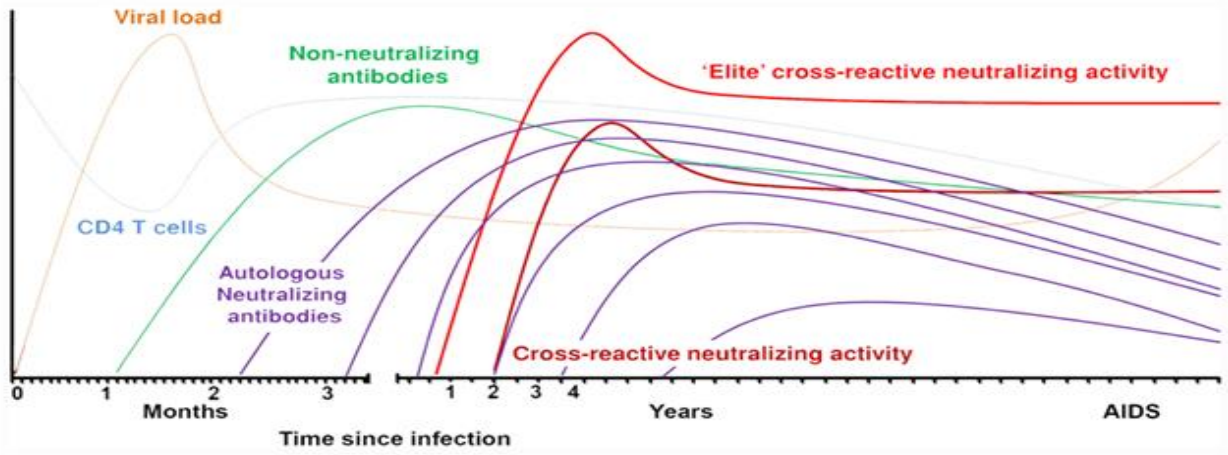

Figure 1.4: Typical B cell response to HIV-1 during the course of infection. Binding and non-Nabs are the earliest to be elicited after HIV-1 infection (green line) followed by a decline in viral loads (orange line) and a rebound in peripheral $CD4^+$ T cells (blue line). Several months later, strain specific autologous Nabs develop (purple line). About 2-4 years after seroconversion, heterologous antibodies (dark red) capable of neutralizing a broad range of circulating strains are generated in a subset of infected individuals (~20%). Even fewer infected individuals (~1%) develop "elite" cross-reactive bNAbs (bright red). HIV-1 variants that are more resistant to these host-generated Nabs are rapidly selected for and the host's viral set point is maintained. Modified from reference (130).

trimer: the CD4 binding site (CD4bs), N-linked glycans on the V1/V2 loops and the V3 loop, the MPER, and the newly described gp120/gp41 interface (**Figure 1.5 & 1.3b**). The time to develop bNAbs varies in different individuals, indicating that the differences in infection, genetic factors, and maturation of the antibody response to HIV-1 may be necessary for the generation of bNAbs. Nucleotide insertions and/or deletions in heavy and light chain genes as well as affinity maturation through extensive somatic hypermutation, a crucial process for the generation of many HIV-1 bNAbs, are rare in other antibodies. Many bNAbs also express unusually long third complementarity determining region of the antibody heavy chain (CDRH3) loops necessary to penetrate the glycan shield surrounding Env (discussed below). These processes may be delayed due to reduced $CD4^+$ T cell help during HIV-1 infection (131). The development of bNAbs is not only correlated with increased time after seroconversion, but also with higher plasma viral load. Therefore, bNAb activities develop over time and are fostered by chronic antigen exposure (126-128). Moreover, some potent bNAbs such as antibodies to the CD4bs, V1/V2, and MPER have been shown to be weakly poly- or self-reactive against self antigen. Antibodies that are self-reactive are typically selected against in healthy individuals. While the majority of the potent, naturally occurring HIV-1 bNAbs are not polyreactive and/or autoreactive (132, 133), the intensive somatic hypermutation and polyreactivity of antibodies in addition to the time it takes

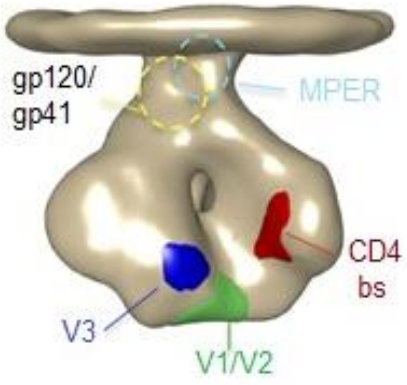

Figure 1.5: Epitopes recognized by broadly neutralizing antibodies. Dominant sites recognized by bNAbs are shown in the context of an EM tomogram from an HIV-1 viral spike. The viral membrane is positioned at the top of the spike. Approximate locations of bNAb binding sites including the CD4bs (red), V1/V2 loops (green), V3 loop (blue), membrane proximal external region (MPER, light blue), and gp120/gp41 interdomain (black) are shown in their respective colors and/or dotted lines. Modified from reference (134).

to generate bNAbs may constitute roadblocks for the immune system to generate bNAbs during a natural infection or immunization strategy.

HIV-1 Strategies for Evading Neutralizing Antibodies

As stated previously, Nabs are directed against the Env because it is the only viral protein displayed on the surface and it is highly immunogenic. HIV-1 uses various strategies to avoid or divert recognition by antibodies including: carbohydrate shielding and shifting (15, 135, 136); conformational masking (137); diverting the immune response from functional targets to nonfunctional envelope spikes such as gp120-gp41 monomers, gp41 stumps, or uncleaved gp160 precursors **(Figure 1.2)** (138, 139); steric occlusion (140); and low density of functional trimers on the viral surface (64). However, as stated previously, autologous Nabs are generated in infected individuals that can prevent infection of new $CD4^+$ T cells. This drives the generation of escape mutants to form the quasispecies of HIV-1 in a single host, the diversity among the nine subtypes of HIV-1 (A, B, C, D, F, G, H, J, and K), and the diversity of more than 35 circulating recombinant forms that can vary up to 20% within a subtype and 35% between subtypes (141). Escape from Nabs occurs through several pathways, including amino acid variations created by mutation processes such as deletions, insertions, and substitutions (17, 142, 143). The mutants are generated by RT during reverse transcription. The RT enzyme is an error-prone polymerase that can tolerate many mutations and generates fit variants that persist despite diverse host selection pressures. This enzyme is estimated to make about $3.4x10^{-5}$ mutations per site per generation and typically impacts the genetic diversity of Env.

Surprisingly, mutations in the *env* gene during acute infection are unexpectedly sparse, do not generally map to known neutralization epitopes, and instead primarily involve changes in the number and position of N-linked glycans (15). Despite enormous variation in HIV-1 viruses in

general, there are typically close to 25 potential N-linked glycan sites (PNLG) on the Env of primary isolates (144), suggesting strong selective pressure to maintain the high number of glycans on Env. The surface subunit gp120 is highly glycosylated with ~50% of the protein's molecular mass accounted for by O-linked and N-linked glycans (52, 145). These glycans contribute to Env folding as well as binding of the virus to the host cell surface. (146, 147). In addition, the N-linked glycans can mediate epitope occlusion and steric hindrance, which physically prevents Nab from accessing the antigenic polypeptide surface of the HIV-1 Env. These glycans can also modulate the exposure or stability of Nab epitopes, including highly conserved epitopes, resulting in viral escape (15, 148, 149). Other N-linked glycans are highly conserved across diverse isolates and are subsequently targeted by Nabs (150, 151). Virus mutations that modulate the position and number of glycans allow the Env to continue to bind necessary receptors, but potentially prevent Nab binding (15), play a role in virus evasion from host immune responses, and contribute to the overall diversity of HIV-1.

While the virus employs a multitude of methods to escape from recognition by Nab, there is evidence that the virus might have limits to its mutational tolerance because of inherent fitness costs resulting from mutation, especially to conserved regions that are necessary for infection. The error-prone RT can impact virus proteins required for viability, leading to a less fit or non-viable virus. However, despite the error prone RT and the presence of autologous Nabs in infected individuals, viremia persists, in part, due to the viral DNA being stably integrated into the host genome. This generates a latent viral reservoir as early as three days after transmission (152) that evades immune responses as the lack of viral protein expression protects it from antibody and cytopathic immune responses (153). Because of the presence of a viral reservoir and the generation of escape mutants, even infected patients who generate bNAbs do not clear

the infection (15, 124), indicating that an effective HIV-1 vaccine needs to prevent early infection events and the formation of a viral reservoir. This is a challenge since vaccine strategies historically do not prevent infection, but instead rely on memory cells developed during vaccination to rebound and subsequently clear the pathogen (154, 155). However, if a protective Nab response is induced early, it could potentially prevent early HIV-1 infection events.

Evidence for a Protective Role of Antibodies

Many licensed vaccines targeting viral pathogens elicit protective antibodies (156). Therefore, generating an HIV-1 vaccine capable of eliciting protective Nabs and non-Nabs is a major goal for the field. Bearing in mind that HIV-1 is primarily a sexually transmitted disease, it is important to consider designing strategies to block mucosal transmissions of the virus, potentially including the production of mucosa-associated immunoglobulin A (IgA) and immunoglobulin G (IgG). HIV-infected individuals and HIV-exposed individuals that are persistently seronegative can develop mucosal HIV-specific IgAs capable of blocking viral translocation through epithelial cells (157, 158). Furthermore, gp41-vaccinated macaques that developed gp41-specifc vaginal IgGs with neutralizing and/or ADCC activities and IgAs that block HIV-1 translocation were protected against repeated-high dose vaginal challenges of simian-human immunodeficiency virus (SHIV), a virus combining parts of the HIV and SIV genomes (159), indicating a protective role for mucosal anti-HIV neutralizing and non-Nabs.
In addition to mucosal antibodies, non-Nabs have been associated with protection in individuals termed long-term non-progressors who naturally control HIV-1 infection (160, 161). Non-Nabs that mediate ADCC and/or ADCVI potentially reduce the risk of mother-to-child transmission of HIV-1 (162) and passive transfer of non-neutralizing monoclonal antibodies or hyperimmune

serum provided partial protection against a challenge virus in macaques (163-166). Therefore, it may be beneficial to generate an HIV-1 vaccine that induces protective non-Nabs.

While the protective effect of non-Nabs needs further study, bNAbs have demonstrated efficient and long lasting therapeutic or prophylactic properties in animal models. Numerous studies have established that prophylactic passive transfer of bNAbs to macaques protects them from SHIV infection (167-178). Moreover, macaques and humanized mice were protected against mucosal challenge when bNAbs were expressed via vector-mediated gene transfer (179, 180), but it is unknown if HIV-1 Nabs correlate with protection in humans. Indeed, passive transfer of NAbs failed to control viral rebound in infected patients due to the emergence of HIV-1 escape variants (181, 182). However, next-generation human HIV-1 bNAbs are currently being tested in therapeutic human trials. While bNAbs need further testing to determine their therapeutic capacity, these data support the need for a prophylactic HIV-1 vaccine to generate bNAbs that potentially protect against infection of highly diverse circulating virus variants.

Approaches for Vaccine Development

Generating vaccine-mediated protection is a complex challenge. Long-term protection requires the persistence of vaccine antibodies and/or the generation of immune memory cells capable of rapid and effective reactivation after exposure to a pathogen. Effective vaccines typically require multiple immunizations in the form of prime-boost. Traditionally, the same vaccines are given multiple times as homologous boosts, but heterologous boosts using unmatched vaccine delivery methods while using the same antigen can be more immunogenic than homologous prime-boosts, potentially due to the heterologous nature of the immunogens used (183).

B and T cells are essential to the induction of high-affinity antibodies and immune memory that results in protection against the pathogen. B lymphocytes produce antibodies while other effectors, including cytotoxic $CD8^+$ T cells, may limit the spread of infectious agents by killing infected cells or secreting antiviral cytokines. Additionally, $CD4^+$ T cells provide growth factors and signals to help to maintain and generate B and $CD8^+$ T cell responses. Most vaccines trigger both B and T cell responses (184, 185).

In general, prophylactic vaccines against HIV-1 attempted to either prevent establishment of infection through generation of Nabs, or generate T cell responses that attenuate disease progression once infection occurs (186). Many approaches have been considered in an attempt to generate an effective vaccine against HIV-1 including live-attenuated, inactivated/killed, and subunit platforms. Live-attenuated vaccines using whole inactivated HIV-1 have not been seriously considered due to concerns of incomplete inactivation that would result in life-long infection. For instance, while vaccination of macaques with live, *nef* deleted SIV has shown protection against SIV infection (187-189), in vivo *nef* repair and reversion can occur (190). Therefore, vaccine efforts have been directed towards strategies that employ envelope protein subunits, HIV-1 protein expression via recombinant vectors, or DNA vectors.

Because of previous success with viral vaccines utilizing subunit proteins, some of the first clinical trials attempted, but ultimately failed, to generate bNAbs with recombinant HIV-1 Env protein subunits (191) (discussed below). Because of their failure, alternative proteins, including Env immunogens comprised of the ectodomain of gp120 and gp41 termed gp140 **(Figure 1.3A)**, have been used as immunogens. Alternatively, DNA vaccination strategies were also explored. Early attempts using naked DNA plasmid vaccines were effective at generating T cell responses, but, when given alone were poorly immunogenic and responses lacked durability

(192-195). Alternative strategies use DNA as a prime or boost in an immunization regimen, potentially in combination with subunit proteins or viral vector constructs such as adenovirus or poxvirus.

The use of recombinant adenovirus constructs expressing HIV-1 proteins has also been extensively tested in non-human primates. Adenovirus serotypes, including adenovirus type 5 (Ad5) and those that are less commonly associated with human disease, that express HIV-1 proteins have been shown to induce T cell mediated protection in non-human primates (196-198) (discussed below). However, preexisting immunity to adenovirus vectors may affect the magnitude and quality of immune responses if these vectors are used as vaccines. On the other hand, only the magnitude of the response is attenuated when using poxvirus vectors (199-201). Poxviruses have been used to express HIV-1 proteins because of their large capacity for integration of foreign DNA and their ability to undergo genome transcription in the cytoplasm of cells thanks to poxvirus-encoded RNA polymerase (202). Several different vaccinia vectors have been developed and tested in immunization regimens: Modified Vaccinia Ankara (MVA) virus, New York attenuated vaccinia virus (NYVAC), avipox virus canarypox (ALVAC), and Vaccinia New York virus (v-NY). Most of these vectors are good vaccine vector candidates because they are replication incompetent in mammalian cells; however, v-NY is capable of replication. These poxviruses have not only been used alone and in prime-boost regimens, but using an ALVAC vector-gp120 boost strategy in a clinical trial generated the most effective protection against HIV-1 acquisition to date (see below).

Previous Clinical Vaccination Studies

The development of a safe, effective, preventive HIV-1 vaccine remains among the highest global health priorities. Using the approaches for vaccine development listed above,

many HIV-1 vaccine trials have been completed, but very few have made it to Phase IIb/III efficacy trials (186, 203). I will discuss several Phase II or III clinical trials below.

The first Phase III efficacy trail of a prophylactic HIV-1 vaccine (VAX004) attempted to use recombinant subunit gp120 (rgp120) derived from MN, a laboratory-grown strain, and an envelope derived from a clade B primary isolate (GNE8) to elicit Nabs against HIV-1. Several different sites recruited male and female study participants at high risk for heterosexual transmission. The participants were immunized with placebo or the rgp120 vaccine formulated in alum at months 0, 1, 6, 12, 18, 24, and 30. Infection rates among vaccinated and placebo groups were similar. There were little to no protective effects for secondary end points such as viral load, $CD4^+$ T cell count, or time to ART initiation (204-206), indicating that there was no vaccine efficacy.

Another Phase III efficacy trail of a vaccine (VAX003/AIDSVAX B/E) containing two different rgp120 antigens, one from subtype B MN and the other from a primary isolate CRF_AE (A244), was conducted in Thailand, a developing country, among intravenous drug users. The participants were immunized with placebo or the rgp120 vaccine formulated in alum at months 0, 1, 6, 12, 18, 24, and 36. Vaccine efficacy was estimated at 0.1% and no effect was observed on previously mentioned secondary end points (207, 208).

In an effort to overcome the ineffective immunogen capacity of rgp120s and instead, harness T cell responses as a potential protective mechanism, a Phase IIb vaccine trial tested a recombinant, replication-defective Ad5 containing clade B *gag*, *pol*, and *nef* gene inserts. This study attempted to generate T cell mediated immunity instead of antibody immunity. The trial enrolled volunteers from diverse populations at high risk of HIV-1 infection. Volunteers were given placebo or vaccine on day 1, week 4, and week 26. While there was controversial evidence

of viral control in non-human primates challenged with SHIV (196, 209, 210), the trial was stopped when there was no effect on postinfection viral RNA levels (211). In fact, there seemed to be an enhancement of infection among Ad5-positive, uncircumcised men who have sex with men (MSM) (212).

The only successful HIV-1 vaccine trail to date had a modest 31.2% efficacy 42 months post vaccination. Instead of using one type of immunogen, this trial used a pox prime-protein boost strategy (13). Vaccine recipients from the general population of Thailand were primed at 0, 1, 3, and 6 months with a recombinant, replication incompetent canarypox virus, ALVAC-HIV vCP205, expressing gp120 from 92TH023 gp120 (subtype CRF01_AE) in addition to Gag, a portion of the protease gene, and the gp41 from HIV-1 LAI. Participants were boosted with AIDSVAX B/E at 3 and 6 months. Vaccine efficacy was initially 60% at 12 months post vaccination, but diminished over time (213). Interestingly, high levels of Env-specific IgA antibodies correlated with reduced vaccine efficacy, perhaps because these antibodies may have interfered with the vaccine induced protective responses (214). Instead, protection against acquisition was correlated with V1/V2 IgG binding titers and high levels of ADCC (214-216). A targeted sieve analysis performed on breakthrough viruses from RV144 vaccinees found that the vaccine induced differential acquisition of HIV-1 based on the viral sequence in the V2 region (217). These data support the hypothesis that V1/V2 responses are associated with vaccine-induced protection. Only easy to neutralize (Tier 1) isolates were neutralized by vaccinees and little to no neutralization of more difficult to neutralize (Tier 2) isolates was achieved (218). This indicates that the vaccination strategy could potentially be improved by inducing Nabs in addition to V1/V2 directed non-Nabs. In an effort to improve upon the promising results from

the RV144 trial through rational vaccine design, my work focused on understanding how specific N-linked glycans impact the antigenicity and immunogenicity of Env.

Modulation of Antigenicity and Immunogenicity of Env by a Conserved N-linked glycan

It has previously been demonstrated that glycans can modulate the antigenicity of Env and the sensitivity of virus to Nabs (150, 151, 219-226), but relatively few have addressed the potential role glycans have on the immunogenicity of HIV-1 Env. Glycan modifications typically have little effect on the immunogenicity of the wild-type (WT) Env (227-230) and removal of multiple glycans often resulted in a poor immunogen (227-231). However, some studies have demonstrated that removal of specific N-linked glycans proximal to the CD4-binding region of Env enhanced the ability of Env to induce Nabs (148). Our lab was interested in understanding how single N-linked glycan modifications alter the antigenicity and immunogenicity of Env.

To determine the antigenic and immunogenic role of specific N-linked glycans on HIV-1 Env proteins, previous investigators in our group focused on mutants that met the following criteria: (1) mutant Env was expressed and processed normally; (2) mutant Env retained overall functional integrity as indicated by successful rescue of viable virus; and (3) mutant virus had enhanced susceptibility to bNAbs. Previous studies in the Hu lab determined that removal of a single, conserved N-linked glycan on the C-terminal stem of V2 loop located at amino acid N197 (N7) resulted in significantly increased sensitivity of a single virus, 89.6, to Nabs that recognize the CD4bs, V3 loop, and a CD4-induced site exposed in CD4-bound Env (232). Additionally, the removal of the N7 glycan increased the CD4 independent infection of 89.6. This indicates that the N7 glycan masks the CD4bs and the coreceptor binding sites. In contrast to earlier studies that failed to elicit an enhanced immune response with deglycosylated Env, removal of the N7 glycan enhanced the ability of 89.6 to generate cross-reactive serum (226). This led me to

hypothesize that Env modifications that enhance the exposure of conserved epitopes could represent a class of immunogens that produce qualitatively different antibody responses to HIV-1. Since the N7 glycan is highly conserved (19, 148, 233) and because our previous studies were based on a single Env, it is of interest to determine if the N7 glycan impacts the antigenicity, function, and immunogenicity of diverse HIV-1 Env.

Goals of This Dissertation

The ability of the N7 glycan to modulate the antigenic and immunogenic property of Env was previously demonstrated using Env from a single isolate (226). It is unclear whether the findings by this study reflected a conserved functional role of the N7 glycan in modulating the antigenicity and immunogenicity of Env or if the results are due to the specific isolate tested. The overall goal of this dissertation is to determine if the N7 glycan modulates the antigenicity and immunogenicity of diverse Env. In the third chapter of this dissertation, I explore whether the N7 glycan alters the antigenic property of a panel of Env. Using antibodies as probes, I determined how the N7 glycan impacts the exposure or stability of different epitopes on Env. In the fourth chapter of this dissertation, I address our hypothesis that Env modifications that enhance the exposure of conserved epitopes can be used as immunogens that produce qualitatively different antibody responses to HIV-1. Using a small animal model, I explored whether a poxvirus prime-gp120 protein boost regimen could generate antibody responses that have been associated with protection in the RV144 trial. In addition, I examined how these responses may be influenced by the specific Env immunogen used and if the presence or absence of the N7 glycan on Env alters the immune response.

Chapter Two

Materials and Methods

Cells

TZM-bl cells (catalog no. 8129, NIH AIDS reagent program), an African green monkey kidney cell line (BSC40; ATCC catalog no. CRL2761), 293T cells (ATCC catalog no. 11268) and a human osteosarcoma cell line that lacks a functional TK gene (TK- 143B; ATCC catalog no. CRL-8303) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% of Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (complete DMEM). TK- cells were cultured in complete DMEM containing 25 μg/ml of 5-bromodeoxyuridine.

Monoclonal Antibodies and Polyclonal Serum

The following monoclonal antibodies were obtained from NIH ARRRP: MPER-targeted antibody 4E10 (234) and mannan-targeted antibody 2G12 (235, 236) were from Dr. Hermann Katinger; CD4bs-targeted antibody IgG1b12 was from Drs. Dennis Burton and Carlos Barbas (237-240); V3 loop-targeted antibody 447-52D (241-246) and V2 loop-targeted antibody 697-30D (246-248) were from Dr. Susan Zolla-Pazner; and CD4-induced antibody 17b (249-254) was from Dr. James E. Robinson. Recombinant soluble CD4-183, obtained from Pharmacia, Inc, contains the first two domains of human CD4 produced in *Escherichia coli* (255). CD4bs-targeted antibody VRC01 (256) was obtained from Dr. John Mascola, V1/V2 loop-targeted antibodies PG9 and PG16 (150) were from Dr. Dennis Burton. The CD4-IgG2 was from Progenics Pharmaceuticals and C5-targeted antibody D7324 was purchased from Aalto BioReagents. The pooled clade B serum was from NABI and the pooled clade C serum was a gift from Dr. Charles Wood.

Envelopes

Plasmid encoding QA255.662J Env were kindly gifted by Dr. Julie Overbaugh (257). Plasmids encoding 89.6 and ADA were gifted by Dr. Joseph Sodroski (258, 259). Plasmids encoding B33, LN40 (260) and JR-FL (261) Envs were gifted by Dr. Paul Clapham. A plasmid encoding SF162 was gifted by Drs. Leonidas Stamatatos and Cecilia Cheng-Mayer (262) and 1084i by Dr. Ruth Ruprecht (263). The following Env-encoding plasmids were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: TRO.11 (264, 265), 6535.3, SS1196, QH0692.42, SC422661.8, AC10.0.29, and PVO.4 from Drs. David Montefiori and Feng Gao (265); RHPA4259.7, REJO4541.67, and WITO4160.33 from Drs. Hahn and Salazar-Gonzalez (265); THRO4156.18 and CAAN5342.A2 from Drs. Hahn and Kothe (265); TRJO4551.58 from Drs. Hahn, Wei, and Shaw (265); Bal.26 (266) and DJ263.8 (267) from Dr. John Mascola; CRF02_AG Clone 271 from Drs. Ellenberger, Li, Callahan, and Butera; CE1176_A3 from Drs. Ronald Swanstrom, Li-Hua Ping, Jeffrey Anderson, David Montefiori (264); and murine leukemia virus (MuLV) from Drs. Nathaniel Landau and Dan Littman (268).

Construction of Env Mutants

Plasmids containing the *rev-env* gene of individual HIV-1 isolates were used as the template for site-directed mutagenesis using a QuikChange mutagenesis kit (Stratagene). CE1176 mutants were generated by Genewiz or by using a the QuikChange mutagenesis kit (225). Briefly, the indicated amino acid residue was substituted with the indicated residue by site-specific mutagenesis to generate CE1176 mutants that are resistant or sensitive to specified antibodies. To generate glycan mutants of Env, the asparagine (N) residue in the PNLG sequon (N-X-S/T) of most *env* genes was substituted with glutamine (Q) by site-specific mutagenesis. B33 N197 was replaced with glutamic acid (E) because the N197Q mutant Env did not generate

viable virus. The N197 PNLG sequon was restored in QA255 by replacing the proline (P) at amino acid 199 with serine (S) and in JR-FL Env by substituting the aspartic acid (D) at amino acid 197 with N. Conditions for the mutagenesis included a two-step PCR: 95°C for 2 minutes followed by 30 cycles of annealing and extension at 55°C for 30 seconds and denaturing at 95°C for 20 seconds, and finally annealing at 65°C for 6 min. The mutated *rev-env* genes were then subcloned into mammalian expression vector pSVIIIΔEnv (269). The results of site-directed mutagenesis were verified by DNA sequencing.

Infectivity and Neutralization Assays

Stocks of Env-pseudotyped viruses were prepared by co-transfection of Env and backbone plasmid in 293T cells (265). pSG3ΔEnv backbone was used to generate clade B Env-pseudotyped virus and Q23ΔEnv backbone was used to generate clade A and C Env-pseudotyped virus. All assay stocks were titrated in TZM-bl cells as previously described (265). Infectivity of Env-pseudotyped virus was measured by luciferase reporter gene expression in TZM-bl cells. Neutralization as measured by reduction of luciferase gene expression was performed as previously described (270, 271). Briefly, indicator virus containing 150 50% tissue culture infective doses ($TCID_{50}$) was incubated with single sera dilutions or serial threefold dilutions of antibody or serum samples in duplicates in a total volume of 150 μl for 1.5 hours at 37°C in 96-well flat-bottom culture plates before being added to TZM-bl cells (10,000 cells in 100 μl of growth medium containing DEAE dextran). One set of control wells received cells plus virus (virus control), and another set received cells only (background control) in addition to wells that received antibodies with known ID_{50} values (positive control). After a 72-hour incubation, 100 μl of cells were transferred to a 96-well white solid plate (Costar) for assays of luciferase activities using BrightGlo substrate solution as described by the supplier (Promega).

Neutralization activity was expressed as the concentration of antibodies or reciprocal serum dilution that resulted in 50% reduction of RLU (IC_{50}).

In peptide competition experiments the same neutralization protocol described above was applied, except, prior to the exposure of sera to pseudovirus, the sera were incubated for 30 minutes at 37^{o}C with 30 μg/mL of overlapping peptides spanning the V3 loop of a consensus B (ConB) HIV-1 subtype Env or scrambled V3 peptide. Percent neutralization was by determined by measuring the reduction in RLU relative to wells that contain the same dilution of corresponding pre-immune sera.

Neutralization of the global Tier 2 virus panel and mutant CE1176_A3 was performed at Duke University (Dr. David Montefiori) as previously described (264, 272). Percent neutralization activity was expressed as the reciprocal serum dilution that resulted in 50% reduction of RLU (ID_{50}).

Fortebio Octet Bio-layer Interferometry

Fortebio was performed as previously described (273). Briefly, anti-Human IgG Fc Capture tips (Fortebio) were prewet for 5 min in HBS-EP running buffer (GE Healthcare) immediately prior to use. The microplates used in the Octet were filled with 200 μl of sample or buffer per well and agitated at 1000 rpm. Tips were then saturated with 10 μg/mL CD4-IgG2, then washed with running buffer before being transferred into two fold dilution series of gp120 where the ka (1/Ms) was read. The tips were then transferred into running buffer to measure kd (1/s). The tips were regenerated using 0.1 M Glycine, pH 1.5 (GE Healthcare) before being used on subsequent samples. Values from a CD4-IgG2 coated tip that was transferred into running buffer alone were subtracted from all test values. Additionally, one uncoated tip was incubated with gp120 alone to ensure there were no interactions between Env and the capture tips. All

interaction analyses were conducted at 25 °C.

Viral Protein Capture Assays

The p24 concentration of a given pseudovirus preparation was measured with an HIV-1 p24 ELISA (Zeptometrix) and compared to its $TCID_{50}$. Env capture assays using D7324 were carried out as previously described (243, 274) with slight modifications. In brief, 96-well plates were coated with 10 µg/mL sheep anti-gp120 capture antibody D7324 (Aalto Bio Reagents Ltd, Dublin, Ireland) overnight at room temp, blocked with PBS 0.05% Tween/5% milk, washed with PBS 0.05% Tween and incubated for 2 hours at 37°C with WT or N7 mutant Env from one of three sources: gp120 generated by recombinant vaccinia infected cells (275), detergent lysed pseudovirus, or whole pseudovirus. SF162 or JR-FL Env captured on plates was washed before monoclonal antibody (mAb), 697-30D (at 30 µg/mL or 3 µg/mL, respectively), was added and incubated for 1.5 hours at 37°C. After washing, the bound mAbs were detected using HRP conjugated anti-human IgG (ThermoFisher) followed by incubation with TMB substrate (Sigma). After 2M H_2SO_4 was used to stop the reaction, mAb binding was quantified by optical density readings at 450nm (OD450). Binding of antibody to each virus was normalized to OD450 readings obtained with HIV-positive polyclonal serum for the same virus. This controlled for possible differences in the number of virions captured. Separate wells included supernatant from parental vaccinia virus infected cells or ΔV2 gp120 as negative controls (data not shown). Binding of WT virus was included on each plate and set as 100% binding. All experiments were repeated three times in duplicates.

Recombinant Vaccinia Virus (rVV)

A plaque-purified, replication-competent vaccinia virus (VV) generated from the New York City Board of Health strain (v-NY) was used as the vector to express full length gp160 or

subunit gp120, generated by the introduction of one or two stop codons at the primary gp120-gp41 cleavage site, of either N7+ or N7– JR-FL or PVO.4 Env as previously described (276-278). In brief, Env transgenes were inserted into the thymidine kinase (TK) gene of the v-NY strain of VV by homologous recombination. Two rounds of plaque purification were performed under negative selective conditions using media containing 24 μg/mL of 5-bromodeoxyuridine on TK- 143B cells before a third round of purification was performed under non-selective conditions on BSC40 cells. Plaques were screened for transgenes by PCR using a primer set specific to the TK gene and Env. Recombinant viruses were expanded and propagated on BSC40 cells to generate crude virus stocks. Virus stocks that expressed gp160 were subsequently concentrated by sucrose density sedimentation as previously described (278, 279). Subunit gp120s were purified from BSC40 cells gp120-expressing recombinant vaccinia infected BSC40s as previously described (275).

Expression of the transgene, under the control of a synthetic early-late promoter of VV, was verified by Western blot analysis as previously described (275, 280). Briefly, monolayers of BSC40 cells were grown to confluency and subsequently infected with recombinant or v-NY parental virus at a multiplicity of infection of 0.01. At 48 hours post-infection, cells were harvested and resuspended in PBSAM buffer (PBS, 10 mM $MgCl_2$, 0.01% BSA). After two freeze-thaw cycles, cells were sonicated, lysed with NuPage reducing agent (Invitrogen, catalog no NP0004), and fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After being electro-transferred to nitrocellulose membrane, immunoreactive proteins were detected with pooled HIV-positive serum (NABI).

Immunization

Twenty-four female New Zealand White rabbits were purchased and maintained at Covance (Denver, PA). All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee. Rabbits were divided into groups and were sedated with Ketamine (40 mg/kg) and Zylazine (5 mg/kg), then primed with ~10^8 plaque forming units of recombinant VV at weeks 0 and 8 by scarification. Virus was applied to two shaved sites on the back of the animals. Each area was pricked 20 times, penetrating the skin, with a bifurcated needle. Animals remained sedated until both scarification sites were dry. At weeks 35 and 48, animals were boosted intramuscularly at two sites (50 μg each) with 100 μg of cognate gp120 formulated in alum. Blood for processing to serum was collected from each rabbit on the day of, and two weeks after each priming or boosting event. Blood samples were processed to serum according to Covance test facility SOP. Proper immunization was verified by visualization of vaccinia virus lesions, vaccinia neutralization assays, and antibody capture assays.

Antibody Capture Assays

HIV-1 Env-specific antibody titers in serum were determined by ELISA as previously described (281). WT JR-FL or WT PVO.4 gp120 produced from Chinese Hamster Ovary (CHO) cells (kindly gifted by Dr. Shan Lu), or WT SF162 gp120 (Immune-tech catalog no. 001-0028P) were used as the capture antigen (100 ng gp120 per well). Endpoint ELISA titer of binding antibodies is defined as the reciprocal of the serum dilution that resulted in OD readings greater than the mean OD readings of the pre-immune sera at 1:100 dilution plus three times the standard deviation. The limit of detection was considered as the starting dilution (1:100) of the test sera. All experiments were repeated three times in duplicates.

Bioplex assays against peptides were performed as previously described (282). Briefly, 15-mer peptides overlapping by 11-mers corresponding to the variable regions 1-3 (V1-V3) or conserved regions 1 or 5 (C1 or C5 respectively) were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Consensus Subtype B Env Peptide Set. 10 μg of each peptide was coupled to Bio-Plex COOH beads (BioRad) by primary amine coupling according to the manufacturer's protocol. Immune and pre-bleed sera were diluted 1:20 in 1% blotto-PBS and mixed with peptide-coupled beads (~500 beads per peptide) and incubated at room-temp for 1 ½ hours with shaking. To avoid potential competition, only beads coupled to peptides with *no* overlapping segments were included in the same well. After washing, phycoerytherin-labeled secondary antibody diluted 1:100 in 1% blotto was added for 1 hour with shaking. Following washing, the beads were analyzed for binding on a Bioplex 100 (Bio-Rad) and the net mean fluorescence intensity (MFI) against individual peptides from each pool is reported, minus the net MFI of matched pre-bleed samples. Reactivity of 447-52D (from the NIH ARRRP and Dr. Susan Zolla-Pazner) to V3 peptide (241-246) and coupled beads with no antibody or sera added were used as positive and negative controls respectively. All experiments were repeated at least twice in duplicates.

Binding of serum to V1/V2-gp70 fusion proteins was performed at Duke University (Dr. Georgia Tomaras). Sera HIV-1 specific antibodies to a panel of V1/V2 scaffolds were measured by binding antibody multiplex assay similar to previously described (122, 214), with the following modifications: secondary antibody was goat anti-rabbit IgG, biotinylated (Thermo Fisher Scientific, Rockford, IL). Antibody measurements were acquired on a Bio-Plex instrument (Bio-Rad, Hercules, CA) using 21CFR Part 11 compliant software with the primary readout as MFI. Samples with MFI values of 1) at least 100, 2) higher than the antigen specific

cutoffs (95 percentile MFI of all pre-immune sera for each V1V2 scaffold), and 3) >3-fold of the pre-immune serum from the same animal, were considered positive.

Statistical Analysis

Prism (GraphPad Software, Inc.) was employed for all statistical analyses, all using two-sided tests. The Wilcoxon matched-pairs signed-rank test was used to compare K_D, pg p24/$TCID_{50}$, percent binding values, MFI binding, and ID_{50} values against the global Tier 2 panel. The Mann-Whitney test was used to compare reciprocal serum ID_{50} values and percent neutralization against Tier 1 viruses. The percent of the global Tier 2 panel neutralized was compared between groups using Fisher's exact test and within the same groups using McNemar's test for proportions for paired values.

Chapter Three

The Conserved Role of an N-linked Glycan on the Surface Antigen of HIV-1 Modulating Virus Sensitivity to Broadly Neutralizing Antibodies Against the Receptor and Coreceptor Binding Sites

Abstract

HIV-1 establishes persistent infection in part due to its ability to evade host immune responses. Occlusion by glycans contributes to masking conserved sites that are targets for some broadly neutralizing antibodies (bNAbs). Previous work has shown that removal of a highly conserved potential N-linked glycan (PNLG) site at amino acid residue 197 (N7) on the surface antigen gp120 of HIV-1 increases neutralization sensitivity of the mutant virus to CD4 binding site (CD4bs)-directed antibodies as compared to its WT counterpart. However, it is not clear if the role of the N7 glycan is conserved among diverse HIV-1 isolates and if other glycans in the conserved regions of HIV-1 Env display similar functions. In this work, we examined the role of PNLGs in the conserved region of HIV-1 Env, particularly the role of the N7 glycan in a panel of HIV-1 representing different clades, tissue origins, coreceptor usage, and neutralization sensitivity. We demonstrate that the absence of the N7 glycan increases the sensitivity of diverse HIV-1 isolates to CD4bs- and V3 loop- directed antibodies, indicating that the N7 glycan plays a conserved role masking these conserved epitopes. However, the effect of the N7 glycan on virus sensitivity to neutralizing antibodies directed against the V2 loop epitope is isolate dependent. These findings indicate that the N7 glycan plays an important and conserved role modulating the structure, stability or accessibility of bNAb epitopes in the CD4bs and coreceptor-binding region, thus representing a potential target for the design of immunogens and therapeutics.

Importance

N-linked glycans on the HIV-1 envelope protein have been postulated to contribute to viral escape from host immune responses. However, the role of specific glycans in the conserved regions of HIV-1 Env in modulating epitope recognition by broadly neutralizing antibodies has not been well defined. We show here that a single N-linked glycan plays a unique and conserved role among conserved glycans on HIV-1 gp120 in modulating the exposure or the stability of the receptor and coreceptor binding site without affecting the integrity of the Env to mediate viral infection or the ability of the mutant gp120 to bind to CD4. The observation that the antigenicity of the receptor- and coreceptor-binding sites can be modulated by a single glycan indicates that select glycan modification offers a potential strategy for the design of HIV-1 vaccine candidates.

Introduction

Although the role of neutralizing antibodies has yet to be determined in the only clinical trial of human immunodeficiency virus type 1 (HIV-1) vaccines that has shown a modest degree of protection, it is generally believed that it would be advantageous for a vaccine to elicit broadly neutralizing antibodies (bNAbs) against diverse primary isolates. Passive administration of neutralizing monoclonal antibodies or bNAbs derived from HIV-1 infected patients has been shown to protect macaques from simian-human immunodeficiency virus (SHIV) infection (167-170, 173). A fraction of HIV-1 infected individuals (~20-30%) generate bNAbs within 2-4 years of initial infection (203, 283-286). However, generation of bNAbs by active immunization has been a challenge as no HIV-1 vaccine candidate has successfully elicited antibodies with similar neutralizing breadth (203, 287).

Nevertheless, broadly neutralizing monoclonal antibodies isolated from select individuals have helped define the targets of bNAbs. These bNAbs are directed against one of five conserved epitopes on HIV-1 envelope glycoprotein (Env); the CD4-binding site (CD4bs), the membrane-

proximal ectodomain region (MPER), carbohydrates on the outer domain, a quaternary structure in the V1/V2 loops, and a newly described epitope present only in cleaved envelope trimers (134, 284, 287, 288). However, HIV-1 has evolved many protective mechanisms to evade host immune responses, including occlusion of potential targets by glycans (14-20). These glycans account for ~50% of the molecular mass of the Env surface antigen (gp120) and may mask nearly the entire surface of Env, including the conserved epitopes targeted by some bNAbs, rendering it difficult to elicit antibodies targeting these sites

We and others previously reported that removal of a single N-linked glycan located at amino acid position N197 (N7) on gp120 resulted in significantly increased sensitivity of HIV-1 to neutralization (148, 226, 270) and enhanced the ability of Env to induce neutralizing antibodies in macaques (226). However, these studies were based on a small number of isolates. Since the N7 glycan is highly conserved across diverse HIV-1 and SIV isolates (19, 148, 233), it is of interest to determine if the N7 glycan plays a conserved role in the antigenicity, immunogenicity, and function of HIV-1 Env.

In the present study, we sought to extend earlier observations by examining if the role of the N7 glycan is unique among potential N-linked glycans in the conserved region of gp120 and determine if the effect of the N7 glycan on Env antigenicity is conserved among diverse isolates of HIV-1. Specifically, we used broadly neutralizing monoclonal antibodies with defined epitope specificities to study the effects of the N7 glycan on the antigenicity of Env from a panel of HIV-1 representing diverse clades, tissue origin, coreceptor usage, and neutralization sensitivities. Results from this study indicate that the N7 glycan has a unique and conserved role modulating the exposure or stability of conserved epitopes in the CD4bs and variable loops.

Results

The N7 glycan is unique among PNLGs in the conserved regions of gp120 in its ability to modulate the sensitivity of HIV-1 to CD4bs-specific antibodies

Previous work demonstrated that the N7 glycan modulates sensitivity of HIV-1 to CD4bs-specific neutralizing antibodies (148, 226, 270). To examine if the same effect is exerted by other conserved glycans, we introduced mutations substituting the asparagine (N) residue with glutamine (Q) at each of the PNLG sequons (N-X-T/S) in the conserved regions of the surface antigen gp120 of HIV-1 89.6. For this study, we focused on mutant Env that are expressed and processed normally and retain overall functional integrity as indicated by successful rescue of viable virus (data not shown). Thus, the N262 glycan mutant did not generate viable virus and was not included in further experiments. Neutralization sensitivity of pseudotyped viruses bearing WT or mutant Env was examined in a TZM-bl cell assay against sCD4, CD4bs, and MPER-directed antibodies as well as pooled HIV-positive serum. Results in **Figure 3.1** show that the N7 glycan is unique among PNLGs in the conserved regions of gp120 in its ability to modulate virus sensitivity to CD4bs-specific neutralizing antibodies. Removal of select single N-linked glycans modified the sensitivity of virus to certain antibodies; e.g. the N361 mutant was modestly more sensitive to IgG1b12. However, only removal of the N7 glycan increased the sensitivity of 89.6 to all CD4bs-directed antibodies tested as well as HIV-positive pooled serum (226). This indicates that the N7 glycan plays a unique role among conserved glycans on gp120 modulating the stability or access to the CD4bs in the 89.6 Env.

Increased sensitivity of N7 mutant viruses to CD4bs-specific neutralizing antibodies is conserved among diverse isolates of HIV-1

Previous work only examined the role of the N7 glycan in a limited number of isolates (148, 226, 270). Because the sequon for the N7 glycan is present in greater than 95% of available

HIV and SIV sequences (19, 148, 233), we examined if the N7 glycan plays a conserved role in diverse isolates of HIV-1. To test this, we modified the N7 glycan site on the Env from a panel of HIV-1 representing different clades, tissue origin, coreceptor usage, and neutralization sensitivity **(Table 3.1)**, including two viruses, QA255 and JR-FL (257, 261), that lack the N7 glycan site in the Env of WT viruses. To determine the effects of the N7 glycan on the panel of Env, we used site-directed mutagenesis to remove the N7 glycan site of most Env by replacing the asparagine (N) with a glutamine (Q), or to restore the N7 glycan site for those Env missing this site in their WT sequences (see Materials and Methods). Plasmids expressing WT or N7-modified Envs were then used to generate pseudoviruses. Neutralization sensitivity of these pseudoviruses was first tested using antibodies that recognize CD4bs or CD4 induced (CD4i) epitopes **(Table 3.2)**.

All blood and lymph node isolates tested demonstrated increased sensitivity to sCD4 and neutralizing antibodies directed against the CD4bs when the N7 glycan was absent. This includes VRC01, which has a small footprint (256, 289), indicating that the N7 glycan affects the access to or stability of the core residues in the CD4bs.

While the absence of the N7 glycan increased the sensitivity of blood- and lymph node-derived HIV-1 to sCD4, such an effect was not observed in CNS-derived viruses **(Table 3.2)**. However, the absence of the N7 glycan did increase the sensitivity of JR-FL and B33 to VRC01. The same effect was observed for all CNS isolates tested against IgG1b12, indicating that the N7 glycan controls the access to or the stability of the CD4bs in CNS isolates as well as blood and lymph node isolates.

To confirm these effects are due to the absence of the N7 glycan rather than the specific amino acid present at the PNLG site, we introduced different amino acid substitutions at the N7

sequon of a blood isolate, ADA, and a lymph node isolate, LN40. Substitution of N197 with serine in LN40 (LN40 N197S) resulted in a similar increased sensitivity to CD4bs antibodies compared to the N197Q mutation, indicating that the effect is not specific to the amino acid change. However, the same N197S substitution in ADA Env resulted in increased neutralization resistance to b12 and no change in sensitivity to VRC01. Examination of Env sequence of ADA showed that the serine substitution at N197 created an upstream PNLG site at N195 **(Fig. 3.2)**. Thus, the restoration of neutralization resistance resulting from this upstream PNLG is consistent with the role of a glycan near the N7 site capable of modulating CD4bs exposure.

It has been reported that glycan modification in conserved regions of HIV-1 gp120 reduced viral infectivity (148, 226, 290). To determine if the increased sensitivity of N7 mutants to CD4bs antibodies is associated with reduced infectivity of the mutant viruses, we examined the infectivity of the N7 glycan mutant viruses that showed the greatest increase in neutralization sensitivity to CD4bs antibodies as compared to their WT versions. We measured the $TCID_{50}$ of these pseudotyped viruses normalized to their p24 content. Results in **Figure 3.3** show that the removal of the N7 glycan did not impact the infectivity of these viruses, supporting the notion that the increased neutralization sensitivity of N7 glycan mutants to CD4bs antibodies was not due to their decreased infectivity.

Removal of the N7 glycan does not alter the binding kinetics of gp120 to CD4

To determine if N7 glycan removal alters the affinity of Env protein to its receptor, we examined the binding kinetics of CD4-IgG2 to gp120 proteins with or without the N7 glycan. Monomeric gp120 from ADA, JR-FL, and PVO.4 were purified as previously described (275) and their binding kinetics to CD4-IgG2 were compared by bio-layer interferometry **(Table 3.3)**. Different gp120 showed different K_D (M) values, in agreement with observations from previous

studies (291-293). However, the presence or absence of the N7 glycan did not affect the K_D (M) values of gp120, indicating that N7 glycan mutations did not alter the binding properties of monomeric gp120 to its receptor, consistent with the lack of effect of the N7 glycan on viral infectivity.

Effect of the N7 glycan on virus sensitivity to CD4-induced neutralizing antibodies

It has been demonstrated that removing the N7 glycan not only increases the sensitivity of HIV-1 to CD4bs-directed antibodies, but also to CD4-induced (CD4i) neutralizing antibodies, potentially by enhancing the stability of or access to epitopes recognized by CD4i antibodies (270). Furthermore, CD4-independent viruses, which presumably have Env in an "open" conformation upon CD4 binding, are significantly more sensitive to neutralization by sCD4, similar to our finding with N7-deglycosylated viruses (226, 270). Therefore, we examined the sensitivity of the panel of HIV-1 to CD4i antibody 17b in the presence or absence of subneutralizing concentrations of sCD4 **(Table 3.2)**. While the N7 glycan modulated sensitivity of virus to CD4bs antibodies, its effect on viral sensitivity to CD4i antibodies appeared to be isolate-dependent. Increased neutralization sensitivity to 17b was only observed in N7 mutants of 89.6, ADA, and LN40 N197S. The N7 mutant of a Tier 3 isolate, PVO.4 (294), was only neutralized by 17b with IC_{50} values close to 20 µg/ml, which was the upper limit of the assay, making it difficult to determine if the N7 glycan has any effect on neutralization sensitivity to 17b. All other blood and lymph node viruses tested, either with or without the N7 glycan, were resistant to neutralization by 17b, either in the presence or absence of sCD4.

Of the few CNS isolates tested, N7 glycan removal did not modulate the neutralization sensitivity to 17b; however, we also observed an isolate-dependent effect of the N7 glycan on virus sensitivity to neutralization by 17b. The presence of subneutralizing concentrations of

sCD4 augmented the neutralizing sensitivity of B33 to 17b, but no change was seen in SF162 or JR-FL. JR-FL was resistant to neutralization by the highest concentration of 17b used in the presence or absence of sCD4.

We also investigated if the increased sensitivity to CD4i antibodies correlated with the ability of the mutant viruses to infect CD4-negative cells. Infectivity of pseudotyped viruses bearing WT or mutant Env on CD4-negative cells (NNP2-CCR5 for R5-tropic viruses or U87-CXCR4 for 89.6; (226)) was determined using inocula normalized for their infectivity in isogenic CD4-expressing cells (NP2-CCR5-CD4 or U87-CXCR4-CD4, respectively) **(Fig. 3.4**). Increased infectivity in CD4-negative cells was only observed in ADA N197S and 89.6 N7 glycan mutants. Therefore, the ability of N7 mutant Env to mediate CD4-independent viral entry appears to be isolate specific and dependent on the specific amino acid substitutions at the PNLG site.

Effect of the N7 glycan on neutralization sensitivity to V3-specific monoclonal antibodies

The increased susceptibility of the N7 mutants to CD4i antibodies indicates the possibility of enhanced exposure or stability of the bridging sheet. Indeed, previous work has shown that removing the N7 glycan results in increased sensitivity of HIV-1 to antibodies targeting the V3 loop and MPER (226, 270). Additionally, the crystal structure of soluble, cleaved trimers indicates that the crown of the V3 loop is buried under the N7 glycan of an adjacent protomer (295, 296). We examined if the removal of the N7 glycan correlated with increased exposure or stability of neutralizing epitopes in the V3 loop, which is a major determinant of coreceptor usage. Neutralization sensitivity to 447-52D, an antibody that recognizes the tip of the V3 loop (242), was determined for pseudotyped viruses with or without the N7 glycan **(Table 3.4)**. Interestingly, removal of the N7 glycan increased the susceptibility of

all viruses neutralized by 447-52D, including the CNS derived isolates. Even PVO.4 and JR-FL, two isolates that are resistant to 50 µg/mL of 447-52D when the N7 glycan is present, were readily neutralized by the same antibody when the N7 glycan was removed. This indicates that the N7 glycan not only alters the accessibility or stability of the CD4bs, but also the coreceptor binding site.

Effect of the N7 glycan on neutralization sensitivity to monoclonal antibodies directed to quaternary neutralizing epitopes in V2

Since the N7 glycan is located at the C-terminal stem of the V2 loop, we examined if the removal of the N7 glycan would affect virus sensitivity to monoclonal antibodies, PG9 and PG16, which recognize a glycan-dependent, quaternary neutralizing epitope in V2 (151). Most of the isolates in the test panel lack the N160 glycan recognized by PG9 and PG16 and are resistant to neutralization by these antibodies **(Fig. 3.2)**. Therefore, we focused on isolates that are sensitive to PG9 and PG16. Results in **Table 3.4** show that the N7 glycan also affected the sensitivity of HIV-1 to these monoclonal antibodies, but in an isolate-dependent manner. PVO.4 showed increased sensitivity to PG9 and PG16 when the N7 glycan was absent, whereas the other N7 mutant viruses became more resistant. Interestingly, the introduction of a PNLG site upstream of the N7 site in ADA N197S restored sensitivity to PG9 and partially so to PG16, again indicating that a glycan at or around the N7 glycan site may play a role maintaining the quaternary structure recognized by PG9/PG16. This isolate-dependent effect of the N7 glycan on neutralization sensitivity to PG9/PG16 is in contrast to its effect on the sensitivity to CD4bs and V3-directed neutralizing antibodies where an increased sensitivity was observed in all isolates tested. In this context, it is of interest to note that the panel of isolates tested have varying V2

loop sequences, amino acid length, and number of glycosylation sites, including the presence or absence of N160 **(Fig. 3.2)**, which is a part of the epitope targeted by PG9/PG16.

In order to determine if the effect of the N7 glycan on sensitivity to V2-specific antibodies is limited to those isolates that maintain the N160 PNLG, we examined WT and N7 mutant Env from SF162 and JR-FL for reactivity to monoclonal antibody 697-30D, which also recognizes a conformation-dependent epitope in V2, but does not rely upon the quaternary form of Env, nor the presence of the N160 glycan **(Fig. 3.5)** (247). The binding of 697-30D to WT and mutant Env was determined by antigen-capture assays using three forms of the Env that differ in their conformational and oligomeric structures: monomeric gp120s, lysed pseudovirus, and whole pseudovirus, with the latter exhibiting the most sterically constrained forms of Env (56, 110, 140, 297, 298). Binding of 2G12 and HIV-positive serum was detected with all three forms of Env tested (data not shown) and was therefore used as positive controls. Vaccinia virus infected cell lysate was included as a negative control. All values described were normalized to binding by HIV-positive serum and were expressed as percentages of binding to the WT Env.

N7-deglycosylated SF162 Env from lysed virions and monomeric gp120s showed significantly reduced binding to 697-30D compared to their glycosylated counterparts ($p \leq 0.01$) **(Fig. 3.5A)**. However, there was no difference in 697-30D binding between glycosylated or deglycosylated Env when whole SF162 virions were used, indicating that the N7 glycan may alter the conformation of the V2 loop in the less constrained gp120, but not in the trimeric form on whole virions where there is reduced local flexibility. However, no differences were observed between N7-glycosylated or deglycosylated forms of JR-FL Env, as presented in gp120, whole virions, or lysed virions **(Fig. 3.5B)**, possibly as a result of the more constrained nature of the V2

loop in JR-FL as compared to that in SF162. Again, these finding support the notion that the N7 glycan affects the antigenicity of the V2 loop in an isolate-specific manner.

Effect of the N7 glycan on neutralization sensitivity to monoclonal antibodies directed to epitopes in the mannan cluster and the MPER

To examine the effect of the N7 glycan on the antigenicity of distal parts of the Env, we tested the sensitivity of WT and N7 mutant virus to mannan-dependent and MPER-specific antibody, 2G12 and 4E10 respectively (234-236). There was little to no change in 2G12 or 4E10 sensitivity when the N7 glycan was removed **(Table 3.4)**. While QA255, 89.6, ADA, and LN40 showed ~3 fold sensitivity difference to 2G12 and PVO.4 and SF162 exhibited a modest (~4 fold) increase in sensitivity to 4E10 when the N7 glycan was removed, other viruses demonstrated no change. These data indicate that the N7 glycan plays less of a role in these distal epitopes compared to those in the CD4bs and V2/V3 loops.

The N7 glycan modulates virus sensitivity to antibodies from HIV-positive individuals.

The highly conserved nature of the N7 glycan indicates an important role for virus survival. To demonstrate this potential role, we examined the sensitivity of the WT and N7 mutant viruses in the panel of HIV-1 isolates against pooled serum samples from HIV-positive individuals. As expected, there was a wide spectrum of neutralization sensitivity among the viruses tested against the clade B and clade C serum pools **(Table 3.4)**. However, most of the viruses tested, regardless of their neutralization sensitivity, showed 3- to 60-fold increased sensitivity to either the clade B or clade C HIV-positive pooled sera when the N7 glycan was absent, consistent with a conserved role of the N7 glycan in the evasion of host neutralizing antibody responses. The only exception was a clade A isolate, QA255, where the N7-

glycosylated form was highly resistant to the clade B serum used (1:20), making it difficult to determine any increase in sensitivity for the N7-deglycosylated virus.

Discussion

Glycans on HIV-1 envelope proteins have been shown or postulated to play an important and multi-faceted role in viral replication, including structural integrity of the virus, coreceptor usage, evasion of host immune responses, and targets for bNAbs (148, 150, 151, 226, 299). Here, we report that the conserved role for the N-linked glycan at amino acid position 197 (N7) in modulating the antigenicity of the CD4bs and epitopes in the V3 loop, a major determinant for coreceptor usage. This role is conserved among a panel of viruses tested, including isolates of different clades, tissue origin, coreceptor usage, and neutralization sensitivity **(Table 3.2 & 3.4)**. The N7 glycan is also unique among PNLGs in the conserved regions of HIV-1 in modulating the sensitivity to CD4bs-directed neutralizing antibodies while maintaining the integrity of distal epitopes (2G12, MPER). The increased sensitivity of N7 mutants was not due to a decrease in viral infectivity **(Fig. 3.3)**, nor any defect in the ability of the N7 mutant gp120 to bind the CD4 receptor **(Table 3.3)**. The observation that N7 mutants also showed increased sensitivity to pooled HIV-positive serum is consistent with a role for the N7 glycan in viral evasion from the host immune responses *in vivo* **(Table 3.4)**.

It is of interest to note that the absence of the N7 glycan in CNS versus blood/lymph node isolates resulted in differential sensitivity to sCD4. While the blood/lymph node isolates demonstrated increased sensitivity to all CD4bs-directed antibodies when the N7 glycan is absent, the same mutation in CNS isolates showed no change in sensitivity to sCD4 **(Table 3.2)**. This differential sensitivity may be derived from the immune-privileged environment, from which CNS isolates were obtained, i.e., there is little selective pressure for CNS isolates to evade

antibodies against the CD4bs (300, 301). CNS isolates typically display a higher avidity for CD4, requiring lower levels to infect cells, and are more resistant to blocking by anti-CD4 antibodies compared to spleen-derived envelopes (302). Interestingly, the V1/V2, V3, and C2 regions, which include the N7 glycan site, determine the low CD4 dependence and high avidity for CD4 in CNS isolates (303). These factors may contribute to the unchanging sensitivity of CNS isolates to sCD4 when the N7 glycan is removed. However, removal of the N7 glycan did result in increased sensitivity of CNS viruses to other CD4bs-directed antibodies, e.g., VRC01 or IgG1b12, and to V3-directed antibody, 447-52D **(Table 3.2 & 3.4)**, thus conforming to the conserved role of the N7 glycan masking these conserved epitopes.

While the role we ascribe to the N7 glycan is through inference by site-specific mutation with a conserved amino acid substitution (N to Q) at the PNLG sequon, several observations support our notion that the effect of the N7 mutant is not due to the amino acid substitution *per se*, but rather to the absence of the glycan at this sequon. As shown in **Table 3.2**, both N197Q and N197S mutants resulted in similar changes in the sensitivity of LN40 to sCD4 and CD4bs-specific antibodies VRC01 and IgG1b12. Interestingly, the same amino acid substitutions in ADA resulted in divergent phenotypes, with the N197S mutant exhibiting comparable sensitivity to the WT virus to VRC01 and increased *resistance* to IgG1b12, while the N197Q mutant showed increased sensitivity to these antibodies. It is noteworthy that the substitution at amino acid 197 with S resulted in the creation of a new upstream PNLG sequon at N195 **(Fig. 3.2)**. Thus, we interpret the divergent phenotype of the N197Q and N197S mutants as evidence supporting a conserved role for an N-linked glycan at the base of the V2 loop: either the N7 glycan in the case of the WT ADA, or the newly created glycan at N195 in the case of N197S. The differences in the apparent molecular weights of WT and N7 mutant Env expressed in

recombinant vaccinia viruses also support the occupancy of the N7 PNLG in all the isolates tested (data not shown).

Although the N7 glycan modulates the receptor and coreceptor binding site in a conserved manner, its effect on the ability of the Env to mediate CD4-independent entry is specific to the isolate tested and the specific amino acid change introduced at the PNLG site. Data from this and previous studies showed ADA N197S and 89.6 as the only two isolates that display enhanced ability to mediate CD4-independent entry when the N7 glycan is absent **(Fig. 3.4)** (226, 270). In these cases, the enhanced ability to mediate CD4-independent entry correlated with increased sensitivity to CD4i antibody, 17b **(Table 3.2)** (270). However, in other isolates tested, the presence or absence of the N7 glycan does not affect 17b sensitivity, even though increased susceptibility to a V3 antibody, 447-52D, was observed **(Table 3.4)**. Furthermore, as in the case of ADA, the creation of an upstream PNLG sequon in the N197S mutant resulted in the acquisition of CD4-independent phenotype, whereas the absence of the N7 glycan in N197Q retained CD4-dependence **(Fig. 3.4)**. Thus, even though the N7 glycan modulates the access or stability of epitopes in the CD4bs and coreceptor binding sites, neither the N7 glycan nor susceptibility to 17b *per se* is predictive of CD4 independence in an isolate-dependent or sequence-specific manner.

The N7 glycan also modulates the sensitivity of virus to neutralizing antibodies recognizing a quaternary structure in V1/V2. Interestingly, this effect appears to be isolate dependent **(Table 3.4)** (304). PVO.4 was the only isolate that demonstrated increased sensitivity to PG9/PG16 antibodies when the N7 glycan was removed, while the same glycan mutant in every other virus tested showed increased resistance. Since PVO.4 has the longest V2 loop length and the highest number of potential N-linked glycan sites, we speculate that PVO.4 may

have a higher degree of structural constraint in the V2 loop compared to other isolates tested. If so, the conformation-dependent epitope in V2 recognized by PG9/PG16 may be better preserved in PVO.4 than the other isolates. Thus, when the N7 glycan is removed, the better preserved PG9/PG16 epitope in PVO.4 Env may be better exposed (and thus the higher sensitivity to these mAb), whereas the greater flexibility in other isolates may result in the loss of this epitope (and thus greater resistance). Furthermore, we note that the isolate-dependent effect of the N7 glycan on the epitopes recognized by V2-specific mAb is not dependent on the presence or the nature of the N160 glycan in the isolates tested. This is because the N7 glycan also affects Env binding to a V2-specific, conformation-dependent antibody, 697-30D, the epitope of which is not dependent on the N160 glycan **(Fig. 3.5)**. Thus, the isolate-dependent effect of the N7 glycan on V2 antigenicity is likely mediated through its effect on the positioning or the conformation of the V1/V2 loop.

Currently, we do not have a clear understanding of the basis of the effect of the N7 glycan reported in this study. As shown in **Table 3.3**, the presence or absence of the N7 glycan does not affect the affinity of gp120 to CD4-IgG2. This observation is consistent with previous publications demonstrating that the CD4 binding site on gp120 is not modified by carbohydrates (249, 305). Others have demonstrated that the N7 glycan may help to stabilize the native Env by occluding the V3 loop, shielding V3 epitopes, and inhibiting its premature release prior to CD4 binding (295, 296). These findings, together with our data reported here support the possibility that the biological property of the N7 glycan may only be discernible in the trimeric form of Env. Furthermore, we have little information on the nature of the glycans on the N7 site and how that may affect its role in different isolates. Further studies will be needed to provide a better understanding of the structural and biophysical basis of our observations.

We report here the conserved phenotype of an N-linked glycan in masking epitopes in the CD4bs and coreceptor binding sites, epitopes targeted by some of the most potent and broadly reactive neutralizing antibodies. Because glycans on HIV-1 play an important role in the structure and function of Env (290, 306, 307) and because multiple studies have shown that carbohydrates modulate Env antigenicity (148, 150, 151, 219-224, 226, 299), glycan modification may represent a rational approach for immunogen design. Although multiple studies have shown little or negative impact of glycan modifications on Env immunogenicity (227-231), we previously demonstrated that removal of specific glycans may enhance the ability of HIV Env to elicit neutralizing antibody responses (226). Similar observations were also made by the removal of specific N-linked glycans proximal to the CD4-binding region (148). Mutant Env with specific glycans removed also binds to the unmutated common ancestors of broadly neutralizing mAbs better than glycosylated versions (222, 308). Furthermore, glycan modification may play a role in modifying the immunogenicity of other vaccine candidates, including hemorrhagic fever (309), and feline immunodeficiency virus (310). Taken together, our finding of the conserved nature of the N7 glycan in masking the receptor and coreceptor binding sites of HIV-1 Env may inform the design of immunogens more capable of eliciting bNAbs against these important targets.

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Table 3.1: Select HIV-1 isolates representing different clades, coreceptor usage and tissue origin

Isolate	Clade	Tropism	Source	Reference	Accession Number
QA255	A	R5	PBMC	(257)	N/A
89.6	B	R5/X4	PBMC	(311)	U39362
ADA	B	R5	PBMC	(312)	AY426119
B33	B	R5	Brain	(260)	ADD17640
LN40	B	R5	Lymph Node	(260)	ADD17641
JR-FL	B	R5	Brain	(261)	AAB05604
PVO.4	B	R5	PBMC	(265)	AAW64259
SF162	B	R5	Brain	(313)	P19550
1084i	C	R5	PBMC	(263)	AAV80387

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Table 3.2: Neutralization sensitivity of WT and N7 mutant pseudotyped viruses to monoclonal antibodies targeting CD4bs or CD4-induced (CD4i) epitopes

Env	N7 Glycan[b]	IC50 (μg/mL)[a]									
		CD4 binding site epitopes						CD4-induced (CD4i) epitopes			
		sCD4	Fold[c] change	VRC01	Fold[c] change	IgG1b12	Fold[c] change	17b w/o sCD4	Fold[c] change	17b with sCD4[d]	Fold[c] change
Blood Isolates											
QA255	+	>30		5.29		N.D.		>20		>20	
	—	5.88	>5.1	0.44	12	N.D.		>20	-	>20	-
89.6	+	0.16		1.3		N.D.		>20		>20	
	—	0.03	5.3	0.27	4.8	N.D.		0.85	>23.5	0.72	>27.8
ADA	+	0.18		0.94		0.13		0.56		2.7	
	—	0.03	6	0.15	6.3	0.01	13	0.03	18.7	0.02	135
	N197S	0.02	9	1.26	-	>30	>230	0.003	187	0.18	15
LN40	+	>30		0.14		>30		>20		>20	
	—	11.02	>2.7	0.02	7	0.07	>429	>20	-	>20	-
	N197S	27.24	>1.1	0.02	7	0.13	>231	>20	-	0.007	2857
PVO.4	+	7.8		1.16		N.D.		>20		>20	
	—	0.18	43.3	0.05	23.2	N.D.		12.3	>1.6	15.5	>1.3
1084i	+	>30		1.87		N.D.		>20		>20	
	—	4.5	>6.7	0.41	4.6	N.D.		>20	-	>20	-
CNS Isolates											
B33	+	2.1		0.47		0.3		>20		2	
	—	0.76	-	0.05	9.4	0.008	37.5	>20	-	2.09	-
JR-FL	+	2.19		1.16		0.16		>20		>20	
	—	3.03	-	0.21	5.5	0.018	8.9	>20	-	>20	-
SF162	+	0.12		0.45		0.033		2.19		2.47	
	—	0.04	3	0.3	-	0.006	5.5	3.84	-	2.54	-

[a] Input virus was normalized by their infectivity at 150 $TCID_{50}$ in TZM-bl cells. The column subheadings indicate the respective monoclonal antibodies and epitopes they recognize. N.D., not determined. Results represent the average of two or more experiments, each performed in duplicates.

[b] The presence (+) or absence (–) of the N7 glycan is indicated. Unless otherwise indicated, the N residue in the PNLG sequon was substitute with Q. N197S indicates an N to S substitution at the same 197 amino acid residue.

[c] Fold change was calculated as the ratio between the IC_{50} values with N7– and N7+ viruses. Only values greater than or equal to 3 are shown. *Increased* neutralization sensitivity greater than 4-fold is highlighted in red; *decreased* sensitivity greater than 4-fold is highlighted in green.

[d] A subneutralizing concentration of sCD4 was used for these assays. Because the viruses exhibit different sensitivities to sCD4, 20% of the IC_{50} concentration was used for each individual virus. Reproduced with permission from reference (225).

Table 3.3: Binding affinity of N7-glycosylated and deglycosylated gp120 to CD4-IgG2.

Env	N7 Glycan[a]	K_D (M)	ka (1/Ms)	kd (1/s)	p-value[b]
ADA	+	4.74E-09	5.13E+04	2.46E-04	0.25
	–	6.85E-09	4.21E+04	2.91E-04	
JR-FL	+	3.64E-09	7.19E+04	2.61E-04	0.25
	–	2.72E-09	6.69E+04	1.81E-04	
PVO.4	+	4.02E-08	4.59E+04	1.80E-03	>0.99
	–	4.01E-08	5.52E+04	2.20E-03	

[a] N7-glycosylated (+) and deglycosylated (–) gp120s were used.

[b] p-value for K_D(M) obtained with N7-glycosylated or deglycosylated gp120 is calculated by the Wilcoxon matched-pairs signed-rank test.

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Table 3.4: Neutralization sensitivity of WT and N7 mutant pseudotyped viruses to HIV-positive serum and monoclonal antibodies targeting V1/V2, V3, mannan and MPER epitopes

Env	N7 Glycan[b]	IC_{50} (μg/mL)[a]													
		V1/V2				V3		Mannan		MPER		HIV-Positive Serum			
		PG9	Fold[c] change	PG16	Fold[c] change	447-52D	Fold[c] change	2G12	Fold[c] change	4E10	Fold[c] change	Clade B	Fold[c] change	Clade C	Fold[c] change
Blood Isolates															
QA255	+	0.31		0.06		>50		25.8		22.74		<20		448.07	
	--	>30	>96.8	>30	>500	>50	-	7.99	3.2	15.35	-	39	>2.0	518	-
89.6	+	N.D.		N.D.		0.37		2.28		5.21		2416		235	
	--	N.D.		N.D.		0.02	18.5	0.75	3	3.8	-	18049	7.5	4099	17.4
ADA	+	1.09		0.05		0.75		27.6		0.35		2411		490	
	--	7.86	7.2	0.88	17.6	0.02	37.5	10.5	-	0.2	-	14114	5.9	3275	6.7
	N197S	1.7	-	0.25	5	0.01	75	10	-	0.06	5.8	91634	38	30764	62.8
LN40	+	N.D.		N.D.		12.49		0.71		2.28		1060		1917	
	--	N.D.		N.D.		3.46	3.6	2.14	3	2.94	-	2581		644	3
	N197S	N.D.		N.D.		7.66	-	1.2	-	0.99	-	3608	3.4	1648	-
PVO.4	+	6.92		3.02		>50		1.63		3.22		248		486	
	--	0.78	8.9	0.79	3.8	0.02	>2500	2.77	-	0.87	3.7	3418	13.8	1539	3.2
1084i	+	0.11		1.13		>50		>50		3.22		170		271	
	--	1.67	15.2	>30	26.5	>50	-	>50	-	6.01	-	462		1374	5.1
CNS Isolates															
B33	+	N.D.		N.D.		6.85		>50		6.69		157		309	
	--	N.D.		N.D.		0.55	12.5	>50	-	2.64	-	624	4	345	-
JR-FL	+	N.D.		N.D.		>50		1.65		18.65		81		122	
	--	N.D.		N.D.		17.86	>2.8	0.82	-	9.79	-	929	11.5	125	-
SF162	+	N.D.		N.D.		0.35		1.57		3.63		16329		1951	
	--	N.D.		N.D.		0.03	11.7	1.6	-	1.08	3.4	71721	4.4	4158	-

[a, b & c] See legends to **Table 3.2**.

Reproduced with permission from reference (225).

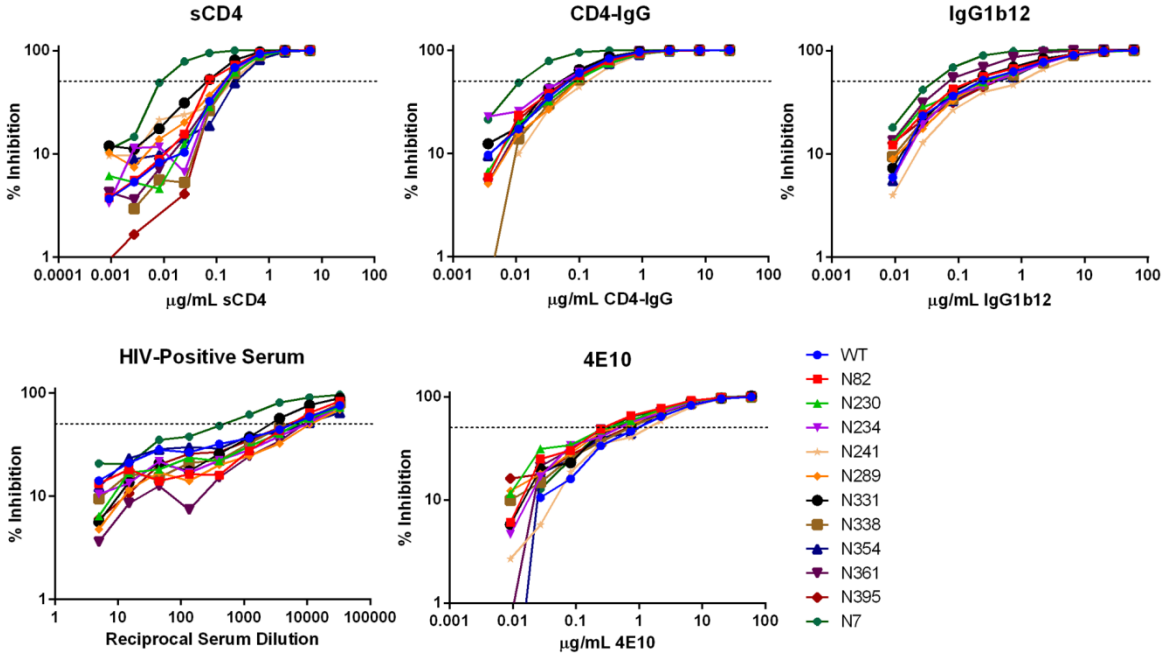

Figure 3.1: Neutralization sensitivity of viruses pseudotyped with WT 89.6 ENV or PNLG mutants in the conserved regions of Env. Mutants were designated by the amino acid position of the asparagine residue of the PNLG sequon. N197 mutant is abbreviated as N7. Neutralization assay was performed using sCD4, CD4-IgG, broadly neutralizing monoclonal antibodies, or pooled HIV-positive serum at concentrations or serum dilutions as indicated. Neutralization was quantified as the percent inhibition of viral infectivity measured by RLU expressed in TZM-bl indicator cells. Dotted line denotes the concentration or reciprocal serum dilutions that resulted in 50% inhibition of viral infectivity (IC_{50}). Reproduced with permission from reference (225).

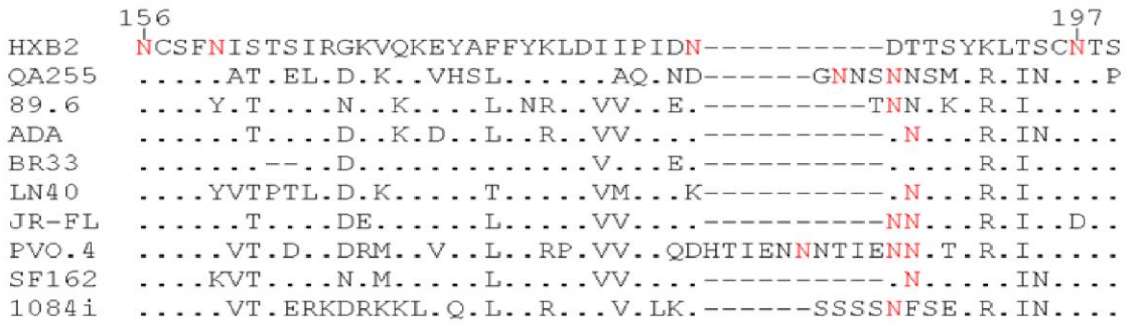

Figure 3.2: V2-loop amino acid sequence of the panel of HIV-1 isolates. The amino acid sequence of the V2 loop for each Env (257, 260, 261, 263, 265, 311-313) is aligned to the HXB2 reference sequence (314) using ClustalW. Numbering is based on the HXB2 amino acid sequence. Identical amino acid residues are indicated by dots. Dashes indicate gaps. PNLG sites in each isolate are indicated by a red letter N. The N7 PNLG site is at amino acid position 197. Reproduced with permission from reference (225).

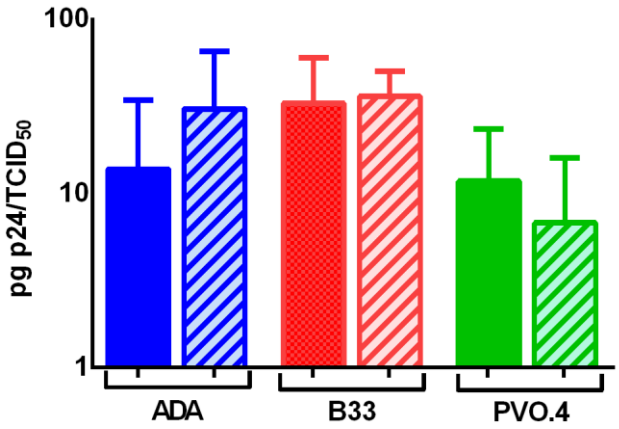

Figure 3.3: Infectivity of viruses pseudotyped with WT or mutant N7 Env. The relative infectivity of viruses pseudotyped with WT or N7 mutant of Env from ADA, B33, or PVO.4 was measured as the quantity of p24 (in picograms) normalized to the infectivity ($TCID_{50}$) of the respective viruses. Solid boxes indicate viruses pseudotyped with the N7-glycosylated form of Env and hatched boxes, the N7-deglycosylated forms. Results for all viruses are shown with the average and standard deviation obtained from three independent experiments performed in duplicates. Reproduced with permission from reference (225).

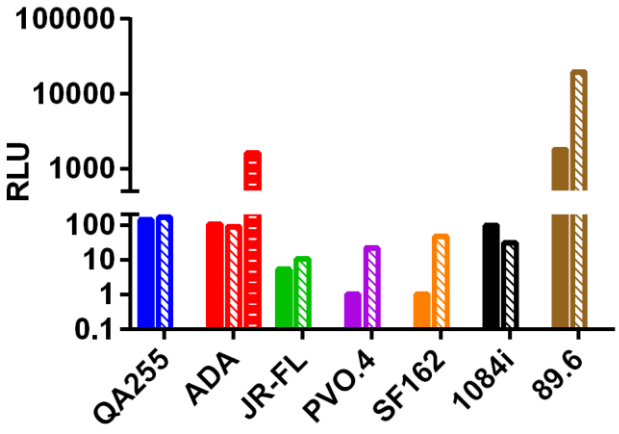

Figure 3.4: CD4 dependence of infection by WT and N7 glycan mutants. The infectivity of WT and N7 mutants were compared in cells expressing coreceptors CCR5 or CXCR4, with or without co-expression of CD4. Infectivity of R5 tropic viruses was determined in NP2 cells expressing CCR5. Infectivity of 89.6 was determined using U87 cells expressing CXCR4 (adapted from Li *et. al*. (226)). The inoculum used for each virus was normalized by its infectivity in isogenic cells expressing the CD4 receptor (200,000 RLU measured in NP2-CCR5-CD4 or U87-CXCR4-CD4 cells). Solid boxes indicate infectivity of viruses with the N7 PNLG present, hatched boxes indicate N197Q mutants, and the striped box indicate ADA N197S mutant virus. The results in all panels represent the average from at least two independent experiments performed in duplicates. Reproduced with permission from reference (225).

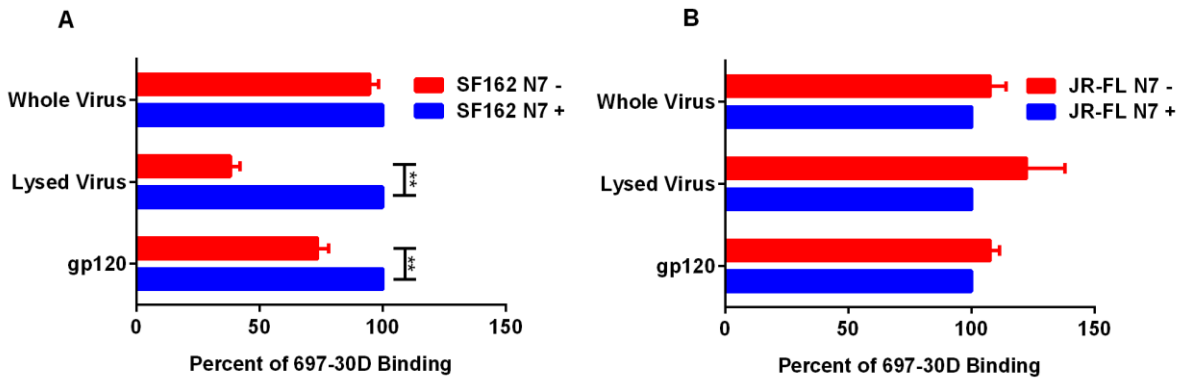

Figure 3.5: Effect of N7 glycan on the binding of a monoclonal antibody recognizing a conformation-dependent epitope in the V2 loop. Binding of monoclonal antibody 697-30D to the WT or N7 mutant forms of SF162 (A) or JR-FL (B) Env was measured by antigen capture assays. Three different forms of Env were tested: (1) whole virions pseudotyped with the indicated Env, (2) pseudovirions lysed with triton-x 100, or (3) gp120 expressed in recombinant vaccinia virus infected cells (275). Binding to 697-30D was normalized to binding obtained with HIV-positive serum and values obtained with the N7-deglycosylated Env (red bars) were expressed as a percentage of the values obtained with the N7-glycosylated Env (blue bars). The results represent the average and standard deviation obtained from three individual experiments performed in duplicates. p-values were calculated by the Wilcoxon matched-pairs signed-rank test (**$p \leq 0.01$). Reproduced with permission from reference (225).

Chapter Four

Induction of Heterologous Tier 2 HIV-1 Neutralizing and Cross-Reactive V1/V2-Specific Antibodies in Rabbits by Prime-Boost Immunization

Abstract

The RV144 poxvirus prime-protein boost remains the only immunization strategy shown to elicit a modest level of protection against HIV-1 acquisition in humans. Although neutralizing antibodies (Nab) were generated, they were against sensitive viruses, not the more resistant "Tier 2" isolates that dominate circulating strains. Instead, risk reduction correlated with non-broadly Nab recognizing epitopes in the V1/V2 region of HIV-1 envelope glycoprotein (Env). Here, we examined if Tier 2-virus Nab and V1/V2-specific non-Nab could be elicited by a poxvirus prime-gp120 boost strategy in a rabbit model that included antigenically diverse envelope proteins. We studied two Clade B Envs that differ in multiple parameters, including their tissue origin, neutralization sensitivity, and the presence of the N197 (N7) glycan that was previously shown to modulate the exposure of conserved epitopes on Env. We demonstrate that immunized rabbits generated cross-reactive neutralizing activities against >50% of Tier 2 global HIV-1 isolates tested. Some of these activities were directed against the CD4 binding site (CD4bs). These rabbits also generated antibodies that recognized protein scaffolds bearing V1/V2 sequences from diverse HIV-1 isolates. However, there are subtle differences in the specificity and the response rate of V1/V2-specific antibodies between animals immunized with JR-FL or PVO.4 Env, and the presence or absence of the N7 glycan. These findings demonstrate that antibody responses that have been correlated with protection against HIV-1 acquisition in humans can be elicited in a preclinical model by a poxvirus prime-gp120 boost strategy and that improvements

to these responses may be achievable by optimizing variables such as the nature of the priming and boosting immunogens.

Importance

The only vaccine approach shown to date to elicit any protective efficacy against HIV-1 acquisition is based on a poxvirus prime-protein boost regimen (RV144 Thai trial). Reduction of risk was associated with non-broadly Nab targeting the V1/V2 loops of the envelope protein gp120. However, the modest efficacy (31.2%) achieved in this trial highlights the need to examine approaches and factors that may improve vaccine-induced responses, including cross-reactive neutralizing activities. We show here that rabbits immunized with a novel recombinant vaccinia prime-gp120 protein boost regimen generated V1/V2 binding antibodies and neutralizing activities against heterologous Tier 2 HIV-1 isolates. The observation that such responses can be generated in an animal model offers the possibility that the protective efficacy of the prime-boost immunization approach can be further improved. The results described here could inform the design of future HIV-1 vaccine candidates.

Introduction

While many vaccine approaches have been tested in the clinic, all but one have failed to elicit protection against HIV-1 acquisition (203, 287). Only the RV144 trial achieved a modest efficacy of 31.2% using a prime-boost strategy with non-replicative recombinant canarypox virus and bivalent gp120 protein (13). Antibodies against variable loops 1 and 2 (V1/V2) and high levels of antibody-dependent cellular cytotoxicity (ADCC) activities were found to inversely correlate with the risk of HIV-1 acquisition (214-216). Nabs were generated, but were primarily against Tier 1 isolates, with little or no Tier 2 neutralizing activity detected (218). Despite these limitations, results of the RV144 trial provide a starting point to examine factors in the prime-

boost strategy that may improve vaccine efficacy, including the generation of antibodies that may neutralize Tier 2 viruses.

Passively administered Nab have been shown to protect against primate lentivirus infection in animal models (174, 178, 203, 285-287); therefore it remains a major goal for HIV-1 vaccines to elicit these antibodies. Recent studies described vaccine-induced Tier 2 virus Nab in immunized animals; however, these responses are limited, sporadic, and primarily against the autologous Tier 2 isolates (315-317). Novel immunogens are being examined in hopes that they may elicit cross-reactive Tier 2 Nab (203, 287, 318, 319). We previously reported that removal of a single N-linked glycan at amino acid N197 (N7) of gp120 enhanced the ability of Env to generate cross-reactive neutralizing responses (226, 315). This study was based on a single isolate, 89.6. Since the N7 glycan and its effect on Env antigenicity are highly conserved (148, 225, 226, 270, 320), it is of interest to determine if the effects of the N7 glycan on Env immunogenicity can be observed in isolates other than 89.6.

In the present study, we sought to examine if antibody responses that have been correlated with protection against HIV-1 acquisition in humans can be elicited in a preclinical model by a poxvirus prime-gp120 protein boost strategy. Specifically, we used a replication-competent vaccinia virus vector for priming and two Clade B Envs (JR-FL and PVO.4) for boosting. These Env differ in multiple parameters, including their tissue origin, neutralization sensitivity, and the presence of the N7 glycan that was previously shown to modulate the exposure of variable loop 3 (V3) and CD4 binding sites (CD4bs) on Env (225, 226, 261, 265). Using this prime-boost immunization regimen, we were able to induce cross-reactive binding antibodies against V1/V2 fusion proteins and neutralizing responses against heterologous Tier 2 isolates. However, contrary to our previous finding in 89.6, the N7 glycan had little or no impact

on Env immunogenicity in the context of the poxvirus prime-gp120 boost used, indicating the need for further improvements in immunization strategy by optimizing the nature of the priming and boosting immunogens.

Results

Immunogens and immunization regimen

To determine if antibody responses elicited by a poxvirus prime-gp120 protein boost strategy are affected by the choice of the Env immunogen used, we studied two Clade B Envs (JR-FL and PVO.4) that differ in multiple parameters including: their tissue origin, neutralization sensitivity, and the presence of the N7 glycan previously shown to modulate the exposure of the CD4 and coreceptor binding sites on Env (225, 226, 261, 265). JR-FL, is a brain isolate that lacks the N7 glycan sequon (261) and shows Tier 2 neutralization sensitivity, whereas PVO.4 is a blood isolate that retains the highly conserved N7 glycan sequon and shows a Tier 3 neutralization resistant phenotype (225, 265, 294).

To increase the efficacy of priming with these Env immunogens, we took advantage of an attenuated, but replication-competent vaccinia virus vector, v-NY, which was used in the first clinical trials of the poxvirus-protein boost strategy (276, 321). We generated recombinant vaccinia viruses that express full-length Envs with (N7+) or without (N7–) the sequon of the N197 glycan **(Fig. 4.1)**. As reported previously, over-expression of full-length HIV-1 Env resulted in the accumulation of unprocessed gp160. However, these Env are processed, albeit inefficiently, and are fully functional in engaging the CD4 receptor and mediating membrane fusion (280, 322).

New Zealand White rabbits were primed at weeks 0 and 8 with recombinant vaccinia virus expressing one of four full-length gp160s: JR-FL N7 deglycosylated (JR-FL N7–), JR-FL

N7 glycosylated (JR-FL N7+), PVO.4 N7 glycosylated (PVO.4 N7+), or PVO.4 N7 deglycosylated (PVO.4 N7–) Env **(Fig. 4.1)**. Animals were boosted with the cognate gp120 Env protein formulated in alum at weeks 35 and 48 **(Fig. 4.2)**. Animal sera collected two weeks after each boosting event (week 37 and 50 respectively) were tested for antibody binding and neutralizing capabilities compared to pre-immune sera.

All immunized animals generated Env-specific antibodies

In order to determine the potency and cross-reactivity of the Env-specific antibodies, we tested the ability of week 37 and 50 sera to bind homologous wild type (WT) JR-FL and PVO.4 gp120 protein as well as heterologous SF162 gp120 protein in an ELISA **(Fig. 4.3A)**. Immune sera showed endpoint binding titers of ~10^5 against SF162 gp120 and up to ~10^6 titers against homologous gp120. All sera demonstrate similar endpoint binding titers to heterologous gp120. Maximal endpoint titers were achieved after the first gp120 boost, with no further increase obtained after the second boost. This indicates that the prime-boost immunization regimen, regardless of the Env used in this study, was able to generate high titers of cross-reactive Env-specific antibodies.

Effect of gp120 boost on the specificity of anti-Env responses

Previous studies with subunit and prime-boost immunization have elicited higher antibody titers against V3, constant region 1 (C1), and constant region 5 (C5) compared to responses against V1 and V2 (323-325). To determine the epitope-specificity of the antibody responses in immunized animals, we used a Bioplex assay to examine the ability of week 37 and 50 sera to bind overlapping peptides derived from consensus B (ConB) Env spanning C1, C5, V1, V2, and V3 epitopes **(Fig. 4.3B)**. Consistent with previous studies and regardless of which immunogen was used, high levels of serum reactivity were detected against C1, C5, or V3

peptides compared to V1 and V2 peptides. All animals generated antibodies that recognize peptides that span the mid-region of the V2 loop (HXB2 numbering sequence 169-184, data not shown), a region recognized by antibodies generated by protected RV144 vaccinees (326). While there was no significant difference in the overall gp120-binding antibody titer between the week 37 and 50 sera **(Fig. 4.3A)**, the second gp120 immunization significantly boosted antibody reactivity to all peptides tested **(Fig. 4.3B)**, indicating a preferential expansion of antibodies that recognize linear epitopes resulting from the gp120 boosts.

Cross-reactive antibodies that recognize recombinant V1/V2 fusion proteins

Antibodies against the V1/V2 loop, specifically those that recognize V1/V2-gp70 fusion proteins, have been shown to correlate with protection in the RV144 trial (214-216). Since all animals generated antibodies against peptides corresponding to V1 and V2 **(Fig. 4.3B)**, we tested the rabbit sera's ability to recognize a cross-clade panel of V1/V2 fusion proteins derived from different Envs in a multiplex binding antibody assay **(Fig. 4.4)**. By week 37, all 12 JR-FL and 9/12 PVO.4 immunized animals generated antibodies that recognize V1/V2 fusion proteins derived from multiple isolates of different clades. Reactivity to V1/V2 fusion proteins is comparable between week 37 and week 50 sera, indicating that the priming followed by one gp120 boost was sufficient to generate V1/V2 binding antibodies. The JR-FL N7+ immunized animals generated some of the highest mean florescence intensity (MFI) values against clade B V1/V2 fusion proteins compared to other groups. Regardless of the presence or absence of the N7 glycan, immunization with JR-FL Env consistently generated V1/V2 fusion binding antibodies capable of recognizing clade B, C, and AE Env. This contrasts with the more sporadic reactivity observed with PVO.4 Env immunization. These results indicate the prime-boost

strategy effectively induced V1/V2-directed antibodies, but the profile of their V1/V2-reactivity differed between animals that received the JR-FL or the PVO.4 immunogens.

Neutralization of standard Tier 1B and Tier 2 clade B isolates

In order to determine their neutralization capability, we tested immune sera against a number of Tier 1 HIV-1 and a standard panel of primary clade B isolates **(Table 4.1)**. At 1:15 dilution, all sera neutralized SF162, a Tier 1A isolate, and most sera were capable of neutralizing some Tier 1B isolates, including DJ263.8, a clade A isolate (267), indicating the presence of cross-neutralizing activities. However, CRF02_AG_271, a clade AG isolate, was not as readily neutralized by the sera. Sera from JR-FL N7+ or PVO.4 N7+ immunized animals neutralized over 50% of the Tier 2 and Tier 3 isolates on the standard clade B panel; whereas those from N7– Env-immunized animals failed to do so. Notably, two animals in the PVO.4 N7+ group, animal 643 and 645, showed neutralizing activities against nearly the entire panel of Tier 1, 2, and 3 isolates tested.

Previous studies have shown that sera neutralizing Tier 1 isolates are primarily directed against the V3 loop. Therefore, we tested if linear ConB V3 peptides would compete with the ability of immune sera to neutralize Tier 1 viruses **(Fig. 4.5)**. Regardless of the immunogen used, neutralizing activities of immune sera against SF162 and Bal.26 was fully or partially competed out with the addition of V3 peptides **(Fig. 4.5A&B)**, indicating that V3 directed antibodies contributed to the neutralizing activities in the sera. However, neutralization of DJ263.8 was unchanged with the addition of V3 peptides **(Fig. 4.5C)**, indicating that the neutralizing activities could not be competed with the V3 peptides used, or the Nab may recognize epitopes other than V3 on the clade A Env.

Cross-clade neutralization of a panel of global Tier 2 isolates

To further test the breadth of the neutralizing reactivity of the immune sera, we tested week 37 sera's ability to neutralize a standard panel of global Tier 2 viruses **(Fig. 4.6)**. Interestingly, each virus on the panel was neutralized by at least one animal sera tested. All animals, except one in the JR-FL N7– group (animal 631), generated sera capable of neutralizing at least one virus on this global panel (data not shown). Notably, a Tier 2 clade C isolate, CE1176_A3, was neutralized by sera from all but one animal, with titers reaching ~1:100 for some sera **(Fig. 4.6A)**. However, animals immunized with N7+ immunogens generated sera with significantly higher neutralization titers against the global Tier 2 isolate panel compared to animals immunized with their N7– counterparts **(Fig. 4.6B).** JR-FL N7+ immunized animals generated higher ID_{50} titers against the global virus panel and clade B virus panel compared to JR-FL N7– immunized animals. PVO.4 N7+ immunized animals generated higher ID_{50} titers against the clade B panel and a trend ($p=0.08$) toward increased ID_{50} titers against the global panel compared to PVO.4 N7– immunized animals. In fact, over 50% of the isolates in the global Tier 2 panel was neutralized by week 37 sera from the JR-FL N7+ group, significantly higher than the 18% neutralized by sera from the JR-FL N7– group (data not shown?). Sera from PVO.4 N7+ immunized animals neutralized 45% of the panel while sera from PVO.4 N7– immunized animals neutralized 36% of the panel. However, the percent of neutralization against the global panel diminished significantly after a second gp120 boost for JR-FL N7+ and PVO.4 N7– groups, indicating that repeated immunization with gp120 did little to boost the neutralizing antibody response against heterologous Tier 2 isolates tested.

Characterization of neutralizing activities in immune sera against CE1176_A3

To determine the epitope specificity of the Tier 2 neutralizing activities in the immune sera, we generated a panel of site-specific mutants of CE1176_A3 that are resistant to one or

more broadly neutralizing monoclonal antibodies (bNMab) of known specificities. CE1176_A3 *env* was used to generate these mutants because all animals but one could neutralize this isolate better than other isolates on the global panel **(Fig. 4.6A)**. Previous work has defined the epitopes targeted by bNMabs by mutational analysis: mutants N280D or G458Y are resistant to CD4bs-directed antibodies VRC01, CH31, and 3BNC117 (327); N160K mutant is resistant to V2-directed antibodies CH01 PG9, and PG16 (151, 328, 329), N332A mutant is resistant to V3 glycan-directed antibody PGT128 (150), W672A mutant is resistant to MPER-directed antibodies 4E10, 2F5, or 10E8 (330, 331), and a double mutant, N611A/N625A, is resistant to antibody PGT151 directed to gp120/gp41 interface epitopes (134, 332). Based on this information, a panel of single-site and multiple-site mutants was generated and their sensitivity profile tested against a panel of bNMab listed above. Results in **Fig. 4.7** confirm the expected neutralization sensitivity profile of the following mutants: (1) single-site mutants N280D or G458Y were resistant to CD4bs-directed monoclonal antibodies (Mab) VRC01, CH31, and 3BNC117, but sensitive to Mab directed to all other sites **(Fig. 4.7A & B)**; (2) a quintuple mutant (N160K.N332A.N611A.N625A.W672A) retained sensitivity to CD4bs antibodies, but was resistant to antibodies directed to all other sites **(Fig. 4.7C)**; (3) a quadruple mutant (N332A.N611A.N625A.W672A) was sensitive to CD4bs- and V2-directed antibodies, but resistant to V3, MPER, and gp120/gp41 interface antibodies **(Fig. 4.7D)**; (4) a triple mutant (N160K.N332A.W672A) was sensitive to CD4bs- and gp120/gp41 interface-directed antibodies, but resistant to V2-, V3-, and MPER-directed antibodies **(Fig. 4.7E)**. Thus, these mutants demonstrated the expected neutralization sensitivity profile against the panel of bNMab tested.

Having characterized the neutralization sensitivity profile of these mutants, we examined the epitope specificity of the neutralizing activity in a subset of immune sera against WT and

mutant CE1176_A3. Most sera had similar neutralizing potency against WT CE1176_A3 and mutant viruses, thus showing no identifiable specificity but confirming their neutralizing activities against this Tier 2 virus. Notably, two animals, 640 and 647, showed clearly reduced neutralization potency when tested against CD4bs resistant mutants **(Fig. 4.8)**. Serum from animal 640 was unable to neutralize CE1176_A3 mutants N280D and G458Y, both of which are resistant to CD4bs-directed antibodies, while retaining neutralizing activities against all other mutants tested **(Fig. 4.8A)**. Serum from animal 647 also showed reduced neutralization potency against the N280D, but not the G458Y mutant, consistent with the idea that the antibodies in this animal's serum targeted epitope(s) dependent on N280, but not G458 **(Fig. 4.8B)**. Together these data indicate that CD4bs-directed antibodies may contribute to the neutralizing activities in the immune sera from these rabbits against a heterologous Tier 2 virus, CE1176_A3. Interestingly, animal 647 sera also showed reduced potency against three multiple-site mutants tested (**Fig. 4.8B**), indicating neutralizing activities directed against multiple targets.

Discussion

We demonstrate here a poxvirus prime-gp120 boost immunization regimen elicited, in a rabbit model, immune responses that have been shown to correlate with protection against HIV-1 acquisition in humans or other preclinical animal models. Specifically, we showed that immunized rabbits generated cross-clade V1/V2-specific antibodies, a response found to correlate with risk reduction of HIV-1 acquisition in the RV144 trial (214, 215, 326). In addition, all the immunized rabbits in this study showed neutralizing activities against one or more of the HIV-1 isolates tested, including a number of heterologous Tier 2 isolates in the standard TZM-bl assay, albeit at low titers (1:20 to ~1:100). These observations, if confirmed in other preclinical

models, may provide a starting point to examine factors in the prime-boost immunization strategy that may improve upon the modest efficacy achieved by the RV144 trial.

The profile of V1/V2-specific responses varied between animals immunized with JR-FL or PVO.4 Env **(Fig. 4.4)**. All JR-FL-immunized animals generated antibodies that recognized V1/V2 fusion proteins from diverse isolates, whereas a number of PVO.4-immunized animals failed to show any detectable V1/V2 reactivity. The V1/V2 loop of different HIV-1 isolates varies significantly in their amino acid length and the number of potential N-linked glycan sites (17, 67, 68). Whether the differential immunogenicity in V1/V2 responses observed in this study reflects the accessibility or structural stability of the V1/V2 loop in these Env is yet to be determined. Nevertheless, our finding indicates that Env from some HIV-1 isolates may be better than others at inducing antibodies that recognize the conformation-dependent epitopes in the V1/V2 loop. While the functional role of the V1/V2 specific antibodies remains unclear, immunized animals in this our findings indicate that the recombinant vaccinia prime-gp120 protein boost regimen described in this study was able to generate, in a preclinical animal model, antibodies with epitope-specificity that have been correlated with protection in the RV144 trial (214, 326).

Furthermore, immunized rabbits described here also generated cross-reactive neutralizing activities, not only against Tier 1A and some Tier 1B viruses, but also against heterologous Tier 2 viruses, with up to 50% neutralization of the global Tier 2 panel tested **(Table 4.1 & Fig. 4.6)**. This neutralizing activity was reproducible **(Fig. 4.8 and data not shown)** and was not due to non-specific activities as indicated by the lack of neutralization against MuLV **(Table 4.1)**. However, the nature and the specificity of the neutralizing responses observed in these animals remain to be defined. Some, but not all, of the Tier 1 neutralizing activities can be competed out

by V3 peptides **(Fig. 4.5)**, indicating that antibodies directed to non-V3 or non-linear epitopes in V3 may contribute to the neutralization of these easy-to-neutralize isolates. Analysis with epitope-specific mutants of CE1176 indicates some animals generated CD4bs-directed antibodies, albeit with differential and overlapping specificities, that contribute to the neutralization against this Tier 2 virus **(Fig. 4.8)**. This is highly surprising, since cross-reactive neutralizing antibodies directed against the CD4bs typically take 2-4 years after seroconversion to develop in infected individuals (218, 271, 286, 287). Without further substantiation, we cannot attribute the CD4bs-directed neutralizing activities in the immunized rabbits to bNab as defined by Mab such as VRC01. Nevertheless, our findings support the notion that the prime-boost immunization approach as described here can induce Nab responses against heterologous Tier 2 isolates, albeit with limited breadth and modest titers. Future studies will be needed to better define the nature of these responses, and if and how these responses could be improved.

It is interesting to note that repeated gp120 boosting increased the ability of immune sera to bind certain peptides, including those that were also recognized by RV144 vaccinees **(Fig. 4.3B)** (214-216). However, the increase in peptide-specific responses was not accompanied by increased titers to gp120 **(Fig. 4.3A)**. Therefore, the second boost may preferentially increase responses to linear peptides presented by the gp120 proteins. Additionally, repeated gp120 immunization failed to boost and, in some cases, resulted in diminished neutralization responses **(data not shown).** This observation is consistent with the idea that the first subunit protein immunization may boost antibodies that were generated after the priming event, while the second subunit protein boost preferentially expanded antibodies induced by the first protein immunization that were limited in their neutralizing activities (333). If so, gp120 may be a

suboptimal immunogen for a prime-boost immunization regimen aimed at generating neutralizing antibody responses.

While the N7 glycan has been shown to increase the stability or exposure of the CD4bs and V3 epitopes in a conserved manner (225, 226), results reported here suggest that the N7 glycan may not play a conserved role in modulating Env immunogenicity, at least in the context of the vaccinia prime-gp120 boost regimen. As shown in **Table 4.1 & Fig. 4.6,** animals immunized with N7+ Env showed similar or slightly broader neutralization and higher titers of neutralizing activity compared to those immunized with their N7– counterparts. The failure to observe any difference is not due to any masking effect of the N7 glycan that may have resulted from suboptimal immunization in N7– immunized animals since all groups have similar vaccinia neutralizing (data not shown) and gp120 binding titers (**Fig. 4.3)**. This is in direct contrast to our previous study in macaques demonstrating that N7– 89.6 Env induced broader and more potent neutralizing serum compared to its glycosylated counterpart (226). While these results may be related to the Env immunogen (89.6 vs. JR-FL or PVO.4), or the animal species (rabbits vs. macaques) used, the potential effect of the N7 glycan on Env immunogenicity could also depend on a number of other factors. For example, while the removal of the N7 glycan enhanced the ability of 89.6 Env to mediate CD4 independent entry (226), no such effect was observed for N7– versions of JR-FL or PVO.4 Env (225). Additionally, our earlier study in macaques used 89.6 gp140 as the boosting immunogen, whereas the current study used gp120 protein, in an attempt to better simulate the RV144 regimen. Since the N7 glycan is predicted to shield the adjacent protomer's V3 loop (295, 296), the biological property of the N7 glycan may be dependent on the trimeric structure of Env. In fact, our V3 peptide competition data demonstrates no difference between the ability of N7+ or N7– Env to generate V3-neutralizing antibodies in

the context of gp120 boost **(Fig. 4.5)**. Therefore, gp120 may not be the appropriate immunogen to determine if the N7 glycan has any impact on Env immunogenicity. Indeed, other studies have demonstrated improved neutralization responses in animals immunized with trimeric gp140 compared to animals immunized with gp120 monomers (334-337). Our finding that repeated gp120 boosting skewed antibody responses to linear epitopes **(Fig. 4.3B)** may also contribute to the lack of improved neutralization responses observed in N7– immunized animals **(data not shown)**. It is therefore of interest to explore immunogens that can better preserve the trimer structure, such as SOSIP trimers, to test the potential role of the N7 glycan in modulating Env immunogenicity.

Despite many attempts (315-317, 338-340), generation of bNab against Tier 2 primary HIV-1 isolates by vaccination remains an unmet goal. Several recent studies succeeded in demonstrating vaccine-induced Nab against autologous Tier 2 virus, or a panel of mutated Tier 2 isolates with increased CD4 or V3 epitope exposure (225, 315-317). Our finding of neutralizing responses against heterologous Tier 2 global isolates, albeit of modest potency and breadth, point to the possibility of generating such responses through the prime-boost immunization platform. The basis for the apparently improved response reported here is not clear. Several factors may contribute our findings, including the possible role of a replication competent virus for priming (276, 321). Replication competent viruses have been shown to be more effective protecting against certain viral infections and disease than immunization by replication incompetent viruses or subunit vaccines (341-343). Alternatively, differences in vaccine responses may also result from the specific Env, the nature of the boosting immunogens, and the immunization regimen used. Further studies will be needed to determine the potential contribution of these factors,

including the role of specific glycans, to improve the protective efficacy achievable by the prime-boost immunization regimen.

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Group	Virus	SF162	6535.3[b]	BAL.26	SS1196	DJ263.8	CRF02_AG Clone 271	JR-FL	QH0692.42[b]	SC422661.8[b]	TRO.11[b]	AC10.0.29[b]	RHPA4259.7[b]	THRO4156.18[b]	REJO4541.67[b]	WITO4160.33[b]	CAAN5342.A2[b]	PVO.4[b]	TRJO4551.58[b]	MuLV
	Clade	B	B	B	B	A	AG	B	B	B	B	B	B	B	B	B	B	B	B	
	Animal Number	Tier 1A	Tier 1B	Tier 1B	Tier 1B	Tier 1B	Tier 1B	Tier 2	Tier 2	Tier 2	Tier 2	Tier 2	Tier 2	Tier 2	Tier 2	Tier 2	Tier 2	Tier 3	Tier 3	
JR-FL N7–	630	95	71	91	84	65	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	631	96	63	93	80	78	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	632	95	-	96	81	65	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	633	94	57	79	75	59	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	634	93	63	95	81	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	635	95	57	87	78	79	-	-	-	-	-	-	-	-	-	-	-	-	-	-
JR-FL N7+	636	95	57	92	66	84	-	55	-	-	-	-	-	51	-	-	-	-	-	-
	637	90	-	93	75	51	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	638	98	-	98	82	78	56	-	-	-	-	-	-	-	-	-	-	-	-	-
	639	94	81	88	72	85	-	52	55	-	-	-	-	51	55	-	-	-	-	-
	640	97	91	96	83	90	53	90	70	-	-	73	60	-	60	-	-	-	-	-
	641	99	58	99	86	93	57		58	-	-	-	-	-	51	-	-	-	-	-
PVO.4 N7+	642	97	-	94	75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	643	98	84	96	86	87	69	87	83	78	50	75	81	72	74	62	-	68	-	-
	644	96	-	90	64	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	645	98	92	57	56	86	-	81	81	78	72	81	84	74	76	63	63	59	56	-
	646	93	-	55	-	-	-	70	-	-	-	-	-	-	-	-	-	-	-	-
	647	99	90	98	84	-	58	66	51	-	-	-	56	-	-	-	-	-	-	-
PVO.4 N7–	648	73	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-	-	-	-
	649	96	-	89	63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	650	97	67	94	68	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	651	90	-	58	-	54	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	652	98	-	97	60	59	56	-	-	-	-	-	-	-	-	-	-	-	-	-
	653	89	-	-	-	-	-	69	-	-	-	-	-	-	-	-	-	-	-	-

% Neutralization of HIV-1 Pseudovirus Tested with 1:15 Ratio of Week 37 Serum[a]

Table 4.1: Neutralization of Tier 1 and Tier 2 viruses by animal sera.

[a]Input virus was normalized by their infectivity at 150 $TCID_{50}$ in TZM-bl cells. The column subheadings specify the indicator viruses and their respective clades. Percent neutralization was determined in TZM-bl cells by measuring the reduction in RLU relative to wells that contain the same dilution of corresponding pre-immune sera. 50-74% neutralization is highlighted in pink, ≥75% neutralization is highlighted in red. Results represent the average of two or more experiments, each performed in duplicates.

[b]Isolates included in the standard clade B panel are indicated (265)(265)(265).

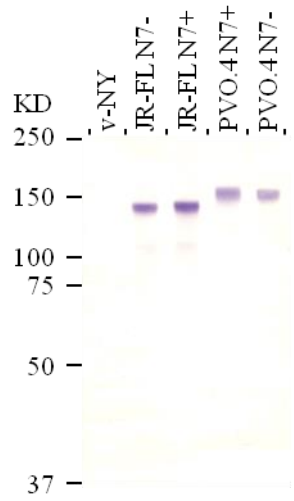

Figure 4.1: Western blot analysis of gp160 expressed by recombinant vaccinia virus.

Lysates of African green monkey kidney cells (BSC-40) infected with parental vaccinia virus, v-NY, or recombinant viruses expressing the full-length Env of HIV-1 JR-FL or PVO.4 were resolved by SDS-PAGE and immunoreactive protein gp160 was detected by HIV+ human serum. N7- or N7+ denotes the absence or presence of the sequon for the N-linked glycan at amino acid 197.

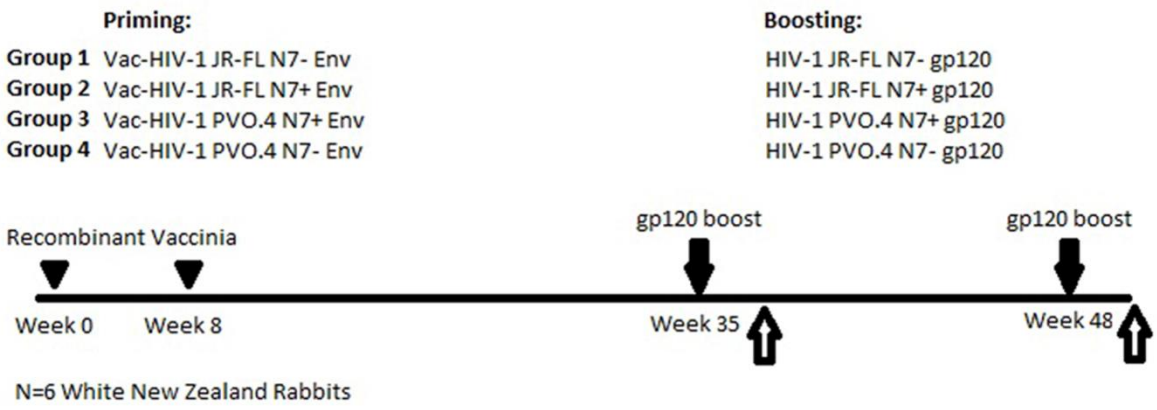

Figure 4.2: Study design and immunization schedule. Rabbits were immunized with recombinant vaccinia virus expressing full length gp160 at weeks 0 and 8 followed by a cognate gp120 protein boost formulated in alum at weeks 35 and 48 as described in Materials and Methods. Sera were collected before and two weeks after each immunization event. Time points for the collection of week 37 and 50 sera used for the analyses described in this study are marked by open arrows.

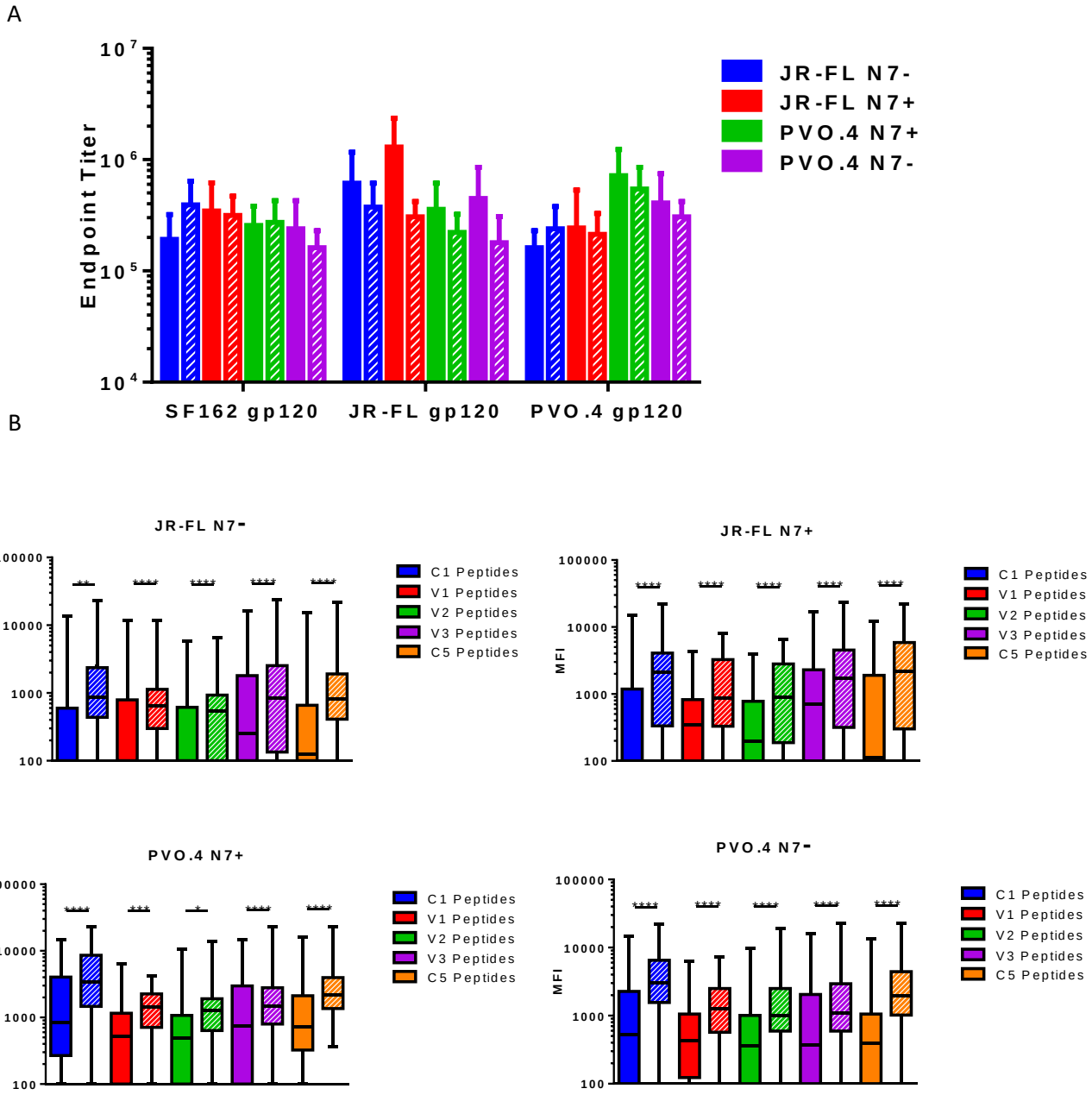

Figure 4.3: gp120-specific antibody response in immunized animals. (A) Endpoint titers of Env-specific antibodies in week 37 (solid boxes) or week 50 (hatched boxes) sera were determined by ELISA against a heterologous gp120 (WT SF162), or homologous gp120's (WT JR-FL or PVO.4 gp120) denoted on the X axis. Error bars indicate standard deviations within the group. (B) Reactivity of antibody responses targeting single, overlapping linear peptides

corresponding to the variable (V1-V3) or conserved (C1 & C5) regions of gp120 as indicated was reported as the mean fluorescence intensity (MFI) in the Bioplex assay. Solid boxes indicate week 37 serum and hatched boxes represent week 50 serum. The boxplots indicate the median as well as the range of responses against individual peptides in each pool, including the maximum, minimum, and the 75% and 25% quartiles as indicated. p-values (* $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$, **** $p \leq .0001$) were calculated by the Wilcoxon matched-pairs signed-rank test.

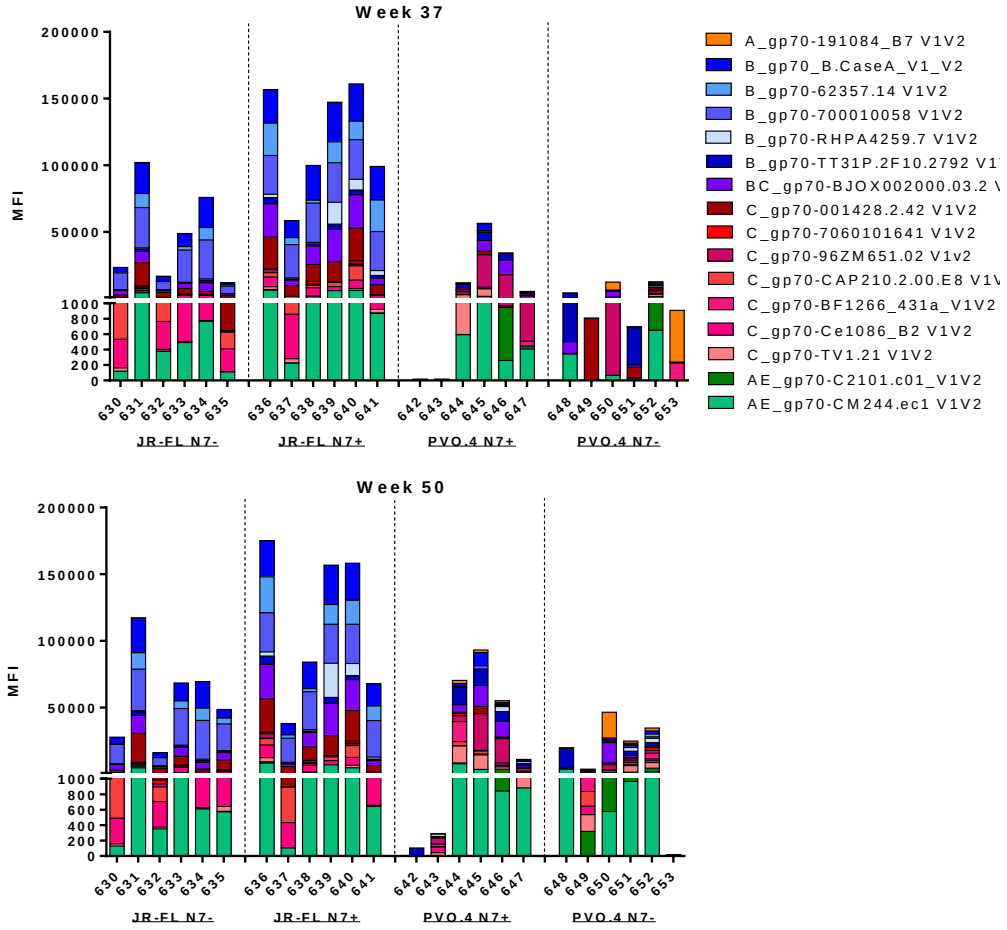

Figure 4.4: Cross-reactivity of anti-V1/V2 antibodies in immunized animals. V1/V2 specific antibodies in week 37 and 50 sera at a 1:500 dilution were measured against a cross-clade panel (color coded by clade) of gp70-V1/V2 MuLV scaffold proteins in a binding antibody multiplex assay (BAMA). The animal number and immunization group is listed on the X axis. Dotted lines separate the groups.

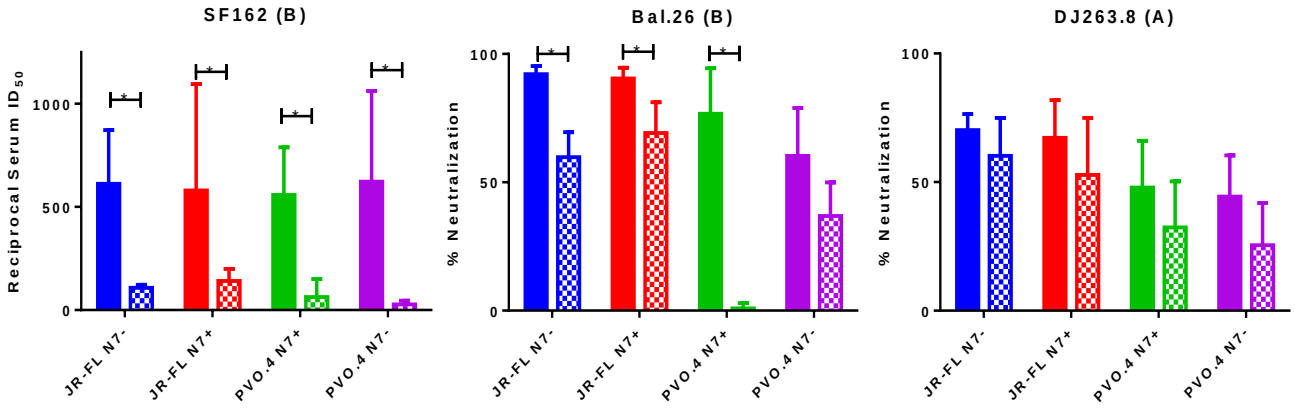

Figure 4.5: Competition of Tier 1 virus-neutralizing activities by V3 peptides. Effect of V3 peptide on the neutralizing activity of week 37 sera against Tier 1A (SF162) and Tier 1B (Bal.26 and DJ263.8) viruses was evaluated. The clade designation of the indicator viruses is shown in parentheses. Neutralizing activities were shown either as reciprocal serum dilution that resulted in 50% reduction of infectivity (ID_{50} panel A), or as percent neutralization of virus infectivity by test sera at 1:15 dilution (panels B & C). Neutralizing activities detected in the presence of a scrambled V3 peptide are shown by solid boxes, and activities detected in the presence of overlapping peptides corresponding to the consensus clade B V3 sequence shown by checkered boxes. Error bars indicate standard deviations of data from at least two independent experiments performed in duplicates. p-values (* $p \leq .05$) were calculated by two-tailed Mann-Whitney test.

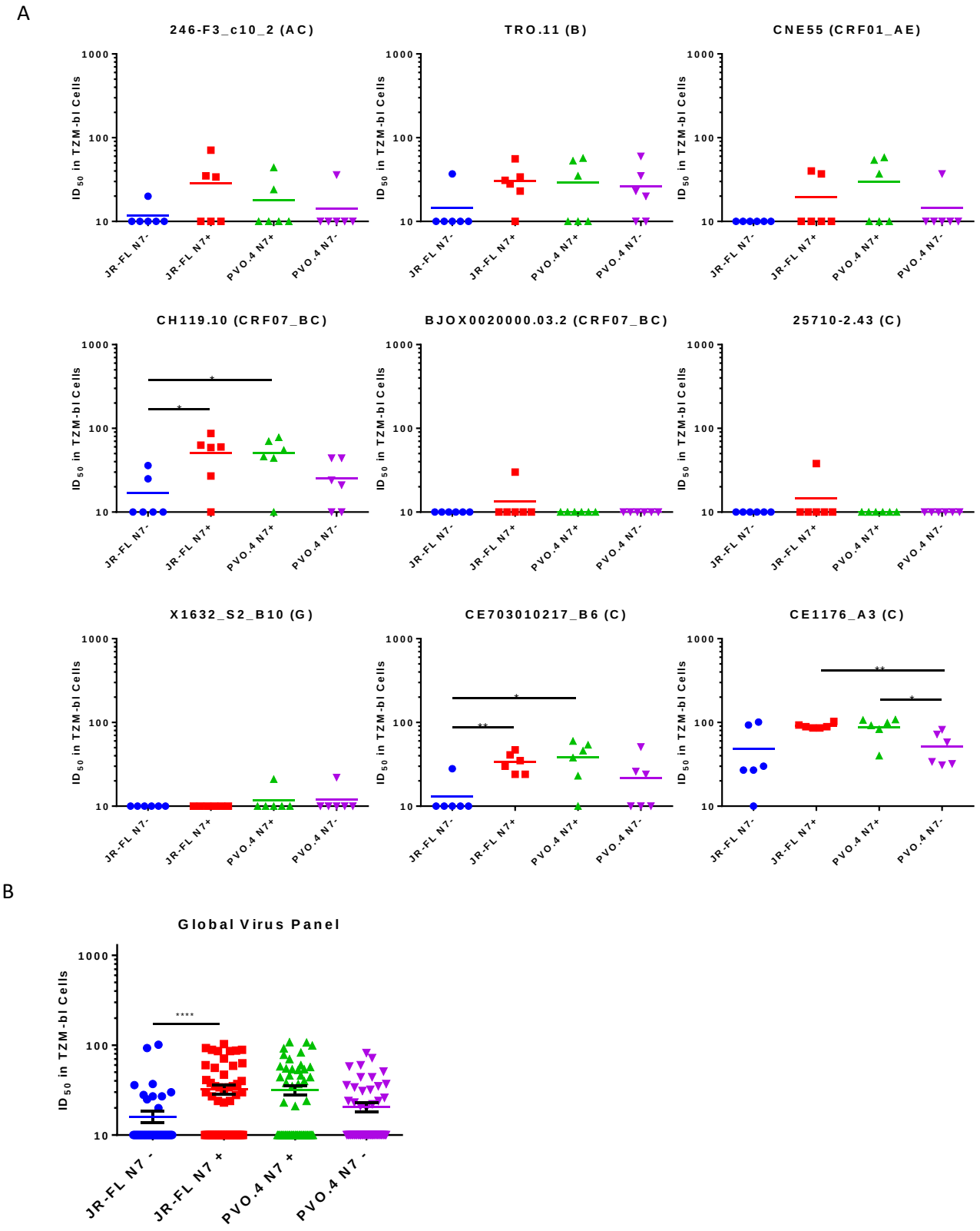

Figure 4.6: Neutralization of Tier 2 viruses. (A) Each graph shows the reciprocal dilution of

week 37 sera from each immunized animal that resulted in 50% reduction of infectivity (ID_{50}) against the indicator virus shown on the heading of the graphs. The clade designation of the indicator viruses is shown in parentheses. Values above 10 were considered as positive neutralization. p-values (* $p \leq .05$, ** $p \leq .01$) were calculated by two-tailed Mann-Whitney test. (B) The neutralization ID_{50} titers of individual animal sera against the global Tier 2 virus panel was determined for each group. The mean is indicated by colored bars and the standard error mean is indicated by black error bars. p-values (* $p \leq .05$, **** $p \leq .0001$) were calculated by Mann-Whitney test for paired values.

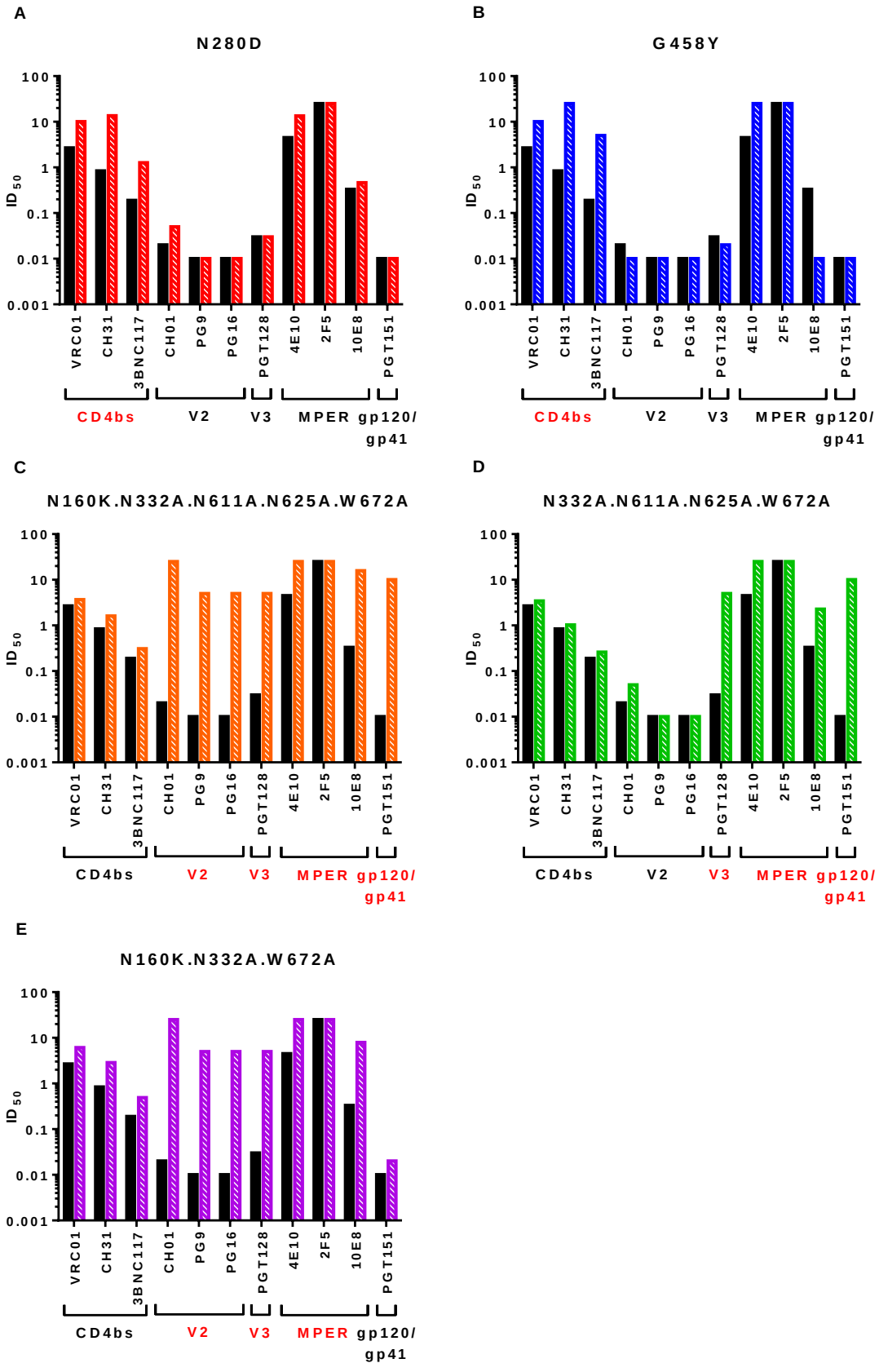
A
N280D
B
G458Y
C
N160K.N332A.N611A.N625A.W672A
D
N332A.N611A.N625A.W672A
E
N160K.N332A.W672A
ID50
100
10
1
0.1
0.01
0.001
VRC01
CH31
3BNC117
CH01
PG9
PG16
PGT128
4E10
2F5
10E8
PGT151
CD4bs
V2
V3
MPER
gp120/
gp41

Figure 4.7: Neutralization sensitivity profile of CE1176_A3 mutants determined by a panel of broadly neutralizing monoclonal antibodies. The ID_{50} values for a given neutralizing antibody are shown against the WT CE1176_A3 (solid, black bar) and the indicated mutants (hatched, colored bar). The epitope recognized by each antibody is indicated at the bottom of the panel. Maximum antibody concentration used for VRC01 or PGT151 is 10 μg/mL; 25 μg/mL for CH31, CH01, 4E10, 2F5, or 10E8; and 5 μg/mL for 3BNC117, PG9, PG16, or PGT128. The lower limit of detection is 0.01 μg/mL of antibody. Increased height of the colored hatch bar compared to the black bar indicates increased resistance of the mutant virus to the antibody tested as compared to the WT virus. Resistance of the mutant to any Mab tested is indicated by red letterings at the bottom of the panel.

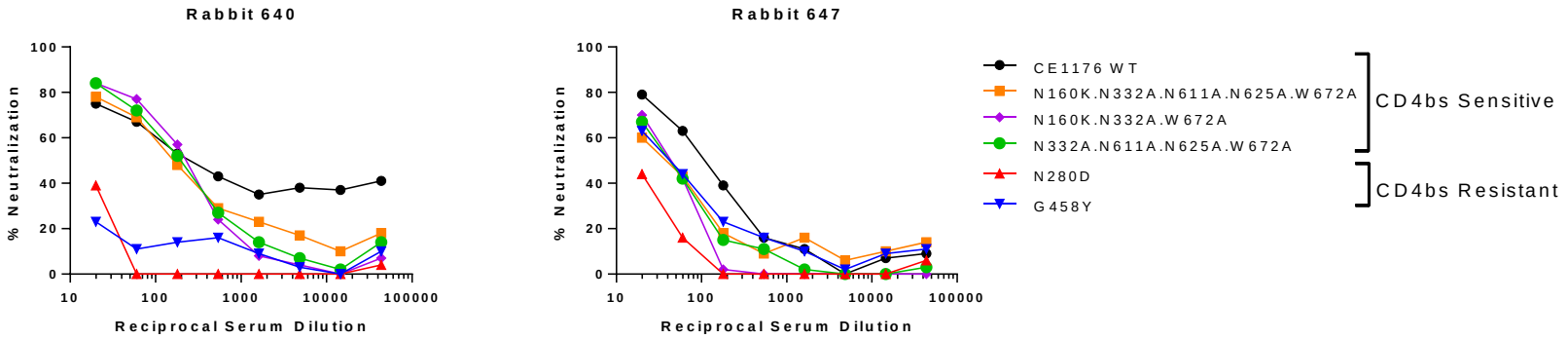

Figure 4.8: Specificity of neutralizing activities in immune sera determined by sensitivity to epitope-specific mutants of CE1176_A3. Week 37 sera from two immunized rabbits were tested for neutralizing activities against WT CE1176_A3 and a panel of epitope-specific mutants. The three multiple-site mutants shown retain their sensitivity to CD4bs directed antibodies, whereas the two single-site mutants are resistant. The X-axis indicates the reciprocal dilutions of the immune sera tested (three-fold serial dilution starting at a 1:20). The Y-axis indicates the percent of viral infectivity neutralized by a given serum dilution against the WT or mutant viruses. Decreased percent neutralization against a mutant virus compared to the WT indicates a loss of neutralizing activities due to the mutation(s) introduced to specific epitope(s) in the indicator virus.

Chapter Five

Conclusions

It is generally believed that to prevent infection, an effective HIV-1 vaccine will have to elicit antibodies that can neutralize virus currently circulating among humans. However, thus far, no HIV-1 vaccine has successfully elicited these Nabs in a clinical trial. One major limitation to discovering an HIV-1 vaccine that would induce protective antibody responses is the lack of understanding of the dichotomy between the antigenic and immunogenic characteristics of Env and how antigens lead to the generation of cross-reactive Nabs. In order to elucidate how the antigenic property of a particular Env relates to its immunogenic property. I focused my thesis work on studying how a single N-linked glycan impacts the antigenicity and immunogenicity of Env. The results of this dissertation could inform future vaccine designs.

Glycan Modification of Env Immunogens

Glycan modification has previously been shown to play a role in modifying the antigenicity and immunogenicity of other vaccine candidates, including hemorrhagic fever and feline immunodeficiency virus (309, 310), possibly by unmasking epitopes. As previously mentioned, glycan modifications have been used to modulate the antigenic properties of HIV-1 Env, but glycan modifications typically have little impact on the immunogenic properties of Env (227-230). However, a few studies have demonstrated that removal of highly conserved N-linked glycans improved the ability of Env to induce Nabs (148, 226). This may be due to the highly conserved nature of the N-linked glycans used in these studies, suggesting that these glycans play an important role on the Env, possibly protecting sites of vulnerability that are exposed upon removal of the glycan. In our studies, we used a set of criteria to select specific glycans whereby glycan mutants maintained functional integrity but exhibited increased sensitivity to

Nabs, indicating more exposed or stabilized epitopes. It is hoped that the exposure of these conserved epitopes will prompt the immune system to target these vulnerable areas.

Alternative criteria can be considered that may also result in altered antigenic and immunogenic Env properties. One approach targets conserved glycans that surround the CD4bs on Env (148). Removal of these glycans affects viral infectivity and improves neutralizing antibody induction. Another immunogen candidate includes glycan modified Env that demonstrates enhanced binding to unmutated common ancestors (UCA) of bNAbs compared to the glycosylated version (222, 308). It is postulated that using immunogens that bind to UCA of bNAbs will guide the B cell response to generate antibodies that resemble bNAb UCA. These bNAb UCA could then be further directed to generate bNAbs with subsequent immunizations using antigen that is more readily recognized by bNAbs. All of these methods of improving the immunogenic property of Env by glycan removal, including a combined approach, can be used to determine the best immunogens for vaccine design. However, the removal of multiple glycans on an Env immunogen typically results in a poor immunogen (227-231). These immunogens potentially induce antibodies that recognize epitopes that are buried under glycans on WT virus. This indicates that there is a limit to HIV-1 Env immunogen modification that needs to be considered when designing vaccine candidates. Therefore, our studies focused on single N-linked glycan modification of Env.

The N7 Glycan Masks Conserved Epitopes on Env

Glycans on HIV-1 have been shown to play diverse roles in viral replication, coreceptor usage, evasion of immune responses, and as targets for bNAbs (148, 150, 151, 226, 299). The sequon for the N7 glycan is present in the majority of available HIV and SIV sequences (19, 148, 233), suggesting strong selective pressure to maintain this particular glycan. Because previous

work examined the role of the N7 glycan on the antigenic and immunogenic properties of Env in only a limited number of isolates (148, 226, 270), we examined if the N7 glycan plays a conserved role on the antigenicity of Env in diverse isolates of HIV-1. In the third chapter of this dissertation, we were able to demonstrate that the N7 glycan plays a conserved role modulating the antigenicity of the CD4bs and V3 loop, a component of the coreceptor binding site, while maintaining the integrity of distal epitopes. These data, in addition to the observation that N7 glycan removal increases sensitivity of virus to pooled HIV-positive sera, leads to the conclusion that the N7 glycan plays a role in virus evasion from host immune responses. Indeed, N7 glycan removal could potentially be a useful tool in vaccine immunogens designed to expose or stabilize conserved epitopes that are targeted by bNAbs.

While the N7 glycan modulates the receptor and coreceptor binding sites in a conserved manner (225, 226), there are some isolate dependent antigenic effects. For instance, the N7 glycan differentially modulates sensitivity of Env to V1/V2 antibodies. Only the Env with the longest and most heavily glycosylated Env, PVO.4, demonstrated increased sensitivity to these antibodies when the N7 glycan was removed. This is in contrast to all other isolates tested that demonstrated increased resistance to V1/V2 antibodies when the N7 glycan was removed. It would be interesting to determine if this is due to a higher degree of structural constraint in the presence of a longer and more heavily glycosylated V2 loop. We hypothesized that greater flexibility in other isolates with shorter and less glycosylated V2 loop may result in the loss of the V1/V2 loop epitope, and thus the greater resistance. This hypothesis can be tested using other isolates that have longer and more glycosylated V2 loops or by adding glycans and amino acids to Env that demonstrate increased resistance to V1/V2 antibodies when the N7 glycan is removed. In addition to the isolate specific differences to V1/V2 antibody sensitivity, the CD4-

independence of Env, typically associated with a more "open" Env conformation (344), is modulated by the N7 glycan in an isolate and sequence-specific manner. To understand the conformational dynamics of Env, single-molecule fluorescence resonance energy transfer (smFRET) analysis, which was previously used to visualize dynamic changes in neutralization-sensitive and neutralization-resistant Env conformations (345), can be used to determine if the N7 glycan or V2 loop length and glycosylation impact the "openness" or flexibility of trimers.

While isolate specificities can impact the antigenic property of Env, results reported in chapter three suggest that the N7 glycan does impact the exposure or stability of some conserved epitopes in all isolates tested. Removal of a single N-linked glycan on Env is a relatively small change that leads to significant differences in the antigenicity of Env. The N7 glycan modified Env represents an attractive immunogen because only a single N-linked glycan is removed that exposes or stabilizes conserved epitopes while preserving other epitopes recognized by Nabs. The knowledge gained in the third chapter about the antigenic properties of Env offers us an opportunity to try and understand how these properties relate to the immunogenic properties of Env demonstrated in chapter four.

Differential Impact of N7 Glycan on Immunogenicity of Env

While the N7 glycan has been shown to have a conserved antigenic role modulating the exposure or stability of the CD4bs and V3 epitopes in the third chapter (225), we described how the N7 glycan had little or no impact on immunogenicity of Env in the context of the poxvirus prime-gp120 boost used in the fourth chapter of this dissertation. This is in direct contrast to our previous findings demonstrating that N7 glycan removal improved the immunogenic property of 89.6 Env (226). Using the results gained from the antigenicity studies, we can attempt to understand why the effect of the N7 glycan on immunogenicity of Env varies.

The differences between the two studies may be attributed to an intrinsic property of the Env immunogen used. One difference between the Env immunogens is their coreceptor usage. While JR-FL and PVO.4 Env utilize the CCR5 coreceptor, 89.6 Env is dualtropic and, therefore, capable of using both CCR5 and CXCR4 coreceptors. This indicates that the portion of Env that mediates coreceptor usage, particularly the V3 loop (310), differ in their phenotypes and genotypes. It would be interesting to use other Env that are dualtropic in immunogen studies to determine what effects, if any, coreceptor usage plays in the immunogenicity of Env.

Another difference between the N7 glycosylated or deglycosylated versions of JR-FL and PVO.4 or 89.6 Env is their ability to mediate CD4 independent entry (225). The removal of the N7 glycan enhanced the ability of 89.6 to enter cells that express CXCR4 (226), while the presence or absence of the N7 glycan does not alter the CD4 independence of JR-FL or PVO.4. This indicates that the N7 deglycosylated version of 89.6 is potentially in a CD4 "bound" conformation with a more exposed coreceptor binding site (226). It would be of interest to design future vaccine studies to determine if CD4 independent isolates have different immunologic properties compared to CD4 dependent isolates.

Another difference between the two studies to consider is the usage of different animal models; macaques versus rabbits. Among the multitude of differences between the species, one major advantage of using macaques is the ability to infect the animals with SHIV due to the presence of $CD4^+$ T cells (346). On the other hand, rabbits naturally lack cells that express CD4 (347) , the primary receptor for HIV-1 Env. This not only means that rabbits are not readily infected with HIV-1, but they may escape the regulatory mechanism of B-cell self-tolerance against self-reactive antibodies that are typical of some HIV-1 bNAbs (126-129), including those that target the CD4bs. This can possibly lead to qualitatively different antibody responses elicited

by the two species. A future vaccine study in macaques could be designed to recapitulate the JR-FL/PVO.4 rabbit immunogen study to determine if the same immunization regimen generates similar immune responses in macaques. This could also elucidate any immunologic variations elicited between the different species that can potentially account for the divergent responses to N7 glycosylated and deglycosylated 89.6 compared to JR-FL and PVO.4 immunogens.

One major difference between the two studies was the boosting immunogen. Our earlier study in macaques used 89.6 gp140 (a derivative of gp160 made by deleting the transmembrane and cytoplasmic domains) as the boosting immunogen (226), whereas the rabbit study used gp120 protein in an attempt to more closely follow the RV144 regimen. These two different immunogens have been shown to adopt different structures (348), possibly exposing different epitopes. It has been previously reported that immunization with gp140 generates qualitatively different antibody responses compared to immunization with gp120 monomers (334-337). Additionally, our observations in chapter four indicate that boosting with gp120 skews the antibody response to linear epitopes and potentially diminishes neutralizing antibody responses. The strongly immunogenic and ineffective epitopes on monomeric gp120 could distract the immune system from targeting the more relevant, broadly neutralizing epitopes. Antibodies elicited by gp120 typically react with epitopes that are poor neutralization targets and are presumable occluded on the native form of Env on HIV-1 isolates (333).

Not only are there differences between these boosting immunogens, but the N7 glycan differentially impacts the antigenic properties of gp140 and gp120. In the third chapter, we described how the N7 glycan does not affect the affinity of gp120 to CD4-IgG2. Instead, previous observations demonstrate that the N7 glycan may help stabilize the trimeric form of Env by occluding the V3 loop, shielding V3 epitopes, and inhibiting its premature release prior

to CD4 binding (295, 296). Therefore, the biological property of the N7 glycan may only be discernible in the trimeric form of Env. Consequently, the benefit of removing the N7 glycan, whereby it exposes conserved epitopes, may be lost when using gp120 to boost antibody responses compared to using gp140, a boosting immunogen that can potentially form trimers. This could explain the differential impact of the N7 glycan on the immunogenic properties of 89.6 and JR-FL/PVO.4 Envs. Future studies will need to be conducted to better understand the potential role of the N7 glycan in modulating the immunogenicity of gp120 protein, gp140 protein, and potentially immunogens that can better preserve the trimeric structure of Env.

V1/V2 Antibody Responses in Poxvirus Prime-gp120 Boosted Animals

As previously discussed, the most successful HIV-1 vaccine to date generated V1/V2 directed non-Nabs that were correlated with protection (214). In chapter four of this dissertation, we describe how immunization with a poxvirus prime-gp120 boost regimen in a small animal model elicited V1/V2-specific antibodies in addition to sera capable of neutralizing heterologous Tier 2 isolates. These observations could provide a starting point to examine factors that may improve upon the efficacy of the RV144 trial.

It is interesting to note how immunization with the different Env (JR-FL or PVO.4) generated different V1/V2-specifc profiles, indicating that Env from some isolates may be better than others at inducing antibodies that recognize the V1/V2 loop. Use of PVO.4, an Env with a longer and more heavily glycosylated V2 loop, generated more sporadic V1/V2 antibody responses compared to immunization with JR-FL, which generated consistent cross-reactive V1/V2 antibody responses in all animals tested. This may be due to the amino acid variation and glycosylation profile of the different Env. It is of interest to note that immunization with PVO.4 N7–, an Env that was shown in chapter three to have a modestly more exposed or stabilized

V1/V2 site compared to PVO.4 N7+ Env, did not impact the generation of V1/V2 directed antibodies compared to immunization with PVO.4 N7+ Env. This indicates that different Env show different immunogenic properties in their V1/V2 region and the antibody response cannot be predicted based on the number of glycans or V2 loop length. Future studies will need to examine factors that influence the immunogenic property of the V1/V2 region to inform the selection of appropriate Env as vaccine immunogens best suited to generate V1/V2 specific antibodies.

While the vaccinia prime-gp120 boost immunization regimen did generate V1/V2 binding antibodies, the functional role of the V1/V2 specific responses remains unclear. The need to better understand the functional quality of the antibody responses that are induced is underscored by the success of the RV144 trial and the failure of the VAX003 trial. The VAX003 clinical trial contained the same bivalent gp120 protein immunogen as RV144, but lacked the ALVAC prime and did not show protection despite the presence of V1/V2 binding antibodies and higher vaccine-elicited Nabs (207, 218). The lack of efficacy could be because the quality of the V1/V2 IgG responses and the context of other immune responses were important (216, 218, 349). More V1/V2 IgG3 responses and antibodies correlated with antiviral function were induced by the RV144 vaccine regiment compared to the VAX003 regimen (216). Additionally, RV144 vaccine-induced plasma IgA that bound to Env correlated directly with the rate of infection, possibly because these antibodies mitigate the effects of potentially protective antibodies by blocking the binding of IgG that recognize overlapping epitopes (214, 350). While rabbits have different Ig subclasses that may result in different functional roles compared to humans, it would be of interest to further test the subclass of antibodies generated in this study and compare their effector functions to those antibodies elicited in the RV144 and VAX003 trial.

It is not yet known whether the V1/V2 binding IgG antibodies were directly responsible for protection against HIV-1 infection in the RV144 trial, but determining the differences in the quality of the antibody responses to different immunogens is critical for designing immunogens to test in future efficacy studies.

One way we can test the quality of the antibody response is to test the ADCC capability of the sera. Because the RV144 trial induced robust HIV-1specific ADCC responses and some of these antibody epitopes mapped to V1/V2 Env sites (214, 216, 351), it would be beneficial to test the rabbit sera's ability to mediate ADCC activity. Testing the ability of sera to mediate ADCC is currently underway in a collaborator's lab. Preliminary data indicates that the animals generated antibodies that can mediate ADCC (data not shown). This indicates that the vaccinia prime gp120-boost vaccine regimen is capable of inducing these ADCC mediating antibody responses similar to those that have been correlated with protection in a clinical trial.

Neutralization Capabilities of Animal Sera

As previously stated, the RV144 trial only generated Tier 1 neutralizing capabilities (218). However, the pox virus prime-gp120 boosted animals generated heterologous Tier 2 neutralization capabilities shown in chapter four. To the best of our knowledge, neutralization of the Tier 2 global panel by vaccinee sera, albeit at modest titers, has not been previously reported. This indicates that the prime-boost immunization regimen used here may be more effective at inducing Nab responses compared to other reported immunization regimens. The basis for the improved responses is not clear, but priming with a replication competent virus may be a beneficial factor. Replication competent viruses have been shown to provide more effective protection against different viral infections and disease compared to immunization with replication incompetent viruses or subunit vaccines (341-343). Priming with replication

competent virus may also offer the benefit of prolonged antigen exposure. As stated previously, it typically takes 2-4 years after seroconversion to generate cross-reactive sera in infected patients (129) and this is possibly due to the necessity of continuous stimulation of the immune response with Env (126-128). While the antigen exposure elicited by vaccinia is not as extensive as HIV-1 infection, the replication competent virus could express antigen for a longer period of time compared to replication incompetent vaccinia virus. This extended antigen exposure in the context of vaccinia infection may be advantageous when the immune system elicits initial antibody responses after priming. Further studies are needed to understand if replication competent viruses that lead to extended antigen exposure improve the antibody responses elicited against Env.

Additional studies in chapter four attempted to determine the epitope specificities of antibodies in neutralization competent sera. Some Tier 1 neutralization capability was competed out with linear V3 peptides, indicating that the sera contain V3 directed neutralizing antibodies that recognize linear epitopes. While this is a typical response to HIV-1 immunization, V3 directed neutralizing antibodies can be cross-reactive, albeit within the same clade (242, 243). In fact, up to 60% of the V3 loop is conserved (121, 352); representing an attractive target for neutralizing antibody responses. Thus, cross-reactive Nabs directed against the V3 loop can be generated.

In order to map antibodies that recognize more complex epitopes, mutant CE1176_A3 viruses were generated with either single-site mutations or multiple-site mutations. For instance, results from testing the sera's ability to neutralize certain mutants indicated that some animals generated CD4bs directed antibodies capable of neutralizing CE1176_A3, a heterologous Tier 2 isolate. The CD4bs is another desirable target for HIV-1 vaccine design because it is highly

conserved and some CD4bs directed antibodies can be cross-reactive (126-128). However, in most cases, testing these mutants with other sera failed to give definitive antibody epitope specificities. This may be due to the sera demonstrating a polyclonal response whose epitope specificity is not easily determined via single epitope specificities, but is dependent on multiple epitopes. Testing the sera with other mutants, especially single mutants, is needed and currently being explored. This panel of CE1176_A3 mutants can be used to further define the epitope specificities of neutralizing antibodies in rabbit sera described here as well as sera antibody samples from other studies.

Informing and Improving Vaccine Designs

A vaccine will most likely need to induce cross-reactive neutralizing antibodies instead of autologous neutralizing antibodies. Indeed, breakthrough virus strains infected vaccinated macaques despite the presence of potent autologous neutralizing antibody responses (353). Additionally, HIV-1 infected patients can become superinfected with a different T/F virus (354), indicating that autologous neutralizing antibodies are not sufficient at preventing HIV-1 infection. In light of the difficulty to induce Nab against any Tier 2 viruses and the shortcoming of autologous neutralization capability, it is notable that the sera described here can neutralize up to 50% of the Tier 2 global panel.

While the animals generated heterologous Tier 2 neutralization capabilities, these were low titer responses. In order to protect against HIV-1 acquisition, these titers may need to be improved. Previous studies in non-human primates typically show that high titers of passively transferred Nabs are required to protect against SHIV acquisition in high-dose viral challenges (174-178). In contrast, more recent studies demonstrate that protection against SHIV challenge is provided at lower neutralizing titers when viral exposure is via the mucosal route (174, 355), as

is the case for the vast majority of HIV-1 infections, or with low-dose repeated challenges (172). In fact, plasma dilutions as low as 1:100 to 1:200 have been associated with protection in nonhuman primate studies (355, 356). Therefore, even modest levels of cross-reactive Nabs elicited by immunization could potentially protect against HIV-1 infection. This is encouraging considering some of the animals described in this dissertation demonstrated neutralizing capabilities against a heterologous Tier 2 isolate at ~1:100 sera dilutions. However, these neutralizing antibody responses can possibly be improved.

Perhaps one way to improve Nab responses is to use different boosting immunogens, including gp140 protein. As previously mentioned, immunization with trimeric gp140 generates qualitatively different antibody responses compared to immunization with gp120 monomers (334-337). Our study showed that a second gp120 protein boost did not improve neutralizing antibody responses and skewed the binding antibody response to linear epitopes. Consequently, it may be beneficial to test trimeric gp140 boosting immunogens. In order to maintain the trimeric structure of an Env antigen that is more readily recognized by bNAbs, future studies can include the use of a soluble, cleaved, stable trimeric form of the gp140 Env protein known as SOSIP trimers (357). They are typically difficult to generate and not all Envs readily generate trimeric SOSIP proteins, but recent work indicates that alternative purification methods and Env modifications improve the ability to generate SOSIP trimers (358, 359). These stabilized trimers express multiple epitopes for bNAbs, but not epitopes for non-Nabs, making them an attractive immunogen. A previous study demonstrated that immunization of rabbits with SOSIP trimers elicited homologous Tier 2 neutralizing antibodies (317, 360). While the neutralization breadth elicited with SOSIP trimer immunization alone was limited, combining the SOSIP trimers in a

vaccinia prime-protein boost regimen may improve the neutralizing antibody responses we described in this dissertation.

Using SOSIP trimers could also clarify if the N7 glycan impacts the immunogenicity of trimeric Env. As discussed earlier, we saw little to no difference in the Nab and binding antibody responses using either N7 glycosylated or N7 deglycosylated Env when animals were boosted with gp120 protein. Early results reported in chapter four suggest that there are no detectable differences in neutralizing antibody epitope specificities between animals that were immunized with N7 glycosylated or deglycosylated Env. This indicates that the antigenic properties of Env, whereby N7 glycan removal resulted in more exposed or stabilized epitopes (225), is not predictive of the immunogenic properties of Env in this study. Because data from chapter three indicate that the biological property of the N7 glycan may only be discernible in the trimeric form of Env, boosting with N7 glycosylated and deglycosylated SOSIP trimers may result in an altered immune response compared to boosting with gp120. Future studies should include immunization with SOSIP trimers and rationally designed immunogens in an effort to better understand how the B cells recognize key Env epitopes and generate antibodies to specific sites.

It is generally agreed that an HIV-1 vaccine will need to not only generate cross-reactive antibodies and potentially non-Nabs, but T-cell mediated immunity as well. Fortunately, recombinant vaccinia virus induces T cell responses to Env (361), and can be used to express other viral genes that help generate T cell mediate immunity, including *gag*, and *pol*. Therefore, replication competent vaccinia is a useful immunization vector that can possibly induce T cell responses in addition to non-Nab and Nab responses. Moreover, when recombinant vaccinia viruses expressing *gag* or *env* are used in combination, virus-like particles can form (362). This generates more antigenic targets for the immune system to respond to and possibly boosts the

immune responses. While the main focus of this dissertation was to determine the neutralizing antibody responses elicited in immunized animals, future studies with promising Env immunogens can include vaccinia that express other HIV-1 proteins in an effort to improve immune responses.

Overall, this dissertation reports the conserved phenotype of an N-linked glycan masking epitopes in the CD4bs and coreceptor binding sites. Because glycans can modulate the antigenicity of Env, glycan modification may represent a rational approach for immunogen design. We also demonstrated that using a poxvirus prime-gp120 boost generates V1/V2 binding and Tier 2 neutralizing antibody responses. Findings described in this dissertation points to specific directions for future research that may result in improved vaccine design. These include the role of specific glycans, the nature of the priming and boosting immunogens, and methodologies to dissect the specificity of vaccine induced responses.

References

1. **Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868-871.
2. **Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S, Leibowitch J, Popovic M.** 1983. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science **220:**865-867.
3. **Weiss RA.** 1993. How does HIV cause AIDS? Science **260:**1273-1279.
4. **Hahn BH, Shaw GM, De Cock KM, Sharp PM.** 2000. AIDS as a zoonosis: scientific and public health implications. Science **287:**607-614.
5. **Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, Cummins LB, Arthur LO, Peeters M, Shaw GM, Sharp PM, Hahn BH.** 1999. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature **397:**436-441.
6. **Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR.** 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature **339:**389-392.
7. **Marx PA, Li Y, Lerche NW, Sutjipto S, Gettie A, Yee JA, Brotman BH, Prince AM, Hanson A, Webster RG, et al.** 1991. Isolation of a simian immunodeficiency virus related to human immunodeficiency virus type 2 from a west African pet sooty mangabey. J Virol **65:**4480-4485.
8. **Peeters M, Sharp PM.** 2000. Genetic diversity of HIV-1: the moving target. AIDS **14 Suppl 3:**S129-140.
9. **Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany AB, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mlotshwa M, Morris L, Taylor D.** 2010. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science **329:**1168-1174.
10. **Auvert B, Taljaard D, Lagarde E, Sobngwi-Tambekou J, Sitta R, Puren A.** 2005. Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 Trial. PLoS Med **2:**e298.
11. **Bailey RC, Moses S, Parker CB, Agot K, Maclean I, Krieger JN, Williams CF, Campbell RT, Ndinya-Achola JO.** 2007. Male circumcision for HIV prevention in young men in Kisumu, Kenya: a randomised controlled trial. Lancet **369:**643-656.
12. **Mansoor LE, Abdool Karim Q, Mngadi KT, Dlamini S, Montague C, Nkomonde N, Mvandaba N, Baxter C, Gengiah TN, Samsunder N, Dawood H, Grobler A, Frohlich JA, Abdool Karim SS.** 2014. Assessing the implementation effectiveness and safety of 1% tenofovir gel provision through family planning services in KwaZulu-Natal, South Africa: study protocol for an open-label randomized controlled trial. Trials **15:**496.
13. **Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, Premsri N, Namwat C, de Souza M, Adams E, Benenson M, Gurunathan S, Tartaglia J, McNeil JG, Francis DP, Stablein D, Birx DL, Chunsuttiwat S, Khamboonruang C, Thongcharoen P, Robb ML, Michael NL, Kunasol P, Kim JH.** 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med **361:**2209-2220.
14. **Overbaugh J, Rudensey LM.** 1992. Alterations in potential sites for glycosylation predominate during evolution of the simian immunodeficiency virus envelope gene in macaques. J Virol **66:**5937-5948.
15. **Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307-312.

16. **Derdeyn CA, Decker JM, Bibollet-Ruche F, Mokili JL, Muldoon M, Denham SA, Heil ML, Kasolo F, Musonda R, Hahn BH, Shaw GM, Korber BT, Allen S, Hunter E.** 2004. Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. Science **303:**2019-2022.
17. **Sagar M, Wu X, Lee S, Overbaugh J.** 2006. Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity. J Virol **80:**9586-9598.
18. **Reitter JN, Means RE, Desrosiers RC.** 1998. A role for carbohydrates in immune evasion in AIDS. Nat Med **4:**679-684.
19. **Zhang M, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C, Korber B.** 2004. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology **14:**1229-1246.
20. **Frost SDW, Wrin T, Smith DM, Pond SLK, Liu Y, Paxinos E, Chappey C, Galovich J, Beauchaine J, Petropoulos CJ, Little SJ, Richman DD.** 2005. Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection. Proc Natl Acad Sci U S A **102:**18514-18519.
21. **Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM.** 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci U S A **105:**7552-7557.
22. **Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM, Wang S, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL, Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA, Allen S, Hunter E, Markowitz M, Hraber P, Perelson AS, Bhattacharya T, Haynes BF, Korber BT, Hahn BH, Shaw GM.** 2009. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. J Exp Med **206:**1273-1289.
23. **Hladik F, McElrath MJ.** 2008. Setting the stage: host invasion by HIV. Nat Rev Immunol **8:**447-457.
24. **Hladik F, Sakchalathorn P, Ballweber L, Lentz G, Fialkow M, Eschenbach D, McElrath MJ.** 2007. Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1. Immunity **26:**257-270.
25. **Boggiano C, Littman DR.** 2007. HIV's vagina travelogue. Immunity **26:**145-147.
26. **Lackner AA, Veazey RS.** 2007. Current concepts in AIDS pathogenesis: insights from the SIV/macaque model. Annu Rev Med **58:**461-476.
27. **Weiss HA, Dickson KE, Agot K, Hankins CA.** 2010. Male circumcision for HIV prevention: current research and programmatic issues. AIDS **24 Suppl 4:**S61-69.
28. **Lee HY, Giorgi EE, Keele BF, Gaschen B, Athreya GS, Salazar-Gonzalez JF, Pham KT, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Hahn BH, Shaw GM, Korber BT, Bhattacharya T, Perelson AS.** 2009. Modeling sequence evolution in acute HIV-1 infection. J Theor Biol **261:**341-360.
29. **Emau P, Jiang Y, Agy MB, Tian B, Bekele G, Tsai CC.** 2006. Post-exposure prophylaxis for SIV revisited: animal model for HIV prevention. AIDS Res Ther **3:**29.
30. **Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, Nguyen PL, Khoruts A, Larson M, Haase AT, Douek DC.** 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J Exp Med **200:**749-759.

31. **Veazey RS, Lackner AA.** 1998. The gastrointestinal tract and the pathogenesis of AIDS. AIDS **12 Suppl A:**S35-42.
32. **Veazey RS, DeMaria M, Chalifoux LV, Shvetz DE, Pauley DR, Knight HL, Rosenzweig M, Johnson RP, Desrosiers RC, Lackner AA.** 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science **280:**427-431.
33. **Mehandru S, Dandekar S.** 2008. Role of the gastrointestinal tract in establishing infection in primates and humans. Curr Opin HIV AIDS **3:**22-27.
34. **Fiebig EW, Wright DJ, Rawal BD, Garrett PE, Schumacher RT, Peddada L, Heldebrant C, Smith R, Conrad A, Kleinman SH, Busch MP.** 2003. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS **17:**1871-1879.
35. **Pantaleo G, Graziosi C, Fauci AS.** 1993. New concepts in the immunopathogenesis of human immunodeficiency virus infection. N Engl J Med **328:**327-335.
36. **Levesque MC, Moody MA, Hwang KK, Marshall DJ, Whitesides JF, Amos JD, Gurley TC, Allgood S, Haynes BB, Vandergrift NA, Plonk S, Parker DC, Cohen MS, Tomaras GD, Goepfert PA, Shaw GM, Schmitz JE, Eron JJ, Shaheen NJ, Hicks CB, Liao HX, Markowitz M, Kelsoe G, Margolis DM, Haynes BF.** 2009. Polyclonal B cell differentiation and loss of gastrointestinal tract germinal centers in the earliest stages of HIV-1 infection. PLoS Med **6:**e1000107.
37. **Rodriguez B, Sethi AK, Cheruvu VK, Mackay W, Bosch RJ, Kitahata M, Boswell SL, Mathews WC, Bangsberg DR, Martin J, Whalen CC, Sieg S, Yadavalli S, Deeks SG, Lederman MM.** 2006. Predictive value of plasma HIV RNA level on rate of CD4 T-cell decline in untreated HIV infection. JAMA **296:**1498-1506.
38. **Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature **373:**123-126.
39. **Highleyman L.** 2010. Inflammation, immune activation, and HIV. BETA **22:**12-26.
40. **Hill M, Tachedjian G, Mak J.** 2005. The packaging and maturation of the HIV-1 Pol proteins. Curr HIV Res **3:**73-85.
41. **Kogan M, Rappaport J.** 2011. HIV-1 accessory protein Vpr: relevance in the pathogenesis of HIV and potential for therapeutic intervention. Retrovirology **8:**25.
42. **Gibellini D, Vitone F, Schiavone P, Re MC.** 2005. HIV-1 tat protein and cell proliferation and survival: a brief review. New Microbiol **28:**95-109.
43. **Blissenbach M, Grewe B, Hoffmann B, Brandt S, Uberla K.** 2010. Nuclear RNA export and packaging functions of HIV-1 Rev revisited. J Virol **84:**6598-6604.
44. **Gottlinger HG.** 2001. The HIV-1 assembly machine. AIDS **15 Suppl 5:**S13-20.
45. **Freed EO.** 2015. HIV-1 assembly, release and maturation. Nat Rev Microbiol **13:**484-496.
46. **Rose KM, Marin M, Kozak SL, Kabat D.** 2004. The viral infectivity factor (Vif) of HIV-1 unveiled. Trends Mol Med **10:**291-297.
47. **Gonzalez ME.** 2015. Vpu Protein: The Viroporin Encoded by HIV-1. Viruses **7:**4352-4368.
48. **Geyer M, Fackler OT, Peterlin BM.** 2001. Structure--function relationships in HIV-1 Nef. EMBO Rep **2:**580-585.
49. **Checkley MA, Luttge BG, Freed EO.** 2011. HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. J Mol Biol **410:**582-608.
50. **Freed EO, Martin MA.** 1995. The role of human immunodeficiency virus type 1 envelope glycoproteins in virus infection. J Biol Chem **270:**23883-23886.
51. **Hunter E, Swanstrom R.** 1990. Retrovirus envelope glycoproteins. Curr Top Microbiol Immunol **157:**187-253.

52. **Bernstein HB, Tucker SP, Hunter E, Schutzbach JS, Compans RW.** 1994. Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides. J Virol **68:**463-468.
53. **Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ.** 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J Biol Chem **265:**10373-10382.
54. **Earl PL, Doms RW, Moss B.** 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc Natl Acad Sci U S A **87:**648-652.
55. **Pinter A, Honnen WJ, Tilley SA, Bona C, Zaghouani H, Gorny MK, Zolla-Pazner S.** 1989. Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. J Virol **63:**2674-2679.
56. **Weiss CD, Levy JA, White JM.** 1990. Oligomeric organization of gp120 on infectious human immunodeficiency virus type 1 particles. J Virol **64:**5674-5677.
57. **Otteken A, Earl PL, Moss B.** 1996. Folding, assembly, and intracellular trafficking of the human immunodeficiency virus type 1 envelope glycoprotein analyzed with monoclonal antibodies recognizing maturational intermediates. J Virol **70:**3407-3415.
58. **Stanley P, Schachter H, Taniguchi N.** 2009. N-Glycans. *In* Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME (ed), Essentials of Glycobiology, 2nd ed, Cold Spring Harbor (NY).
59. **Egan MA, Carruth LM, Rowell JF, Yu X, Siliciano RF.** 1996. Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the Pr55gag precursor protein. J Virol **70:**6547-6556.
60. **Rowell JF, Ruff AL, Guarnieri FG, Staveley-O'Carroll K, Lin X, Tang J, August JT, Siliciano RF.** 1995. Lysosome-associated membrane protein-1-mediated targeting of the HIV-1 envelope protein to an endosomal/lysosomal compartment enhances its presentation to MHC class II-restricted T cells. J Immunol **155:**1818-1828.
61. **Moore JP, McKeating JA, Weiss RA, Sattentau QJ.** 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. Science **250:**1139-1142.
62. **Moore PL, Crooks ET, Porter L, Zhu P, Cayanan CS, Grise H, Corcoran P, Zwick MB, Franti M, Morris L, Roux KH, Burton DR, Binley JM.** 2006. Nature of nonfunctional envelope proteins on the surface of human immunodeficiency virus type 1. J Virol **80:**2515-2528.
63. **Burton DR, Mascola JR.** 2015. Antibody responses to envelope glycoproteins in HIV-1 infection. Nat Immunol **16:**571-576.
64. **Zhu P, Liu J, Bess J, Jr., Chertova E, Lifson JD, Grise H, Ofek GA, Taylor KA, Roux KH.** 2006. Distribution and three-dimensional structure of AIDS virus envelope spikes. Nature **441:**847-852.
65. **Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, Wolf H, Parks ES, Parks WP, Josephs SF, Gallo RC, et al.** 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell **45:**637-648.
66. **Willey RL, Rutledge RA, Dias S, Folks T, Theodore T, Buckler CE, Martin MA.** 1986. Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. Proc Natl Acad Sci U S A **83:**5038-5042.
67. **Chohan B, Lang D, Sagar M, Korber B, Lavreys L, Richardson B, Overbaugh J.** 2005. Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. J Virol **79:**6528-6531.

68. **Curlin ME, Zioni R, Hawes SE, Liu Y, Deng W, Gottlieb GS, Zhu T, Mullins JI.** 2010. HIV-1 envelope subregion length variation during disease progression. PLoS Pathog **6:**e1001228.
69. **Kitrinos KM, Hoffman NG, Nelson JA, Swanstrom R.** 2003. Turnover of env variable region 1 and 2 genotypes in subjects with late-stage human immunodeficiency virus type 1 infection. J Virol **77:**6811-6822.
70. **Masciotra S, Owen SM, Rudolph D, Yang C, Wang B, Saksena N, Spira T, Dhawan S, Lal RB.** 2002. Temporal relationship between V1V2 variation, macrophage replication, and coreceptor adaptation during HIV-1 disease progression. AIDS **16:**1887-1898.
71. **Shioda T, Oka S, Xin X, Liu H, Harukuni R, Kurotani A, Fukushima M, Hasan MK, Shiino T, Takebe Y, Iwamoto A, Nagai Y.** 1997. In vivo sequence variability of human immunodeficiency virus type 1 envelope gp120: association of V2 extension with slow disease progression. J Virol **71:**4871-4881.
72. **Kowalski M, Potz J, Basiripour L, Dorfman T, Goh WC, Terwilliger E, Dayton A, Rosen C, Haseltine W, Sodroski J.** 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237:**1351-1355.
73. **Lasky LA, Nakamura G, Smith DH, Fennie C, Shimasaki C, Patzer E, Berman P, Gregory T, Capon DJ.** 1987. Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell **50:**975-985.
74. **Olshevsky U, Helseth E, Furman C, Li J, Haseltine W, Sodroski J.** 1990. Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. J Virol **64:**5701-5707.
75. **Chen B, Vogan EM, Gong H, Skehel JJ, Wiley DC, Harrison SC.** 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. Nature **433:**834-841.
76. **Clapham PR, McKnight A.** 2001. HIV-1 receptors and cell tropism. Br Med Bull **58:**43-59.
77. **Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, Wu L, Mackay CR, LaRosa G, Newman W, Gerard N, Gerard C, Sodroski J.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135-1148.
78. **Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661-666.
79. **Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA.** 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature **381:**667-673.
80. **Feng Y, Broder CC, Kennedy PE, Berger EA.** 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science **272:**872-877.
81. **Ochsenbauer C, Edmonds TG, Ding H, Keele BF, Decker J, Salazar MG, Salazar-Gonzalez JF, Shattock R, Haynes BF, Shaw GM, Hahn BH, Kappes JC.** 2012. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. J Virol **86:**2715-2728.
82. **Weinberger AD, Perelson AS.** 2011. Persistence and emergence of X4 virus in HIV infection. Math Biosci Eng **8:**605-626.
83. **Freed EO, Myers DJ, Risser R.** 1991. Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. J Virol **65:**190-194.
84. **Hwang SS, Boyle TJ, Lyerly HK, Cullen BR.** 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science **253:**71-74.
85. **Shioda T, Levy JA, Cheng-Mayer C.** 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature **349:**167-169.

86. **Wyatt R, Sullivan N, Thali M, Repke H, Ho D, Robinson J, Posner M, Sodroski J.** 1993. Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. J Virol **67:**4557-4565.
87. **Pollard SR, Rosa MD, Rosa JJ, Wiley DC.** 1992. Truncated variants of gp120 bind CD4 with high affinity and suggest a minimum CD4 binding region. EMBO J **11:**585-591.
88. **Cann AJ, Churcher MJ, Boyd M, O'Brien W, Zhao JQ, Zack J, Chen IS.** 1992. The region of the envelope gene of human immunodeficiency virus type 1 responsible for determination of cell tropism. J Virol **66:**305-309.
89. **Matsushita S, Robertguroff M, Rusche J, Koito A, Hattori T, Hoshino H, Javaherian K, Takatsuki K, Putney S.** 1988. Characterization of a Human Immunodeficiency Virus Neutralizing Monoclonal-Antibody and Mapping of the Neutralizing Epitope. J Virol **62:**2107-2114.
90. **O'Brien WA, Koyanagi Y, Namazie A, Zhao JQ, Diagne A, Idler K, Zack JA, Chen IS.** 1990. HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. Nature **348:**69-73.
91. **Yokoyama M, Naganawa S, Yoshimura K, Matsushita S, Sato H.** 2012. Structural dynamics of HIV-1 envelope Gp120 outer domain with V3 loop. PLoS One **7:**e37530.
92. **Dragic T, Trkola A, Thompson DA, Cormier EG, Kajumo FA, Maxwell E, Lin SW, Ying W, Smith SO, Sakmar TP, Moore JP.** 2000. A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. Proc Natl Acad Sci U S A **97:**5639-5644.
93. **Doms RW, Moore JP.** 2000. HIV-1 membrane fusion: targets of opportunity. J Cell Biol **151:**F9-14.
94. **Shang L, Yue L, Hunter E.** 2008. Role of the membrane-spanning domain of human immunodeficiency virus type 1 envelope glycoprotein in cell-cell fusion and virus infection. J Virol **82:**5417-5428.
95. **Freed EO, Martin MA.** 1996. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. J Virol **70:**341-351.
96. **Gabuzda DH, Lever A, Terwilliger E, Sodroski J.** 1992. Effects of deletions in the cytoplasmic domain on biological functions of human immunodeficiency virus type 1 envelope glycoproteins. J Virol **66:**3306-3315.
97. **Murakami T, Freed EO.** 2000. Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail. J Virol **74:**3548-3554.
98. **Wyss S, Berlioz-Torrent C, Boge M, Blot G, Honing S, Benarous R, Thali M.** 2001. The highly conserved C-terminal dileucine motif in the cytosolic domain of the human immunodeficiency virus type 1 envelope glycoprotein is critical for its association with the AP-1 clathrin adaptor [correction of adapter]. J Virol **75:**2982-2992.
99. **Murakami T, Freed EO.** 2000. The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions. Proc Natl Acad Sci U S A **97:**343-348.
100. **Affranchino JL, Gonzalez SA.** 2006. Mutations at the C-terminus of the simian immunodeficiency virus envelope glycoprotein affect gp120-gp41 stability on virions. Virology **347:**217-225.
101. **Yu X, Yuan X, McLane MF, Lee TH, Essex M.** 1993. Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions. J Virol **67:**213-221.
102. **Kalia V, Sarkar S, Gupta P, Montelaro RC.** 2003. Rational site-directed mutations of the LLP-1 and LLP-2 lentivirus lytic peptide domains in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41 indicate common functions in cell-cell fusion but distinct roles in virion envelope incorporation. J Virol **77:**3634-3646.

103. **Wilk T, Pfeiffer T, Bosch V.** 1992. Retained in vitro infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. Virology **189:**167-177.
104. **Lee SJ, Hu W, Fisher AG, Looney DJ, Kao VF, Mitsuya H, Ratner L, Wong-Staal F.** 1989. Role of the carboxy-terminal portion of the HIV-1 transmembrane protein in viral transmission and cytopathogenicity. AIDS Res Hum Retroviruses **5:**441-449.
105. **Freed EO, Martin MA.** 1995. Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. J Virol **69:**1984-1989.
106. **Cao J, Bergeron L, Helseth E, Thali M, Repke H, Sodroski J.** 1993. Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. J Virol **67:**2747-2755.
107. **Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, Moore JP, Stiegler G, Katinger H, Burton DR, Parren PW.** 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J Virol **75:**10892-10905.
108. **Huang J, Ofek G, Laub L, Louder MK, Doria-Rose NA, Longo NS, Imamichi H, Bailer RT, Chakrabarti B, Sharma SK, Alam SM, Wang T, Yang Y, Zhang B, Migueles SA, Wyatt R, Haynes BF, Kwong PD, Mascola JR, Connors M.** 2012. Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. Nature **491:**406-412.
109. **Tan K, Liu J, Wang J, Shen S, Lu M.** 1997. Atomic structure of a thermostable subdomain of HIV-1 gp41. Proc Natl Acad Sci U S A **94:**12303-12308.
110. **Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC.** 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature **387:**426-430.
111. **Freed EO, Myers DJ, Risser R.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proc Natl Acad Sci U S A **87:**4650-4654.
112. **Salzwedel K, West JT, Hunter E.** 1999. A conserved tryptophan-rich motif in the membrane-proximal region of the human immunodeficiency virus type 1 gp41 ectodomain is important for Env-mediated fusion and virus infectivity. J Virol **73:**2469-2480.
113. **Chan DC, Fass D, Berger JM, Kim PS.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89:**263-273.
114. **Bosch ML, Earl PL, Fargnoli K, Picciafuoco S, Giombini F, Wong-Staal F, Franchini G.** 1989. Identification of the fusion peptide of primate immunodeficiency viruses. Science **244:**694-697.
115. **Freed EO, Delwart EL, Buchschacher GL, Jr., Panganiban AT.** 1992. A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. Proc Natl Acad Sci U S A **89:**70-74.
116. **Dubay JW, Roberts SJ, Brody B, Hunter E.** 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. J Virol **66:**4748-4756.
117. **Munoz-Barroso I, Salzwedel K, Hunter E, Blumenthal R.** 1999. Role of the membrane-proximal domain in the initial stages of human immunodeficiency virus type 1 envelope glycoprotein-mediated membrane fusion. J Virol **73:**6089-6092.
118. **Lu M, Blacklow SC, Kim PS.** 1995. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nat Struct Biol **2:**1075-1082.
119. **Brasseur R, Vandenbranden M, Cornet B, Burny A, Ruysschaert JM.** 1990. Orientation into the lipid bilayer of an asymmetric amphipathic helical peptide located at the N-terminus of viral fusion proteins. Biochim Biophys Acta **1029:**267-273.

120. **Frey G, Chen J, Rits-Volloch S, Freeman MM, Zolla-Pazner S, Chen B.** 2010. Distinct conformational states of HIV-1 gp41 are recognized by neutralizing and non-neutralizing antibodies. Nat Struct Mol Biol **17:**1486-1491.
121. **Zolla-Pazner S.** 2004. Identifying epitopes of HIV-1 that induce protective antibodies. Nat Rev Immunol **4:**199-210.
122. **Tomaras GD, Yates NL, Liu P, Qin L, Fouda GG, Chavez LL, Decamp AC, Parks RJ, Ashley VC, Lucas JT, Cohen M, Eron J, Hicks CB, Liao HX, Self SG, Landucci G, Forthal DN, Weinhold KJ, Keele BF, Hahn BH, Greenberg ML, Morris L, Karim SS, Blattner WA, Montefiori DC, Shaw GM, Perelson AS, Haynes BF.** 2008. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. J Virol **82:**12449-12463.
123. **Robinson HL.** 2013. Non-neutralizing antibodies in prevention of HIV infection. Expert Opin Biol Ther **13:**197-207.
124. **Richman DD, Wrin T, Little SJ, Petropoulos CJ.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci U S A **100:**4144-4149.
125. **Moog C, Fleury HJ, Pellegrin I, Kirn A, Aubertin AM.** 1997. Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficiency virus type 1-infected individuals. J Virol **71:**3734-3741.
126. **Doria-Rose NA, Klein RM, Manion MM, O'Dell S, Phogat A, Chakrabarti B, Hallahan CW, Migueles SA, Wrammert J, Ahmed R, Nason M, Wyatt RT, Mascola JR, Connors M.** 2009. Frequency and phenotype of human immunodeficiency virus envelope-specific B cells from patients with broadly cross-neutralizing antibodies. J Virol **83:**188-199.
127. **Sather DN, Armann J, Ching LK, Mavrantoni A, Sellhorn G, Caldwell Z, Yu X, Wood B, Self S, Kalams S, Stamatatos L.** 2009. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. J Virol **83:**757-769.
128. **Simek MD, Rida W, Priddy FH, Pung P, Carrow E, Laufer DS, Lehrman JK, Boaz M, Tarragona-Fiol T, Miiro G, Birungi J, Pozniak A, McPhee DA, Manigart O, Karita E, Inwoley A, Jaoko W, Dehovitz J, Bekker LG, Pitisuttithum P, Paris R, Walker LM, Poignard P, Wrin T, Fast PE, Burton DR, Koff WC.** 2009. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. J Virol **83:**7337-7348.
129. **Gray ES, Madiga MC, Hermanus T, Moore PL, Wibmer CK, Tumba NL, Werner L, Mlisana K, Sibeko S, Williamson C, Abdool Karim SS, Morris L.** 2011. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. J Virol **85:**4828-4840.
130. **Anonymous.** 2015. Observation of the rare B(s)(0) -->micro+micro- decay from the combined analysis of CMS and LHCb data. Nature **522:**68-72.
131. **Haynes BF, Moody MA, Verkoczy L, Kelsoe G, Alam SM.** 2005. Antibody polyspecificity and neutralization of HIV-1: a hypothesis. Hum Antibodies **14:**59-67.
132. **Mouquet H, Nussenzweig MC.** 2012. Polyreactive antibodies in adaptive immune responses to viruses. Cell Mol Life Sci **69:**1435-1445.
133. **Verkoczy L, Diaz M.** 2014. Autoreactivity in HIV-1 broadly neutralizing antibodies: implications for their function and induction by vaccination. Curr Opin HIV AIDS **9:**224-234.
134. **Huang J, Kang BH, Pancera M, Lee JH, Tong T, Feng Y, Imamichi H, Georgiev IS, Chuang GY, Druz A, Doria-Rose NA, Laub L, Sliepen K, van Gils MJ, de la Pena AT, Derking R, Klasse PJ, Migueles SA, Bailer RT, Alam M, Pugach P, Haynes BF, Wyatt RT, Sanders RW, Binley JM, Ward**

AB, Mascola JR, Kwong PD, Connors M. 2014. Broad and potent HIV-1 neutralization by a human antibody that binds the gp41-gp120 interface. Nature **515:**138-142.

135. **Binley JM, Ban YE, Crooks ET, Eggink D, Osawa K, Schief WR, Sanders RW.** 2010. Role of complex carbohydrates in human immunodeficiency virus type 1 infection and resistance to antibody neutralization. J Virol **84:**5637-5655.
136. **Moore PL, Gray ES, Wibmer CK, Bhiman JN, Nonyane M, Sheward DJ, Hermanus T, Bajimaya S, Tumba NL, Abrahams MR, Lambson BE, Ranchobe N, Ping L, Ngandu N, Abdool Karim Q, Abdool Karim SS, Swanstrom RI, Seaman MS, Williamson C, Morris L.** 2012. Evolution of an HIV glycan-dependent broadly neutralizing antibody epitope through immune escape. Nat Med **18:**1688-1692.
137. **Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, Steenbeke TD, Venturi M, Chaiken I, Fung M, Katinger H, Parren PW, Robinson J, Van Ryk D, Wang L, Burton DR, Freire E, Wyatt R, Sodroski J, Hendrickson WA, Arthos J.** 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature **420:**678-682.
138. **Poignard P, Moulard M, Golez E, Vivona V, Franti M, Venturini S, Wang M, Parren PW, Burton DR.** 2003. Heterogeneity of envelope molecules expressed on primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies. J Virol **77:**353-365.
139. **Klein JS, Gnanapragasam PN, Galimidi RP, Foglesong CP, West AP, Jr., Bjorkman PJ.** 2009. Examination of the contributions of size and avidity to the neutralization mechanisms of the anti-HIV antibodies b12 and 4E10. Proc Natl Acad Sci U S A **106:**7385-7390.
140. **Labrijn AF, Poignard P, Raja A, Zwick MB, Delgado K, Franti M, Binley J, Vivona V, Grundner C, Huang CC, Venturi M, Petropoulos CJ, Wrin T, Dimitrov DS, Robinson J, Kwong PD, Wyatt RT, Sodroski J, Burton DR.** 2003. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. J Virol **77:**10557-10565.
141. **Walker BD, Korber BT.** 2001. Immune control of HIV: the obstacles of HLA and viral diversity. Nat Immunol **2:**473-475.
142. **Li B, Decker JM, Johnson RW, Bibollet-Ruche F, Wei X, Mulenga J, Allen S, Hunter E, Hahn BH, Shaw GM, Blackwell JL, Derdeyn CA.** 2006. Evidence for potent autologous neutralizing antibody titers and compact envelopes in early infection with subtype C human immunodeficiency virus type 1. J Virol **80:**5211-5218.
143. **Moore PL, Ranchobe N, Lambson BE, Gray ES, Cave E, Abrahams MR, Bandawe G, Mlisana K, Abdool Karim SS, Williamson C, Morris L.** 2009. Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection. PLoS Pathog **5:**e1000598.
144. **Myers G, Lenroot R.** 1992. HIV glycosylation: what does it portend? AIDS Res Hum Retroviruses **8:**1459-1460.
145. **Feizi T, Larkin M.** 1990. AIDS and glycosylation. Glycobiology **1:**17-23.
146. **Li H, Chien PC, Jr., Tuen M, Visciano ML, Cohen S, Blais S, Xu CF, Zhang HT, Hioe CE.** 2008. Identification of an N-linked glycosylation in the C4 region of HIV-1 envelope gp120 that is critical for recognition of neighboring CD4 T cell epitopes. J Immunol **180:**4011-4021.
147. **Raska M, Novak J.** 2010. Involvement of envelope-glycoprotein glycans in HIV-1 biology and infection. Arch Immunol Ther Exp (Warsz) **58:**191-208.
148. **Huang X, Jin W, Hu K, Luo S, Du T, Griffin GE, Shattock RJ, Hu Q.** 2012. Highly conserved HIV-1 gp120 glycans proximal to CD4-binding region affect viral infectivity and neutralizing antibody induction. Virology **423:**97-106.
149. **Montefiori DC, Robinson WE, Jr., Mitchell WM.** 1988. Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A **85:**9248-9252.

150. **Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, Wang SK, Ramos A, Chan-Hui PY, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong CH, Phogat S, Wrin T, Simek MD, Koff WC, Wilson IA, Burton DR, Poignard P.** 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature **477:**466-470.
151. **Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, Wrin T, Simek MD, Fling S, Mitcham JL, Lehrman JK, Priddy FH, Olsen OA, Frey SM, Hammond PW, Kaminsky S, Zamb T, Moyle M, Koff WC, Poignard P, Burton DR.** 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science **326:**285-289.
152. **Deng K, Siliciano RF.** 2014. HIV: Early treatment may not be early enough. Nature **512:**35-36.
153. **Mansky LM, Temin HM.** 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol **69:**5087-5094.
154. **Banatvala J, Van Damme P, Oehen S.** 2000. Lifelong protection against hepatitis B: the role of vaccine immunogenicity in immune memory. Vaccine **19:**877-885.
155. **Lambert PH, Liu M, Siegrist CA.** 2005. Can successful vaccines teach us how to induce efficient protective immune responses? Nat Med **11:**S54-62.
156. **Amanna IJ, Messaoudi I, Slifka MK.** 2008. Protective immunity following vaccination: how is it defined? Hum Vaccin **4:**316-319.
157. **Friedman J, Alam SM, Shen X, Xia SM, Stewart S, Anasti K, Pollara J, Fouda GG, Yang G, Kelsoe G, Ferrari G, Tomaras GD, Haynes BF, Liao HX, Moody MA, Permar SR.** 2012. Isolation of HIV-1-neutralizing mucosal monoclonal antibodies from human colostrum. PLoS One **7:**e37648.
158. **Tudor D, Derrien M, Diomede L, Drillet AS, Houimel M, Moog C, Reynes JM, Lopalco L, Bomsel M.** 2009. HIV-1 gp41-specific monoclonal mucosal IgAs derived from highly exposed but IgG-seronegative individuals block HIV-1 epithelial transcytosis and neutralize CD4(+) cell infection: an IgA gene and functional analysis. Mucosal Immunol **2:**412-426.
159. **Bomsel M, Tudor D, Drillet AS, Alfsen A, Ganor Y, Roger MG, Mouz N, Amacker M, Chalifour A, Diomede L, Devillier G, Cong Z, Wei Q, Gao H, Qin C, Yang GB, Zurbriggen R, Lopalco L, Fleury S.** 2011. Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. Immunity **34:**269-280.
160. **Ackerman ME, Crispin M, Yu X, Baruah K, Boesch AW, Harvey DJ, Dugast AS, Heizen EL, Ercan A, Choi I, Streeck H, Nigrovic PA, Bailey-Kellogg C, Scanlan C, Alter G.** 2013. Natural variation in Fc glycosylation of HIV-specific antibodies impacts antiviral activity. J Clin Invest **123:**2183-2192.
161. **Baum LL, Cassutt KJ, Knigge K, Khattri R, Margolick J, Rinaldo C, Kleeberger CA, Nishanian P, Henrard DR, Phair J.** 1996. HIV-1 gp120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression. J Immunol **157:**2168-2173.
162. **Mabuka J, Nduati R, Odem-Davis K, Peterson D, Overbaugh J.** 2012. HIV-specific antibodies capable of ADCC are common in breastmilk and are associated with reduced risk of transmission in women with high viral loads. PLoS Pathog **8:**e1002739.
163. **Burton DR, Hessell AJ, Keele BF, Klasse PJ, Ketas TA, Moldt B, Dunlop DC, Poignard P, Doyle LA, Cavacini L, Veazey RS, Moore JP.** 2011. Limited or no protection by weakly or nonneutralizing antibodies against vaginal SHIV challenge of macaques compared with a strongly neutralizing antibody. Proc Natl Acad Sci U S A **108:**11181-11186.
164. **Van Rompay KK, Berardi CJ, Dillard-Telm S, Tarara RP, Canfield DR, Valverde CR, Montefiori DC, Cole KS, Montelaro RC, Miller CJ, Marthas ML.** 1998. Passive immunization of newborn rhesus macaques prevents oral simian immunodeficiency virus infection. J Infect Dis **177:**1247-1259.
165. **Xiao P, Zhao J, Patterson LJ, Brocca-Cofano E, Venzon D, Kozlowski PA, Hidajat R, Demberg T, Robert-Guroff M.** 2010. Multiple vaccine-elicited nonneutralizing antienvelope antibody

activities contribute to protective efficacy by reducing both acute and chronic viremia following simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. J Virol **84:**7161-7173.

166. **Hidajat R, Xiao P, Zhou Q, Venzon D, Summers LE, Kalyanaraman VS, Montefiori DC, Robert-Guroff M.** 2009. Correlation of vaccine-elicited systemic and mucosal nonneutralizing antibody activities with reduced acute viremia following intrarectal simian immunodeficiency virus SIVmac251 challenge of rhesus macaques. J Virol **83:**791-801.
167. **Pegu A YZ, Chen X, Todd JP, McKee K, Rao S, Mascola J, Nabel G.** 2011. VRC01 provides sterilizing protection to non human primates from mucosal SHIV challenges. The Journal of Immunology **186:**11.
168. **Ruprecht RM.** 2009. Passive immunization with human neutralizing monoclonal antibodies against HIV-1 in macaque models: experimental approaches. Methods Mol Biol **525:**559-566, xiv.
169. **Ruprecht RM, Ferrantelli F, Kitabwalla M, Xu W, McClure HM.** 2003. Antibody protection: passive immunization of neonates against oral AIDS virus challenge. Vaccine **21:**3370-3373.
170. **Haigwood NL, Montefiori DC, Sutton WF, McClure J, Watson AJ, Voss G, Hirsch VM, Richardson BA, Letvin NL, Hu SL, Johnson PR.** 2004. Passive immunotherapy in simian immunodeficiency virus-infected macaques accelerates the development of neutralizing antibodies. J Virol **78:**5983-5995.
171. **Hessell AJ, Hangartner L, Hunter M, Havenith CE, Beurskens FJ, Bakker JM, Lanigan CM, Landucci G, Forthal DN, Parren PW, Marx PA, Burton DR.** 2007. Fc receptor but not complement binding is important in antibody protection against HIV. Nature **449:**101-104.
172. **Hessell AJ, Rakasz EG, Poignard P, Hangartner L, Landucci G, Forthal DN, Koff WC, Watkins DI, Burton DR.** 2009. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS Pathog **5:**e1000433.
173. **Hessell AJ, Rakasz EG, Tehrani DM, Huber M, Weisgrau KL, Landucci G, Forthal DN, Koff WC, Poignard P, Watkins DI, Burton DR.** 2010. Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L. J Virol **84:**1302-1313.
174. **Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, Lewis MG.** 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med **6:**207-210.
175. **Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S, Cavacini LA, Posner MR, Katinger H, Stiegler G, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Lu Y, Wright JE, Chou TC, Ruprecht RM.** 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat Med **6:**200-206.
176. **Mascola JR, Lewis MG, Stiegler G, Harris D, VanCott TC, Hayes D, Louder MK, Brown CR, Sapan CV, Frankel SS, Lu Y, Robb ML, Katinger H, Birx DL.** 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol **73:**4009-4018.
177. **Parren PW, Marx PA, Hessell AJ, Luckay A, Harouse J, Cheng-Mayer C, Moore JP, Burton DR.** 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J Virol **75:**8340-8347.

178. **Shibata R, Igarashi T, Haigwood N, Buckler-White A, Ogert R, Ross W, Willey R, Cho MW, Martin MA.** 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat Med **5:**204-210.
179. **Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D.** 2012. Antibody-based protection against HIV infection by vectored immunoprophylaxis. Nature **481:**81-84.
180. **Saunders KO, Wang L, Joyce MG, Yang ZY, Balazs AB, Cheng C, Ko SY, Kong WP, Rudicell RS, Georgiev IS, Duan L, Foulds KE, Donaldson M, Xu L, Schmidt SD, Todd JP, Baltimore D, Roederer M, Haase AT, Kwong PD, Rao SS, Mascola JR, Nabel GJ.** 2015. Broadly Neutralizing Human Immunodeficiency Virus Type 1 Antibody Gene Transfer Protects Nonhuman Primates from Mucosal Simian-Human Immunodeficiency Virus Infection. J Virol **89:**8334-8345.
181. **Mehandru S, Vcelar B, Wrin T, Stiegler G, Joos B, Mohri H, Boden D, Galovich J, Tenner-Racz K, Racz P, Carrington M, Petropoulos C, Katinger H, Markowitz M.** 2007. Adjunctive passive immunotherapy in human immunodeficiency virus type 1-infected individuals treated with antiviral therapy during acute and early infection. J Virol **81:**11016-11031.
182. **Trkola A, Kuster H, Rusert P, Joos B, Fischer M, Leemann C, Manrique A, Huber M, Rehr M, Oxenius A, Weber R, Stiegler G, Vcelar B, Katinger H, Aceto L, Gunthard HF.** 2005. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat Med **11:**615-622.
183. **Lu S.** 2009. Heterologous prime-boost vaccination. Curr Opin Immunol **21:**346-351.
184. **Sallusto F, Lanzavecchia A, Araki K, Ahmed R.** 2010. From vaccines to memory and back. Immunity **33:**451-463.
185. **Plotkin SA.** 2008. Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis **47:**401-409.
186. **Douek DC, Kwong PD, Nabel GJ.** 2006. The rational design of an AIDS vaccine. Cell **124:**677-681.
187. **Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK, Desrosiers RC.** 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science **258:**1938-1941.
188. **Joag SV, Liu ZQ, Stephens EB, Smith MS, Kumar A, Li Z, Wang C, Sheffer D, Jia F, Foresman L, Adany I, Lifson J, McClure HM, Narayan O.** 1998. Oral immunization of macaques with attenuated vaccine virus induces protection against vaginally transmitted AIDS. J Virol **72:**9069-9078.
189. **Wyand MS, Manson K, Montefiori DC, Lifson JD, Johnson RP, Desrosiers RC.** 1999. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. J Virol **73:**8356-8363.
190. **Whatmore AM, Cook N, Hall GA, Sharpe S, Rud EW, Cranage MP.** 1995. Repair and evolution of nef in vivo modulates simian immunodeficiency virus virulence. J Virol **69:**5117-5123.
191. **Pantophlet R, Burton DR.** 2006. GP120: target for neutralizing HIV-1 antibodies. Annu Rev Immunol **24:**739-769.
192. **MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, Bagarazzi ML, Chattergoon MA, Baine Y, Higgins TJ, Ciccarelli RB, Coney LR, Ginsberg RS, Weiner DB.** 1998. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. J Infect Dis **178:**92-100.
193. **MacGregor RR, Boyer JD, Ciccarelli RB, Ginsberg RS, Weiner DB.** 2000. Safety and immune responses to a DNA-based human immunodeficiency virus (HIV) type I env/rev vaccine in HIV-infected recipients: follow-up data. J Infect Dis **181:**406.
194. **Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, Jones TR, Hobart P, Margalith M, Ng J, Weiss WR, Sedegah M, de Taisne C, Norman JA, Hoffman SL.** 1998. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science **282:**476-480.

195. **Roy MJ, Wu MS, Barr LJ, Fuller JT, Tussey LG, Speller S, Culp J, Burkholder JK, Swain WF, Dixon RM, Widera G, Vessey R, King A, Ogg G, Gallimore A, Haynes JR, Heydenburg Fuller D.** 2000. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine **19:**764-778.

196. **Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, Zhang ZQ, Simon AJ, Trigona WL, Dubey SA, Huang L, Harris VA, Long RS, Liang X, Handt L, Schleif WA, Zhu L, Freed DC, Persaud NV, Guan L, Punt KS, Tang A, Chen M, Wilson KA, Collins KB, Heidecker GJ, Fernandez VR, Perry HC, Joyce JG, Grimm KM, Cook JC, Keller PM, Kresock DS, Mach H, Troutman RD, Isopi LA, Williams DM, Xu Z, Bohannon KE, Volkin DB, Montefiori DC, Miura A, Krivulka GR, Lifton MA, Kuroda MJ, Schmitz JE, Letvin NL, Caulfield MJ, Bett AJ, Youil R, et al.** 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature **415:**331-335.

197. **Wilson NA, Keele BF, Reed JS, Piaskowski SM, MacNair CE, Bett AJ, Liang X, Wang F, Thoryk E, Heidecker GJ, Citron MP, Huang L, Lin J, Vitelli S, Ahn CD, Kaizu M, Maness NJ, Reynolds MR, Friedrich TC, Loffredo JT, Rakasz EG, Erickson S, Allison DB, Piatak M, Jr., Lifson JD, Shiver JW, Casimiro DR, Shaw GM, Hahn BH, Watkins DI.** 2009. Vaccine-induced cellular responses control simian immunodeficiency virus replication after heterologous challenge. J Virol **83:**6508-6521.

198. **Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM, Abbink P, Coffey RT, Grandpre LE, Seaman MS, Landucci G, Forthal DN, Montefiori DC, Carville A, Mansfield KG, Havenga MJ, Pau MG, Goudsmit J, Barouch DH.** 2009. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. Nature **457:**87-91.

199. **Priddy FH, Brown D, Kublin J, Monahan K, Wright DP, Lalezari J, Santiago S, Marmor M, Lally M, Novak RM, Brown SJ, Kulkarni P, Dubey SA, Kierstead LS, Casimiro DR, Mogg R, DiNubile MJ, Shiver JW, Leavitt RY, Robertson MN, Mehrotra DV, Quirk E.** 2008. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. Clin Infect Dis **46:**1769-1781.

200. **Barouch DH.** 2010. Novel adenovirus vector-based vaccines for HIV-1. Curr Opin HIV AIDS **5:**386-390.

201. **Gudmundsdotter L, Nilsson C, Brave A, Hejdeman B, Earl P, Moss B, Robb M, Cox J, Michael N, Marovich M, Biberfeld G, Sandstrom E, Wahren B.** 2009. Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity. Vaccine **27:**4468-4474.

202. **Paoletti E.** 1996. Applications of pox virus vectors to vaccination: an update. Proc Natl Acad Sci U S A **93:**11349-11353.

203. **Saunders KO, Rudicell RS, Nabel GJ.** 2012. The design and evaluation of HIV-1 vaccines. AIDS **26:**1293-1302.

204. **Harro CD, Judson FN, Gorse GJ, Mayer KH, Kostman JR, Brown SJ, Koblin B, Marmor M, Bartholow BN, Popovic V.** 2004. Recruitment and baseline epidemiologic profile of participants in the first phase 3 HIV vaccine efficacy trial. J Acquir Immune Defic Syndr **37:**1385-1392.

205. **Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF.** 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis **191:**654-665.

206. **Gilbert PB, Peterson ML, Follmann D, Hudgens MG, Francis DP, Gurwith M, Heyward WL, Jobes DV, Popovic V, Self SG, Sinangil F, Burke D, Berman PW.** 2005. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. J Infect Dis **191:**666-677.

207. **Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, Hu D, Tappero JW, Choopanya K.** 2006. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J Infect Dis **194:**1661-1671.
208. **Pitisuttithum P.** 2008. HIV vaccine research in Thailand: lessons learned. Expert Rev Vaccines **7:**311-317.
209. **Casimiro DR, Chen L, Fu TM, Evans RK, Caulfield MJ, Davies ME, Tang A, Chen M, Huang L, Harris V, Freed DC, Wilson KA, Dubey S, Zhu DM, Nawrocki D, Mach H, Troutman R, Isopi L, Williams D, Hurni W, Xu Z, Smith JG, Wang S, Liu X, Guan L, Long R, Trigona W, Heidecker GJ, Perry HC, Persaud N, Toner TJ, Su Q, Liang X, Youil R, Chastain M, Bett AJ, Volkin DB, Emini EA, Shiver JW.** 2003. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. J Virol **77:**6305-6313.
210. **Wilson NA, Reed J, Napoe GS, Piaskowski S, Szymanski A, Furlott J, Gonzalez EJ, Yant LJ, Maness NJ, May GE, Soma T, Reynolds MR, Rakasz E, Rudersdorf R, McDermott AB, O'Connor DH, Friedrich TC, Allison DB, Patki A, Picker LJ, Burton DR, Lin J, Huang L, Patel D, Heindecker G, Fan J, Citron M, Horton M, Wang F, Liang X, Shiver JW, Casimiro DR, Watkins DI.** 2006. Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. J Virol **80:**5875-5885.
211. **Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, Gilbert PB, Lama JR, Marmor M, Del Rio C, McElrath MJ, Casimiro DR, Gottesdiener KM, Chodakewitz JA, Corey L, Robertson MN.** 2008. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet **372:**1881-1893.
212. **D'Souza MP, Frahm N.** 2010. Adenovirus 5 serotype vector-specific immunity and HIV-1 infection: a tale of T cells and antibodies. AIDS **24:**803-809.
213. **Kim JH, Excler JL, Michael NL.** 2015. Lessons from the RV144 Thai phase III HIV-1 vaccine trial and the search for correlates of protection. Annu Rev Med **66:**423-437.
214. **Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, Evans DT, Montefiori DC, Karnasuta C, Sutthent R, Liao HX, DeVico AL, Lewis GK, Williams C, Pinter A, Fong Y, Janes H, DeCamp A, Huang Y, Rao M, Billings E, Karasavvas N, Robb ML, Ngauy V, de Souza MS, Paris R, Ferrari G, Bailer RT, Soderberg KA, Andrews C, Berman PW, Frahm N, De Rosa SC, Alpert MD, Yates NL, Shen X, Koup RA, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Rerks-Ngarm S, Michael NL, Kim JH.** 2012. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med **366:**1275-1286.
215. **Zolla-Pazner S, deCamp A, Gilbert PB, Williams C, Yates NL, Williams WT, Howington R, Fong Y, Morris DE, Soderberg KA, Irene C, Reichman C, Pinter A, Parks R, Pitisuttithum P, Kaewkungwal J, Rerks-Ngarm S, Nitayaphan S, Andrews C, O'Connell RJ, Yang ZY, Nabel GJ, Kim JH, Michael NL, Montefiori DC, Liao HX, Haynes BF, Tomaras GD.** 2014. Vaccine-induced IgG antibodies to V1V2 regions of multiple HIV-1 subtypes correlate with decreased risk of HIV-1 infection. PLoS One **9:**e87572.
216. **Yates NL, Liao HX, Fong Y, deCamp A, Vandergrift NA, Williams WT, Alam SM, Ferrari G, Yang ZY, Seaton KE, Berman PW, Alpert MD, Evans DT, O'Connell RJ, Francis D, Sinangil F, Lee C, Nitayaphan S, Rerks-Ngarm S, Kaewkungwal J, Pitisuttithum P, Tartaglia J, Pinter A, Zolla-Pazner S, Gilbert PB, Nabel GJ, Michael NL, Kim JH, Montefiori DC, Haynes BF, Tomaras GD.** 2014. Vaccine-induced Env V1-V2 IgG3 correlates with lower HIV-1 infection risk and declines soon after vaccination. Sci Transl Med **6:**228ra239.

217. **Rolland M, Edlefsen PT, Larsen BB, Tovanabutra S, Sanders-Buell E, Hertz T, deCamp AC, Carrico C, Menis S, Magaret CA, Ahmed H, Juraska M, Chen L, Konopa P, Nariya S, Stoddard JN, Wong K, Zhao H, Deng W, Maust BS, Bose M, Howell S, Bates A, Lazzaro M, O'Sullivan A, Lei E, Bradfield A, Ibitamuno G, Assawadarachai V, O'Connell RJ, deSouza MS, Nitayaphan S, Rerks-Ngarm S, Robb ML, McLellan JS, Georgiev I, Kwong PD, Carlson JM, Michael NL, Schief WR, Gilbert PB, Mullins JI, Kim JH.** 2012. Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2. Nature **490:**417-420.
218. **Montefiori DC, Karnasuta C, Huang Y, Ahmed H, Gilbert P, de Souza MS, McLinden R, Tovanabutra S, Laurence-Chenine A, Sanders-Buell E, Moody MA, Bonsignori M, Ochsenbauer C, Kappes J, Tang H, Greene K, Gao H, LaBranche CC, Andrews C, Polonis VR, Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Self SG, Berman PW, Francis D, Sinangil F, Lee C, Tartaglia J, Robb ML, Haynes BF, Michael NL, Kim JH.** 2012. Magnitude and breadth of the neutralizing antibody response in the RV144 and Vax003 HIV-1 vaccine efficacy trials. J Infect Dis **206:**431-441.
219. **Back NK, Smit L, De Jong JJ, Keulen W, Schutten M, Goudsmit J, Tersmette M.** 1994. An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization. Virology **199:**431-438.
220. **Clevestig P, Pramanik L, Leitner T, Ehrnst A.** 2006. CCR5 use by human immunodeficiency virus type 1 is associated closely with the gp120 V3 loop N-linked glycosylation site. J Gen Virol **87:**607-612.
221. **Kang SM, Quan FS, Huang C, Guo L, Ye L, Yang C, Compans RW.** 2005. Modified HIV envelope proteins with enhanced binding to neutralizing monoclonal antibodies. Virology **331:**20-32.
222. **Ma BJ, Alam SM, Go EP, Lu X, Desaire H, Tomaras GD, Bowman C, Sutherland LL, Scearce RM, Santra S, Letvin NL, Kepler TB, Liao HX, Haynes BF.** 2011. Envelope deglycosylation enhances antigenicity of HIV-1 gp41 epitopes for both broad neutralizing antibodies and their unmutated ancestor antibodies. PLoS Pathog **7:**e1002200.
223. **McCaffrey RA, Saunders C, Hensel M, Stamatatos L.** 2004. N-linked glycosylation of the V3 loop and the immunologically silent face of gp120 protects human immunodeficiency virus type 1 SF162 from neutralization by anti-gp120 and anti-gp41 antibodies. J Virol **78:**3279-3295.
224. **Reynard F, Fatmi A, Verrier B, Bedin F.** 2004. HIV-1 acute infection env glycomutants designed from 3D model: effects on processing, antigenicity, and neutralization sensitivity. Virology **324:**90-102.
225. **Townsley S, Li Y, Kozyrev Y, Cleveland B, Hu SL.** 2015. Conserved Role of an N-Linked Glycan on the Surface Antigen of Human Immunodeficiency Virus Type 1 Modulating Virus Sensitivity to Broadly Neutralizing Antibodies against the Receptor and Coreceptor Binding Sites. J Virol **90:**829-841.
226. **Li Y, Cleveland B, Klots I, Travis B, Richardson BA, Anderson D, Montefiori D, Polacino P, Hu SL.** 2008. Removal of a single N-linked glycan in human immunodeficiency virus type 1 gp120 results in an enhanced ability to induce neutralizing antibody responses. J Virol **82:**638-651.
227. **Benjouad A, Gluckman JC, Montagnier L, Bahraoui E.** 1993. Specificity of antibodies produced against native or desialylated human immunodeficiency virus type 1 recombinant gp160. J Virol **67:**1693-1697.
228. **Bolmstedt A, Hinkula J, Rowcliffe E, Biller M, Wahren B, Olofsson S.** 2001. Enhanced immunogenicity of a human immunodeficiency virus type 1 env DNA vaccine by manipulating N-glycosylation signals. Effects of elimination of the V3 N306 glycan. Vaccine **20:**397-405.
229. **Bolmstedt A, Sjolander S, Hansen JE, Akerblom L, Hemming A, Hu SL, Morein B, Olofsson S.** 1996. Influence of N-linked glycans in V4-V5 region of human immunodeficiency virus type 1

glycoprotein gp160 on induction of a virus-neutralizing humoral response. J Acquir Immune Defic Syndr Hum Retrovirol **12:**213-220.

230. **Quinones-Kochs MI, Buonocore L, Rose JK.** 2002. Role of N-linked glycans in a human immunodeficiency virus envelope glycoprotein: effects on protein function and the neutralizing antibody response. J Virol **76:**4199-4211.
231. **Burke B, Derby NR, Kraft Z, Saunders CJ, Dai C, Llewellyn N, Zharkikh I, Vojtech L, Zhu T, Srivastava IK, Barnett SW, Stamatatos L.** 2006. Viral evolution in macaques coinfected with CCR5- and CXCR4-tropic SHIVs in the presence or absence of vaccine-elicited anti-CCR5 SHIV neutralizing antibodies. Virology **355:**138-151.
232. **Li Y, Cleveland B, Klots I, Travis B, Richardson B, Anderson D, Montefiori D, Polacino P, Hu S-L.** 2008. Removal of a single N-linked glycan in human immunodeficiency virus type 1 gp120 results in an enhanced ability to induce neutralizing antibody responses. Journal of virology **82:**638-651.
233. **Pikora C, Wittish C, Desrosiers RC.** 2005. Identification of two N-linked glycosylation sites within the core of the simian immunodeficiency virus glycoprotein whose removal enhances sensitivity to soluble CD4. J Virol **79:**12575-12583.
234. **Stiegler G, Kunert R, Purtscher M, Wolbank S, Voglauer R, Steindl F, Katinger H.** 2001. A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. AIDS Res Hum Retroviruses **17:**1757-1765.
235. **Buchacher A, Predl R, Strutzenberger K, Steinfellner W, Trkola A, Purtscher M, Gruber G, Tauer C, Steindl F, Jungbauer A, et al.** 1994. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res Hum Retroviruses **10:**359-369.
236. **Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, Srinivasan K, Sodroski J, Moore JP, Katinger H.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol **70:**1100-1108.
237. **Barbas CF, 3rd, Bjorling E, Chiodi F, Dunlop N, Cababa D, Jones TM, Zebedee SL, Persson MA, Nara PL, Norrby E, et al.** 1992. Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. Proc Natl Acad Sci U S A **89:**9339-9343.
238. **Burton DR, Barbas CF, 3rd, Persson MA, Koenig S, Chanock RM, Lerner RA.** 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc Natl Acad Sci U S A **88:**10134-10137.
239. **Burton DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, Parren PW, Sawyer LS, Hendry RM, Dunlop N, Nara PL, et al.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024-1027.
240. **Roben P, Moore JP, Thali M, Sodroski J, Barbas CF, 3rd, Burton DR.** 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J Virol **68:**4821-4828.
241. **Cook RF, Berger SL, Rushlow KE, McManus JM, Cook SJ, Harrold S, Raabe ML, Montelaro RC, Issel CJ.** 1995. Enhanced sensitivity to neutralizing antibodies in a variant of equine infectious anemia virus is linked to amino acid substitutions in the surface unit envelope glycoprotein. J Virol **69:**1493-1499.
242. **Gorny MK, Conley AJ, Karwowska S, Buchbinder A, Xu JY, Emini EA, Koenig S, Zolla-Pazner S.** 1992. Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. J Virol **66:**7538-7542.

243. **Gorny MK, VanCott TC, Hioe C, Israel ZR, Michael NL, Conley AJ, Williams C, Kessler JA, 2nd, Chigurupati P, Burda S, Zolla-Pazner S.** 1997. Human monoclonal antibodies to the V3 loop of HIV-1 with intra- and interclade cross-reactivity. J Immunol **159:**5114-5122.
244. **Gorny MK, Xu JY, Karwowska S, Buchbinder A, Zolla-Pazner S.** 1993. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. J Immunol **150:**635-643.
245. **Nyambi PN, Gorny MK, Bastiani L, van der Groen G, Williams C, Zolla-Pazner S.** 1998. Mapping of epitopes exposed on intact human immunodeficiency virus type 1 (HIV-1) virions: a new strategy for studying the immunologic relatedness of HIV-1. J Virol **72:**9384-9391.
246. **Zolla-Pazner S, O'Leary J, Burda S, Gorny MK, Kim M, Mascola J, McCutchan F.** 1995. Serotyping of primary human immunodeficiency virus type 1 isolates from diverse geographic locations by flow cytometry. J Virol **69:**3807-3815.
247. **Gorny MK, Moore JP, Conley AJ, Karwowska S, Sodroski J, Williams C, Burda S, Boots LJ, Zolla-Pazner S.** 1994. Human anti-V2 monoclonal antibody that neutralizes primary but not laboratory isolates of human immunodeficiency virus type 1. J Virol **68:**8312-8320.
248. **Israel ZR, Gorny MK, Palmer C, McKeating JA, Zolla-Pazner S.** 1997. Prevalence of a V2 epitope in clade B primary isolates and its recognition by sera from HIV-1-infected individuals. AIDS **11:**128-130.
249. **Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648-659.
250. **Sullivan N, Sun Y, Sattentau Q, Thali M, Wu D, Denisova G, Gershoni J, Robinson J, Moore J, Sodroski J.** 1998. CD4-Induced conformational changes in the human immunodeficiency virus type 1 gp120 glycoprotein: consequences for virus entry and neutralization. J Virol **72:**4694-4703.
251. **Thali M, Moore JP, Furman C, Charles M, Ho DD, Robinson J, Sodroski J.** 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J Virol **67:**3978-3988.
252. **Trkola A, Dragic T, Arthos J, Binley JM, Olson WC, Allaway GP, Cheng-Mayer C, Robinson J, Maddon PJ, Moore JP.** 1996. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. Nature **384:**184-187.
253. **Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, Hendrickson WA, Sodroski JG.** 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature **393:**705-711.
254. **Wyatt R, Moore J, Accola M, Desjardin E, Robinson J, Sodroski J.** 1995. Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. J Virol **69:**5723-5733.
255. **Garlick RL, Kirschner RJ, Eckenrode FM, Tarpley WG, Tomich CS.** 1990. Escherichia coli expression, purification, and biological activity of a truncated soluble CD4. AIDS Res Hum Retroviruses **6:**465-479.
256. **Wu X, Yang ZY, Li Y, Hogerkorp CM, Schief WR, Seaman MS, Zhou T, Schmidt SD, Wu L, Xu L, Longo NS, McKee K, O'Dell S, Louder MK, Wycuff DL, Feng Y, Nason M, Doria-Rose N, Connors M, Kwong PD, Roederer M, Wyatt RT, Nabel GJ, Mascola JR.** 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science **329:**856-861.
257. **Bosch KA, Rainwater S, Jaoko W, Overbaugh J.** 2010. Temporal analysis of HIV envelope sequence evolution and antibody escape in a subtype A-infected individual with a broad neutralizing antibody response. Virology **398:**115-124.

258. **Karlsson GB, Halloran M, Li J, Park IW, Gomila R, Reimann KA, Axthelm MK, Iliff SA, Letvin NL, Sodroski J.** 1997. Characterization of molecularly cloned simian-human immunodeficiency viruses causing rapid CD4+ lymphocyte depletion in rhesus monkeys. J Virol **71:**4218-4225.
259. **Kolchinsky P, Mirzabekov T, Farzan M, Kiprilov E, Cayabyab M, Mooney LJ, Choe H, Sodroski J.** 1999. Adaptation of a CCR5-using, primary human immunodeficiency virus type 1 isolate for CD4-independent replication. J Virol **73:**8120-8126.
260. **Peters PJ, Bhattacharya J, Hibbitts S, Dittmar MT, Simmons G, Bell J, Simmonds P, Clapham PR.** 2004. Biological analysis of human immunodeficiency virus type 1 R5 envelopes amplified from brain and lymph node tissues of AIDS patients with neuropathology reveals two distinct tropism phenotypes and identifies envelopes in the brain that confer an enhanced tropism and fusigenicity for macrophages. J Virol **78:**6915-6926.
261. **Koyanagi Y, Miles S, Mitsuyasu RT, Merrill JE, Vinters HV, Chen IS.** 1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science **236:**819-822.
262. **Cheng-Mayer C, Liu R, Landau NR, Stamatatos L.** 1997. Macrophage tropism of human immunodeficiency virus type 1 and utilization of the CC-CKR5 coreceptor. J Virol **71:**1657-1661.
263. **Grisson RD, Chenine AL, Yeh LY, He J, Wood C, Bhat GJ, Xu W, Kankasa C, Ruprecht RM.** 2004. Infectious molecular clone of a recently transmitted pediatric human immunodeficiency virus clade C isolate from Africa: evidence of intraclade recombination. J Virol **78:**14066-14069.
264. **deCamp A, Hraber P, Bailer RT, Seaman MS, Ochsenbauer C, Kappes J, Gottardo R, Edlefsen P, Self S, Tang H, Greene K, Gao H, Daniell X, Sarzotti-Kelsoe M, Gorny MK, Zolla-Pazner S, LaBranche CC, Mascola JR, Korber BT, Montefiori DC.** 2014. Global panel of HIV-1 Env reference strains for standardized assessments of vaccine-elicited neutralizing antibodies. J Virol **88:**2489-2507.
265. **Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, Voss G, Goepfert P, Gilbert P, Greene KM, Bilska M, Kothe DL, Salazar-Gonzalez JF, Wei X, Decker JM, Hahn BH, Montefiori DC.** 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J Virol **79:**10108-10125.
266. **Li Y, Svehla K, Mathy NL, Voss G, Mascola JR, Wyatt R.** 2006. Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolate trimeric and monomeric envelope glycoproteins in selected adjuvants. J Virol **80:**1414-1426.
267. **Li Y, Migueles SA, Welcher B, Svehla K, Phogat A, Louder MK, Wu X, Shaw GM, Connors M, Wyatt RT, Mascola JR.** 2007. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. Nat Med **13:**1032-1034.
268. **Landau NR, Page KA, Littman DR.** 1991. Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J Virol **65:**162-169.
269. **Gao F, Morrison SG, Robertson DL, Thornton CL, Craig S, Karlsson G, Sodroski J, Morgado M, Galvao-Castro B, von Briesen H, Beddows S, Weber J, Sharp PM, Shaw GM, Hahn BH.** 1996. Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. The WHO and NIAID Networks for HIV Isolation and Characterization. J Virol **70:**1651-1667.
270. **Kolchinsky P, Kiprilov E, Bartley P, Rubinstein R, Sodroski J.** 2001. Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. J Virol **75:**3435-3443.
271. **Montefiori DC.** 2005. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Curr Protoc Immunol **Chapter 12:**Unit 12 11.
272. **Montefiori DC.** 2009. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol Biol **485:**395-405.

273. **Abdiche Y, Malashock D, Pinkerton A, Pons J.** 2008. Determining kinetics and affinities of protein interactions using a parallel real-time label-free biosensor, the Octet. Anal Biochem **377:**209-217.
274. **Mayr LM, Cohen S, Spurrier B, Kong XP, Zolla-Pazner S.** 2013. Epitope mapping of conformational V2-specific anti-HIV human monoclonal antibodies reveals an immunodominant site in V2. PLoS One **8:**e70859.
275. **Guo W, Cleveland B, Davenport TM, Lee KK, Hu SL.** 2013. Purification of recombinant vaccinia virus-expressed monomeric HIV-1 gp120 to apparent homogeneity. Protein Expr Purif **90:**34-39.
276. **Cooney EL, McElrath MJ, Corey L, Hu SL, Collier AC, Arditti D, Hoffman M, Coombs RW, Smith GE, Greenberg PD.** 1993. Enhanced immunity to human immunodeficiency virus (HIV) envelope elicited by a combined vaccine regimen consisting of priming with a vaccinia recombinant expressing HIV envelope and boosting with gp160 protein. Proc Natl Acad Sci U S A **90:**1882-1886.
277. **Graham BS, Matthews TJ, Belshe RB, Clements ML, Dolin R, Wright PF, Gorse GJ, Schwartz DH, Keefer MC, Bolognesi DP, et al.** 1993. Augmentation of human immunodeficiency virus type 1 neutralizing antibody by priming with gp160 recombinant vaccinia and boosting with rgp160 in vaccinia-naive adults. The NIAID AIDS Vaccine Clinical Trials Network. J Infect Dis **167:**533-537.
278. **Thippeshappa R, Tian B, Cleveland B, Guo W, Polacino P, Hu SL.** 2015. Oral Immunization with Recombinant Vaccinia Virus Prime and Intramuscular Protein Boost Provides Protection against Intra-Rectal SHIV Challenge in Macaques. Clin Vaccine Immunol doi:10.1128/CVI.00597-15.
279. **Joklik WK.** 1962. The purification fo four strains of poxvirus. Virology **18:**9-18.
280. **Hu SL, Kosowski SG, Dalrymple JM.** 1986. Expression of AIDS virus envelope gene in recombinant vaccinia viruses. Nature **320:**537-540.
281. **Hu SL, Zarling JM, Chinn J, Travis BM, Moran PA, Sias J, Kuller L, Morton WR, Heidecker G, Benveniste RE.** 1989. Protection of macaques against simian AIDS by immunization with a recombinant vaccinia virus expressing the envelope glycoproteins of simian type D retrovirus. Proc Natl Acad Sci U S A **86:**7213-7217.
282. **Carbonetti S, Oliver BG, Glenn J, Stamatatos L, Sather DN.** 2014. Soluble HIV-1 envelope immunogens derived from an elite neutralizer elicit cross-reactive V1V2 antibodies and low potency neutralizing antibodies. PLoS One **9:**e86905.
283. **Burton DR, Poignard P, Stanfield RL, Wilson IA.** 2012. Broadly neutralizing antibodies present new prospects to counter highly antigenically diverse viruses. Science **337:**183-186.
284. **Kwong PD, Mascola JR.** 2012. Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies. Immunity **37:**412-425.
285. **Haynes BF, Kelsoe G, Harrison SC, Kepler TB.** 2012. B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study. Nat Biotechnol **30:**423-433.
286. **Mikell I, Sather DN, Kalams SA, Altfeld M, Alter G, Stamatatos L.** 2011. Characteristics of the earliest cross-neutralizing antibody response to HIV-1. PLoS Pathog **7:**e1001251.
287. **Mascola JR, Montefiori DC.** 2010. The role of antibodies in HIV vaccines. Annu Rev Immunol **28:**413-444.
288. **Blattner C, Lee JH, Sliepen K, Derking R, Falkowska E, de la Pena AT, Cupo A, Julien JP, van Gils M, Lee PS, Peng W, Paulson JC, Poignard P, Burton DR, Moore JP, Sanders RW, Wilson IA, Ward AB.** 2014. Structural delineation of a quaternary, cleavage-dependent epitope at the gp41-gp120 interface on intact HIV-1 Env trimers. Immunity **40:**669-680.
289. **Zhou T, Georgiev I, Wu X, Yang ZY, Dai K, Finzi A, Kwon YD, Scheid JF, Shi W, Xu L, Yang Y, Zhu J, Nussenzweig MC, Sodroski J, Shapiro L, Nabel GJ, Mascola JR, Kwong PD.** 2010. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. Science **329:**811-817.

290. **Li Y, Rey-Cuille MA, Hu SL.** 2001. N-linked glycosylation in the V3 region of HIV type 1 surface antigen modulates coreceptor usage in viral infection. AIDS Res Hum Retroviruses **17:**1473-1479.
291. **Brighty DW, Rosenberg M, Chen IS, Ivey-Hoyle M.** 1991. Envelope proteins from clinical isolates of human immunodeficiency virus type 1 that are refractory to neutralization by soluble CD4 possess high affinity for the CD4 receptor. Proc Natl Acad Sci U S A **88:**7802-7805.
292. **Ivey-Hoyle M, Culp JS, Chaikin MA, Hellmig BD, Matthews TJ, Sweet RW, Rosenberg M.** 1991. Envelope glycoproteins from biologically diverse isolates of immunodeficiency viruses have widely different affinities for CD4. Proc Natl Acad Sci U S A **88:**512-516.
293. **Mulligan MJ, Ritter GD, Jr., Chaikin MA, Yamshchikov GV, Kumar P, Hahn BH, Sweet RW, Compans RW.** 1992. Human immunodeficiency virus type 2 envelope glycoprotein: differential CD4 interactions of soluble gp120 versus the assembled envelope complex. Virology **187:**233-241.
294. **Seaman MS, Janes H, Hawkins N, Grandpre LE, Devoy C, Giri A, Coffey RT, Harris L, Wood B, Daniels MG, Bhattacharya T, Lapedes A, Polonis VR, McCutchan FE, Gilbert PB, Self SG, Korber BT, Montefiori DC, Mascola JR.** 2010. Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. J Virol **84:**1439-1452.
295. **Lyumkis D, Julien JP, de Val N, Cupo A, Potter CS, Klasse PJ, Burton DR, Sanders RW, Moore JP, Carragher B, Wilson IA, Ward AB.** 2013. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. Science **342:**1484-1490.
296. **Julien JP, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, Klasse PJ, Burton DR, Sanders RW, Moore JP, Ward AB, Wilson IA.** 2013. Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science **342:**1477-1483.
297. **Poignard P, Saphire EO, Parren PW, Burton DR.** 2001. gp120: Biologic aspects of structural features. Annu Rev Immunol **19:**253-274.
298. **Chen L, Kwon YD, Zhou T, Wu X, O'Dell S, Cavacini L, Hessell AJ, Pancera M, Tang M, Xu L, Yang ZY, Zhang MY, Arthos J, Burton DR, Dimitrov DS, Nabel GJ, Posner MR, Sodroski J, Wyatt R, Mascola JR, Kwong PD.** 2009. Structural basis of immune evasion at the site of CD4 attachment on HIV-1 gp120. Science **326:**1123-1127.
299. **O'Rourke SM, Schweighardt B, Phung P, Mesa KA, Vollrath AL, Tatsuno GP, To B, Sinangil F, Limoli K, Wrin T, Berman PW.** 2012. Sequences in glycoprotein gp41, the CD4 binding site, and the V2 domain regulate sensitivity and resistance of HIV-1 to broadly neutralizing antibodies. J Virol **86:**12105-12114.
300. **Hanly A, Petito CK.** 1998. HLA-DR-positive dendritic cells of the normal human choroid plexus: a potential reservoir of HIV in the central nervous system. Hum Pathol **29:**88-93.
301. **Muldoon LL, Alvarez JI, Begley DJ, Boado RJ, Del Zoppo GJ, Doolittle ND, Engelhardt B, Hallenbeck JM, Lonser RR, Ohlfest JR, Prat A, Scarpa M, Smeyne RJ, Drewes LR, Neuwelt EA.** 2013. Immunologic privilege in the central nervous system and the blood-brain barrier. J Cereb Blood Flow Metab **33:**13-21.
302. **Martin-Garcia J, Cao W, Varela-Rohena A, Plassmeyer ML, Gonzalez-Scarano F.** 2006. HIV-1 tropism for the central nervous system: Brain-derived envelope glycoproteins with lower CD4 dependence and reduced sensitivity to a fusion inhibitor. Virology **346:**169-179.
303. **Rossi F, Querido B, Nimmagadda M, Cocklin S, Navas-Martin S, Martin-Garcia J.** 2008. The V1-V3 region of a brain-derived HIV-1 envelope glycoprotein determines macrophage tropism, low CD4 dependence, increased fusogenicity and altered sensitivity to entry inhibitors. Retrovirology **5:**89.

304. **Gorny MK, Stamatatos L, Volsky B, Revesz K, Williams C, Wang XH, Cohen S, Staudinger R, Zolla-Pazner S.** 2005. Identification of a new quaternary neutralizing epitope on human immunodeficiency virus type 1 virus particles. J Virol **79:**5232-5237.
305. **Myszka DG, Sweet RW, Hensley P, Brigham-Burke M, Kwong PD, Hendrickson WA, Wyatt R, Sodroski J, Doyle ML.** 2000. Energetics of the HIV gp120-CD4 binding reaction. Proc Natl Acad Sci U S A **97:**9026-9031.
306. **Haggerty S, Dempsey MP, Bukrinsky MI, Guo L, Stevenson M.** 1991. Posttranslational modifications within the HIV-1 envelope glycoprotein which restrict virus assembly and CD4-dependent infection. AIDS Res Hum Retroviruses **7:**501-510.
307. **Hansen JE, Witzke NM, Nielsen C, Mathiesen LR, Teglbjaerg LS, Nielsen CM, Nielsen JO.** 1990. Derivatives of amphotericin inhibit infection with human immunodeficiency virus in vitro by different modes of action. Antiviral Res **14:**149-159.
308. **McGuire AT, Hoot S, Dreyer AM, Lippy A, Stuart A, Cohen KW, Jardine J, Menis S, Scheid JF, West AP, Schief WR, Stamatatos L.** 2013. Engineering HIV envelope protein to activate germline B cell receptors of broadly neutralizing anti-CD4 binding site antibodies. J Exp Med **210:**655-663.
309. **Dowling W, Thompson E, Badger C, Mellquist JL, Garrison AR, Smith JM, Paragas J, Hogan RJ, Schmaljohn C.** 2007. Influences of glycosylation on antigenicity, immunogenicity, and protective efficacy of ebola virus GP DNA vaccines. J Virol **81:**1821-1837.
310. **Willett BJ, McMonagle EL, Logan N, Samman A, Hosie MJ.** 2008. A single site for N-linked glycosylation in the envelope glycoprotein of feline immunodeficiency virus modulates the virus-receptor interaction. Retrovirology **5:**77.
311. **Reimann KA, Li JT, Voss G, Lekutis C, Tenner-Racz K, Racz P, Lin W, Montefiori DC, Lee-Parritz DE, Lu Y, Collman RG, Sodroski J, Letvin NL.** 1996. An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. J Virol **70:**3198-3206.
312. **Pastore C, Ramos A, Mosier DE.** 2004. Intrinsic obstacles to human immunodeficiency virus type 1 coreceptor switching. J Virol **78:**7565-7574.
313. **Cheng-Mayer C, Quiroga M, Tung JW, Dina D, Levy JA.** 1990. Viral determinants of human immunodeficiency virus type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. J Virol **64:**4390-4398.
314. **Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalski JA, Whitehorn EA, Baumeister K, et al.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature **313:**277-284.
315. **Crooks ET, Tong T, Chakrabarti B, Narayan K, Georgiev IS, Menis S, Huang X, Kulp D, Osawa K, Muranaka J, Stewart-Jones G, Destefano J, O'Dell S, LaBranche C, Robinson JE, Montefiori DC, McKee K, Du SX, Doria-Rose N, Kwong PD, Mascola JR, Zhu P, Schief WR, Wyatt RT, Whalen RG, Binley JM.** 2015. Vaccine-Elicited Tier 2 HIV-1 Neutralizing Antibodies Bind to Quaternary Epitopes Involving Glycan-Deficient Patches Proximal to the CD4 Binding Site. PLoS Pathog **11:**e1004932.
316. **Bradley T, Fera D, Bhiman J, Eslamizar L, Lu X, Anasti K, Zhang R, Sutherland LL, Scearce RM, Bowman CM, Stolarchuk C, Lloyd KE, Parks R, Eaton A, Foulger A, Nie X, Karim SS, Barnett S, Kelsoe G, Kepler TB, Alam SM, Montefiori DC, Moody MA, Liao HX, Morris L, Santra S, Harrison SC, Haynes BF.** 2016. Structural Constraints of Vaccine-Induced Tier-2 Autologous HIV Neutralizing Antibodies Targeting the Receptor-Binding Site. Cell Rep **14:**43-54.
317. **Sanders RW, van Gils MJ, Derking R, Sok D, Ketas TJ, Burger JA, Ozorowski G, Cupo A, Simonich C, Goo L, Arendt H, Kim HJ, Lee JH, Pugach P, Williams M, Debnath G, Moldt B, van Breemen MJ, Isik G, Medina-Ramirez M, Back JW, Koff WC, Julien JP, Rakasz EG, Seaman MS, Guttman M, Lee KK, Klasse PJ, LaBranche C, Schief WR, Wilson IA, Overbaugh J, Burton DR, Ward AB,**

Montefiori DC, Dean H, Moore JP. 2015. HIV-1 VACCINES. HIV-1 neutralizing antibodies induced by native-like envelope trimers. Science **349:**aac4223.
318. **Stamatatos L, Morris L, Burton DR, Mascola JR.** 2009. Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? Nat Med **15:**866-870.
319. **Day TA, Kublin JG.** 2013. Lessons learned from HIV vaccine clinical efficacy trials. Curr HIV Res **11:**441-449.
320. **Kolchinsky P, Kiprilov E, Sodroski J.** 2001. Increased neutralization sensitivity of CD4-independent human immunodeficiency virus variants. J Virol **75:**2041-2050.
321. **Graham BS, Belshe RB, Clements ML, Dolin R, Corey L, Wright PF, Gorse GJ, Midthun K, Keefer MC, Roberts NJ, Jr., et al.** 1992. Vaccination of vaccinia-naive adults with human immunodeficiency virus type 1 gp160 recombinant vaccinia virus in a blinded, controlled, randomized clinical trial. The AIDS Vaccine Clinical Trials Network. J Infect Dis **166:**244-252.
322. **Lifson JD, Feinberg MB, Reyes GR, Rabin L, Banapour B, Chakrabarti S, Moss B, Wong-Staal F, Steimer KS, Engleman EG.** 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature **323:**725-728.
323. **Kim YB, Han DP, Cao C, Cho MW.** 2003. Immunogenicity and ability of variable loop-deleted human immunodeficiency virus type 1 envelope glycoproteins to elicit neutralizing antibodies. Virology **305:**124-137.
324. **Qin Y, Banasik M, Kim S, Penn-Nicholson A, Habte HH, LaBranche C, Montefiori DC, Wang C, Cho MW.** 2014. Eliciting neutralizing antibodies with gp120 outer domain constructs based on M-group consensus sequence. Virology **462-463:**363-376.
325. **Qin Y, Shi H, Banerjee S, Agrawal A, Banasik M, Cho MW.** 2014. Detailed characterization of antibody responses against HIV-1 group M consensus gp120 in rabbits. Retrovirology **11:**125.
326. **Karasavvas N, Billings E, Rao M, Williams C, Zolla-Pazner S, Bailer RT, Koup RA, Madnote S, Arworn D, Shen X, Tomaras GD, Currier JR, Jiang M, Magaret C, Andrews C, Gottardo R, Gilbert P, Cardozo TJ, Rerks-Ngarm S, Nitayaphan S, Pitisuttithum P, Kaewkungwal J, Paris R, Greene K, Gao H, Gurunathan S, Tartaglia J, Sinangil F, Korber BT, Montefiori DC, Mascola JR, Robb ML, Haynes BF, Ngauy V, Michael NL, Kim JH, de Souza MS.** 2012. The Thai Phase III HIV Type 1 Vaccine trial (RV144) regimen induces antibodies that target conserved regions within the V2 loop of gp120. AIDS Res Hum Retroviruses **28:**1444-1457.
327. **Lynch RM, Wong P, Tran L, O'Dell S, Nason MC, Li Y, Wu X, Mascola JR.** 2015. HIV-1 fitness cost associated with escape from the VRC01 class of CD4 binding site neutralizing antibodies. J Virol **89:**4201-4213.
328. **Wu X, Changela A, O'Dell S, Schmidt SD, Pancera M, Yang Y, Zhang B, Gorny MK, Phogat S, Robinson JE, Stamatatos L, Zolla-Pazner S, Kwong PD, Mascola JR.** 2011. Immunotypes of a quaternary site of HIV-1 vulnerability and their recognition by antibodies. J Virol **85:**4578-4585.
329. **Honnen WJ, Krachmarov C, Kayman SC, Gorny MK, Zolla-Pazner S, Pinter A.** 2007. Type-specific epitopes targeted by monoclonal antibodies with exceptionally potent neutralizing activities for selected strains of human immunodeficiency virus type 1 map to a common region of the V2 domain of gp120 and differ only at single positions from the clade B consensus sequence. J Virol **81:**1424-1432.
330. **Brunel FM, Zwick MB, Cardoso RM, Nelson JD, Wilson IA, Burton DR, Dawson PE.** 2006. Structure-function analysis of the epitope for 4E10, a broadly neutralizing human immunodeficiency virus type 1 antibody. J Virol **80:**1680-1687.
331. **Kim AS, Leaman DP, Zwick MB.** 2014. Antibody to gp41 MPER alters functional properties of HIV-1 Env without complete neutralization. PLoS Pathog **10:**e1004271.
332. **Falkowska E, Le KM, Ramos A, Doores KJ, Lee JH, Blattner C, Ramirez A, Derking R, van Gils MJ, Liang CH, McBride R, von Bredow B, Shivatare SS, Wu CY, Chan-Hui PY, Liu Y, Feizi T, Zwick MB,**

Koff WC, Seaman MS, Swiderek K, Moore JP, Evans D, Paulson JC, Wong CH, Ward AB, Wilson IA, Sanders RW, Poignard P, Burton DR. 2014. Broadly neutralizing HIV antibodies define a glycan-dependent epitope on the prefusion conformation of gp41 on cleaved envelope trimers. Immunity **40:**657-668.

333. **Burton DR, Desrosiers RC, Doms RW, Koff WC, Kwong PD, Moore JP, Nabel GJ, Sodroski J, Wilson IA, Wyatt RT.** 2004. HIV vaccine design and the neutralizing antibody problem. Nat Immunol **5:**233-236.
334. **Kovacs JM, Nkolola JP, Peng H, Cheung A, Perry J, Miller CA, Seaman MS, Barouch DH, Chen B.** 2012. HIV-1 envelope trimer elicits more potent neutralizing antibody responses than monomeric gp120. Proc Natl Acad Sci U S A **109:**12111-12116.
335. **Grundner C, Li Y, Louder M, Mascola J, Yang X, Sodroski J, Wyatt R.** 2005. Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins. Virology **331:**33-46.
336. **Yang X, Wyatt R, Sodroski J.** 2001. Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. J Virol **75:**1165-1171.
337. **Kim M, Qiao ZS, Montefiori DC, Haynes BF, Reinherz EL, Liao HX.** 2005. Comparison of HIV Type 1 ADA gp120 monomers versus gp140 trimers as immunogens for the induction of neutralizing antibodies. AIDS Res Hum Retroviruses **21:**58-67.
338. **Bowles EJ, Schiffner T, Rosario M, Needham GA, Ramaswamy M, McGouran J, Kessler B, LaBranche C, McMichael AJ, Montefiori D, Sattentau QJ, Hanke T, Stewart-Jones GB.** 2014. Comparison of neutralizing antibody responses elicited from highly diverse polyvalent heterotrimeric HIV-1 gp140 cocktail immunogens versus a monovalent counterpart in rhesus macaques. PLoS One **9:**e114709.
339. **Vaine M, Wang S, Crooks ET, Jiang P, Montefiori DC, Binley J, Lu S.** 2008. Improved induction of antibodies against key neutralizing epitopes by human immunodeficiency virus type 1 gp120 DNA prime-protein boost vaccination compared to gp120 protein-only vaccination. J Virol **82:**7369-7378.
340. **Vaine M, Wang S, Hackett A, Arthos J, Lu S.** 2010. Antibody responses elicited through homologous or heterologous prime-boost DNA and protein vaccinations differ in functional activity and avidity. Vaccine **28:**2999-3007.
341. **Querec TD, Akondy RS, Lee EK, Cao W, Nakaya HI, Teuwen D, Pirani A, Gernert K, Deng J, Marzolf B, Kennedy K, Wu H, Bennouna S, Oluoch H, Miller J, Vencio RZ, Mulligan M, Aderem A, Ahmed R, Pulendran B.** 2009. Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. Nat Immunol **10:**116-125.
342. **Gaucher D, Therrien R, Kettaf N, Angermann BR, Boucher G, Filali-Mouhim A, Moser JM, Mehta RS, Drake DR, 3rd, Castro E, Akondy R, Rinfret A, Yassine-Diab B, Said EA, Chouikh Y, Cameron MJ, Clum R, Kelvin D, Somogyi R, Greller LD, Balderas RS, Wilkinson P, Pantaleo G, Tartaglia J, Haddad EK, Sekaly RP.** 2008. Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. J Exp Med **205:**3119-3131.
343. **Nakaya HI, Pulendran B.** 2012. Systems vaccinology: its promise and challenge for HIV vaccine development. Curr Opin HIV AIDS **7:**24-31.
344. **White TA, Bartesaghi A, Borgnia MJ, Meyerson JR, de la Cruz MJ, Bess JW, Nandwani R, Hoxie JA, Lifson JD, Milne JL, Subramaniam S.** 2010. Molecular architectures of trimeric SIV and HIV-1 envelope glycoproteins on intact viruses: strain-dependent variation in quaternary structure. PLoS Pathog **6:**e1001249.

345. **Munro JB, Gorman J, Ma X, Zhou Z, Arthos J, Burton DR, Koff WC, Courter JR, Smith AB, 3rd, Kwong PD, Blanchard SC, Mothes W.** 2014. Conformational dynamics of single HIV-1 envelope trimers on the surface of native virions. Science **346:**759-763.
346. **Li J, Lord CI, Haseltine W, Letvin NL, Sodroski J.** 1992. Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. J Acquir Immune Defic Syndr **5:**639-646.
347. **Dunn CS, Mehtali M, Houdebine LM, Gut JP, Kirn A, Aubertin AM.** 1995. Human immunodeficiency virus type 1 infection of human CD4-transgenic rabbits. J Gen Virol **76 (Pt 6):**1327-1336.
348. **Pritchard LK, Vasiljevic S, Ozorowski G, Seabright GE, Cupo A, Ringe R, Kim HJ, Sanders RW, Doores KJ, Burton DR, Wilson IA, Ward AB, Moore JP, Crispin M.** 2015. Structural Constraints Determine the Glycosylation of HIV-1 Envelope Trimers. Cell Rep **11:**1604-1613.
349. **Excler JL, Ake J, Robb ML, Kim JH, Plotkin SA.** 2014. Nonneutralizing functional antibodies: a new "old" paradigm for HIV vaccines. Clin Vaccine Immunol **21:**1023-1036.
350. **Tomaras GD, Ferrari G, Shen X, Alam SM, Liao HX, Pollara J, Bonsignori M, Moody MA, Fong Y, Chen X, Poling B, Nicholson CO, Zhang R, Lu X, Parks R, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Gilbert PB, Kim JH, Michael NL, Montefiori DC, Haynes BF.** 2013. Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG. Proc Natl Acad Sci U S A **110:**9019-9024.
351. **Bonsignori M, Pollara J, Moody MA, Alpert MD, Chen X, Hwang KK, Gilbert PB, Huang Y, Gurley TC, Kozink DM, Marshall DJ, Whitesides JF, Tsao CY, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Kim JH, Michael NL, Tomaras GD, Montefiori DC, Lewis GK, DeVico A, Evans DT, Ferrari G, Liao HX, Haynes BF.** 2012. Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family. J Virol **86:**11521-11532.
352. **Zolla-Pazner S, Cardozo T.** 2010. Structure-function relationships of HIV-1 envelope sequence-variable regions refocus vaccine design. Nat Rev Immunol **10:**527-535.
353. **Burton SL, Kilgore KM, Smith SA, Reddy S, Hunter E, Robinson HL, Silvestri G, Amara RR, Derdeyn CA.** 2015. Breakthrough of SIV strain smE660 challenge in SIV strain mac239-vaccinated rhesus macaques despite potent autologous neutralizing antibody responses. Proc Natl Acad Sci U S A **112:**10780-10785.
354. **Smith DM, Richman DD, Little SJ.** 2005. HIV superinfection. J Infect Dis **192:**438-444.
355. **Hessell AJ, Poignard P, Hunter M, Hangartner L, Tehrani DM, Bleeker WK, Parren PW, Marx PA, Burton DR.** 2009. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat Med **15:**951-954.
356. **Shingai M, Donau OK, Plishka RJ, Buckler-White A, Mascola JR, Nabel GJ, Nason MC, Montefiori D, Moldt B, Poignard P, Diskin R, Bjorkman PJ, Eckhaus MA, Klein F, Mouquet H, Cetrulo Lorenzi JC, Gazumyan A, Burton DR, Nussenzweig MC, Martin MA, Nishimura Y.** 2014. Passive transfer of modest titers of potent and broadly neutralizing anti-HIV monoclonal antibodies block SHIV infection in macaques. J Exp Med **211:**2061-2074.
357. **Sanders RW, Vesanen M, Schuelke N, Master A, Schiffner L, Kalyanaraman R, Paluch M, Berkhout B, Maddon PJ, Olson WC, Lu M, Moore JP.** 2002. Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. J Virol **76:**8875-8889.
358. **Pugach P, Ozorowski G, Cupo A, Ringe R, Yasmeen A, de Val N, Derking R, Kim HJ, Korzun J, Golabek M, de Los Reyes K, Ketas TJ, Julien JP, Burton DR, Wilson IA, Sanders RW, Klasse PJ, Ward AB, Moore JP.** 2015. A native-like SOSIP.664 trimer based on an HIV-1 subtype B env gene. J Virol **89:**3380-3395.

359. **Sanders RW, Derking R, Cupo A, Julien JP, Yasmeen A, de Val N, Kim HJ, Blattner C, de la Pena AT, Korzun J, Golabek M, de Los Reyes K, Ketas TJ, van Gils MJ, King CR, Wilson IA, Ward AB, Klasse PJ, Moore JP.** 2013. A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS Pathog **9:**e1003618.

360. **de Taeye SW, Ozorowski G, Torrents de la Pena A, Guttman M, Julien JP, van den Kerkhof TL, Burger JA, Pritchard LK, Pugach P, Yasmeen A, Crampton J, Hu J, Bontjer I, Torres JL, Arendt H, DeStefano J, Koff WC, Schuitemaker H, Eggink D, Berkhout B, Dean H, LaBranche C, Crotty S, Crispin M, Montefiori DC, Klasse PJ, Lee KK, Moore JP, Wilson IA, Ward AB, Sanders RW.** 2015. Immunogenicity of Stabilized HIV-1 Envelope Trimers with Reduced Exposure of Non-neutralizing Epitopes. Cell **163:**1702-1715.

361. **Zarling JM, Morton W, Moran PA, McClure J, Kosowski SG, Hu SL.** 1986. T-cell responses to human AIDS virus in macaques immunized with recombinant vaccinia viruses. Nature **323:**344-346.

362. **Haffar O, Garrigues J, Travis B, Moran P, Zarling J, Hu SL.** 1990. Human immunodeficiency virus-like, nonreplicating, gag-env particles assemble in a recombinant vaccinia virus expression system. J Virol **64:**2653-2659.

Curriculum Vitae

Education

Doctor of Philosophy in Microbiology
University of Washington (2016)
Dissertation: Modulating the Antigenicity and Immunogenicity of HIV-1 by a Specific, Conserved N-Linked Glycan

Bachelor of Science in Biology
Indiana University-Purdue University Indianapolis (IUPUI) (2009)
Senior Thesis: Histone Acetyltransferase and Histone Deacetylase Inhibitors Activate and Deactivate Gene Expression Needed for Axolotl Limb Regeneration

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Research Experience

University of Washington, Seattle, Washington (2010-2016)
Modulation of antigenic and immunogenic properties of Env by an N-linked glycan

I showed that a single N-linked glycan alters the antigenic properties of HIV-1 Env. I also examined if correlates of protection from an HIV-1 vaccine study could be generated in rabbits with a vaccinia prime-gp120 boost regimen.

IUPUI, Indianapolis, IN (2006-2009)
Histone Acetyltransferase and Histone Deacetylase Inhibitors Activate and Deactivate Gene Expression Needed for Axolotl Limb Regeneration

I examined how histones acetylation and deacetylation altered limb regeneration in Axolotls. I was able to show that histone deacetylase inhibitors prevent limb bud formation in amputated Axolotl limbs. Therefore, the deacetylation of histones may be necessary for limb regeneration.

Stress Response Proteins in Mitochondria, a Model of Resistant Kidney Injury

I examined the protein differences in two rat models that demonstrate sensitivity or resistance to acute ischemic injury. I showed that the heat shock proteins in the mitochondria differ and may contribute to the differential injury responses reported.

Effects of Doxycycline on *Xenopus Laevis* Eye Development

I showed that doxycycline does not impact the growth and development of the *Xenopus Laevis* eye. Therefore, doxycycline can be used to induce genes that are transferred into developing *Xenopus Laevis.*

Awards and Honors

Keystone Symposia Underrepresented Trainee Scholarship, Keystone Meeting, 2013
Outstanding Poster Award, Non-human primate meeting, 2011
Ronald E. McNair Program, 2008

IUPUI Dean's list, 2006-2009
Freshman Sam H. Jones Community Service Scholar, 2006

Service

Recruitment chair, Department of Microbiology, 2012
Student Retreat chair, Department of Microbiology, 2014-2015

Teaching

University of Washington, Department of Microbiology

Guest lecture: Western blot and array technology, MICROM 431 (Recombinant DNA Techniques), 2014
Guest lecture: *in vitro* site-directed mutagenesis, MICROM 431 (Recombinant DNA Techniques), 2014
Teaching assistant, MICROM 411 (Gene Action Laboratory), 2012
Teaching assistant, MICROM 302 (General Microbiology Laboratory), 2011

Publications

Townsley S, Li Y, Kozyrev Y, Cleveland B, Hu SL. 2015. Conserved Role of an N-Linked Glycan on the Surface Antigen of Human Immunodeficiency Virus Type 1 Modulating Virus Sensitivity to Broadly Neutralizing Antibodies against the Receptor and Coreceptor Binding Sites. J Virol **90:**829-841