Efficiently cleaved HIV-1 envelopes: can they be important for vaccine immunogen development?

Supratik Das, Rajesh Kumar*, Shubbir Ahmed*, Hilal Ahmad Parray* and Sweety Samal*

Abstract: The enormous diversity of HIV-1 is a significant impediment in selecting envelopes (Envs) that can be suitable for designing vaccine immunogens. While tremendous progress has been made in developing soluble, trimeric, native-like Env proteins, those that have elicited neutralizing antibodies (Abs) in animal models are relatively few. A strategy of selecting naturally occurring Envs suitable for immunogen design by studying the correlation between efficient cleavage on the cell surface and their selective binding to broadly neutralizing Abs (bNAbs) and not to non-neutralizing Abs (non-NAbs), properties essential in immunogens, may be useful. Here we discuss some of the challenges of developing an efficacious HIV-1 vaccine and the work done in generating soluble immunogens. We also discuss the study of naturally occurring, membrane-bound, efficiently cleaved (naturally more sensitive to furin) Envs and how they may positively add to the repertoire of HIV-1 Envs that can be used for vaccine immunogen design. However, even with such Envs, the challenges of developing well-folded, native-like trimers as soluble proteins or using other immunogen strategies such as virus-like particles with desirable antigenic properties remain, and are formidable. In spite of the progress that has been made in the HIV-1 vaccine field, an immunogen that elicits neutralizing Abs with significant breadth and potency in vaccines has still not been developed. Efficiently cleaved Envs may increase the number of available Envs suitable for immunogen design and should be studied further.

Keywords: broadly neutralizing antibodies (bNAbs), efficiently cleaved Env, envelope (Env), HIV-1, immunogen, NFL, non-neutralizing antibodies, SOSIP, vaccine

Introduction

HIV-1 infection resulting in AIDS (acquired immunodeficiency syndrome) is one of the leading causes of morbidity and mortality in the world. Although highly active antiretroviral therapy has considerably reduced mortality and improved quality of life, a sterilizing/broadly protective vaccine immunity and/or a cure will be required to eradicate the HIV-1 pandemic. The HIV-1 envelope (Env) glycoprotein, which protrudes from the viral membrane and is required for attaching to the host plasma membrane, leading to conformational changes resulting in viral entry, is the sole target of neutralizing antibodies that are broad and potent and develop spontaneously in a fraction of HIV-1-infected patients. One of the strategies for developing a vaccine against HIV-1 is to stimulate the humoral immune response of the vaccinees, using Env-based immunogens, such that they develop broadly neutralizing antibodies (bNAbs) by recapitulating the bNAb maturation pathway. Development of a vaccine that confers sterilizing protection by B-cell-mediated immunity against HIV-1 infection is one of the most important challenges of biomedical research. This is primarily due to the...
The Env gp160 precursor polypeptide is proteolytically processed by the host enzyme furin into two constituent proteins gp160 (SU; soluble subunit) and gp41 (TM; transmembrane subunit), which are then rearranged into a trimer of heterodimers to form the functional HIV-1 viral spike. The Env spikes on the viral membrane are relatively sparse with only about 11–14 units on each virus. They are required for the virus to attach to the host plasma membrane and enter the cell through interactions with the host CD4 receptor and engagement with either CCR5 or CXCR4 co-receptors and involves intricate conformational changes, which ultimately lead to viral and host membrane fusion and release of contents of the virus into the cytoplasm.4–9 A big challenge in developing HIV-1 vaccine immunogens has been in obtaining stable Env proteins that mimic the native-like, trimeric structure in the correct conformation as is on the viral membrane. The handling of the functional HIV-1 Env
protein in its recombinant form is tricky due to the metastable and labile nature of the protein. Initially monomeric gp120 proteins were purified\textsuperscript{10,11} and when such proteins were used for immunization in animals they yielded only strain-specific antibodies.\textsuperscript{12,13} In some studies, in non-human primates, encouraging protective results were obtained with gp120.\textsuperscript{14–18} Subsequently, however, efficacy trials in humans with vaccines containing recombinant gp120 also did not confer protection against infection.\textsuperscript{19–21} Attempts to make Env soluble proteins by mutating the cleavage site and adding a domain at the C-terminus of gp41 that naturally form trimers, for example, foldon did not give desirable results as it was found to be largely present in the non-native form.\textsuperscript{3,22–24} After nearly a decade of research, primarily in John Moore’s laboratory, it was finally possible to stabilize an Env protein in what is now called the SOSIP form (Table 1) (Figure 2). This was first achieved with the clade A Env BG505 and subsequently with other Envs. John Moore and Rogier Sanders’ group designed the soluble, cleaved SOSIP.664 gp140 Env trimers based on the Env from the transmitted/founder clade A strain BG505.\textsuperscript{25,26} The design went through a number of iterations before the final product was developed.\textsuperscript{25,26–28} The protein BG505SOSIP.664 was created by truncating the Env gp160 polypeptide at position amino acid 664 which removes the entire C-terminal tail (CT), transmembrane domain and membrane proximal external region (MPER) domain and helps prevent aggregation in the absence of detergent.\textsuperscript{28} It also contains the SOS intermolecular disulphide bond (A501C and T605C mutations\textsuperscript{25}) that links gp120 and gp41 and the I559P (isoleucine to proline substitution) mutation in the N-terminal heptad repeat region of gp41, that destabilizes the post-fusion form of the protein,\textsuperscript{27} but these changes help in forming a properly folded, proteolytically cleaved protein that

### Table 1. Some examples of strategies used for developing HIV-1 Env-based soluble immunogens.

| Name             | Strategy                                                                 | Reference                                      |
|------------------|--------------------------------------------------------------------------|------------------------------------------------|
| SOSIP            | A501C, T605C mutation–disulphide bond; I559P mutation, REKR to RRRRRR (R6) mutation; stop codon at aa 664 (no MPER) | Sanders et al.; Pugach et al.; Guenaga et al.; Julien et al.; de Taeye et al.; Ahmed et al.\textsuperscript{26,39–43} |
| NFL              | REKR cleavage site replaced with Gly–Ser linkers; I559P mutation; stop codon at aa 664 (no MPER) | Sharma et al.\textsuperscript{23} |
| SC-gp140         | REKR cleavage site replaced with Gly–Ser linkers; SOSIP mutations        | Georgiev et al.\textsuperscript{44} |
| UFO              | Rational redesign of HR1; SOS mutations; cleavage site replaced with Gly–Ser linker | Kong et al.\textsuperscript{45} |
| ConC\_base0      | Repair and stabilize; repair strain-specific residues with more prevalent ones to create a consensus followed by structure-based stabilization in prefusion closed conformation | Rutten et al.\textsuperscript{46} |
| DS-SOSIP chimera | Additional stabilizing 201C, 433C disulphide bond and portions of BG505  | Joyce et al.\textsuperscript{47} |
| eOD-GT6/eOD-GT8  | Germline CD4bs-directed bNAb-binding, minimal gp120 outer domain variants obtained by computation-guided engineering and directed-library screening | Jardine et al.\textsuperscript{48} |
| FP8-KLH          | Eight N-terminal amino acid of fusion peptide linked to KLH              | Xu et al.\textsuperscript{49} |

bNAb, broadly neutralizing antibody; Env, envelope; Gly–Ser, glycine–serine; KLH, keyhole limpet hemocyanin; NFL, native flexibly linked.
consists of significant amounts of trimers with appropriate antigenic properties. In addition, BG505SOSIP.664 gp140 contains a REKR to RRRRRR (R6) substitution at the cleavage site that enhances cleavage and a T332N mutation that restores binding to bNAbs targeting epitopes containing the N332 glycan. This protein in its native, trimeric form was relatively easy to purify, first through affinity columns like CNBr-activated Sepharose 4B-beads covalently linked to 2G12 bNAb or a Galanthus nivalis-lectin column, followed by size exclusion chromatography. The trimeric protein was more thermally stable than its gp120 monomer counterpart in differential scanning calorimetry studies and showed homogeneous, compact, propeller-shaped trimers in electron microscopy studies. Furthermore, the purified trimer but not its corresponding gp120 monomer binds efficiently to a panel of bNAbs but weakly to non-NAbs in enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance assays. Purification of this stable, trimeric, soluble, native-like Env protein allowed determination of its crystal structure in association with the bNAb PGT122 and cryo-electron microscopy reconstruction of the Env with the CD4bs (CD4 binding site) targeted bNAb PGV04, which for the first time generated information about the nature of the Env–bNAb interaction at the structural level. Soon BG505SOSIP.664 gp140 became a potent reagent for different types of studies, including isolation of new bNAbs. Subsequently, positive-selection affinity chromatography, using quaternary epitope-targeting bNAbs PGT151 and PGT145-bound columns, were used to purify SOSIP variants of the Envs B41, 92UG037.8, CZA97.012, whereas a negative-selection purification method using a CD4bs (CD4 binding site)-directed non-NAb-bound column, to remove non-native

Figure 2. Schematic representation of some examples of soluble, stabilized native-like Env designs. The designs shown are SOSIP, BG505 chimera, DS-SOSIP, NFL and chemically cross-linked. Env, envelope; NFL, native flexibly linked.
forms, was used to purify SOSIP Env proteins of JRFL and 16055. Some of the additional SOSIP trimers that have been purified and characterized are for Envs 4-2.J41, AMC008, AMC011, DU422, and ZM197M. Later, several other strategies led to the development of soluble, native-like Env immunogens, some of which are shown in Table 1. Unlike, their non-native counterpart, native-like Env trimers display specifically broadly neutralizing epitopes [Figure 3(a)] and occlude non-neutralizing epitopes, whereas other forms such as gp120 retain binding to non-neutralizing antibodies [Figure 3(b)].

**Other strategies for generating soluble Env-based immunogens**

One of the issues with the SOSIP design is the necessity to coexpress the protease furin, required for proteolytic processing of the precursor Env. Therefore, a more simplified soluble Env protein was designed by attaching the gp120 subunit with the gp41 truncated fragment using a flexible linker composed of glycine and serine residues. These cleavage-independent trimers are different in structure and property from uncleaved Envs, the latter being largely non-native and do not discriminate between binding to bNAbs and non-NAbs. The “native flexibly linked” (NFL) (Richard Wyatt’s group), SC (Peter Kwong’s group) and UFO trimers belong to the family of cleavage-independent trimers (Table 1) (Figure 2). In the NFL trimer design, the REKR cleavage site is replaced with a glycine-serine (G4S) flexible peptide linker that allows sufficient conformational flexibility at the linked terminus of the two subunits to mimic structurally and conformationally the termini of the cleaved state of the unlinked subunits and form native-like but joined Env trimers. The amino acid sequence in this design was truncated at residue 664 and the I559P mutation of the BG505SOSIP design was retained. The single-chain BG505 gp140 NFL2P trimers resembled the cleaved BG505SOSIP trimers in electron microscopy (EM) analysis and antigenic profile suggesting that this design does generate native-like trimers. Similar properties were observed with the JRFL gp140 NFL2P trimers. The crystal structure of the BG505NFL.664 trimer has been solved and it exhibits quaternary protein structure resembling those of BG505SOSIP.664 and the glycan profile of the two trimers are comparable but also have some differences, for example, the N160 site of BG505SOSIP.664 contains mixed populations of oligomannose and complex glycans, whereas BG505NFL.664 contains exclusively oligomannose glycans. This is despite the fact that BG505NFL.664 undergoes furin cleavage-independent maturation during transport to the cell surface. Other BG505 stabilizing designs include introducing additional disulphide bonds. For a recent review of other similar designs see Sanders et al.
The first generation, recombinant, soluble Env trimers were further improved as the SOSIP and NFL designs in their original form works best for only a subset of Env sequences. This involved redesign of the Env sequence in order to confer greater structural stability, greater homogeneity of the purified protein and enhanced antigenicity. Using sequence and the high resolution BG505SOSIP crystal structure, certain amino acid residues in JRFL and 16055 Envs, especially those proximal to the gp120-gp41 interface, were reverted back to amino acids that are present in BG505 Env and these trimer-derived (TD) residues enhanced well-ordered trimer formation, improved antigenic profile and increased thermal stability. In addition, an engineered intraprotomer disulphide bond further prevented CD4-induced conformational changes. Targeted glycine substitutions in gp41 at helix-to-coil transitions allowed further stabilization and solution of the crystal structure of clade C Env. Similarly, antigenic and structure-guided targeted mutagenesis strategies have been used to improve yield and proportion of native-like trimers with other Envs that form native, soluble proteins with difficulty. The proportion of closed native-like trimers can be increased and thermal stability and antigenicity improved by introducing additional disulphide bonds. Mutagenesis has also been used to reduce the effect of the V3 immunodominant epitope and CD4-induced structural changes. In general it appears that in addition to the basic SOSIP and single-chain trimer designs different Envs may require different stabilization strategies to further improve the yield of thermally stable, soluble, native-like Envs that bind to bNAbs with high to very high affinities, bind weakly to non-NAbs, do not expose V3 immunodominant or CD4-induced epitopes and have a very high proportion of closed native trimers. Some other examples of stabilized trimers include the repair-and-stabilize approach and trimers stabilized by using additional disulphide bond and different portions of BG505 (transmembrane subunit) proteins is directly correlated with its ability to specifically and efficiently recognize bNAbs but weakly to non-NAbs when expressed on the cell surface. These are the characteristics of functional Envs and are essential in immunogens suitable for developing a vaccine as well, as the purpose of immunization will be to specifically elicit bNAbs and not non-NAbs. Furthermore, antigenic characteristics of Envs, when incorporated into pseudotyped viruses, in neutralization assays like TZM-bl assay does not always match those on the cell surface. For example, the clade B Env, YU2 is uncleaved on the cell surface although it is potently neutralized by the trimer-selective, cleavage-specific bNAb PGT145 and shows high differential neutralization by the bNAb VRC01 as compared to non-NAb F105 in pseudovirus neutralization assays. In addition, site-specific N-glycosylation of Envs, many of which determine binding to most bNAbs, from

**Naturally occurring, efficiently cleaved, membrane-bound Envs**

The practical difficulty of choosing Envs, which can be used for immunogen design, given the enormous diversity of HIV-1, led to the devise of a strategy to limit the pool to Envs that are functional and hence are more likely to be suitable for immunogen design. For this purpose, a six-step strategy was devised by Bimal Chakrabarti’s group based on an earlier study by Richard Wyatt's group, which showed, using the well-known clade B Env, JRFL that efficient cleavage of the gp160 precursor Env polypeptide into its constituent gp120 (soluble subunit) and gp41 (transmembrane subunit) proteins is directly correlated with its ability to specifically and efficiently recognize bNAbs but weakly to non-NAbs when expressed on the cell surface. These are the characteristics of functional Envs and are essential in immunogens suitable for developing a vaccine as well, as the purpose of immunization will be to specifically elicit bNAbs and not non-NAbs. Furthermore, antigenic characteristics of Envs, when incorporated into pseudotyped viruses, in neutralization assays like TZM-bl assay does not always match those on the cell surface. For example, the clade B Env, YU2 is uncleaved on the cell surface although it is potently neutralized by the trimer-selective, cleavage-specific bNAb PGT145 and shows high differential neutralization by the bNAb VRC01 as compared to non-NAb F105 in pseudovirus neutralization assays. In addition, site-specific N-glycosylation of Envs, many of which determine binding to most bNAbs, from
infectious viruses match closely those in membrane-bound, recombinant trimers but have notable differences with soluble recombinant Envs.\textsuperscript{71,72} Furthermore, in order to develop immunogens to be delivered as virus-like particle or through viral vectors and plasmid DNA, antigenic status of the Env on cell surface has to be determined.\textsuperscript{73} While antigenicity of virus-like particles can be directly investigated, as has been done with JRFL, pre-checking the efficiency of cleavage on the cell surface improves the chances of the virus-like particle approach to immunogen design. Therefore, it is important to determine the cell surface antigenicity of Envs to be taken up for immunogen design.

Initially, differential binding to cleavage nonspecific bNAb VRC01 in comparison to non-NAb F105 using FACS (fluorescence activated cell sorting)-based cell surface antibody-binding assays was used to screen a panel of Indian clade C Envs and determine the ratio of binding.\textsuperscript{74} Subsequently, differential binding to cleavage-dependent bNAbs like PGT151 and PGT145 in comparison to non-NAbs like F105 was found to be a better method to screen Envs for efficient cleavage on the cell surface as binding to these bNAbs is considerably reduced in uncleaved or cleavage-defective Envs.\textsuperscript{73–75} Envs that showed the highest ratio of binding (bNAb versus non-NAb) were chosen for further characterization.\textsuperscript{73–75} Efficiently cleaved Envs bind potently to a majority of bNAbs targeting different epitopes but weakly to non-NAbs in FACS-based cell surface antibody-binding assays and are potently neutralized by these bNAbs but weakly by non-NAbs.\textsuperscript{73–75} The presence of neutralization antibody epitopes are verified by pseudovirus neutralization assays, whereas those of non-neutralizing antibody epitopes are verified by cell surface staining of the uncleaved versions of Envs with non-neutralizing antibodies, which expose these epitopes.\textsuperscript{73–75} In addition, they are efficiently cleaved on the cell surface when tested using gp120 shedding assay, neutavidin-agarose pull-down assay of cell surface biotinylated proteins and immunoprecipitation assay of isolated plasma membrane fractions using cleavage nonspecific bNAb (VRC01), cleavage-dependent bNAb (PGT151) and non-NAb (F105); all experiments being carried out using transfected cells.\textsuperscript{73–76} Mutating the REKR cleavage site to the cleavage-resistant SEKS significantly reduces binding to the cleavage-dependent bNAb PGT151.\textsuperscript{74} Using these experimental strategies several efficiently cleaved, membrane-bound Envs with desirable antigenic properties suitable for immunogen design were identified from clades A (A5; QB726.70M.ENV.C4), B (JRCSF), C (4-2.J41) and B/C (LT5.J4b12C) (Table 2), which comprise about 75% of circulating HIV-1 strains. Significantly, it was also observed that the membrane-bound form of the well-characterized clade A Env BG505 is also efficiently cleaved and specifically binds to bNAbs but weakly to non-NAbs.\textsuperscript{73} Thus, efficient cleavage of these Envs into the constituent subunits is directly correlated with their ability to specifically bind bNAbs as opposed to non-NAbs and this correlation may be a general property of functional Envs. These observations suggest that these Envs can also be delivered as virus-like particles or through viral vectors and plasmid DNA. Uncleaved Envs bind to both cleavage nonspecific bNAbs and non-NAbs efficiently.\textsuperscript{2,73–75} The only exception to this rule with efficiently cleaved Envs found so far is the chimeric B/C recombinant Env LT5.J4b12C, which binds efficiently to PGT151 and PGT145 even in its cleavage-defective form.\textsuperscript{76} One possibility is that faster migration of the gp120 of this Env in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) compared with JRFL suggests that it may be less glycosylated and that may in turn affect its binding to cleavage-dependent bNAbs like PGT151 and PGT145.\textsuperscript{76} However, this hypothesis requires experimental validation. A list of efficiently cleaved Envs with their properties is shown in Table 2. As described in the following section, efficiency of cleavage on the cell surface and their ability to selectively bind bNAbs over non-NAbs is necessary but not sufficient to select Envs suitable for immunogen design.\textsuperscript{73}

**Role of C-terminal tail in determining antigenic properties of efficiently cleaved Envs and their soluble proteins**

The CT of HIV-1 Envs has been implicated in a number of functions of the protein such as trafficking, proper surface expression, membrane fusion, virion replication and budding.\textsuperscript{77–82} Two alternative models have been proposed on the orientation of the cytoplasmic tail primarily the Kennedy epitope (KE).\textsuperscript{83} In one model, the KE is
exposed on the virion surface and in the other it is intracytoplasmic. However, the role of the CT in HIV-1 Env function is not completely understood. The long CT of Env is frequently deleted in order to generate soluble proteins and to enhance expression of membrane expressed as well as soluble trimer (Figure 2). While generating soluble SOSIP proteins using the efficiently cleaved Env, 4-2.J41 it was observed that deletion of the CT, as required in this design, was disrupting the conformation and destabilizing the native structure of this protein. This observation was verified on the cell surface and it was found that deletion of the CT of 4-2.J41Env led to enhanced binding to non-NAbs on the plasma membrane suggesting that non-neutralizing epitopes of this envelope get exposed once the CT is truncated. Binding to some conformational and cleavage-specific, trimer-selective bNAbs were also altered. A caveat in this observation is that CT truncation mostly leads to an overall increase in expression of Env, which may lead to differential changes in binding affinity to different antibodies. This effect was not due to defective cleavage of the 4-2.J41ACT Env on the plasma membrane. The SOSIP version of 4-2.J41 Env could only be stabilized by replacing the gp41 subunit of this Env with the gp41 from BG505 Env, up to amino acid 664. This chimeric soluble protein 4-2.J41.gp41(BG505), after purification, showed excellent antigenic properties and the native-like trimeric propeller shape in electron microscopy studies. Thus, it appears that the gp41 of BG505 Env has unique properties that aid in stabilization of soluble Env protein, although whether this strategy will work for other unstable Envs remains to be determined. Besides, although it has not been tested, this study suggests that reinstatement of the transmembrane domain on BG505, after amino acid 664, probably will not alter the native conformation on cell surface.

Subsequent to the observation of the destabilizing effect of CT deletion on 4-2.J41 Env conformation/antigenicity, these studies were extended to the other efficiently cleaved Envs that were identified. Prior to these studies, the effect of CT truncation on antigenicity/conformation of Envs were determined by either using partially cleaved Envs or with Envs whose cleavage status were not reported, which may not accurately reflect properties in functional Envs. The clade BEnv, JRFL had already been shown to retain its native conformation/antigenicity on the cell surface and in pseudovirus neutralization assays even after deletion of the CT. This finding was
again confirmed.\textsuperscript{70} Furthermore, it was found that deletion of the CT of the clade A Env, A5 did not affect its cell surface antigenicity/conformation, but in the case of the clade B Env, JRCSF non-neutralizing epitopes were exposed and binding of some conformational antibodies were altered.\textsuperscript{70} Neither of these Envs showed any alteration in efficiency of cleavage upon CT truncation.\textsuperscript{70} The B/C recombinant, chimeric Env, LT5.J4b12C showed an intermediate effect, that is, non-neutralizing epitopes were partially exposed when its CT was deleted.\textsuperscript{76} One of the characteristics of the LT5.J4b12C Env, both in its SOSIP form as well as on the cell surface, is that it binds very weakly to sCD4 and is therefore resistant to CD4-induced conformational changes, which is also a desirable property for Envs suitable for immunogen design.\textsuperscript{76,90} However, the possibility exists that the weak binding to sCD4 of LT5.J4b12C Env is due to a more open conformation rather than a more compact trimer. It should be noted here that there are other strategies or designs which may limit the exposure of CD4i epitopes or poorly neutralizing antibodies that target the CD4-binding site, for example, DS-SOSIP and the NFL TD trimers.\textsuperscript{47,54} Similar to 4-2.J41 Env, deletion of the CT of LT5.J4b12C Env also has an effect on the ability to form stable, soluble, homogenous SOSIP protein, in that although the SOSIP protein could be generated, purified and characterized, the yield was relatively low compared with BG505SOSIP or 4-2.J41 gp41 (BG505)SOSIP proteins.\textsuperscript{43,90} These studies suggest that in naturally occurring, efficiently cleaved Envs, the cell surface antigenicity/conformation of only a subset of these Envs is affected by deletion of their CT and this in turn may have an effect on their ability to form soluble, native-like SOSIP proteins in significant quantity. Although, preliminary studies suggest that JRCSFN197DSOSIP is generated and displays several bNAb-binding epitopes in crude supernatant of transfected 293T cells,\textsuperscript{75} it remains to be determined whether this protein is stable in its purified form. Besides, it remains to be determined whether efficiently cleaved Envs whose CT determines their ectodomain (ET) antigenicity/conformation can be stabilized using the single-chain trimer strategies such as NFL without resorting to domain swap using BG505 Env gp41 up to amino acid 664. This is especially important in order to develop immunogens that can elicit bNAbs that require wild-type conformation at the gp120–gp41 interface.

Fragments of the CT of 4-2.J41 Env were restored in order to determine which minimal domain or region of the CT of this Env is required to restore wild-type cell surface antigenicity/conformation.\textsuperscript{70} It was observed that a conserved hydrophilic domain (CHD), also called the KE, between amino acids 724 and 745 is necessary and sufficient to restore wild-type antigenicity/conformation in 4-2.J41ACT\textsubscript{753} Env.\textsuperscript{70} The same region/domain in JRCSFACT\textsubscript{750} Env was able to restore wild-type antigenicity/conformation.\textsuperscript{70} However, the properties of 4-2.J41 and JRCSF Envs are largely restricted to the membrane-bound protein as in the context of virus there is only a modest effect of both CT deletion and CHD restoration on neutralization potency of different bNAbs and non-NAbs in pseudovirus neutralization assays.\textsuperscript{70} Based on these observations we proposed that CT deletion in 4-2.J41 and JRCSF Envs probably leads to formation of native, near-native and non-native forms of the Env in a heterogeneous mixture, which is reflected in altered cell surface antigenicity/conformation, but in the context of the virus and therefore in the presence of one or more viral protein(s) only native or near-native forms of the Envs are incorporated into virions by a yet unknown mechanism.\textsuperscript{70}

**Animal models**

Different types of Envs stabilized in different manner or manipulated or in different forms or different delivery methods have been used to study their antibody elicitation properties in different animal models primarily rabbits, mice, guinea pigs, nonhuman primates and recently in cows.\textsuperscript{58–60} An important study was the immunization of rabbits with BG505SOSIP.664 gp140 (T332N knock-in) trimers, which gave an autologous tier 2 and heterologous tier 1 neutralizing antibody response that target different epitopes (conformational epitopes for tier 2 responses and linear V3-directed for tier 1 responses).\textsuperscript{61} In rhesus macaques, BG505SOSIP.664 trimers also elicited antibodies with autologous tier 2 neutralization and limited breadth.\textsuperscript{61,64} Later, the elicitation of antibodies upon simultaneous and sequential immunization with clades A, B and C trimers in rabbits were studied.\textsuperscript{93,91} In one study,
immunization with BG505, AMC008 and ZM197M trimers elicited autologous tier 2 neutralizing response in monovalent, cocktail and sequential regimens, although in the case of AMC008 and ZM197M, cocktail and sequential combinations were less potent than monovalent trimers.63 Boosting with SOSIP.v4 or SOSIP.v5 trimers did not have a difference between BG505 and AMC008 trimers but SOSIP.v4 had a stronger effect than SOSIP.v5 in the case of ZM197M trimers.63 Heterologous tier 1A neutralization with higher titer was observed in both cocktail and sequential combinations while there was more modest effect on heterologous tier 2 neutralization.63 Most immunization studies with Envs have elicited largely strain-specific antibody responses with limited breadth, suggesting that finding the appropriate Env and animal model is challenging. In addition, autologous neutralizing antibodies elicited by these trimers in rabbits and macaques as well as virus-like particles in rabbits target holes in the glycan shield.91–99 Removal of glycans proximal to the CD4 supersite and immunization of macaques with these trimers resulted in potent autologous neutralization and significant heterologous neutralization with CH505 trimers, suggesting a correlation between immunogenicity and protein-surface accessibility to antibody within a limited range.97 Blocking known immunogenic glycan holes and then opening other holes in the glycan shield redirected the antibody response to the new glycan hole epitopes.100 These studies generated a lot of information about the neutralizing response of Env-based trimers that led to other more advanced studies. More recently, several studies have given hope that elicitation of broadly neutralizing antibodies in animal models immunized with Envs is possible. In one such approach, targeting germline precursors with germline-targeting Env-based immunogens was employed. Wild-type Envs usually do not bind germline precursors of bNAbs101–103 and therefore they have to be redesigned to enable priming of germline (GL) precursors and then boosted with successive immunogens with an enhanced affinity gradient towards antibody intermediates that guide antibody maturation through somatic mutation leading to elicitation of bNAbs. In one such study by William Schief’s group, minimal, engineered outer domain (eOD) of gp120 targeting VRC01-class of CD4bs bNAbs precursors was designed by screening of directed libraries of computationally guided mutations using yeast cell surface display method to identify eOD variants that showed increased affinity to GL-VRC01.48 The eOD-GT1 variant was further improved to eOD-GT6 and eOD-GT6 fused to self-assembling nanoparticles was able to potentely induce B-cells expressing both mature and GL VRC01 class antibodies in Ca2+-dependent activation assays.48 An improved version of this GL-targeting priming immunogen eOD-GT8 60 mer was used to immunize mice containing a knock-in of germline-reverted heavy chain of VRC01 coupled with mouse light chains and led to an immune response targeting the CD4bs.65 Recovered IgGs from sorted B-cells and hybridomas mostly were knock-in VRC01 H-chain with some VRC01-class somatic mutations and mouse L-chains with five amino acid CDRL3 length and QQY motif in the Vκ CDRL3 characteristic of VRC01-class LCDR3s.91–99 Removal of glycans proximal to the CD4 supersite and immunization of macaques with these trimers resulted in potent autologous neutralization and significant heterologous neutralization with CH505 trimers, suggesting a correlation between immunogenicity and protein-surface accessibility to antibody within a limited range.97 Blocking known immunogenic glycan holes and then opening other holes in the glycan shield redirected the antibody response to the new glycan hole epitopes.100 These studies generated a lot of information about the neutralizing response of Env-based trimers that led to other more advanced studies. More recently, several studies have given hope that elicitation of broadly neutralizing antibodies in animal models immunized with Envs is possible. In one such approach, targeting germline precursors with germline-targeting Env-based immunogens was employed. Wild-type Envs usually do not bind germline precursors of bNAbs101–103 and therefore they have to be redesignned to enable priming of germline (GL) precursors and then boosted with successive immunogens with an enhanced affinity gradient towards antibody intermediates that guide antibody maturation through somatic mutation leading to elicitation of bNAbs. In one such study by William Schief’s group, minimal, engineered outer domain (eOD) of gp120 targeting VRC01-class of CD4bs bNAbs precursors was designed by screening of directed libraries of
bNAb-specific characteristics by boosting with specifically designed successive immunogens in a sequential regimen. Further refinement of this approach is required to elicit bNAbs upon vaccination consistently with features similar to those obtained from HIV-1-infected individuals. Similar approaches have been used to study elicitation of other bNAbs.

One of the difficulties in HIV-1 vaccine research has been finding appropriate animal models to study elicitation of bNAbs upon immunization. Apart from the transgenic knock-in mice approach, another breakthrough study came from Dennis Burton’s group, wherein immunization of cows with BG505SOSIP led to development of antibodies with greater cross-clade neutralization breadth and potency along with autologous neutralization. Breadth and potency of the neutralizing response increased over later time points with additional boosts of the immunogen. Isolation of cow IgGs led to the identification of one monoclonal antibody NC-Cow1 that showed 72% neutralization breadth in a 117-virus neutralization panel. Specificity of NC-Cow1 was towards the CD4bs and it had low affinity towards the immunodominant V3 loop. Competition ELISA showed that the isolated cow monoclonal antibodies competed with VRC01-class antibodies but not other CD4bs antibodies or antibodies that target other epitopes. This study is significant in that it suggests that selecting an appropriate animal model and a suitable Env can tremendously aid in finding out which Env is suitable for eliciting which class of antibodies so that they can be taken up for further studies. In addition, these studies suggest that immunogens need to be tested in different animal models in order to gain deeper knowledge and come to a consensus about their ability to elicit neutralizing responses such that more evolved immunogens can be designed to ultimately elicit bNAbs by vaccination.

**Perspective**

In this review, we have discussed briefly the salient features of Env-based immunogens developed so far and the identification of efficiently cleaved, membrane-bound, functional Envs from different clades with characteristics suitable for developing immunogens. We have also discussed some of the findings with these Envs, which provide insights into the properties of the Envs that require further studies and may help fine-tune the usage of Envs as immunogens as well as improve our understanding of the biology of HIV-1 Envs. These studies suggest that these naturally occurring, efficiently cleaved Envs require further investigations in order to find out whether they would make good immunogens similar to BG505SOSIP.

Given the enormous viral diversity of HIV-1, choosing Envs suitable for immunogen design is challenging. Previously, approaches used by other researchers have yielded several soluble Env proteins by using either the SOSIP design or the single-chain trimer designs and further modifications. These breakthrough designs have significantly advanced our knowledge of both HIV-1 Envs and allowed testing suitability of these proteins for use as immunogens in different animal models. However, early immunization studies in animal models have been challenging with mostly strain-specific responses and limited breadth. More recently, immunization studies in transgenic mice (GL-bNAb heavy-chain knock-in) with germline-targeting and boosting immunogens have elicited antibodies with several bNAb features and greater breadth. BG505SOSIP.664 immunization of cow has yielded VRC01-class cow antibodies with greater neutralization breadth and significant potency. However, eliciting neutralizing antibodies through vaccination with breadth and potency, closer to that seen for some of the best naturally occurring bNAbs isolated from infected patients, still remains a challenge. A different approach for selecting HIV-1 Envs suitable for immunogen design has been based on a previous study as described above. This approach has focused on the efficient cleavage property of the Env gp160 precursor polypeptide and together with others shown that this property is correlated with the ability of Envs to preferentially bind bNAbs and weakly to non-NAbs, the latter being desirable in immunogens as the purpose of a vaccine will be to specifically elicit bNAbs and not non-NAbs. Using a set of experiments, several naturally occurring, efficiently cleaved Envs with such properties were identified from clades A, B, C and B/C. In addition, it was observed that the CT plays a crucial role in maintaining cell surface antigenicity/conformation in a subset of these Envs and therefore, the effect of the CT on antigenicity/conformation should ideally be investigated before designing immunogens where it is
necessary to truncate the CT. These results also suggest that there may exist a specific mechanism to preferentially incorporate functional Envs into virions. Since the SOSIP and single-chain trimer strategies for designing Env-based soluble immunogens does not work efficiently for all Envs, design strategies specifically tailored for different Envs will have to be used. Different stabilizing strategies, mainly structure-based, have been developed to enlarge the pool of available soluble Env proteins. Determining cleavage property, cell surface antigenicity and effect of CT truncation on antigenicity maybe a relatively straightforward strategy to identify Envs suitable for immunogen design. Whether this approach yields Envs that can be converted easily into soluble SOSIP or single-chain trimers and elicit neutralizing antibodies with significant breadth and potency in animal models will require further experimentation. However, we note with optimism that the membrane-bound form of the Env BG505, whose soluble form is very stable even after CT truncation and has elicited neutralizing antibodies in cow with the improved breadth and potency, is efficiently cleaved on the cell surface. In addition, efficiently cleaved Envs are useful in order to deliver immunogens by other methods such as viral vectors, plasmid DNA or as virus-like particles. The HIV-1 vaccine field is going to be benefited if and when several of these Envs are able to elicit neutralization antibodies with significant breadth and potency in animal models.

Conclusion
Subsequent to the stabilization of BG505SOSIP, 664 gp140 soluble Env protein and its characterization, the field of HIV-1 Env-based, B-cell-mediated immunity-dependent vaccine development has seen rapid advances in our knowledge of HIV-1 Envs, isolation and characterization of bNAbs and a better understanding of the issues that need to be overcome in order to elicit broad and potent bNAbs upon vaccination. These advances have led to significant knowledge of structure–function relationships of several Envs and bNAbs, development of better animal models, testing of Env-based immunogens in different animal models and studying the neutralizing antibodies elicited, antibody germline targeting using modified immunogens and sequential immunization methodologies. However, it is necessary to expand the repertoire of available Envs suitable for immunogen design. A promising strategy could be to identify naturally occurring, efficiently cleaved Envs, as they have the natural property of binding specifically to bNAbs and poorly to non-NAb, properties prerequisite in immunogens, and then develop immunogens based on these Envs and test them in animal models. The CT of these efficiently cleaved Envs also appear to play a role in their cell surface antigenicity/conformation and the effect of CT truncation on Env properties should be studied prior to determining strategies to convert them into immunogens. These studies have the strong possibility of generating Env-based immunogens with properties similar to BG505SOSIP. Future work should involve converting these Envs into immunogens using different methods and then testing them in different animal models to investigate whether they elicit superior neutralizing antibodies.

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ORCID iD
Supratik Das https://orcid.org/0000-0002-7279-938X

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