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

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Chapter 8

Reducing the immunodominant non-neutralizing V3 response to HIV-1 Env trimers

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Abstract
To provide protective immunity against circulating primary HIV-1 strains, a vaccine most likely has to induce broadly neutralizing antibodies (bNAbs) to the HIV-1 envelope glycoprotein spike (Env). HIV-1 Env trimers that closely mimic the native Env spike, of which BG505 SOSIP.664 is the prototype, can induce autologous neutralizing antibodies (NAbs) against relatively resistant (Tier-2) primary viruses, but not bNAbs. Ideally Env immunogens should present bNAb epitopes but limit the presentation of immunodominant non-NAb epitopes that might induce off-target and potentially interfering responses. The V3 loop harbors such immunodominant non-NAb epitopes. Probably as a consequence of conformational flexibility the V3 becomes exposed during vaccination with BG505 SOSIP.664 trimers and is a dominant target for non-NAbs. In an effort to reduce the immunodominance of the V3 loop, we introduced two leucines at positions 306 and 308 in next-generation BG505 SOSIP. v4.1 and SOSIP.v5.2 trimers. The hydrophobic interactions within the V3 loop between 306L, 308L and 316W, and with other nearby residues in the V1V2 domain, sequestered the V3 loop in the prefusion state and stabilized the trimers. These V3 stabilized trimers induced dramatically reduced V3-non-NAbs when compared with the parental SOSIP.v4.1 and SOSIP. v5.2 Env trimers, without impairing the autologous NAb response. Immunization strategies that aim for the induction of bNAbs might benefit from the use of next generation HIV-1 Env trimers on which the exposure of the immunodominant V3 non-NAb epitopes is prevented.
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Introduction
An HIV-1 vaccine that provides protective immunity against the majority of diverse circulating HIV-1 strains will probably be the most effective way to bring the HIV-1 epidemic to a halt [1]. Since neutralizing antibodies (NAbs) are the correlate of protection for most licensed vaccines, inducing such NAbs is an obvious goal for HIV-1 vaccine research [2]. The identification of multiple broadly neutralizing antibodies (bNAbs) from HIV-1-infected individuals, including ones that can neutralize up to 90% of circulating HIV-1 viruses strengthened the support for the search of an HIV-1 vaccine that could elicit bNAbs targeting the HIV-1 envelope glycoprotein (Env) [2–4].

The Env protein complex facilitates HIV-1 infection of immune cells, predominately T-cells, via binding to the CD4 receptor and subsequently one of the chemokine receptors CCR5 or CXCR4 [5]. Env is produced as a gp160 polyprotein precursor and cleaved into two non-covalently linked subunits, gp41 and gp120, by proteases of the furin family [6]. Three gp41 subunits and three gp120 subunits then form a trimer of three heterodimers by non-covalent interactions [7]. The gp120 subunits, which are composed of five conserved (C1-C5) and five variable domains (V1-V5), initiate HIV-1 infection via binding to the receptor and coreceptor, while the transmembrane gp41 subunits harbor the fusion machinery to accomplish fusion of the virus and host membranes [5,8]. The trimeric Env spike is decorated by a dense shield of N-linked glycans that are attached to 20-35 potential N-linked glycosylation sites (PNGS) on gp120 and 3-5 PNGS on each gp41 subunit, the exact number of PNGS depending on the viral isolate [9,10].

To prevent the induction of NAbs, limit the recognition by NAbs, and facilitate escape from NAbs, the HIV-1 Env trimer has acquired several elegant mechanisms that allow evasion from humoral immunity. The conserved protein domains on the Env trimer are masked by the dense glycan shield as well as the flexible variable loops that show extreme sequence diversity [10–12]. Furthermore, during HIV-1 infection, non-functional, misfolded, degraded or unprocessed Env products serve as a decoy for the humoral immune system because these Env forms present epitopes that are not available on the functional native trimeric Env spike, while many bNAb epitopes are absent from them [13–20]. Moreover, the functional Env trimer itself is unstable, disintegrating in to the separate subunit rapidly, and conformationally flexible, sampling closed and more open conformations [21–23].

As a consequence of the defense mechanisms described above, most Abs raised during the early phase of HIV-1 infection are directed against neutralization-irrelevant epitopes in gp41 and against the V3 domain on gp120. Thus, the V3 domain is considered to be an immunodominant epitope on the Env trimer [3,24,25]. Although V3 Abs can neutralize a subset of highly sensitive (termed Tier-1) virus isolates, they are non-neutralizing for the majority of more neutralization-resistant (Tier-2) primary viruses [26]. As a result V3 Abs do not drive the selection of HIV-1 escape mutants [27,28].

Despite these immune evasion strategies, 20-30% of HIV-1 infected individuals develop a bNAb response over time, usually between 1 and 3 years post-seroconversion [29–31]. To overcome the obstacles posed by the evasion mechanisms of Env, bNAbs usually have extraordinary characteristics. They often demonstrate a very high percentage of somatic hypermutation (SHM), have an extraordinary long CDRH3 loop to penetrate the glycan shield, originate from rare germline precursor genes, and/or develop polyreactivity [4,32]. The isolation of hundreds of bNAbs from HIV-1 infected individuals in the last decade
enabled the identification of several sites of vulnerability on the Env trimer. Initially, bNAbs were divided into five distinct groups based on their epitope on the HIV-1 Env trimer: the V2-apex, the oligomannose patch, the gp120-gp41 interface, the CD4 binding site (CD4bs) and the membrane proximal extended region (MPER) [32–34]. However, after mapping and comparing the epitopes of many bNAbs isolated in recent years, it became evident that almost the complete surface of the Env trimer can be targeted by bNAbs [33,35].

The challenge in the development of an HIV-1 vaccine lies in the design of an Env immunogen that can elicit a bNAb response, rather than a non-neutralizing antibody (non-NAb) response, or a strain-specific NAb response. In the last decades many HIV-1 envelope immunogens were tested in animals and humans. These include gp120 monomers, uncleaved gp140 “pseudotrimers” and, more recently, native-like trimers [18,35–45]. When tested in diverse animal species and humans, gp120 monomers and gp140 pseudotrimers raised potent NAbs against highly neutralization sensitive (Tier-1A) viruses, but were unable to elicit Tier-2 NAbs, not even to the autologous sequence-matched viruses [36–42]. Since gp120 monomers and uncleaved gp140 Env trimers do not recapitulate the native-like HIV-1 Env spike [20,46–48], the Ab response in immunized animals is dominated by V3 non-NAbs, resulting in Tier-1 neutralization but not Tier-2 neutralization [41,49,50].

These early generation Env immunogens have recently be superseded by immunogens that more closely mimic the structure of the native-like trimer on the viral membrane, the prototype of which is BG505 SOSIP.664 gp140 [17,43,44,51–56]. Unlike gp120 monomers and gp140 pseudotrimers, they present the epitopes for bNAbs that require native-like quaternary structure such as those at the trimer apex and the gp120-gp41 interface [17,20,43,48,57–62]. Furthermore, non-NAb epitopes are largely occluded by native-like intersubunit interactions. The atomic resolution structures of BG505 SOSIP.664 and other trimers in complex with several bNAbs were solved, facilitating structure-based vaccine design [7,10,23,63–66]. Importantly, BG505 and B41 SOSIP.664 were the first immunogens to raise potent autologous Tier-2 NAbs in rabbits and macaques, which is considered an important step on the way to bNAb induction [18,43,67]. However, despite the induction of autologous Tier-2 NAbs, no bNAbs were induced and the Ab response remained dominated by V3 non-NAbs [18,68].

The V3 region is a highly flexible region on the Env trimer and is part of the co-receptor binding site. In the prefusion state of the native trimer the V3 region is tucked away in a hydrophobic pocket underneath the variable domains 1 and 2 at the trimer apex [7,63,64]. The conformational changes upon CD4 binding break the interactions of the variable domains (V1-V3) at the trimer apex, opening up the trimer and driving the V3 domain to pop out of its springloaded position to engage the co-receptor [21]. HIV-1 Env trimer immunogens undergo similar conformational changes upon CD4 binding but also show spontaneous sampling of more open conformations, a process referred to as ‘breathing’ of the Env trimer [17,21,22,69]. The spontaneous sampling of more open conformations in BG505 SOSIP.664 trimers is thought to expose the immunodominant V3 region, resulting in a strong V3 directed Ab response in animals [17,21,70].

To reduce exposure of the V3 domain on the Env trimer and reduce the exposure of V3 non-NAb epitopes and V3 immunogenicity in vivo, we and others have found strategies to stabilize the closed prefusion conformation [43,44,53,54,71–73]. We identified a stabilizing mutation within the V3 region, A316W, that increases hydrophobic packing of the V3 loop,
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effectively sequestering the V3 loop in its prefusion conformation in the hydrophobic pocket underneath the V1V2 domains [43]. The A316W substitution is included in the next-generation SOSIP.v4 design that we now routinely apply to new Env trimers. When comparing the clade A BG505 SOSIP.v4 and the clade B AMC008 and B41 SOSIP.v4 trimers with their parental SOSIP.664 trimers the stabilized SOSIP.v4 immunogens elicited similar levels autologous NAbs, but substantially reduced levels of V3 dominated Tier-1A NAbs, showing that we achieved a reduction of V3 immunodominance [43]. However, the effect was not absolute and V3-specificities remained abundant.

We hypothesized that HIV-1 vaccine programs aimed at inducing bNAbs by using SOSIP trimers could be assisted by further limiting V3 directed non-NAb responses. To further increase hydrophobic packing of the V3 region and thereby reducing V3 immunogenicity we designed additional hydrophobic mutations in the V3 region. We identified two hydrophobic mutations in the V3 loop of BG505 SOSIP trimers, S306L and R308L, that increased the stability of BG505 SOSIP immunogens, further reduced binding of V3 non-NAbs to the trimers, and severely impaired V3 directed Tier-1A NAb responses in vaccinated rabbits. These new V3 stabilized HIV-1 Env immunogens are useful as platforms for immunogen design aimed at inducing bNAbs.

Results

Design of hydrophobic residues in V3 to prevent V3 exposure

We previously found that the introduction of an A316W mutation in the V3 domain, part of the SOSIP.v4 design, reduced the exposure of the V3 domain and thereby induced significantly less V3 targeting non-NAbs in mice and rabbits. We set out to further decrease the non-NAb V3 response and designed new hydrophobic mutations in the V3 region in the background of the stabilized BG505 SOSIP.v4.1 and BG505 SOSIP.v5.2 trimers that already contained the A316W substitution (Fig.1A; [43, de la Peña et al. manuscript in preparation]). In the prefusion state, the V3 loop is buried in a pocket formed by the V1V2 loop and V1V2 stem of the same protomer and the V1V2 loop of another protomer [7,64,65,74]. The V3 crown predominantly participates in intraprotomer hydrophobic interactions with the V1V2 loop and the N-terminal V1V2 stem in gp120 of the same protomer [74]. Disruption of these hydrophobic interactions with adjacent domains changes the packing fo the V3 loop, destabilizes the position of the V3 loop in the pocket and triggers alternative energetically favorable exposed conformations of the V3 loop [74]. To strengthen the hydrophobic interactions of the V3 loop with adjacent gp120 domains and retain the conformation in which the V3 loop is buried in the pocket formed by the adjacent V1V2 elements, we introduced leucines at position 306 and 308 (Fig. 1A-D). Both residues, a serine at position 306 and an arginine at position 308 in the original sequence, are located upstream of the V3 tip and face the inner-domain of gp120 in a similar way as residue 316 does, which is positioned on the other side of the V3 tip (Fig. 1C). When modeling different hydrophobic mutations at position 306 and 308 in silico, the introduction of a leucine was favored. Thus, leucines introduced a substantial amount of hydrophobic mass without clashing with neighboring residues, as, for example the introduction of aromatic residues would do (Fig. 1D). In silico modeling showed that all three hydrophobic residues, S306L R308L and A316W

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are in close contact (<5 Å) to each other and to other hydrophobic residues with (V120, I307, I309, F317, Y318), supporting the proposition that these mutations strengthen the hydrophobic interactions in the V3 domain (Fig. 1D, Table 1).

We first introduced the S306L, R308L and A316W substitutions in a D7324-tagged version of the BG505 SOSIP.664 trimer alone and in combination and transiently expressed them in 293T cells. We then compared trimer formation and antigenicity of the mutants with that of unmodified BG505 SOSIP.664. All V3 mutants formed trimers efficiently, but the expression of the two double mutants S306L A316W and S306L R308L and the triple mutant S306L R308L A316W was somewhat reduced compared to that of unmodified BG505 SOSIP.664 (Fig. 1E).

To determine the effect of the mutations on the exposure of the V3 loop of BG505 SOSIP.664 we tested binding of three V3 directed non-NAbs (19b, 14e and 39f) and one bNAb recognizing the oligomannose patch (2G12) in a D7324 capture ELISA. Individually, the S306L and R308L did not show a marked effect on the binding of V3 non-Abs to BG505 SOSIP.664 (Fig. 1F). However, together (S306L R308L) and particularly in combination with the A316W mutation (S306L R308L A316W), the binding of V3 Abs 14e, 39f and 19b was greatly reduced or completely abrogated (Fig. 1F). These data suggest that hydrophobic residues at positions 306, 308 and 316 help maintain a sequestered V3 conformation preventing V3 non-NAbs from binding. An alternative explanation is of course that the epitopes of these Abs are directly affected by the substitutions. This will be addressed below. Binding of 2G12 to the hydrophobic V3 mutants and unmodified BG505 SOSIP.664 was similar (Fig. 1F).

Table 1: Contact residues of residues 306, 308 and 316 in BG505 SOSIP.664

<table>
<thead>
<tr>
<th>Residue</th>
<th>Contact residues &lt;4Å</th>
<th>Contact residues &lt;5Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>R308</td>
<td>T162, E164, Q170, I307, I309, G312, Q315</td>
<td>T162, T163, E164, Q170, S306, I307, I309, G312, Q315, A316, F317, N197 (other protomer)</td>
</tr>
<tr>
<td>A316</td>
<td>Q203, I307, G314, Q315, F317, Y318</td>
<td>V120, Q203, I307, R308, I309, G314, Q315, F317, Y318</td>
</tr>
<tr>
<td>A316W</td>
<td>Q203, I307, L308, G314, Q315, F317, Y318</td>
<td>V120, Q203, L306, I307, L308, I309, G312, Q314, Q315, F317, Y318</td>
</tr>
</tbody>
</table>

We determined the contact residues of the three V3 residues both in the unmodified situation (S306, R308 and A316), and after in silico modeling of the hydrophobic mutations (S306L, R308L and A316W). The modeling and analysis of contacts was performed with the crystal structure of BG505 SOSIP.664 (PDB: 5CEZ ; [66]) using Pymol [120]. Residues 306, 308 and 316 are listed in bold text.

Introduction of hydrophobic residues in V3 reduces Env function

To determine the effect of the S306L and R308L substitutions on Env function and viral infectivity, we introduced them in the background of BG505 pseudovirus and assessed the infectivity of TZMbl cells. The substitutions S306L and R308L decreased infectivity of the BG505 pseudovirus by 2- and 5-fold, respectively (Fig. 1G). The combination of substitutions S306L and R308L further decreased infectivity (by 10-fold), to an infectivity level that was...
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Figure 1: Design and preliminary characterization of hydrophobic substitutions in the V3 domain

(A) Linear representation of V3 stabilized BG505 SOSIP variants. The SOSIP.664 mutations are indicated in red, the SOSIP.v4.1 mutations (E64K and A316W) in blue, the SOSIP.v5.2 mutations (A73C-A561C) in purple and the new hydrophobic V3 mutations in light brown [17,43, de la Peña et al. manuscript in preparation]. The assignment of glycans was based on Behrens et al [11]. (B) Side view of the crystal structure of BG505 SOSIP.664 [66] in which two protomers are shown in surface rendering, one in white and one in grey, while the third protomer is represented as cartoon. In this third protomer various subdomains are colored differently: gp41 in dark-yellow, gp120 in green and the V3-loop in red. The hydrophobic V3 mutations, S306L, R308L and A316W were introduced in the crystal structure by in silico mutagenesis using Pymol and are depicted in blue. (C) A detailed view of the V3-loop of the BG505 SOSIP.664 trimer, with residues S306, R308 and A316 illustrated as sticks. (D) A detailed view of the V3-loop of the BG505 SOSIP.664 crystal structure, in which mutations S306L, R308L and A316W were introduced by in silico mutagenesis (shown in blue). (E) BN-PAGE analysis of unpurified V3-modified BG505 SOSIP.664-D7324 variants, followed by westernblotting with 2G12. (F) D7324 capture ELISA with unpurified V3-modified BG505 trimer variants. (G) Infection of TZM-bl cells by BG505.T332N Env-pseudovirus variants with S306L, R308L and/or A316W substitutions.

similar as that of the A316W single mutant ([43], Fig. 1G). Thus, the introduction of hydrophobic residues at positions 306 and 308 in the V3 region severely reduced the
Figure 2: Biochemical characterization of BG505 trimers with hydrophobic substitutions in the V3 domain

BG505 SOSIP.v4.1 and SOSIP.v5.2 trimers variants were purified using PGT145-affinity chromatography and subjected to analysis by BN-PAGE, SDS-PAGE, NS-EM, DSC and ELISA. To improve the readability of the panels we numbered BG505 trimer variants as follows: 1: BG505 SOSIP.664, 2: BG505 SOSIP.v4.1, 3: BG505 SOSIP.v4.1 S306L R308L, 4: BG505 SOSIP.v5.2, 5: BG505 SOSIP.v5.2 S306L R308L. (A-C) Coomassie staining of BG505 trimers (1-5) subjected to BN-PAGE analysis (A), and reducing (left 3 lanes) and non-reducing (right 3 lanes) SDS-PAGE analysis (B, C). SDS-PAGE results for the S306L R308L mutant in the background of the BG505 SOSIP.v4.1 and BG505 SOSIP.v5.2 trimer are displayed in panels B and C, respectively. (D) 2D class averages of NS-EM analyses of BG505 SOSIP.v4.1 S306L R308L and SOSIP.v5.2 S306L R308L trimers. (E) Fit of the thermal melting curves of BG505 SOSIP.v4.1 S306L R308L and SOSIP.v5.2 S306L R308L trimers in comparison with those of SOSIP.664, SOSIP.v4.2 and SOSIP.v5.2, analysed using a two-state model. The data of the latter was adopted from [43, de la Peña et al. manuscript in preparation]. The raw melting curves can be found in Fig. S1. (F) MAb binding to the PGT145-purified BG505 SOSIP.v4.1 S306L R308L and SOSIP.v5.2 S306L R308L trimers and comparator proteins as probed using a D7324 sandwich ELISA.
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infectivity of the BG505 pseudovirus, either by interfering with conformational changes required for Env trimer function or by affecting the interaction of the V3 domain with the co-receptor directly. Both the V3 crown and V3 stem domain participate in the interaction with the CCR5 co-receptor. The V3 crown binds the second extracellular loop (ECL2) of CCR5, while the V3 stem interacts with the amino-terminal domain of CCR5 [75–78].

Introduction of hydrophobic residues in V3 increases Env trimer stability
To further study the two hydrophobic V3 mutations S306L and R308L, we introduced the two substitutions in the background of the well described BG505 SOSIP.v4.1 as well as the further stabilized BG505 SOSIP.v5.2, that both already contain the A316W substitution in V3 [43, de la Peña et al. manuscript in preparation]. The SOSIP.v4.1 S306L R308L and SOSIP.v5.2 S306L R308L proteins as well as their respective parental proteins were expressed in 293F cells. Protein purification via PGT145 affinity chromatography yielded cleaved Env trimers for both constructs as assessed by BN-PAGE and SDS-PAGE (Fig. 2A-C). The combination of the S306L and R308L substitutions did reduce trimer yields after PGT145 purification. The yields were ~1.0 mg/l for BG505 SOSIP.v4.1 and ~0.5 mg/l for BG505 SOSIP.v5.2 compared to ~2.0 mg/l for both the parental SOSIP.v4.1 and SOSIP.v5.2 proteins (Table 2). These results were consistent with the reduced expression observed in the BN-PAGE analysis of unpurified material from 293T cells (Fig. 1E). Negative stain electron microscopy (NS-EM) analyses revealed that all trimers were >95% native-like, indicating that S306L and R308L mutations did not adversely affect the conformation of the trimers (Table 2; Fig. 2D).

### Table 2: Biochemical data on V3 hydrophobic mutants of BG505 SOSIP trimers.

<table>
<thead>
<tr>
<th>Construct</th>
<th>NS-EM</th>
<th>DSC</th>
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<tr>
<td></td>
<td>Yield (mg/l)</td>
<td>% NL trimer</td>
</tr>
<tr>
<td>BG505 SOSIP.v5.664</td>
<td>~2.0</td>
<td>&gt;95</td>
</tr>
<tr>
<td>BG505 SOSIP.v4.1</td>
<td>~2.0</td>
<td>&gt;95</td>
</tr>
<tr>
<td>BG505 SOSIP.v4.1</td>
<td>~1.0</td>
<td>&gt;95</td>
</tr>
<tr>
<td>S306L R308L</td>
<td>~2.0</td>
<td>&gt;95</td>
</tr>
<tr>
<td>BG505 SOSIP.v5.2</td>
<td>~0.5</td>
<td>&gt;95</td>
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</table>

*a Values derived from previous studies [43, de la Peña et al. manuscript in preparation]

*b Reported values are for a His-tagged construct [de la Peña et al. manuscript in preparation]

The biophysical properties of 293F cell-expressed, PGT145-purified SOSIP.664-D7324 trimers were assessed using NS-EM to determine native-like trimer formation, and DSC to quantify thermostability ($T_m$: midpoint of thermal denaturation). The original EM and DSC data are shown in Fig. 2D, 2E and Fig. S1. The DSC data were fitted using a two state model (Fig. 2E). The $T_m$ values and NS-EM data for SOSIP.664, SOSIP.v4.1 and SOSIP.v5.2 have been reported previously and are listed here for comparison [43, de la Peña et al. manuscript in preparation]. Note that all constructs contain a D7324 tag, except for BG505 SOSIP.v5.2, which was produced with a His-tag (de la Peña et al. manuscript in preparation). NL trimers: native-like trimers.
Table 3: Interaction of anti-V3 Abs with residues 306, 308 and 316

<table>
<thead>
<tr>
<th></th>
<th>S/R306</th>
<th>R/H308</th>
<th>A316</th>
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<tbody>
<tr>
<td>447-52D</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>19b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>39f</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14e</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4F5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The dependencies of V3 MAbs on residues 306, 308 and 316 as determined in published reports are listed [78,79,82]. These studies reported that the respective V3 MAbs were dependent (recorded here with +) or not dependent (-) on residues 306, 308 and 316. ND, not determined.

Since the introduction of hydrophobic residues in V3 can increase the overall thermostability of SOSIP trimers [43], we assessed the thermostability of the SOSIP.v4.1 S306L R308L and SOSIP.v5.2 S306L R308L proteins in comparison with their parental proteins by differential scanning calorimetry (DSC). The SOSIP.v4.1 S306L R308L trimer had a midpoint of thermal denaturation ($T_m$) of 72.6°C compared to 69.5°C for SOSIP.v4.1. The SOSIP.v5.2 S306L R308L trimer had a $T_m$ of 78.1°C compared to 74.2°C for SOSIP.v5.2. Thus, the introduction of the S306L and R308L mutations increased thermostability of the SOSIP.v4.1 and SOSIP.v5.2 trimers by 3.2°C and 3.9°C, respectively, suggesting that the two leucines enhanced the hydrophobic packing of the V3 thereby keeping the V3 region in the hydrophobic pocket underneath the V1V2 domain (Table 1; Fig. 2E). To conclude, the introduction of two hydrophobic residues in the V3 region, S306L and R308L, increased the thermostability of BG505 SOSIP.v4.1 and SOSIP.v5.2 trimers, however at the expense of lower trimer yields.

**Introduction of hydrophobic residues in V3 decreases binding of V3 non-NAbs to Env trimers**

To determine if we successfully decreased presentation of V3 non-NAb epitopes, we compared binding of V3-directed non-NAbs 39f, 4F5, 447-52D and 19b to the BG505 SOSIP.v4.1 and SOSIP.v5.2 trimers harboring the S306L and R308L mutations with binding to the parental SOSIP.v4.1 and SOSIP.v5.2 trimers as well as the original SOSIP.664 trimer. All 4 MAbs bound efficiently to the SOSIP.664 trimers, consistent with previous results showing that under ELISA conditions, but not SPR, ITC, or NS-EM conditions, the epitopes for V3 non-NAbs become exposed (Fig. 2F; [17,43]). The 4 MAbs bound 2-10 fold less efficiently to the SOSIP.v4.1 protein and binding of 39F and 447-52d to the SOSIP.v5.2 trimer was undetectable (Fig. 2F). These results are in line with previous results showing that the SOSIP.v4.1 and SOSIP.v5.2 designs reduce V3 exposure [43, de la Peña et al. manuscript in preparation]. When we tested binding of the 4 V3 MAbs to the BG505 SOSIP.v4.1 and SOSIP.v5.2 in which the S306L and R308L substitutions were present, we could not detect any binding of 39F, 4F5 and 447-52D (Fig. 2F) and 19b binding was further reduced.

Obviously, these two substitutions might affect binding of V3-specific MAbs by two mechanisms, first by reducing V3 exposure via enhanced hydrophobic packing, thus indirectly affecting binding, and second by directly affecting the epitope of the V3 MAbs. These experiments do not distinguish between these two possibilities, and probably both mechanisms play a role (see below). Interestingly, in the presence of soluble CD4 the binding of MAb 17b, which interacts with an epitope that becomes exposed upon CD4 binding and requires CD4-induced conformational changes, was substantially reduced when the S306L and R308L substitutions were present, suggesting that the enhanced hydrophobic packing
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of the V3 region interferes with CD4 induced conformational changes in the Env trimer (Fig. 2F). bNAbs targeting other epitopes on the trimer (2G12, VRC01, PGT145, PGT151 and PGT126), including ones dependent on native-like quaternary trimer structure interacted equally well to the S306L R308L double mutant proteins and their unmodified counterparts, indicating that the overall conformation of the trimer was not affected by these changes (Fig. 2F).

Introduction of hydrophobic residues in V3 reduces V3 immunogenicity in rabbits

To determine whether the greatly reduced V3 non-NAb binding to the BG505 SOSIP.v5.2 S306L R308L protein translated into a reduced V3 Ab responses in vivo, we immunized rabbits with BG505 SOSIP.v5.2 or SOSIP.v5.2 S306L R308L at week 0, 4 and 20, and analyzed Ab binding titers as well as neutralization capacity of the sera at week 22, two weeks after the third immunization (Fig. 3A).

We first measured Ab binding titers to the modified BG505 SOSIP.v5.2 S306L R308L trimer and the unmodified BG505 SOSIP.664 trimer. SOSIP.v5.2 S306L R308L immunized rabbits induced similar trimer binding titers compared to the modified and unmodified trimers, indicating that the Abs elicited by the SOSIP.v5.2 S306L R308L immunogen predominantly recognized shared epitopes on the SOSIP.v5.2 S306L R308L trimer and the unmodified BG505 SOSIP.664 trimer (Fig. 3B). Similar results were obtained with the sera from the BG505 SOSIP.v5.2 control group (Fig. 3B).

To assess whether Abs that specifically target the hydrophobic residues in the V3 were induced, we measured Ab titers to the unmodified BG505 V3 peptide as well as to the triple mutant V3 peptide (S306L R308L A316W) by ELISA. The sera from the BG505 SOSIP.v5.2 immunized control animals reacted strongly with the WT V3 peptide, but less efficiently (6-fold difference of the median titer) with the V3 triple mutant peptide, indicating that residues S306, R308 and A316 contributed to V3 targeting Abs that were present in the SOSIP.v5.2 recipient sera (Fig. 3C).

Table 4. Midpoint neutralization titers for sera from rabbits immunized with BG505 SOSIP.v5.2 or SOSIP.v5.2 S306L R308L trimers, tested against a panel of Env-pseudotyped viruses.

<table>
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<tr>
<th>Virus Tier Clade</th>
<th>BG505</th>
<th>SF162</th>
<th>MW.965</th>
<th>AMC008</th>
<th>REJO</th>
<th>WITO</th>
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Table 4. Midpoint neutralization titers for sera from rabbits immunized with BG505 SOSIP.v5.2 or SOSIP.v5.2 S306L R308L trimers, tested against a panel of Env-pseudotyped viruses.

The TZM-bl cell assay was performed at AMC, except for MW.965 neutralization, which was performed at Duke. ID_{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_{50} <40; yellow, weak neutralization, ID_{50} 40-100; orange, moderate neutralization, ID_{50} 100-1000; red, strong neutralization, ID_{50} >1000.
The sera from rabbits immunized with the SOSIP.v5.2 S306L R308L protein showed weaker binding to the WT V3 peptide in ELISA (2-fold reduction in median titer compared to the response induced by SOSIP.v5.2). Furthermore, binding to the V3 triple mutated peptide was equally weak, indicating that the V3 domain was generally less immunogenic and that the introduced hydrophobic residues in the V3 did not create highly immunogenic neo-epitopes (Fig. 3C).

Next, we measured neutralization of the parental Tier-2 virus BG505 (containing a T332N substitution). In the analyses we included the sera from 14 animals that were previously immunized with either BG505 SOSIP.664 (9 animals) or SOSIP.v5.2 (5 animals) according to the same immunization schedule used here [43,70, de la Peña et al. manuscript in preparation]. Strong autologous neutralization of the parental BG505 pseudovirus was observed in the SOSIP.v5.2 immunized and SOSIP.v5.2 S306L R308L immunized rabbits, consistent with the strong autologous neutralization induced in BG505 SOSIP.664 immunized rabbits (Fig. 3D, Table 4) [43,67,70]. We conclude that the introduction of hydrophobic residues in V3 did not compromise the autologous NAb response. This is consistent with observations that the autologous NAb response induced by BG505 SOSIP trimers does not target the V3, but predominantly targets a hole in the glycan shield at position 241 [43,67,68,70].

Since virus isolates that are hypersensitive to neutralization (i.e. Tier-1A viruses) are usually mainly neutralized by V3 Abs, they function as a read out for the elicitation of V3 Abs that are non-neutralizing for primary isolates that have a Tier-2 phenotype such as BG505. BG505 SOSIP.664 trimers induce strong NAb responses against the clade B Tier-1A virus SF162 (median titer of 192; Fig. 3E), and we have previously shown that these responses were predominantly attributable to V3-specificities [43,70]. Neutralization of SF162 by sera from SOSIP.v5.2 immunized rabbits was lower (median ID$_{50}$ of 102). In contrast, SF162 NAb activity was absent from 4 out of 5 animals that received the BG505 SOSIP.v5.2 S306L R308L protein and extremely weak for the fifth animal (ID$_{50}$ of 27; Fig. 3E, Table 4). Overall there was a 10-fold reduction in median ID$_{50}$ for the BG505 SOSIP.v5.2 S306L R308L compared to the BG505 SOSIP.664 recipient animals (p=0.001) and a >5-fold reduction compared to BG505 SOSIP.v5.2 recipient animals (p=0.02). Similar results were obtained with a clade C Tier-1A virus MW.965 (Fig. 3F, Table 4). Thus, while sera from BG505 SOSIP.664 recipients neutralized MW.965 with a median ID$_{50}$ of 221,100, the titer was decreased for BG505 SOSIP.v5.2 recipients (ID$_{50}$ of 289) and virtually abolished for the animals that received BG505 SOSIP.v5.2 S306L R308L (ID$_{50}$ of 27; p=0.02 for the comparison with BG505 SOSIP.664 and p=0.11 for the comparison with SOSIP.v5.2).

We also tested neutralization of heterologous Tier-2 viruses (Table 4). None of the animals induced strong and/or consistent neutralization of any of the Tier-2 viruses tested, except for animal 1834 which received BG505 SOSIP.v5.2 S306L R308L. The sera from this animal neutralized heterologous Tier-2 viruses REJO, WITO, and SHIVp3 with ID$_{50}$ values of 206, 531, and 416. As only one of the BG505 SOSIP.v5.2 S306L R308L recipients developed this cross-neutralizing response we cannot determine whether this was a spurious result or attributable to the introduction of the S306L and R308L substitutions. In summary, the BG505 SOSIP.v5.2 S306L R308L immunized rabbits developed reduced levels of Tier-1A NAbs, which confirms the reduced exposure and immunogenicity of the V3 loop.
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Figure 3: Immunogenicity of BG505 SOSIP.v5.2 S306L R308L trimers in rabbits. (A) Rabbit immunization schedule. Rabbits were immunized with 22 µg of the BG505 SOSIP.v5.2 S306L R308L and comparator proteins at week 0, 4 and 20 and binding titers and neutralization titers were measured in the week 22 sera, i.e. two weeks after the third and last immunization. (B) Trimer binding responses. The midpoint binding titers against D7324-tagged BG505 SOSIP.664 and SOSIP.v5.2 S306L R308L are plotted. (C) Midpoint binding titers against a cyclized WT BG505 V3 peptide and the triple mutant V3 peptide (S306L, R308L and A316W). (D-F) NAb responses. The midpoint neutralization titers against the autologous Tier-2 virus BG505.T332N (D), and heterologous Tier-1A viruses SF162 and MW.965 (E,F), were determined using the TZM-bl assay. Additional control sera from previous rabbit studies were included for comparison. These included sera from 9 BG505 SOSIP.664 recipient animals (Rabbit IDs 1274-1277 and 1569-1573; [18,43]) and from 5 BG505 SOSIP.v5.2 recipients (IDs 1589-1593; de la Peña et al. manuscript in preparation). Mann-Whitney U tests were used to determine whether differences between groups were statistically significant (*, p<0.05; **, p<0.01; ***, p<0.001).

Discussion
The development of an HIV-1 vaccine probably requires the design of (an) Env-based immunogen(s) that elicit(s) bNAb. To achieve that goal it is necessary to increase the immunogenicity of the otherwise subdominant conserved bNAb epitopes and at the same time prevent or reduce the immunogenicity of otherwise immunodominant non-NAb epitopes. The design of native-like trimers that closely mimic the native Env spike, including BG505 SOSIP.664 and next-generation SOSIP trimers, represents one step into this direction,
as on SOSIP trimer many non-NAb epitopes are occluded by trimer interactions while most bNAb epitopes are exposed. As a result, BG505 SOSIP.664 and further stabilized HIV-1 Env trimers induce autologous NAbs against the autologous Tier-2 virus in rabbits and macaques, something not achieved by earlier Env forms that exposed many non-NAb epitopes.

Nevertheless, despite the induction of Tier-2 NAbs, the immune response induced by BG505 SOSIP.664 trimers was dominated by non-NAbs targeting the V3 domain, revealing that the V3 domain became exposed during vaccination with BG505 SOSIP.664 trimers and remained highly immunodominant. We previously showed that the V3-response can be decreased by an A316W substitution in V3. Here, we extended our previous efforts to decrease the exposure and immunogenicity of the immunodominant V3 domain by introducing two additional hydrophobic residues in the V3 domain, S306L and R308L. Rabbits that were immunized with the BG505 SOSIP.v5.2 S306L R308L trimer developed virtually no V3-targeting Tier-1A NAbs, whereas similar autologous Tier-2 NAbs were elicited compared to animals that received BG505 SOSIP.664 or SOSIP.v5.2 trimers. HIV-1 Env immunogens with limited presentation of otherwise immunodominant non-NAb V3 epitopes might facilitate various immunization strategies that aim for the development of bNAbs.

In the prefusion structure of the Env trimer, the metastable V3 loop is sequestered in a hydrophobic pocket below the V1V2 domain. Our primary goal was to stabilize the V3 region in that sequestered prefusion position, preventing or limiting its exposure to the humoral immune system. The findings that the S306L and R308L substitutions reduced binding of V3 MAbs, reduced CD4-induced conformational changes and enhanced the thermostability of BG505 SOSIP trimers, strongly suggest that we achieved that goal. Furthermore, our results illustrate that single amino acid substitutions had only minor effects on the exposure of the V3 domain and that a combination of three hydrophobic substitutions (S306L, R308L and A316W) was necessary to provide the optimal hydrophobic environment to retain the V3 loop in its prefusion conformation. In silico mutagenesis of S306L and R308L and A316W showing that all three introduced hydrophobic residues are in close contact (<5 Å), supports the idea that the hydrophobic interactions between these three residues and neighboring residues is the driving force for the stabilization of the V3 region (Table 1, Fig. 1D). When designing such hydrophobic mutations in or around V3 it is crucial to limit clashes with neighbouring residues. For example, an L125F substitution in the V1V2 stem (L125F) resulted in the clash with an aromatic residue in the V3 loop (F317) and increased the exposure of the V3 domain [74]. We observed a similar increase in V3 loop exposure when we introduced a tryptophan at position 125 (L125W) in the BG505 SOSIP.664 trimer (data not shown).

In addition to reducing the exposure of the V3 domain by stabilizing its hidden conformation, the two leucine mutations might also have a direct effect on the immunogenicity of the V3 region. Thus, the two introduced leucines might directly reduce the interaction of V3 Abs that were induced by a WT V3, and induce Abs that only recognize the mutant V3, but not the WT V3. V3 MAbs 447-52D and 2219 bind the tip of the V3-loop, the GPGR motif, but also interact with residues 306 and 308 [79–81]. Many other V3 non-NAbs target charged residues in or near the GPGR motif at the V3 tip [82], illustrating that the S306L and R308L mutations are in a region that is frequently targeted by V3 non-NAbs (Table 3). A study in which amino-acids in the V3 of a gp120 monomer were replaced by serine showed that V3 immunogenicity was largely reduced when the amino-acids upstream of the
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V3-tip (i.e. residues 306-311) were replaced by serine [83]. Thus, it is likely that S306L and R308L will reduce the immunogenicity of the V3 loop directly, by changing the V3 non-NAb epitopes, and also indirectly, by reducing the exposure of these same non-NAb epitopes. The fine specificities of the epitope of V3 MAb 447-52D and 19b illustrate that probably both mechanisms are involved. While 447-52D interacts with both residue 306 and 308, 19b is not dependent on these residues for the interaction with the V3 loop (Table 3). We did not find evidence that the newly introduced leucines created immunodominant neo-epitopes (Fig. 3C).

The strategy to reduce V3 non-NAb epitope immunogenicity is based on the implicit assumption that reducing immunodominance of such epitopes alleviates the subdominance of (b)NAb epitopes. A central question is therefore whether “off-target” non-NAb responses actually interfere with the development of (b)NAb or whether they are merely irrelevant. Although non-NAb interference has never been directly proven in HIV-1 Env immunization studies, several lines of evidence from other research areas and in vitro B-cell stimulation experiments support the idea that reducing the immunodominance of non-NAb epitopes should be beneficial. For example, non-NAbs have been proposed to interfere with the induction of bNAb against a number of pathogens, such as influenza and malaria [84–89]. The other side of the coin is that bNAb epitopes are usually subdominant. The germline precursor of non-NAbs, i.e. the BCR on naive B-cells, occur frequently in the human repertoire, are easily activated by HIV-1 Env immunogens and need a relatively small number of SHM to acquire high affinity. Instead, the germline precursors of bNAb are rare, difficult to activate by HIV-1 Env immunogens and require extensive SHM to acquire enough affinity to neutralize [90]. In vitro B-cell activation studies suggests that, because germline precursors of non-NAbs have a higher affinity for recombinant HIV-1 Env immunogens, they have a selection advantage in the germinal center over the germline precursors of bNAb [91]. The selection of higher-affinity B cell clones in response to HIV-1 Env immunogens hinders the activation and affinity maturation of low-affinity germline bNAb that compete for the same resources in the germinal center [90–92]. It seems likely that preventing the activation of high-affinity non-NAb precursors will open the way for the activation of bNAb precursors [90,92,93]. However, other strategies including bNAb germline targeting and maturation, as well as particulate immunogen presentation will probably be necessary to initiate and mature bNAb lineages [35,94–96].

In summary, using structure based HIV-1 Env immunogen design we generated next generation BG505 SOSIP trimers with dramatically reduced immunogenicity of otherwise immunodominant non-NAb epitopes in the V3 loop. Two new hydrophobic substitutions S306L and R308L, complemented with the previously described A316W substitution, stabilize the V3 domain in its hidden position underneath the V1V2 domain via hydrophobic interactions. These HIV-1 Env trimers provide improved scaffolds for vaccination strategies aimed at inducing bNAb, and that might involve specific germline-targeting strategies [93,96–100], patient-based lineage strategies [101–106], and particulate immunogen strategies [94,107–110].
Methods

Construct design
The BG505 SOSIP.664 construct has been described elsewhere [18]. It was generated by introducing the following sequence changes: A501C and T605C (gp120-gp41 ECTO disulfide bond; [51]); I559P in gp41 ECTO (trimer-stabilizing; [111]); REKR to RRRRRR in gp120 (cleavage enhancement; [112]); a stop codon at gp41 ECTO residue 664 (improvement of homogeneity and solubility; [113]). SOSIP.664-D7324 trimers contain a D7324 epitope-tag sequence at the C-terminus of gp41 ECTO and were constructed by adding the amino-acid sequence GSAPTKAKRRVVQREKR after residue 664 in gp41 ECTO [17]. Next-generation, further stabilized trimers have the following sequence changes: BG505 SOSIP.v4.1: E64K, A316W [43] and BG505 SOSIP.v5.2: E64K A316W A73C-A561C (de la Peña et al. manuscript in preparation). Point mutants were generated by Quikchange site directed mutagenesis (Agilent, Stratagene), and verified by sequencing. D7324-tagged trimers were used for all experiments, except for immunization experiments, in which untagged trimer variants were used.

Env protein expression
Proteins encoded by the various env genes described above were expressed in adherent (HEK) 293T cells, or in (HEK) 293F cells adapted for suspension cultures, essentially as described [17,43]. All experiments with purified trimers used 293F cell-expressed proteins. The 293T 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 small scale trimer expression, 293T cells were seeded at a density of 5.5×10^4/ml in a 6-well plate. The next day, when the cells had reached a density of 1.0×10^6/ml, they were transfected using polyethyleneimine (PEI), as previously described [114]. Briefly, PEI-MAX (1.0 mg/ml in water) was mixed with expression plasmids for Env and Furin [51] 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 (1 mg/ml) were added in 3 ml of growth media (DMEM supplemented with FCS, penicillin and streptomycin). Culture supernatants were harvested 72 h after transfection. For larger-scale trimer production, Env proteins were produced in 293F cells using a protocol similar to that described previously [43]. Briefly, PEI-MAX (1.0 mg/ml) in water was mixed with the expression plasmids for Env and Furin in OPTI-MEM. For cultures in a 2 l 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 1 l 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 column, essentially as described [17,43,55]. The columns were made by coupling PGT145 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). Protein concentrations were determined using UV280 absorbance and theoretical extinction coefficients via Expasy (ProtParam tool). All experiments were performed with D7324 tagged PGT145-purified trimers.

**SDS-PAGE**

Env proteins were analyzed using SDS-PAGE followed by western blotting or Coomassie blue dye staining [111,115]. 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 2 h 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 (1:2000 dilution, i.e. 0.2 μg/ml), followed by HRP-labeled goat anti-mouse IgG (1:5000; Jackson Immunoresearch) was performed as previously described [111]. 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 (BN)-PAGE**

For BN-PAGE [111,115], the input Env proteins were mixed in a 3:1 ratio with loading dye (500μl 20x MOPS Running Buffer (1 M MOPS + 1 M Tris, pH 7.7), 1000 μl 100% Ultrapure Glycerol (Invitrogen), 50μl 5% Coomassie Brilliant Blue G-250 solution, and 600 μl 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 as the anode buffer 1x NativePAGE Running Buffer (Invitrogen) and as the cathode buffer the same buffer supplemented with 1% NativePAGE Cathode-Buffer Additive (Invitrogen). Western blot 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 [111]. For Coomassie staining BN-PAGE gels were stained using the Colloidal Blue Staining Kit (Life Technologies).

**D7324-capture ELISA**

The methods to perform sandwich ELISAs using D7324-tagged BG505 SOSIP.664 trimers has been described elsewhere [17,33]. 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.
V3 MAb 4F5 was isolated from a patient participating in the Amsterdam Cohort Studies on HIV/AIDS (patient H18877; [116]). The other MAbs used here have been described elsewhere ([57,58,117,118]).

**V3 peptide ELISA**
To determine V3 Ab responses in BG505-immunized rabbits, 96-well MaxiSorp plates were coated overnight at 4°C with 2 µg/ml of a cyclized V3-peptide in PBS. The V3 peptide was based on the sequence of the BG505 SOSIP immunogen, either unmodified (CTRPNNTRKSIRIGPQAFYATGDIIGDIRQAHC) or including the S306L, R308L and A316W changes (CTRPNNTRKLILIGPQWFYATGDIIGDIRQAHC). The V3 peptides were cyclized by a disulfide bond between residues 1 (residue 296 in HXB2 gp160 numbering) and 35 (residue 331). The plates were then washed with PBS supplemented with 0.1% Tween-20 and blocked with PBS supplemented with 0.05% Tween-20, 3.3% FBS, and 2% BSA (PBS-TFB), for 1.5 h at room temperature. After washing with PBS 0.1% Tween, mouse or rabbit serum dilutions were added in PBS-TFB. The subsequent steps in the assay were as described above for the D7324 trimer capture ELISA.

**Negative-stain electron microscopy (EM)**
Purified BG505 trimers were analyzed by negative-stain EM. A 3 µl aliquot containing purified trimer at a concentration of 0.03 mg/ml 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. Data processing methods are described in detail elsewhere, including the closed, partially open, and non-native trimer classification system [33,55].

**Differential scanning calorimetry**
Thermal denaturation of purified Env proteins was studied using a MicroCal VP-Capillary DSC calorimeter (Malvern Instruments) or a nano-DSC calorimeter (TA instruments) as described previously [17,43]. 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 table 2 and Fig. 2E, while the multiple T_m values based on the non-two-state models are in Fig. S1. The DSC experiments were all performed with SOSIP.664-D7324 trimers.
Single-round infection assay and neutralization assay

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). TZM-bl cell neutralization assays using Env-pseudotyped or chimeric molecular clone viruses were performed at two different sites: DUMC, Duke University Medical Center, Durham, NC (for methodology see [119]); AMC, Academic Medical Center, Amsterdam (for methodology see [18,43]). 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 (2×10^5) 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 generate 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 infection, TZM-bl cells (17 × 10^3 cells) were added to a 96-well plate in DMEM containing 10% FCS and penicillin and streptomycin (both at 100 units/ml) and incubated at 37 °C in an atmosphere containing 5% CO₂. A fixed amount of virus (1 ng of CA-p24) was added to the TZM-bl cells (70–80% confluency) in the presence of 400 nM saquinavir (Roche Applied Science) and 40 μg/ml DEAE, in a total volume of 200 µl. To determine neutralization activity of rabbit sera, 1 ng of virus was first 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. 72h later, the medium was removed and the cells were lysed in Lysis Buffer (For 1 l: 1% Triton X-100, 1.8 g anhydrous MgSO₄, 1.88 g EGTA tetrasodium and 3.3 g Glycyglycine in MQ, pH=7.8). Luciferase activity was measured using a Bright-Glo™ Luciferase Assay System (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. 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 6.0.
Rabbit immunization
Rabbits were immunized under subcontract at Covance as described previously, using 22 
µg of PGT145-purified trimers formulated in ISCOMATRIX™ at week 0, 4 and 20, and Ab 
responses were assessed at week 22 [43]. The rabbit sera were assayed for autologous and 
cross-reactive NAbs using Env pseudoviruses in the TZM-bl cell assay [119].

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Supplemental figures

Figure S1: Thermostability of BG505 trimers with hydrophobic substitutions in the V3 domain
DSC analysis of PGT145-purified BG505 SOSIP.v4.1 S306L R308L and SOSIP.v5.2 S306L R308L. The unfolding patterns were fitted using a non-two state model, revealing three individual unfolding peaks. The data were also fitted to a two-state model and the $T_m$ values of those analyses are listed in Table 2 and Fig. 2E.