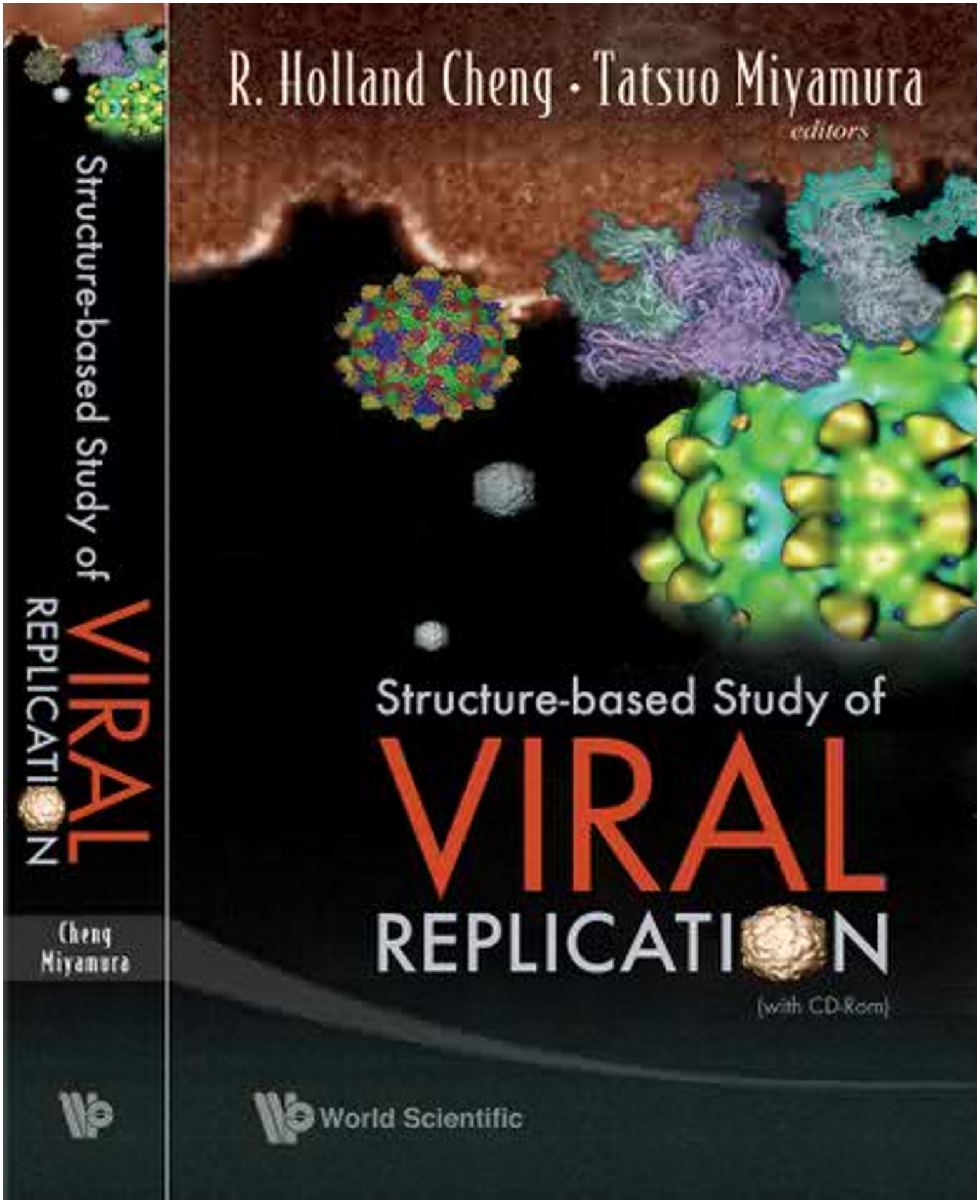
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Chapter 13

Challenges in Designing HIV Env Immunogens for Developing a Vaccine

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Summary

HIV continues to be a major health problem worldwide; however, the situation is particularly serious in Asian and Sub-Saharan countries. Development of an effective HIV vaccine could help to reduce the severity of the disease and prevent infection. Over the last two decades significant efforts have been made toward inducing potent humoral and cellular immune responses by vaccination; however, it appears that either antibodies or CTL may not be sufficient alone for the induction of sterilizing immunity or long-term control of viral replication. Therefore, it is generally believed that both humoral and cellular responses will be needed for an effective HIV vaccine. It has been shown in passive transfer experiments using broadly neutralizing monoclonal antibodies (mAb) such as b12, 2F5, and 2G12 that these mAbs either alone or in combination are effective in conferring

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protection against challenge infection to rhesus macaques. However, for the development of an effective vaccine, it has been a challenge to induce protective antibodies of similar specificities by vaccination. Therefore, efforts are being made by different groups to design an Env immunogen that may be more effective compared to the existing immunogens in inducing potent neutralizing and protective antibody responses by: i) optimizing existing Env structure to enhance the exposure of functional epitopes to focus the responses to these epitopes; ii) obtaining structural information on HIV Env, and using this information for structure-based novel immunogen design; and iii) identifying novel functional epitopes, and designing strategies to incorporate them in potential vaccines. Once potent HIV Env structures have been identified, their effectiveness may be enhanced through the use of adjuvants, delivery systems, and prime and boost strategies to improve the quality and magnitude of neutralizing responses.

Introduction

AIDS continues to be a major health problem throughout the world, with approximately 40 million cases and 20 million deaths recorded so far. In certain parts of the world, such as Sub-Saharan Africa, the prevalence of human immunodeficiency virus (HIV) in the population is estimated to be as high as 35%.[1] If current infection rates and the absence of affordable treatments continue, 60% of the current adolescent population in that region will not live to the age of 60.[1] In the United States today, an estimated 950,000 individuals are living with HIV, and 40,000 to 80,000 new infections occur each year.[1] Moreover, the situation is continuously deteriorating as a result of the rapid emergence of drug resistance against most of the effective antivirals. Therefore, there is an urgent need for an effective anti-HIV vaccine that may be used either alone as a prophylactic vaccine or in conjunction with anti-viral drugs as a therapeutic treatment.

Based on preclinical and early clinical studies, it was concluded that neutralizing antibody responses induced by monomeric HIV Env are not potent enough to protect against the HIV infection.[2,3] Therefore the dogma shifted from the induction of neutralizing

antibodies to the induction of cellular responses. Previous studies used the gag to show the correlation between strong cytotoxic T-cell activity and reduced viral load. However, more recent data obtained in a rhesus macaque challenge model suggest that cellular responses focused on a single epitope may not be sufficient either to effectively control viral replication.[4–7] It remains to be seen if increasing the breadth of CTL responses will have a positive impact on the outcome of challenge infection. Hence, it is now believed that humoral, cellular, and T-helper cell responses, both at peripheral and mucosal sites, are needed for broad and durable protection against HIV. Conceptually, antibodies would serve as a first line of defense by completely preventing infection (Fig. 1A) or reducing the virus inoculum, whereas cellular responses would facilitate the clearance of HIV-infected $CD4^+$ T-cells that escape antibody-mediated neutralization

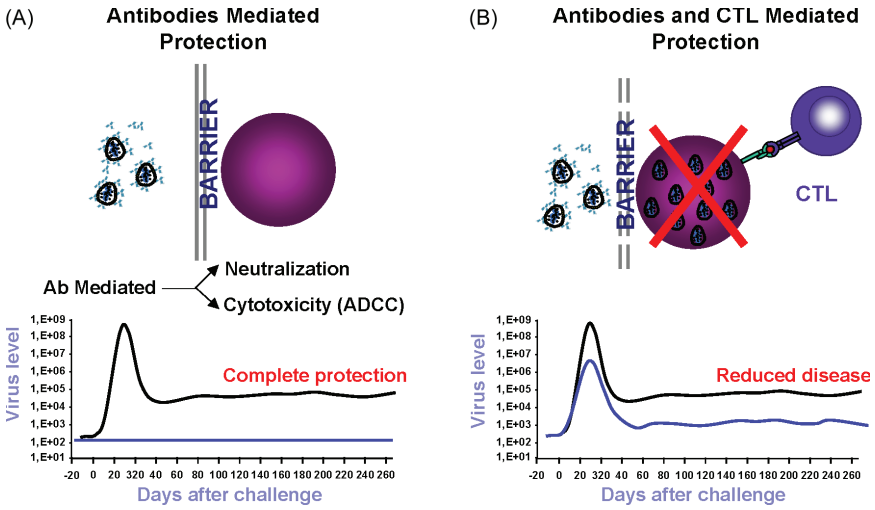


Fig. 1. Two potential outcomes for a preventative HIV vaccine. In the graphs, the black line shows time course of viremia in the absence of vaccination. In panel A, the blue line indicates the virologic outcome in the case of antibody-mediated (neutralization and ADCC) complete protection. In panel B, the blue line indicates the outcome in the case of a vaccine that induces partial protection and reduces the disease where both humoral and cellular responses participated.

(Fig. 1B) and would thereby reduce the severity of the disease. However, Mascola and colleagues have shown that the induction of cellular responses using DNA vaccines in conjunction with passive transfer of neutralizing antibodies did not offer additional protection compared to the protection afforded by passive transfer of neutralizing antibodies alone.[8] Further studies will be needed to demonstrate the advantage of inducing potent cellular and humoral responses by an effective HIV vaccine for inducing the sterilizing immunity or effectively controling the level of viral replication.

For the induction of neutralizing antibody responses, HIV Env glycoprotein is the major antigenic target. However it has proven difficult to induce neutralizing antibody responses of appropriate specificity against diverse primary HIV-1 strains utilizing monomeric HIV Env (i.e. gp120) glycoprotein. Therefore, a major challenge is to develop novel strategies to identify and expose critical epitopes that may be the target for inducing such broadly cross-reactive neutralizing antibodies with protective efficacy. This focus of this review is: a) to capture the progress made in structural analysis of critical neutralizing antibodies, and b) to present strategies to design more potent immunogens that may induce neutralizing antibodies of protective efficacy.

Role of Antibodies in Protection Against HIV

Antibodies serve as a correlate of protection against some of the bacterial[9,10] and viral pathogens,[11] therefore targeting humoral responses has been critical for developing prophylactic vaccines against these targets. In the case of HIV, it has been a daunting task to make an effective vaccine due to a lack of correlates of protection. During the natural course of HIV infection or immunization with purified protein, a very strong humoral response is induced. However, most of these antibodies are binding antibodies, and only a fraction of these antibodies have neutralizing properties. These antibodies are termed as "neutralizing antibodies" (nAb) because they neutralize the infectious virus, and therefore prevent its entry to the cells or reduce the level of inoculum, thereby reducing the severity of the disease.[12]

However, only a proportion of all neutralizing antibodies have protective efficacy. Until recently, it was generally believed that not all Env spikes need to be engaged by these nAbs for them to be efficient in controlling or preventing viral replication. However, Yang and colleagues demonstrated that virus neutralization requires essentially all of the functional trimers to be occupied by at least one antibody. Their model applies to antibodies differing in neutralizing potency and to virus isolates with different neutralization sensitivities.[13] However, recent data suggest that in addition to nAbs, there is another class of antibodies that exert their protective efficacy by inducing antibody dependent cellular cytotoxicity (ADCC); i.e. they direct the killing of infected cells through recognition of viral proteins on cell surfaces.

Possible Steps for Immune Intervention

There are several possible steps during the virus lifecycle (pre- and post-attachment events) where immune intervention is possible and may prevent the infection or control viral replication, as illustrated in Fig 2. Since binding of HIV to its receptor (CD4) and co-receptor (e.g. CCR5) is critical for viral entry in new $CD4^+$ T-cells, a logical and attractive strategy could be to prevent the interaction of the HIV virion with its cellular receptor or co-receptor. Other possible and attractive strategies could be to target other important post-attachment events such as: a) the formation of a coiled-coil structure leading to virus/host membrane fusion[14–17]; b) primary un-coating of the virus in the cytoplasm; c) transcription or virus assembly; and d) budding of the new virion. For HIV-1, inhibition of receptor binding has been seen for different types of antibodies, including: i) those that target carbohydrate of the virus glycoprotein[18,19]; ii) those that target different ***adhesion molecules***; iii) those specific for various regions of gp120 such as the ***CD4 binding domain***, the ***CD4-induced (CD4i) epitope*** (located in the bridging sheet of gp120 that is created or exposed when gp120 interacts with CD4),[20] and the ***variable loops***, V2 and V3; iv) those that target the ***co-receptor binding site***[21]; and (v) another class of mAbs such as 2F5, 4E10, or Z13 that may

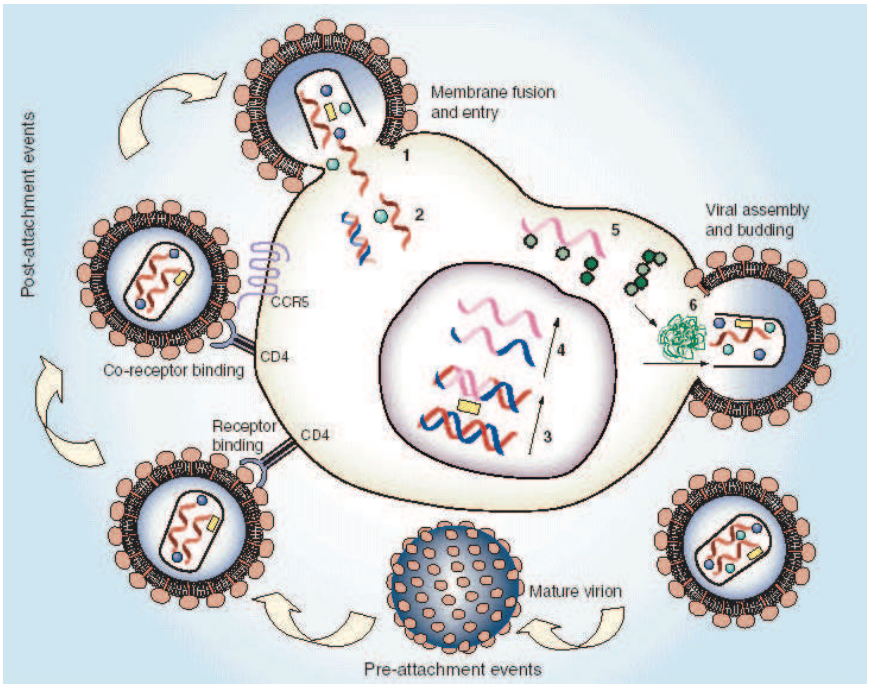


Fig. 2. Lifecycle of HIV-1 virus and potential sites of immune intervention. Step 1: Pre-attachment events: block the binding of virion to its receptor (i.e. CD4); complement mediated lysis and aggregation of infectious virion. Step 2: Post attachment events — involves prevention of binding to co-receptor and prevention of virus/cell fusion. In addition, these neutralizing antibodies may also interfere in downstream events such as: (2-4) uncoating, integration, and reverse transcription after entry; (5) processing and assembly; and (6) maturation and budding of virus particles.

interfere with gp41 coiled-coil formation or fusion of the virus/ host cell membrane. Some of these epitopes in context to Env trimer are presented in Fig. 3. Another mechanism by which antibodies can mediate protection is aggregation of virus particles, thereby reducing the number of infectious virus particles and rendering the virus more susceptible to phagocytosis and subsequent destruction.[22]

Another mechanism by which binding antibodies may exert a protective function is complement-mediated ADCC, where antibodies

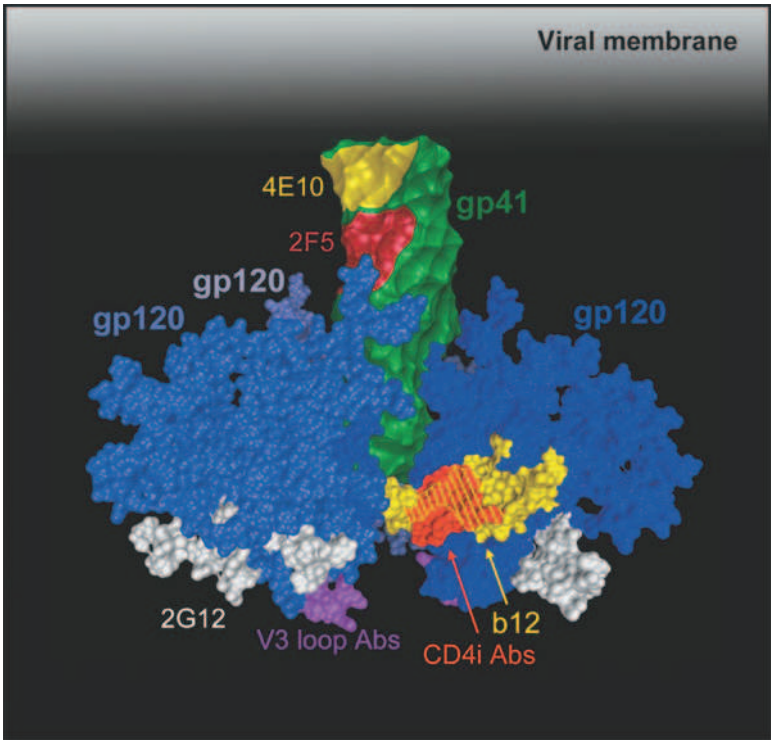


Fig. 3. Neutralizing epitopes in context to trimers. (Figure is adapated from Burton *et al.* (2004) *Nature Immunol* **5**: 233–236.)

act as a bridge between Fc receptor (FcR)-bearing cells and HIV-1-infected cells or other cells that have passively absorbed HIV-1 Env onto their surface. The killing of virus-infected cells by ADCC involves cytotoxic cells (such as NK cells) and may contribute toward the elimination of the virus.[23–26] Alsmadi and Tilley[27] studied the ability of human and chimpanzee mAbs directed against cluster II overlapping epitopes of gp41, CD4 binding site, V3 loop, and C5 domain of gp120 for their ability to induce ADCC. They demonstrated that mAbs directed against conserved epitopes generally exhibited ADCC activity against a broader range of HIV-1 strains than those directed against variable epitopes. Furthermore, it appears that many if not most neutralizing antibodies of high affinity and of the immunoglobulin G1 subclass (IgG1) are capable of exerting ADCC. In a

multi-centered AIDS cohort study, early ADCC responses in patients were associated with higher numbers of $CD4^+$ T-cells and the absence of lymphadenopathy during the first two years of follow up.[28] In two other studies, ADCC responses correlated inversely with plasma viral load and also with $CD4^+$ T-cell counts.[24,29] The presence of neutralizing and ADCC activity in children born to HIV-infected mothers correlated better with the clinical outcome.[30] Finally, it has been shown that ADCC antibodies were present in cervicovaginal fluids in HIV-1 infected women,[31] supporting the hypothesis that this form of immunity can contribute in protection against HIV-1 at the site of virus entry. In a recent study, Gomez-Roman *et al.* have shown that priming with replicating adenovirus type 5 host range mutant-SIV recombinants, followed by boosting with SIV gp120, elicited potent ADCC activity that correlated with protection against the mucosal challenge infection with pathogenic SIVmac251 in rhesus macaques.[32] This is the first study that demonstrates a good correlation between the *in vitro* ADCC activity and *in vivo* reduced viremia; however, further studies will be needed to establish the relationship between the induction of ADCC and protection against HIV. The identification of "ADCC epitopes" may help in designing strategies on means to present and enhance the potency of these epitopes to prevent or control the viral infection.

Neutralizing Epitopes Relevant for Vaccine Development

As mentioned above, Env is the most important target for induction of antibody responses against HIV. In addition, it has several known CTL and helper T-cell epitopes that may provide targets for an effective anti-HIV vaccine. The *env* gene is expressed during the late phase of viral transcription as the gp160 precursor protein. Translation of the precursor protein is dependent on the viral Rev protein, which binds to the rev responsive element (RRE) in Env mRNA and mediates its nuclear export.[33] During the maturation of the virus, the gp160 is proteolytically processed by cellular serine proteases to yield: i) membrane-spanning domain termed gp41 and ii) an extracellular

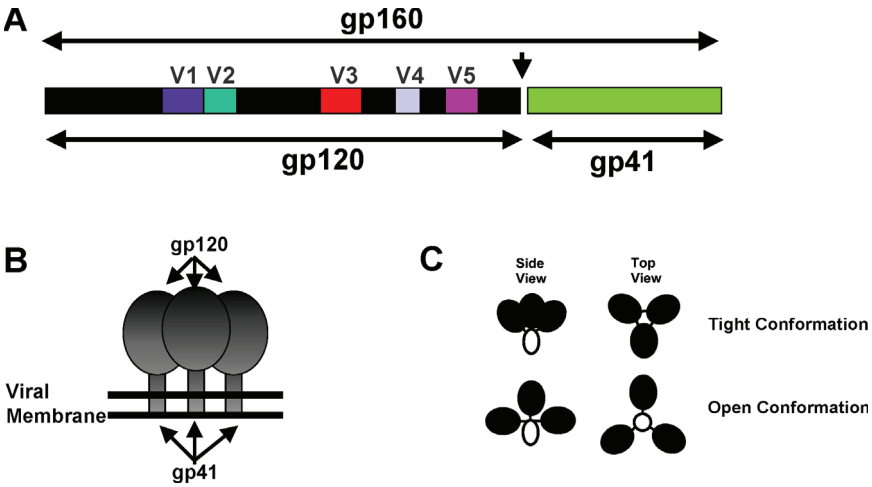


Fig. 4. Linear map of HIV Env (SF162) indicating various conserved and variable domains (Panel A), and large numbers of cysteine residues and extensive glycosylation, likely conformation of Env involving gp120 ectodomain, and gp41 on the surface of the virus (Panel B). Also shown is the schematic of trimers in open and closed confirmation (Panel C). Further structural studies will be needed to demonstrate if the native trimers are in open or closed conformation.

domain termed gp120 (Fig. 4A). Env proteins become heavily glycosylated during their passage through the Golgi apparatus. gp120 and gp41 are non-covalently associated on the viral surface to form trimeric spikes (Fig. 4B) that can bind to the CD4 on T-cells, which is the primary receptor of HIV-1. Comparison of the sequences of the *env* gene from different isolates and clades reveals that it has five hypervariable regions and five conserved regions, and a large number of cysteine residues (Fig. 4A). There are differences between the size of the variable loops and the number of glycosylation sites in V1 and V2 loops among virus isolates of different clades, and between early and late primary viruses. This sequence variation in the hypervariable loops is due to nucleotide changes and subsequent accumulation of point mutations resulting in amino acid substitutions. These mutation-induced changes in Env tertiary structure are selected by the

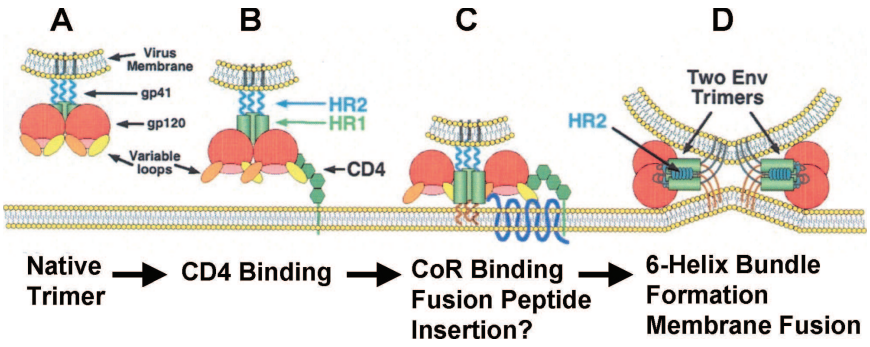


Fig. 5. Receptor and co-receptor mediated entry of virus into $CD4^+$ T-cell. The figure is adapted from Moore and Doms.[34] After CD4 binding, gp120 undergoes a conformational change and exposes the co-receptor binding site on Env (Panel B). The triggered Env binds to a seven-trans-membrane domain co-receptor (third section, CoR). The hydrophobic fusion peptide at the N terminus of gp41 becomes exposed and inserts into the membrane of the cell (Panel C). Whether this results from CD4 binding or co-receptor binding is not known. Co-receptor binding ultimately results in formation of a six-helix bundle in which the helical HR2 domains in each gp41 subunit fold back and pack into grooves on the outside of the triple-stranded HR1 domains (Panel D), bringing the fusion peptide and trans-membrane domain of gp41 (and their associated membranes) into close proximity. It is likely that several Env trimers need to undergo this conformational change in order to form a fusion pore, although here only two trimers are depicted. It is not known whether gp120 remains associated during the fusion process or dissociates from gp41. Although only a single CD4-binding event is shown, multiple CD4-binding events may be needed to activate a single Env trimer.

immune responses of the host and provide a means of immune escape. The receptor and co-receptor dependent entry process of HIV into target cells is depicted in Fig. 5.[34] Interaction of HIV Env with the CD4 is an obligatory step for virus entry into the cell; therefore, as expected, the CD4 binding domain of gp120 is a highly conserved, complex, and conformational dependent region. Hence, the **CD4 binding site of Env (CD4BS)** may be an excellent target for immune intervention. Consistent with this hypothesis, many mAbs

against the CD4 BS have been developed that can neutralize T-cell line-adapted isolates,[35,36] suggesting that these epitopes are relatively well exposed on the virion surface.[37] The most potent and well-characterized monoclonal antibody against the CD4BS, b12,[38] neutralizes a broad range of primary isolates and confirms the critical role of the CD4BS in HIV-1 infection.[39–41] Interestingly, other CD4BS monoclonal antibodies such as 559/64D, 15e, F105, b3, and b6 do not neutralize primary isolates.[42,43] The reasons for this discrepancy are not yet understood, but b12 differs from all the other CD4BS antibodies in its sensitivity to V1-V2 loop deletion.[44] It is not known if b12 contacts the V1-V2 loop or if the sensitivity is due to an indirect effect of conformational rearrangements following V1-V2 deletions. High resolution crystal structure of b12 has been solved, and a key feature of the antibody-combining site is the protruding, finger-like long CDR H3 that can penetrate the recessed CD4-binding site of gp120, which is quite effective in neutralizing the virus. A docking model of b12 and gp120 reveals severe structural constraints that explain the extraordinary challenge in eliciting effective neutralizing antibodies similar to b12. The structure, together with mutagenesis studies, provides a rationale for the extensive cross-reactivity of b12 and is a valuable framework for the design of HIV-1 vaccines capable of eliciting b12-like activity.[45]

After primary attachment of virus to the T-cell surface, gp120 interacts with chemokine receptors CCR5 or CXCR4, which are the most common cellular co-receptors for HIV-1. The interaction of Env to CD4 induces the conformational change in gp120 and gp41 (Fig. 5B). As a result, the co-receptor binding site and fusogenic region of gp41 are displayed (Fig. 5C), leading to the fusion of viral and cellular membranes (Fig. 5D) and, release of the viral core particles in to the cytoplasm of the cell. Therefore ***CD4-inducible epitopes of Env*** may represent another target for immune intervention. Antibodies that show reactivity toward HIV Env when it is complexed with soluble CD4 (sCD4) were found in HIV-1-infected individuals,[46] suggesting that these epitopes are immunogenic. Several such human mAbs have been identified, including 17b, 48D, CG10, 23E, and X5.[47–49] The region recognized by these anti-CD4i monoclonal

antibodies has now been mapped to β-strands in the V1/V2 stem and the C4 region of gp120. These regions are involved in binding of Env to chemokine receptors.[49–52] Surprisingly, 17b and other CD4i mAbs in their complete IgG form do not neutralize most primary isolates.[49] In contrast, Fab and single-chain fragments of these anti-CD4i mAbs neutralize primary isolates,[48,53] suggesting that there may be space constraints between the CD4i epitope on gp120 and the target cell membrane.[54] Therefore, the entire IgG molecule may not be capable of neutralization due to steric hindrance.[55,56] Further studies are needed to confirm the accessibility of these conformational epitopes for targeting them for vaccine application.

Contrary to earlier beliefs that epitopes in the variable domains are isolate-specific (thus may not be appropriate targets for vaccine application), recent structural studies suggest that **V3 loop does contain conserved functional epitopes**, which may be targeted by vaccines. The V3 loop is immunogenic and anti-V3 antibodies are induced early during infection and after immunization.[3,57–59] However, a large proportion of these antibodies are directed against linear epitopes in the V3 loop, which serve as decoys for directing immune responses away from conserved V3 regions. These antibodies neutralize homologous isolates, but they have little or no neutralizing activity against diverse primary isolates.[43,60] However, broadly neutralizing anti-V3 antibodies directed against the conserved conformational epitopes have been described.[61] The most broadly reactive of these neutralizing anti-V3 mAbs (such as 447-52D, 19b, and 2182) can neutralize a large proportion of clade B primary isolates[39,62–64] and also have been shown to neutralize viruses from clades A, F, C, and G,[65] suggesting that the epitopes recognized by these mAbs are conserved across clades.

Structural studies have shown that V3-loop has some constant features, such as a relatively fixed size (30–35 aa), a conserved type-II turn at its crown, a disulfide bond at its base, and a net positive charge.[66,67] These features are required in the V3 loop in order for it to interact with the chemokine receptor.[68,69] It has been shown by Cao *et al.* that deletion of V3 loop renders the virus non-infectious, suggesting that V3 loop is essential.[70] Recently, structural studies have demonstrated that V3 loops in R5 viruses are homologous to the β-hairpin structures

in CC chemokines (CCL3 [MIP-1α], CCL4 [MIP-1 β], and CCL5 [RANTES]), but homologous to the CXC chemokine CXCL12 (SDF-1) in X4 viruses.[71] In addition, Yonezawa *et al.* have replaced the V3 loop of the X4 virus with a 43-amino acid region of SDF-1, which includes the β-hairpin, and demonstrated that infectivity was maintained.[72] Together, these data suggest a critical role for the V3 region in virus infectivity and provide a potential rationale for designing immunogens that induce antibodies to the conserved V3 motif. However, these concepts need to be evaluated in pre-clinical studies for their ability to induce cross-reactive neutralizing antibody responses.

Carbohydrate-dependent Epitopes in gp120

HIV Env is heavily glycosylated, with approximately 50% of its mass due to carbohydrates.[73] It has been demonstrated that glycosylation is critical for the Env-CD4 interaction, since non-glycosylated Env protein (env2-3) does not bind to CD4.[74,75] Thus, carbohydrate moieties on Env appear to provide a functional conformation to Env critical for its interaction with CD4. In addition, based on the crystallization studies of gp120, it appears that the exposed face of gp120 is heavily glycosylated (Fig. 4A). Even in context of trimer, molecular modeling studies suggest that the heavily glycosylated part of the molecule is exposed to the immune system.[76,77] These data suggest that extensive glycosylation may shield critical neutralizing epitopes. Therefore, it is not surprising that antibodies recognizing carbohydrate-dependent epitopes on Env are not readily induced during the course of natural infection. The best-studied ***anti-carbohydrate antibody*** with broadly neutralizing activity is ***mAb 2G12***, which targets a cluster of carbohydrate moieties in gp120.[19,78] This mAb has broad neutralizing activity both against T-cell line-adapted and primary HIV-1 isolates.[19,79] However, its reactivity may be limited, as it does not neutralize subtype C isolates.[80] The unconventional configuration of this mAb[81] and the poor immunogenicity of the epitope recognized by 2G12[79] raise questions about the mode of neutralization (since the epitope recognized by this mAb has been localized on the immunologically silent face of gp120) and how to design an

immunogen with optimal exposure of this epitope. Based on oligomeric modeling studies it seems that the immunologically silent face of Env is likely to face toward the target cell membrane, and thus it is conceptually possible that 2G12 may neutralize the virus by preventing the interaction of the virus with target cells.

As with most pathogens, HIV-1 induces a polyclonal antibody response to a wide array of epitopes on different viral proteins. Studies of polyclonal sera have helped to identify the specificities of antibodies that are associated with protection. It has been shown that sera collected from some HIV-infected individuals neutralize primary isolates (using CCR5 as a co-receptor, R5).[82,83] In addition, several investigators have generated potent neutralizing monoclonal antibodies from the bone marrow of HIV^+ patients against critical functional and conserved epitopes (Table 1), such as the ones in the CD4-binding site (CD4-BS),[38] CD4-inducible epitopes (CD4-i),[46] carbohydrate-dependent epitope,[79] and the epitopes present in variable loops[58,59,84–86] and gp41 regions of Env.[87–89] The relative position of these epitopes compared to the host and target membrane and the complexity of the binding sites is presented in Fig. 3. Furthermore, neutralizing antibodies from the HIV^+ patient's sera can be affinity-purified on a gp120 column,[83,90] suggesting that neutralizing epitopes are present and exposed on gp120. Induction of antibodies to each of these epitopes may ultimately be useful in protecting individuals against HIV-1 infection; however, presentation of each of these epitopes in context to a vaccine is the challenge.

During the past few years, considerable attention has been focused on neutralizing antibodies; therefore, two major points need to be addressed: (i) characterizing the fine specificity of protective antibodies, and (ii) means to elicit these protective antibodies by immunization.

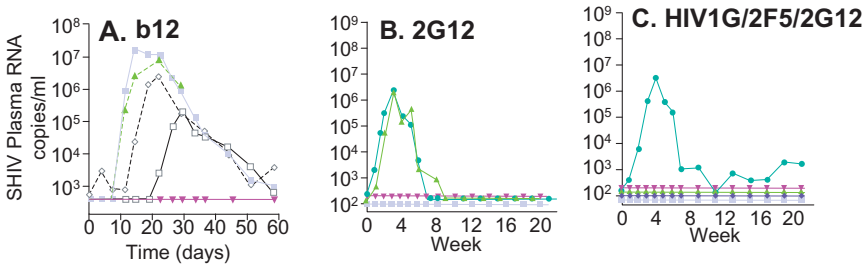
Protective Efficacy of Neutralizing Monoclonal Antibodies in Passive Transfer

It is generally accepted that antibodies are important for protection against HIV infection; however, so far it has been difficult to induce protective antibodies of the appropriate specificity by vaccination. To have a better understanding of the role of neutralizing antibodies

Table 1. Conserved epitopes on the ENV that are targets for developing an HIV vaccine

Env Region	Targeted Epitope	nAbs	Non-nAbs	Characteristics
gp120	CD4-Binding Site (CD4BS)	F105 b12	15e 21h 559/64D 650-D 448D 39.3 b3 b6 830D	These antibodies compete with CD4 for binding to Env. Not all of the CD4BS antibodies neutralize primary isolates.
	CD4-inducible conformational epitope	E51 X5 CG10	17b 48D 23E 49E 21C	Binding of Env to CD4 enhances the exposure of these epitopes. Most of these antibodies neutralize primary isolates as Fab and not as the whole IgG.
	Carbohydrate dependent epitope	2G12		Poorly immunogenic, and binding is dependent upon proper N-linked glycosylation.
gp41	Epitopes in close proximity to viral membrane	2F5 4E10 Z13		These antibodies interfere in membrane fusion and therefore prevent viral entry. To date, these are the most potent neutralizing antibodies identified.
	Cluster I of gp41	Clone 3 246-D		Highly immunogenic epitope, but Clone 3 is the only one of many mAbs specific for this epitope that has neutralizing activity.

in preventing or controlling the HIV infection, the most commonly used approach is to perform passive immunization using neutralizing mAbs in non-human primates. Emini and colleagues have shown that chimpanzees were protected by an anti-gp120 V3 loop-specific



Parren *et al.* (2000); *J Virol* 75: 8340–8347
Mascola *et al.* (2000); *Nature Med* 6: 207–210

Fig. 6. Dose-dependent protection conferred by either single (b12, and 2G12) or a combination of neutralizing antibodies (2F5, and 2G12) to animals in passive transfer experiments as demonstrated Refs. 188 and 189.

antibody[91] against a challenge infection by a TCLA isolate. Subsequently, Conley *et al.* demonstrated the efficacy of mAb 2F5 in partially protecting chimpanzees against intravenous challenge with a primary isolate.[92] The limited availability of non-human primates, particularly chimpanzees, led the investigators to develop an *in vivo* rodent model using severe combined immunodeficient (SCID) mice transplanted with human PBL (hu-PBL-SCID mice) to study the protection afforded by mAbs against HIV-1 infection.[93,94] Using this model it has been shown that passive administration of neutralizing mAbs prior to or shortly after challenge could protect the mice against a challenge infection.[95–97] In this model the antibody concentration needed to protect against the challenge infection *in vivo* was 10 times higher than the concentration needed to neutralize the same isolate *in vitro*.[98] It is interesting, but perhaps not surprising, to observe the differences in the protective efficacy of these neutralizing mAbs *in vivo* and *in vitro*. There are several factors that may influence the protective efficacy of these mAbs, such as dose of the challenge virus and also the growth kinetics of the virus *in vivo* versus *in vitro*. Parren *et al.* demonstrated that higher antibody concentration is required to neutralize primary isolates compared to T-cell adapted isolates.[99] Similar observations were made by Mosier and colleagues

against primary isolates.[100] The use of this model for the evaluation of antibody efficacy is limited due to concerns regarding the route of viral challenge in the mouse model. However, the presence of human cells in the context of the peritoneal and lymph node architecture, complement and phagocytic cells, and the ability to sustain viral infection make it an interesting initial model to test the protective efficacy of passively administered antibodies.

To better understand the qualitative and quantitative characteristics of antibody-mediated protection against HIV-1, Parren and colleagues and Mascola and colleagues used a rhesus macaque challenge model, where animals were passively transfused with neutralizing antibodies and then challenged with the chimeric simian/human immunodeficiency virus (SHIV).[101–103] These viruses contain the Env from HIV-1 and structural proteins from SIV, and are pathogenic in macaques, thereby allowing evaluation of the protective efficacy of antibodies directed against Env. Parren and colleagues passively transferred different concentrations of mAb b12 and then challenged the animals. It was observed that the protection afforded by b12 to rhesus macaques against SHIVSF162P4 is concentration dependent (Fig. 6A). At the concentration of 25 mg/kg of the antibody, all the animals were solidly protected. However, at a 5 mg/ kg level, 50% of the animals were protected. Not surprisingly, at the lowest concentration of 1 mg/kg, none of the animals were protected against the challenge. Mascola and colleagues have demonstrated that a combination of neutralizing antibodies (HIVIG, 2F5, 2G12) was protective against i.v. challenge,[104] where two out of three animals were completely protected while the third animal had a two-log reduction in viral load set point and $CD4^+$ T-cells were also preserved. However, in follow-up experiments it was shown that passive transfer of single antibodies (2F5 or 2G12 or HIVIG) did not protect the animals against the challenge infection because all the animals were infected,[104] suggesting that breadth of immune response may be critical for the protective efficacy. This was contrary to what was observed for b12; however, the challenge virus was different for these studies, and different mAbs were used for passive transfers. Since the primary mode

of transmission for HIV-1 is through mucosal surfaces, efforts have been made to evaluate the efficacy of antibodies for protection against a mucosal challenge.[102] As with systemic challenge, it was demonstrated that complete protection from mucosal challenge was obtained in a majority of animals that received a triple antibody combination (HIVIG/2F5/2G12) (Fig. 6B); however, two out of five animals that received a double combination (2F5/2G12) and two out of four animals that received 2G12 alone were protected against the challenge infection, once again indicating that breadth of the immune response is critical for the protective efficacy (Fig. 6B). To address post-exposure use of neutralizing mAb, Nishimura and colleagues asked the question how soon after virus exposure neutralizing antibodies must be present to block an SIV/HIV chimeric virus infection in pig-tailed macaques.[105] They demonstrated that sterilizing immunity can be achieved in 75% of the animals that received neutralizing IgG six hours after intravenous SIV/HIV chimeric virus inoculation,[105] suggesting that antibodies of appropriate specificity have not only prophylactic but also have therapeutic application.

Ruprecht and colleagues have performed several studies to evaluate the role of neutralizing antibodies in preventing mother-to-infant transmission of virus.[106–108] They demonstrated that passive transfer of a mAb cocktail (F105, 2F5, and 2G12) completely protected pregnant mothers against intravenous SHIV-vpu$^+$ challenge after delivery. The infants subsequently born to these infected mothers who received the mAbs indirectly across the placenta from their mothers and received another dose of antibody cocktail followed by oral challenge with SHIV-vpu$^+$ were also completely protected against the infection.[106] Subsequent experiments have demonstrated that it was possible to protect neonates against a highly pathogenic SHIV 89.6P challenge by passive infusion of a mAb cocktail containing F105, 2F5, 2G12, and b12. Another infusion of the same mAb cocktail an hour after the virus exposure, followed by another dose on day eight, completely protected animals against infection.[106,109] In a recent study, Ferrantelli *et al.*[110] demonstrated complete protection of neonatal rhesus macaques against exposure to pathogenic simian-human immunodeficiency virus (SHIV) by human anti-HIV mAbs. In this study,

eight animals were orally exposed to the pathogenic SHIV and four of them were given post-exposure prophylaxis with three anti-HIV mAbs (2F5, 2G12, and 4E10) at 40 mg/kg dose. All the animals that received mAb therapy were protected from infection ($p = 0.028$) and their plasma, peripheral blood mononuclear cells, and lymph nodes remained free of virus for more than a year. In contrast, all the controls experienced high viral RNA levels and the loss of $CD4^+$ T-cells and died (median survival time 5.5 weeks).

Challenges in Inducing Antibodies of Approriate Specificity with Broadly Neutralizing Activity

During the course of natural infection, HIV triggers antibodies, cytotoxic T-cell (CTL), and $CD4^+$ T-helper immune responses. In general, the primary peak of viremia declines before the appearance of neutralizing antibodies against HIV Env. HIV-infected individuals may generate potent neutralizing antibody responses to autologous isolates, but these responses are slow to develop and take a long time to mature.[3,82,111–116] Interestingly, some long-term non-progressors who remained disease free for more than 10 years after HIV infection developed strong, broadly cross-reactive neutralizing antibody responses, which may have contributed toward their ability to control infection.[117–120] The induction of antibody responses by monomeric Env-based protein subunit vaccines is initially modest and requires multiple boosts to induce strong responses. In addition, these antibodies primarily recognized linear epitopes in the variable domains and neutralized T-cell line-adapted (TCLA) virus isolates at significant dilutions,[121,122] and fail to neutralize primary HIV-1 isolates.[3] Furthermore, these non-conformational anti-gp120 specific antibodies are predominantly subtype-specific and, to some extent, isolate-specific. However, contrary to the earlier neutralization data, a recently published study indicated that using a modified neutralization assay with an extended incubation phase, these first generation of monomeric gp120 vaccines can induce antibodies capable of neutralizing primary isolates.[123] Thus for the fair comparisons of potential vaccine candidates there is an urgent need to develop standardized neutralization assays.

Another factor that has the potential to limit the protective effect of neutralizing antibodies is the emergence of neutralization escape mutants.[124] Richman and colleagues have shown that most patients with primary HIV infection (0–39 months) developed significant neutralizing antibody responses against the autologous virus; however, neutralizing responses against the laboratory-adapted isolates as well as heterologous primary isolates are slow to develop and often lower.[118] The appearance of neutralization-resistant mutants indicates that neutralizing antibody responses have a negative effect on the viral replication during HIV infection. Thus, it appears that the virus is under intense selection pressure to mutate neutralizing epitopes without compromising functional aspects of the Env, such as binding to the receptor and co-receptor to evade immune pressure. This adds additional constraints in development of an effective anti-HIV vaccine. Despite this selective pressure, virus replication proceeds unhindered in infected individuals. Thus, HIV Env can tolerate multiple mutations and most of these mutations are positioned in variable regions that contain several glycosylation sites. In general the number of glycosylation sites in Env glycoprotein is relatively conserved across subtypes and isolates, suggesting a pivotal role for carbohydrates in Env function and structural integrity. This extensive glycosylation provides the virus with some flexibility to modify neutralizing epitopes to evade the immune pressure simply by altering the glycosylation profile (adding, deleting or changing the position), without perturbing the secondary structure.[120]

Strategies to Design Immunogens that may Induce Neutralizing Antibodies of Protective Specificities by Vaccination

It is evident that gp120 contains neutralizing epitopes and that antibodies directed against these epitopes can protect against virus infection. During the course of natural infection, primary neutralizing antibody responses are induced in humans. However, the gp120 monomer has been relatively ineffective at eliciting these primary isolate neutralizing responses. Furthermore, a recently concluded phase III

efficacy trial using a gp120 monomeric vaccine demonstrated that there was no difference in the rate of infectivity between vaccine recipients and the placebo group.[125–127] The ability of this vaccine to raise functional antibody responses such as neutralizing Abs and ADCC has yet to be fully evaluated, but the information gained may help to guide further vaccine development. It will also be critical to understand the barriers in inducing antibodies that recognize similar epitopes to those recognized by 2F5, 4E10, Z13, F105, 2G12, and b12. Several strategies are being evaluated by various investigators to develop and evaluate novel Env immunogens to induce broadly neutralizing antibody responses by vaccination, including: (A) structural optimization targeting conserved neutralization epitopes, and (B) approaches to overcome genetic diversity for vaccine development.

A. *Structural Optimization to Target Conserved Neutralization Epitopes*

The main approaches toward structural optimization of Env for inducing broadly cross-reactive neutralizing antibodies involve the use of: (i) native trimers, (ii) triggered-Envs, (iii) rationally designed Env structures, and iv) mimotopes.

(i) *Native trimers as immunogen*

As mentioned previously, critical neutralizing epitopes are preserved and presented on the Env glycoprotein, but the virus has evolved ways to minimize the immunogenicity of Env, such as by extensive glycosylation of the molecule, to shield the conserved functional regions. Similarly, the receptor-binding site, a likely target for immune intervention, is buried within the molecule and is partially protected from immune recognition. The observation that most broadly reactive neutralizing antibodies isolated so far (i.e. IgG1b12, 2G12, and 2F5) have a stronger affinity for the native envelope (trimer) than for monomeric gp120 or gp41 provides[44,128,129] the impetus to evaluate trimeric Env glycoproteins for their ability to induce broadly cross-reactive, primary isolate neutralizing antibodies. Early immunogenicity

studies demonstrated that antibodies induced by oligomeric Env cross-reacted with HIV Env of other subtypes and neutralized both T cell-adapted and some primary isolates of HIV-1.[130] To use the trimeric Env as an immunogen to elicit functional antibody responses, the first and foremost challenge is to stabilize Env in the trimeric conformation without compromising the structural integrity of the molecule. Several approaches have been tried with reasonable success.[78,131–142] Yang *et al.* used GCN4-stabilized oligomers to demonstrate that antibodies induced by oligomeric gp140 were more effective at neutralizing heterologous primary isolates compared to antibodies elicited by the corresponding monomeric gp120 protein.[143] Earl *et al.* made similar observations in rhesus macaques using a trimeric Env protein from SIV.[135] Recently, we reported the purification and characterization of stable trimeric Env protein from US4 (Subtype B R5 isolates).[140] We also purified and characterized trimeric protein from SF162 and demonstrated that it elicited strong antibody responses in rabbits that neutralized two out of six heterologous subtype-B primary isolates.[144] To further enhance the immunogenicity of trimeric Env, we have introduced a partial deletion of the V2 loop and expressed, purified, and characterized this novel Env immunogen.[139] Using biophysical, biochemical, and immunological techniques, we demonstrated that the purified Env was a trimer, had nanomolar affinity to CD4, and had critical neutralizing epitopes exposed and preserved on the trimer.[139] Deletion of the V2 loop qualitatively altered the immunogenicity of the V3 and V1 loops and rendered the C5 region more immunogenic.[145] The V2 loop deletion in the context of a trimer elicited strong antibodies in rabbits that neutralized five out of six heterologous subtype-B primary isolates, and conferred partial protection upon pathogenic challenge with $SHIV_{SF162P4}$ to rhesus macaques immunized with this V2 loop deleted trimer in a DNA prime and protein boost strategy.[146,147] These animals were followed for more than three years without any sign of disease. In addition, we demonstrated a reasonable correlation between the presence of neutralizing antibodies at the time of challenge in the vaccinated animals and the levels of plasma viremia during acute infection.[148]

This protein is currently being evaluated in a human clinical trial (HVTN049). In a separate study, Quinnan and colleagues have evaluated the efficacy of oligomeric Env for inducing broadly neutralizing antibodies.[149] They primed a group of rhesus macaques with alphavirus replicon particles expressing the Env from R2 strain, and later these animals were boosted with the soluble oligomeric gp140. Concurrently, animals were also immunized with SIV gag/pol genes or no SIV genes to determine the additional protective benefit of inducing cell-mediated immune responses. The antibodies induced by alphavirus priming and oligomeric protein boost neutralized diverse primary isolates *in vitro*. The immunized animals were protected against the challenge infection with SHIVDH12 (Clone7). Furthermore, the protection was associated with neutralizing antibody titers of >1:80 or with cellular responses of 2000 spot-forming cells/10E6 PBMC. More recently, we have extended these studies for the subtype C as well. In a proof of concept study in both rabbits and rhesus macaques, we have demonstrated that HIV envelope DNA vaccines derived from the South African subtype-C TV1 strain can effectively prime for humoral responses against both subtype-B and subtype-C primary R5-tropic HIV-1 isolates in rabbits and rhesus macaques. Priming rabbits with DNA plasmids encoding TV1 gp140 modified in the second hypervariable loop (gp140TV1ΔV2), followed by boosting with oligomeric TV1ΔV2 proteins, elicited more potent HIV-neutralizing antibody responses than DNA vaccines encoding TV1 gp160 or the intact TV1 gp140. Boosting with oligomeric TV1ΔV2 envelope proteins in MF59 adjuvant also elicited higher titers of antibodies with homologous neutralizing activity against TV-1 and heterologous neutralizing activity against the subtype-B SF162 primary isolate. In addition, combining subtype-B and subtype-C V2-deleted immunogens resulted in increased cross-clade neutralizing activity. Importantly, subtype-B-neutralizing antibody responses were also observed after immunizing rhesus macaques with subtype-C TV1ΔV2 immunogens. Our results demonstrate that the modified subtype-C gp140TV1ΔV2 immunogen described herein elicits broad, subtype-B and subtype-C neutralizing antibodies.[150]

(ii) *Triggered Env: targeting conformational epitopes*

Conformational epitopes in Env induced upon interaction with CD4 are attractive targets for inducing neutralizing antibodies. It is well documented that upon binding to CD4, HIV Env protein undergoes significant conformational changes,[151,152] which are critical for membrane fusion and viral entry in the target cells.[152] Furthermore, mAbs such as 17b, 48d, 23e, 49e, 21c, E51, CG10, and X5 are better able to recognize specific epitopes on gp120 after it interacts with soluble CD4 (triggered Env).[46,48,49,153,154] Attempts have been made by various groups to evaluate such triggered Env (gp120-CD4 complexes) as potential vaccine candidates.[47,155] It was demonstrated that gp120-CD4 complexes can induce broadly neutralizing antibody responses, but they need to be stabilized using cross-linking reagents.[155] In a proof of concept study, we have demonstrated that triggered Env (gp120-CD4 complexes) induced strong immune responses against both gp120 and CD4[145] and these antibodies neutralized both a primary isolate (SF162) as well as a T-cell adapted isolate (SF2).[156] To elucidate the contribution of anti-Env antibodies to neutralization, we affinity purified the anti-Env antibodies from anti-CD4 antibodies and showed that these antibodies neutralized two subtype-B and one subtype-C primary HIV-1 isolates that were tested.[156] Since gp120 SF2 alone was not able to induce primary isolate neutralizing antibodies,[144] these preliminary data suggested that certain neutralizing epitopes may be better exposed on triggered Env compared to untriggered gp120.

These studies illustrate the potential utility of the approach; however, the use of full-length CD4 raises the potential for induction of autoimmune responses. One strategy to overcome this problem is to identify the minimal binding domain of CD4. The recently reported crystallographic structure of gp120, in complex with CD4 and the Fab portion of mAb 17b,[157] has demonstrated that a large surface (742 Å) of the domain D1 of CD4 binds to a large depression (800 Å) on gp120. This CD4 interface is composed of 22 residues, contributing to gp120 binding with mixed hydrophobic, electrostatic, and H-bonding interactions. The large size and complexity of this

interface makes the reproduction of such a functional domain into a small non-immunogenic molecule a challenge, and highlights the difficulty in developing high-affinity CD4 mimics. However, despite of the large number of residues present in gp120-CD4 interaction, studies on hormone-receptor systems have shown that few residues might dominate the binding energy at protein-protein interface.[158] Thus, the design of a minimal CD4 mimics may be possible.

The transfer of functional sites to small proteins acting as structural scaffolds has been proposed as a useful strategy to reproduce the structure and function of the target protein in small molecular systems.[159,160] This approach has led to the discovery of scorpion toxin Scyllatoxin fragment as an effective mimic of CD4. A mini-protein, CD4M3, was chemically synthesized, folded efficiently, and presented a circular dichroism spectrum similar to that of native Scyllatoxin. In competitive ELISA, CD4M33 was able to specifically bind gp120 at an IC50 of 40 μM, which is four orders of magnitude higher than that of sCD4. This strategy has been recently applied to the engineering of a mini-protein that mimics the core of the CD4 protein surface that interacts with the gp120 envelope glycoprotein of HIV-1 and, hence, inhibits virus attachment to cells and infection.[161]

The biological performance of this mini-protein was improved using "rational" structure design. In total, five substitutions were introduced (Gln20Ala, Thr25Ala, Leu18Lys, Ser9Arg, and Pro28) and the resulting mini-protein (CD4M9) bound to gp120 at 400 nM, induced conformational changes in Env, and inhibited infection of CD4-expressing cells by different virus isolates. So far, an improved CD4M33 has an affinity for gp120 similar to CD4,[162] and induces a conformational change in gp120 similar to that induced by sCD4X.[162] In an earlier study, Fouts and colleagues have shown that covalently cross-linked complexes of CD4 and HIV Env IIIB induced antibodies that neutralized a wide range of primary isolates.[163] Ig with neutralizing activity was recovered by affinity chromatography using Env/CD4M9 single-chain polypeptide. More recently, using CD4M9, an earlier version of the CD4 mini-protein developed by Vita and colleagues, Fouts *et al.* prepared single-chain constructs of SCBal/M9 and performed immunogenicity studies in rabbits.[164] They showed

that antibodies induced by Env-CD4M9 cross-linked complexes neutralized a broad range of primary isolates. These results demonstrate that: i) a significant portion of gp120 binding surface of CD4 can be reproduced in a mini-protein system, ii) an engineered CD4 mini-protein contains sufficient CD4 structural elements to induce gp120 conformational changes, and iii) these surrogate molecules may be useful in making stable complexes with envelope protein to expose envelope epitopes for the induction of neutralizing antibodies.

In another approach, Guo *et al.* described a small synthetic molecule (termed BMS 378806) that inhibited the interaction of gp120 with cellular CD4 and prevented viral entry.[165] This compound bound to gp120 at a stoichiometry of approximately 1:1 with an affinity similar to that of CD4. Therefore, molecules such as this one should be evaluated for their ability to induce conformational change in Env.

(iii) *Rational design of Env immunogen*

A crystal structure of HIV-1 gp120 core in complex with two N-terminal domains of CD4 and a Fab fragment of mAb 17b that binds CD4-induced co-receptor-binding site was solved by Kwong,[166] and is known as liganded structure. The structure was determined using the gp120 that lacked the V1/V2 and V3 loops as well as 52 and 19 residues from the N- and C-termini. The structure provided some basic, but very important, information about the organization of gp120. The protein is comprised of two domains, the inner and the outer, which are bridged by a mini-domain called a "bridging sheet", a four-stranded, anti-parallel (β-sheet) (Fig. 7). The inner domain is buried internally in the gp120/gp41 trimeric complex; therefore, as expected, antibodies against this region do not neutralize the virus. The outer domain is extensively glycosylated and does not elicit antibody response, and thus it is termed as immunologically silent. The receptor binding site (i.e. CD4 binding pocket) is located in a region that borders the outer and inner domains and the bridging sheet. More recently, crystal structures of HIV-1 gp120 with intact V3 loop have been solved.[167] It appears that V3 loop forms a rod-like structure that protrudes out from the gp120 core. This is not surprising

considering its highly immunogenic properties. The crystal structure of SIV gp120 reveals a number of important facts. **First**, its overall structure is very similar to that of HIV-1 gp120, especially the outer domain of the protein.[76] **Second**, neither the CD4 nor the co-receptor binding site (BS) is properly formed in the unliganded state of the Env. In the unliganded form, the two double-stranded anti-parallel sheets are well separated and many residues that make up the CD4 BS are either masked or in different configuration compared to after binding to CD4. **Third**, there is large displacement and rotation of the inner domain and bridging sheet components upon CD4 binding: (i) the three-strand anti-parallel sheet of the inner domain rotates by 30°, (ii) the four-turn α-helix 1 shifts away from the outer domain, and (iii) the tip of the V1 $\alpha/2$ stem moves by over 40Å. These events result in the formation of the bridging sheet. At present, the structure of the V1/V2 loop is not known. However, it is thought to partially mask the bridging sheet. Deeper insights into the native (i.e. un-truncated) structures of gp120 could facilitate envelope-based vaccine design. Now the efforts are being made by different groups including ours to rationally design novel immunogens that may induce broadly neutralizing antibody responses. So far, the focus has been to optimize engineered Env structure for inducing potent antibody responses against conserved functional epitopes by structure-based targeted deglycosylation, by hyper-glycosylation, or by introducing mutations/deletions in the bridging sheet.

Structure-based Targeted Deglycosylation

The extensive glycosylation of Env is likely to be involved in immune evasion. Based on crystal structure it seems that the outer domain of gp120 is heavily glycosylated, as shown by Wyatt and colleagues[77] (Fig. 8). Despite being most exposed to antibodies, this region of Env is known as "silent face" because it seldom induces neutralizing antibody responses. Thus, the sugar moieties may be shielding critical neutralization epitopes. Malenbaum *et al.* have demonstrated that the removal of glycosylation at position 301 resulted in an increased neutralizing sensitivity of HIV-1 to CD4 BS antibodies.[168] Furthermore,

mutant virus lacking 301 glycan also demonstrated sensitivity to CD4i antibodies. In another study, elimination of N-linked glycosylation in the V1 and V2 loops of pathogenic SIV mac239 rendered the virus more sensitive to host antibody responses.[169] It is apparent from the crystal structure that sugar moieties lie proximal to, but not within, the receptor binding site. Koch and colleagues have performed structure-based deglycosylation of four sites flanking the receptor-binding region (i.e. 197, 276, 301, and 386). Removal of a single glycosylation site at the base of V3 loop (i.e. aa 301) has rendered the mutant virus more sensitive to antibody-mediated neutralization by anti-CD4 BS antibodies. Furthermore, deletion of all 4 glycosylation sites has made the resultant virus sensitive to neutralization by CD4i antibodies. In an other study, McCaffrey *et al.*[170] have demonstrated that removal of sugar moieties at positions 293, 299 329, and also 438 and 454 made the SF162 virus more sensitive to neutralization by CD4BS mAbs and also to V3 loop specific neutralizing mAbs. In addition, the deglycosylation of V3 loop (293, 299, and 329) also made the mutant virus more sensitive to mAbs specific to CD4i epitope. Furthermore, the same group has also demonstrated that deglycosylation at positions 293 (V3 loop), 438 (C4 region), and 454 (V5 loop) also rendered the resultant virus more susceptible to the anti-gp41 mAb 2F5. These studies suggest that carbohydrates at these positions protect the CD4 binding site, V3 loop, co-receptor binding site and also gp41 epitope.[170] Further studies will be needed to evaluate the efficacy of this approach in exposing critical neutralizing epitopes in Env immunogens.

Hyper-glycosylation for Focusing the Immune Response Toward Neutralizing Epitopes Recognized by b12

Burton and colleagues have taken the opposite approach of incorporating additional carbohydrate residues. They have demonstrated that four alanine substitutions on the perimeter of the Phe43 cavity of gp120 reduced the binding of weakly neutralizing CD4 BS antibodies

to gp120,[171] while increasing the binding of a potent broadly neutralizing antibody b12. In further studies, they focused on the reduction of binding of a wide range of non-neutralizing antibodies by incorporating seven more glycosylation sites in addition to the four alanine mutations.[172] It was interesting to note that these hyperglycosylated Env proteins were not recognized by non-neutralizing antibodies directed toward CD4 BS (such as b3, b6, CD4-IgG2, 15e, F91, and F105); however, binding of the neutralizing antibody b12 remained, albeit at lower affinity, largely unaffected. Furthermore, hyperglycosylation affected the exposure of conformational epitopes recognized by mAbs 17b, 48d, and X5. It remains to be determined whether these modified molecules can alter the immunogenicity of Env and, in particular, if the increased b12 reactivity can be correlated with enhanced neutralizing activity.

Directing the Immune Response Toward the CD4 Binding Site by Introducing Deletions in Bridging Sheet

It has been difficult to induce antibody responses directed against the CD4 BS by vaccination with gp120. From gp120 structure analysis, it is apparent that Env is folded into inner and outer domains (Fig. 7). The inner domain (with respect to the N and C terminus) comprises two helices, a small five-stranded β sheet sandwiched at its proximal end, and a projection at the distal end from which the V1/V2 loop emanates. The outer domain is a stalked double barrel that lies alongside the inner domain, such that the outer barrel and inner bundle axes are approximately parallel. The gp120 inner and outer domains are attached by a bridging sheet that further limits the accessibility of the CD4 binding pocket. This bridging sheet is composed of four anti-parallel β strands (namely β-2, β-3, β-20, and β-21) (Fig. 8). The CD4 binding site is buried deep between the inner and outer domains of this molecule and, hence, possibly not accessible to antibodies. Structural data suggest that the V2 loop may fold over the bridging sheet and, due to its three-dimensional position, the bridging sheet is

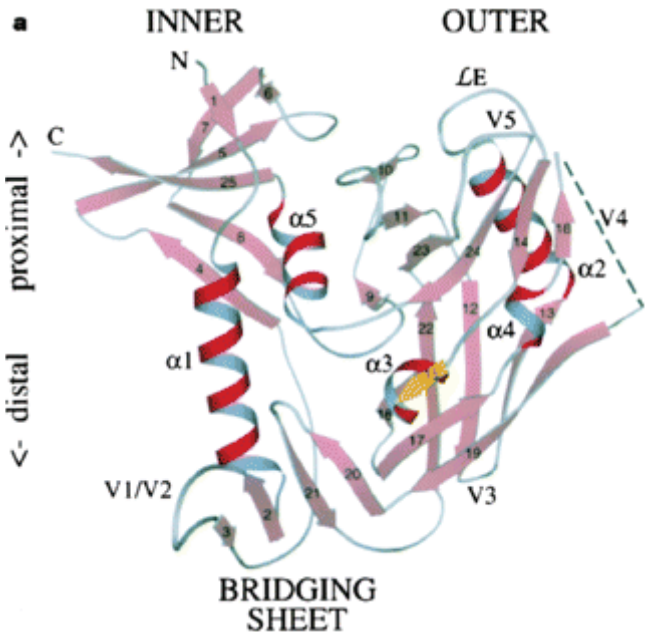


Fig. 7. gp120 core structure. Schematic representation of the tertiary structure of the HIV-1_{HXB-2} Env gp120 polypeptide highlighting the bridging sheet that connects the outer and inner domains as determined by the crystallographic studies.[157]

believed to mask the elements involved in CD4 binding and co-receptor binding. It has been shown that the deletion of V1 or V1V2 loops does not abrogate the functional activities of the envelope glycoprotein because the recombinant V1 or V1V2 deleted viruses are fully replication-competent in human PBMC.[173] Furthermore, these deletions in the context of Env protein also did not alter its ability to bind sCD4.[139,174] This suggests that these modifications alone preserve the integrity of important conserved epitopes on Env. Furthermore, deletion of V1 and V2 loops has made the virus more susceptible to antibody-mediated neutralization; however, no improvement was observed in terms of directing the immune response to the CD4 binding site.[139] Therefore, we introduced additional deletions in the bridging sheet in an attempt to sufficiently expose the CD4 binding

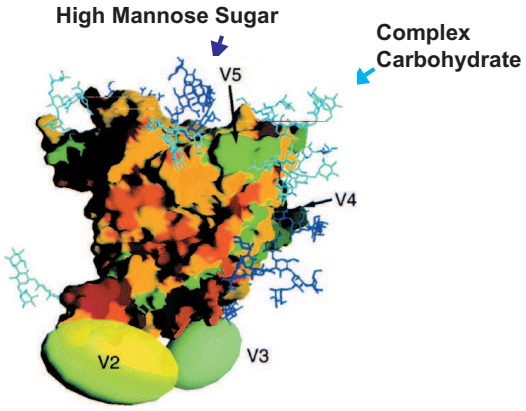


Fig. 8. The molecular surface of the gp120 core, including the modeled N-terminal residues, V4 loop, and carbohydrate structures, are shown as presented by Wyatt *et al.*[77] The outer domain of gp120 is heavily decorated with complex carbohydrates (blue) and terminal mannose sugars (dark blue). The molecular surface of gp120 is color-coordinated to demonstrate the variability in the Env: red indicates residues conserved among all primate immunodeficiency viruses; orange indicates residues conserved in all HIV isolates; yellow indicates some degree of variability; and green indicates significant variability among all HIV isolates.

site without destroying the proper folding and conformation of Env. As mentioned above, there are four β strands: β-2 strand corresponds to approximately amino acid residues Cys-119 to Thr-123, β-3 corresponds to approximately Ser199 to Ile-201, β-20 extends from amino acid residues 422-Gln to 426-Met, and β-21 extends from amino acid residues 431-Gly to 435-Tyr relative to HXB-2. The V1 and V2 loops emanate from the first pair of β strands (Cys-126 to Cys-196) and a small loop extrudes from the second set of β strands. This small loop extends from amino acid Trp-427 to Val-430. The H-bonds between β-2 and β-21 are the only connections between domains of the lower half of the protein (joining helix α-1 to the CD4 BS). Based on these structural features, we have designed a series of deletions within the small loop of the bridging sheet to further expose the CD4 binding site. In addition, we are evaluating deletions in the large loop (i.e. V1 and V2 loops) either alone or in conjunction with

deletions in small loop to enhance the exposure of conserved neutralization epitopes that are shielded from the immune system (unpublished observations).

Structural Information on gp41

Four crystal structures of HIV/SIV gp41 cores are available; all of these structures are similar and represent a post-fusion conformation of gp41.[175] It seems that in prefusion state, in the native and untriggered conformation, the surface of gp41 is shielded by gp120, and thus is not accessible for antibody recognition. Upon binding of CD4 and co-receptor, both gp120 and gp41 undergo extensive conformational changes that expose the gp41 in an extended fusion intermediate conformation to position the fusion peptides for insertion into the host membrane, during which gp41 is perhaps most vulnerable from host antibody binding. In a proof of concept study, LaCasse and colleagues have shown that it is possible to target the fusion intermediate for vaccine application.[176] In the study they immunized the animals with fusion intermediates, and demonstrated that post immunization sera neutralized 24 out of 25 primary isolated tested. Furthermore, it has been shown that the peptides derived from the sequences of HR1 and HR2 blocked the membrane fusion process of HIV.[76,177] Most likely, these peptides bind to the gp41 fusion intermediates during the conformational changes, and therefore prevent the membrane fusion and viral internalization. Now the most important question is how to exploit the potential of fusion intermediates for vaccine development. The most important constraint is exploiting these fusion intermediates for vaccine application is that the structure of gp41 in fusion intermediate conformation is not known. Therefore, structural information of a longer or full-length gp41 protein will be helpful to understand the influence on overall gp41 structure and function of the segments that were omitted in the present crystal structures of gp41 core. In addition, detailed structure studies of gp41 variants in conjunction with functional characterization of antigenicity of the forms can drive a better understanding of the structural epitopes to productively drive antigen development.

Current Structural Information that may be used for Designing new Immunogens

While the efforts were directed to solve the crystal structure of Env trimer, and to address the technical challenges associated with it, two groups have applied a low-resolution technique (i.e. Cryo-EM) to circumvent the problems associated with crystallization and to provide the structural information on the Env trimer.[178,179] Zulleti *et al.* have two best-fitted Cryo-EM three-dimensional desnsities, and both of these structures suggest that: i) all the glycans are pointing outward from the surface of the trimer, and solvent exposed, which is consistent with the glycan shield model proposed by different investigators to protect the virus against the neutralizing antibodies[120,180]; ii) the variable regions V4 and V5 are positioned on the top surface of the complex, an orientation that would facilitate their recognition by antibodies[181]; iii) the V1V2 loop extends outward, implying good solvent exposure, and in addition it is possible that the interaction of V1V2 loop and the co-receptor binding site in the monomer may limit the access of the co-receptor binding site to antibodies; iv) the CD4 binding surface is exposed on the outer edge of each gp120 protomer, and is oriented such that access to membrane-associated CD4 on the target cell is possible; and v) the two models differ significantly with regard to the orientation and exposure of V3 loop, and the orientation of co-receptor binding sites. In the **first model**, the ***V3 base points toward the trimer interface*** running roughly parallel to the 3-fold symmetry axis, being partially exposed in the cavities between the lobes. In this trimer model, the V3 loop would be substantially masked by packing into the trimer axis, potentially reinforced by inter-V3 bonding. In the **second model**, the ***V3 loop is oriented outward in solvent exposed phase***. In addition, V3 seems to be highly flexible in the CD4-unbound trimer. After Env binds to CD4 it induces a conformational change in Env that increases V3 exposure. This is supported experimentally by the increased accessibility of the V3 loop in the Env trimer to antibody binding and enzymic proteolysis subsequent to CD4 engagement.[182,183] It is also consistent with the highly exposed nature of the V3 loop in the structure of the

V3-containing gp120–CD4–Fab complex.[167] The increased exposure of V3 in the CD4-bound conformation could be explained by a rearrangement of the trimer in the first model, or by a conformational change involving the V3 loop in the second model.

The conserved **co-receptor binding site**, comprising the bridging sheet[76] and associated regions, is thought to be largely inaccessible in the trimer. Experimental data support this concept, since most CD4-induced (CD4i) surface-specific mAbs cannot access their epitopes on the CD4-unligated Env trimer, as evidenced by weak or absent neutralization. It is possible that the V3 loop may be involved in protecting the co-receptor binding sites from antibody recognition and binding.

A trimer model has been proposed previously in which the inner domain of gp120 points toward the gp120-gp41 interface and the outer domain extends outward.[76] Such a model is inconsistent with the orientation of the major axis of the ellipsoidal gp120 density within the density corresponding to gp120 in our structure. In this previous model, the V3 loop is exposed to solvent and the co-receptor binding site is buried at the trimer interface. The two fittings proposed by Zanetti *et al.* clearly suggest that either the V3 loop is also pointing toward the trimer interface, or the co-receptor binding site is on the outside of the trimer, and protected from solvent by V3.[178] It is likely that the Env complex exhibits some conformational flexibility.

In the context of trimeric Env, the gp41 stem appears as a compact structure with no obvious separation between the three monomers.[178] The membrane-proximal region of gp41 is characterized by a highly conserved hydrophobic region, which is thought to mediate trimer self-assembly.[184] Furthermore, the density corresponding to the gp41 stem region is shorter and slightly wider than the post-activation coiled-coil conformation,[185] in agreement with the hypothesis that a dramatic conformational change is required for gp41 to extend toward, and insert into, the target cell membrane. The shape of the complex suggests a new model for the Env trimer organization. The volumes are consistent with the assignment of the globular domains to gp120 and the stem to gp41. The gp120

protomers appear to fold over gp41 rather than depart radially from it, contacting each other at the top of the spike. The interaction between gp120 monomers at this contact is likely to be weak, to allow the disassembly of Env ectodomain and extrusion of gp41 for insertion into the host membrane for the membrane fusion to take place.

The two models proposed shed light on several important antigenic and mechanistic features of the Env trimer. First, they provide models consistent with the concept that glycans and immunodominant variable loops are positioned on exposed gp120 surfaces to damp the neutralizing antibody response. Second, they confirm that only a limited gp41 surface is exposed for antibody binding, as has been proposed previously.[92] Third, they provide two possible descriptions of the position of the V3 loop and the co-receptor binding.

B. *Approaches to Overcome Genetic Diversity of HIV Env for Vaccine Development*

HIV is one of the most genetically diverse viral pathogens studied to date. The uneven distribution of the various clades across the globe presents one of the most challenging aspects to HIV vaccine design, as it is unknown whether it will be possible to make an HIV vaccine to cover all clades or whether a tailor-made vaccine based on the most prevalent strain for a given region will be needed. To address the problem of genetic diversity in HIV multivalent vaccine formulations and vaccines based on consensus and ancestral sequences are being evaluated.

Multivalent vaccine approach

The objective of this approach is to use multiple clade-specific immunogens in a vaccine to increase the breadth of the immune responses without compromising the potency against one or more clades. Various groups have recently demonstrated the potential utility of this approach. For example, Shan Lu and colleagues demonstrated that by including monomeric Envs from different clades, improved breadth of neutralizing antibodies against different subtypes

was achieved.[186] Similar observations were made with a DNA prime and rAdeno virus boost regimen by Letvin and colleagues.[187] In a proof of concept study, we immunized a group of rabbits with HIV-1 Envs derived from subtype-B and subtype-C isolates either alone or in combination in a DNA prime and protein boost regimen. All of the animals immunized either alone or with the bivalent B and C vaccine induced comparable levels of antibody responses against subtype-B and subtype-C envelope proteins in ELISA. The antibodies induced in the bivalent group were able to neutralize both homologous subtype-B and subtype-C isolates as well as other B and C strains.[150]

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