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Chapter 9

**Nanoparticle displayed stabilized native-like envelope trimers as immunogens**

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Abstract
Inducing broadly neutralizing antibodies (bNabs) against the HIV-1 envelope glycoprotein (Env) is the major goal of most HIV-1 subunit vaccine approaches. Recently, we have shown that soluble native-like Env trimers can induce neutralizing antibodies (NAbs) against the autologous neutralization-resistant (Tier 2) viruses. Furthermore, we have shown that presenting such Env trimers on ferritin-based nanoparticles increased antibody titers. Here, we have fused ferritin to several stabilized soluble Env trimers from different HIV-1 strains from clades B (AMC008, AMC016, and AMC018) and C (ZM197M). Fusion to ferritin preserved native-like trimer glycosylation, thermostability and antigenicity. We tested whether the AMC008 trimer nanoparticles were able to boost humoral immune responses in rabbits that had previously been immunized with single AMC008 or BG505 trimers. In a second experiment, we used a trivalent cocktail of AMC008, AMC016, and AMC018 trimers on ferritin nanoparticles to boost rabbits that had previously been immunized with BG505 trimers displayed on ferritin. In both experiments we observed enhanced NAb responses after nanoparticle vaccination. Thus, nanoparticles displaying stabilized native-like Env trimers are promising immunogens for the development of HIV-1 vaccines that can induce bNabs.
Introduction

An effective HIV-1 vaccine should probably induce broadly neutralizing antibodies (bNAbs), but raising such antibodies (Abs) is difficult. The target of HIV-1 neutralizing antibodies (NAbs) is the viral envelope glycoprotein (Env) complex, but HIV-1 has evolved several strategies to avoid NAb induction and recognition [1]. Over the course of 30 years, different immunogens have been designed to overcome these evolutionary hurdles, with limited success.

Until recently, no immunogens were able to induce NAbs against neutralization-resistant (Tier 2) primary circulating viruses [2]. However, recombinant soluble proteins based on the BG505 and B41 isolates that adequately mimic the viral Env spike, termed SOSIP.664 trimers [3–6], can induce NAbs against the sequence matched autologous Tier 2 viruses, arguably an important first step towards an effective HIV-1 vaccine [7,8]. Additional soluble native-like Env trimers from different env genes and subtypes have recently been described [8–11]. Such antigens can be used in sequential or cocktail immunizations that select cross-reactive B cells that could generate NAbs with increased breadth [12].

However, SOSIP.664 trimer immunogens also generated Abs against epitopes that are not exposed on the viral Env spike and usually do not neutralize primary circulating viruses [7]. Several lines of evidence suggest that such non-neutralizing Abs (non-NAbs) might interfere with the induction of NAbs, in particular with cross-reactive NAbs [12–16]. Therefore, native-like Env trimer variants were designed that expose less non-neutralizing epitopes [8,17,18] and the first immunogenicity studies show that these trimers indeed induce less non-NAbs when vaccinated in animals [8] (de la Peña et al. manuscript in preparation). The same protein design improvements could be applied to different env isolates which gave rise to an arsenal of soluble Env trimers with higher propensity to fold into native-like trimers and showed more favorable antigenicity [8,17,18]. Arguably, such Env trimers could be important constituents of vaccines which involve multiple immunogens to induce cross-reactive NAbs.

Traditional B cell immunology dictates that multimeric display of antigen is necessary for sufficient B cell activation and potent Ab responses [19,20]. This is exemplified by the fact that the only two licensed viral subunit vaccines (against hepatitis B and human papillomavirus) are virus-like particles [21]. The nanoparticles based on the self-assembling *Helicobacter pylori* ferritin 24-mer have been used to display different viral antigens, such as influenza hemagglutinin (HA) and glycoprotein 350 of Epstein-Barr virus [22–24]. Likewise, we have shown that BG505 SOSIP.664 attached to ferritin nanoparticles is more immunogenic than BG505 SOSIP.664 alone [25]. In an attempt to induce a broader and more potent NAb response we have engineered and characterized ferritin nanoparticles carrying stabilized Env trimers from clades B and C. Some of these ferritin-displayed immunogens were used to boost rabbits using a monovalent or cocktail vaccine containing nanoparticles with Env trimers from subtype B.

Results

Display of AMC008 SOSIP.v4.2 trimers on ferritin nanoparticles

We have recently described a new SOSIP.664 trimer based upon a clade B env gene: AMC008 SOSIP.664. Additional modifications were introduced to obtain stable native-like Env trimers (termed SOSIPv4.2) from this isolate with favorable antigenicity (Fig. 1A) [8].
AMC008 SOSIP.v4.2 was fused to the self-assembling *Helicobacter pylori* ferritin nanoparticle [25] (Fig. 1A). AMC008 SOSIP.v4.2-ferritin was transfected in suspension HEK293F cells and purified via PGT145 affinity chromatography. Typical yields were significantly higher (~1-3 mg/L) compared to those obtained with BG505 SOSIP.664-ferritin (~0.2 mg/L). The SOS bond between C501 and C605 was formed effectively as indicated by the presence a gp140 band in the non-reducing SDS-PAGE analyses (Fig. 1B). The lack of covalently linked dimers and trimers showed that aberrant interprotomer disulfide bonds, commonly found in gp120 and non-native gp140 preparations [26], were absent. Reducing SDS-PAGE analysis revealed that the gp140 moiety of AMC008 SOSIP.v4.2-ferritin was inefficiently cleaved into gp120 and gp41,~\text{SCTD} (~55% cleaved for this particular transfection; range 55-70%), despite the fact that we co-transfected higher amounts of furin expression vector than usual (Fig. 1B). One possible explanation is that access of furin to its cleavage site is sterically obstructed (Fig. 5B). This, as well as its potential implications are considered in the Discussion.

We next explored the structural integrity of AMC008 SOSIP.v4.2-ferritin by a variety of biophysical techniques. Dynamic light scattering (DLS) analyses showed that the hydrodynamic radius ($R_h$) of AMC008 SOSIP.v4.2-ferritin proteins was 151 Å (±7.3 Å), while that of trimeric AMC008 SOSIP.v4.2 was 62 Å (Fig. 1D). Negative stain electron microscopy (NS-EM) and cryo electron microscopy (cryo-EM) analyses confirmed that the size of AMC008 SOSIP.v4.2-ferritin was 30-40 nm diameter and the particles consisted of Env trimers surrounding a ferritin sphere (Fig. 1C). In some of the 2D class average the gp41 and gp120 subunits are discernable. NS-EM is normally used to confirm the native-like appearance of soluble Env trimers [27], but the different orientations of the Env trimers on the ferritin moiety impeded such analyses. The midpoint of thermal denaturation ($T_m$) of AMC008 SOSIP.v4.2-ferritin as assessed by differential scanning calorimetry (DSC) was 69.2°C, compared to 64.0°C for the AMC008 SOSIP.v4.2 trimer alone [8], suggesting that the conjugation to the ferritin 24-mer had a substantial stabilizing effect on AMC008 SOSIP.v4.2 (Fig. 1E and F). We also observed a small additional melting event around 77°C, probably representing denaturation of *H. pylori* ferritin [23].

We analyzed the glycosylation profile of AMC008 SOSIP.v4.2-ferritin and AMC008 SOSIP.v4.2 trimers by hydrophilic interaction liquid chromatography-ultra performance liquid chromatography (HILIC-UPLC) (Fig. 1G) [28]. AMC008 SOSIP.v4.2-ferritin displayed a high oligomannose content (72%), dominated by Man$_9$GlcNAc$_2$ (28%) and Man$_9$GlcNAc$_2$ (19%) glycans, as did trimeric AMC008 SOSIP.v4.2 (69% oligomannose; 39% Man$_9$GlcNAc$_2$; 12% Man$_8$GlcNAc$_2$), resembling the glycosylation profile of the native spike as well as other soluble native-like Env trimers, but not that of gp120 or non-native uncleaved trimers (Fig. 1G) [28].

To determine the antigenicity of AMC008 SOSIP.v4.2 on nanoparticles we used enzyme-linked immunosorbent assays (ELISA) based on lectin capture [25] (Fig. 1H). Generally, there were little or no differences in binding of most bNabs, including those directed against quaternary structure dependent epitopes at the trimer apex (2G12, VRC01, PGT128, PGT145). However, 35O22 and PGT151, bNabs that target the gp120/gp41 interface [29,30] interacted significantly less efficiently with AMC008 SOSIP.v4.2-ferritin compared to AMC008 SOSIP.v4.2 (Fig. 1H). A likely explanation for this finding is that bNabs that bind closer to the nanoparticle core are sterically hindered from accessing their epitopes by neighboring Env trimers (see Discussion; Fig. 5A). Overall, fusion of AMC008 SOSIP.v4.2 to ferritin generated nanoparticles fully occupied with trimers that
Nanoparticle display of stabilized native-like Env trimers interacted well with most bNAbs, had high thermostability, and had a native-like glycan shield.

**Figure 1.** Design and characterization of AMC008 SOSIP.v4.2-ferritin. A. Left: SOSIP.v4.2 mutations [8] indicated on the BG505 SOSIP664 crystal structure (PDB: 5CEZ from [42]). Right: the SOSIP664 crystal structure modeled on the *Helicobacter pylori* ferritin crystal structure (PDB: 3BVE). B. Non-reducing (-DTT) and reducing (+DTT) coomassie stained SDS-PAGE gels showing PGT145-purified AMC008 SOSIP.v4.2 trimer and AMC008 SOSIP.v4.2-ferritin. C. 2D class averages of cryo-EM analysis of AMC008 SOSIP.v4.2-ferritin. The ferritin sphere, gp41ECTO and gp120 subunits are indicated in the top left image. D. Representative DLS graphs showing the size distribution of AMC008 SOSIP.v4.2 and SOSIP.v4.2-ferritin proteins. E. Melting curves of AMC008 SOSIP.v4.2 and SOSIP.v4.2-ferritin measured by DSC. The asterisk denotes a possible additional melting event at ~77°C. F. Summary of the data presented in B, D and E. G. Glycan profiles of AMC008 SOSIP.v4.2 and SOSIP.v4.2-ferritin as determined by HILIC-UPLC. Circle diagrams indicate the relative amounts of Man₉GlcNAc₂ (M5-M9) and complex glycans. H. Antigenicity of AMC008 SOSIP.v4.2 and SOSIP.v4.2-ferritin measured by lectin-capture ELISA. The input concentration needed to get similar protein loading (calibrated by monitoring 2G12 binding) was lower for SOSIP.v4.2-ferritin (0.4 μg/ml) than SOSIP.v4.2 (1.5 μg/ml), probably because of the increased binding avidity of lectin for AMC008 SOSIP.v4.2-ferritin.
Display of AMC016, AMCO18 and ZM197M SOSIP trimers on ferritin nanoparticles

Vaccination with a single Env antigen will probably never generate potent cross-reactive NAbs [7]. Therefore, we also screened different SOSIP.v4.2-ferritin constructs based on different isolates for their ability to form nanoparticles. Ferritin constructs containing AMC016 or AMCO18 SOSIP.v4.2 (van den Kerkhof et al. submitted) could be purified with sufficient yield and purity (1.7-5.1 mg/L; 0.8-3.1 mg/L respectively) (Fig. 2A). Non-reducing SDS-PAGE analysis indicated that the SOS bond was formed efficiently, while aberrant interprotomer disulfide bonds were absent. Reducing SDS-PAGE analysis showed that the trimers on AMC016 and AMCO18 SOSIP.v4.2-ferritin were slightly more efficiently cleaved than AMCO08 SOSIP.v4.2-ferritin (~80% and ~70% cleavage, respectively, compared to ~55% for AMCO08) (Fig. 2A). NS-EM analyses revealed that both AMC016 and AMCO18 SOSIP.v4.2-ferritin formed fully occupied nanoparticles with a diameter of 30-40 nm (Fig. 2B).

**Figure 2.** Characterization of ferritin nanoparticles containing clade B soluble Env trimers. A. Non-reducing (-DTT) and reducing (+DTT) coomassie stained SDS-PAGE gels showing AMC016 and AMCO18 SOSIP.v4.2-ferritin. AMCO08 SOSIP.v4.2 and AMCO08 SOSIP.v4.2-ferritin were used for comparison. B. NS-EM 2D class averages of AMC016 and AMCO18 SOSIP.v4.2-ferritin.

**Figure 3.** Characterization and optimization of ferritin nanoparticles carrying a subtype C (isolate ZM197M) Env trimer. A. Crystal structure of BG505 SOSIP.664 (PDB: 5CEZ from [42]) in with the C73 and C561 positions highlighted in orange spheres. Together with the S35M, S43Q/N, H66R and A316W substitutions (as part of SOSIP.v4.2 [8], in cyan and yellow spheres as in Fig. 1C), the A73C and A561C substitutions constitute the SOSIP.v5.2 design (de la Peña et al. manuscript in preparation). B. NS-EM 2D class averages of ZM197M SOSIP.v4.2-ferritin. C. NS-EM 2D class average of ZM197M SOSIP.v5.2-ferritin.
To obtain a clade C trimer presented on nanoparticles, we designed a SOSIP.v4.2-ferritin construct based on the ZM197M strain [10], following the same design as above for AMC008, AMC016 and AMC018 (Fig. 3). However, the yields of this construct after PGT145 purification were poor (~0.2 mg/L) and NS-EM analyses showed that ZM197M SOSIP.v4.2-ferritin did not assemble into well-organized Env-carrying ferritin nanoparticles (Fig. 3B). The quality of ZM197M SOSIP.v4.2 trimers can be substantially improved by introducing an additional disulfide bond between residue 73 in gp120 and residue 561 in gp41Ecto generating SOSIP.v5.2 trimers (de la Peña et al. manuscript in preparation). When we added the C73-C561 disulfide bond to make ZM197M SOSIP.v5.2-ferritin, we were able to purify bona fide Env-carrying nanoparticles with a diameter of 30-40 nm at decent yields (~0.9 mg/L; Fig. 3C).

Capacity of nanoparticle displayed SOSIP.v4.2 trimers to boost humoral immunity
We examined the capacity of clade B SOSIP.v4.2 trimers presented on ferritin to boost antibody responses in two (uncontrolled) experiments in which rabbits had been previously exposed to either single trimers (AMC008 or BG505 SOSIP.664 trimers or more stabilized variants [8]) or BG505 SOSIP.664-ferritin [25] (Fig. 4).

In the first experiment, rabbits had previously received BG505 or AMC008 SOSIP trimers, or stabilized versions of those (described extensively in [8] (de la Peña et al. manuscript in preparation)). We specifically selected animals that that were low responders at week 22 and evaluated their responsiveness to AMC008 SOSIP.v4.2-ferritin given at weeks 36 and 52. The NAb responses at week 22 against the Tier 1B primary virus AMC008 were absent or low (50% inhibitory dilution (ID_{50}) <100) for the 6 animals that had previously received BG505 SOSIP variant trimers, and moderate (ID_{50} between ~100-300) for the 6 AMC008 SOSIP trimer recipients (Fig. 4A) (also reported in [8] (de la Peña et al. manuscript in preparation). In the BG505 SOSIP-primed animals, the median AMC008 ID_{50} values increased from 29 (week 22) to 57 and 226 (weeks 38 and 54, respectively), while in the AMC008 SOSIP-primed rabbits, they increased from 230 (week 22) to 241 (week 38) and 524 (week 54) (Fig. 4A). None of the rabbits displayed a strong heterologous NAb responses at the Tier 2 level after AMC008 SOSIP.v4.2-ferritin vaccination, but sporadic low titer NAb responses were observed in some rabbits (Fig. 4B).

In an attempt to induce more consistent NAbs against heterologous Tier 2 viruses, we boosted 5 animals that had previously been immunized with the clade A BG505 SOSIP.664-ferritin [25], with a single immunization of a cocktail of nanoparticles displaying either AMC008, AMC016, or AMC018 SOSIP.v4.2 trimers (Fig. 4C). Two weeks after this boost we detected moderate NAb responses against the autologous Tier 1B virus AMC008 (median ID_{50} value: 439) and the autologous Tier 2 virus AMC018 (median ID_{50} value: 97) viruses, but virtually none against the autologous Tier 2 virus AMC016 (Fig. 4C). Notably, all 5 rabbits displayed weak neutralization against the heterologous Tier 2 viruses AMC011 and REJO (median ID_{50} values of 63 and 50, respectively; Fig. 4C). Together these results show that Env trimers displayed on ferritin nanoparticles are immunogenic and can boost NAb responses.
Figure 4. NAb responses following clade B SOSIP.v4.2-ferritin boosts in immunized rabbits. A. Rabbits were first immunized with (stabilized versions of) BG505 or AMC008 SOSIP trimers at weeks 0, 4 and 20, also described in [8](de la Peña et al. manuscript in preparation). This was followed by two boosts of AMC008 SOSIP.v4.2-ferritin at weeks 36 and 52. The 50% inhibitory serum dilution (ID$_{50}$) against the autologous Tier 1B AMC008 virus were measured two weeks after each of the immunizations (weeks 22, 38 and 54). Statistically significant differences between the week 22 and 54 ID$_{50}$ titers were determined with the Wilcoxon matched-pair signed rank test for the BG505 or AMC008 primed groups together (top) or separately (bottom).

B. ID$_{50}$ titers of the rabbit sera in A from week 54 against a panel of different viruses. Note that the neutralizing activity against a negative control virus (mouse leukemia virus (MLV)) was unusually high (ID$_{50}$ >20) for some sera. Therefore, ID$_{50}$ titers of HIV isolates were only considered meaningful (indicated with yellow or orange shades) when the titers were 3-fold higher than the corresponding titers against MLV.

C. Midpoint neutralization titers of sera obtained two weeks after the rabbits received a single cocktail boost containing AMC008, AMC016 and AMC018 SOSIP.v4.2-ferritin following earlier DNA prime and protein boosts of BG505 SOSIP664-ferritin (extensively described in [25]).

Discussion

Presenting an antigen on nanoparticles can increase the immunogenicity of that antigen [31]. Moreover, vaccines containing antigens isolated from different pathogen strains can increase cross-reactivity of the humoral response [12]. In an attempt to generate cross-reactive HIV-1 NAb, we produced several ferritin-based nanoparticles that carry native-like Env trimers with favorable antigenicity. Specifically, we were able to fuse AMC008, AMC016, and AMC018 SOSIP.v4.2 ferritin in contrast, attempts to do so failed for other isolates, such as B41 and AMC009 SOSIP.v4.2 [8,9](van den Kerkhoff et al. manuscript in preparation).
preparation (not shown). For the clade C isolate ZM197M, fusion of SOSIPv4.2 to ferritin was unsuccessful, but adding an extra disulfide bond between gp120 and gp41\textsubscript{ECTO} (i.e., generating SOSIPv5.2) improved the formation of ferritin nanoparticles dramatically (Fig. 3C). This is in line with research describing that the addition of the C73-C561 disulfide bond improved antigenicity and thermostability of most SOSIP trimers, but more dramatically for ZM197M SOSIP trimers (de la Peña et al. manuscript in preparation). Collectively, these results suggest that assembly of well-formed SOSIP-carrying ferritin nanoparticles depends on env gene isolate and the presence of stabilizing mutations.

The Env trimers were tightly packed on the ferritin core with the apexes of two Env trimers separated by \sim 10 \text{ nm}. Since the optimal epitope distance for B cell activation has been suggested to be \sim 10 \text{ nm} [32], these particles might be optimal for the activation of apex NAbs. On the other hand, the particle geometry leaves little space for bNAbs that bind to epitopes closer to the ferritin nanoparticle core, such as 35O22 and PGT151 (Fig. 1H), although we note that the decreased binding of PGT151 we observed could also be affected by the lower degree of SOSIP cleavage on ferritin [29]. We also note that HA carrying ferritin nanoparticles could induce Abs that target the HA stem close to the ferritin core [22]. However, the accessible area and possible angles of approach for such Abs are larger than for Abs that would target the gp120/gp41 interface or gp41\textsubscript{ECTO} of SOSIP664 on ferritin (Fig. 5A). Thus, presenting Env trimers on ferritin particles might affect the relative immunogenicity of bNAb epitopes and the Ab specificites induced by vaccination.

The dense configuration of Env trimers on ferritin might also restrict access of furin to the gp140 moieties leading to less efficient gp140 cleavage (Fig. 5B). Cleavage of gp140 is important for the formation of native-like trimers [27, 33], but not always absolutely required [27]. For example, 50% of BG505 SOSIP664 trimers that have an inactivated furin cleavage site appear to have a native-like structure [27]. Moreover, uncleaved single-chain Env trimers that lack the furin cleavage site, but instead contain a flexible linker, consist mostly or solely of native-like Env trimers, although the env sequence, the presence of stabilizing mutations and the linker length all influence the phenotype [17, 34, 35].

**Figure 5.** Structural models of SOSIP664 (PDB: 4TVP) and the influenza HA spike (PDB: 3SM5) on ferritin (3BVE) viewed down one of the fourfold axes of the nanoparticle. A. An Ab that targets the gp120/gp41 interface of SOSIP664, similar to 35O22 or PGT151 bNAbs [29, 30], will probably clash with a neighboring trimer on ferritin (left). Analogously, an Ab that targets the stem region of the HA spike should have enough leeway to bind without clashing with another HA trimer on ferritin (right). B. The same SOSIP664-ferritin structural model as in A with the crystal structure of mouse furin (PDB: 1P8J; Asp108-Pro582) modeled at the approximate site of the furin cleavage site of the Env trimer.
principle, the protein backbone of six arginine residues of uncleaved AMC008 SOSIP.v4.2 span the same distance as the protein backbone of the six amino acid long flexible linkers of native-like single-chain BG505 trimers [35], suggesting that uncleaved AMC008 SOSIP.v4.2 on ferritin could have similar native-like conformations. Furthermore, the glycosylation profile, the absence of aberrant interprotomer disulfide bonds and binding of PGT145 (a bNAb that is specific for native-like Env trimers only) in ELISA suggested that the majority of individual AMC008 SOSIP.v4.2-ferritin trimers assume a native-like conformation, which must then include the majority of uncleaved AMC008 SOSIP.v4.2-ferritin trimers.

When measuring the thermostability by DSC, we observed that AMC008 SOSIP.v4.2-ferritin had a significantly higher midpoint of thermal denaturation than its trimeric AMC008 SOSIP.v4.2 counterpart. Thus, ferritin provided additional thermostability to the trimer complex, possibly by sequestering and/or stabilizing the “loose ends” at the C-terminus of AMC008 SOSIP.v4.2 and/or by forcing AMC008 SOSIP.v4.2 in its trimeric conformation. Higher thermostability might enhance the antigen’s half-life in vivo thereby increasing the chances for productive encounters with B cells that have the capacity to produce bNAbs. Furthermore, high thermostability might mitigate problems with the cold-chain, when distributing vaccines in low-income countries [36,37].

The (uncontrolled) immunogenicity studies in rabbits, conducted at an ad-hoc basis, showed that AMC008 SOSIP.v4.2-ferritin vaccination boosted NAb responses against the autologous AMC008 virus, while a single cocktail boost consisting of clade B AMC008, AMC016 and AMC018 SOSIP.v4.2-ferritin induced consistent weak neutralization against some heterologous clade B Tier 2 viruses. In both experiments, the rabbits were already primed with other Env-based immunogens and we cannot ascertain how the priming immunogens influenced the ensuing NAb responses. For example, the BG505 SOSIP.664-ferritin DNA prime given to the rabbits in the second immunogenicity study [25] (Fig. 4C) could have generated a subpopulation of misfolded proteins that induced non-NAbs that might have interfered with the response against the subsequent nanoparticle boosts.

Ferritin-conjugated Env trimers were expressed and subsequently purified with an affinity chromatography column using PGT145, a bNAb that is specific for native-like Env trimers [33,38]. Because purification might occur through the interaction of PGT145 with a minimum of one trimer per particle, the positive selection power of PGT145 is reduced as up to 7 out of 8 trimers per particle might not undergo positive selection through PGT145. If positive selection through PGT145-purification is desirable, one could develop nanoparticle platforms that permit conjugation of purified native-like Env trimers, for example, by attaching them to preformed nanoparticles or use two-component systems in which individual trimers are made and purified, and nanoparticle assembly is induced by adding a second component. Such more versatile systems could also allow the presentation of native-like Env trimers from different isolates on the same nanoparticle, which might aid in activating cross-reactive B cells more efficiently.

Here, we have shown that it is possible to present stabilized native-like Env trimers from different isolates on ferritin nanoparticles. When used as immunogens these nanoparticles enhanced autologous NAb responses and induced weak neutralization against some heterologous Tier 2 viruses in rabbits that were previously exposed to other immunogens. These results warrant additional immunization studies with ferritin-fused Env trimers and demonstrate that ferritin and other nanoparticles are promising platforms to increase the immunogenicity of native-like Env trimers.
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Methods

Plasmids and design

The DNA sequence of Helicobacter pylori ferritin (GenBank accession no. NP_223316), with a N19Q mutation to remove a potential glycosylation site [22] was placed at the 3’ end of DNA encoding SOSIP constructs, separated by a short Gly-Ser-Gly linker, as described before [25]. The sequences of the AMC008, AMC016, AMC018 and ZM197M env genes and additional SOSIP.v4.2 modifications are reported in [8,10] (van den Kerkhof et al. manuscript in preparation). The A73C and A561C substitutions yielding SOSIP.v5.2 are described elsewhere (de la Peña et al. manuscript in preparation). All sequences were codon optimized for mammalian cell expression and mutations were verified by sequencing.

Protein production and purification

Suspension HEK293F cells were maintained in FreeStyle medium (Life Technologies) and transfected using PEImax at a density of 0.8 × 10⁶–1.2 × 10⁶ cells/ml with a plasmid expressing trimer constructs or the different ferritin-fused Env trimer designs. A plasmid encoding furin was co-transfected in a 1:1 ratio to have optimal gp140 cleavage [25]. The supernatant was harvested 6 or 7 days after transfection, centrifuged and filtered using Steritops (0.22 µm pore size, Millipore, Amsterdam, The Netherlands). CNBr-activated sepharose 4B beads (GE Healthcare) carrying PGT145 were added to the supernatant and put on a roller at 4°C for the duration of the Lord of the Rings Trilogy, Extended edition. Subsequently, supernatant and beads were passed over a Econo-Column chromatography column (Bio-Rad) and beads were washed extensively with washing buffer (0.5M NaCl, 20mM TrisHCl, pH 8.0). Protein was eluted with 3.0 M MgCl₂, pH 7.5 and immediately buffer exchanged into TN75 buffer (75 mM NaCl and 20 mM Tris HCl, pH 8.0) using a 100 kDa cutoff Vivaspin 20 filter (Sartorius, Goettingen, Germany).

Electron microscopy

Protein samples were imaged and processed as described before [25]. For cryo-electron microscopy on AMC008 SOSIP.v4.2-ferritin, the protein was concentrated to ~3.0 mg/mL and grids were frozen manually at 4 °C, using 2/C-Flat holey grids. Image processing was performed as described previously [39].

SDS and BN PAGE

Proteins were analyzed by SDS-PAGE and BN-PAGE as described previously [8]. Protein bands were visualized with Coomassie Blue staining (Bio-Rad, Veenendaal, The Netherlands).

Enzyme-linked immunosorbent assays (ELISA)

PGT145-purified AMC008 SOSIP.v4.2 and AMC008 SOSIP.v4.2-ferritin were diluted in Tris-buffered saline (TBS)/10% fetal calf serum (FCS) to 1.5 and 0.4 µg/ml respectively. Proteins were immobilized for 2 h at room temperature on half-well 96-well plates in TBS/10% FCS (Greiner) precoated with Galanthus nivalis lectin (Vector Laboratories) at 20 µg/ml in 0.1 M NaHCO₃ overnight. Subsequent steps were performed as previously described for D7324-tag ELISAs [3].
Neutralization assays
Virus stocks were produced in HEK293T cells and the neutralization assays were performed at the Academic Medical Center in Amsterdam according to published procedures [8]. The TZM-bl reporter cell line used for the neutralization assays 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).

Glycan analysis
Glycan profiling was performed exactly as described elsewhere [28]. In short, Env proteins were resolved by SDS-PAGE. Bands corresponding to gp140 were excised and the N-linked glycans attached to gp140 protein were released by N-glycosidase F (PNGase F; NEB). Glycans were labelled with 2-aminobenzoic acid (2-AA) [40]. Fluorescently labelled glycans were resolved by hydrophilic interaction liquid chromatography-ultra performance liquid chromatography (HILIC-UPLC) using a 2.1 mm × 10 mm Acquity BEH Amide Column (1.7 µm particle size) (Waters, Elstree, UK) as described before [28]. Fluorescence was measured using an excitation wavelength of 250 nm and a detection wavelength of 428 nm. Data processing was performed using Empower 3 software (Waters Corporation, Milford, MA, USA). The percentage abundance of oligomannose-type glycans was calculated by integration of the relevant peak areas before and after Endoglycosidase H digestion, following normalization [28].

Dynamic light scattering (DLS)
DLS measurements were performed at 20°C using a Dynapro Nanostar instrument (Wyatt Technologies), with 10 acquisitions of 5 s each. Each sample was centrifuged at 10,000 x g for 10 min prior to the DLS measurement to remove any trace aggregates or dust from the sample. The hydrodynamic radii (R_h) were calculated using the Dynamics Analysis software (Wyatt Technologies), assuming a spherical model.

Differential scanning calorimetry (DSC)
Thermal denaturation was studied using a nano-DSC calorimeter (TA instruments). Proteins were first extensively dialyzed against PBS, and the protein concentration was then adjusted to 0.1 mg/ml for AMC008 SOSIP.v4.2 and 0.7 mg/ml for AMC008 SOSIP.v4.2-ferritin to correct for the increased molecular weight. 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 Tm values reported correspond to the temperature associated with the peak of the melting curve, since reliable modeling of the AMC008 SOSIP.v4.2-ferritin melting curve was not possible.

Immunizations
The initial phase of both immunization experiments have been described before [8,25](de la Peña et al. manuscript in preparation). Both studies and their extensions described here were approved by the Covance Institutional Animal Care and Use Committee and IACUC (permit numbers 0022-15 and 0082-14). In study 0022-15 twelve New Zealand White rabbits received 22 µg of PGT145-purified BG505 or AMC008 SOSIP.664, SOSIP.v4 or SOSIP.v5 in 75 units of ISCOMATRIX™ adjuvant by intramuscular immunizations at weeks 0, 4 and 20 [8,41](de la Peña et al. manuscript in preparation). This was followed by intramuscular immunizations of 22 µg of PGT145-pure AMC008 SOSIP.v4.2-ferritin in ISCOMATRIX™ adjuvant at weeks 36 and 52. In study 0082-14, five New Zealand White rabbits received 200 µg of a non-adjuvanted DNA plasmid (BG505 SOSIP.664-ferritin) via electroporation of the quadriceps at weeks 0, 4, and 12, followed by intramuscular protein boost (17 µg of BG505 SOSIP.664-ferritin in 75 units ISCOMATRIX™) at week 24 and 30, described before [25]. This was followed by an additional protein boost at week 50 with an intramuscular immunization of a cocktail containing PGT145-purified AMC008, AMC016 and AMC018 SOSIP.v4.2-ferritin (22 µg total protein per rabbit, i.e. 7.5 µg of each immunogen) 75 units ISCOMATRIX™.

References


