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

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Chapter 9

Improving the immunogenicity of native-like HIV-1 envelope trimers by hyperstabilization

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Abstract
The production of native-like recombinant versions of the HIV-1 envelope glycoprotein (Env) trimer requires overcoming the natural flexibility and instability of the trimer. The engineered BG505 SOSIP.664 trimer mimics the structure and antigenicity of native Env but is capable of improvement. Here, we describe how the introduction of new disulfide bonds between the gp120 and gp41 subunits of SOSIP trimers of the BG505 and other genotypes improves their stability and antigenicity, reduces their conformational flexibility, and helps maintain them in the unliganded conformation. The resulting next generation SOSIP.v5 trimers induce strong autologous Tier-2 neutralizing antibody (NAbs) responses in rabbits. In addition, the BG505 SOSIP.v6 trimers consistently induced weak heterologous NAb responses against a subset of Tier-2 viruses that were not elicited by the prototype BG505 SOSIP.664 immunogen. These new stabilization methods can be applied to trimers from multiple genotypes for use as components of multivalent vaccines aimed at inducing broadly neutralizing antibodies (bNAbs).
Introduction

Despite many attempts, no experimental vaccine has induced strongly protective immunity against HIV-1 infection. One approach to this problem is the generation of an envelope glycoprotein (Env) based vaccine that induces broadly neutralizing antibodies (bNAbs) [1]. A major obstacle to creating such a vaccine is the instability of the Env trimer, which for many years hindered the generation of recombinant, soluble proteins that adequately mimicked the functional Env trimer on virions.

We have described a soluble, recombinant Env trimer, BG505 SOSIP.664, that is stabilized by a disulfide bond between gp120 and gp41 and an Ile-to-Pro substitution at position 559 in gp41 [2–4]. This trimer is based on the BG505 clade A founder virus isolated from a 6-week old infant [5]. Negative-stain electron microscopy (EM) and cryo-EM studies show that it closely resembles the native, membrane-associated trimer at the structural level [4]. The BG505 SOSIP.664 trimer, and others of the same design based on different genotypes, display the epitopes for most bNAbs but few non-NAbs [4,6–11]. In immunogenicity studies in rabbits, the BG505, AMC008 and B41 SOSIP.664 trimers induced NAbs against the corresponding autologous viruses [12,13]. Several BG505 trimer structures, determined by crystallography and cryo-EM, have provided new insights into the architecture and function of HIV-1 Env [14–19].

Although SOSIP.664 trimers are stable enough to produce and purify, we hypothesized that their performance as immunogens could be improved by reducing their conformational flexibility and the consequent exposure of immunodominant, potentially distractive non-NAb epitopes [13]. In addition, further stabilization of the trimer may be beneficial by increasing its half-life in vivo and hence its chances of encountering the rare naïve B cells that recognize bNAb epitopes. Finally, as human vaccine candidates, high thermal stability would be beneficial in practical contexts such as storage conditions and shelf life [20].

The increasingly high-resolution structures of SOSIP trimers greatly facilitate the design of stabilization strategies. BG505 SOSIP.664 trimers have already been further stabilized by adding an intra-gp120 disulfide bond linking residues 201 and 433, which fixes the bridging sheet in its ground state and thereby reduces the exposure of non-NAb epitopes [21,22]. Combining two different substitutions in gp120, E64K or H66R plus A316W, increases the stability of SOSIP.v4 trimers of various genotypes, reduces the exposure of non-NAb CD4i and V3 epitopes (i.e. 17b, 19b and 14e), and decreases the induction of V3-directed non-NAbs in immunized rabbits [13]. We have also introduced a disulfide bond between gp120 residue-49 of one protomer and gp41 residue-555 of a second protomer to increase thermostability of the resulting BG505 SOSIP.664 E49C-L555C trimers [23]. Forming a complex with the quaternary-structure dependent bNAb, PGT145, can also improve trimer stability [24]. Finally, by comparing the BG505 sequence with others that form stable trimers poorly, several substitutions were identified that increase the stability of JR-FL and 16055 SOSIP.664 trimers [22]. The most stable native-like trimer reported to date is BG505 SOSIP.664 E49C-L555C, with a midpoint of thermal denaturation ($T_m$) of 75.2°C (i.e., an increase of 7.6°C over the SOSIP.664 prototype) [23]. Strong precedents underpin this approach as disulfide bonds play a well established role in protein stability [25,26]. For example, disulfide bonds can be up to 17-fold more abundant in proteins from thermophilic archaea and bacteria, compared to mesophiles, and the number of bonds correlates with the
maximum growth temperature [27,28].

Here, we described the structure-guided introduction of additional disulfide bonds between gp120 and gp41 that further stabilize the resulting SOSIP.v5 trimers in their unliganded, closed state. Furthermore, we have combined these inter-subunit bonds to create hyperstable SOSIP.v6 trimers. When BG505 SOSIP.v6 trimers were tested as immunogens in rabbits, they induced strong autologous responses as well as weak but consistent NAbs against a subset of heterologous Tier-2 viruses. In the latter regard the new trimers were superior to the SOSIP.664 prototype.

Results

Addition of a second disulfide bond between gp120 and gp41
To reduce the flexibility and increase the stability of BG505 SOSIP.664 trimers, we designed, screened and produced variants containing an additional engineered intra-protomer disulfide bond, i.e., between the gp120 and gp41 subunits. Since the disulfide bond between residue 501 of gp120 and residue 605 of gp41 (i.e., the SOS bond) used to create SOSIP trimers is located near the base of the trimer [2,16], we sought locations nearer to the trimer apex for a second intra-protomer bond. Initially, new disulfide bonds were evaluated in the absence of the SOS bond, but promising candidates were subsequently combined with SOS to make double disulfide-bond variants (Figs. S1 and S2). Based on favorable biochemical and antigenic properties, we selected two such variants, designated SOSIP.664 H72C-H564C and SOSIP.664 A73C-A561C, for further analyses (Figs. S1 and S2). To provide additional stability, we also introduced two point substitutions: A316W to improve hydrophobic packing of V3 residues and prevent sporadic, unwanted V3 exposure from its hidden location below V1V2; and E64K to impede the occasional spontaneous and reversible sampling of the CD4-bound conformation (Fig. 1A; [13]). SOSIP.664 trimers containing the E64K and A316W substitutions and either an H72C-H564C or A73C-A561C disulfide bond are referred to as SOSIP.v5.1 or SOSIP.v5.2 variants, respectively (Fig. 1A; see also Table 1).

Biochemical and biophysical properties of stabilized BG505 SOSIP.v5 trimers
The two variants of BG505 SOSIP.v5 trimers were expressed in 293F cells and purified via PGT145-affinity chromatography [13]. Non-reducing SDS-PAGE analysis showed that the SOSIP.v5 proteins migrated more slowly than their SOSIP.664 counterparts (Fig. S3A), consistent with a decrease in SDS uptake when a protein becomes more compact. We used tandem mass spectrometry (MS/MS) to confirm that, in addition to the 10 canonical disulfide bonds and the SOS bond, the new bond was also formed in both SOSIP.v5 variants (Table S5).

PGT145-purified BG505 SOSIP.664 and SOSIP.v5 trimers have a typical native-like conformation (98% of the population) when visualized by NS-EM (Table 1; Fig. S3C). PGT145-purified, native-like BG505 SOSIP.664 trimers have a more open configuration, on average, compared to ones purified by 2G12 columns, typically ~30% closed vs. >95%. The E64K and A316W substitutions partially reverse this opening effect, the resulting SOSIP.v4.1 trimers being ~70% closed [13] (Table 1). The addition of the H72C-H564C and A73C-A561C bonds has a further effect, to 85% closed for SOSIP.v5.1 and 91% for SOSIP.v5.2 trimers (Table 1.
Figure 1. Design, antigenicity, structure and dynamics of BG505 SOSIP.v5 trimers containing novel disulfide bonds between gp120 and gp41. (a) Linear schematic of the BG505 SOSIP.664, SOSIP.v4, SOSIP.v5, SOSIP.v5.2 I201C-A433C and SOSIP.v6 constructs. Modifications that create the SOSIP.664 construct are indicated in red, and include the T332N glycan insertion, the SOS disulfide bond (Cys501-Cys605), and the I559P and R6 substitutions [4]. The E64K and A316W substitutions added to make the SOSIP.v4 construct are colored blue [13]. The newly engineered disulfide bonds in the remaining constructs, are shown in purple. There are two sub-variants of the SOSIP.v5 construct: The v5.1 design has a disulfide bond between Cys72 and Cys564, whereas SOSIP.v5.2 has the bond between Cys73 and Cys561. The Cys201-Cys433 and Cys49-Cys555 disulfide bonds, previously described by Do Kwon et al. and Garces et al., respectively, introduced into the SOSIP.v5.2 construct are shown in grey [21,23]. The resulting constructs are designated SOSIP.v5.2 I201C-A433C and SOSIP.v6, respectively. (b) SPR analysis of the binding of bNAbs PG16, PGT145, PGT151 and 35022 to quaternary epitopes (upper panel), and of CD4-IgG2 and the 17b CD4i epitope non-NAb +/- the prior addition of CD4-IgG2 (lower panel), to the indicated BG505 trimer variants. (c) Crystal structure of the quaternary complex of BG505 SOSIP.664 72C-564C trimer (cyan), in complex with PGT122 Fab (pink), 35O22 Fab (orange) and NIH 45-46 scFv (deep blue). The three antibody fragments are represented as surfaces, and the trimer’s N-linked glycans as spheres. One of the three protomers is highlighted for clarity. The gp41 HR1 location of the C-terminus of the engineered 72C-564C disulfide bond is marked with a rectangle. (d) Detail of the interaction between gp41 HR1 and gp120. A 2Fo-Fc composite omit map contoured at 1.0σ around gp41 HR1 (blue mesh) reveals more ordered electron density (green mesh) for gp41 residues 547-569 (dotted lines), implying that the 72C-564C disulfide bond may help to stabilize this region of the trimer. The Fig. was rendered using Pymol. (e) Differences in H/D exchange rates between the unliganded and CD4-bound forms of the indicated SOSIP trimer variants. The net difference in H/D exchange as a sum of all time points (in Da) is plotted for each observable peptide. Only differences that were outside the error range were included in the summation process. The same set of peptides was used for each trimer construct. The individual exchange plots for each observable peptide are shown in Fig. S4d.
Table 1. Biophysical characterization of stabilized SOSIP trimers from BG505, AMC008, B41 and ZM197M isolates

<table>
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<th>Isolates</th>
<th>New substitutions added to SOSIP.664</th>
<th>SOSIP version</th>
<th>Yield (µg/l)</th>
<th>Morphology (NS-EM)</th>
<th>Thermostability (DSC)</th>
<th>Glycan composition [HILIC-UPLC]</th>
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<sup>a</sup> The yields of PGT145-purified trimers are listed, together with the percentages that have a native-like conformation and that are in the closed form, as determined by negative stain EM. The TM values were derived using trimer variants without a C-terminal tag, and are shown in Supplementary Fig. 5c. The percentage of Man<sub>8</sub>GlcNAc<sub>2</sub> and Man<sub>9</sub>GlcNAc<sub>2</sub> glycans, as well as the total percentage of oligomannose glycans are provided for each trimer (Supplementary Fig. 5e). The TM values are based on two-state model fitting. TM values for His-tagged trimers (BG505) were consistently ~0.9-1.0°C higher than the values for the same trimers without a tag. TM values obtained with D7324-tagged trimers (AMC008, B41 and ZM197M) were up to 0.4°C higher than those without a tag.

<sup>b</sup> An overview of the modifications made to the stabilized trimer variants is shown in Table S1.

<sup>c</sup> NS-EM and glycan composition data for SOSIP.4.1 and SOSIP.4.2 trimers were previously reported in De Taeye et al., 2015 [13].

<sup>d</sup> Data for this trimer variant were previously described in DoKwon et al., 2015 and Guenaga et al., 2015 [21,22].

<sup>e</sup> The TM values for each construct were obtained by DSC using a two-state model (Fig. S5d).

<sup>f</sup> The TM values for each construct were obtained by DSC using a two-state model (Fig. S5d). The percentages of Man<sub>8</sub>, Man<sub>9</sub>, and total oligomannose glycans are given for each trimer (Fig. S5e).
and Fig. S3C). DLS studies support the conclusion that the SOSIP.v5 trimers are both more compact (see Table S2 and Fig. S3D for these and other biophysical analyses, including SAXS data).

We then assessed BG505 trimer thermostability using differential scanning calorimetry (DSC). The midpoints of thermal denaturation ($T_m$) of the SOSIP.664 H72C-H564C and A73C-A561C trimers were 72.0°C and 72.5°C, respectively, i.e. 4.4°C and 4.9°C higher than for SOSIP.664 (Table 1). The introduction of either disulfide bond in the SOSIP.v4.1 context again increased trimer thermostability, the $T_m$ values of 75.0°C and 75.3°C for SOSIP.v5.1 and SOSIP.v.5.2, respectively, representing increases of 4.3°C and 4.6°C over SOSIP.v4.1 and 7.4°C and 7.7°C over SOSIP.664 (Table 1, Fig. S3E).

The glycosylation profiles of the SOSIP.664 H72C-H564C, SOSIP.664 A73C-A561C and both SOSIP.v5 trimers were all similar to those of SOSIP.664 and SOSIP.v4.1, with oligomannose glycoforms dominating (61%-64%) and in particular Man$_8$GlcNAC$_2$ and Man$_9$GlcNAc$_2$ glycans (Table 1, Fig. S3F). The high density of unprocessed oligomannose glycans is a hallmark of native-like Env trimers [29–31].

**Antigenicity of stabilized BG505 SOSIP.v5 trimers**

We used a panel of bNAbs to assess the antigenicity of His-tagged versions of the stabilized BG505 trimer variants by ELISA. All the tested bNAbs bound comparably to the SOSIP.664, SOSIP.v4 and SOSIP.v5 trimers, implying that the stabilization changes had not compromised antigenicity (Table 2, Fig. S3G). Surface Plasmon Resonance (SPR) studies confirmed that the quaternary structure-dependent epitopes at the trimer apex (PG16 and PGT145) and the gp120/gp41 interface (35O22 and PGT151) were fully preserved on both versions of SOSIP.v5 trimers (Fig. 1B). CD4-IgG2 bound substantially less well to the SOSIP.v5 trimers than to their SOSIP.664 precursor in ELISA (Table 2). An SPR analysis showed that the rate of CD4-IgG2 dissociation from both SOSIP.v5 variants was markedly greater than for SOSIP.664 (Fig. 1B). The latter finding is consistent with a report that stabilizing BG505 SOSIP.664 trimers with a disulfide bond between gp120 positions 201 and 433 leads to a faster rate of sCD4 dissociation.

BG505 SOSIP.664 trimers bind 17b, a non-NAb against a CD4-inducible (CD4i) epitope, minimally but detectably in the absence of CD4 [4]. Under the same conditions, neither SOSIP.v5 trimer bound 17b detectably when CD4-IgG2 was absent, and the extent of CD4i-epitope induction by CD4-IgG2 was less than seen with SOSIP.664 and SOSIP.v4.1 (Table 2, Fig. S3G). An SPR analysis confirmed and extended these results (Fig. 1B). Moreover, the CD4bs non-NAb b6 also bound less well to the SOSIP.v5 trimers than to SOSIP.664 and SOSIP.v4.1 (Table 2, Fig. S3G). Some of the reductions in non-NAb epitope exposure are attributable to the A316W substitution that is present in SOSIP.v4 trimers [13]. However, the new disulfide bonds in the SOSIP.v5 trimers confer additional benefit (Table 2, Fig. S3G).

Taken together, the antigenicity studies show that the SOSIP.v5 trimers, each further stabilized by an additional disulfide bond, preserve the desired bNAb reactivity profiles of their SOSIP.664 and SOSIP.v4.1 precursors, while minimizing the display of non-NAb epitopes associated with V3, the CD4bs and the CD4i site. Their binding of CD4-IgG2 is also reduced, while VRC01 binding is unchanged.

**X-ray structure of a stabilized BG505 SOSIP trimer**

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To assess the impact of an extra disulfide bond on trimer structure we expressed the BG505 SOSIP.664 H72C-H564C trimer in 293S cells and purified it by 2G12-affinity chromatography followed by size exclusion chromatography (SEC); its crystal structure was then determined as a complex with bNAbs PGT122 Fab, 35O22 Fab and NIH45-46 scFv (Fig. 1C). Although the crystals only diffracted to 7Å resolution, the availability of higher resolution structures for other BG505 SOSIP trimers allowed a clear interpretation of the lower resolution electron density maps, as exemplified by good refinement statistics (Table S3). The structure showed that the overall architecture of the trimer is preserved upon addition of the 72C-564C inter-subunit disulfide bond (Fig. 1C). In addition, bNAbs against three distinct sites of vulnerability (PGT122, N332; 35O22, gp41/gp120 interface; NIH45-46, CD4bs) interacted with their epitopes in a manner similar to their recognition of the SOSIP.664 trimer (Fig. 1C). Residues 547-569 in the gp41 HR1 N-terminal region near the site of the 72C substitution in gp120 also have stronger electron density, especially in one of the three protomers (Fig. 1D). Thus, the added H72C-H564C inter-subunit linkage may contribute to stabilizing the ground state of the trimer.

Dynamics of BG505 SOSIP.v5 trimers
Protein domains that adopt a stable secondary structure and/or are buried are protected from deuterium exchange, while exposed flexible domains undergo rapid exchange. We therefore used Hydrogen-Deuterium Exchange (HD-X) coupled with mass spectrometry (MS) to explore the impact of the new trimer-stabilizing changes on protein dynamics and conformational flexibility. The resulting exchange profiles for the BG505 SOSIP.664 A73C-A561C, SOSIP.v5.1 and SOSIP.v5.2 trimers were similar to the SOSIP.664 precursor (Fig. S4). We used the same method to study CD4-induced conformational changes. Adding a substantial molar excess of sCD4 to the SOSIP.664 trimers resulted in less protection in the V2 and V3 loops but more protection of the CD4bs, in gp120 layers 1-3, and in gp41 HR1, in agreement with previous studies (Fig. 1E and S4) [32]. In contrast, all the above changes were greatly diminished or completely abolished in the various stabilized trimers (Fig. 1E and S4). The SOSIP.v5.2 variant responded the least to sCD4, in that the CD4-induced exposure of V2 and V3 was now entirely abrogated (Fig. 1E and S4). Thus, the extra inter-subunit disulfide bond and the E64K and A316W substitutions together impede the opening of the apex of the SOSIP.v5.2 trimers when sCD4 is present. We conclude that, by all available measures, the BG505 SOSIP.v5.2 trimers are completely trapped in the closed, ground state.

Stabilizing SOSIP trimers from clades B and C
To assess the generality of the stabilization method, we introduced the H72C-H564C or A73C-A561C disulfide bonds into the B41 (clade B) SOSIP.v4.1 construct and the AMC008 (clade C) and ZM197M (clade C) SOSIP.v4.2 constructs [13,33,34].

The resulting B41 AMC008, and ZM197M SOSIP.v5 variants were purified by PGT145-affinity chromatography and analyzed by SDS-PAGE and BN-PAGE analysis. While AMC008 SOSIP.v5 and B41 SOSIP.v5 yielded similar amounts of trimers to their comparably produced SOSIP.664 and SOSIP.v4 counterparts, the yields for ZM197M were considerably improved (1.0 mg/l for SOSIP.v5.1 and v5.2 vs. ~0.3 mg/l for SOSIP.664 and SOSIP.v4) (Table 1). All the various purified SOSIP.v5 trimers were cleaved efficiently (Figs. S5A and S5B), and they all migrated more slowly on non-reducing SDS-PAGE gels, as seen
Table 2. Antigenic characterization of stabilized trimers from BG505, AMC008, B41 and ZM197M isolates

- Binding of bNAbs and non-NAbs was determined using a Ni-NTA (BG505 and AMC008) or D7324- (B41 and ZM197M) capture ELISA. Half-maximal binding concentrations (EC50, in µg/ml) are shown for SOSIP.664 trimers (in bold). Antibody binding to the various stabilized trimers is expressed as percentages of the binding to SOSIP.664 (defined as 100%). The values are representative of at least 2 independent experiments, and the experimental error is <25%. The ELISA curves from one representative experiment are shown in Supplementary Fig. 5f. Color code: green, >130% binding; orange 10-70% binding; red <10% binding. ND: Not determined.

- An overview of the modifications made to the stabilized trimers variants is shown in Fig. 1A.

- The SOSIP.v4.1 and SOSIP.v4.2 trimer variants were described in de Taeye et al., 2015 [13].
for the BG505 constructs (Fig. S5A). Negative-stain EM confirmed that the PGT145-purified SOSIP.v5 trimers were again invariably native-like, and also more likely to be in the closed conformation (Table 1, Fig. S5C). As for BG505, oligomannose glycoforms dominated (63-70%, in particular Man$_{n}$GlcNAc$_{2}$ and Man$_{n}$GlcNAc$_{3}$) the glycan compositions of the B41, AMC008 and ZM197M SOSIP.v5 variants (Table 1; Fig. SSE) [29–31]. The additional disulfide bond improved the thermostability of each trimer construct. For AMC008, the $T_m$ increased from 60.2°C (SOSIP.664) to 68.3-5°C (SOSIP.v5.1 and v5.2), i.e., by 8.1 and 8.3°C (Table 1; Fig. SSD). Increases in $T_m$ of between 4 and 7°C compared to SOSIP.664 were also seen for the various B41 and ZM197M SOSIP.v5.1 and SOSIP.v5.2 trimers (Table 1; Fig. SSD). Overall, we conclude that introducing a second inter-subunit disulfide bond confers additional stability on SOSIP.v5 trimers from three different clades.

### Antigenic properties of B41, AMC008 and ZM197M SOSIP.v5 trimers

In general, the antigenicity properties of the SOSIP.v5.1 and v5.2 trimers of all three genotypes were again comparable to the corresponding SOSIP.v4 and/or SOSIP.664 trimers; bNAb binding was retained while non-Nab reactivity with V3 and CD4i epitopes was reduced (Table 2, Fig. S5F). As seen with BG505, CD4-IgG2 binding to the AMC008, B41 and ZM197M SOSIP.v5 variants was reduced compared to SOSIP.v4 (Table 2). An unexpected observation was that the introduction of an additional disulfide bond into the B41 and ZM197M SOSIP.v5 constructs improved the binding of the quaternary structure-dependent bNAbs PG16, PGT145 and PGT151, compared to SOSIP.664 and SOSIP.v4 trimers (Table 2, Fig. S5F). The effect was particularly noticeable for B41, for which PGT151-binding was negligible for the SOSIP.664 trimers but strong for both the SOSIP.v5 versions (Table 2, Fig. S5F). Bearing in mind that PGT151 neutralizes the B41 virus, the extra disulfide bond must favorably modify the local conformation of the gp120–gp41 interface to restore the PGT151 epitope that is missing from the SOSIP.664 trimer [34].

### Comparison and combination with other trimer stabilization approaches

Two other SOSIP trimer stabilization strategies are outlined in Fig. 1A. First, introducing a disulfide bond between residues 201 and 433 (substitutions I201C and A433C) in the gp120 bridging sheet has been reported to increase the thermostability and reduce the conformational flexibility of these BG505 trimers [21]. Second, a disulfide bond between gp120 and gp41 of different protomers (substitutions E49C and L555C) also makes BG505 trimers more thermostable [18]. BG505 SOSIP trimer variants containing either of these disulfide bonds have a reduced exposure of CD4i and V3 non-NAb epitopes [18].

We introduced the above disulfide bonds into BG505 SOSIP.664 or SOSIP.v5.2 constructs and determined the yields, antigenic profiles and thermal stability of the resulting trimers. The SOSIP.v6 construct contains the E49C-L555C substitutions on the SOSIP.v5.2 background, while the SOSIP.v5.2 I201C-A433C construct includes the 201C-433C disulfide bond (Figs. 1A, S3B). The yields of PGT145-purified SOSIP.v5.2 I201C-A433C and SOSIP.v6 trimers (~1.5 and ~0.8 mg/l, respectively) were lower than for SOSIP.664 and SOSIP.v5.2 (~2.0 mg/l) (Table 1). Non-reducing SDS-PAGE analysis showed that SOSIP.v6 proteins migrated as a trimer, which is consistent with the formation of inter-protomer disulfide bonds (Fig. S3A). However, two sub-populations were visible on the gels. Based on a comparison with the migration patterns of various control proteins, we propose that the slower migrating
variant, ~60% of the total, contains all three engineered disulfide bonds (A501C-T605C, A73C-A561C, E49C-L555C), while the faster migrating species lacks the A73C-A561C bond. It may be that the presence of the E49C-L555C bond prevents the A73C-A561C bond from forming in a sub-population of trimers (Fig. S3A).

The quaternary antibodies PGT151, PGT145 and VRC026.09 bound more strongly to the BG505 SOSIP.v5.2 I201C-A433C and SOSIP.v6 trimers than to their precursors, while the binding of non-NAbs to V3 and CD4i epitopes continued to be very low (Table 2, Fig. S3G). Both new trimers resembled their precursors in containing mostly oligomannose glycans, specifically in the form of Man₈GlcNAc₂ glycans (Table 1, Fig. S3F).

The new disulfide bonds increased BG505 trimer thermostability. Adding the 201C-433C bond to the SOSIP.v5.2 construct increased the $T_m$ from 75.3°C to 80.7°C. The SOSIP.v6 trimer was particularly thermostable; the $T_m$ of the majority sub-population was 92.2°C, and a substantial unfolding event was detected at 78.8°C, which we propose reflects the minority of trimers in which the 73C-561C linkage is not formed. The $T_m$ of 92.2°C represents a dramatic improvement in stability (i.e. by 24.6°C) compared to the original BG505 SOSIP.664 trimer ($T_m = 67.6°C$).

**Immunogenicity of stabilized BG505 SOSIP trimers in rabbits**

We compared the immunogenicity of various prototypic and stabilized BG505 SOSIP trimers in rabbits using a previously described protocol, measuring the antibody responses 2 weeks after the third immunization (Fig. 2A) [12,13]. The immunogens, all tested under comparable conditions, are summarized and color-coded in Fig. 2B, and the antibody responses are shown in Fig. 2C-G.

All the BG505 trimer-immunized animals had high and comparable titers of binding antibodies to the corresponding trimers, as measured in ELISA (Fig. 2C). The sera from the SOSIP.664 trimer recipients consistently neutralized the autologous Tier-2 BG505.T332N virus in a titer (IC$_{50}$) range of 100-10,000, which is consistent with our earlier reports (Fig. 2D) [12,13]. The corresponding gp120 monomer was almost entirely ineffective in this regard, with only one of five animals responding weakly (Fig. 2D). The autologous NAb responses (median IC$_{50}$ values) elicited by the different trimers were generally comparable (SOSIP.664, 4432; SOSIP.v4.1, 4503; SOSIP.v5.1 + SOSIP.v5.2 pooled, 3457; SOSIP.v6, 7798), except for the SOSIP.v5.2 I201C-A433C hyper-stabilized trimer (median IC$_{50}$, 226; not significant compared to SOSIP.664) (Fig. 2D). Thus, with the possible exception of the I201C-A433C change to the SOSIP.v5.2 construct, the modifications used to create new, more stable trimers do not impair the induction of the autologous BG505.T332N NAB response.

One of the goals of trimer-stabilization projects is to reduce the antigenicity and immunogenicity of epitopes for non-NAbs and Tier-1 NAbs, the latter dominated by V3-directed antibodies, and thereby focus the immune response on more productive targets [13,21,35]. Compared to the BG505 SOSIP.664 trimers and, more so, the gp120 monomers, NAb titers against the Tier-1A SF162 virus were reduced by 3-fold for the combined SOSIP.v5.1 and SOSIP.v5.2 groups (p=0.0175 vs. SOSIP.664) and were also lower for SOSIP.v5.2 201C-433C (not statistically significant vs. SOSIP.664). In contrast, SF162 NAb titers were higher in the SOSIP.v6 group compared to SOSIP.664 and the combined SOSIP.v5 groups (p=0.0031)(Fig. 2E).

Sera from the various BG505 trimer-immunized rabbits were generally only weakly
Figure 2. Immunogenicity of BG505 and ZM197M SOSIP.v5 trimers in rabbits. (a) Schematic representation of immunization schedule. (b) Color coding for the immunogens tested. Panels c–h show data for BG505-based immunogens; panels i–n for ZM197M. (c, i) Midpoint antibody binding titers (EC$_{50}$) as measured by D7324-capture ELISA for the trimer variants indicated on the x-axis at the foot of the Fig., and by the color-coding scheme outlined in (b). (d–g, i–m) Neutralization of HIV-1 viruses in the TZM-bl assay by sera from animals immunized with BG505 or ZM197M trimer variants. The plots show ID$_{50}$ values, the serum dilution at which infectivity is inhibited by 50%. (d and j) Autologous viruses; (e and k) SF162 heterologous Tier 1A; (f and g) 92RW heterologous Tier 2 virus; (l and m) SHIV162P3 heterologous Tier 2 virus. (h and n) The numbers of heterologous viruses neutralized by each serum with an IC$_{50}$ >40 are shown. Note that the data for the 5 BG505 SOSIP.664 and SOSIP.v4.2 control animals have been published previously, but are re-plotted here for comparison as the immunization and assay conditions are comparable [13].
and sporadically active against a panel of heterologous Tier 2 viruses (Table S4). However, the clade A virus 92RW and the clade B virus SHIV162P3 were occasionally neutralized in the titer range 40-200 (Figs. 2I and 2K). Here, the most consistently immunogenic trimer was SOSIP.v6, with median IC_{50} values against 92RW and SHIV162P3 of 46 and 66, respectively (p=0.0142 and p=0.0021, respectively, for SOSIP.v6 compared with the combined SOSIP.664, SOSIP.v4.1 and SOSIP.v5 groups). For each individual rabbit, we analyzed the number of heterologous Tier-2 viruses that were neutralized with IC_{50} values >40. Neutralization at this titer level was only sporadic for the SOSIP.664, SOSIP.v4.1 or SOSIP.v5 groups; the majority of the sera were inactive against all heterologous Tier-2 viruses. In contrast, sera from the SOSIP.v6 group neutralized one (n=2), two (n=1) or three (n=2) of the viruses in the test panel at a titer >40 (Fig. 2H). When the SOSIP.v6 group was compared with the SOSIP.664, SOSIP.v4.1, SOSIP.v5.1 + SOSIP.v5.2 and SOSIP.5.2 I201C-A433C groups, the number of heterologous Tier-2 viruses neutralized with IC_{50} values >40 was significantly higher (n = 10 for SOSIP.v6 vs. n = 0, 4 and 3, respectively; Kruskal-Wallis p<0.001 in each case) (Fig. 2H).

Immunogenicity of stabilized ZM197M SOSIP trimers in rabbits
In a similar study, we tested the ZM197M SOSIP.664, SOSIP.v4.2 and SOSIP.v5.2 trimers in rabbits (Fig. 2I-N). The autologous trimer binding antibody titers were again comparable among the groups, but ~2 to 5-fold lower than those induced by BG505 trimers (Fig. 2I). The autologous NAb response to the ZM197M trimers, in general, was also markedly weaker and less consistent than seen with their BG505 counterparts (compare Fig. 2I with 2C, and 2J with 2D). The ZM197M SOSIP.v5.2 trimer was clearly the most immunogenic for the autologous NAb response (5/5 responders, with a median IC_{50} of 114, compared to 3/10 responders for the combined SOSIP.664 and SOSIP.v4.2 groups; p<0.0001 by χ^2 test, Fig. 2K). The three ZM197M trimers induced SF162 Tier-1A NAb titers to comparable extents.

As seen in the BG505 study, sera against the various ZM197M trimers only sporadically neutralized heterologous Tier-1B and Tier-2 viruses (Table S4). Overall, the most frequent NAb responses to 92RW and SHIV162P3 were induced by the ZM197M SOSIP.v5.2 variant. Thus, 3/5 sera from this group neutralized both viruses at titers >40 (p=0.007 and non-significant for 92RW and SHIV162P3, respectively, compared to SOSIP.664 plus SOSIP.4.2 combined; Figs. 2L and 2M). Three of the 5 SOSIP.v5.2 sera neutralized three heterologous Tier-2 viruses at a titer >40, while none of the SOSIP.664 or SOSIP.v4.2 sera did so (Kruskal-Wallis p>0.001; Fig. 2N). Assessed across the entire heterologous Tier-2 panel, the ZM197M SOSIP.v5.2 sera neutralized significantly more viruses (IC_{50} values >40) compared to the SOSIP.664 or SOSIP.v4.2 groups (n = 9 vs. 0 or 0, respectively; Fischer-Freeman p<0.0001, Fig. 2N).

In conclusion, the hyperstable BG505 SOSIP.v6 trimers induced significantly broader Tier-2 NAb responses than the SOSIP.664, SOSIP.v4 and SOSIP.v5 variants. For the ZM197M genotype, the SOSIP.v5.2 construct was the most broadly immunogenic for Tier-2 NAbs of those tested. Overall, the still inconsistent heterologous Tier-2 NAb responses induced by the BG505 SOSIP.v6 and ZM197M SOSIP.v5.2 trimers constitute a new baseline for further trimer design and delivery improvements.

Conclusions
We describe the creation of native-like SOSIP trimers that are stabilized and antigenically
improved by the introduction of additional disulfide bonds. The most stable variant, BG505 SOSIP.v.6, has a $T_m$ of 92.2°C, an increase of ~25°C over the prototypic SOSIP.664 design. The most stable trimers contain three new disulfide bonds per gp120-gp41 protomer. The 30 intermolecular disulfide bonds naturally present in the trimer are located within the inner or outer domains of gp120 or, in one case, the immunodominant loop of gp41. In contrast, the engineered bonds are all inter-domain: i.e., between the gp120 inner and outer domains (C201-C433), between the gp120 and gp41 subunits of one protomer (C501-C605 and C73-C561), and between gp120 and gp41 on different protomers (C49-C555).

The inter-subunit disulfide bond linking gp120 residue-501 to gp41 residue-605 (the “SOS bond”) was positioned without the availability of structural information on the gp120-gp41 interface [2]. The cryo-EM structure of the native, membrane-associated trimer confirms the accuracy with which this bond was placed [36]. The newly introduced gp41 cysteine residues that successfully form inter-subunit disulfide bonds are located between the α6 and α7 segments that were not well resolved in the initial BG505 SOSIP.664 trimer structures [14,16,37]. More recent structures and HD-X experiments imply that this region might be quite dynamic and, hence, capable of adopting multiple conformations [18,32].

The finding that cysteine residues at multiple positions in gp41 HR1 can pair with gp120 residues 72 or 73 is consistent with this region being flexible when the engineered proline residue is present at position 559 [36]. However, the trimer structure predicted various other positions for cysteine substitutions that did not, in practice, lead to the efficient formation of new disulfide bonds. One explanation is that the presence of additional cysteine residues can sometimes interfere with oxidative folding and disulfide bond isomerization in the endoplasmic reticulum, leading to the production of mis-folded proteins that are subsequently degraded [38].

The BG505 SOSIP.v6 and ZM197M SOSIP.v5.2 trimers have encouraging immunogenicity properties in rabbits; compared to the SOSIP.664 prototypes, their abilities to elicit autologous NAbS was preserved, Tier-1 NAb titers were reduced or comparable and there was an improved, although still weak, induction of heterologous NAbS against a subset of Tier-2 viruses. The potential of these stabilized trimers, and others based on different genotypes, should be further evaluated in various immunization strategies aimed at inducing bNAbs. From a wider perspective, the reduced conformational flexibility of stabilized trimers may help maintain them in the ground state for longer in vivo, thereby maximizing the presentation of bNAb epitopes and increasing the probability of a successful encounter with the rare B cells that recognize bNAbs. The reduced accessibility of unwanted, potentially distracting non-NAb sites may also be advantageous. How these various factors intersect to drive the induction of higher titer autologous and heterologous Tier-2 NAb titers is likely to be also influenced by the genotype of the trimer, as well as its design. An additional virtue of a more stable vaccine immunogen is an increased shelf life and simplified storage capacity under real-world conditions [20].

Methods

Construct design
The constructs expressing BG505, B41, AMC008 and ZM1097M SOSIP.664 proteins have
been described elsewhere [4,13,33,34]. To improve the formation of soluble trimers, these constructs contained the following changes compared to the original Env sequence: a TPA signal sequence; A501C and T605C; I559P; REKR to RRRRRR at the C-terminus of gp120; a stop codon after residue 664 [2,39–42]. In addition, we introduced substitutions to restore glycan-dependent bNAb epitopes: T332N in BG505 and L543N in ZM197M [4,13,33,34]. We refer to these constructs as SOSIP.664. We further introduced the E64K or H66R and A316W trimer-stabilizing changes [13], as well as the I/V535M and L543N trimer-improving changes to gp41, where necessary [13]. We also constructed AMC008, B41 and ZM197M trimer variants bearing either a His-tag (BG505) or a D7324 epitope-tag sequence at the C-terminus of residue 664 (GSGSGGSGHHHHHHHHH or GSAPTKAKRRVQREKR, respectively), as described in Sanders et al. 2013 [4]. Point mutations were made using Quickchange site-directed mutagenesis kit (Agilent Technologies, La Jolla, CA, USA) and constructs were verified by sequencing prior to use.

**Protein expression and purification**
The Env proteins were transiently expressed in adherent 293T cells or suspension 293F cells in the presence of excess co-transfected furin and purified using PGT145-affinity chromatography as described previously [4,13,33,34,43]. All the exploratory experiments using unpurified Env from supernatant of transfected cells made use of 293T cell-expressed proteins, while purified trimers were derived from 293F cells. SDS-PAGE and BN-PAGE analyses were performed as previously described [4,13,15,34].

**Surface Plasmon Resonance (SPR), ELISA and thermostability ELISA**
SPR analyses were performed as previously described [4,6,44]. D7324-capture and Ni-NTA capture ELISAs have been described elsewhere [4,6].

**Antibodies and Fabs**
MAbs were obtained as gifts, or purchased, from the following sources: John Mascola and Peter Kwong (VRC01); Dennis Burton (PG9, PG16, PGT121, PGT145, PGT151, b6); Polymun Scientific (2G12); Michel Nussenzweig (3BC315); Mark Connors (35022); and James Robinson (17b, 19b, 14e).

**Differential scanning calorimetry (DSC)**
To probe the thermostability of SOSIP.664 trimers we used a Nano-DSC (TA Instruments, New Castle, DE, USA) and analyzed the data using NanoAnalyze Software v.3.3.0 (TA Instruments). The data were fitted using an independent non-two-state model, as the asymmetry of some of the peaks suggested the presence of unfolding intermediates. However, for simplification we also analyzed the data using a two-state scaled model. We report the $T_m$ values derived from the two-state scaled model in the main manuscript, and the multiple $T_m$ values based on the independent non-two-state models in the SI section. All the DSC data were derived using tagged trimers. When some comparative studies were performed, we found that the $T_m$ values obtained with His-tagged trimers were consistently ~0.9-1.0°C higher than those for the same trimers without tag (data not shown), and 0.5°C higher than those for D7324-tagged trimers [13]. For example, PGT145-purified His-tagged BG505 SOSIP.664 trimers have a $T_m$ of 67.6°C, while the corresponding non-tagged trimers have a $T_m$ of 66.7°C (data
not shown), and the D7324-tagged versions have a $T_m$ of 67.1°C [13]. The implication is that the His-tag has a modest stabilizing effect on the trimer.

**Negative stain electron microscopy**

Purified BG505 SOSIP.664 trimers were analyzed by negative stain EM. A 3 µl aliquot containing ~0.03 mg/mL of the 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 Uranyl formate or Nano-W (Nanoprobes) for 30 s. Data were collected using a FEI Tecnai F20 or T12 electron microscope operating at 120 keV, with an electron dose of ~55 e/Å² and a magnification of 52,000x that resulted in a pixel size of 2.05Å at the specimen plane. Images were acquired with a Gatan US4000 CCD or Tietz TemCam-F416 CMOS camera using a nominal defocus range of 900 to 1300 nm.

**Image processing**

Particles were picked automatically using DoG Picker and put into a particle stack using the Appion software package. Initial, reference-free, two-dimensional (2D) class averages were calculated using particles binned by five via the Xmipp Clustering 2D Alignment and sorted into classes. Particles corresponding to trimers or complexes were selected into a substack and binned by four before another round of reference-free alignment was carried out using the Xmipp Clustering and 2D alignment and IMAGIC software systems.

**Small Angle X-ray Scattering (SAXS)**

SAXS measurements were conducted on Beam Line 4-2 at the Stanford Synchrotron Radiation Laboratory [45]. The focused 11 keV X-ray beam irradiated a thin-wall quartz capillary cell, placed at 2.5 m upstream of the MX 225HE detector (Rayonix, Evanston, IL, USA). Aliquots containing 50 µl of BG505 SOSIP.664 constructs (1 – 2 mg/ml) were injected onto a high resolution Sepharose 200 column (GE Healthcare, Wilmington, MA, USA) with a flow rate of 50 µl/min in a buffer comprising 20 mM Na₃PO₄ pH 7.4, 150 mM NaCl, 0.02% NaN₃, 1 mM EDTA. The flow from the column passed through a UV detector cell and into the quartz capillary cell. Exposures of 1 s duration were collected every 5 s throughout the run, with a circulating water bath maintaining the capillary cell temperature at 8°C. The detector pixel numbers were converted to the momentum transfer via the equation $q = 4p \times \sin(q/2)/l$, where 2q is the scattering angle and l the X-ray wavelength of 1.127 Å, and 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), which was subtracted from all subsequent exposures during the elution profile. The $R_g$ and I(0) parameters for each frame were batch analyzed using autoRg, and frames with stable $R_g$ values were merged in primus for the final scattering curve [46]. The real space distance distribution function was calculated from the merged data sets using GNOM [47].

**Dynamic Light Scattering (DLS)**

Proteins in PBS at 1 – 2 mg/ml were centrifuged at 15000 rcf for 10 min at 4°C immediately before light scattering measurements. Data were collected on a Dynapro instrument (Wyatt Technologies, Goleta, CA, USA), with 30 acquisitions of 10 s each at 20°C, and analyzed with the manufacturer’s software (Dynamics, Wyatt Technologies). We note that the
hydrodynamic radius \( (R_h) \) of 6.9 nm derived using DLS for BG505 SOSIP.664 trimers that we report here and elsewhere (Supplementary Table 1; [13]) is slightly lower than the previously reported value of 8.1 nm [15]. The latter value was derived using flow mode size-exclusion chromatography (SEC) coupled with static and quasi-elastic light scattering detectors, and fitted using a monomodal model. Calculated hydrodynamic radius values derived using this method represent the mean and distribution of diffusion constants.

X-ray crystallography
The BG505 SOSIP.664 72C-564C trimer was expressed and purified as previously described [15]. Purified trimers were mixed with a molar excess of the PGT122 Fab and 35O22 Fab and treated with EndoH (New England BioLabs). Subsequently, the complex was mixed with NIH45-46 scFv in molar excess and the quaternary complex was purified to size homogeneity using a Superose 6 10/30 gel filtration column (GE Healthcare). The complex was tested in crystallization trials at a protein concentration of 4 mg/ml, using an Oryx8 crystallization robot (Douglas Instruments). Crystals grew in sitting drop experiments from a condition containing 8% (w/v) polyethylene glycol 8000, 0.1 M Tris, pH 8.5. A complete dataset was obtained to 7.0 Å from a single flash-frozen crystal that was cryo-protected with 30% glycerol. Data were processed using XDS [48], and data collection and processing statistics are reported in Supplementary Table 2. For molecular replacement in PHASER [49], a hybrid model was generated using the BG505 SOSIP trimer + PGT122 Fab + 35022 Fab from PDB ID: 4TVP and NIH45-46 scFv superposed from PDB ID: 5D9Q. The resulting hybrid model was used as the search model. Iterative rigid body and grouped B-factor refinements were carried-out with non-crystallographic symmetry (NCS) in PHENIX and inspected in COOT [50,51]. Refinement statistics are summarized in Supplementary Table 2.

Hydrogen-Deuterium Exchange (HD-X)
Soluble two-domain CD4 (sCD4) [52] was obtained from the NIH AIDS reagents program. All proteins were SEC-purified using a Superdex S200 column (GE Healthcare) and a PBS buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.02 % sodium azide) immediately before HDX-MS analysis. Complexes were formed by an overnight incubation at 4°C with sCD4, which was present at a 3-fold molar excess relative to each protomer of the trimer. Native gels were run for each sample to monitor sCD4-trimer complex formation (Supplementary Fig. 4d). BG505 SOSIP.664 trimers and its variants (15 µg) were diluted 10-fold into deuterated PBS buffer at room temperature. After incubation periods of 3 s, 1 min, 30 min and 20 h the exchange reactions were quenched by mixing with an equal volume of cold 200 mM 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 and stored at -80°C. Differences in deuterium exchange profiles that exceeded the error of the measurement were visualized on the trimer structure using custom macros in PyMOL [53].

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**References**

Hyperstabilization of HIV-1 Env trimers from different clades


Hyperstabilization of HIV-1 Env trimers from different clades

Supplementary Figures and Tables

A

Supplementary Figure 1: Introducing a novel disulfide bond between gp120 and gp41. (A) Five regions in gp120 and gp41 that are in reasonably close contact are shown in the crystal structure of BG505 SOSIP664. These regions constitute residues in C1, C2 and C3 in gp120 and HR1 in gp41. The gp120 subunit of a BG505 heterodimer is shown in grey and its gp41 in dark red. A close view of the interface is represented in the inset. Residues in gp120 that might contact residues in gp41 are shown in blue spheres. (B) A panel of unpurified His-tagged BG505 SOSIP664 gp140 proteins was screened in which the original disulfide bond was replaced by two new cysteine residues in the regions highlighted in panel a. Screening was based on recently published BG505 crystal structures [15,16]. Trimerization efficiency was analyzed by BN-PAGE followed by western blotting. The majority of the mutants showed less efficient trimerization. The antigenic profile of the newly generated BG505 gp140 trimers was determined by His-tag ELISA. Since BG505 gp140 lacking a disulfide bond between gp120 and gp41 falls apart in its constituents resulting in loss of reactivity with anti-gp120 MAbs [4,7,54], binding of anti-gp120 MAb (2G12) was evidence for the formation of an intermolecular disulfide bond. A number of mutant proteins, particularly those with a new cysteine at position 72 or 73 in the loop between α and β0 combined with one in residues 554-568 in the loop between α6 and α7, showed strong reactivity with 2G12 suggesting that they formed a covalent bond (i.e. new disulfide bond) between gp120 and gp41. Two bNAbs that recognize distinct quaternary epitopes (PGT145: V1V2-apex; and PGT151: gp120-gp41 interface) were used to assess the antigenic structure of the mutants. Four mutants showed binding of PGT145 and PGT151 similar to the original BG505 SOSIP664 trimer: H72C-H564C, A73C-A558C, A73C-A561C A73C-L568C, suggesting that these cysteine pairs preserved the antigenic structure of the BG505 gp140 trimers. The antigenic profile of these four proteins was assessed using a larger panel of bNAbs. Quaternary structure dependent bNAbs 35022, 3BC315 and PG16 bound efficiently to these BG505 proteins. Spontaneous opening up of the trimer was investigated with the binding of CD4i-Abs 17b. Binding of these antibodies was abrogated and the presence of sCD4 partially restored ability to bind. Non-NAb F240 binding to the disulfide loop region of gp41 showed a slight increased binding compared to original BG505 SOSIP664, suggesting that the absence of the S01-605 in this domain opens up the epitope of F240. F240 is directed to an epitope overlapping with residue 605 [55]. Color code: red: no binding, orange: moderate binding and green: strong binding. Binding ability is also scaled from no binding (-) to strong binding (+++). The summaries are based on at least 2-3 experiments. (C) The effect on stability of the Env mutants was evaluated by a novel thermal melting assay that can be used on unpurified Env trimers [13]. The proteins were incubated for 1 h at varying temperatures in a graded PCR machine and the residual binding of 2G12 was assessed by ELISA (right panel). The first derivative reveals the unfolding pattern (right panel). (D) The midpoints of thermal denaturation (Tm) for each mutant were determined based on the melting profiles in panel C. Mutants containing the disulfide bond between H72C-H564C and A73C-A561C showed an increase in Tm by 1.3°C and 2.0°C compared to BG505 SOSIP trimer, respectively, suggesting that locating the disulfide bond at the core of the trimer, as opposed to the membrane proximal end, increases its stability. The Tm values for double disulfide proteins are included here for reference (see Supplementary Fig. 2).
Supplementary Figure 2: Combining two disulfide bonds between gp120 and gp41. (A) Cleavage and trimerization efficiency of supernatant produced gp140 proteins that incorporated the original disulfide bond at position 501-605, or a new disulfide bond at positions H72C-H564C or A73C-A561C, or both disulfide bonds, were determined by reducing SDS-PAGE (left panel) and BN-PAGE (right panel). All the mutants were properly cleaved, but showed slightly less efficient trimerization compared to original BG505 SOSIP.664 protein. (B) The antigenic phenotype of unpurified BG505 gp140 variants was assessed by ELISA and summarized. bNAbs 2G12, VRC01, PGT145 and PGT151 bound very strongly to all variants. The BG505 gp140s containing only the H72C-H564C or A73C-A561C disulfide bond but not the original C501-C605 bond showed decreased reactivity with non-NAb 17b to a CD4i-epitope, but increased reactivity with non-NAb F240, which is directed to an epitope overlapping with residue 605 [55], suggesting that the replacement of the original bond with the new bonds closed up non-NAb epitopes in the membrane distal parts of the trimer, but opened-up non-NAb epitopes at in gp41. The double disulfide mutants adopted a combined phenotype. Thus, binding of CD4i non-NAb 17b were abrogated, a property shared with the H72C-H564C and A73C-A561C mutants, and the binding of the gp41 non-NAb F240 was also very low, a property conferred by the original C501-C605 disulfide bond. (C) Additional Cys substitutions at residues 558-569 were screened for their ability to pair with 72C and 73C, and improve native like BG505 SOSIP.664 trimers that also contained the C501-C605 disulfide bond. Each unpurified protein was tested for reactivity with 2G12, PGT145 and PGT151 and its thermostability was tested as described in Supplementary Figure 1. A number of different disulfide bonds were tolerated without disturbing the overall conformation of the trimer, pointing at a high degree of flexibility in this region. In general, the mutants that were binding quaternary-dependent bNAbs to a level similar or higher than the original BG505 SOSIP.664 gp140 also showed high trimer stability. The presence of an extra disulfide bond in the core of the trimer slightly enhanced trimer stability. Color code: red: no binding, orange: moderate binding and green: strong binding. Binding ability is also scaled from no binding (-) to strong binding (+++). The summaries are based on 3 experiments.
Supplementary Figure 3: Biochemical, biophysical and antigenic characterization of PGT145-purified stabilized BG505 SOSIP.664 trimers. (A) Cleavage efficiency and disulfide bond formation was determined by SDS-PAGE under reducing and non-reducing conditions, respectively. Under reducing conditions all proteins showed a gp120 band, indicating that they were completely cleaved. Under non-reducing conditions the proteins all showed a gp140 band, indicating that they were not completely cleaved. (B) BG505 SOSIP.664, SOSIP.v4.1 and SOSIP.v5 gp140 proteins purified by PGT145 affinity chromatography were exclusively trimeric as determined by BN-PAGE. (C) The formation of native-like trimers was assessed by negative stain electron microscopy. The 2D reference free class averages of the two double disulfide bond proteins (BG505 SOSIP.v5.1 and BG505 SOSIP.v5.2) compared to BG505 SOSIP.664 and SOSIP.v4.1 are shown. The percentage of closed native-like and open native-like trimers [34] is shown in green and the percentage of non-native trimers in red. (D) SAXS scattering curves (left panel) and Kratky plots (right panel) show the consistency of the scattering pattern among the wild-type BG505 and disulfide mutants, indicating that they have the same solution structure.
Supplementary Figure 3: Biochemical, biophysical and antigenic characterization of PGT145-purified stabilized BG505 SOSIP.664 trimers. (E) The thermal stability of BG505 SOSIP.664, BG505 SOSIP.v4.1 and BG505 SOSIP.v5 was measured by DSC. The independent non-two state best-fit curves are depicted in dashed red line and the \( T_m \) values of each peak are given in the graphs. See materials and methods section for more details on curve modeling. (F) Glycan profiles of BG505 variants were determined by HILIC-UPLC. Man$_5$-GlcNAc$_2$ glycans are indicated as M5-M9 above the panel. (G) The antigenicity of BG505 SOSIP.664, BG505 SOSIP.664 H72C-H564C or A73C-A561C, BG505 SOSIP.664 E64K A316W (BG505 SOSIP.v4.1) and BG505 SOSIP.664 E64K A316W H72C-H564C (BG505 SOSIP.v5.1) or A73C-A561C (BG505 SOSIP.v5.2) was determined by Ni-NTA ELISA. Binding curves of a panel of bNAb and non-NAb antibodies are shown.
Supplementary Figure 4: HDX-MS profiles of PGT145 purified wild-type and stabilized BG505 SOSIP.664. Butterfly plots comparing the HDX-MS profiles of SOSIP.664 with A73C-A561C (A), BG505 SOSIPv5.2 (B), BG505 SOSIPv5.1 (C) and comparing (D) BG505 SOSIP.664, (E) SOSIP.664 A73C-A561C, (F) SOSIPv5.2 (G) SOSIPv5.1 upon sCD4 binding. The percent exchange for each observable peptide is plotted at the position of the center of the peptide on the primary sequence for each time point (3 s to 20 h). The difference plots below each primary plot reveal regions undergoing slower exchange (more protected, above the zero) and faster exchange (less protected, below the zero). Differences are mapped onto one lobe of trimer crystal structure (PDB: 4VTP, [16]). Segments unresolved in the crystal structure (V2, V4, and a portion of gp41) are shown as dashed lines. Point mutations are indicated and shown as green spheres.
Supplementary Figure 4: HDX-MS profiles of PGT145 purified wild-type and stabilized BG505 SOSIP.664. 
(H) Individual exchange plots for SOSIP.664 peptides. The percent exchanged at each time point (3s, 1min, 30min, 20hr) is shown for BG505 SOSIP.664 wild-type (black), wild type + sCD4 (red), A73C-A561C (green), A73C-A561C + sCD4 (orange), BG505 SOSIP. v5.2 (blue), and BG505 SOSIP.v5.2 + sCD4 (purple), BG505 SOSIP.v5.1 (gray), and BG505 SOSIP.v5.1 + sCD4 (tan). Error bars show the standard deviation from duplicate measurements.
Supplementary Figure 4: HDX-MS profiles of PGT145 purified wild-type and stabilized BG505 SOSIP.664.

(H) Individual exchange plots for SOSIP.664 peptides. The percent exchanged at each time point (3s, 1min, 30min, 20hr) is shown for BG505 SOSIP.664 wild-type (black), wild type + sCD4 (red), A73C-A561C (green), A73C-A561C + sCD4 (orange), BG505 SOSIP. v5.2 (blue), and BG505 SOSIP.v5.2 + sCD4 (purple), BG505 SOSIP.v5.1 (gray), and BG505 SOSIP.v5.1 + sCD4 (tan). Error bars show the standard deviation from duplicate measurements.
Supplementary Figure 5. Biochemical biophysical and antigenic characterization of PGT145-purified stabilized clade-B (AMC008 and B41) and clade C (ZM197M) SOSIP.664 trimers. (A) Cleavage efficiency and disulfide bond formation was determined by SDS-PAGE under reducing and non-reducing conditions, respectively. Under reducing conditions all proteins showed a gp120 band, indicating that they were completely cleaved. Under non-reducing conditions the proteins all showed a gp140 band, but the proteins containing two disulfide bonds migrated slower through the gels. (B) Stabilized subtype B (AMC008 and B41) and subtype C (ZM197M) gp140 proteins purified by PGT145 affinity chromatography were exclusively trimeric as determined by BN-PAGE. (C) The formation of native-like trimers was assessed by negative stain electron microscopy. The 2D reference free class averages of the two double disulfide bond proteins (SOSIP.v5) compared to its wild-type SOSIP are shown. The percentage of closed native-like and open native-like trimers [34] is shown in green and the percentage of non-native trimers in red. (D) The thermal stability of the disulfide mutants compared to its wild-type was measured by DSC for AMC008, B41 and ZM197M trimers. The independent non-two state best-fit curves are depicted in dashed red line and the $T_m$ values of each peak are given in the graphs. See materials and methods section for more details on curve modeling.
Supplementary Figure 5. Biochemical biophysical and antigenic characterization of PGT145-purified stabilized clade-B (AMC008 and B41) and clade C (ZM197M) SOSIP.664 trimers. (E) Glycan profiles of AMC008, B41 and ZM197M variants were determined by HILIC-UPLC. Man5-9GlcNAc2 glycans are indicated as M5-M9 above the panel. (F) The antigenicity of AMC008, B41 and ZM197M SOSIPs was determined by D7324-ELISA. Representative curves of a panel of bNAbs and non-NAbs are shown. The plots are representative of two or three experiments.
Hyperstabilization of HIV-1 Env trimers from different clades

[Graphical representation of data showing the stabilization of HIV-1 Env trimers from different clades, with various markers and lines representing different samples and controls.]
Table S1. Nomenclature for stabilized SOSIP trimers. Modifications introduced to stabilize the SOSIP.664 trimer. In green: modifications present; in red, not present. No division between cells indicates one or the other mutation.

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*Described in de Taeye et al., 2015

Table S2. Biophysical properties of BGS05 SOSIP.664 trimers. DLS and SAXS analysis were performed in stabilized SOSIP trimer variants.

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a Standard deviation differs in +/- 0.3-0.5Å
b Standard deviation is +/- 0.2

Table S3. X-ray data collection and refinement statistics

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Table S4. Midpoint neutralization titers of sera from rabbits immunized with stabilized SOSIP trimers. Week 22 sera were tested against a panel of pseudoviruses from different subtypes and titers. ID₅₀ values at week 22 are shown. In white, no neutralization; ID₅₀ < 20; in yellow, weak neutralization, 40 < ID₅₀ < 100; in orange, moderate neutralization, 100 < ID₅₀ < 1000; in red, strong neutralization, ID₅₀ > 1000. The TZM-bl cell assay was performed at the AMC. The data on animals 1569-1578 (BG505 SOSIP.664 and BG505 SOSIP.v4.1) have been published elsewhere [1] but are included here for comparison.

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Table S5. Midpoint neutralization titers of sera from rabbits immunized with stabilized SOSIP trimers. Week 22 sera were tested against a panel of pseudoviruses from different subtypes and titers. ID₅₀ values at week 22 are shown. In white, no neutralization; ID₅₀ < 20; in yellow, weak neutralization, 40 < ID₅₀ < 100; in orange, moderate neutralization, 100 < ID₅₀ < 1000; in red, strong neutralization, ID₅₀ > 1000. The TZM-bl cell assay was performed at the AMC. The data on animals 1569-1578 (BG505 SOSIP.664 and BG505 SOSIP.v4.1) have been published elsewhere [1] but are included here for comparison.
Table S5. Midpoint neutralization titers of sera from rabbits immunized with stabilized SOSIP trimers. Week 22 sera were tested against a panel of pseudoviruses from different subtypes and tiers. ID<sub>50</sub> values at week 22 are shown. In white, no neutralization; ID<sub>50</sub> < 20; in yellow, weak neutralization, 40 < ID<sub>50</sub> < 100; in orange, moderate neutralization, 100 < ID<sub>50</sub> < 1000; in red, strong neutralization, ID<sub>50</sub> > 1000. The TZM-bl cell assay was performed at DUMC. The data on animals 1569-1578 (BG505 SOSIP.664 and BG505 SOSIP.v4.1) have been published elsewhere [1] but are included here for comparison.
Table S6. Disulfide bond formation in BG505 SOSIP variants. Location of disulfide bonds was assessed by MS-MS-UPLC. CS is Charged State. NA is not applicable.