Stabilizing HIV-1 envelope glycoprotein trimers to induce neutralizing antibodies

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Abstract

An effective HIV-1 vaccine probably will need to be able to induce broadly neutralizing HIV-1 antibodies (bNAbs) in order to be efficacious. The many bNAbs that have been isolated from HIV-1 infected patients illustrate that the human immune system is able to elicit this type of antibodies. The elucidation of the structure of the HIV-1 envelope glycoprotein (Env) trimer has further fueled the search for Env immunogens that induce bNAbs, but while native Env trimer mimetics are often capable of inducing strain-specific neutralizing antibodies (NAbs) against the parental virus, they have not yet induced potent bNAb responses. To improve the performance of Env trimer immunogens, researchers have studied the immune responses that Env trimers have induced in animals; they have evaluated how to best use Env trimers in various immunization regimens; and they have engineered increasingly stabilized Env trimer variants. Here, we review the different approaches that have been used to increase the stability of HIV-1 Env trimer immunogens with the aim of improving the induction of NAbs. In particular, we draw parallels between the various approaches to stabilize Env trimers and ones that have been used by nature in extremophile microorganisms in order to survive in extreme environmental conditions.

Background

The development of an effective and safe vaccine against HIV-1 requires a detailed understanding of the virological and immunological characteristics of HIV-1 infection. The virus has the ability to mutate very quickly, resulting in great viral diversity and making the development of an effective vaccine very challenging. Therefore, many research groups in the HIV-1 vaccine field pursue the development of a vaccine that can induce broadly neutralizing antibodies (bNAbs), i.e. antibodies that can target the functional envelope glycoprotein (Env) on many different virus isolates.

A focus of vaccine design is the generation of soluble Env trimer mimetics that can induce such antibodies and much progress has been made over the last few years in generating recombinant Env trimers that resemble the native Env spike. This required negating the inherent instability and flexibility of the native Env trimer and was accomplished by molecular design, resulting in soluble stable Env trimers, of which SOSIP.664 trimers were the prototype [1–4]. The clade A BG505 SOSIP.664 trimer, now the gold standard in HIV native-like trimer immunogen design, allowed the determination of the high-resolution structure of the Env trimer [5–7]. A recent structure of the membrane-derived JR-FL trimer confirmed that the soluble and stabilized BG505 trimer resembled the native Env trimer present on the viral membrane [8]. Moreover, the SOSIP.664 design could be extrapolated to HIV-1 isolates other than BG505, thereby expanding the toolkit for HIV-1 vaccine design [9–14]. When used as immunogens in animal trials, SOSIP.664 proteins from various strains elicited autologous (strain-specific) Tier-2 neutralizing antibodies (NAbs); however, these immunogens failed to elicit potent bNAbs in most animals [15–18].

Here, we describe several approaches that have been pursued in order to increase the performance of soluble Env trimer mimetics as immunogens to induce NAbs. First, we review different methods that have been used to improve the stability of HIV-1 Env trimers, including forced viral evolution, structure-based design, high throughput screening of mutant trimers and selection...
of improved trimers by mammalian cell display. We also review which epitopes on Env trimer mimetics are targeted by the immune system, and we assess different immunization strategies in which Env trimer immunogens can be employed, including cocktail and sequential vaccination regimens.

Generating and validating mimetics of the native Env spike
The native Env trimer is unstable and flexible (conformationally heterogeneous), and the same applies to early generation soluble Env trimer derivatives. As a consequence it took many years to elucidate its high-resolution structure by X-ray crystallography and cryo-electron microscopy (EM) techniques [19–21]. Initial low resolution cryo-electron tomography reconstructions of membrane-bound and soluble trimers provided new insights [22, 23], but high-resolution structures of the trimer were solved by using BG505 SOSIP.664 and the wide assortment of potent bNAbs that became available over the last decade [5, 24, 25]. Large gains in resolution were obtained with the first Env trimer crystal structure (4.7 Å resolution), which included a complex of the BG505 SOSIP.664 trimer with the V3-glycan bNAb PGT122 [20], and the first cryo-EM derived model of the same trimer in complex with the CD4 binding site bNAb PGV04 at a resolution of 5.8 Å [19]. In addition to providing lattice contacts to facilitate crystallization and 3D features to facilitate EM reconstruction, these bNAbs also provided validation of the structures, as the respective bNAb epitopes were clearly present.

The next step was to improve the resolution of the trimer structure by complexing the trimer with a combination of several new bNAbs. The use of the 35O22 bNAb directed to the gp120-gp41 interface and antibodies of the PGT121-family increased the resolution to ~3.5 Å and then 3.0 Å, and provided new details of the prefusion conformation of gp41, especially in HR1, a partially disordered region [6, 7, 26]. The SOSIP platform has been applied to trimers from different HIV-1 clades and their structures in complex with diverse bNAbs have also been elucidated, providing valuable new information for structure-based vaccine design [12, 21, 27–30]. Overall, the structures of all SOSIP trimers showed a highly similar trimer core, but revealed some differences in the variable loops that emanate from the core [21].

Another breakthrough came with the elucidation of the cryo-EM structure of a membrane-derived JR-FL trimer that was stabilized by the bNAb PGT151, but not by SOSIP mutations [8]. The overall structural features of the membrane-derived trimer as well as bNAb epitopes agree well to those of the soluble SOSIP trimers. However, subtle differences were observed in the HR1 region of gp41, where the I559P substitution in the soluble trimer breaks a helix that is present in the full-length Env structure, exactly as it was meant to do [1, 8]. The high similarity of the membrane-derived and the soluble version of the Env confirm the value of the SOSIP design for generating soluble Env spike mimetics. A modification of the SOSIP design involves the introduction of a flexible Gly-Ser linker between gp120 and gp41 to replace the furin cleavage site, sometimes with additional modifications, effectively resulting in single chains trimers that do not require furin cleavage [31–33].

Designing next-generation Env trimers: learning from HIV-1 itself
A strategy to stabilize the Env trimers is by understanding and exploiting stability on the virus. To protect Env from NAbs, the virus evolves in a Darwinian way by selecting mutations in Env, in particular its variable loops, and by masking the protein surface with a shifting glycan shield. Virus evolution can also be exploited in the lab to obtain valuable information about mutations that can stabilize the Env trimer while retaining its functionality [34–37]. Such mutations can then be used to stabilize recombinant Env vaccine candidates.

By culturing HIV-1 virus under harsh conditions such as unphysiological temperatures (45–55 °C) or incremental concentrations of denaturant (GuHCl), Leaman and colleagues identified a more stable Env mutant that contained seven mutations compared to its wild-type counterpart. Most of the mutations were located in the gp120-gp41 interface, including positions 535 and 543 (Fig. 1, Table 1) [34]. These substitutions were also identified by an earlier study in which the sequence of the early generation but relatively stable KNH1144 SOSIP protein was compared to that of the unstable JR-FL SOSIP [38]. De Taeye et al. introduced, when not present, the 535M and 543N mutations into distinct clade B (AMC008 and B41) and clade C trimers (ZM197M) in order to increase their trimerization and stability [10].

Other substitutions that can improve native-like trimers were selected based on studies on how the virus becomes dependent on the entry inhibitor VIR165, and how HIV-1 can adapt to cold [39, 40]. These substitutions are located in C1 domain of gp120 (E64K, H66R and H66A; Fig. 1, Table 1) and likely keep the virus in the prefusion conformation by impeding steps towards the CD4-bound conformation by interacting with the HR1 region in gp41 [10, 41]. Thus, mutations that increase the stability of the native Env spike on virions can also be useful for the development of stable soluble native-like Env immunogens.
Designing next-generation Env trimers: learning from extremophile organisms

SOSIP trimers based on most virus isolates other than BG505 initially did not form stable native-like trimers efficiently. However, the available trimer structures provided sufficient structural details to design modifications that improve the structure and stability of Env trimers, and that allowed generating stable trimers from many different isolates and clades.

When considering how to stabilize vaccine antigens, much can be learnt from nature. Extremophile bacteria and archea, which thrive in extreme environmental conditions such as high and low temperatures (between 45–122 °C and below −15 °C, respectively) or alkaline and acidic conditions (pH > 11 and pH < 1, respectively) [42–44], have evolved highly stable proteins compared to their mesophilic homologues [43, 45]. In extremophile organisms, natural evolution has applied six methods of protein stabilization. Several of these methods have been applied, either intentionally or not, to HIV-1 Env trimer vaccine design.

First, thermophilic proteins often have an increased number of hydrophobic residues at domain and oligomer interfaces, facilitating tighter packing of protein domains [46, 47]. A similar strategy was applied to HIV-1 Env trimers to stabilize the trimer and prevent the exposure of non-NAbs [48–53]. For example, de Taeye et al. avoided the spontaneous exposure of the V3 loop by increasing the hydrophobic interactions within the V3 domain and between the V3 and V1V2 domains, by

Fig. 1 Amino acid substitutions that help stabilize soluble native-like trimers. Crystal structure of the BG505 SOSIP664 trimer (5CEZ, [7]) displaying amino acid substitutions that stabilize native-like soluble trimers (see text for details). Two protomers are colored in white and one protomer is colored according to different regions. In gp120, V1V2 in cyan, V3 in magenta, inner domain layer 1 in blue, layer 2 in yellow, layer 3 in orange, N- and C-termini in green. Gp41 is colored in red. Boxes show detailed views of regions of the Env trimer that contain stabilizing amino acid substitutions. The substitutions were modeled by using the mutagenesis tool in Pymol molecular graphics system version 2.0.6 [102].
## Table 1  Amino acid substitutions that stabilize soluble native-like trimers

|       | Hydrophobic | Aromatic | Proline/glycine | Disulfide bonds | Charged | Other |
|-------|-------------|----------|----------------|-----------------|---------|-------|
| C1    |             |          |                | E49C-L555Ca     | E47D<sup>b</sup> | E106T<sup>b</sup> |
|       |             |          |                | H72C-H564Ca     | K49E<sup>b</sup> |       |
|       |             |          |                | A73C-A561Ca     | E64K<sup>c</sup> |       |
|       |             |          |                |                 | V65K<sup>o</sup> |       |
|       |             |          |                |                 | H66R<sup>c</sup> |       |
|       |             |          |                |                 | T106E<sup>d</sup> |       |
| V1    | L154M<sup>e</sup> |          | Y177W<sup>e</sup> |                 |         |       |
| V2    | I165L<sup>k</sup> | I172V<sup>k</sup> |                | I201C-A433C<sup>mn</sup> |         |       |
|       |             |          |                |                 | Q171K<sup>f</sup> |       |
|       |             |          |                |                 | I192R<sup>f</sup> |       |
| C2    | A204F<sup>p</sup> | I203F<sup>h</sup> |                |                 |         |       |
|       | M271I<sup>d</sup> | F223W<sup>d</sup> |                |                 |         |       |
|       | T290A<sup>d</sup> |          |                |                 |         |       |
| V3    | N300M<sup>m</sup> | N302Y<sup>f</sup> | A316W<sup>e</sup> |                | H308R<sup>f</sup> |       |
|       | N302M<sup>m</sup> | A319Y<sup>e</sup> |                |                 |         |       |
|       | R304Y<sup>d</sup> |          |                |                 |         |       |
|       | S306L<sup>i</sup> |          |                |                 |         |       |
|       | R308L<sup>i</sup> |          |                |                 |         |       |
|       | T320L<sup>i</sup> |          |                |                 |         |       |
| C3    |             |          |                | I201C-A433C<sup>mn</sup> | E429R<sup>b</sup> | N363Q<sup>d</sup> |
| C4    | Y420M<sup>e</sup> |          |                | A501C-T605C<sup>g</sup> | A500R<sup>b</sup> | R432Q<sup>b</sup> |
| C5    |             |          |                | F519R<sup>j</sup> | FS19R<sup>i</sup> | FS165<sup>d</sup> |
| FP    |             |          |                | L520R<sup>j</sup> | L520R<sup>j</sup> |       |
| HR1<sup>a</sup> | DS89V<sup>g</sup> | T538F<sup>h</sup> | L556P<sup>p</sup> | E49C-L555Ca | L568D<sup>g</sup> | IS35N/M<sup>g</sup><sup>a</sup> |
|       |             | I548F<sup>b</sup> | I556G/P<sup>g</sup> | H72C-H564Ca | K388E<sup>e</sup> | LS43N<sup>g</sup><sup>c</sup> |
|       | I573F<sup>b</sup> | A561P<sup>d</sup> | L568G<sup>e</sup> | A73C-A561Ca | G588R<sup>p</sup> | N553S<sup>g</sup> |
|       |             |          | T569G<sup>p</sup> |                 | V570H<sup>f</sup> | R385H<sup>f</sup> |
|       |             |          |                 |                 | K388Q<sup>f</sup> |       |
| DSL<sup>i</sup> | K655F<sup>d</sup> | E647F<sup>p</sup> | M629P<sup>h</sup> | A501C-T605C<sup>g</sup> |         |       |
| HR2<sup>a</sup> | K658F<sup>d</sup> | N651F<sup>q</sup> | S636G<sup>i</sup> |         |         |       |
|       | E662A<sup>b</sup> |          |                 |                 |         |       |

<sup>a</sup> Torrents de la Pena et al. Cell Rep. 2017  
<sup>b</sup> Guenaga et al. J Virol. 2015  
<sup>c</sup> de Taeye et al. Cell. 2015  
<sup>d</sup> Steichen et al., Immunity. 2016  
<sup*e</sup> Chuang et al. J Virol. 2017  
<sup>f</sup> Ringe et al. J Virol. 2017  
<sup>g</sup> Rutten et al. Cell Rep. 2018  
<sup>h</sup> Sullivan et al. J Virol. 2017  
<sup>i</sup> de Taeye et al. J Biol Chem. 2017  
<sup>j</sup> Guenaga et al. Immunity. 2017  
<sup>k</sup> Binley et al. J Virol. 2000  
<sup>l</sup> Sanders et al. J Virol. 2002  
<sup>m</sup> Guenaga et al. Plos Path. 2015  
<sup>n</sup> Do Kwon et al., Nat. Struct. Mol. Biol. 2015  
<sup&o</sup> Dey et al., Virol. 2007  
<sup>p</sup> Fusion peptide  
<sup>q</sup> Heptad repeat 1  
<sup>r</sup> Disulfide loop  
<sup>s</sup> Heptad repeat 2
introducing two Leu residues (S306L, R308L) in the V3 loop (Fig. 1, Table 1) [53]. Similarly, Chuang et al., Kulp et al., Steichen et al. and Rutten et al., introduced hydrophobic mutations in the trimer core (A204I, T320L, E381M, Q422L) or the trimer stem (D589V, K655I, K658V, E662A) using structure-based design and mammalian cell display, which resulted in increased Env packing and reduced flexibility (Fig. 1, Table 1) [49–51, 54].

Second, extramembrane proteins contain a higher number of aromatic amino acids, which can enhance protein thermostability through ring stacking interactions as well as hydrophobic packing [55–57]. In structure-based HIV-1 immunogen design, several groups used the same principle and introduced aromatic residues to reduce V3 exposure (A316W, A319Y), and to increase stability of the trimer apex (Y177W, N302Y, N302F), the trimer base (E647F, N651F) and the trimer interface (gp120-gp41 interface: A223W, T538F and I548F; gp41-gp41 interface: I573F) (Fig. 1, Table 1) [10, 48–51, 54]. Overall, the introduction of hydrophobic and aromatic residues accounts for ~ 45% of the total number of mutations that are described in the literature to increase Env trimer stability.

Third, proteins from thermophilic organisms tend to have an increased number of charged residues involved in internal ion pairing and hydrogen bonding, as well as an increased number of positively charged residues at the solvent-exposed surface to provide stability at high temperatures [57]. For HIV-1 Env trimers the introduction of charged amino acids at the gp120 and gp41 interface also contributed to formation of well-ordered native-like trimers from different clades with enhanced thermostability (A500R, A558R) (Fig. 1, Table 1) [13, 58].

Fourth, proteins from thermophilic organisms usually contain many more predicted disulfide bonds than mesophilic organisms, which increases protein stability dramatically [45, 59, 60]. In mesophiles, proteins with many disulfide bonds are rare. As a consequence, there is a strong positive correlation between the number of disulfide bonds in proteins and the maximum growth temperature of thermophilic organisms [45, 59, 60]. Some viruses, such as influenza and vaccinia viruses, contain a disulfide bond that links the two Env subunits together, but HIV-1 Env naturally does not have such a disulfide bond, resulting in shedding of the gp120 subunit. The first step of generating stable native-like trimers was therefore the introduction of a disulfide bond between the gp120 and gp41 subunits (A501C-T605C) (Fig. 1, Table 1) [2]. To stabilize the flexible trimer interface, additional disulfide bonds have been introduced in the Env trimer: an intersubunit disulfide bond (A73C-A561C) and an interprotomer disulfide bond (E49C-L555C) (Fig. 1, Table 1) [7, 61]. Furthermore, an intrasubunit disulfide bond (I201C-A433C) described by Kwon et al. and Guenaga et al. also stabilized the trimer in its pre-fusion state (Fig. 1, Table 1) [62, 63]. Combining three non-native disulfide bonds (A501C-T605C + A73C-A561C + I201C-A433C or A501C-T605C + A73C-A561C + E49C-L555C) resulted in hyperstable trimers that reached melting temperatures of up to 81 °C and 92 °C, respectively [61].

Fifth, thermostable organisms increase the number of proline and glycine residues in loops to provide conformational rigidity to the protein [43]. In the HIV field, similar approaches have been used to generate soluble Env trimers. Since the HR1 region forms a helix in the post-fusion state and it adopts a partially disordered conformation in the pre-fusion state, we introduced the I559P mutation in the loop of HR1 to destabilize the post-fusion state of gp41 and stabilize the pre-fusion state [1]. Similarly, the introduction of glycine or proline residues in the HR1 and HR2 (N554G, L556P, A558P, I559G, T569I, T569P, T569G and S563G) further stabilized soluble HIV-1 Env trimers (Fig. 1, Table 1) [1, 54, 58]. Kong et al. computationally modeled HR1 loops with low Gibbs free energy that resulted in increased numbers of proline residues and rigidification of the HR1 loop [64].

A last mechanism that thermophilic organisms apply to survive at high temperatures is the reduction of asparagine and glutamine residues to prevent deamidation. This strategy has not been (intentionally) used for HIV vaccine design yet.

Thus, strategies to stabilize Env trimers from BG505 and other isolates using high throughput screening, selection by mammalian display, and structure-based design, in many ways mirror what extremophiles have achieved in nature to survive under extreme conditions. The resulting improvements in stability of soluble Env trimers allow us to use these immunogens in immunogenicity studies by facilitating the generation of a toolkit of trimers from different clades. Several of these trimers have been evaluated as immunogens and some studies have suggested that in some cases increased thermostability translates into increased immunogenicity [61, 65]. Furthermore, by increasing the trimer shelf life and avoiding cold chain transportation and storage will help to eventually produce a vaccine that is globally available.

**Evaluating Env trimers in vivo: learning from immunization experiments**

Native-like Env trimers have been tested as immunogens in small animals, mostly rabbits, and nonhuman primates. These studies indicated that native-like trimers consistently induced, for the first time, NAb responses against hard-to-neutralize (Tier 2) primary HIV-1
isolates. However, heterologous primary isolates were not, or only weakly and sporadically neutralized. Highly stable native-like trimers have been designed to improve the immunogenicity of the trimer by increasing its half-life in vivo and thus the presentation of bNAb epitopes. Immunogenicity studies with the highly stable trimers did not increase the generation of autologous NAb responses, but they induced weak heterologous Tier 2 responses in some cases. While trimer thermostability in vitro is a useful parameter that can be linked to in vivo observations [61, 65], it will also be important to investigate additional stability parameters such as trimer stability in serum at 37 °C.

Immunization with SOSIP trimers also induced strong non-neutralizing antibody (non-NAb) responses against V3 epitopes and neo-epitopes at the bottom of the trimer [10, 15, 16, 66]. Heterologous primary isolates were not, or only weakly and sporadically neutralized, pointing to possible directions of further research to improve native-like trimer immunogens.

First, it has recently been shown that the NAb responses in animals immunized with BG505 SOSIP trimers are dominated by specificities targeting a hole in the glycan shield, specifically the peptidic surface surrounding amino acids at positions 241 and 289, where most virus isolates have N-linked glycans [17, 67]. While autologous NAb responses might in some cases be a starting point for generating bNAb responses [7, 68], they could also distract or compete for such responses. If the latter scenario were true, one might want to dampen immunodominant isolate-specific, glycan-hole directed NAb responses. One strategy to counteract the immunogenicity of the BG505 specific glycan hole would be to immunize with trimers that contain glycans at positions N241 and N289. Previous studies have shown that immunizations with trimers based on isolates with a denser glycan shield (AMC008 and ZM197M) induced a broader heterologous NAb response compared to trimers from isolates with large holes in the glycan shield (BG505 and B41), which supports the pursuit of this strategy [69].

Second, immunization with BG505 SOSIP.664 trimers induced a strong response against non-NAb V3 epitopes [10, 50, 53, 70], leading to the hypothesis that this immunodominant V3-response interfered with the generation of bNAb responses. When rabbits were immunized with an improved version of the trimer, the BG505 SOSIP.v4 trimer, which contained the A316W mutation that sequestered the V3 epitope, these SOSIP trimers induced weaker anti-V3 responses and V3-directed Tier 1A virus NAb responses, without affecting the autologous NAb response [10, 16]. In a next iteration of trimer design, two additional hydrophobic residues were incorporated in the V3 loop of the BG505 SOSIP.v4 trimer (R306L and R308L) to completely abolish the responses against the V3 loop [53]. Although these modifications reduced V3 immunogenicity, they did not improve the autologous NAb responses, nor did they result in a broadening of the NAb response. Similar results were recently obtained by Kulp et al. using different V3 designs [16, 50].

Third, the generation of soluble Env trimers resulted in the exposure of neo-epitopes at the bottom of the trimer, which is occluded by the viral membrane when the Env trimer is presented on virions. It has been suggested that the bottom of the trimer presents another immunodominant non-NAb epitope that could interfere with NAb responses [66, 70]; M. J. van Gils, C. A. Cottrell, A. B. Ward, R. W. Sanders unpublished data). To prevent the exposure of this epitope one could hide it, for example by placing the trimer on a nanoparticle.

Although interference by V3 and trimer bottom non-NAb responses is an attractive hypothesis, there is no formal proof yet that these non-NAb responses interfere with more desirable NAb and bNAb responses. However, the V3 and trimer bottom non-NAb epitopes are usually solely of peptidic nature. B cells recognizing such epitopes are much more frequent in the naïve B cell repertoire and probably have higher affinity than naïve B cells recognizing composite peptide-glycan bNAb epitopes [70]. Higher affinity B cells might have a selective advantage over the lower affinity B cells targeting bNAb epitopes, because they might bind and process more antigen and, as a consequence, receive more T cell help. This will make it unlikely that B cells with the intrinsic capacity to mature into bNAb will thrive in an environment that favors B cells targeting non-NAb or strain-specific glycan hole NAb epitopes. However, these arguments are somewhat theoretical in the HIV-1 context and the immune responses raised against Env trimers in animal and human vaccination experiments should be dissected in more detail to address these concerns.

To improve our understanding of the fate of Env trimers in vivo a number of studies focused on the germinal center responses against Env trimers. Macaques were immunized with stable Env trimers and germinal center cells from the lymph nodes were collected over time using fine needle aspirates (FNA), thereby avoiding the need to take lymph node biopsies and thereby blunting the response in that lymph node [18, 70]. While all the macaques generated immune responses against the trimer, the NAb responses correlated quantitatively with GC B cell frequencies. These studies provide a frame-of-reference for further studies on germinal center B cells and Tfh cells and their roles in epitope immunodominance and subdominance. Furthermore, insights in the amount of Env that enters the lymph nodes and the half-life of the Env protein in circulation would help efforts to
study how the immunogen is delivered to B cells and how this can be improved. Previous work on other immunogens, including on gp120, suggest that it is worthwhile to exploit fluorescently tagged native-like trimers and to answer some of these questions, especially whether highly stable trimers show longer trimmer half life in the presence of serum and proteases [71–73].

**Evaluating Env trimers in vivo: learning from different immunization regimens**

Until now, monovalent immunization with soluble HIV-1 Env trimers has only induced strong NAb responses against autologous viruses, and only weak and sporadic heterologous Tier 2 NAb responses. One strategy to increase neutralization breadth involves exploring different vaccine regimens such as cocktails of different immunogens. HIV-1 is a highly diverse pathogen, as is influenza virus. For influenza virus we use annually updated vaccines composed of a trivalent or tetravalent cocktail of different inactivated influenza viruses. However, annual influenza vaccination only protects against viral variants that are closely related to the vaccine strains, which exemplifies how difficult it is to induce a bNAb response against highly diverse viruses. The search for a universal flu vaccine shares similarities with the search for a bNAb-inducing HIV-1 vaccine.

To increase neutralization breadth, we have explored the use of cocktail and sequential regimens [17, 69]. We observed that immunization with a combination of immunogens in a cocktail formulation or in sequence did not induce bNAb, but merely autologous NAb responses. Furthermore, the autologous NAb responses were prominent against the most immunodominant trimer of the cocktail [69]. Thus, the immune response shows narrow specificity, similar to what has been reported for influenza vaccines [74]. These results indicate that an HIV-1 Env vaccine based on a cocktail or sequence of randomly chosen trimers is unlikely to induce bNAb.

An alternative to the cocktail and sequential formulations could be to guide naïve B cell lineages towards bNAb activity by rational design. Since in natural infection the bNAbs develop through the co-evolution of the virus and the antibodies, one strategy that is being pursued is immunization with longitudinal Env sequences from patients that developed a bNAb response [75–80]. This strategy aims to recapitulate the evolutionary path of the virus and assumes that the development of the bNAb response largely depends on viral characteristics. Another, but somewhat related strategy, termed germline-targeting, focuses on the activation of rare subsets of naïve B cell that express B cell receptors (germline precursors) that have the intrinsic capacity to develop into bNAb. SOSIP trimers generally do not bind inferred germline versions of bNAb and several groups are designing immunogens that bind specifically to germline antibodies to guide the B cell responses towards the development of broadly neutralizing antibodies [51, 81–87].

Trimmers can also be used to boost responses that are primed by epitope-specific immunogens. For example, Xu et al. applied trimers in an immunization regimen aimed at focusing immune responses to the fusion peptide. They immunized guinea pigs and macaques with a fusion peptide coupled to the carrier protein KLH, and boosted the responses with stabilized BG505 SOSIP trimers. This immunization strategy induced autologous NAb responses in all the animals and substantial NAb responses against heterologous Tier-2 viruses in some animals [88]. When they isolated the antibodies that were responsible for the broad neutralization they could confirm that these antibodies targeted the fusion peptide on both autologous and heterologous viruses [88].

Another strategy to overcome the low affinity of the immunogens to the desired but rare germline precursors of bNAb is to multimerize the antigen, thereby increasing the potency of the Ab response by cross-linking the B cell receptors. The use of liposomes and ferritin nanoparticles that present Env trimers on their surface indeed improve the NAb response [89–91]. The flexibility of the nanoparticle system would allow the incorporation of trimers from different clades or lineages to enhance NAb responses against conserved B cell epitopes.

**Applying the lessons learnt to other viral pathogens**

We described how to make stable HIV-1 Env trimers for structural and immunological studies and how to use them in the quest for an HIV-1 vaccine. However, the lessons learnt in the HIV-1 field can also be applied to other viruses and *vice versa*. Similar to HIV-1 Env, other viral fusion proteins, such as the respiratory syncytial virus (RSV) F protein, are intrinsically metastable and easily switch from the pre-fusion to the post-fusion form. While a lot of efforts had to be invested to produce a stable soluble HIV-1 Env trimmer, the influenza HA protein is comparatively stable and can be easily expressed. In contrast, the RSV F protein is, similarly to HIV Env, quite unstable and it adopts the post-fusion conformation when purified as soluble protein. While McLellan and colleagues introduced a disulfide bond and hydrophobic residues to keep the RSV glycoprotein in the pre-fusion state [92], Krarup et al. prevented the transition of this protein to the post-fusion state by introducing helix-breaking prolines in the refolding region 1, quite similar to what has been done for HIV-1 Env [93].
Recently, high-resolution structures of other viral glycoproteins were solved, including those of human parainfluenza virus 5, ebola virus, lassa virus, human betacoronavirus HKS1, lymphocytic choriomeningitis virus, herpes simplex virus 1 and severe fever with thrombocytopenia syndrome virus [92, 94–100]. The above-mentioned strategies that worked for HIV-1 Env have also benefited the stabilization and native-like pre-fusion forms of several of these glycoproteins. To keep the Middle East respiratory syndrome coronavirus (MERS-CoV) glycoprotein in the pre-fusion state, Pallesen et al. introduced two prolines at the start of the central helix of the protein, similarly to the I559P substitution introduced in the HIV-1 Env trimer [1, 96]. Similarly, to retain the lassa virus glycoprotein in the pre-fusion conformation, Hastie and colleagues incorporated a proline in the HR1 domain [98]. To further improve the stability the authors introduced a disulfide bond between the two subunits and improved the cleavage site as previously done for the HIV-1 Env trimer. Thus, the general strategy is to retain the viral glycoprotein in the pre-fusion conformation by structure-based design [2, 92, 96].

To further improve the immunogenicity of Env trimers, we can also learn from the recombinant vaccines against viral pathogens that are currently available. Hepatitis B virus, hepatitis E virus and human papillomavirus use recombinant virus-like particles as the immunogen [101]. These vaccines are self-assembling nanoparticles that mimic the native virions and expose neutralizing epitopes on their surface. As previously discussed, improvement of the nanoparticle design in the HIV-1 vaccine field is being pursued by several groups including us. In short, the strategies used to improve HIV-1 immunogen design provide a template to design vaccine candidates for other viruses and vice versa.

**Conclusion**

Here, we reviewed the latest design strategies to stabilize the soluble HIV-1 Env trimers as well as different immunization strategies maximize their value. The development of native-like trimers as immunogens, the availability of high-resolution structures, the design of different immunization strategies, the promise of germline-targeting and nanoparticle presentation, combined with an increased understanding of the host immunological responses against Env trimers, should advance the field of HIV-1 trimer vaccination. These efforts should advance the HIV-1 field and provide lessons for subunit vaccines against other viruses for which diversity is an issue, such as, but not limited to, influenza virus, dengue virus and hepatitis C virus.
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