Immunogenicity of a Prefusion HIV-1 Envelope Trimer in Complex with a Quaternary-Structure-Specific Antibody

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ABSTRACT

The HIV-1 envelope trimer (Env) is the target of broadly neutralizing antibodies and is being explored as a vaccine candidate to elicit protective antibodies. One of the most promising antigenic and structural mimics of HIV-1 Env is the SOSIP.664-stabilized soluble trimer from the clade A strain BG505, which is preferentially recognized by broadly neutralizing antibodies. Trimer immunization elicits high-titer neutralization of the autologous tier 2 BG505 strain; however, breadth is limited, and substantial interest has focused on understanding and improving trimer immunogenicity. We sought to improve the antigenic specificity of BG505 SOSIP.664 by reducing recognition of the variable loop 3 (V3) region, which elicits only weakly neutralizing antibodies. To stabilize the trimer in its prefusion closed conformation, we complexed trimeric BG505 SOSIP.664 with the antigen-binding fragment (Fab) of PGT145, a broadly neutralizing quaternary-structure-specific antibody. Compared to the ligand-free trimer, the PGT145 Fab-BG505 SOSIP.664 complex displayed increased melting temperature stability and reduced V3 recognition. In guinea pigs, immunization with the PGT145 Fab-BG505 SOSIP.664 complex elicited ~100-fold lower V3-directed binding and neutralization titers than those obtained with ligand-free BG505 SOSIP.664. Both complexed and ligand-free BG505 SOSIP.664 elicited comparable neutralization of the autologous BG505 virus, and in both cases, BG505 neutralization mapped to the outer domain of gp120 for some guinea pigs. Our results indicate that it is possible to reduce immune recognition of the V3 region of the trimer while maintaining the antigenic profile needed to induce autologous neutralizing antibodies. These data suggest that appropriate modifications of trimer immunogens could further focus the immune response on key neutralization epitopes.

IMPORTANCE

HIV-1 Env trimers have been proposed as preferred HIV-1 vaccine immunogens. One version, BG505 SOSIP.664, a soluble stabilized trimer, was recently shown to elicit high-titer autologous neutralizing antibodies (NAbs) in rabbits. Here we compared two immunogens: the ligand-free BG505 SOSIP.664 trimer and the same trimer bound to the antigen-binding fragment (Fab) of the PGT145 antibody, a broadly neutralizing antibody which recognizes the trimer at its membrane-distal apex. We hypothesized that the Fab-bound complex would stabilize BG505 SOSIP.664 in its prefusion closed conformation and limit reactivity to weakly neutralizing antibodies targeting the variable loop 3 (V3) region. In guinea pigs, the Fab-complexed trimer induced 100-fold lower responses to the V3 region, while both ligand-free and Fab-complexed trimers elicited similar levels of autologous NAbs. Our findings demonstrate the potential to reduce “off-target” immunogenicity while maintaining the capacity to generate autologous NAbs.

As the sole viral antigen on the surfaces of HIV-1 virions, the envelope trimer (Env) mediates virus entry into host cells and is the target of virus-directed neutralizing antibodies (NAbs) (1–3; reviewed in references 4 and 5). Env comprises three gp120 receptor-binding subunits noncovalently associated with three gp41 transmembrane subunits. Similar to the transmembrane subunits of other type 1 fusion proteins, the gp41 subunit transitions between prefusion, intermediate, and postfusion states as part of its entry-related fusion of viral and target cell membranes (2, 6, 7). However, unlike other type 1 fusion machines, HIV-1 Env transitions between at least three distinct prefusion states (8). One of these, the prefusion closed conformation, is the target of most broadly neutralizing antibodies (2, 3, 5, 8, 9), and it has been proposed that HIV-1 Env fixed in the prefusion closed state may be a preferred HIV-1 immunogen (2, 3, 5, 9, 10).

A soluble recombinant glycoprotein mimic of the HIV-1 spike, named BG505 SOSIP.664 for the HIV-1 strain (BG505) and the stabilizing mutations involved (SOSIP.664), was recently described (11). An important antigenic characteristic of this protein is preferential recognition by neutralizing antibodies versus non-

or poorly neutralizing antibodies (11, 12). Immunization with BG505 SOSIP.664 induces high titers of NAbs against the autologous BG505 virus in rabbits and induces similar but weaker responses in monkeys (10), while it does not induce autologous NAbs in mice (13). The elicitation of such NAbs is an important advance in the HIV-1 vaccine field, especially if such autologous

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neutralization could be extended to include neutralization of heterologous tier 2 viruses.

Because careful optimization of antigenic specificity for broadly neutralizing antibodies versus non- or poorly neutralizing antibodies was critical to the development of BG505 SOSIP.664 (11), it seemed reasonable to consider additional improvements in the antigenic specificity of this soluble recombinant Env trimer. One potential area of improvement is via the variable loop 3 (V3) region on Env, which is targeted by weakly neutralizing antibodies unable to neutralize most tier 2 viral isolates (14). The V3 region on BG505 SOSIP.664 is partially exposed (11), as indicated by reactivity to V3 NAb and by the elicitation of V3 antibodies when this protein is used as an immunogen (9, 10). Here we sought to further stabilize BG505 SOSIP.664 in its prefusion closed state and thereby also to minimize V3 exposure and reactivity. We purified the BG505 trimer through an affinity column procedure involving complexation to the antigen-binding fragment (Fab) of the V1V2-directed PGT145 antibody (15), which we previously observed in single-molecule fluorescence resonance energy transfer (smFRET) experiments to show strong enhancement of the prefusion closed conformation (8). We compared the stabilities, antigenicities, and, most importantly, immunogenicities of wild-type BG505 SOSIP.664 (here termed the BG505 trimer) and the antibody-bound complex of the BG505 trimer with PGT145 Fab (named the PGT145-trimer complex). The results provide insight into the immunogenic consequences of improving the stability and antigenic specificity of the BG505 SOSIP.664 trimer, which is important to further developing the BG505 trimer as a vaccine immunogen.

MATERIALS AND METHODS

Protein expression and purification. BG505 SOSIP.664 was generated as previously described (2, 11), with a glycan site at residue 332 (T332N), a 6R cleavage site, and a truncated C terminus at residue 664 (HxB2 numbering). The supernatant from transiently transfected 293F cells was passed over a 2G12 antibody affinity column, and bound protein was eluted with ~30 to 40 ml of 3 M MgCl₂, 10 mM Tris, pH 8.0, concentrated to ~1 to 2 ml, and injected onto a Superdex 200 gel filtration column in phosphate-buffered saline (PBS). The PGT145-trimer complex was obtained by on-column purification as described previously (16). Briefly, ~5 mg of PGT145 IgG (containing a human rhinovirus 3C protease [HRV3C] cleavage site in the hinge region) was loaded onto ~5 ml of protein A (Pierce) resin. BG505 SOSIP.664 supernatant was flowed over the protein A-PGT145 IgG column. The column was washed with 5 column volumes of PBS and incubated overnight at 4°C with protease HRV3C. The flowthrough was collected, concentrated, and loaded onto a Superdex 200 column in PBS. Fractions containing the complex of trimeric BG505 SOSIP.664 with PGT145 Fab were pooled, concentrated, aliquoted, frozen in liquid nitrogen, and stored at -80°C prior to use.

Proteins used for enzyme-linked immunosorbent assay (ELISA), i.e., BG505 SOSIP.664-D7324 trimer, a D7324-tagged trimer (11), and for neutralization competition assays, including the ΔeOD-GT6 monomer (a CD4-binding site [CD4bs] knockout mutant of eOD-GT6 with D279K and D368V mutations [17]), monomeric BG505 D368R OD (outer domain amino acids 252 to 482; HxB2 numbering), and its GV3B, GV4B, and GV5B mutants (detailed in Fig. 8; each with the D368R mutation), were produced in transiently transfected Expi293 cells (Invitrogen) and purified with *Galanthus nivalis* lectin columns followed by Superdex 200 gel filtration chromatography. Monomeric forms of BG505 SOSIP.664 D368R were purified similarly to the BG505 SOSIP.664 trimer (2G12 affinity and gel filtration chromatography), except that the monomeric form was collected after gel filtration chromatography.

Immunizations. Two-month-old Hartley guinea pigs were immunized with either the BG505 trimer or the PGT145-trimer complex. Injections consisted of 25 μg of BG505 trimer or 25 μg of PGT145-trimer complex formulated in 400 μl of PBS, with 100 μg of poly(I-C) adjuvant (HMW; Invivogen). Equal weights of protein were used in the immunizations to allow for accurate quantification of the immunogens. The immunizations were administered intramuscularly as two separate injections of 200 μl into each quadriceps muscle. Poly(I-C) adjuvant was prepared by making a 2-mg/ml stock solution in saline, heating the necessary amount for 10 min at 70°C, and cooling it at room temperature for 1 h prior to injection. Immunizations were performed on weeks 0, 4, 8, 12, and 22. Blood draws for immune assessment included a prebleed week -1 sample and blood draws performed 2 weeks after each immunization. All animal experiments were reviewed and approved by the Animal Care and Use Committee of the Vaccine Research Center, NIAID, NIH, and all animals were housed and cared for in accordance with local, state, federal, and institute policies in an American Association for Accreditation of Laboratory Animal Care-accredited facility at the NIH. Collected sera were heat inactivated for 1 h at 56°C before being analyzed.

DSF. A CFX96 real-time PCR thermal cycler system (Bio-Rad) was used to perform differential scanning fluorimetry (DSF) assessments of the BG505 trimer and the PGT145-trimer complex. Sypro orange (Molecular Probes) at a stock concentration of 5,000× was diluted to a concentration of 300× in PBS, and 1.25 μl was added to 23.75 μl of a 500 nM solution of BG505 trimer, PGT145-trimer complex, or PGT145 Fab diluted in PBS. Samples were equilibrated at 25°C for 3 min in the thermal cycler before undergoing a melting curve analysis from 35 to 95°C that increased 0.5°C per cycle, with each cycle lasting 10 s. To determine melting temperature (T_m) values, CFX Manager software (Bio-Rad) was used to find the melting transition temperature range, and this range was fit to a sigmoidal Boltzmann curve in GraphPad Prism as previously reported (18). All samples were run in duplicate.

Biolayer interferometry (BLI) antigenicity analysis. An Octet HTX instrument (ForteBio) was used to assess complex binding pre- and post-PGT145 Fab antibody binding. Purified monoclonal antibody (MAb) VRC01 or 447-52D and other IgGs were loaded (15 μg/ml until saturation) onto anti-human Fc biosensors (AHC; ForteBio). Following a buffer (0.05% bovine serum albumin [BSA] and 0.02% Tween 20 in PBS) wash step, the trimer was associated at 6 different concentrations (2-fold dilutions from 200 nM to 6.25 nM) and then allowed to dissociate in buffer. A 1:1 ratio of trimer to Fab was assumed for the PGT145-trimer complex to calculate the molar concentration, and an irrelevant glycoprotein was used as a negative control in all experiments. All Octet experiments were performed three times after regeneration of tips with 0.3 M low-pH glycine solutions. Values for reference wells containing only buffer (0.05% BSA and 0.02% Tween 20 in PBS) were subtracted from values for sample wells. All experiments were performed at 30°C and with a plate shaking speed of 1,000 rpm. Octet analysis software was used to analyze all data, and curve fits are shown as black lines where relevant; we deemed that no fit was observed if fewer than 3 curves could be fit with an R^2 value of ≥ 0.95 .

ELISAs for detection of anti-PGT145 Fab, anti-OD, and anti-V3-peptide responses. Ninety-six-well plates (Reacti-Bind; Pierce) were coated with 100 μl/well of 2 μg/ml PGT145 Fab, OD, or peptide in PBS overnight at 4°C (MW965.26 V3 peptide, TRPNNTTRKSIRIGPGQTFYATG; and BG505 V3 peptide, TRPNNTTRKSIRIGPGQAFYATG [the amino acid difference is underlined]). For each consecutive step following coating, plates were washed 5 times with PBS-T (PBS plus 0.05% Tween) and incubated at 37°C for 1 h. After coating, plates were blocked with 200 μl/well of blocking buffer (B3T; 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 3.3% fetal bovine serum [FBS], 2% BSA, 0.07% Tween 20, 0.02% thimerosal). Next, guinea pig sera were diluted in B3T and added to the plates in 5-fold serial dilutions. A goat anti-guinea pig antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, MD) was then added to each well at a 1:10,000 dilution

FIG 1 On-column purification of PGT145-trimer complex. (A) Schematic of on-column complex formation between PGT145 and the BG505 trimer. PGT145 IgG containing a protease (HRV3C) cleavage site in the hinge region was loaded onto protein A resin. The supernatant from 293F cells expressing BG505 SOSIP.664 was flowed over the protein A-PGT145 IgG column. The PGT145-trimer complex was cleaved off the column by adding HRV3C. The complex was further purified by Superdex 200 gel filtration. (B) Gel filtration profile of the eluate from panel A showing the PGT145-trimer complex and excess PGT145 Fab. (C) Gel filtration profile of the PGT145-trimer complex (in red), using complex fractions from panel B, and an overlay with the profile of the 2G12 affinity-purified BG505 trimer. Proteins were detected by the A₂₈₀ (y axis), and elution volumes are shown on the x axis. (D) Coomassie blue-stained SDS-PAGE gel under reducing (R) and nonreducing (NR) conditions, showing the gel filtration-purified ligand-free Env, Fab-Env complex, and PGT145 Fab. MW, molecular weight markers.

in B3T. Tetramethylbenzidine (TMB) substrate (SureBlue; KPL, Gaithersburg, MD) was used to develop plates for 10 min before 1 N sulfuric acid was added to stop the reaction, without washing beforehand. Plates were read at 450 nm (SpectraMax instrument using SoftMax Pro 5 software; Molecular Devices), and the final optical density was determined after the horseradish peroxidase nonspecific background binding was subtracted.

D7324-capture ELISAs for detection of anti-BG505 trimer responses. Ninety-six-well plates (Reacti-Bind; Pierce) were coated with 100 μ l/well of 2 μ g/ml anti-D7324 antibody (Aalto Bioreagents, Dublin, Ireland) in PBS overnight at 4°C. After coating, plates were blocked for 1 h at room temperature with PBS plus 5% skim milk (Difco, Becton, Dickinson and Company). Plates were then washed 5 times with PBS-T before the addition of 0.5 μ g/ml of BG505 SOSIP.664-D7324 (D7324-tagged) trimer (11) diluted in PBS plus 10% FBS for 2 h at room temperature. After the addition of the trimer, subsequent procedures mimicked those of the anti-V3-peptide ELISAs, except that dilutions were made in PBS-T instead of B3T.

Antigenic characterization of BG505 trimer and PGT145-trimer complex by lectin-capture ELISAs. Lectin-capture ELISAs were performed to measure the binding of V3-specific antibodies to the trimer proteins as described previously (19). Following overnight coating at 4°C with snowdrop lectin from *Galanthus nivalis* (Sigma-Aldrich), 96-well MaxiSorp plates (Thermo Fisher Scientific) were blocked using PBS-T plus 5% skim milk and 1.5% BSA. All incubation and washing steps were performed as previously described for the D7324-capture ELISAs. BG505 trimer or PGT145-trimer complex proteins diluted in PBS plus 10% FBS

to 2 μ g/ml were captured on the plates for 2 h at room temperature. V3-specific monoclonal antibodies were serially diluted starting at 10 μ g/ml and transferred to the 96-well plates. The bound monoclonal antibodies were recognized using horseradish peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch) at a 1:5,000 dilution. The plates were then developed and read as described previously.

HIV-1 neutralization assays. Sera from immunized guinea pigs were assessed for virus neutralization as previously described, using a single-round infection assay where a reduction in luciferase luminescence indicates neutralization activity (20). Target cells were TZM-bl cells, which are a clonal HeLa cell line expressing CD4, CXCR4, and CCR5. Upon infection, the HIV-1 protein Tat induces a luciferase reporter gene, whose expression is measured in relative light units. Data are presented as the reciprocal inhibitory dilutions of sera required to inhibit either 50% or 80% of infection (ID₅₀ or ID₈₀, respectively), calculated using a regression fit as previously described (20). In the competition assays, V3 peptides and competitor proteins were incubated with sera at 37°C for 30 min, virus was added to this mixture, and neutralization assays were carried out as described above. The final concentration of competitor peptides and proteins in the mixture of sera and virus was 25 μ g/ml.

Flow cytometry-based CD4-HIV Env blocking assay. TZM-bl cells (HeLa cells overexpressing CD4) at 100% confluence were incubated at 37°C and 5% CO₂ with 9 nM EDTA in PBS until cells were lifted. To make soluble Env probes, BG505 SOSIP.664 avi-tagged trimers were biotinylated and conjugated with the fluorochrome allophycocyanin (trimer-

FIG 2 Thermal stability of ligand-free and Fab-bound Env trimers. (A) Differential scanning fluorimetry (DSF) showed the fluorescence of Sypro orange dye as it bound to the BG505 trimer (blue), the PGT145-trimer complex (red), or PGT145 Fab (green) in response to increases in temperature and trimer unfolding. The assay was run on a thermal cycler (Bio-Rad), with the dye binding to hydrophobic pockets in response to thermally induced unfolding. (B) The melting temperature (T_m) of each sample was determined by comparison of data obtained as described for panel A from the start to the end of melting (BG505 trimer, 61 to 68.5°C; and PGT145-trimer complex, 65 to 74°C), as determined by CFX Manager software (Bio-Rad). The T_m value is displayed as the halfway point of each curve (black horizontal lines) and was determined by Boltzmann sigmoidal curve fitting (black dashed lines). RFU, relative fluorescence units.

APC). The trimer-APC probe was incubated (at a concentration that provided optimal staining separation between stained and unstained cells as determined by an initial titration) with guinea pig sera, monoclonal antibody controls, or nothing for 1 h at room temperature in 50 μ l of 10% FBS in PBS. The guinea pig sera were added at a final dilution of 1:50, and monoclonal antibodies were added at the indicated concentrations, from 0.008 μ g/ml to 1 μ g/ml. After incubation, the 50- μ l volumes were transferred to 5×10^5 TZM-bl cells for 30 min on ice before washing with PBS, fixing with 0.5% paraformaldehyde, and running of samples on a BD LSR flow cytometer.

MSD-ECLIA analysis. Mesoscale discovery-electrochemiluminescence immunoassay (MSD-ECLIA) analysis was performed as described previously (9). Briefly, standard 96-well bare multiarray MSD plates were coated with a panel of anti-HIV neutralizing and nonneutralizing monoclonal antibodies (30 μ l/well) at a concentration of 10 μ g/ml overnight at 4°C. The following day, plates were washed and blocked with 150 μ l of blocking buffer and then incubated for 1 h on a vibrational shaker at 650 rpm. After the incubation with blocking buffer was complete, the plates were washed, and serially 2-fold-diluted BG505 trimer or PGT145-trimer complex (starting at 4 μ g/ml) was transferred (25 μ l/well) to the MSD plates and incubated for 2 h on the vibrational shaker at 650 rpm. The plates were washed, and the bound trimer was measured with an MSD Sulfoltag-labeled 2G12 antibody on an MSD Sector Imager 2400 instrument.

RESULTS

Column purification of BG505 trimer and PGT145-trimer complex. A standard method for purifying the BG505 trimer employs

a 2G12 affinity column followed by size-exclusion chromatography to isolate the trimeric fraction (1, 21). These procedures generate mostly well-folded trimers that bind tightly to most HIV-1 broadly neutralizing antibodies (11, 12), including those preferentially reacting with the quaternary conformation of Env, such as the V1V2 apex-directed MAbs PGT145 and CAP256-VRC26. However, these purified trimers also bind to the weakly neutralizing V3-directed 447-52D antibody (9), which recognizes the V3 tip (22, 23). The BG505 trimer used in this study was purified following the above-described procedure. Additionally, in an effort to further stabilize the BG505 trimer in its prefusion closed conformation, we generated a complex of BG505 bound to the Fab of PGT145. To do this, we employed an on-column antibody purification step (Fig. 1A), as we have in the past (16), which does not require low-pH or high-salt elution steps that could affect trimer folding. All binding, washing, and eluting steps of the purification process were carried out in PBS to avoid disruption of the trimer structure and to remove or lower the amount of aberrantly folded trimers. The purified complex showed a slightly shifted single peak compared to the ligand-free trimer, and there was no free PGT145-Fab peak by gel filtration column chromatography (Fig. 1B and C). SDS-PAGE analysis showed that the complex contained both the gp140 BG505 trimer and the PGT145 Fab at the correct estimated molecular weights (Fig. 1D).

The PGT145-trimer complex displays increased stability relative to that of ligand-free Env. To explore the physical properties of the BG505 trimer and the PGT145-trimer complex, we measured their melting point temperatures by using differential scanning fluorimetry (DSF) (18) (Fig. 2). The ligand-free trimer and the PGT145-trimer complex showed similar patterns of melting, although the trimer complex showed a higher melting temperature (68°C) than that of the BG505 trimer (65°C) (Fig. 2A). In this assay, the majority of fluorescence was due to the binding of the Sypro orange dye to exposed hydrophobic residues of the BG505 trimer, as PGT145 alone generated low levels of fluorescence (Fig. 2B). These data suggest the PGT145-trimer complex to be more thermally stable than the ligand-free trimer. We observed a slight difference in the fluorimetry-determined melting temperature for the BG505 trimer compared to previously reported results (9, 11), which we attribute to methodological differences between the fluorimetry-based assay used here and the differential scanning calorimetry assay used in the published reports.

The PGT145-trimer complex displays a more favorable antigenic profile than ligand-free Env. The antigenic properties of the purified proteins were assessed by BLI (Fig. 3), MSD-ECLIA (Fig. 4A, B, and D), and ELISA (Fig. 4C). Compared to the BG505 trimer, the PGT145-trimer complex displayed a substantial reduction in binding to the V3 MAb 447-52D, while maintaining similar levels of binding to the trimer-preferring MAbs PGT151 and 35O22 and the CD4bs MAb VRC01. The PGT145-trimer complex also displayed reduced binding to weakly or poorly neutralizing antibodies F105, 17b, and 447-52D in the absence or presence of soluble CD4 (Fig. 3 and 4), suggesting its greater stability in the closed conformation. We also tested an expanded panel of V3 MAbs, including 2219, 2557, 2558, 3037, 1006-15D, and 3074 (24, 25), targeting regions of the V3 peptide, by MSD-ECLIA (Fig. 4B) and lectin-capture ELISA (Fig. 4C). The PGT145-trimer complex also showed reduced binding to these MAbs com-

FIG 3 Antigenic comparison of ligand-free BG505 trimer and PGT145-trimer complex by biolayer interferometry. The binding of the ligand-free BG505 trimer (blue) and the PGT145-trimer complex (red) was assessed by capture with the following panel of antibodies: VRC01 (CD4bs directed), PGT145 (V1V2 directed), PGT151 (gp120-gp41 interface directed), 447-52D (V3 directed), F105 (CD4bs directed), and 17b (CD4-induced epitope directed). The dashed lines represent the end of the association step (300 s), and black lines represent curve fit data as calculated by Octet analysis software. When fewer than 3 curves could be fit, no fit was determined. Six concentrations from 6.25 nM to 200 nM were tested in 2-fold serial dilutions.

pared to the ligand-free trimer. As expected, compared to the ligand-free trimer, the PGT145-trimer complex displayed reduced binding to the IgG form of PGT145, confirming that the PGT145-binding site is partially blocked in the PGT145-trimer

complex. Overall, the antigenicity results obtained using BLI, MSD-ECLIA, and ELISA were concordant and indicated that the PGT145-trimer complex had increased specificity for broadly neutralizing antibodies versus the ligand-free trimer.

FIG 4 Antigenic comparison of ligand-free BG505 trimer and PGT145-trimer complex by MSD-ECLIA, lectin-capture ELISA, and effect of adjuvant. (A and B) Antigenicities of the ligand-free and complex trimeric proteins as measured by MSD-ECLIA. Area under the curve (AUC) values (see Materials and Methods) are shown for a panel of neutralizing (green) and nonneutralizing (red) antibodies and antibodies in the presence of CD4 (orange). (C) Antigenicities of BG505 trimer and PGT145-trimer complex as measured with a panel of V3-specific monoclonal antibodies by using lectin-capture ELISA. Monoclonal antibodies were 5-fold serially diluted starting at 10 μ g/ml, and their binding to lectin-captured trimer proteins was determined. AUC values were determined with ELISA optical density values at 450 nm for different antibody dilutions. (D) Antigenicities of the ligand-free and complex trimeric proteins in the presence of poly(I:C) adjuvant as measured by MSD-ECLIA. The color style is the same as that in panel A.

Additionally, the antigenicities of both proteins in the presence of poly(I:C) adjuvant were determined by MSD-ECLIA. Although there was a modest reduction in antibody binding in the presence of the adjuvant, the overall trend of recognition by neutralizing antibodies and nonneutralizing antibodies was preserved (Fig. 4D).

PGT145-trimer complexes elicit high autologous tier 2 virus-neutralizing activity and reduced V3-dependent tier 1 virus-neutralizing activity. To assess whether stabilizing the BG505 trimer with the quaternary-structure-specific antibody PGT145 *in vitro* translated into altered immunogenicity, we immunized guinea pigs five times intramuscularly (at weeks 0, 4, 8, 12, and 22)

FIG 5 Guinea pig immune responses as measured by ELISA. (A) The BG505 SOSIP.664-D7324 (D7324-tagged) trimer was captured on an ELISA plate to measure the serum response elicited by the BG505 trimer (blue) and the PGT145-trimer complex (red). Each line indicates the ELISA titers for an individual animal. Immunizations, labeled by weeks, are shown by arrows above the graph, with the first immunization performed at week 0. There was a statistically significant difference between the two immunization groups at weeks 17 and 24 (*, $P < 0.05$; two-tailed Mann-Whitney test). (B) V3-directed serum responses measured by V3 peptide ELISA. ELISA titers against the V3 peptide from the tier 1 MW965.26 virus are shown, as elicited by BG505 trimer (blue)- and PGT145-trimer complex (red)-immunized guinea pigs. The responses to the MW965.26 V3 peptide were lower in the PGT145-trimer complex group than in the ligand-free trimer group (*, $P < 0.05$; two-tailed Mann-Whitney test). (C) Anti-PGT145 Fab antibody response elicited by the BG505 trimer or the PGT145-trimer complex at prebleed (green) or week 24 (blue), as measured by ELISA. *, $P < 0.05$ (two-tailed Mann-Whitney test).

with either the PGT145-trimer complex or the BG505 trimer. Sera from both immunization groups displayed robust responses to the BG505 trimer as assessed by ELISA (Fig. 5A), with the BG505 trimer generating 5- to 10-fold higher titers than the PGT145-trimer complex at weeks 17 and 24. The BG505 trimer also induced ~100-fold higher geometric mean titers of anti-V3-peptide responses than the PGT145-trimer complex ($P < 0.05$ at time points post-week 6; Mann-Whitney test) (Fig. 5B). Thus, consistent with its antigenic profile, the PGT145-trimer complex reduced the V3 immunogenicity of the Env trimer significantly post-week 6. As expected, the PGT145-trimer complex also induced high titers of anti-PGT145 Fab antibodies at week 24 (Fig. 5C). Although cross-reactive anti-PGT145 Fab antibodies were detected in some of the guinea pigs immunized with the BG505 trimer at week 24, their titers were not significantly higher than those in the prebleed samples ($P > 0.05$). The neutralizing activity in sera of the immunized animals was assessed against diverse neutralization-sensitive (tier 1A) and more resistant (tier 1B and tier 2) viral strains. Sera from all of the BG505 trimer- and PGT145-trimer complex-immunized groups achieved autologous neutralization of the BG505 virus (BG505.W6M.C2), although the timing and final titer varied. At week 24, 2 weeks after

the fifth immunization, the levels of autologous BG505 virus neutralization for each group were generally similar as measured by ID₅₀ or ID₈₀ values (Tables 1 and 2 and Fig. 6A) (PGT145-trimer complex group ID₅₀ range of 40 to 5,720 versus BG505 trimer group ID₅₀ range of 19 to 480; Mann-Whitney test; $P = 0.3$). Neutralization of many tier 1 viral strains was also observed (Tables 1 and 2). The highest titers were against the MW965.26 strain, which is known to be highly sensitive to anti-V3 antibodies (26). The PGT145-trimer complex elicited an almost 100-fold lower titer against this virus than that seen with the BG505 trimer at week 24 (ID₅₀ range of 51 to 640 versus 5,247 to 24,765; $P = 0.03$; Mann-Whitney test) (Fig. 6B). Overall, animals immunized with the PGT145-trimer complex elicited an immune response with comparable autologous tier 2 virus neutralization at week 24 but reduced V3-directed neutralization of more sensitive tier 1 viruses. This was also shown by the lower relative neutralization activity (ratio of the ID₅₀ titer for the tier 1 virus MW965 to the ID₅₀ titer for tier 2 autologous viruses) at week 24. The anti-V3 response measured by ELISA correlated positively with MW965 virus neutralization (Fig. 6C, left panel) ($P = 0.0001$; $R^2 = 0.95$), and V3 peptide competition assays verified that the neutralization

TABLE 1 Elicitation of neutralizing antibodies in guinea pigs by the BG505 trimer and the PGT145-trimer complex as assessed by neutralization assay^a

				ID ₅₀								ID ₈₀								
Immunogen, WK 0, 4, 8, 12, 22				BG505 trimer				PGT145-trimer complex				BG505 trimer				PGT145-trimer complex				
Animal ID				GP-1	GP-2	GP-3	GP-4	GP-5	GP-6	GP-7	GP-8	GP-1	GP-2	GP-3	GP-4	GP-5	GP-6	GP-7	GP-8	
Clade A	BG505.W6M.C2	Tier 2	WK 6	33	<10	<10	<10	<10	<10	<10	<10	13	<10	<10	<10	<10	<10	<10	<10	
			WK 10	157	30	<10	<10	<10	26	<10	495	55	12	<10	<10	<10	<10	<10	158	
			WK 14	358	47	<10	<10	43	72	13	1788	86	11	<10	<10	<10	12	<10	456	
			WK 17	311	36	11	<10	20	59	13	1933	102	13	<10	<10	<10	12	<10	548	
	MG505.W0M.A2	Tier 2	WK 24	482	42	19	19	154	103	40	5720	130	12	<10	<10	28	28	<10	1477	
			WK6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	
			WK 10	11	37	<10	<10	<10	<10	<10	<10	<10	13	<10	<10	<10	<10	<10	<10	
			WK 14	27	49	<10	34	17	25	13	<10	10	21	<10	<10	11	<10	<10	<10	
	Q23.17	Tier 1B	WK 17	21	38	12	16	18	15	25	<10	<10	13	<10	<10	<10	<10	<10	<10	
			WK 24	42	72	15	35	62	10	78	<10	13	18	<10	10	20	<10	<10	<10	
Q168.a2	Tier 2	WK 24	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
		WK 6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
RW020.2	Tier 1B*	WK 10	<10	<10	<10	<10	<10	15	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
		WK 14	51	15	15	<10	45	40	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
		WK 17	28	25	30	43	12	37	35	28	<10	<10	<10	<10	<10	<10	<10	<10		
		WK 24	70	70	33	75	61	48	124	284	<10	<10	<10	<10	<10	<10	<10	<10		
Clade B	ADA.DG	Tier 1	WK 6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
			WK 10	<10	<10	21	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
			WK 14	547	153	190	20	14	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
			WK 17	396	<10	<10	<10	14	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
	BaL.26	Tier 1B	WK 24	626	<10	79	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
			WK 6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
			WK 10	<10	33	<10	<10	<10	<10	<10	<10	<10	13	<10	<10	<10	<10	<10		
			WK 14	43	34	<10	<10	<10	<10	<10	<10	11	11	<10	<10	<10	<10	<10		
	Bx08.16	Tier 1B	WK 17	46	14	<10	<10	<10	<10	<10	<10	11	<10	<10	<10	<10	<10	<10		
			WK 24	143	25	<10	<10	<10	<10	<10	<10	21	<10	<10	<10	<10	<10	<10		
WK 6			<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10			
WK 10			<10	<10	<10	<10	<10	<10	<10	12	<10	<10	<10	<10	<10	<10	<10			
JR-FL	Tier 2	WK 14	45	23	15	<10	<10	<10	<10	25	<10	<10	<10	<10	<10	<10	<10			
		WK 17	66	<10	<10	<10	<10	<10	<10	13	<10	<10	<10	<10	<10	<10	<10			
SF162	Tier 1A	WK 24	138	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
		WK 24	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10			
		WK 6	129	53	44	<10	<10	<10	<10	<10	28	18	16	<10	<10	<10	<10			
		WK 10	1012	985	144	72	<10	<10	181	667	113	201	25	12	<10	<10	39	90		
TRO.11	Tier 2	WK 14	2213	1153	133	340	21	<10	72	528	392	254	37	46	<10	<10	26	92		
		WK 17	1146	529	159	192	21	<10	220	673	181	115	42	29	<10	<10	38	113		
		WK 24	1811	782	204	254	10	<10	47	589	244	107	27	19	<10	<10	11	57		
		WK 24	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
Clade C	CAP256.2.06.C9	Tier 2	WK 6	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10		
			WK 10	<10	<10	<10	<10	<10	14	<10	<10	<10	<10	<10	<10	<10	<10	<10		
			WK 14	<10	<10	<10	<10	28	42	<10	<10	<10	<10	<10	<10	<10	<10	<10		
			WK 17	<10	<10	<10	22	<10	26	18	25	<10	<10	<10	<10	<10	<10	<10		
	MW965.26	Tier 1A	WK 24	<10	17	12	46	37	29	48	145	<10	<10	<10	<10	<10	<10	<10		
			WK 6	6729	3474	3762	409	23	<10	<10	<10	2142	1055	855	79	<10	<10	<10	<10	
			WK 10	15076	20968	14890	4677	438	37	56	61	5785	7921	5293	1650	147	<10	25	22	
			WK 14	25449	20065	13606	7116	1250	43	190	84	10539	8709	4977	2598	462	14	77	32	
	Non-HIV	SIVmac251.30	SVA-MLV	WK 17	22672	12407	10974	4698	1145	61	423	114	8417	5297	4428	1684	457	21	134	35
				WK 24	24765	9379	8604	5247	640	51	217	84	6630	3146	2868	1555	166	22	64	25

^a Reciprocal inhibitory dilution values at 50% (ID₅₀) and 80% (ID₈₀) neutralization are reported, with potency represented as follows: green, values of 10 to 99; yellow, values of 100 to 999; and red, values of >1,000. The number of weeks (WK) after initial immunization is indicated. *, internally determined to be a tier 1B virus.

of MW965 was largely dependent on anti-V3 antibodies (Fig. 6C, right panel).

In addition to neutralization of the autologous BG505.W6M.C2 virus, seven of the eight sera at weeks 17 and 24 had low titers of neutralizing activity against a virus closely related to BG505.W6M.C2, i.e., MG505.W0M.A2, which was cloned from the infected mother of patient BG505 (27). Some sera also displayed low titers of neutralization against the tier 2 CAP256.2.06.C9 virus, though this

neutralization was not evident at the ID₈₀ level (Table 2). None of the sera neutralized the control simian immunodeficiency virus (SIV) or SVA-MLV (murine leukemia virus Env-pseudotyped virus) strain (Tables 1 and 2).

Autologous tier 2 virus-neutralizing sera prevent soluble trimer Env binding to CD4. Soluble HIV-1 gp140 BG505 trimers generated high titers of autologous virus neutralization in rabbits (10), while such neutralizing activity was undetectable in mice

TABLE 2 Neutralization ID₅₀ and ID₈₀ values at week 24^a

		ID ₅₀							
Virus	Tier	BG505 trimer				PGT145-trimer complex			
		GP-1	GP-2	GP-3	GP-4	GP-5	GP-6	GP-7	GP-8
ADA.DG	1	626	<10	79	<10	<10	<10	<10	<10
BaL.26	1B	143	25	<10	<10	<10	<10	<10	<10
Bx08.16	1B	138	<10	<10	<10	<10	<10	<10	<10
MW965.26	1A	24765	9379	8604	5247	640	51	217	84
Q23.17	1B	<10	<10	<10	<10	<10	<10	<10	<10
RW020.2	1B	70	70	33	75	61	48	124	284
SF162	1A	1811	782	204	254	10	<10	47	589
BG505.W6M.C2	2	482	42	19	19	154	103	40	5720
MG505.W0M.A2	2	42	72	15	35	62	10	78	<10
CAP256.2.06.C9	2	<10	17	12	46	37	29	48	145
JR-FL	2	<10	<10	<10	<10	<10	<10	<10	<10
Q168.a2	2	<10	<10	<10	<10	<10	<10	<10	<10
TRO.11	2	<10	<10	<10	<10	<10	<10	<10	<10
SIVmac251.30	n/a	<10	<10	<10	<10	<10	<10	<10	<10
SVA-MLV	n/a	<10	<10	<10	<10	<10	<10	<10	<10

		ID ₈₀							
Virus	Tier	BG505 trimer				PGT145-trimer complex			
		GP-1	GP-2	GP-3	GP-4	GP-5	GP-6	GP-7	GP-8
ADA.DG	1	<10	<10	<10	<10	<10	<10	<10	<10
BaL.26	1B	21	<10	<10	<10	<10	<10	<10	<10
Bx08.16	1B	<10	<10	<10	<10	<10	<10	<10	<10
MW965.26	1A	6630	3146	2868	1555	166	22	64	25
Q23.17	1B	<10	<10	<10	<10	<10	<10	<10	<10
RW020.2	1B	<10	<10	<10	<10	<10	<10	<10	<10
SF162	1A	244	107	27	19	<10	<10	11	57
BG505.W6M.C2	2	130	12	<10	<10	28	28	<10	1477
MG505.W0M.A2	2	13	18	<10	10	20	<10	<10	<10
CAP256.2.06.C9	2	<10	<10	<10	<10	<10	<10	<10	<10
JR-FL	2	<10	<10	<10	<10	<10	<10	<10	<10
Q168.a2	2	<10	<10	<10	<10	<10	<10	<10	<10
TRO.11	2	<10	<10	<10	<10	<10	<10	<10	<10
SIVmac251.30	n/a	<10	<10	<10	<10	<10	<10	<10	<10
SVA-MLV	n/a	<10	<10	<10	<10	<10	<10	<10	<10

^a Reciprocal inhibitory dilution values at 50% (ID₅₀) and 80% (ID₈₀) neutralization are reported, with potency represented as follows: green, values of 10 to 99; yellow, values of 100 to 999; and red, values of >1,000. n/a, not applicable.

(13) and relatively low in monkeys (10). Here we observed the reproducible induction of autologous neutralization in another animal model, the guinea pig, when animals were immunized with the BG505 trimer or the PGT145-trimer complex. To investigate the molecular basis of the observed neutralization activity, we utilized a flow cytometry-based assay to assess whether sera from immunized animals were capable of blocking the binding of HIV-1 Env to CD4. In this assay, fluorescently labeled BG505 trimers were incubated with control monoclonal antibodies, week 24 immune sera, or no competitor before being incubated with TZM-bl cells, which display CD4 on their surfaces. As expected, the CD4bs-directed antibody VRC01 was able to prevent soluble trimers from binding to TZM-bl cells in a concentration-dependent manner, while no competition was observed with the V3-directed antibody 447-52D (Fig. 7A). Different levels of inhibition of CD4-Env trimer binding were observed for sera from both immunization groups (Fig. 7B), with guinea pig 8 (GP-8) from the PGT145-trimer complex-immunized group achieving the highest degree of blocking. Interestingly, the sera from the same guinea pig also had the highest titer of BG505 virus neutralization (Table 1). Indeed, the potency of each serum to inhibit CD4 binding correlated with the ID₅₀ titer of BG505 virus neutralization (Fig. 7C) ($P = 0.001$; $R^2 = 0.84$).

Autologous neutralization mapping. To further probe the

elicited neutralizing response, we attempted to determine the epitope(s) responsible for autologous BG505 neutralization. We performed neutralization competition assays using guinea pig sera for which reciprocal neutralization titers were higher than 100 (GP-1 from the BG505 trimer-immunized group and GP-5, GP-6, and GP-8 from the PGT145-trimer complex-immunized group) (Table 1). The BG505 gp120 outer domain (OD; amino acids 252 to 482), containing a D368R mutation to abrogate CD4 binding, almost completely outcompeted the neutralization activity in week 24 sera from GP-1 and GP-8 (Fig. 8A) but did not compete with the neutralization activity in GP-5 and GP-6 sera. Thus, in GP-1 and GP-8 sera, BG505 virus neutralization appeared to be mediated, in large part, by antibodies to the outer domain region of gp120, while this did not appear to be true for GP-5 and GP-6 sera. As the control OD in the neutralization competition assay, we used an OD that was previously optimized for VRC01-class antibody germ line binding, eOD-GT6, which is derived from a clade B HxB2 Env (17). As expected, for all these sera, no neutralization competition was observed with eOD-GT6 (Fig. 8A).

To further map the neutralizing activity in GP-1 and GP-8, we constructed three hybrid OD mutants by using the BG505 OD backbone and replacing the BG505 OD sequence with that of eOD-GT6 at the C2-V3, C3-V4, and C4-V5 regions to create OD

FIG 6 Immunogenicities of ligand-free BG505 trimer and PGT145-trimer complex as assessed by virus neutralization. (A) Comparison of neutralizing activities of sera from guinea pigs immunized with the BG505 trimer (blue) and the PGT145-trimer complex (red) against the autologous BG505.W6M.C2 virus (left) and the neutralization-sensitive MW965.26 virus (right). (B) Week 24 neutralization titers (geometric means with 95% confidence intervals). Animals immunized with the PGT145-trimer complex elicited an immune response with comparable autologous BG505 neutralization at week 24 ($P = 0.03$; two-tailed Mann-Whitney test) but reduced tier 1 virus neutralization at week 24 postvaccination ($P = 0.03$; two-tailed Mann-Whitney test). The response elicited with the PGT145-trimer complex was more biased toward autologous neutralizing activity, as shown by a lower ratio of the ID_{50} for MW965.26 to the ID_{50} for BG505.W6M.C2 for each animal ($P = 0.03$; two-tailed Mann-Whitney test). (C) Tier 1 neutralizing activity is directed to the V3 region of HIV-1 Env. (Left) Neutralization of the tier 1 virus MW965.26 correlated with anti-MW965.26 V3 peptide ELISA endpoint titers for both immunization groups at week 24. The black line represents the linear regression for all 8 animals. (Right) The MW965.26 V3 peptide was able to compete with the serum neutralization activity for MW965.26 virus in the BG505 trimer control group ($P = 0.0009$; two-tailed Mann-Whitney test).

GV3B, OD GV4B, and OD GV5B, respectively, with the D368R mutation (Fig. 8B).

We then tested the recognition of these various ODs by week 24 immune sera (Fig. 9). With the exception of GP-3 sera, there was

little or no binding to eOD-GT6. The sera from the trimer-immunized group (GP-1 to GP-4) showed similar binding profiles for BG505 OD D368R and the hybrid ODs. In contrast, sera from GP-5, GP-7, and GP-8 in the PGT145-trimer complex-immu-

FIG 7 Effects of elicited immune responses on Env trimer binding to CD4. Immune sera blocked Env trimer binding to CD4-expressing cells, with inhibition correlating with autologous virus neutralization activity. (A) BG505 Env trimer binding to CD4-expressing cells in the presence of various concentrations of monoclonal antibodies 447-52D (left) and VRC01 (right) in a flow cytometry-based CD4 blocking assay. (B) BG505 trimer binding to CD4-expressing cells in the presence of sera (1:50 dilution; week 24) from guinea pigs immunized with the BG505 trimer (left) and guinea pigs immunized with the PGT145-trimer complex (right) in the same flow cytometry-based CD4 blocking assay. Guinea pig numbers correspond to the names in [Tables 1 and 2](#). (C) Serum neutralization of the autologous BG505 virus in both immunization groups correlates with the ability of sera to block CD4 binding. The values shown in the tabular part of the figure are neutralization ID₅₀ values and percent inhibition of CD4 binding. Percent competition was calculated as the mean fluorescence on the R660 channel, with 100% competition corresponding to the fluorescence of unstained cells. The lower panel shows a graphical display of the data with a linear regression line.

FIG 8 Mapping of autologous BG505 virus neutralization. (A) Neutralization competition assays were performed with the indicated protein at 25 $\mu\text{g}/\text{ml}$, as described in Materials and Methods, using week 24 sera with an ID_{50} of >100 . All competitors had mutations (CD4bs knockout mutations) to avoid attachment to cell surface CD4 in the neutralization assay. Data are shown for the sera from guinea pig 1 (GP-1), from the BG505 trimer immunization group, and GP-5, -6, and -8, from the PGT145-trimer complex immunization group. The BG505 outer domain (OD; gray) outcompeted neutralization in both GP-1 and GP-8, while eOD-GT6 (red) did not. None of the ODs competed significantly with the neutralizing activity in sera from GP-5 and GP-6. (B) Hybrid BG505 OD/eOD-GT6

FIG 9 Mapping of immune responses to the OD region of the trimer by ELISA. Binding of guinea pig IgG antibodies from week 24 immune sera to BG505 OD, eOD-GT6, or hybrid ODs was determined by ELISA. The optical densities at 450 nm are shown for different serum dilutions.

nized group showed more binding to BG505 OD D368R than to the hybrid ODs, indicating that antibodies specific to the C2-V3, C3-V4, and C4-V5 regions of BG505 OD were present. Also, GP-6 sera showed a small amount of binding to BG505 OD and minimal binding to hybrid ODs but had similar or higher binding titers for the trimer (Fig. 5A) than the other sera in the same group, suggesting that the major epitopes targeted may not be located in the monomeric OD region.

We then performed neutralization competition assays with the hybrid ODs on GP-1 and GP-8 sera (Fig. 8C). The OD hybrid GV4B, which contained the eOD-GT6 C3-V4 sequence, did not compete with the neutralizing activity. This narrowed the critical epitope responsible for autologous neutralization to the region spanning C3 to V4 (amino acids 331 to 418; HxB2 numbering) of BG505 OD for GP-1 sera and GP-8 sera. Interestingly, the CD4-binding loop (centered around D368) is located in this C3 region. The OD hybrids GV3B and GV5B competed efficiently with the neutralizing activity of GP-1 sera, indicating that the C2-V3 and C4-V5 regions of BG505 OD were not critical for neutralizing activity of the sera (Fig. 8C). For guinea pig GP-8 sera, both OD hybrids GV3B and GV5B partially competed with the neutralizing activity, indicating these regions to also be important for neutralization in this animal. Overall, these data indicate that more than one epitope is likely responsible for mediating autologous neutralization.

DISCUSSION

The soluble BG505 trimeric protein immunogen can elicit NABs to the autologous tier 2 virus but also elicits substantial antibodies against the V3 region of Env, which mediate neutralizing activity

mainly against tier 1 viruses. Here we examined the consequences of increasing the antigenic specificity of the BG505 trimer to broadly neutralizing antibodies through complex formation with the Fab of the V1V2-directed quaternary-structure-specific antibody PGT145. Our rationale for using the PGT145-trimer complex as an immunogen was 3-fold: (i) it allowed purification of the Env trimer without exposure to harsh conditions, such as low pH or high salt (3 M MgCl₂); (ii) it used the quaternary-structure-specific antibody PGT145, which was previously shown to stabilize the prefusion closed (ground) state of Env (8) while still exposing neutralizing epitopes; and (iii) it provided for a more stabilized Env trimer, as shown by its increased thermostability. Note that several prior studies suggested that antibody-bound complexes can enhance the quality of the immune response compared to noncomplexed immunogen counterparts (28–30). Anti-CD4bs antibodies have been used to form complexes with gp120 to enhance immune responses to V3 (28, 29, 31).

Various purification methods for similar SOSIP.664 trimers have been reported and include supernatant purification over lectin, negative selection with weakly neutralizing CD4bs antibody, and gel filtration (32). We recently found that negative selection by V3-directed antibodies was able to remove the trimer fraction recognized by V3-directed antibodies (9). Positive selection of HIV-1 gp140 SOSIP.664 trimers by use of PGT145 has also been shown to be an effective way to prevent V3 from being accessible to protease digestion in the epitope region recognized by 447-52D (33, 34). However, the observation that Env trimers purified over 2G12 affinity or lectin columns still bind V3-directed weakly neutralizing antibodies, such as 447-52D, indicates that a population

construct sequences and locations on the HIV-1 gp120 (dark gray)/gp41 (light gray) protomer. The diagram gives a sequence representation of the BG505 SOSIP.664 construct and the construction of hybrid ODs. The OD contains amino acids 252 to 482, and eOD-GT6 is shown in the original amino acid sequence order of gp120. Hybrid OD mutants were made by using the BG505 backbone and eOD-GT6 sequences placed at either the C2-V3, C3-V4, or C4-V5 region, for GV3B, GV4B, or GV5B, respectively. The locations of the C2-V3 (GV3B), C3-V4 (GV4B), and C4-V5 (GV5B) regions are shown in ribbon representations of a BG505 SOSIP.664 protomer (one gp120 and one gp41), using the structure from PDB entry 4TVF. Each region inserted to make the hybrid OD is shown in red. The region that interacts with CD4 is colored yellow. (C) Neutralization competition assays as described for panel A, with hybrid OD mutants. Only GV4B OD did not compete efficiently (brown) with the neutralizing activity in immune sera from GP-1 and GP-8, suggesting that the neutralizing epitopes are proximal to the C3-V4 region.

of these trimers are likely to be in an undesirable open conformation with the V3 loop exposed, antigenically similar to what is observed with a CD4-induced trimer or a gp120 monomer (35).

Previous reports have shown that broadly neutralizing antibodies, particularly PGT145, hold HIV-1 Env in its prefusion closed conformation (8, 36), and our data support this observation. Since envelope spikes of tier 2 viruses appear to dwell predominantly in the prefusion closed conformation, the stabilized conformation of the Env trimer may be needed to induce antibodies able to neutralize most tier 2 viruses. We hypothesize that binding of the PGT145 Fab stabilized the Env trimer in its prefusion closed state by reducing the “flickering” of the Env trimer into conformations that expose V3. Additionally, the PGT145 Fab purification process may result in removal of aberrantly folded trimers (9). While the exact mechanism of reduced V3 reactivity is not known at this time, weakly neutralizing antibodies, such as 447-52D, are not structurally compatible with the SOSIP trimer in its closed prefusion conformation (9). Thus, it is difficult to know if the lower V3 reactivity we observed was partially due to steric effects (PGT145 blocking access of weakly V3-directed antibodies to an “open” conformation of the SOSIP trimer) or due solely to stabilization of the prefusion state as it has been described elsewhere (8). The physical stabilization against unfolding of Env by complexation to the PGT145 Fab supports the latter possibility. Note that the PGT145-trimer complex contained about 11% less trimer than the ligand-free trimer at an equal weight. This slight difference of dosage is unlikely to account for the 100-fold reduction of the anti-V3-peptide response. The V3 response in both groups reached a maximum after three immunizations, suggesting that immunization dose was not a limiting factor. The fate of the PGT145-trimer complex *in vivo* after intramuscular injection is unknown. The PGT145-trimer complex is not covalently linked, and the bound PGT145 Fab can be exchanged with PGT145 IgG, as shown in Fig. 3. It may be informative to use a covalently linked complex as well as antibodies other than PGT145 to form other complexes to dissect the impact on trimer immunogenicity. PGT145 is a human monoclonal antibody, and anti-PGT145 Fab antibodies were detected in the PGT145-trimer complex group. Such immune responses to the Fab or the junction of the Fab and the trimer may account for the lower anti-trimer responses observed in the PGT145-trimer complex group than in the BG505 trimer group. The use of species-matched antibodies would likely overcome this issue.

Our immunization data from guinea pigs verified that the PGT145-trimer complex induced lower titers of antibodies to the V3 region than those induced by the ligand-free trimer, as measured by both binding and neutralization. Despite this reduction in antibody binding to the V3 region, the PGT145-trimer complex maintained the ability to elicit high titers of autologous virus neutralization, especially in GP-8. Among the four guinea pigs in the PGT145-trimer complex group, GP-8 generated the lowest anti-V3-peptide response, as measured by ELISA, and the lowest neutralizing activity against the tier 1A MW965.26 virus. Sera from GP-8 strongly neutralized the tier 1B RW020.2 virus and also displayed some neutralizing activity against the tier 2 CAP256.2.06.C9 virus, suggesting that these sera could target a neutralization epitope present on some tier 2 viruses. Further studies will be needed to confirm this observation.

Our mapping experiments indicated that the elicited BG505 neutralization in some immune sera was directed to the C3-V4

region of Env. Since this region contains main contacts with CD4, it is possible that antibodies directed to this region would sterically obstruct CD4 binding. Of the tier 2 isolates we tested, the autologous BG505.W6.C2 virus was the only virus neutralized at a high titer (Table 1), suggesting that the neutralization epitopes mediating this neutralization, such as the C3-V4 region targeted by the immune sera, may not be conserved or accessible on other tier 2 viruses. The immune sera from the guinea pigs that strongly neutralized BG505.W6M.C2 (GP-1, GP-5, GP-6, and GP-8) showed low or no activity against the closely related MG505.W0M.A2 virus. The Env sequences of the two viruses differ at only 16 amino acid positions. Five of them are located in the C3-V4 region, at amino acid positions 343, 357, 358, 360, and 396. The amino acids at these positions, especially at positions 357, 358, and 360, which were identified in a previous study (10), may be important for neutralization of the autologous virus. Some immune sera appeared to mediate autologous neutralization through other epitopes, such as those within the C2-V3 and C4-V5 regions of the outer domain or regions outside the outer domain. In these immune sera, antibodies specific to the trimer may be responsible, and such antibodies could trigger allosteric changes to Env.

The immunogenicity results obtained for guinea pigs immunized with the BG505 trimer immunogen are in agreement with recently reported data observed for rabbits (10). In both animal models, the BG505 trimer immunogen elicited high titers of V3-directed tier 1 and OD-directed tier 2 autologous virus-neutralizing activity. These results suggest a common mechanism for the elicitation of these neutralizing antibodies and similar hierarchies for dominant immune epitopes in both animal species. In contrast, in macaques, the neutralizing activity elicited with the same immunogen was substantially lower (10), perhaps related to the triggering of Env to be in its open conformation by macaque CD4 (37). It was recently shown that the BG505 trimer immunogen failed to elicit tier 2 autologous virus-neutralizing antibodies in mice (13). Therefore, the immunogenicity of Env trimer immunogens may depend on the animal model used, and definitive data on the immunogenicity of Env trimers may require human clinical trials.

In summary, we improved the antigenic specificity of the BG505 trimer for neutralizing antibodies by complex formation with PGT145. This significantly reduced (100-fold) the elicitation of V3-directed antibodies, though this did not translate into a marked improvement in autologous or heterologous virus neutralization: neither the potency nor the breadth of tier 2 neutralization was significantly improved. One limitation of our study is the small number of animals, which may not have allowed detection of more subtle improvements in tier 2 neutralization. Note that the extended development time of most broadly reactive neutralizing antibodies (many appear only after years of infection [38, 39]) suggests the potential importance of reducing initial responses against poorly or nonneutralizing responses that are immunodominant, such as those against V3. Overall, our results indicate that it is possible to reduce the immunogenicity of the V3 region of the Env trimer, and it is possible that—in the context of additional improvements in immunogen design or prime-boost immunization strategies—this will help to focus the immune response on crucial neutralization epitopes.

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