Stabilization of HIV-1 envelope glycoprotein trimers to induce neutralizing antibodies

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_Citation for published version (APA):_
Chapter 7

Immunogenicity of stabilized HIV-1 envelope trimers with reduced exposure of non-neutralizing epitopes


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Cell, 163(7):1702-15
Abstract
The envelope glycoprotein trimer mediates HIV-1 entry into cells. The trimer is flexible, fluctuating between closed and more open conformations and sometimes sampling the fully open, CD4-bound form. We hypothesized that conformational flexibility could hinder the induction of broadly neutralizing antibodies (bNAbs). We therefore modified soluble Env trimers to stabilize their closed, ground states. The trimer variants were indeed stabilized in the closed conformation, with a reduced ability to undergo receptor-induced conformational changes and a decreased exposure of non-neutralizing V3-directed antibody epitopes. In rabbits, the stabilized trimers induced similar autologous Tier-1B or Tier-2 NAb titers to those elicited by the corresponding wild-type trimers, but lower levels of V3-directed Tier-1A NAbs. Stabilized, closed trimers might therefore be useful components of vaccines aimed at inducing bNAbs.

Graphical Abstract
Introduction

The mature, proteolytically cleaved envelope glycoprotein trimer (Env) mediates HIV-1 entry into target cells by undergoing a complex series of conformational changes initiated by binding to the CD4 receptor and the CCR5 or CXCR4 co-receptor. Defining how Env functions during cell entry has major implications for the rational, structure-guided design of trimer-based vaccines aimed at inducing broadly neutralizing antibodies (bNAbs) against highly divergent primary HIV-1 strains. One promising approach is to use recombinant, soluble trimers [1,2] as tools to increase our understanding of these coordinated conformational changes, via X-ray and cryo-EM structures and biophysical analyses [3–8].

Creating a soluble, native-like trimer is complicated by the instability of the association between the gp120 and gp41 subunits, and between the individual gp120-gp41 protomers. The native trimer is inherently metastable because it must undergo profound conformational rearrangements during virus entry [9]). One successful stabilization strategy involves introduction of an intermolecular disulfide bond (SOS) to link gp120 and gp41, a point substitution (I559P, i.e. IP) to maintain the gp41 subunits in their prefusion form, and truncation at residue 664 to improve trimer solubility [1,10–13]. The resulting trimers are termed SOSIP.664. Soluble SOSIP trimers based on the clade A BG505 env gene have been used to generate high resolution X-ray and cryo-EM structures [4,5,7,14], to isolate new bNAbs that recognize quaternary structure-dependent epitopes, and to characterize known bNAbs [1,15–20]. In addition to BG505, native-like SOSIP.664 trimers have also been produced from the B41, ZM197M and DU422 clade B or C env genes, as well as other sequences [21–25]. As immunogens, the BG505 and B41 SOSIP.664 trimers induce NAbs to the neutralization-resistant (Tier-2) autologous virus in rabbits and/or macaques [2].

While the induction of consistent NAb responses against the autologous Tier-2 viruses by the BG505 and B41 SOSIP.664 trimers is an unprecedented achievement, it is the first among several steps towards the induction of bNAbs. It is highly unlikely that any single Env antigen will induce bNAbs. Instead it is probably necessary to devise more sophisticated vaccination regimens that include germline targeting, evolutionary lineages, multivalent immunogens, alone or more likely in combination [16,26–31].

Limiting the exposure of non-NAb epitopes is also likely to be necessary for optimal immunogenicity. On BG505 SOSIP.664, non-NAb epitopes in V3 are particularly immunogenic [2]. Non-NAbs and narrow specificity NAbs have been proposed to interfere with the induction of bNAbs against many pathogens, including influenza, malaria, HIV-1, and others [30,32–37]. One mechanistic explanation for this phenomenon is that high affinity non-NAbs may enter germinal centers and block antigen binding to lower affinity B cell receptors with specificity for bNAb epitopes [30,38]. In an in vitro study, McGuire et al. showed that when HIV-1 Env antigens were added to a mixture of B cells bearing the germline precursors of bNAbs and non-NAbs, including those for V3 non-NAbs, the latter were preferentially activated. However, modification of the Env antigen to reduce non-NAb epitope presentation (including deletion of the V3 region) facilitated the activation of lower affinity bNAb precursors [30]. Refocusing Ab responses away from V3 towards other epitopes has also been reported [34]. Moreover, Eggink et al. showed that inhibiting the immunogenicity of the influenza hemagglutinin head domain enhanced responses to the normally subdominant stalk domain, such that more broadly reactive NAbs were elicited [33]. Overall, it is highly plausible that limiting the exposure of non-NAb epitopes on native-
like Env trimers will improve their use as a platform for inducing bNAbs.

The native Env trimer and its SOSIP.664 mimics are conformationally flexible; they can “breathe” such that they fluctuate between closed and more open forms in a dynamic equilibrium [3,6,14,22,39]. While unliganded BG505 SOSIP.664 trimers have a high propensity to remain in the closed, ground state conformation, the equilibrium for their B41, DU422 and ZM197M counterparts is shifted. Thus, a greater proportion of partially open, but still fully native-like, trimers can be visualized by negative stain electron microscopy (NS-EM) [22,23]. Another manifestation of this conformational isomerism is the binding of some non-NAbs to a small fraction (presumably the partially open forms) of the SOSIP.664 trimer population; these non-NAbs recognize V3, CD4-induced (CD4i) and a subset of CD4-binding site (CD4bs) epitopes, which are all normally only accessible on neutralization-sensitive (Tier 1) viruses [1]. How conformational flexibility influences the immunogenicity of Env trimers is not yet known. However, we hypothesize that a trimer stabilized in the closed, unliganded state would allow the antibody response to be focused on the desired NAb epitopes with minimal induction of unwanted, and perhaps even interfering or distractive, non-NAbs, such as those directed against V3.

Here, we describe the modification of SOSIP.664 trimers to reduce the rate or extent of “breathing” and impede conformational changes involved in the transition to more open conformations that expose non-NAb epitopes.

**Results**

**AMC008 SOSIP.664 trimers as a basis for studying stabilization strategies**

For initial studies of how to improve the frequency of closed SOSIP.664 trimers at the expense of partially open or non-native forms, we selected the AMC008 clade B env gene, an early sequence from an elite neutralizer enrolled in the Amsterdam Cohort Studies on HIV/AIDS, as the test case. The parental AMC008 virus was categorized as a Tier-1B virus by the Duke Central Laboratory, suggesting that the native trimer on the virus is relatively open and could expose V3- and/or CD4i-epitopes (not shown). When the AMC008 SOSIP.664 Env proteins were expressed, ~50% migrated as trimers on a BN-PAGE gel (Fig. S1A), but only ~35% of the 2G12/SEC-purified AMC008 trimers were visibly native-like in NS-EM images (Fig. S2C). We have previously described how unliganded native-like SOSIP.664 trimers can exist in two conformations that are distinguishable by NS-EM: a compact “closed” conformation and partially open conformation [22]. While the partially open trimers that we have described have a higher degree of loop and/or domain mobility, no major decrease in density at the center of the trimer could be observed, suggesting that the protomers do not become separated at the interface between them. Additionally, the transition from the closed to the partially open conformation is at least partially reversible, and in a thermodynamic equilibrium [6]. These rapidly interchanging configurations differ from the open conformation described by Scharf et al., which is more analogous to the CD4-induced fully open state that arises when the trimer undergoes a major reorganization and that is likely to be irreversible [14,39].

We found that ~25% of the AMC008 SOSIP.664 trimers were in the closed, native-like form, with ~10% partially open, but the remaining ~65% adopted heterogeneous,
splayed out shapes that resemble uncleaved, non-native gp140 proteins [40,41]. The midpoint of thermal denaturation ($T_m$), determined by differential scanning calorimetry (DSC), for AMC008 SOSIP.664 trimers was 59.6°C, which is much lower than for their BG505 counterparts (67.3°C; Table 1). Finally, the antigenicity profile of the AMC008 SOSIP.664 trimers showed that they bound quaternary structure-dependent bNAbs very poorly, but several non-NAbs efficiently (Data S1A). As such defects are fairly typical of what we have seen with many Env sequences, the AMC008 SOSIP.664 trimers seemed suitable for testing structure-guided stabilization strategies. To assess their general applicability, we also tested the same strategies on the prototype BG505, B41 and ZM197M trimers.

**Positive selection of native-like trimers by PGT145 purification**

We first investigated whether we could isolate native-like AMC008 SOSIP.664 trimers by affinity chromatography using the quaternary structure-dependent bNAb PGT145, which does not recognize non-native Env proteins [22,24]. Even without a subsequent SEC step, PGT145-purification yielded homogeneous, fully cleaved trimers (Fig. S2A&B) that were ~100% native-like when visualized by NS-EM (Fig. S2C). Oligomannose glycoforms dominated (~67%) the glycan profile. The high Man$_8$GlcNAc$_2$ and Man$_9$GlcNAc$_2$ content (~14% and ~34%, respectively) is a hallmark of native trimers (Table 1; Fig. S3D) [41–43]. However, NS-EM imaging also showed that most (~85%) of the PGT145-purified trimers were in a partly open conformation (Table 1, Fig. S2C), and their thermal stability was not substantially improved ($T_m$ = 60.2°C; Table 1).

PGT145 purification also yielded a homogeneous population of fully native-like BG505 SOSIP.664 trimers (Fig. S3A). The proportion of trimers in the closed conformation however was reduced to ~35%, compared to >95% after 2G12/SEC purification. Thus, binding to and/or elution from PGT145 causes the BG505 trimers to partially open up, which may be attributable to the known, albeit quite subtle, allosteric effects of PGT145 on the trimer structure [44]. The stability of these PGT145-purified BG505 trimers ($T_m$ = 66.7°C) was also subtly reduced compared to 2G12/SEC ($T_m$ = 67.3°C; Table 1), but their glycan content (Table 1) [41] and antigenicity profiles were both unaltered, compared to 2G12/SEC-purified trimers [44]. B41 SOSIP.664 trimers, on the other hand, were similar after purification by PGT145 or 2G12/SEC, in that they were partly open (45-60%) in both cases [22]. For ZM197M, the closed trimer content was reduced from 80% (2G12/SEC) to ~15% (PGT145) (Table 1, Fig. S2A-C) [23].

**Mutation of gp41 residues 535 and 543: SOSIP.v3 trimers**

A previous comparison of JR-FL, a clade B Env that forms SOSIP trimers inefficiently, with KNHI144, a clade A Env that does so more efficiently, showed that a methionine at position 535 (M535) and glutamine at 543 (Q543) in HR1 of gp41 benefit trimer formation (Fig. 1E, Table S2) [45]. Similar findings were made when HIV-1 was cultured under harsh conditions [46]. We noted that the BG505 sequence contains M535 and N543. Based on the high quality of BG505 trimers, we considered that N543 might have the same stabilizing effect as Q543 on the gp41 prefusion structure. In contrast, the AMC008 sequence contains neither M535 nor N/Q543, but instead the unfavorable I535 and L543 (Fig. 1E, Table S2). We therefore constructed AMC008 SOSIP.664 trimer mutants containing one or more of the I535M, L543N and L543Q substitutions (Fig. S1A). All three individual changes improved trimer formation,
Figure 1. Design of amino-acid substitutions to stabilize SOSIP.664 trimers. (A) Crystal structure of BG505 SOSIP.664 trimer [6], showing the locations of amino-acid substitutions relevant to this study. One protomer is colored according to sub-regions: gp41 in red; V1V2 in cyan; V3 in purple; gp120 inner domain layer 1 in blue; layer 2 in yellow; layer 3 in orange; outer domain and N- and C- termini of gp120 in green. (B) Detailed view of V3 and surrounding regions showing the A316W substitution. (C) Detailed view of layers 1 and 2 of the gp120 inner domain, showing how E64 and H66 face the unstructured region of gp41 (residues 548-568) between α6 and α7. (D) Detailed view of a CD4-ligated gp120 monomer structure [50] highlighting the same region as in (C) and showing how the E64 and H66 side chains face gp120 layer 2. (E) Depiction of how the α6-α9 helices of gp41 encircle the N- and C-termini of gp120, with M535 and N543 located in the middle and at the C-terminus of α6, respectively. (F) Infection of TZM-bl cells by BG505.T332N Env-pseudoviruses with an A316W, E64K or H66R substitution. (G) Summary of 19b binding thermodynamics (ΔTIC). (H) Summary of CD4 binding kinetics (ΔBLI).
substitution. (G) V3 Fab 19b binding to AMC008, BG505 and B41 SOSIP.664 and SOSIPv4 trimers was assessed by ITC. The enthalpy changes (ΔH), dissociation constants (K_D) and stoichiometries of binding (molar ratio; N) are listed in panel (H). (I) Biolayer interferometry analysis of CD4 binding to AviB-tagged mutant wild-type BG505 SOSIP.664 trimers. (J) SPR analysis of ligand binding to BG505 SOSIP.664-His trimers and both versions of SOSIPv4-His trimers. Top row: CD4-IgG2 (left panel), 17b (middle panel), CD4-IgG2 followed by 17b (right panel); bottom row, PG16, PGT145, PGT151 and 35O22. Antibodies and CD4-IgG2 were injected at 500 nM. See also figure S1, table S2 and S3.

which was also the case for the I535M+L543N combination (Fig. S1A). When this double mutant was PGT145-purified, NS-EM analysis showed the percentage of closed vs. open trimers was unchanged (~15% closed; Table 1), but its thermostability was slightly increased from 60.2°C (wild-type) to 61.6°C (Table 1). The I535M+L543N substitutions also modestly improved binding of bNAbs PGT145, 35O22 and PGT151, but otherwise did not affect trimer antigenicity (Table 2, Fig. Data S1A&E).

The B41 and ZM197M sequences also both lack one of the beneficial amino acids at positions 535 and 543, so we introduced the L543N substitution into B41 and V535M into ZM197M. The L543N substitution improved PGT145 binding to B41 SOSIP.664 trimers in ELISA (Data S1C). From here on, we refer to SOSIP.664 proteins containing the optimal amino acids at position 535 (i.e. methionine) and 543 (i.e. asparagine or glutamine) as SOSIP version 3 (SOSIP.v3). Specifically, SOSIPv3.1 contains the 543Q change, and SOSIP.v3.2 contains 543N. For more details on nomenclature, see Table S3.

Reducing V3 exposure by enhancing hydrophobic packing
Stabilizing the closed conformation of SOSIP.664 trimers requires the V3 region to be sequestered and the exposure of non-NAb epitopes (for Tier-2 viruses), including CD4i epitopes, to be reduced. Although the V3 region is tucked beneath the V1V2 domain in the BG505 SOSIP.664 structure, it can become transiently exposed on some or all trimers as they breathe in vitro [1]. V3 is also immunogenic in vivo [2]. One way to gauge V3 exposure is MAb reactivity with D7324-tagged SOSIP.664 trimers under ELISA conditions in which V3 epitopes become readily accessible [1].

We designed an A316W substitution to strengthen hydrophobic interactions at the apex, thereby decreasing the propensity for V3 to flip out of its ground-state location (Fig. 1A&B). For AMC008 and BG505 SOSIP.664 proteins, the A316W change improved trimer formation and also increased the thermostability of the BG505 trimers as assessed by an assay that can be used with unpurified Env proteins (Fig. S1D-G). The V3 non-NAbs 447-52D, 39F, 14e and 19b bound less well to all the A316W variant trimers compared to wild-type (Data S1E-H). For example, 14e and 19b binding to PGT145-purified BG505 SOSIP.664 A316W trimers was reduced by ~80% and ~50%, respectively; similarly, binding of the CD4i non-NAbs 17b and 412d was ~50% lower (Table 2, Data S1F). As the A316W substitution did not affect binding of the same non-NAbs to isolated V3 peptides, we conclude that it works indirectly on the trimer by impeding exposure of V3 epitopes (Fig. S1H). In contrast, the A316W substitution had no adverse effect on the binding of multiple bNAbs to the AMC008, BG505, B41 and ZM197M SOSIP.664 trimers (Data S1E-H). Other bulky hydrophobic amino acids (Tyr/Phe/Val/Ile) also decreased V3 exposure (>3-fold), but other substitutions had little effect (Fig. S1I).

When BG505 SOSIP.664 A316W trimers were purified on PGT145 columns and viewed by NS-EM, ~80% were in the closed conformation, an increase compared to the
~35% value for the wild-type BG505 trimers. Their thermostability was also greater ($T_m = 69.0°C$ vs. 66.7°C; Table 1). When introduced into the BG505 Env-pseudovirus, the A316W substitution reduced infectivity by ~98%, implying either that conformational flexibility is important during entry or that residue A316 plays a direct role in co-receptor binding (Fig. 1F).

Reducing spontaneous sampling of the CD4-bound conformation
We next evaluated two substitutions (E64K and H66R) in layer 1 of the gp120 inner domain that were identified in a study of how HIV-1 becomes resistant to, and eventually dependent on, the entry inhibitor VIR165 (Fig. 1A, C, D) (D.E., S.W.dT., I.B., P.J.K., B.B., R.W.S., unpublished data). In brief, the resistance changes appear to impede native trimers from assuming the CD4-bound conformation, but when VIR165 is present the trimers can undergo CD4-induced conformational changes. In the unliganded BG505 SOSIP.664 trimmer, the side chains of residues 64 and 66 are positioned to interact with HR1 of gp41 [7,8,47], but the high crystallographic B-values around residues 64 and 66 imply some local flexibility (Fig. 1C) [7]. Hydrogen-deuterium exchange (HDX) studies confirmed that this region does not adopt a stable secondary structure, but becomes ordered after CD4-binding [3]. Indeed, in the structure of a CD4-ligated gp120 core protein, the side chains of residues 64 and 66 have reoriented to interact with gp120 layer 2 (Fig. 1D) [48]. Thus, these residues may be part of a switch that transduces CD4-induced conformational changes from the CD4bs and layers 3 and 2 of gp120 to gp41 via layer 1. We hypothesize that the E64K and H66R substitutions impede this allosteric process.

Exploratory studies on unpurified culture supernatants indicated that the E64K and H66R changes each substantially reduced, or even eliminated, the binding of the CD4i non-NAbs to AMC008, BG505, B41 and ZM197M Env proteins (Fig. S1J&K, Data S1A-D). When mutant BG505 SOSIP.664 trimers were PGT145-purified and visualized by NS-EM, the percentage of closed trimers was substantially increased for the E64K mutant (~85% closed vs. 35% for wild-type), but not H66R (~30% closed; Table 1). Each substitution had only a minor effect on thermostability ($T_m = 67.9°C$ for E64K, 67.5°C for H66R vs. 66.7°C for wild-type). In an ELISA, bNAb binding was unaltered or sometimes enhanced (e.g., for the quaternary structure-dependent bNAbs PG16 and PGT145), whereas the 17b and 412d non-NAbs no longer bound (Table 2, Data S1F).

Combining A316W with E64K or H66R substitutions: SOSIP.v4 trimers
The E64K or H66R substitutions were combined with A316W to make double mutant AMC008, BG505, B41 and ZM197M variants based on the SOSIP.v3 design (Table S3). In each case, pilot studies on unpurified Env proteins (culture supernatants) indicated that the E64K+A316W and H66R+A316W double mutants from all four genotypes had acquired the beneficial properties associated with each individual change. Each double mutant was expressed efficiently, formed fully cleaved trimers and was slightly more thermostable than wild-type (Fig. S1A-G). Moreover, in all four cases, the binding of non-NAbs to CD4i-epitopes and V3 epitopes was diminished compared to wild type, or even abolished, while bNAb epitopes were mostly unaffected (Data S1A-D).

For simplicity, we here introduce the following nomenclature: SOSIP.v4 trimers contain the optimal amino acids at position 535 and 543 (SOSIP.v3; see above) and, in
addition, the A316W change and either the E64K (designated SOSIP.v4.1) or the H66R (SOSIP.v4.2) substitution (Table S3).

Table 1. Biophysical properties of stabilized AMC008, BG505, B41 and ZM197M SOSIP.664 trimers.
The biophysical properties of 293F cell-expressed, PGT145-purified SOSIP.664-D7324 trimers were assessed using NS-EM to determine native-like trimer formation, DSC to quantify thermostability ($T_m$), and glycan profiling to measure glycan content. For comparison, previously reported $T_m$ values and glycan profiles for 2G12-SEC purified SOSIP.664 trimers are included [1–4]. The unprocessed EM, DSC and glycan profiling data are shown in Fig. S2 and S3. The DSC data were fitted using both two state and non-two state models (Fig. S3B). The $T_m$ values based on two-state model fitting are listed here, while values based on the alternative model fitting are in Table S1.

<table>
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<th>Stabilizing mutations / SOSIP version</th>
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<th>Glycan analysis</th>
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* Values for AMC008 SOSIP.664 expressed in 293S cells

b Values derived from previous studies [1, 22, 23]

$T_m$ for 2G12/SEC purified BG505 SOSIP.664-D7324 expressed in 293F cells. Previously, a $T_m$ of 68.1°C was reported for BG505 SOSIP.664 expressed in 293S cells [1].

d Glycan profile of BG505 SOSIP.664 expressed in 293T cells [41]
Table 2. Antigenicity of stabilized AMC008, BG505, B41 and ZM197M SOSIP trimers.

<table>
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Binding of bNAbS and non-NAbS to PGT145-purified SOSIP.v4 and SOSIP.664 trimers was assessed by D7324-capture ELISA (Data S1E-H). Half maximal binding concentrations (EC_{50} in μg/ml) or area under the curve (AUC) values are shown. AUC values were used for some bNAbS and non-NAb 17b because plateau values differed substantially (>20%) between trimer variants or binding curves did not reach plateau levels (17b). Values determined with AUC values are underlined. Ab binding to each SOSIP.v4 trimer is expressed as a percentage of binding to the corresponding SOSIP.664 trimer (= 100%). Whenever possible, the comparison was based on EC_{50} values. OD glycan is a reference to outer domain glycans. The experimental error is within ± 20% of the recorded value.

Biophysical properties of stabilized SOSIP.v4 trimers

The various PGT145-purified SOSIP.v4 trimers were fully cleaved and highly homogeneous, as assessed by reducing and non-reducing SDS-PAGE and BN-PAGE analysis (Fig. S2A&B). The proportion of closed trimers was increased in each case compared to wild-type, most notably for AMC008 SOSIP.v4.2 (~85% closed vs. ~15%; Table 1, Fig. S2C). Except for ZM197M SOSIP.v4 (no change), the SOSIP.v4 trimers were also more thermostable by 2-4°C, compared to wild-type (Table 1, Fig. S3B). The glycosylation profiles differed little from their wild-type counterparts, with oligomannose glycans again predominating (Table 1, Fig. S3D). Dynamic light scattering (DLS) experiments showed that, like the BG505 wild-type trimers, both SOSIP.v4 variants were monodisperse and had the same hydrodynamic radius (R_h) of ~69 Å (Fig. S3C). Furthermore, in small angle x-ray scattering (SAXS) studies, these trimers were all well folded, with a radius of gyration (R_g) of ~53 Å (Fig. S3C).

Antigenicity of stabilized SOSIP.v4 trimers

D7324-tagged versions of wild-type (i.e., SOSIP.664) and stabilized trimer variants were PGT145 affinity-purified and studied by ELISA. The AMC008, BG505, B41, ZM197M SOSIP.v4 trimers all retained the ability to bind bNAbS (2G12, PGT135, PGT121, PGT126, PGT145, 35O22, VRC01 and CH103) (Table 2, Data S1E-H). This outcome was confirmed by surface plasmon resonance (SPR) for BG505 SOSIP.v4 and the quaternary structure-dependent
bNAbs PG16, PGT145, 35O22 and PGT151 (Fig. 1J). However, in ELISA, there was a marked reduction in binding of the V3 non-NAbs 14e and 19b and the CD4bs non-NAb b6, while the CD4i non-NAbs 17b and 412d bound to a negligible extent (Table 2, Data S1E-H). Furthermore, although the SOSIP.v4 trimers still bound CD4-IgG2, they did not undergo CD4-induced conformational changes efficiently, as judged by the lack of induction of the 17b and 412d CD4i epitopes (Table 2, Data S1E-H).

In previous isothermal titration calorimetry (ITC) studies, the V3 non-NAb 19b IgG bound minimally to the BG505 SOSIP.664 trimers, with a stoichiometry of 0.2 (i.e., on average one IgG per 5 trimers), which suggest that a subpopulation of the trimers exposes V3, or that all trimers expose V3 transiently [1,22]. In contrast, 19b binding to the BG505 SOSIP.v4.1 mutant was abolished (Fig. 1G&H). Reactivity of 19b was also decreased for AMC008 SOSIP.v4 compared to wild-type; the stoichiometry of the 19b-trimer complex was 0.3 for wild-type (i.e., ~1 IgG per 3 trimers), but 0 (i.e., no complexes were detected) for SOSIP.v4.1 and negligible (~0.03; i.e., ~1 IgG per 30 trimers) for SOSIP.v4.2 (Fig. 1G&H). 19b binding to B41 SOSIP.v4 was also abolished (no detectable binding, compared to a stoichiometry of 0.2 for B41 SOSIP.664; Fig. 1G&H). Hence, the V3 region is effectively sequestered on SOSIP.v4 trimers.

To confirm their overall native-like structure and bNAb epitope presentation, we prepared complexes of the AMC008 SOSIP.v4.2 trimers with bNAbs PGV04 and 35O22 (added as Fabs), and visualized them by NS-EM. The stabilized trimers were all compact entities that were indistinguishable from previously published low-resolution reconstructions of BG505 and B41 SOSIP.664 trimers [1,22] (Fig. 3, Fig. S5).

**CD4 binding and CD4A-induced conformational changes**

In ELISA, CD4-IgG2 binding to AMC008, B41 and ZM197 SOSIP.v4 trimers was slightly reduced compared to SOSIP.664, and more markedly so for the BG505 trimers, while there was no significant change in binding of CD4bs bNAbs CH103 and VRC01. An SPR analysis showed that CD4-IgG2 reached lower plateaus earlier for the SOSIP.v4 trimers than for the wild-type trimers, and the ligand dissociated markedly faster from the SOSIP.v4 trimers (Fig. 1J). This finding is consistent with a report that the introduction of the H66N substitution into a gp120 core protein leads to a faster rate of sCD4 dissociation but no change in the association kinetics [49]. Biolayer interferometry data confirmed that the affinity of sCD4 for various stabilized, BG505 SOSIP.664 trimers was similar or slightly lower, with the greatest reduction (2.4-fold) seen with the A316W single mutant and the SOSIP.v4.2 double mutant (Fig. 1I).

The ELISA studies also showed that the sCD4-induction of the 17b and 412d CD4i epitopes was negligible for the AMC008, B41, ZM197M and BG505 SOSIP.v4 trimers (5-11% compared to 100% for their SOSIP.664 counterparts; Table 2, Data S1E-H). When 17b was added to CD4-IgG2-trimer complexes in an SPR study, it did not bind detectably to the BG505 SOSIP.v4 trimers, in marked contrast to its strong binding to the wild-type BG505 SOSIP.664 trimers under the same conditions (Fig. 1J). Thus, the stabilizing substitutions in the SOSIP.v4 trimers impede CD4-induced conformational changes.

**Conformational flexibility assessed by hydrogen-deuterium exchange**

We used hydrogen-deuterium exchange coupled with mass spectrometry (HDX-MS) to study
Chapter 7

Figure 2. HDX-MS analysis of BG505 SOSIP.664 and SOSIP.v4 trimers. Butterfly plots of PGT145-purified BG505 (A) SOSIP.664, (B) SOSIP.v4.1, (C) SOSIP.v4.2 trimers, comparing deuterium exchange levels in the presence and absence of sCD4. The percent exchange at each time point for each peptide is shown at its position along the primary sequence of the protein. Regions that are less (red) or more (blue) protected upon CD4-binding are mapped on the BG505 SOSIP.664 crystal structure [6], to depict CD4-induced conformational changes. The differences in CD4-induction between the SOSIP.664 and SOSIP.v4.1 or SOSIP.v4.2 trimers were also plotted on the crystal structure (rightmost panels of B and C). The exchange kinetics of individual peptides are shown in Fig. S4 and Data S1.
Figure 3. EM reconstruction of AMC008 SOSIP.v4.2 trimers in complex with PGV04 and 35O22 Fabs. Top and side view of AMC008.v4.2 trimers (blue) in complex with PGV04 (yellow) and 35O22 (red) Fabs. The reconstruction shows that AMC008.v4.2 trimers are compact and native-like. At the resolution obtained here, there is a very high degree of structural similarity with BG505 SOSIP.664 trimers in complex with 35O22 [EMDB-2672]. See also figure S5.

were very similar to those obtained previously for 2G12/SEC-purified BG505 SOSIP.664 trimers with only minor changes in exchange patterns proximal to the stabilizing mutations (Fig. 2, Fig. S4A&B) [3]. Hence, the E64K+A316W and H66R+A316W substitutions did not alter the overall structure and conformational dynamics of the trimers.

When sCD4 was added in molar excess, the deuterium-exchange patterns now differed markedly for SOSIP.v4 trimers compared to wild-type SOSIP.664 (Fig. 2, Data S1I). sCD4-binding to wild-type trimers resulted in greater protection of the CD4bs, but also of layers 1-3 in the gp120 inner domain and HR1 of gp41 (Fig. 2A). Thus, sCD4-binding orders and/or buries these regions, as reported previously [3]. Conversely, sCD4-binding led to decreased protection of the V2 and V3 loops as well as in several gp41 regions, which is consistent with a sCD4-induced opening of the trimer apex that eventually propagates and increases the accessibility of the gp41 fusion machinery (Fig. 2A). Regions of the trimer that are less (red) or more (blue) protected upon sCD4 binding are mapped onto the BG505 SOSIP.664 crystal structure (Fig. 2A-C).

In contrast, the above sCD4-induced changes were greatly attenuated or entirely abrogated when the SOSIP.v4 trimers were studied. The stabilizing substitutions appear, therefore, to block the ability of sCD4 to induce ordering of residues 370-382 (CD4bs), 245-256 and 476-483 (layer 3), 206-226 (layer 2), 53-92 (layer 1) and 566-592 (HR2; α7) (Fig. 2B&C, Data S1I). Furthermore, the substitutions reduced the sCD4-induced disorder of residues 165-181 (V2), 286-320 (V3), 520-537 (α6) and 593-628 (gp41 disulfide loop). Thus, the E64K+A316W and H66R+A316W substitutions restrain the sCD4-induced opening of the trimer apex, as well as the conformational cascade that spreads from the CD4bs via layer 3, layer 2 and layer 1 to gp41 HR1 (Fig. 2B&C), described as the allosteric “priming” network [3].
Reduced V3 antibody and Tier-1A neutralizing antibody responses to SOSIP.v4 trimers in mice and rabbits

Our principal goal was to reduce the exposure, and by inference the immunogenicity, of non-NAb epitopes such as V3. Accordingly, we immunized mice with BG505 SOSIP.664 or SOSIP.v4.1 trimers and analyzed the trimer- and V3-specific Ab binding titers two weeks after the third immunization (week 18; Fig. 4A). While trimer binding titers were similar (Fig. S6A), V3 binding Ab responses, as measured by a direct V3-peptide ELISA, were approximately 100-fold lower in SOSIP.v4.1-immunized mice compared to the SOSIP.664 recipients (p=0.039, Fig. 4B).

We also immunized rabbits with AMC008, BG505 and B41 SOSIP.664 trimers and their SOSIP.v4 counterparts, and quantified the Ab responses by ELISA two weeks after the third immunization (week 22; Fig. 4A). All the rabbit sera contained high titers of binding Abs against the corresponding SOSIP.664 and SOSIP.v4 trimers (Fig. S6B-D). When we used the direct V3 peptide ELISA we found that the V3 Ab responses were significantly reduced in the SOSIP.v4-immunized rabbits compared to SOSIP.664 (p=0.0015 for the comparison of all SOSIP.664 vs. SOSIP.v4; Fig. 4C&D). Similar results were obtained with a V3 peptide-competition ELISA (Fig. S6E).

Since Tier-1A NAb responses to BG505 SOSIP.664 trimers are dominated by V3-specificities [2], we expected that SOSIP.v4 trimers would induce lower, or less V3-dependent, titers of such NAbs. That was indeed the case; the titers of NAbs against the Tier-1A virus SF162 were significantly lower in SOSIP.v4 immunized rabbits compared to SOSIP.664 (by 14-, 8- and 10-fold for BG505, AMC008 and B41, respectively; p=0.0001 for the combined datasets; Fig. 4E&F; Tables S4 and S5). In contrast, there was no such reduction in the autologous NAb titers. Overall, strong autologous neutralization (ID50 > 500) was observed in 9 of 10 BG505 SOSIP.v4-immunized rabbits, with a weaker response in the 10th, and in 3 of 5 BG505 SOSIP.664 recipients (Fig. 4G&H; Table S5). Autologous NAb titers were observed in 4 out of 5 rabbits from each of the B41 SOSIP.664 and SOSIP.v4.1 immunized groups (Fig. 4G&H; Table S5). An autologous NAb response was also seen in the majority of the AMC008 trimer recipients. However, the AMC008 NAb titers were lower than the corresponding autologous responses to the BG505 and B41 trimers, despite AMC008 being the more sensitive virus (i.e., Tier-1B vs. Tier-2) (Fig. 4G&H; Table S5). Autologous neutralization strongly correlated with BG505 and AMC008 SOSIP.v4 trimer binding titers (Fig. S6F-I). For each of the trimer genotypes, the ratio of the SF162 (Tier-1A) and autologous NAb titers show that the Ab responses in the SOSIP.v4 immunized rabbits were directed away from the V3 region, compared to the responses elicited by the SOSIP.664 trimers (Fig. 4I&J; Table S4 &5). We also tested all the rabbit sera against a large panel of heterologous viruses and found that cross-neutralization of Tier-2 viruses was weak and sporadic, which is consistent with our findings using BG505 SOSIP.664 trimers [2].

Discussion

We re-designed native-like SOSIP.664 trimers to reduce their intrinsic “breathing”. The E64K/H66R and A316W substitutions stabilized the trimers in the closed, pre-fusion ground state, impeding or even preventing their spontaneous sampling of the CD4-induced conformation, and reducing the accessibility of V3. Two gp41 substitutions, I/V535M and L543N/Q, increased trimer formation. Together, these four substitutions improve the
Figure 4. Immunogenicity of AMC008, BG505 and B41 SOSIP.v4 trimers. (A) Immunization schedules. Mice were immunized at weeks 0, 4 and 16 (black arrows) and the Ab responses were analyzed at week 18 (red arrow). Rabbits were immunized at weeks 0, 4 and 20 (black arrows) and the Ab responses were analyzed at week 22 (red arrow). Blue symbols represent BG505 trimer-immunized animals, red symbols AMC008 and green symbols B41. The SOSIP.664 recipients are shown by closed circles, SOSIP.v4 by squares. Statistical comparisons between groups were performed using a two-tailed Mann-Whitney U test (*p>0.05, **p>0.01, ***p>0.001). (B) V3 peptide ELISA endpoint binding Ab titers in mouse sera are plotted. (C, D) V3 peptide binding responses were determined by ELISA. The midpoint binding Ab titers in rabbit sera are plotted in (C), and the normalized values of all SOSIP.664 and SOSIP.v4 values, compared to the mean of the corresponding SOSIP.664 group, are shown in (D). The midpoint (E, F) SF162 (Tier-1A), and (G, H) autologous Tier-2 (BG505 and B41) or Tier-1B (AMC008) neutralization titers were determined using the TZM-bl assay (Tables S4 and S5). Specifically, the serum dilutions at which HIV-1 infectivity is inhibited by 50% (ID$_{50}$) are plotted in (E) and (G), and the normalized values of all SOSIP.664 and SOSIP.v4 values, compared to the mean of the corresponding SOSIP.664 group, are shown in (F) and (H). (I) The ratios of autologous/SF162 NAb titers are shown for sera from the various SOSIP.664 and SOSIP.v4 immunized rabbits. (J) The normalized values derived from the data in (I) are plotted. See also figure S6 and table S4 and S5.
current SOSIP.664 design [1] and the probability of generating stable, native-like closed trimers for a range of Env sequences. These trimer improvement strategies allowed us to generated native-like trimers from a new Env sequence derived from a patient that developed a bNAb response. The AMC008 SOSIP.v4 trimers might be suitable starting points for lineage vaccines designed to mimic the development of neutralization in the patient.

Other mutation strategies, such as an I201C-A433C intra-gp120 disulfide bond and a A433P point substitution, also reduce non-NAb binding to BG505 SOSIP.664 trimers, increase their thermostability and constrain their conformational flexibility [8]. The immunogenicity of these modified trimers has not yet been reported. It is also not yet known whether these strategies are effective for trimers from other genotypes, or whether they can improve intrinsically lower quality trimers such as AMC008 SOSIP.664.

Our results provide insights into the functions of the Env trimer. The A316W, E64K and H66R changes do not affect the folding or overall structure of soluble, SOSIP.664 trimers as the resulting structures are very similar to wild-type, but they do ablate infectivity when introduced into viral Env. The likely explanation is that residues E64 and H66 are critical for facilitating CD4-induced conformational changes and the formation of the CD4i epitopes associated with the co-receptor binding site that are essential for virus-cell entry but not important in the soluble trimer context. Other substitutions at position 66 (H66N and H66A) are known to increase the resistance of HIV-1 to low-temperature inactivation by decreasing spontaneous sampling of the CD4-induced conformation, which was previously described as “intrinsic Env reactivity” [49–51].

In the context of an infectious virus, V3 needs to be accessible and dissociate from the V1V2 domain, perhaps as an integral part of the entry process. Native-like SOSIP.664 trimers appear to retain this natural plasticity, such that their V3 regions become exposed under certain conditions [1,2]. The same flexibility in the orientation of the V1, V2 and V3 loops at the trimer apex may underpin the transitions between fully closed and partially open versions of some native-like SOSIP.664 trimers (e.g., B41) that we have described elsewhere [22]. We now show that these spontaneous conformational transitions in soluble trimers can be impeded by the introduction of stabilizing changes.

In conclusion, by preventing the conformational changes normally required for Env function, we have further stabilized recombinant, native-like trimers now designated SOSIP.v4. The principal rationale for the stabilization strategy was that it might be beneficial for generating protective humoral immune responses. Antigenicity studies showed that bNAb epitopes, in particular those that are dependent on quaternary structure, were better presented on the SOSIP.v4 trimers while non-NAb epitopes such as V3, which might serve as immunological decoys, were occluded. The reduced antigenicity of the V3 region had an immunological correlate, in that the SOSIP.v4 trimers induced weaker anti-V3 responses in rabbits and, as a result, lower titers of V3-dependent NAb against the Tier-1A virus SF162. This outcome was achieved without compromising the autologous NAb response. As noted earlier, a single native-like trimer of any genotype will probably not be sufficient to induce bNAb. However, vaccination regimens that include germline targeting, evolutionary lineages, and/or multivalent trimers are strategies towards bNAb generation that merit pursuing [16,26–30,52]. In all of these vaccination scenarios, it is likely that reducing the immunogenicity of immunodominant non-NAb epitopes in favor of the subdominant bNAb epitopes, such as through the generalizable trimer-stabilizing mutations we describe here,
The improved stability of SOSIP.v4 trimers might also improve their in vivo half-life or how they are affected by certain adjuvants, thereby maximizing the opportunity for naïve B cells to encounter bNAb epitopes. The added resistance to CD4-induced opening, which may occur in vivo when macaques or humans (but not rabbits) are immunized, would further help maintain the closed, immunologically relevant conformation of the stabilized trimer. Overall, we propose that the stabilized SOSIP.v4 trimers are promising Env vaccine candidates for evaluation in various strategies intended to induce heterologous Tier-2 NAb responses.

Methods and Experimental Procedures

Trimer design, expression and purification
The AMC008 env gene is derived from a subtype B Tier-1B virus, isolated 8 months post-seroconversion from an elite neutralizer in the Amsterdam Cohort Studies on HIV/AIDS (patient H18818 in reference [53]). The design of AMC008 SOSIP.664 trimers is identical to the BG505, B41 and ZM197M SOSIP.664 constructs described elsewhere [1,22,23]. For more details see SI Methods.

Antigenicity assays
Antigenicity was determined using tagged SOSIP.664 trimers for SPR or D7324-capture ELISA, as described elsewhere [1,44,54]. In ELISA, the D7324 antibody was attached to the solid phase and used to capture the epitope-tagged trimers, which were then recognized by various solution-phase MAbs. ITC was used to generate data on MAb-trimer interactions in solution [18,55].

Thermostability assays
As a screening assay, we used a PCR machine-based system to expose unpurified SOSIP.664-D7324 trimers to a range of temperatures, before using D7324-capture ELISA to assess when the 2G12 epitope had been lost. A conventional DSC assay was used to quantify the melting of purified trimers, as described previously [22]. The two methods yielded data on multiple trimers that correlated well.

Biophysical techniques
Multiple biophysical assays were used to analyze the properties of various SOSIP.664 trimers, including DLS, SAXS and HDX-MS as described elsewhere [3,56]. Images of purified SOSIP.664 trimers, either alone or as Fab complexes, were generated by NS-EM following previously described procedures [1]. A more detailed description of the EM process can be found in SI Methods.

Mouse and rabbit immunizations
Mice and rabbits were immunized with 10 or 22 µg of trimer, respectively, formulated with ISCOMATRIX™, at weeks 0, 4 and 16 (mice) or 0, 4, and 20 (rabbits). For details see SI Methods.
Acknowledgments

We thank Hermann Katinger, Mark Connors, James Robinson, Dennis Burton, John Mascola, Peter Kwong and William Olson for donating antibodies and reagents directly or through the NIH AIDS Research and Reference Reagent Program. The Amsterdam Cohort Studies on HIV infection and AIDS, a collaboration between the Amsterdam Health Service, the Academic Medical Center of the University of Amsterdam, Sanquin Blood Supply Foundation, Medical Center Jan van Goyen and the HIV Focus Center of the DC-Clinics, are part of the Netherlands HIV Monitoring Foundation and financially supported by the Center for Infectious Disease Control of the Netherlands National Institute for Public Health and the Environment. The EM reconstruction data reported in this paper have been deposited in the Electron Microscopy Data Bank, www.emdatabank.org (EMDB ID code EMDB-6500).

Funding

This work was supported by National Institutes of Health Grants P01 AI82362 and P01 AI110657, R21 AI112389, UM1 AI100663 (Scripps CHAVI-ID) and NIAID Contract # HHSN27201100016C (to DCM); by the International AIDS Vaccine Initiative (IAVI); by the Bill and Melinda Gates Foundation through the Collaboration for AIDS Vaccine Discovery (CAVD) and by the Aids fonds Netherlands, Grant #2012041. R.W.S. is a recipient of a Vidi grant from the Netherlands Organization for Scientific Research (NWO) and a Starting Investigator Grant from the European Research Council (ERC-StG-2011-280829-SHEV). The electron microscopy data were collected at The Scripps Research Institute Electron Microscopy Facility.

Author Contributions

Conceived and designed the experiments: SWdT, GO, ATdP, TvdK, PP, IB, JH, HA, JS, WCK, HS, DE, BB, HD, CL, SC, MC, DCM, PJK, KKL, JPM, IAW, ABW, RWS
Performed the experiments: SWdT, GO, ATdP, MG, JPJ, JLT, LKP, JAB, AY, CL, JC
Analyzed the data: SWdT, GO, ATdP, MG, JPJ, SC, PJK, JPM, IAW, ABW, RWS
Wrote the paper: SWdT, GO, JPM, IAW, ABW, RWS
Edited the paper: SWdT, GO, JPM, IAW, ABW, RWS, MG, KKL, MC, LKP, PJK, JPI, WCK, BB, CL

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8. Do Kwon, Y., Pancera, M., Acharya, P., et al. Crystal structure, conformational fixation and entry-related...


Cell

Supplemental Information

Immunogenicity of Stabilized HIV-1 Envelope Trimers with Reduced Exposure of Non-neutralizing Epitopes

Figure S1. Screening of stabilizing mutations using unpurified AMC008, BG505, B41 and ZM197M SOSIP.664 proteins, related to figure 1. BN-PAGE analysis of unpurified (A) AMC008, (B) BG505, (C) B41 SOSIP.664 variants, followed by Western blotting with 2G12. In the AMC008 SOSIP.664 context, the A316W, I535M and L543N substitutions make a substantial contribution to trimer formation, both individually and collectively. We note that introducing the reverse N543L substitution into BG505 SOSIP.664 reduced trimer formation (data not shown). (D) Use of a novel thermostability assay to screen unpurified BG505 SOSIP.664-D7324 variants. Culture supernatants from 293T cells were incubated for 30 min at temperatures ranging from 39–77°C, then analyzed by D7324-capture ELISA with 2G12 detection. The binding of 2G12 is plotted as a function of temperature, with the OD value at 39°C set at 100%. The A316W substitution increases thermostability. (E) The unfolding pattern of the variant proteins was visualized by plotting the first derivative of the graph in (D) using GraphPad prism 5. (F) The Tm values were derived from the peaks in the first derivative curves shown in (D). The Tm values obtained in this assay are highly reproducible (standard deviation of ± 0.3°C for BG505 SOSIP.664-D7324). (G) Correlation plot between Tm values obtained using unpurified BG505 SOSIP.664-D7324 proteins in the above thermostability assay and Tm values obtained using PGT145-purified BG505 SOSIP.664-D7324 trimers in a DSC assay (Table 1; Fig. S2E). (H) Competition ELISA using wild-type and A316W-substituted BG505-V3 peptides. Increasing concentrations of wild-type (light green) or A316W-substituted (dark green) peptides were incubated with the V3 non-NAb 39F (1.0 µg/ml) prior to addition to immobilized, PGT145-purified BG505 SOSIP.664-D7324 trimers. As the two peptides were equally effective at binding 39F and impeding its binding to the trimers, the A316W substitution does not directly affect the 39F epitope. Similar results were obtained with two other V3 non-NAbs, 19b and 14e (data not shown). (I) High-throughput mutagenesis screen at position 316 of BG505 SOSIP.664-D7324, with a capture ELISA endpoint. Wild-type or position 316-mutated variant Env proteins in culture supernatants from transiently transfected 293T cells were captured via D7324. The bound proteins were detected using either PGT145 (0.11 µg/ml; to detect native trimers) or 14e (0.5 µg/ml; to detect V3 exposure), and the ratios of the OD signals were plotted. The wild-type residue (alanine) is shown in grey, the designed mutant (tryptophan) in red and other substituted amino acids in black. Only bulky hydrophobic amino acids reduce V3 non-NAb binding. (J, K) Spontaneous sampling of the CD4i conformation was assessed using a D7324-capture ELISA. CD4i epitope exposure was assessed by measuring the binding of high concentrations (10 µg/ml) of non-NAbs (J) 17b and (K) 412d. The OD values obtained using the wild-type BG505 SOSIP.664-D7324 protein were set at 100% and used to normalize the values for the E64K and H66R mutants. Mock indicates blank transfection supernatant.
Stabilization of HIV-1 Env trimers from different clades

Figure S2. The effect of stabilizing mutations on the biochemical and biophysical properties of AMC008, BG505, B41 and ZM197M SOSIP.664 proteins, related to table 1. (A) The PGT145-purified SOSIP.664 proteins were analyzed by BN-PAGE analysis and found to be exclusively trimeric. (B) Reducing (+DTT) and non-reducing (-DTT) SDS-PAGE analysis of the same proteins. The complete conversion of gp140 bands into gp120 when DTT is present indicates gp120-gp41 cleavage is efficient; there are also no indications that the purified trimers form higher m.wt. disulfide-linked oligomers. (C) Negative-stain EM analyses of 2G12/SEC-purified (top left panel) or PGT145-purified (all other panels) AMC008, BG505, B41 and ZM197M SOSIP.664 trimer variants. The 2D class averages are shown. Based on loop-movement, compactness and angles between individual protomers, the trimers are classified as closed native-like, partially open native-like or non-native [1]. Native-like trimers are regularly shaped and have the highest concentration of electron density at the particle center (usually shaped like a triangle because Env is trimeric). The absence or presence of additional density around this center of mass determines whether trimers are classified as closed native-like or partially open native-like, respectively. Non-native forms are often elongated and no triangular center of density is visible. The classifications are quantified below each panel. The percentages of closed and partially open native-like trimers are in green, and of non-native forms in red. The total number of particles classified is defined as 100%. We observe +/-5% variation between experiments.
Figure S3. The effect of stabilizing mutations on the biochemical and biophysical properties of AMC008, BG505, B41 and ZM197M SOSIP.664 proteins, related to table 1. (A) SEC profiles of 2G12- or PGT145-purified BG505 SOSIP.664 and SOSIP.v4.1 trimers. (B) DSC analysis of PGT145-purified AMC008, BG505, B41 and ZM197M SOSIP.664 trimer variants. The unfolding patterns were fitted using a non-two state model, revealing three individual unfolding peaks. The $T_m$ values of the individual peaks are listed in Table S1. For simplification, the data were also fitted to a two-state model and the $T_m$ values of those analyses are provided in Table 1. (C) SAXS analysis of PGT145-purified BG505 SOSIP.664 and SOSIP.v4 trimers. The merged SAXS data are shown for the BG505 trimers (upper panel). Lower panel: summary of SAXS and DLS derived structural parameters. The radii of gyration ($R_g$) were derived from SAXS experiments using both Guinier analysis with a $q^*$, $R_g$ range of 1 - 1.3, and from the particle distance distribution plots obtained from indirect transformation using GNOM [19]. The standard deviations of the $R$ and $R_g$ values were small, and similar between the different constructs (+/- 0.2-0.5 Å). (D) Glycan profiles of PGT145-purified SOSIP.664 variants as determined by HILIC-UPLC. The percentages of Man$_5$-GlcNAc$_2$ glycans (M5-M9), as a proportion of the total glycan population, are listed in Table 1.
Figure S4. The effect of stabilizing mutations on HDX profiles of BG505 SOSIP.664 trimers, related to figure 2. (A, B) Left panels: Butterfly plots of HDX profiles in the absence of CD4. (A) PGT145-purified BG505 SOSIP.664 vs. SOSIP.v4.1 trimers; (B) PGT145-purified BG505 SOSIP.664 vs. SOSIP.v4.2 trimers. Right panels: The differences in exchange patterns are mapped onto the higher resolution BG505 SOSIP.664 structure (Pancera et al., 2014). Regions that are less or more protected from deuterium exchange are colored red and blue, respectively. The positions of the stabilizing mutations are indicated in green. (C) BN-PAGE gel-shift analysis of BG505 SOSIP.664 wild-type and double mutant trimers in the presence of sCD4 (0.55 mg/ml; i.e., in 9-molar excess per trimer. For all three trimers, there is a complete band-shift in the presence of sCD4, which shows that saturating binding of CD4 occurs in each case. However, note that the wild-type trimers dimerize when CD4 binds, but the two double mutants do not. The large conformational changes upon CD4 binding in the wild-type protein might result in dimerization via the flexible variable loops, which is not the case for the mutants, as conformational changes are largely prevented upon CD4 binding.
Figure S5. Negative-Stain EM of PGV04 and 35O22 Fabs bound to AMC008 SOSIP.v4.2, related to figure 3. (A) 2D class averages and (B) 2D projections of the 3D model. (C) Fourier shell correlation (FSC) curve of the refined map. The resolution of the final map calculated from the FSC function at 0.5 is 15 Å.
Stabilization of HIV-1 Env trimers from different clades

Figure S6: Ab responses in SOSIP.664 or SOSIPv4.1 immunized mice and rabbits, related to figure 4. (A) Ab responses to BG505 SOSIP.664 trimers were quantified in mouse sera drawn at week-18 by D7324-capture ELISA, using BG505 SOSIP.664-D7324. The mice were immunized with PGT145-purified BG505 SOSIPv4.1 or BG505 SOSIP664 trimers at weeks 0, 4, and 16. Midpoint binding Ab titers in rabbit sera were also measured by D7324-capture ELISA, using (B) SOSIP.664-D7324 or (C) SOSIP.v4-D7324 trimers as the test antigen. Rabbits were immunized at week 0, 4 and 20 and the Ab responses were analyzed at week 22. Blue symbols represent BG505 trimer-immunized animals, red symbols AMC008. The SOSIP.664 recipients are shown by closed circles, SOSIP.v4 by squares. (D) The ratios of the SOSIP.664/SOSIP.v4 midpoint titers are plotted. (E) As an alternative to direct V3-peptide ELISA, the V3 Ab response was also determined by pre-incubating the sera with a cyclized V3 peptide before determining the residual anti-trimer binding titers by ELISA (see methods). The relative V3 Ab responses (as a % of the total anti-trimer Ab responses) were calculated by comparing the midpoint titers in the absence and presence of the V3 peptide. Correlation plots between autologous BG505 NAb titers and either (F) BG505 SOSIP.664 binding Ab titers, or (G) BG505 SOSIP.v4 binding Ab titers. The correlation was slightly stronger when the NAb titer comparison was with SOSIP.v4-D7324 binding Ab titers. Correlation plots between autologous AMC008 NAb titers and either (H) AMC008 SOSIP.664 binding Ab titers, or (I) AMC008 SOSIP.v4 binding Ab titers. The strong correlation with the binding Ab titers to the AMC008 SOSIP.v4-D7324 trimers implies that AMC008 SOSIP.v4 trimers present neutralization-relevant epitopes in vivo more efficiently than their SOSIP.664 counterparts. The r and p values for non-parametric Spearman correlations are shown. Statistical comparisons between groups were performed using a two-tailed Mann-Whitney U test (*p>0.05, **p>0.01, ***p>0.001).
Supplementary Tables

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<th>Stabilizing mutations</th>
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Table S1: Detailed DSC analysis of PGT145-purified SOSIP trimer variants, related to table 1. In addition to the simplified two-state model analysis, the unfolding patterns were fitted using a non-two state model (see Fig. S3B), revealing three individual unfolding peaks. The T_m values of the individual peaks are given in °C.
Table S2. Amino acids present at positions 535 and 543 of the α6 helix in gp41, related to figure 1. The amino acids present in the wild-type sequences of clones JR-FL, KNH1144, BG505, B41, ZM197M and AMC008 at positions 535 and 543, as well as the amino acids optimal for Env stability (in green; [33,34]) are shown. The right-most column specifies the amino acid changes that have now been incorporated into the various SOSIP.664 constructs to optimize trimer formation (resulting in the SOSIP.v3 trimers).

Table S3. Nomenclature for new stabilized SOSIP.664 trimers, related to figure 1. An overview of the modifications made to the stabilized SOSIP.664 trimer variants. Green, present; red, not present. SOSIP.681 and SOSIP.664 were renamed to SOSIP.v1 and SOSIP.v2 respectively. R6 refers to an improved hexa-arginine furin cleavage site [7].
Table S4. Midpoint neutralization titers for sera from rabbits immunized with SOSIP.664 or SOSIP.v4 trimers tested against a panel of Env-pseudotyped viruses, related to figure 4.

Table S5. Midpoint neutralization titers for sera from immunized rabbits with SOSIP.664 or SOSIP.v4 trimers and tested against a panel of Env-pseudotyped viruses, related to figure 4. The TZM-bl cell assay was performed at DUMC. ID\textsubscript{50} values, i.e., the week-22 serum dilution at which infectivity was inhibited by 50%, are recorded, with the boxes colored according to their magnitude. White, no neutralization, ID\textsubscript{50} <40; yellow, weak neutralization, ID\textsubscript{50} 40-100; orange, moderate neutralization, ID\textsubscript{50} 100-1000; red, strong neutralization, ID\textsubscript{50} >1000.
SI Experimental Procedures

Construct design
The BG505, B41, and ZM197M SOSIP.664 constructs have been described elsewhere [1–3]. The AMC008 env gene is derived from subtype B virus, isolated 8 months post-seroconversion from an individual in the Amsterdam Cohort Studies on HIV/AIDS who eventually developed broadly neutralizing antibodies (patient H18818 in [4]). The AMC008 SOSIP.664 gp140 construct was designed as previously described [1–3], by introducing the following sequence changes: A501C and T605C (gp120-gp41 disulfide bond; [5]); I559P in gp41 (trimer-stabilizing; [6]); REKR to RRRRRRR in gp120 (cleavage enhancement; [7]); a stop codon at gp41 residue 664 (improvement of homogeneity and solubility; [8]). SOSIP.664-D7324 trimers contain a D7324 epitope-tag sequence at the C-terminus of gp41 and were made by adding the amino-acid sequence GLNDIFEAQKIEWHE after residue 664 in gp41 [2]. Similarly, biotinylated SOSIP.664-aviB trimers were generated by adding the avidin (avi) sequence GLNDFEQQKIEWHE after residue 664, followed by biotinylation (aviB) as described [9]. Point mutants were made by Quikchange site directed mutagenesis (Agilent, Stratagene), and verified by sequencing. All experiments used D7324-tagged trimers, except for DLS, SAXS, HDX-MS and ITC studies (untagged trimers), SPR (His-tagged trimers) and Octet assays (aviB-tagged trimers).

Construction of an AMC008-LAI chimeric molecular clone
The molecular clone of LAI was used as the backbone to construct a chimeric virus [10]. This LAI clone contains a unique SacI restriction site 434 nucleotides upstream of the env start codon and a unique BamH1 site at the codons specifying amino acids G751 and S752 in LAI gp160 (HXB2 numbering). A DNA fragment was synthesized that contained the LAI sequences between the SalI site and the env start codon, followed by the AMC008 env sequence up to the BamH1 site (Genscript). The fragment was then cloned into the LAI molecular clone backbone using SalI and BamH1 as digestion enzymes. The resulting molecular clone encodes the complete AMC008 gp160 sequence, except for the C-terminal 106 amino acids of the cytoplasmic tail, which are derived from LAI gp160. The authenticity of the clone was verified by sequencing. Infectious virus was produced from the clone by transfection and used in virus infectivity and neutralization assays based on TZM-bl cells as described below. When the neutralization-sensitivity of the AMC008 –LAI chimeric virus was assessed using a panel of human sera and MAbs and a panel of test viruses at the Duke University Medical Center, it was classified as being in Tier-1B (not shown). Note that the various AMC008 SOSIP.664 constructs are based on the same env sequence that is present in the AMC008 chimeric molecular clone.

Env protein expression
Proteins encoded by the various env genes described above were expressed in adherent 293T cells, or in 293F or 293S (GntI−) variants adapted for suspension cultures, essentially as described [2,11]. All experiments with purified trimers used 293F cell-expressed proteins, except for ITC assays when the producer cell was, in most cases, 293S. The 293T or 293S cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml). For trimer expression on a small-scale, 293T cells were seeded at a density of 5.5×10⁶/ml in a 6-well plate. The next day, when the cells had reached a density of 1.0×10⁶/ml, they were transfected using polyethyleneimine (PEI), as previously described [12]. Briefly, PEI-MAX (1.0 mg/ml) in water was mixed with expression plasmids for Env and Furin [5] in OPTI-MEM (Gibco). For one well, 3.75 µg of Env plasmid, 1.25 µg of Furin plasmid and 12.5 µl PEI-MAX (1mg/ml) were added in 3 ml of growth media (DMEM + 10% FCS + penicillin and streptomycin). Culture supernatants were harvested 72 h after transfection. For larger-scale production, Env proteins were produced in 293F cells using a protocol similar to that described previously [2,11]. Briefly, PEI-MAX (1.0 mg/ml) in water was mixed with expression plasmids for Env and Furin in OPTI-MEM. For cultures in a 2L disposable Nalgene flask (VWR), 250 µg of Env plasmid, 62.5 µg of Furin plasmid and 0.94 mg of PEI-MAX were added to 1L of pre-warmed Free-style 293 expression medium (Life Technologies). 293F cells were cultured for 6–7 days at 37°C, in an atmosphere containing 8% CO₂ and at a rotation speed of 125 rpm.

Trimer purification
Env proteins were purified from transfection supernatants by affinity chromatography using a PGT145- or a 2G12-column, essentially as described [1,2,11]. The columns were made by coupling PGT145 or 2G12 to CNBr-activated Sepharose 4B beads (GE Healthcare). Briefly, supernatants were vacuum filtered through 0.2-µm filters and passed (0.5–1 ml/min flow rate) through the column, which was then washed with 2 column volumes of buffer (0.5 M NaCl, 20 mM Tris, pH 8.0). Bound Env proteins were eluted using 1 column volume of 3 M MgCl₂ and then immediately buffer-exchanged into 75 mM NaCl, 10 mM Tris, pH 8.0, using Vivaspin-20 tubes. The proteins were concentrated using Vivaspin columns with a 30-kDa cut off (GE Healthcare). 2G12-purified Env proteins were
Further fractionated by size exclusion chromatography (SEC) to obtain pure trimers, whereas the PGT145 column yielded pure trimers without the need for SEC. Protein concentrations were determined using UV absorbance and theoretical extinction coefficients via Expasy (ProtParam tool). All experiments were performed with PGT145-purified trimers except for ITC and Octet studies, which used 2G12/SEC-purified trimers.

Neutralization assays
The TZM-bl reporter cell line, which stably expresses high levels of CD4 and the co-receptors CCR5 and CXCR4 and contains the luciferase and β-galactosidase genes under the control of the HIV-1 long-terminal-repeat promoter, was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (John C. Kappes, Xiaoyun Wu, and Tranzyme Inc., Durham, NC). The assays at DUMC were performed essentially as described in protocols at: http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm. Assays performed at AMC had the following modifications. For use in virus production, 293T cells (2×10⁵) were seeded in a 6-well tissue culture plate (Corning) in 3 ml of DMEM (Gibco) containing 10% FCS, penicillin (Sigma) and streptomycin sulphate (Gibco) (both at 100 U/ml) per well. The culture was refreshed after 1 d by adding 3 ml of culture medium when the cells had reached a confluence of 90–95% and were ready for transfection. To make Env-pseudotyped viruses, the following expression plasmids were added to 240 µl of OPTI-MEM (Gibco) and 10 µl of lipofectamine 2000 (Invitrogen) per well: 1.6 µg of BG505.T332N Env plasmid and 2.4 µg of pSG3ΔEnv plasmid (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. Durham, NC)). To make infectious chimeric viruses by transfection the same procedure was used except that 4 µg of a molecular clone plasmid was added per well. After incubation for 20 min at room temperature, the transfection mixture was added to the cells, and the culture supernatants were harvested 48 h later as the source of Env-pseudotyped or infectious chimeric viruses for infection and/or neutralization experiments.

One day prior to virus infection, 1.7×10⁵ TZM-bl cells per well were seeded in a 96-well plate in DMEM containing 10% FCS, 1× MEM nonessential amino acids, penicillin and streptomycin (both at 100 U/ml), and incubated at 37°C for 24 h in an atmosphere containing 5% CO₂. To determine neutralization activity of rabbit sera, a fixed amount of virus (500 pg of p24-antigen equivalent) was incubated for 1 h at room temperature with heat-inactivated sera (3-fold serial dilutions starting at 1:20). The mix was then added to the cells in the presence of 40 µg/ml DEAE-Dextran (Sigma) and Saquinavir, in a total volume of 200 µl. Three days later, the medium was removed and the cells were washed once with PBS (150 mM NaCl, 50 mM sodium phosphate, pH 7.0) and lysed in Reporter Lysis Buffer (Promega). Luciferase activity was measured using a Luciferase Assay kit (Promega) and a Glomax Luminometer according to the manufacturer’s instructions (Turner BioSystems). All infections were performed in duplicate. Uninfected cells were used to correct for background luciferase activity. To determine the V3 specificity of the NAb responses, sera were first incubated for 1 h at room temperature with 1mg/ml of a V3 peptide (BG505: TRPNNTRRKSIIRGQPQAFYATGDIRGDIQRQAH or AMC008: TRPNNTRRKSIINIGPGRAFYTTGEIIGDIRQAH). The residual NAb titers were then quantified as described above. The infectivity of each virus in the absence of serum was set at 100%. Nonlinear regression curves were determined and the 50% inhibitory serum dose (ID₅₀) was calculated using a sigmoid function in Prism software version 5.0.

SDS-PAGE
Env proteins were analyzed using SDS-PAGE followed by western blotting or Coomassie blue dye staining [6,14]. The input material was mixed with loading dye (25 mM Tris, 192 mM Glycine, 20% v/v glycerol, 4% m/v SDS, 0.1% v/v bromophenol blue in milli-Q water) and incubated at 95°C for 5 min prior to loading on a 4-12% or 8% Tris-Glycine gel (Invitrogen). For reducing SDS-PAGE, dithiothreitol (DTT; 100 mM) was included in the loading dye. The gels were run for 2h at 125 V (0.07 A) using 50 mM MOPS, 50 mM Tris, pH 7.7 as the running buffer (Invitrogen). Western blot analysis of SDS-PAGE gels using mouse MAb ARP3119 (1:2,000 dilution, i.e. 0.2 µg/ml), followed by HRP-labeled goat anti-mouse IgG (1:5000; Jackson Immunoresearch) was performed as previously described [6]. The Western Lightning ECL system (PerkinElmer Life Sciences) was used for luminometric detection. Coomassie blue staining of SDS-PAGE gels was performed using the PageBlue Protein Staining Solution (Thermo Scientific).

Blue Native-PAGE
For BN-PAGE [6,14], the input Env proteins were mixed with loading dye (500µl 20x MOPS Running Buffer (1M MOPS + 1M Tris, pH 7.7)+ 1000µl 100% Ultrapure Glycerol (Invitrogen cat#15514-011)+ 50µl 5% Coomassie Brilliant Blue G-250 + 600µl milli-Q water) and directly loaded onto a 4–12% Bis-Tris NuPAGE gel. The gels were run for 1.5 h at 200 V (0.07 A) using Anode-Buffer (20x NativePAGE Running Buffer (Invitrogen) in milli-Q water) and Cathode-Buffer (1% NativePAGE Cathode-Buffer Additive in Anode-Buffer; both from Invitrogen). Western blot analysis of SDS-PAGE gels using mouse MAb ARP3119 (1:2,000 dilution, i.e. 0.2 µg/ml), followed by HRP-labeled goat anti-mouse IgG (1:5000; Jackson Immunoresearch) was performed as previously described [6]. The Western Lightning ECL system (PerkinElmer Life Sciences) was used for luminometric detection. Coomassie blue staining of SDS-PAGE gels was performed using the PageBlue Protein Staining Solution (Thermo Scientific).
analysis of BN-PAGE gels was carried out using human MAb 2G12 (0.1 µg/ml), followed by HRP-labeled goat anti-human IgG (1:5,000 dilution, Jackson Immunoresearch and the Western Lightning ECL system (PerkinElmer Life Sciences), essentially as described previously [6]. BN-PAGE gels were stained using the Colloidal Blue Staining Kit (Life Technologies).

Surface plasmon resonance (SPR)
SPR was performed on a Biacore 3000 instrument at 25°C, using HBS-EP (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.002% P20 surfactant) as the running buffer (GE Healthcare). The D7324 antibody (Aalto BioReagents) was coupled to the chip as previously described [15], to an immobilization level of 9,000 resonance units (RU). The flow rate was adjusted to 10 µl/min. The ligands, i.e., D7324-tagged BG505 SOSIP.664, v4.1 and v4.2 trimers, diluted in running buffer to 20 µg/ml, were then captured, giving immobilization levels (Rₙ values) of 300 RU. A control channel into which no trimers were injected was used for background subtraction. In the simple binding assessment, the analytes, i.e., the test MAb or CD4-IgG2 (500 nM, diluted in HBS-EP), were allowed to associate with the trimers for 5 min before dissociation was recorded for 10 min. The flow rate was 50 µl/min throughout each run. After each cycle, the surface was regenerated by a 60 s injection of 10mM Glycine [pH 2.0], at a flow rate of 30 µl/min. Induction of conformational changes by CD4 was studied in the SPR format by injecting two analytes in a single cycle. The first analyte (CD4-IgG2; 500 nM) was injected for 200 s followed by the second (17b; 500 nM) for a further 200 s, both at a flow rate of 30 µl/min. After each cycle, the surface was regenerated as described above, before fresh trimers were captured for the next run.

D7324-capture ELISA
This method has been described elsewhere [2,16]. Microlon-600 96-well, half-area plates (Greiner Bio-One) were coated overnight with Ab D7324 at 10 µg/ml in 0.1 M NaHCO₃, pH 8.6 (50 µl/well). After washing and blocking steps, purified BG505 SOSIP.664-D7324 trimers were added at 2 µg/ml in TBS for 2 h. Unbound trimers were removed by 2 wash steps with TBS before various concentrations of test Abs were added for 2 h. After 3 washes with TBS, HRP-labeled goat anti-human IgG (Jackson Immunoresearch) was added at a 1:3000 dilution in TBS/2% skimmed milk for 1 h, followed by 5 washes with TBS/0.05% Tween-20. Colorimetric detection was performed using a solution containing 1% 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich), 0.01% H₂O₂, 100 mM sodium acetate and 100 mM citric acid. Color development (absorption at 450 nm) was stopped using 0.8 M H₂SO₄ (25 µl) when a plateau value was reached in the two wells containing the highest Ab concentration. In some experiments (Fig. S1, Data S1), MAb 17b or 412d were added to the immobilized Env proteins in the presence of two-domain scCD4 (1.0 µg/ml). In other experiments (Fig. S1H), a fixed concentration of 14e (0.1 µg/ml), 19b (0.6 µg/ml) or 39F (1.0 µg/ml) was incubated in solution with escalating concentrations of V3 peptides (Genscript) before adding the mixture to the ELISA plate. The V3 peptides (wild-type: CTRPNNNTRKSIRIGPQAFYATGDIGDIRQAHC; A316W: CTRPNNNTRKSIRIGPQQWYATGDIGDIRQAHC) were cyclized by a disulfide bond between residues 1 and 35. To quantify antibody responses in immunized rabbits, sera (from week-22) were serially diluted in 3-fold steps from a 1:100 start point, using 40% sheep serum (Biotrading) and 2% milk powder in TBS as the buffer. When V3-directed Ab responses were analyzed, the sera were incubated in solution with a V3 peptide (1 µg/ml) for 1 h prior to adding the mixture to the test wells. The peptide sequences were identical to the V3 region of the immunogen. As with the BG505 V3 peptides described above, the peptides were cyclized by a disulfide bond between residues 1 and 35. The sequences of the AMCN08 peptides were (wild-type: CTRPNNNTRSKIINGPQARFTGGEIGDIRQAHC; A316W: CTRPNNNTRSKINGPQARFTGGEIGDIRQAHC). The secondary antibody was goat anti-rabbit IgG (Jackson Immunoresearch), and the color development procedures were as described above.

V3 peptide ELISA
To determine V3 Ab responses in BG505-immunized mice and rabbits, 96-well MaxiSorp plates were coated with 2 µg/ml of a cyclized V3-peptide in PBS, by incubation overnight at 4°C. The V3 peptide was based on the BG505, AMC008 or B41 sequence, as appropriate, either unmodified or including the A316W change present in SOSIP.v4 trimers. To quantify antibody responses in immunized rabbits, sera (from week-22) were serially diluted in 3-fold steps from a 1:100 start point, using 40% sheep serum (Biotrading) and 2% milk powder in TBS as the buffer. When V3-directed Ab responses were analyzed, the sera were incubated in solution with a V3 peptide (1 µg/ml) for 1 h prior to adding the mixture to the ELISA plate. The V3 peptides (wild-type: CTRPNNNTRKSIRIGPQAFYATGDIGDIRQAHC; A316W: CTRPNNNTRSKINGPQARFTGGEIGDIRQAHC) were cyclized by a disulfide bond between residues 1 and 35. The sequences of the AMCN08 peptides were (wild-type: CTRPNNNTRSKIINGPQARFTGGEIGDIRQAHC; A316W: CTRPNNNTRSKIINGPQARFTGGEIGDIRQAHC). The secondary antibody was goat anti-rabbit IgG (Jackson Immunoresearch), and the color development procedures were as described above.

ELISA-based thermostability assay
As a screening assay for trimer stability, we devised a new thermal melting assay suitable for use on culture supernatants containing unpurified BG505 SOSIP.664-D7324 trimers (SI Fig. 1D-G). Supernatants (typically 60 µl containing ~15 µg/ml of total Env protein) were incubated for 30 min in a temperature gradient ranging from 39-77°C using a G-storm PCR machine (GRI Lab Care). The supernatants were then transferred to a 96-well plate
and a D7324-capture ELISA was performed as described above. The 2G12 detection MAb was used at 0.1 µg/ml, a concentration that gives ~75% of the maximal signal in this ELISA format and, hence, allows any temperature-dependent loss of 2G12 reactivity to be quantified. When multiple, unpurified BG505 SOSIP.664-D7324 trimer variants were tested, the results from this screening assay correlated well with the outcome of DSC experiments using the same (but purified) trimers (Fig. S1G). However, the midpoints of thermal denaturation ($T_m$) in the present assay were consistently 3-5°C lower than obtained via DSC, probably because the 2G12 epitope unfolds or is perturbed at a lower temperature than the bulk of the trimer.

**Biolayer interferometry (BLI)**

BLI assays were performed using the Octet Red96 instrument (Pall FortéBio). C-terminally His-tagged two-domain scCD4 (domains 1 and 2, expressed in 293F cells and purified via Ni-NTA affinity chromatography and SEC) was immobilized onto Dip and Read Ni-NTA-coated biosensors for 120 s followed by a 60 s baseline measurement in Kinetics Buffer (phosphate-buffered saline [PBS] pH 7.2 supplemented with 0.01% w/v bovine serum albumin and 0.002% v/v Tween 20). The probes were then dipped for 300 s into wells containing 200 nM of BG505 SOSIP.664-avIB wild-type or BG505 SOSIP.664-AviB containing single or double stabilizing mutations (E64K, H66R, A316W, version 4.1, or 4.2), in the same buffer. After the association step, the probes were placed into wells containing only the buffer for 600 s to measure dissociation. An inter-step correction was applied to align the end of the association curve to the beginning of the dissociation curve, and a single binding site model was used to determine on- and off-rates from the aligned curves.

**Differential scanning calorimetry (DSC)**

Thermal denaturation was studied using a nano-DSC calorimeter (TA instruments). All Env protein samples were first extensively dialyzed against PBS, and the protein concentration then adjusted to 0.1–0.3 mg/ml. After loading the sample into the cell, thermal denaturation was probed at a scan rate of 60°C/h. Buffer correction, normalization and baseline subtraction procedures were applied before the data were analyzed using NanoAnalyze Software v.3.3.0 (TA Instruments). The data were fitted using both two-state and non-two-state models, as the asymmetry of some of the peaks suggested that unfolding intermediates were present. We report the $T_m$ values derived from the two-state model in the main text, while the multiple $T_m$ values based on the non-two-state models are in the SI section. The DSC experiments were all performed with SOSIP.664-D7324 trimers, but we determined that the presence of the D7324-tag does not alter the $T_m$ values compared to the corresponding non-tagged trimers (data not shown). By running multiple DSC runs with the same protein (BG505 SOSIP.664-D7324), we determined that the standard deviation of the nano-DSC melting temperatures is +/- 0.3°C.

**Dynamic light scattering (DLS)**

DLS measurements were performed at 20°C using a Dynapro Nanostar instrument (Wyatt Technologies), with 40 acquisitions of 5 s each. Each sample was spun at 10,000 x g for 10 min prior to the DLS measurement to remove any trace aggregates or dust from the sample. The hydrodynamic radius ($R_h$) and the molecular weight (MW) were calculated using the Dynamics Analysis software (Wyatt Technologies), assuming a spherical model. To determine the Rh of stabilized SOSIP.664 trimers more precisely, we opted for DLS instead of the SEC/quasi-elastic light scattering (QELS) method used previously [11]. The reason is that the use of flow mode SEC coupled to static and QELS detectors is limited by fitting using a mono-modal model. Calculated Rh values derived from this method thus represent the mean and distribution of diffusion constants. For a protein sample containing a small amount of polydispersity that is attributable to the presence of larger species, the resulting $R_h$ value can be artificially elevated. As some preparations of BG505 SOSIP.664 trimers can contain up to ~5% of higher-order aggregates that are not completely separated from the trimer by SEC, their presence may account for the higher Rh values (i.e. 8.1 nm for the BG505 SOSIP.664 trimer [11]) than are presented here. Overall, because DLS measurements allow for multi-modal fitting models, we considered them to be a better way to measure Rh values for the wild-type and stabilized SOSIP.664 trimers.

**Small angle X-ray scattering (SAXS)**

SAXS measurements were conducted on Beam Line 4-2 at the Stanford Synchrotron Radiation Laboratory [17]. The focused 11 keV X-ray beam irradiated a thin-wall quartz capillary cell, placed 2.5 m upstream of the Rayonix MX 225HE detector (Evanston, IL). A 50 µl sample of various PGT145-purified BG505 SOSIP.664 trimer variants (1 – 2 mg/ml) were injected onto a high resolution Sepharose-200 column (GE Healthcare) with a flow rate of 50 µl/min in 20 mM Na$_2$PO$_4$, pH 7.4, 150 mM NaCl, 0.02 % NaN$_3$, 1 mM EDTA. The column eluate passed through a UV detector cell and into the quartz capillary cell. X-ray exposures were collected for 1 s every 5 s throughout the run, during which a circulating water bath maintained the capillary cell temperature at 8°C. The detector pixel numbers were converted to the momentum transfer using the formula: $q = 4\pi \sin \theta / l$, where $l$ is the X-ray wavelength of 1.127 Å and $2\theta$ is the scattering angle calibrated using a silver behenate powder standard placed at the capillary...
position. A background scattering curve was obtained from the first 100 exposures (before the void volume), and was subtracted from all subsequent exposures during generation of the sample elution profile. The radius of gyration values ($R_g$) and I(0) for each frame were batch-analyzed using AutoRg, and frames with stable $R_g$ values were merged using Primus [18] for the final scattering curve. The real space distance distribution functions were calculated from the merged data sets by indirect transformation using the program GNOM [19].

Hydrogen-deuterium exchange coupled with mass spectrometry (HDX-MS)

Two domain soluble CD4 (scCD4) [20] was obtained from the NIH AIDS Reagents Repository. Immediately before HDX-MS analysis, all proteins were SEC-purified using a Superdex S200 column (GE Healthcare) and a PBS-based elution buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl with 1 mM EDTA and 0.02 % sodium azide). Complexes were formed by overnight incubation at 4°C with a 9-fold molar excess of scCD4 (relative to one trimer). Native gels were run for each sample to assess complex formation (SI Fig. 4C). Various PGT145-purified BG505 SOSIP.664 trimers (15 µg) were diluted 10-fold into deuterated PBS buffer and incubated at room temperature. The deuterium exchange reactions were quenched after 3 s, 1 min, 30 min and 20 h by mixing with an equal volume of cold 200 mM Tris-(2-Carboxyethyl)phosphine hydrochloride (TCEP), 0.2 % formic acid (final pH 2.5). The samples were subsequently digested with pepsin (0.15 mg/ml) for 5 min on ice, flash frozen in liquid nitrogen, stored at -80°C and analyzed by LC-MS as described previously [21]. Differences in exchange profiles that exceeded the error, based on the standard deviation from duplicate measurements, were visualized on the trimer structure using custom macros in PyMOL [22].

Negative-stain electron microscopy (EM)

Purified BG505, B41, ZM197M and AMC008 SOSIP.664 trimers, either alone or as Fab complexes (with PGV04 and 35O22), were analyzed by negative-stain EM. To form complexes, a 6-10 molar excess of each Fab was incubated with trimers overnight at room temperature. A 3 µl aliquot containing ~0.03 mg/ml of a trimer or Fab-trimer complex was applied for 5 s onto a carbon-coated 400 Cu mesh grid that had been glow discharged at 20 mA for 30 s, then negatively stained with 2% (w/v) uranyl formate for 60 s. Data were collected on either an FEI Tecnai T12 electron microscope operating at 120 keV, with an electron dose of ~25 e/Å² and a magnification of 52,000x that resulted in a pixel size of 2.05 Å at the specimen plane, or an FEI Talos electron microscope operating at 200 keV, with an electron dose of ~25 e/Å² and a magnification of 92,000x that resulted in a pixel size of 1.57 Å at the specimen plane. Images were acquired with a Tietz TemCam-F416 CMOS camera (FEI Tecnai T12) or FEI Ceta 16M camera (FEI Talos) using a nominal defocus range of 1000-1500 nm.

Image processing and 3D reconstruction

Data processing methods are described in detail elsewhere, including the closed, partially open, and non-native trimer classification system [1,16]. Briefly, a total of 24,522 particles were included in the final reconstruction for the 3D average of AMC008 SOSIP.v4.2 in complex with PGV04 and 35O22 Fabs. An initial common-lines model was generated using 2D class averages in EMAN2 [23] followed by refinement against all particles in Sparx [24]. The resolution of the final reconstruction is ~15 Å based on a Fourier shell correlation of 0.5 (Fig. S4A-C).

Isothermal titration calorimetry (ITC)

ITC was performed using an Auto-ITC 200 instrument (GE Healthcare) using a protocol similar to one described previously [2,11]. Briefly, prior to conducting the titrations, proteins were dialyzed against Tris-saline buffer (150 mM NaCl, 20 mM Tris, pH 8.0). Absorbance at 280 nm using calculated extinction coefficients was used to determine and adjust protein concentrations. The ligand present in the syringe was 19b IgG at a concentration of 10-20 µM, while BG505, B41 or AMC008 SOSIP .664 trimers were present in the cell at a concentration of 4-6 µM. Data analysis was performed using Origin 7.0 software. Binding constants ($K_d$), the molar reaction enthalpy ($\Delta H$), and the stoichiometry of binding (N), by fitting the integrated titration peaks via a single-site model or a two-site model, as appropriate.

Glycan profiling

Env trimers (10 µg) were resolved by SDS-PAGE under non-reducing conditions, followed by staining with Coomassie Blue. Bands corresponding to gp140 were excised from the gels and washed alternately with acetoniitrile and water, five times. N-linked glycans were then released by addition of protein N-glycosidase F (PNGase F) at 5000 U/ml and incubation at 37°C for 16 h, according to the manufacturer’s instructions (New England Biolabs). The released glycans were subsequently eluted from gel bands by extensive washing with water, and then dried using a SpeedVac concentrator. Released glycans were labelled with 2-aminobenzoic acid (2-AA) as previously described [25]. Briefly, glycans were resuspended in 30 µl of water followed by addition of 80 µl of labelling mixture (comprising 30 mg/ml 2-AA and 45 mg/ml sodium cyanoborohydride in a solution of sodium acetate trihydrate [4% w/v] and boric acid to a final concentration of 10-20 µM, while BG505, B41, ZM197M and AMC008 SOSIP .664 trimers were present in the cell at a concentration of 4-6 µM. Data analysis was performed using Origin 7.0 software. Binding constants ($K_d$), the molar reaction enthalpy ($\Delta H$), and the stoichiometry of binding (N), by fitting the integrated titration peaks via a single-site model or a two-site model, as appropriate.

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acid [2% w/v in methanol]. Samples were then incubated at 80°C for 1 h. Excess label was removed using Speed Amide-2 cartridges, as previously described [25]. Fluorescently labelled glycans were resolved by hydrophilic interaction liquid chromatography-ultra performance liquid chromatography (HILIC-UPLC) using a 2.1 mm x 10 mm Acquity BEH Amide Column (1.7 μm particle size) (Waters). The following gradient was run: time = 0 min (t = 0): 22.0% A, 78.0% B (flow rate of 0.5 ml/min); t = 38.5: 44.1% A, 55.9% B (0.5 ml/min); t = 39.5: 100% A, 0% B (0.25 ml/min); t = 44.5: 100% A, 0% B (0.25 ml/min); t = 46.5: 22.0% A, 78.0% B (0.5 ml/min); t = 48: 22.0% A, 78.0% B (0.5 ml/min), where solvent A was 50 mM ammonium formate, pH 4.4, and solvent B was acetonitrile. Fluorescence was measured using an excitation wavelength of 250 nm and a detection wavelength of 428 nm. Data processing was performed using Empower 3 software.

The percentage abundance of oligomannose-type glycans was calculated by integration of the relevant peak areas before and after Endoglycosidase H digestion, following normalization. Digestions were performed on free glycans at 37°C for 16 h. The digested glycans were purified using a polyvinylidene fluoride (PVDF) protein-binding membrane plate (Millipore) prior to HILIC-UPLC analysis.

**Immunizations**

Groups of 6-week old Balb/cJ or 129S1/SvlmJ mice were immunized as described [26] with 10 μg of BG505 SOSIP.664 or SOSIPv4.1 trimers in 0.2-0.4 μg ISCOMATRIX™ adjuvant (CSL Ltd) via footpad injections. The first immunization (week 0) was followed by booster immunizations at weeks 4 and 16. For the final immunization (week 16), the mice were given 20 μg of trimers in ISCOMATRIX™, and they were then bled at week 18.

Rabbits (5 per group) were immunized as described with 22 μg of PGT145-purified trimers at week 0, 4 and 20, and NAb responses were assessed at week 22 [27]. The rabit sera were assayed for autologous and cross-reactive NABS using Env-pseudoviruses in the TZM-bl cell assay [13], and for trimer-binding antibodies by D7324-capture ELISA [2]. The Env-pseudotyped viruses and their Tier classifications have been described elsewhere [28–32]. When the neutralization-sensitivity of the parental AMC008 virus was assessed using a panel of human sera and MAbs and a panel of test viruses at the Duke University Medical Center, it was classified as being in Tier-1B (not shown), while the BG505.T332N virus has a Tier-2 phenotype [27]. Note that the autologous AMC008 and BG505. T332N viruses do not contain the stabilizing mutations described in this manuscript.

**References**


