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AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

Abstract

Human immunodeficiency virus type 1 (HIV-1) is a rapidly evolving pathogen and causes the acquired immunodeficiency syndrome (AIDS) in humans. There are ~30-35 million people infected with HIV around the world, and ~25 million have died since the first reported cases in 1981. Additionally, each year 2-3 million people become newly infected and more than one million die of AIDS. An HIV-1 vaccine would help halt an AIDS pandemic, and efforts to develop a vaccine have focused on targeting the HIV-1 envelope, Env, found on the surface of the virus. A number of chronically infected individuals have been shown to produce antibodies, called broadly neutralizing antibodies (bnAbs), that target many strains of HIV-1 by binding to Env, thus suggesting promise for HIV-1 vaccine development. BnAbs to take years to develop and have a number of traits that inhibit their production; thus, a number of researchers are trying to understand the pathways that result in bnAb production so that they can be elicited more rapidly by vaccination. This review discusses results and implications from two HIV-1 infected individuals studied longitudinally who produced bnAbs against two different sites on HIV-1 Env, and immunization studies that used Envs deriving from those individuals.

Keywords:

HIV-1, Env, immunogen, epitope, conformation, co-evolution, heterologous neutralization

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)
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Introduction

HIV-1, the etiological agent of AIDS, is a rapidly evolving pathogen. The complex nature of the HIV envelope, Env, has impeded the development of an effective HIV-1 vaccine.[1] Env is a trimeric gp160 assembly that is processed into gp120 (CD4 receptor-binding subunit (CD4bs)) and gp41 (transmembrane subunit) by furin cleavage.[2] Gp120 sometimes is "shed", leaving behind gp41 "stumps", or other oligomers, which are incapable of cell entry[3] and can favor the production of strain-specific, or non-neutralizing, antibodies.[4, 5] An added level of humoral immune evasion stems from the dynamic nature of HIV Env and conformational masking.[6-12] Upon binding to the host cell receptor CD4, gp120 undergoes large conformational changes to an "open" state that permits subsequent binding of a co-receptor, triggering further conformational changes and ultimately host-cell entry mediated by gp41 rearrangements.[13] These different conformations of Env present immunodominant epitopes, misdirecting the humoral immune response to produce non-protective antibodies.

HIV also evades the immune system by rapid mutation[14], particularly within the five variable loops (V1-V5)[15], and extensive glycosylation[16-18], of Env. V1–V3 are found at the apex of the trimer (**Fig. 1**), and are important for trimer association and cell entry.[19, 20] V5 is found near the CD4 binding site. Env glycosylation, which can be quite heterogeneous because of steric effects against glycan processing enzymes[21, 22], serves to shield a large portion of gp120 since carbohydrate moieties may appear as "self" to the immune system.

HIV-1 broadly neutralizing antibodies (bnAbs), which target >50% viral variants, are produced in up to 25% of infected individuals[23-26], suggesting promise for HIV-1 vaccine development[27]. The epitopes of currently identified bnAbs cluster on six predominant immunogenic regions on Env[28, 29]. Moreover, bnAbs develop over a course of at least 4-5 years of infection. It is now possible to obtain longitudinal virus and antibody sequences from HIV-infected donors because of improvements in single B-cell sorting technologies[30], single-genome amplification and sequencing[31] and computational approaches that allow the inference of progenitor antibodies[32](**Fig. 2**). These data allow the investigation of virus-antibody co-evolutionary pathways through the development of bnAbs[33-35,13,14] to provide

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

information necessary to develop vaccines to elicit bnAbs along shorter, dominant pathways.[36]

A number of structural analyses have been performed on HIV-1 bnAb-producing donors studied longitudinally[33-35, 37, 38], but Envs from only two of those cases have been tested in animals (**Fig. 2**). In this review, we will discuss the two longitudinal cases and the related immunization studies. We will focus on lessons learned from the infected donors, structural features of the immunogens used, and considerations for future studies.

HIV-Antibody "Arms Race" Analyses for B-cell Immunogen Design Approaches

Infection with an HIV transmitted founder (T/F) virus[31] causes the immune system to produce strain-specific antibodies against the T/F Env.[39] The virus "escapes" recognition by mutating its Env rapidly. Processes of somatic hypermutation (SHM) and recombination in B-cells results in antibodies with a greater affinity for escape virus Envs[40], which then mutate to yield new escape virus Envs.[41] Because they mutate rapidly, most circulating viruses are resistant to antibodies from the same time point. These co-evolutionary trajectories produce a virus-antibody "arms race", which in some cases results in antibodies that can target heterologous tier-2 (difficult to neutralize) viruses[42, 43] and bnAbs.

Understanding the patterns of antibody affinity maturation resulting in bnAb development could lead to the design of immunogens that increase the likelihood of maturation along the desired, yet disfavored, pathways leading to bnAbs[36, 44, 45]. A vaccination protocol based on a B-cell lineage would prime with one immunogen, and boost with a sequence of several different immunogens.[46] To do this, one would design immunogens with an enhanced affinity for the unmutated common ancestor (UCA)[47] and intermediate antibodies using their paratopes as structural templates. This relies on available structural information – ideally, crystal structures would be determined of the complex of the mature antibody Fab with antigen, and of the UCA and one or more intermediate antibodies. A structure of a UCA–antigen or intermediate antibody–antigen complex would also be helpful, but the native antigen may not bind tightly enough to the

UCA to enable structure determination, and thus may require indirect methods to determine how to target the UCA.

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

CH505: CD4bs bnAb development

The CH103 Broadly Neutralizing Antibody Lineage

Liao et al.[38] tracked the co-evolution of the CH103 CD4bs bnAb lineage and the clade C CH505 HIV-1 Env isolated from an African patient, CH505, starting approximately 4 weeks after infection through the development of bnAbs. Interestingly, all CH103 lineage members, even the UCA, could bind the T/F virus Env. Mutations in the autologous CH505 virus Env did, however, escape recognition by earlier members of the antibody clonal lineage. Moreover, as antibody maturation proceeded, the more mature members of the lineage could bind with increasing affinity to heterologous virus Envs.

The crystal structure of the complex formed between the bnAb CH103 Fab fragment and a gp120 outer domain showed that CH103 interacted with the gp120 CD4-binding loop, V5 loop, and D loop[38]. The interaction was dominated by the heavy chain complementarity determining region (CDR) 3 loop of CH103, which interacts with the CD4bs. The UCA CDRH3 loop was more flexible than that of more mature members, which likely explains its lack of breadth. To understand affinity maturation, unliganded Fab structures were analyzed by superposing heavy-chain variable domains (V_H) on that of CH103 because the V_H domain contacts the conserved CD4 binding loop.[48] The superposition showed that during affinity maturation, the orientation of the light-chain variable domain (V_L) shifted with respect to V_H, likely to accommodate gp120 V5 loop insertions that occurred during the first year of infection, and which would relieve unfavorable contacts with the UCA light chain. The shift was observed between intermediate antibodies I3 and I2, which corresponded to the point at which heterologous neutralization occurred. Thus, the shift in orientation could account for the ability of CH103 bnAbs to bind heterologous Envs with larger V5 loops.

Results from these studies suggested that binding for a T/F Env to a UCA B-cell receptor (BCR) of a bnAb lineage like CH103 can induce bnAbs and be used as a potential

starting vaccination immunogen to elicit CD4bs bnAbs. Since several key changes in antibodies occurred between the UCA and I3, and between I3 and I2, Envs that bind I3 and I2 should be used as boosts to trigger I3- and I2-like antibodies to drive forward a CH103-like lineage. This bnAb lineage is an especially promising lineage to attempt to mimic via sequential vaccination because its natural evolution required fewer mutations compared to other CD4bs bnAbs to become broad.

The CH235 Cooperating Lineage

Viruses typically mutate to escape immune pressures, but CH103 was observed to bind Envs with D-loop mutations more avidly. Why the virus mutated to become more sensitive to CH103-lineage antibodies turned out to be the consequence of a "cooperating" antibody lineage, called CH235, another bnAb lineage produced in the CH505 donor.[49, 50] The CH235 and CH103 lineages demonstrated cooperative evolution throughout the course of HIV-1 infection: the virus developed an escape mutation in response to the CH235 lineage, which drove forward the development of the CH103 bnAb lineage. A hallmark of this cooperative evolution is that both antibodies interact at the same site on Env to drive their development.

Unlike CH103, the V_H domain of the CH235 lineage antibodies mimicked CD4 binding to gp120.[51, 52] Additionally, unlike CH103, CH235 had very few interactions with the V5 loop, but had significant interactions with the D loop[49]. To compensate for escape D-loop mutations, the antibody strengthened its binding to the C4 region, which is on the opposite face of gp120. Negative stain EM 3D reconstructions showed that CH235 members bound to a closed Env trimer with an approach angle similar to that of VRC01[53] and other CD4-mimicking bnAbs.

Studies from the CH505 donor suggest that an immunization strategy could use the CH505 T/F Env to induce naive B-cell responses. The presence of cooperating lineages suggests that a strategy for simultaneous induction of CDRH3 and VRC01-class CD4bs bnAbs as elicited here, could be to follow up by sequential immunizations using D loop mutant Envs.

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)
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AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

CH505 Immunization Studies

Williams et al.[54] studied the development of HIV-1 targeted antibodies induced by vaccination using four CH505 Envs as gp120s: the T/F Env and Envs from weeks 53, 78, and 100. Sequential immunizations of rhesus macaques (RMs) with the four gp120s in their natural order produced the DH522 antibody clonal lineage. This lineage was the most similar to that of CH103, but only had autologous tier-1 (neutralization-sensitive), heterologous tier-1 and weak heterologous tier-2 neutralization activities.[42] A crystal structure of the DH522.2 Fab in complex with a gp120 core, together with a superposition on the BG505 Env SOSIP.664 (gp140) trimer revealed that the antibody bound to the CD4bs in a way that the antibody would clash with a nearby gp120 subunit.[54] A negative stain EM 3D reconstruction showed that the antibody bound to an open conformation of the Env trimer. The inability to bind the closed trimer explains the limited neutralization breadth of these clonal lineage members, and suggests that future immunizations should use closed trimers, such as disulfide stabilized SOSIPs.[29, 55]

In another CH505 immunization study Saunders et al.[56] vaccinated rabbits with CH505 T/F chimeric SOSIP trimers containing a BG505 gp41 region to help stabilize the closed Env conformation.[18] Additional Env mutations were included to eliminate exposure of V3 loop epitopes and co-receptor binding sites.[57] Overall results were better when using stabilized CH505 Envs versus non-stabilized ones.[56] Rabbits receiving these stabilized Envs produced autologous tier-2 neutralizing plasma. Deletion of CD4bs shielding glycans from immunogens resulted in more CD4bs antibodies, including ones that could neutralize heterologous tier-2 viruses. While the antibody epitope(s) were not determined, these studies show the importance of Env stabilization in the induction of tier-2 neutralizing antibodies. Moreover, since removing glycans from certain sites on Env could induce larger antibody responses[58], immunization strategies could use enlarged “glycan hole” Envs as primes, and then boost with sequential Envs that have smaller “glycan holes” to concentrate the antibody response towards a more desirable site. Some germline-targeted trimer-based vaccine approaches have used this approach. For example, others have focused on creating glycan holes and changing variable loop lengths and residues around a particular epitope on the BG505 SOSIP trimer that was modified to target the germline of

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)
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the PGT121 glycan supersite bnAb. Knock-in mice immunized by this trimer and boosted with successive trimers in which the native surface of Env was gradually restored resulted in cross-reactive antibodies [59,60]. These results suggest that similar results could be achieved with suitable trimers based on lineage studies, such as from CH505.

Four CH505 Envs from similar time-points were also used in rhesus macaque sequential immunization studies conducted by Saunders et al.[56] In this case, cleavage deficient forms of the gp140 trimer were used, since these were previously shown to be immunogenic, and induced low levels of antibodies to certain tier-2 HIV-1 strains in guinea pigs[61] and in macaques[62, 63]. One macaque produced tier-2 HIV-1 neutralizing plasma, but against the V1V2 region, even though CD4bs-targeting immunogens were used. These results demonstrated that V1V2-directed antibodies are more readily induced than those against the CD4bs. This is consistent with the fact that CH235.12 had a favorable orientation in binding the CD4bs, but still needed high degrees of SHM (>20%) to develop breadth[49, 50]. Studies done in knock-in mice suggest receptor editing and different antibody gene usage in macaques versus humans could also inhibit CD4bs bnAbs development.[54] Thus, it may be easier to induce bnAbs against other epitopes.

CH848: V3-glycan bnAb development

The DH270 Broadly Neutralizing Antibody Lineage

V3-glycan bnAbs penetrate the Env glycan “shield” and contact both carbohydrate and protein components at the V3 loop base, which includes the highly conserved GDIR motif[64]. These bnAbs require the glycan at N332 for binding and neutralization, and some also require other glycans, such as the one at N301. Thus, members of this class of bnAbs have different Env footprints[65-68]. For example, in contrast to other V3-glycan bnAbs, DH270-lineage bnAbs have shorter CDRs but are still able to penetrate the glycan shield at a different approach angle, as demonstrated by a negative stain EM 3D reconstruction[37]. This binding diversity, together with the limited SHM required for bnAb development against this site, suggests that this class of bnAbs may be easier to elicit by a vaccine.

To better understand V3-glycan bnAb development, the co-evolution of the DH270 bnAb lineage and the clade C CH848 HIV-1 Env isolated from an African patient, CH848,

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

was analyzed over a 5-year period.[36, 69] These studies made use of a synthetic Man_9-V3 glycopeptide, which was designed based on the epitope of the V3-glycan bnAb PGT128[67] and includes both N301 and N332 glycans. It was demonstrated that Man_9-V3 structurally mimics the V3 region of an intact trimer[69] and binds to antibodies with a similar affinity as gp120 or trimers[69], suggesting its promise for studying antibody-Env interactions, and its use as an immunogen. A similar peptide was used to isolate the DH270.6 bnAb and DH475 cooperating antibody (discussed later) from this patient[37].

In contrast to the CH103 lineage, the DH270 UCA did not bind to the autologous T/F virus Env.[37] Heterologous breadth and potency of DH270 lineage antibodies increased with the accumulation of VH mutations, with improbable mutations playing a critical role in affinity maturation.[31,59] Improbable mutations are rare events that occur at cold spot microsequences[71, 72] and once they occur, they are likely to remain fixed in a lineage. Crystal structures of DH270.6 and DH270.3, which is less potent/broad, in complex with Man_9-V3 and the Man_9 glycan, respectively, revealed that two low-probability mutations were needed for specific anchoring at two complementary positions on Env and therefore breadth.[65] The $G57R_H$ mutation, which occurred early, allowed intermediate IA4 (or a precursor) to latch onto the V3 loop backbone near the GDIR motif in a different manner from other V3-glycan bnAbs[35, 66, 67]; the later $R98T_H$ mutation in the DH270.6 branch, and absent in the DH270.3 branch, relieved the conserved $D115_H$ residue from a salt bridge within the antibody, so it could bind a high mannose N332 glycan moiety, similar to what was observed in other V3-glycan bnAbs[35, 67]. Thus, repeated immunization with modified Envs to select for the improbable mutations, such as $G57R_H$ and $R98T_H$, may be essential for bnAb induction.

A challenge in initiating a similar lineage as DH270 by vaccination is the fact that its UCA did not bind any sequenced Env trimers or free glycans. The DH270 UCA did, on the other hand, bind to the Man_9-V3 glycopeptide and its aglycone form (preferential binding to the glycopeptide form increased throughout affinity maturation).[37] One explanation for this is that some altered form of CH848 T/F Env (such as a shed gp120 or fragment of it) exposed the V3 loop that stimulated the germline BCR to produce this lineage. This

suggests that a possible immunization regimen should start by priming the bnAb UCA with the synthetic Man_9-V3 glycopeptide, before boosting with an Env trimer.

The DH272 and DH475 Cooperating Lineages

DH270 bnAb lineage development was aided by two autologous neutralizing cooperating lineages, DH272 and DH475, which also bind the V3-glycan region in an N332 glycan-dependent manner.[37] DH272 and DH475 targeted viruses in the first year and selected viral escape mutants that would become sensitive to DH270 bnAb lineage members. After 51 weeks, a V1 loop deletion (34 to ~16 V1 loop residues) resulted in a switch in neutralization sensitivity from the DH272 and DH475 lineages to the DH270 lineage. Due to the proximity of the V1 and V3 loops (**Fig. 1**)[73], the V1 loop deletion could either alter the conformation of V3, or minimize steric interference that longer V1 loops present. There was a restoration of longer V1 loops later in infection and later DH270 antibodies adapted to viruses with longer V1 loops, similarly to other V3-glycan bnAbs, allowing recognition of a broader spectrum of Envs and enhancing breadth.[37]

Exactly how DH475 and DH272 bind to Env to cause the escape mutations in V1 has yet to be determined, which would guide immunogen design to engage similar responses. The action of cooperating lineages for bnAb development suggests that immunogen strategies will require multiple Envs and boosting regimens. These results specifically suggest that a vaccine regimen should include boosts with Env trimers with progressive V1 lengths.

CH848-Related Immunization Studies

Alam et al.[70] and others (unpublished data) are exploring the use of the Man_9-V3 glycopeptide as an immunogen since it was found to interact with the DH270 UCA of CH848. Not only would such a peptide mimic the bnAb epitope and bind a germline antibody, but it will also minimally present dominant strain-specific epitopes[74], suggesting promise as an immunogen. Rhesus macaques were immunized with the Man_9-V3 glycopeptide to test whether antibodies against the V3-glycan epitope could be induced.[70] Three antibodies were isolated that could selectively bind Man_9-V3 and not its aglycone version. Two of these could also bind to CH848 T/F and consensus C gp120s, were sensitive

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)
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AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

to the presence of the N332 glycan, and relied on high mannose glycans for binding. These data showed that Man_9-V3 immunization could induce antibody responses against the V3-glycan bnAb region.

While the monomeric Man_9-V3 glycopeptide is not a potent immunogen, its potency could be increased by multimerization of Man_9-V3, such as mounting it on a nanoparticle as was done for the engineered outer domain targeting B-cell precursors of the VRC01 bnAb[75] and for the short chain oligosaccharides for producing anti-glycan antibodies against Streptococcal challenges. [76] Such multimerization would increase multivalency to more effectively increase activation of the necessary B cell pools.

It still remains to be determined whether an immunization strategy that primes with the Man_9-V3 glycopeptide and boosts with trimers would be effective at producing bnAbs. A recent study with fusion peptide immunizations followed by boosts with the BG505 SOSIP trimer have yielded cross-clade neutralizing responses[77], suggesting that a similar approach with a V3 glycopeptide may yield promising results as well. The antibodies isolated from rhesus macaque immunizations with Man_9-V3 have not been structurally determined in complex with the glycopeptide or Env, thus it is unknown yet whether other considerations are needed when using Man_9-V3. Additional structural information would help guide such immunization studies.

Other Considerations

While the variability of the antibody repertoire among individuals could pose a challenge for B-cell immunogen design, for some viral-neutralizing epitopes the relevant immunoglobulin repertoire consists of a small number of VH families, suggesting that different individuals may produce similar maturation pathways.[78, 79] There have also been examples of convergent evol-ution of human antibodies, in which antibodies with similar gene usage can be isolated from different subjects[80-83], or of antibodies that could arise with similar CDR structures using different VH families[84-88], lending support for longitudinal studies in HIV vaccine development.

Moreover, much of the emphasis in the field has been on elucidating bnAb-Env interactions[89] and determining changes in antibody structure during affinity maturation.[33-

AIDS Research and Human Retroviruses
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[35, 37, 48] Affinity enhancing mutations in antibodies or virus escape mutations could alter thermostability or dynamics[90-94], suggesting these features could also play a role in virus-antibody co-evolution and should be considered in immunogen design strategies. Moreover, there is also a lack of Env structures throughout virus evolution and glycosylation patterns of T/F Envs may be different from the more difficult to neutralize tier-2 and tier-3 Envs.[42, 95, 96] Thus, structures of autologous virus Envs throughout their evolution should be determined.

There is also evidence that truncating the Env gp41 tail, as is done for SOSIP trimers, can alter the antigenicity of the trimer[97], suggesting that use of a full length Env may be necessary for structural studies and immunization trials. Together with the use of nanodiscs[98-100], which will provide a stabilizing environment for the transmembrane region of Env, advances in cryo-electron microscopy[101-104] and single molecule fluorescence microscopy[105] are likely to play important roles in structurally characterizing Envs and providing insights for immunization trials.

Conclusions

A goal of HIV-1 immunogen design is to combine other bnAb specificities to achieve a broad coverage of T/F viruses. Additional co-evolution studies, as the ones described here, will help provide greater insights to achieve this goal. To account for both B-cell ontogeny and co-evolution, it will be important to consider both the conformation of Env and the "rate-determining" steps to induce bnAb development via prime-boost regimens. Of note, it may not be ideal to study bnAb development in rhesus macaques using human derived data, since rhesus macaques do not use the same antibody genes and since immunization studies have not yet resulted in bnAbs [62, 106, 107]. Exploring virus-antibody co-evolution and performing immunization trials in the same model would help circumvent this problem. An example of this would be to study infection in SHIVs[108], and follow up with immunization trials in rhesus macaques to determine if vaccination could result in similar antibodies as those produced in SHIV infections.

While Env trimers may better mimic the trimer on an intact virion than a monomeric gp120 and mask non-neutralizing epitopes, such trimers may not bind to the

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

UCA of a bnAb lineage as was demonstrated by the DH270 UCA. Synthetic peptides that interact with the progenitor antibodies and mimic the natural epitope, like the Man_9-V3 glycopeptide, could be used as a prime in such cases. Furthermore, such a synthetic immunogen would be helpful in displaying an epitope that might otherwise be a heterogeneous in the context of a trimer. Chemical synthesis can overcome this hurdle by generating glycopeptides with homogeneous glycosylation patterns[109]. Moreover, such a peptide could present the bnAb epitope without interference from other surface features—e.g., the nearby gp120 V1V2 loop, which can interfere with V3 loop recognition. The absence of distracting epitopes in Man_9-V3 suggests that minimal antigens of this kind might be suitable candidate immunogens in regimens involving long-term antigenic exposure. To enhance viral clearance, it might be useful to use Man_9-V3 in conjunction with a monomer containing a T-cell helper epitope, such as a glycoconjugate of the non-natural pan DR epitope[110]. Moreover, to improve immunogenicity, the peptide could be conjugated to a ligand such as the Toll-like receptor 2 lipopeptide as was described elsewhere for a V3-glycopeptide-based immunization study.[111] Trimers may be used in succession, especially if cooperating lineages recognizing other Env regions are necessary for bnAb development. Trimers used in germline-targeted immunization approaches have shown promise for inducing cross-reactive antibodies[59,60] and thus should be considered in lineage-based immunogen design efforts as well.

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AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

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AIDS Research and Human Retroviruses
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AIDS Research and Human Retroviruses
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AIDS Research and Human Retroviruses
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AIDS Research and Human Retroviruses
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Figure Legends

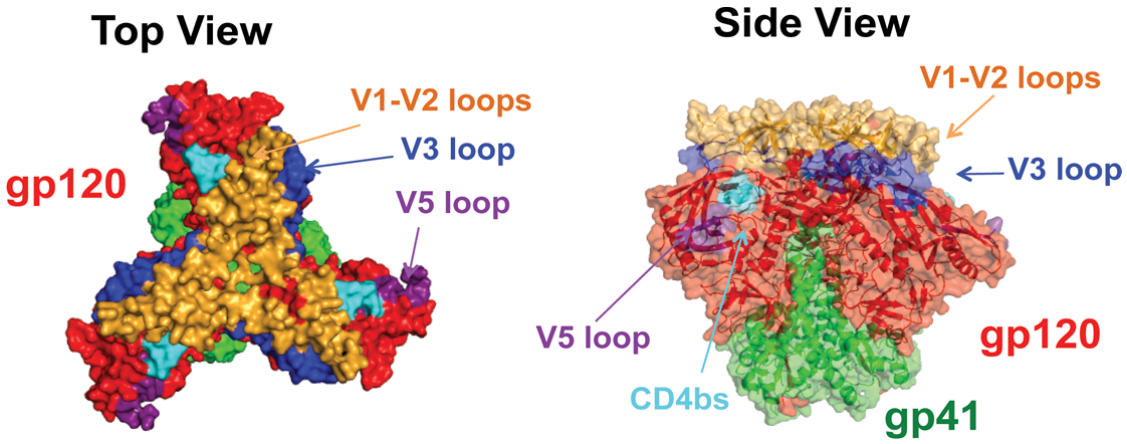

Figure 1. HIV Env Structure. The receptor-binding gp120 (red) and the transmembrane bound gp41 (green), which are targets for vaccine design, are shown as a surface diagram. Variable loops (V1V2, V3, and V5) and the CD4bs are colored as indicated in the figure.

AIDS Research and Human Retroviruses
Structural Insights from HIV-Antibody Co-Evolution and Related Immunization Studies (DOI: 10.1089/AID.2018.0097)

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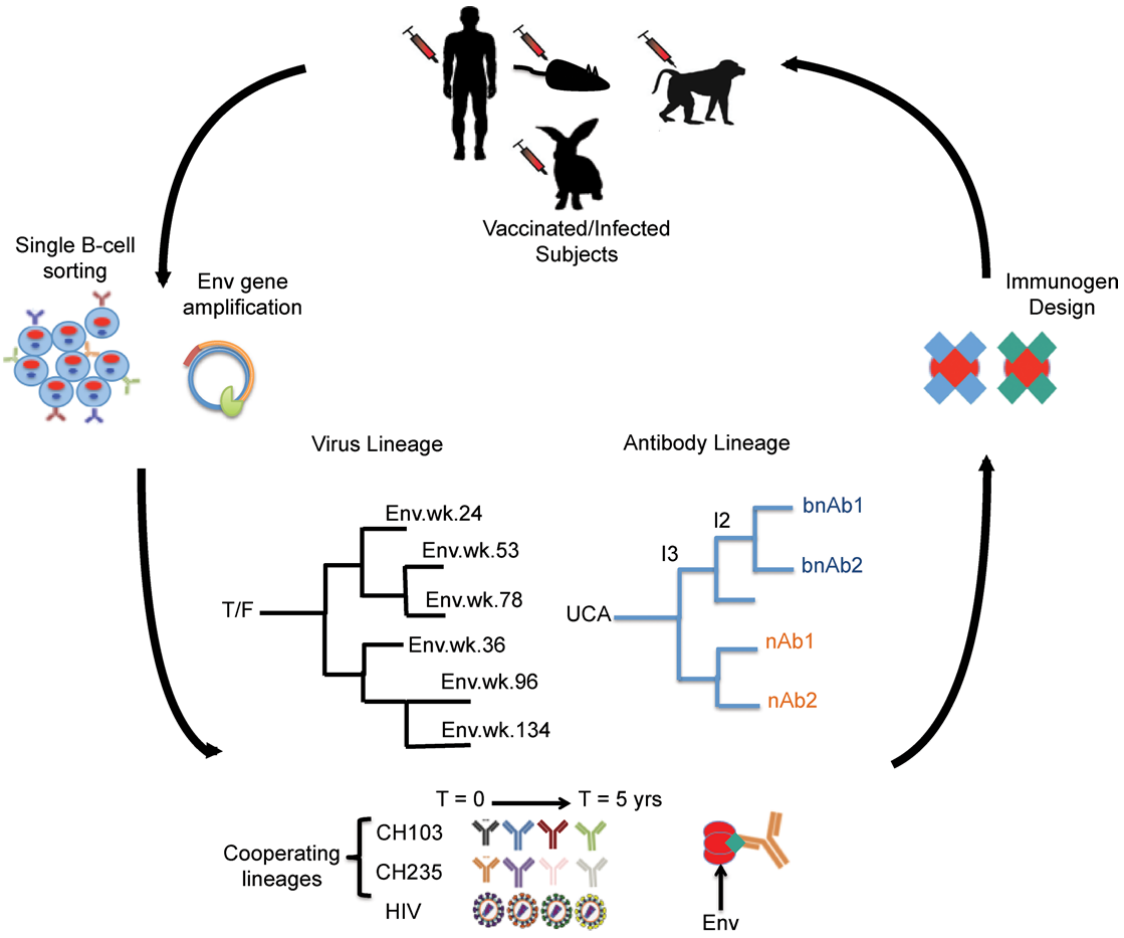

Figure 2. Scheme of workflow for analyzing HIV-antibody arms races for immunogen design purposes. HIV-1 infected donors are recruited and longitudinal sequences of HIV and antibodies are determined using single B-cell sorting and Env gene amplification and sequencing. Phylogenetic trees are constructed from available sequence data and computational approaches, and then structural analysis is done to determine key structural changes that might identify rate-limiting steps, important for immunogen design. Immunogens are then introduced into vaccinated or infected individuals and the process is repeated.